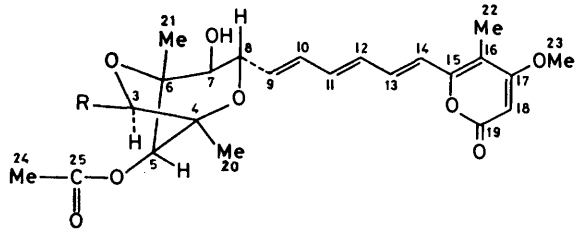
Biosynthesis of the Aurovertins B and D. The Role of Methionine and Propionate in the Simultaneous Operation of Two Independent Biosynthetic Pathways [1]

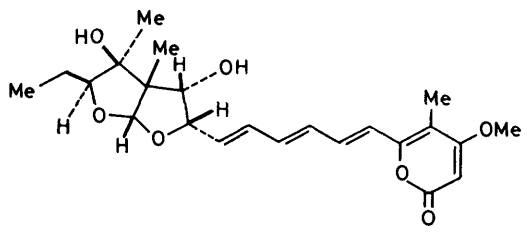
By **Pieter S. Steyn, Robert Vleggaar,* and Philippus L. Wessels,** National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria 0001, Republic of South Africa

The complete assignment of the natural abundance ^{13}C n.m.r. spectra of the aurovertins B (1) and D (2), toxic metabolites isolated from *Calcarisporium arbuscula* NRRL 3705, permitted a study of their biosynthetic origin using the following ^{13}C-labelled precursors: [1-^{13}C]-, [2-^{13}C]-, [1,2-^{13}C]-, and [2-^{13}C, 2-$^{2}H_3$]-acetate, (2*S*)-[*methyl*-^{13}C]methionine, [2-^{13}C]malonate, and [1-^{13}C]- and [3-^{13}C]propionate. The results show that both the aurovertins B and D can be formed *via* two biosynthetic pathways which are distinguishable by the different origins of C(1)—C(3). The first pathway involves the *C*-methylation of a C_{20}-polyketide precursor at C_{18}, followed by the loss of the chain-initiating acetate unit, C_{19}–C_{20}; C(1) in the aurovertins is thus derived from methionine and C(2) and C(3) from malonate. The second pathway involves a C_{19}-precursor, formed from a propionate chain-initiating unit and eight malonate units; C(1)—C(3) are derived from propionate. The simultaneous operation of two independent pathways in the biosynthesis of the aurovertins B and D is unique amongst fungal metabolites.

AUROVERTINS B (1) and D (2), metabolites from *Calcarisporium arbuscula* NRRL 3705,[2,3] are members of a group of toxic substances which act as inhibitors of ATP-synthesis and ATP-hydrolysis catalysed by mitochondrial enzyme systems.[4] Our isolation of asteltoxin (4), a related metabolite from *Aspergillus stellatus* [5] which inhibits wild-type *E. coli* ATP-ase with a potency *ca.* 10-times lower than aurovertin D,[6] prompted us to investigate the biosynthesis of these compounds.



(1) R = $CH_2^2Me^1$
(2) R = CH(OH)Me
(3) R = Me



(4)

Several plausible mechanisms, some without firm precedent in fungal polyketide biosynthesis, can be formulated for the biosynthesis of aurovertin B (and D) and can be distinguished by the different origins of C(1)—C(3).† (*a*) Pathway 1 involves a C_{18}-polyketide precursor † and requires the introduction of a methyl group from the C_1-pool at the methyl carbon atom of the chain-initiating acetate unit; C(1) is thus derived from methionine and C(2) from [2-C]acetate. A similar methylation has been postulated to explain the formation of the ethyl side-chain of barnol [7] and is indicated for the biosynthesis of stellatin.[8] (*b*) Pathway 2 requires the loss of the methyl carbon atom, C_{20}, of the chain-initiating acetate unit of a C_{20}-polyketide; C(1) is thus derived from [1-C]acetate and C(2) from [2-C]acetate. (*c*) Alternatively (Pathway 3), the C_{20}-polyketide is methylated at C_{18}, followed by the loss of the starter-acetate unit, C_{19}—C_{20}; C(1) is thus derived from methionine and C(2) from [2-C]malonate. (*d*) Incorporation of propionate [9] into aurovertin B can occur *via* pathway 4, in which propionate is used as a chain-initiating unit; C(1), C(2), and C(3) are thus derived from propionate. The participation of propionate in the biosynthesis of a polyketide-derived metabolite, *viz.* homo-orsellinic acid has been reported.[10]

^{13}C N.m.r. spectroscopy provides an efficient tool for distinguishing between the different, postulated, biosynthetic pathways and identifying a particular one. A study of the incorporation of different ^{13}C-labelled precursors into the aurovertins B and D was therefore undertaken.

N.M.R. Assignments.—The assignment of the ^1H n.m.r. spectra of the aurovertins B (1) and D (2), as presented in Table 1, is based on chemical-shift considerations and homonuclear-decoupling experiments, as well as n.O.e. experiments. The previously reported assignment for aurovertin B[3] and our results are in good agreement. Also, an unequivocal assignment of the C(13) proton resonance, as outlined below, facilitated the analysis of the ^{13}C n.m.r. spectrum of the two metabolites. Pronounced upfield shifts were recorded for 18-H ($\Delta\delta$ 0.27), 22-H (0.24), 23-H (0.51) (all protons loca-

† Subscript numbers, *e.g.* C_n, refer to the carbon atom *n* of the original polyketide whereas C(*n*) denotes a particular carbon atom in the final metabolite.

TABLE 1

N.m.r. data for Aurovertin B (1) and D (2)

Carbon atom	δ_C [a]	^1J(CH)/Hz	$^{>1}J$(CH)/Hz	J(CC)/Hz	δ_H [b]	J(HH)/Hz
	Aurovertin B					
1	11.7 Qm	122.0		S	1.09t	7.4
2	20.1 Tm	123.7		39.4	1.70m	
3	85.4 Dm	140.2		40.2	3.93dd	4.9; 8.2
4	82.6 S [c]	—		39.4		
5	80.6 D	154.4	—	38.8	4.80s	
6	83.4 S [c]	—		38.6		
7	76.2 D	144.2	—	37.6	3.30d	7.9
8	77.6 D	145.0		50.3	4.17t [c]	7.3
9	134.3 D	158.0	—	50.8	5.93dd	6.2; 13.9
10	131.2 Dm	151		56.2	} 6.3—6.6	
11	137.0 Dm	155		56.5		
12	131.7 Dm	153		56.1 [d]		
13	135.5 Dm	153.5		56.8 [d]	7.17dd	10.3; 15.0
14	119.3 Dm	156.9		70.6	6.35d	15.0
15	154.1 Sdqn	—	8.5; 5.9	69.7		
16	107.9 Sqn	—	6.5	62.1		
17	170.4 Sq	—	3.6	62.4		
18	88.6 D	168.0	—	78.6	5.50s	
19	163.4 S	—	—	78.5		
20	16.4 Q	127.8	—	S	1.19s	
21	15.0 Q	127.8	—	S	1.27s	
22	8.8 Q	129.4	—	S	1.96s	
23	56.1 Q	146.2	—	S	3.83s	
24	20.7 Q	129.8	—	60.0	2.17s	
25	169.6 Sdq	—	3.8; 6.4	60.0		
	Aurovertin D					
1	21.9 Qd	126.3	1.6	S	1.36d	6.2
2	64.8 Dm	143.9		45.3	4.20dq	9.3; 6.2
3	86.6 Dm	151		45.6	3.76d	9
4	82.8 Sm	—		38.6		
5	81.1 Dm	157.1		38.5	4.78s	
6	83.3 Sm	—		37.9		
7	76.0 Dm	145.2		37.6	3.32d	8.3
8	77.3 Dm	144.6		50.1	4.14dd	6.0; 8.3
9	134.3 D	158.7	—	50.7	5.90dd	6.0; 14.6
10	131.1 Dm	154		56.2	} 6.2—6.6	
11	136.9 Dm	150		56.3		
12	131.6 Dm	154		56.2 [f]		
13	135.4 Dm	155		*f,g*	7.18dd	10.0; 15.0
14	119.2 Dm	160		70.4	6.35d	15.0
15	154.0 Sdqn	—	8.2; 5.3	70.1		
16	108.0 Sqn	—	6.5	62.3		
17	170.4 Sq	—	3.6	62.2		
18	88.5 D	168.3	—	78.6	5.49s	
19	163.6 S	—	—	78.3		
20	17.7 Q	128.1	—	S	1.37s	
21	15.0 Q	128.0	—	S	1.24s	
22	8.7 Q	129.4	—	S	1.94s	
23	56.1 Q	146.2	—	S	3.81s	
24	20.6 Q	129.8	—	60.0	2.13s	
25	169.6 Sdq	—	3.8; 6.8	60.0		

[a] Relative to internal Me_4Si; solvent CD_2Cl_2. Measured from internal CD_2Cl_2 and corrected by using the expression $\delta(Me_4Si) = \delta(CD_2Cl_2) + 53.8$. Capital letters refer to the pattern resulting from directly bonded (C,H) couplings [^1J(CH)] and small letters to that from (C,H) couplings over more than one bond [$^{>1}J$(CH)]. S = singlet, D or d = doublet, T or t = triplet, Q or q = quartet, qn = quintet, and m = multiplet. [b] Relative to internal Me_4Si; solvent $CDCl_3$. [c] Overlapping with other resonances. [d] ^1J(CC) 56.7 Hz, calculated from the inner lines of the AB system and using the natural-abundance ^{13}C resonances as the chemical shift values (see text). [e] The centre line of the triplet is noticeably broader than the other lines. [f] Calculated value, ^1J(CC) 56.5 Hz (see the text and footnote *d*). [g] One transition obscured.

ted on the α-pyrone moiety), and 24-H (0.29) of aurovertin B upon change of the solvent from CD_2Cl_2 to CD_2Cl_2–C_6D_6 (1 : 2 v/v). Only one of the olefinic proton resonances, the doublet at δ 6.35 (J 15.0 Hz), experienced an upfield shift (Δδ 0.15) and must therefore be assigned to 14-H. INDOR experiments indicated that 14-H undergoes spin-spin coupling with the proton resonating at δ 7.17, *i.e.* 13-H.

The ^1H n.m.r. spectrum of aurovertin D was assigned by analogy with that of aurovertin B and, once again, extensive use was made of homonuclear-decoupling experiments. Irradiation at the resonance position of the C(1) methyl protons changes the multiplet at δ 4.20 (2-H) to a doublet (J 9.3 Hz). A similar coupling is observed for the 3-H signal, when the obscuring signal of the methoxy-protons shifts upfield upon change of solvent from $CDCl_3$ to $CDCl_3$–C_6D_6 (1 : 1 v/v). Irradiation at δ 3.32 (7-H) or 5.90 (9-H) collapses the multiplet at δ 4.14 (8-H) to a doublet with J 6.0 and 8.3 Hz, respectively. An unambiguous assignment of the C(20)

and C(21) methyl protons could only be achieved by heteronuclear ^{13}C-{^1H} selective-population inversion (SPI) experiments [11] (see below).

The assignment of the natural abundance ^{13}C n.m.r. spectrum of aurovertin D (2) (Table 1) is based on the results obtained from single frequency n.O.e., proton-noise-decoupled (p.n.d.), off-resonance proton-decoupled, and selective proton-decoupled spectra and from SPI experiments.[12] The p.n.d. natural abundance ^{13}C n.m.r. spectrum of aurovertin D is shown in Figure 1a.

The residual splittings observed in a series of off-resonance proton-decoupled ^{13}C experiments enabled us to correlate the signals of all the proton-bearing carbon atoms with specific proton resonances.[13] With this relationship, the resonances due to C(1)—C(3), C(7)—C(9), C(13), and C(23) could be unambiguously assigned. The magnitude of the observed, directly bonded (C,H) coupling constants (Table 1) support these assignments. The resonances at δ 81.1 and 88.5, which were correlated with the proton singlets at δ_H 4.78 and 5.49, exhibit directly bonded (C,H) coupling constants of 157.1 and 168.3 Hz, respectively, which allow their respective assignment to C(5) and C(18).

The remaining methyl and quaternary carbon resonances were assigned mainly from the results of heteronuclear ^{13}C-{^1H} SPI experiments. The main parameters used in SPI studies are (C,H) couplings and, normally, only one-, two-, and three-bond (C,H) couplings are observed. The reported (C,H) couplings over four bonds are of the order of 1 Hz [14] and need not be considered at the power levels used in the SPI experiments (*ca.* 5 Hz).

Chemical-shift considerations [15] dictate that the four resonances in the δ 154—171 region must be attributed to the two carbonyl [C(19) and C(25)] and the two oxygen-bearing [C(15) and C(17)] sp^2 carbon atoms. The resonances at δ 82.8 and 83.3 are due to the two aliphatic quaternary carbon atoms C(4) and C(6). The signal at δ 107.9 is assigned to C(16). Figure 1b shows the spectrum of a heteronuclear ^{13}C-{^1H} SPI experiment when a π-pulse (τ 0.102 s) is applied at δ_H 1.89 (*i.e.* 5 Hz to high-field of the singlet due to the

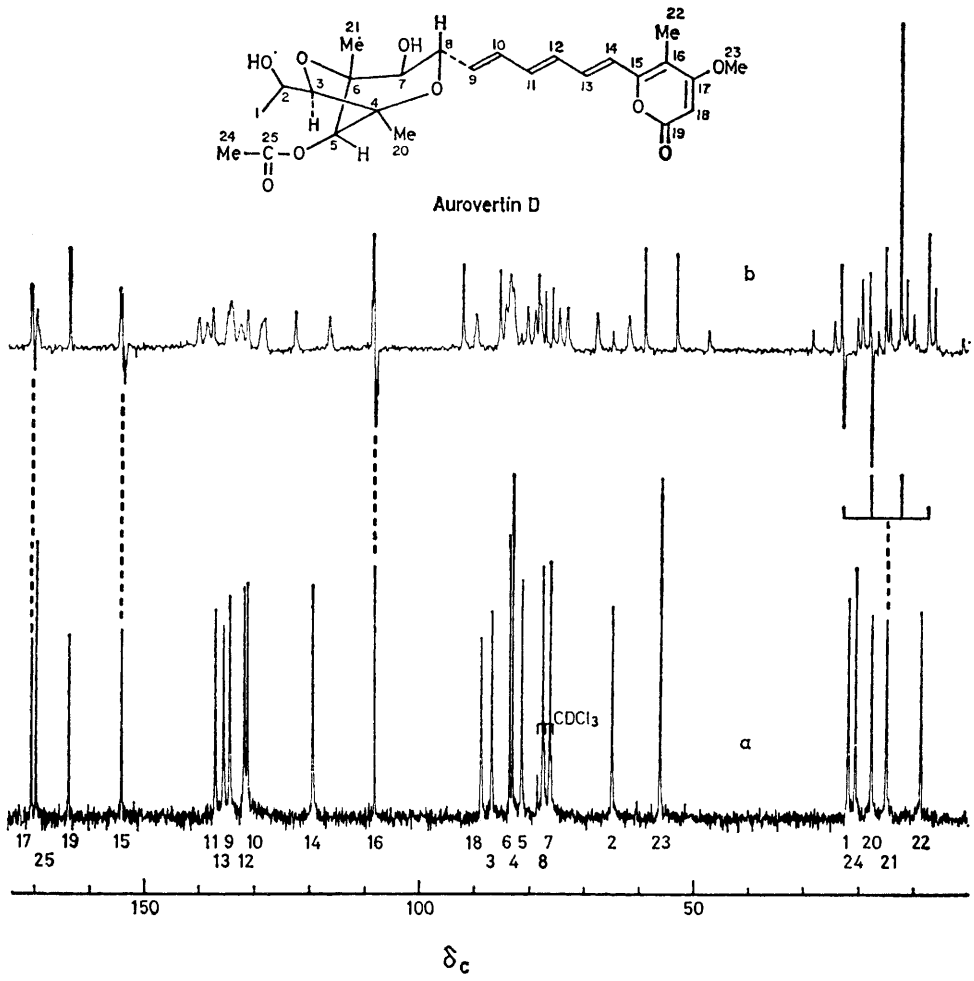


FIGURE 1 The natural-abundance 25.2-MHz ^{13}C n.m.r. spectra of aurovertin D (2) in $CDCl_3$ (spectral width 5 000 Hz; 90° r.f. pulse of 54 μs duration; acquisition time 0.8 s). (a) Proton-noise-decoupled (pulse delay 2 s; transients 1 024); (b) after a low-field 22-H transition has been selectively pulsed (pulse delay 1 s; π-pulse of 0.102 s; transients 25 664).

methyl protons, at δ_H 1.94, 100 MHz). The resonances at δ 107.9 [C(16)], 170.4, and 154.0 are affected. The transition(s) which was (were) selectively pulsed must arise from the C(22) protons, as C(16) is two bonds removed from these protons. Selective decoupling of 13-H (δ_H 7.18) simplifies the multiplet at δ 154.0 thereby assigning it to C(15). As a consequence, the resonance at δ 170.4 could be allocated to C(17). The singlet at δ_H 2.13 (3 H) is assigned to the C(24) methyl protons. Application of a selective π-pulse to a 24-H transition (5 Hz to low-field of the signal at δ_H 2.13) influences the multiplet at δ 169.6 [C(25)]. The remaining resonance, *viz.* that at δ 163.4 is therefore due to the lactone carbonyl carbon atom, C(19).

When a 2-H transition is selectively pulsed in an SPI experiment the resonance at δ 82.8 is affected. As C(4) is three bonds removed from 2-H [compared with a four-bond separation for C(6)] the resonances at δ 82.8 and 83.3 are assigned to C(4) and C(6), respectively.

At this stage it was possible to assign unambiguously the ^{13}C and ^{1}H resonances of the C(20) and C(21) methyl groups. Application of a π-pulse with $\gamma H_2 = 5$ Hz at a position 5 Hz to high-field of the methyl singlet at δ_H 1.24 influenced the C(6) resonance at δ 83.3. The affected proton transition must therefore belong to 21-H. In the spectrum shown in Figure 1b the low-field ^{13}C satellite transition of these protons was also selectively pulsed at δ_H 1.88 [$\nu_0 + \frac{1}{2}\,{}^1J(C,H) = 124 + 64$ Hz, 100 MHz] and proved that the ^{13}C resonance at δ 15.0 arises from C(21). Conversely, when a selective π-pulse is applied 5 Hz to low field of the methyl resonance at δ_H 1.37, the C(4) resonance is affected.

The four remaining olefinic carbon resonances, at δ 131.1, 136.9, 131.6, and 119.2 due to C(10)—C(12), and C(14), could not be assigned from the data obtained from the natural-abundance spectrum. Analysis of the ^{13}C n.m.r. spectra of aurovertin D derived from [1-^{13}C]- and [1,2-^{13}C]-acetate enabled us to assign these carbon resonances (see below).

The resonances observed in the p.n.d. natural abundance ^{13}C n.m.r. spectrum of aurovertin B (Figure 2a) were assigned by the same methodology as outlined for aurovertin D. Our assignments, as presented in Table 1, differ considerably from those reported by Mulheirn *et al.*[3]

Biosynthetic studies. Cultures of *C. arbuscula* were grown in the dark at 23 °C in a stationary culture on an F14 medium at pH 6.2.[16] In their original work on the isolation of aurovertin B, Osselton *et al.*[2] reported the presence of at least nine aurovertins in mycelial extracts of *C. arbuscula*; aurovertin B (1),[3] C (3),[17] and D (2)[3] have been characterized by Linnett and his co-workers at Sittingbourne.[17] In our experiments, the organism formed only the aurovertins B and D; in fact, in a few cases when older culture material, obtained through repetitive subculturing, was used, only aurovertin B was produced.

Studies of the course of fermentation indicated that aurovertin production commenced on day 7 and reached a maximum 14 days after the inoculation of the medium. Preliminary feeding experiments with [1-^{14}C]acetate as the precursor established conditions which would give a suitable ^{13}C enrichment at each individual, acetate-derived carbon atom of the aurovertins on feeding [^{13}C]-acetate. Satisfactory dilution values * for the aurovertins B (15.7) and D (18.0) (assuming 10 labelled positions) and good incorporations (0.8% for each metabolite) were obtained by pulsing cultures of *C. arbuscula* every 12 h from day 7 to day 13 with sodium acetate to a total amount of 1.5 g l^{-1}.

The p.n.d. ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived aurovertin B (Figure 2b) showed 10 enhanced signals, attributed to C(3), C(5), C(7), C(9), C(11), C(13), C(15), C(17), C(19), and C(25), whereas the spectrum of aurovertin B derived from [2-^{13}C]acetate showed enhanced signals representative of C(2), C(4), C(6), C(8), C(10), C(12), C(14), C(16), C(18), and C(24) (see Figure 2c). High enrichment factors † were obtained for both the [1-^{13}C]- and [2-^{13}C]-acetate-derived carbon atoms (average 7.6 and 5.6, respectively). In our initial feeding experiment with [1-^{13}C]acetate the derived aurovertin B exhibited a disparity in the enrichment of the carbonyl carbon atom of the *O*-acetate group, C(25). This signal showed a three-fold enrichment over those of the other [1-^{13}C]acetate-derived carbon atoms. A similar effect, but of lesser magnitude, was observed for the methyl carbon atom of the *O*-acetate group, C(24), in some of the [2-^{13}C]acetate-enriched aurovertin B samples.

The arrangement of intact acetate units in both the aurovertins B and D and thus in the original polyketide progenitor was studied by addition of [1,2-^{13}C]acetate to cultures of *C. arbuscula*. All the signals in the p.n.d. ^{13}C n.m.r. spectra of the aurovertins B and D (Figure 3) derived from [1,2-^{13}C]acetate, with the exception of those for C(1), C(20), C(21), C(22), and C(23), exhibited ^{13}C–^{13}C spin-spin coupling. The measured $^1J(CC)$ values are given in Table 1 and prove the presence of the following intact acetate units, *viz.* C(2)–C(3), C(4)–C(5), C(6)–C(7), C(8)–C(9), C(10)–C(11), C(12)–C(13), C(14)–C(15), C(16)–C(17), C(18)–C(19), and C(24)–C(25).

The yield of aurovertin B derived from [1,2-^{13}C]-acetate in our first feeding experiment was poor owing to deterioration of the fungus and this resulted in some difficulty in observing the ^{13}C–^{13}C coupling satellites. This difficulty was compounded by the severe overlap of some of the ^{13}C signals. Once again, a disparity in the enrichment of the carbon atoms of the *O*-acetate group

* Dilution values obtained from ^{14}C precursor experiments are defined as in equation (1)

$$D = \frac{\text{specific activity (precursor)}}{\text{specific activity (product)}} \times \frac{m\ \text{(product)}}{n\ \text{(precursor)}} \quad (1)$$

where n and m are the appropriate number of labelled sites.

† % Enrichment = 1.1 (Enrichment factor) − 1.1. The enrichment factor for a specific carbon atom is obtained by dividing the signal height in the spectrum of the enriched compound by the corresponding signal height in the natural-abundance spectrum recorded under identical experimental conditions.

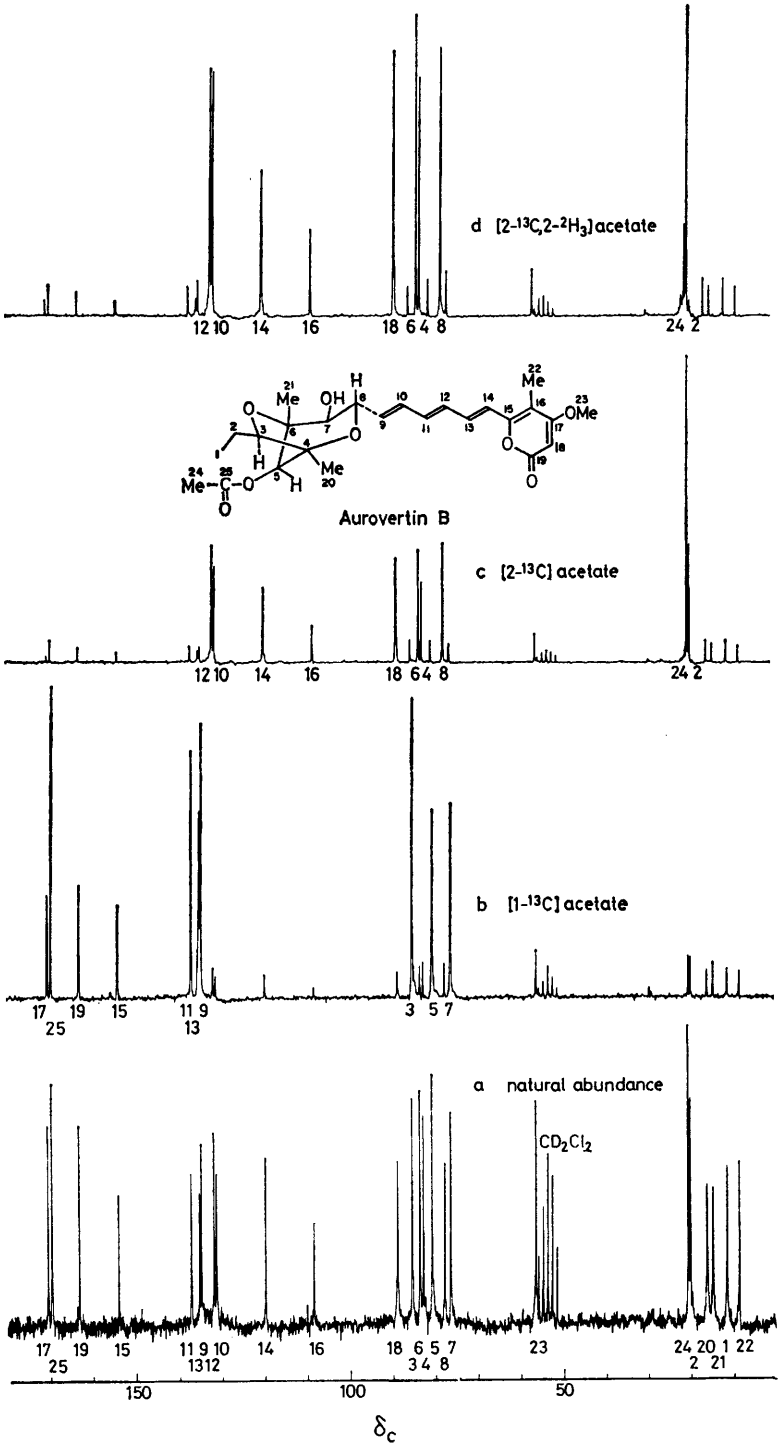


FIGURE 2 The proton-noise-decoupled 25.2-MHz ^{13}C n.m.r. spectra of aurovertin B (1) in CD_2Cl_2 (spectral width 5 000 Hz; 90° r.f. pulse of 54 μs duration; acquisition time 0.8 s; pulse delay 5 s) derived from (a) natural abundance (transients 7 268); (b) [1-^{13}C]acetate (transients 2 958); (c) [2-^{13}C]acetate (transients 5 402); and (d) [2-^{13}C, 2-$^{2}H_3$]acetate (transients 10 638).

was observed, since both C(24) and C(25) exhibited a three-fold enrichment over those of the other acetate-derived carbon atoms. In a subsequent experiment a much improved yield of aurovertin B (and D) was obtained. The appearance of a number of AB spin systems in the 25.2-MHz p.n.d. ^{13}C n.m.r. spectra of the aurovertins B and D derived from [1,2-^{13}C]acetate is actually advantageous since these spin systems are useful

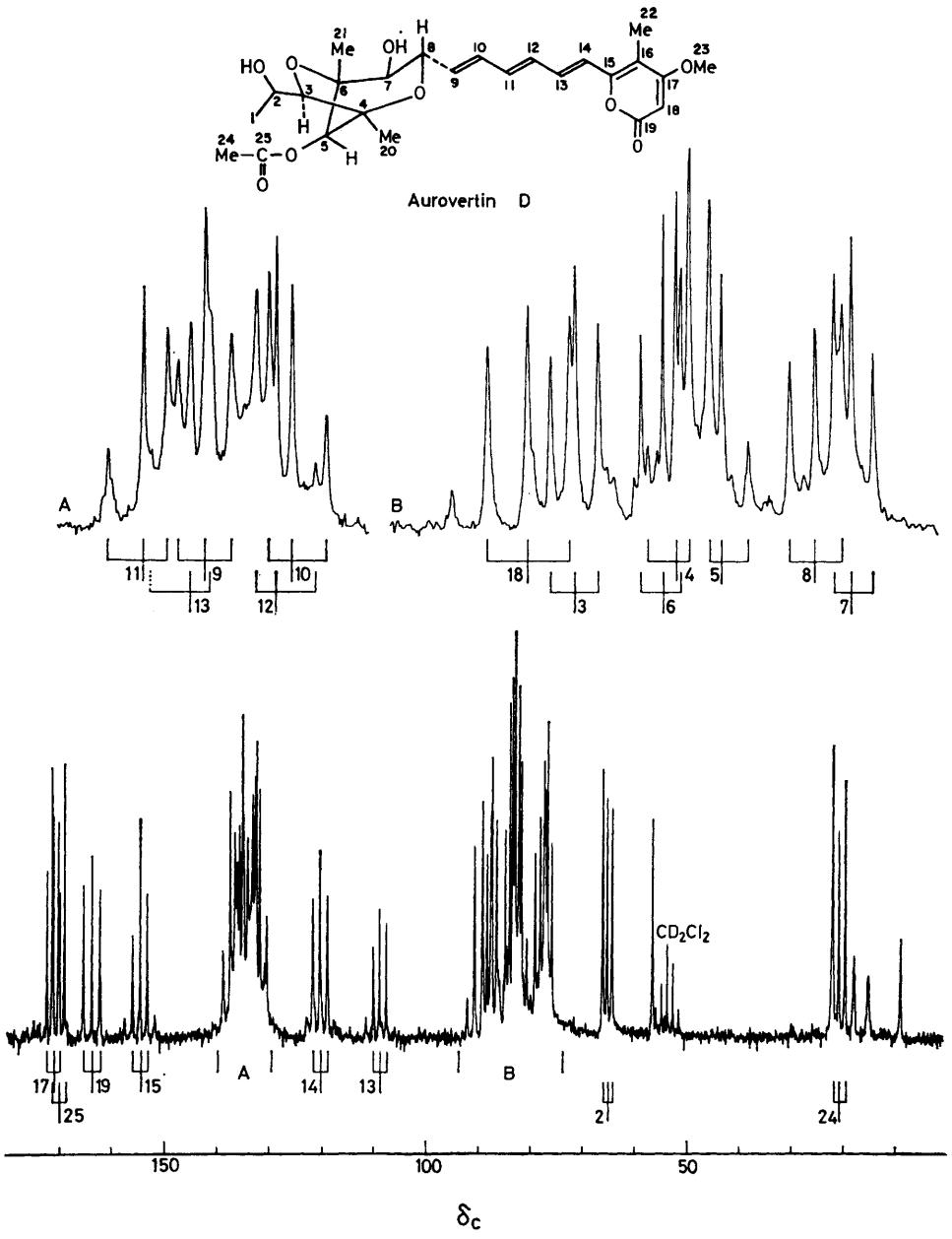


FIGURE 3 The proton-noise-decoupled 25.2-MHz ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived aurovertin D (2) in CD_2Cl_2 (spectral width 5 000 Hz; 90° r.f. pulse of 54 μs duration; acquisition time 0.8 s; pulse delay 5 s; transients 7 033)

for assignment purposes as well as for determining pairs of coupled carbon atoms with similar $^1J(CC)$ values. Valuable information may be lost when ^{13}C n.m.r. spectra of [1,2-^{13}C]acetate-enriched compounds are recorded at higher field strengths.

In analysing AB spin systems, four resonances are required to determine the chemical shifts of nuclei A and B as well as the coupling constant $J(AB)$.[18] The chemical shift of contiguous carbon atoms derived from [1,2-^{13}C]acetate can be obtained from the natural-abundance ^{13}C n.m.r. signals. The ^{13}C isotope effect on a neighbouring ^{13}C atom is normally to higher field and is small (less than 0.04 p.p.m.).[19] The distances of the inner and outer lines of an AB system from the chemical-shift positions depend on the chemical-shift difference $\Delta\nu(AB)$ and the coupling constant $J(AB)$. Only for an AX spin system are the inner and outer lines equidistant from the chemical-shift position of the natural-abundance signal. The inner lines of the AB spin system must be equidistant from the natural-abundance ^{13}C signals; this observation is also valid for the outer lines. For two pairs of ^{13}C atoms with identical coupling constants these distances will differ if the chemical-shift difference, $\Delta\nu(AB)$, of the two pairs is not the same; thus carbon

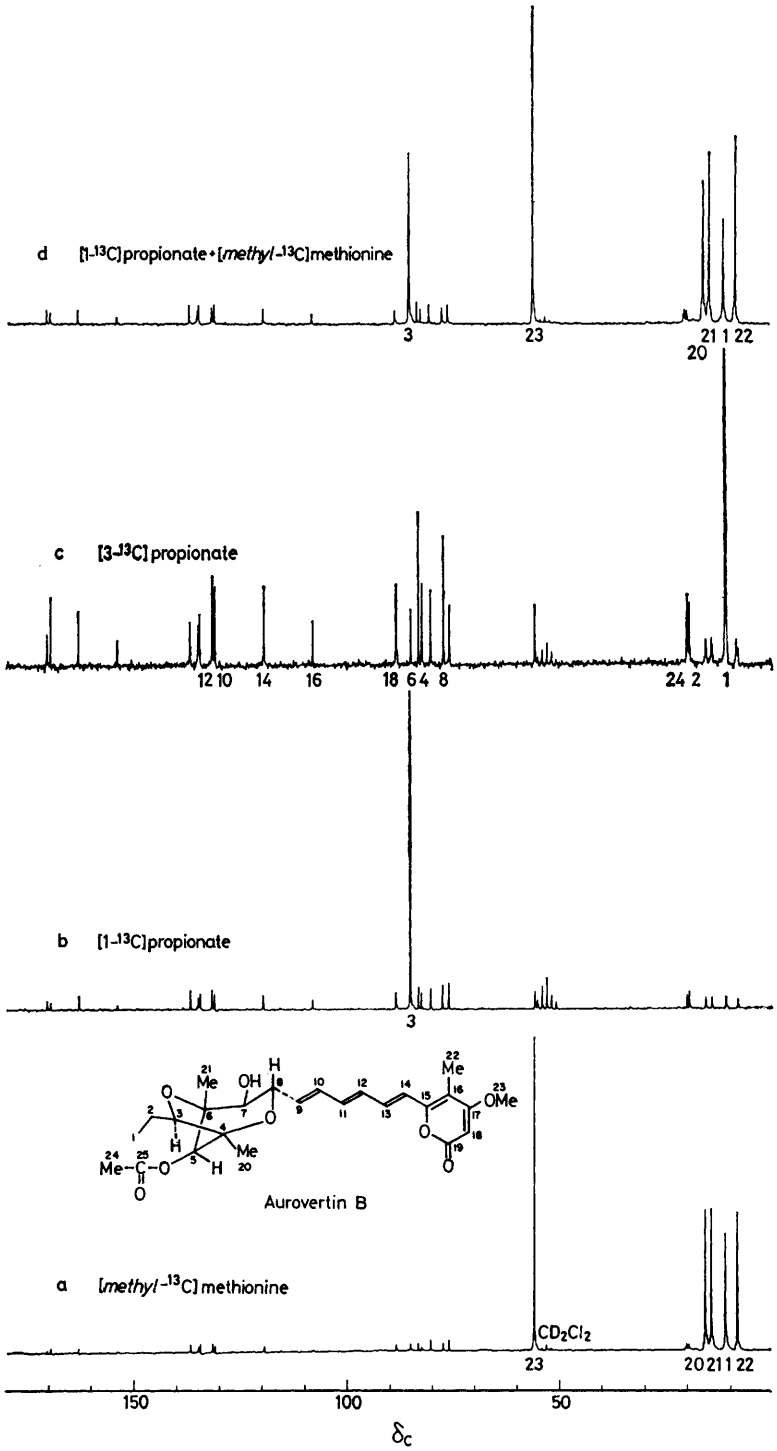


FIGURE 4 The proton-noise-decoupled 25.2-MHz ^{13}C n.m.r. spectra of aurovertin B (1) (solvent CD_2Cl_2; spectral width 5 000; 90° r.f. pulse of 54 μs duration; acquisition time 0.8 s; pulse delay 5 s) derived from (a) [*methyl*-^{13}C]methionine (transients 1 504); (b) [1-^{13}C]propionate (transients 12 099); (c) [3-^{13}C]propionate (transients 1 492); and (d) [1-^{13}C]propionate and [*methyl*-^{13}C]methionine (transients 816).

atoms which are spin-spin coupled can be identified. When the chemical-shift difference is very small the outer lines of the AB system may be almost indistinguishable above the spectral noise level. In this situation the intensity of the inner lines is increased, thereby facilitating the detection of lower enrichment levels. The value of ^1J(CC) is then calculated from equation (2) where x is

$$J(\mathrm{AB}) = \{[\Delta\nu(\mathrm{AB})]^2 - x^2\}/2x \qquad (2)$$

the distance in Hz between the inner lines.

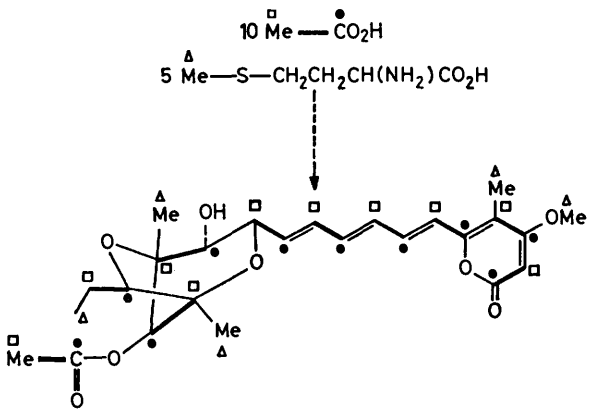
The chemical shifts for C(4) and C(5) in [1,2-^{13}C]acetate-enriched aurovertin D differ by −0.3 and −0.1 Hz, respectively, from the observed natural-abundance resonances. A value of 38.47 Hz for ^1J(CC) is obtained using the value of the chemical-shift difference [Δν(AB) 43.9 Hz] determined from the natural-abundance signals and the distance between the inner lines of the AB system (x 19.9 Hz). The average observed coupling constant is 38.55 Hz.

The general features of the observed AB systems in aurovertin D (and B) derived from [1,2-^{13}C]acetate were used in the assignment of the olefinic carbon atoms C(10)—C(12) and C(14). In the spectrum of aurovertin D (Figure 3) the resonance at δ 119.4 exhibits a (C,C) coupling of 70.4 Hz and forms an AB spin system with the signal at δ 154.0 which has been assigned to C(15) (see above). The resonance at δ 119.4 is therefore assigned to C(14). In an earlier communication,[3] this resonance in aurovertin B and probably also in asteltoxin (4) [5] was erroneously assigned to C(9). In fact, C(9) forms part of an AX spin system. The previously assigned C(13) resonance at δ 135.4 (see above) and the resonance at δ 131.6 constitute a strongly coupled AB spin system and the latter signal is therefore assigned to C(12). The signals of the remaining AB system at δ_A 136.9 and δ_B 131.1 are due to C(11) and C(10), respectively, as the former signal is enhanced in the p.n.d. ^{13}C spectrum of aurovertin D derived from [1-^{13}C]acetate. Conversely, the signal at δ 131.1 is enhanced when [2-^{13}C]acetate is used as the precursor.

The above results, obtained from feeding experiments using ^{13}C-labelled acetate, account for the origin of 20 of the 25 carbon atoms in aurovertin B. The next step was to establish the origin of the remaining five carbon atoms. (2*S*)-Methionine is an excellent source of one-carbon units in nature and in preliminary experiments (2*S*)-[*methyl*-^{14}C]methionine, at different concentration levels, was efficiently incorporated into aurovertin B (absolute incorporation 3—6%). No inhibitory effect was observed up to a concentration of 1 g l^{-1}. Feeding experiments at a level of 375 mg l^{-1} and using 400 ml of medium were chosen in order to obtain sufficient material for ^{13}C n.m.r. spectra, as well as a low dilution value (and thus a satisfactory enrichment). On feeding (2*S*)-[*methyl*-^{14}C]-methionine (specific activity 12.42 μCi mmol^{-1}) to cultures of *C. arbuscula*, aurovertin B with specific activity of 3.52 μCi mmol^{-1} was obtained. On the basis of this dilution value (17.6) for each labelled position (assuming the presence of five labels) it was anticipated that feedings of (2*S*)-[*methyl*-^{13}C]methionine (90 atom %) would give the metabolite with 5.1 atom % ^{13}C at each labelled position, *i.e.* an enrichment factor of 5.6.

In our first feeding experiment with (2*S*)-[*methyl*-^{13}C]methionine as the precursor, the fungus had deteriorated and only aurovertin B was formed in low yield. The p.n.d. ^{13}C n.m.r. spectrum of the metabolite showed enhancement of the signals attributed to C(1), C(20), C(21), C(22), and C(23) (average enrichment factor 5.6). In a subsequent experiment, enrichment factors of 22.7, 25.1, 23.5, 20.5, and 25.5, respectively, were observed for the same signals (Figure 4a). The ^{1}H n.m.r. spectrum of this enriched aurovertin B showed the expected satellites owing to (C,H) coupling. Integration of the signals due to the methoxy-group protons and its satellites indicated the presence of 26.4 atom % ^{13}C at C(23), which corresponds to an enrichment factor of 25.0 and a dilution value of 3.4 for this labelled position. Analysis of the molecular-ion cluster in the mass spectrum of this enriched material indicated the presence of multiple labelled molecules, *viz.* $^{13}C_0$ 49%, $^{13}C_1$, 16.5%, $^{13}C_2$ 9%, $^{13}C_3$ 9.5%, $^{13}C_4$ 10%, and $^{13}C_5$ 6%.

The results obtained from the feeding experiments with ^{13}C-labelled acetate and methionine (see Scheme 1)



SCHEME 1 Biosynthesis of aurovertin B *via* the acetate–polymalonate route.

exclude the formation of aurovertin B *via* pathway 2 which requires that C(1) originates from [1-^{13}C]acetate. The above evidence, however, points to an acetate–polymalonate origin for the metabolite and as such two routes, *viz.* pathways 1 and 3, can be formulated. The two pathways differ in only one respect, in that a starter-acetate unit is either present [pathway 1; C(2) and C(3)] or absent (pathway 3) in the resulting metabolite.

^{13}C-Labelled malonate has been used successfully to detect acetate-starter effects in herquinone,[20] sclerin,[21] and phomazarin.[22] A significant lower extent of labelling of the carbon atoms of the acetate-starter unit allowed its identification in these polyketide-derived metabolites.

Administration of [2-^{13}C]malonate (250 mg) admixed

with unlabelled sodium acetate [22,23] (400 mg) to cultures of *C. arbuscula* gave the aurovertins B and D in good yield. The p.n.d. ^{13}C n.m.r. spectrum of both labelled metabolites showed high enhancement (average enrichment factor 3.5) of the signals due to C(2), C(4), C(6), C(8), C(10), C(12), C(14), C(16), and C(18) and, more important, all were labelled to a similar extent. Partial decarboxylation of the [2-^{13}C]malonate, *i.e.* malonyl–CoA ⟶ acetyl–CoA interconversion, results in the enrichment of C(24), the methyl carbon of the *O*-acetate group, but only to one-eighth of the extent of the other skeletal carbon atoms. In a study using [2-^{13}C]-malonate the malonate-derived carbon atoms in the ansa-chain of rifamycin S were enriched, with no significant enrichment of the carbon atoms of the acetoxy-substituent being observed.[24] As no significant lower extent of enrichment could be demonstrated, it must be concluded that the starter unit of the original polyketide is lost in the biosynthesis of the aurovertins B and D; pathway 1 is thus not a viable proposition.

Additional evidence in favour of pathway 3 was obtained by feeding experiments with [2-^{13}C, 2-$^{2}H_3$]-acetate. The incorporation of this doubly labelled precursor into aurovertin B *via* pathway 1 should result in the retention of two (or less) deuterium atoms at C(2), whereas no deuterium should be present at C(2) if pathway 3 is operative. Experimental evidence has shown that the methyl hydrogens of acetyl–CoA are incorporated into fatty acids in varying degrees; the predominant species (*ca.* 80%) at the terminal methyl group (*i.e.* the methyl group of the starter unit), using [2-^{13}C, 2-$^{2}H_3$]acetate as precursor, is $^{13}C^{2}H_3$.[25,26] Little loss of deuterium from the precursor occurs before the incorporation.[26] A similar result was demonstrated for the starter-acetate unit in griseofulvin.[27,28]

The presence of carbon atoms bearing deuterium atoms can be investigated with ^{13}C n.m.r. spectroscopy. The ^{13}C signals of ^{13}C–^{2}H species appear as triplets shifted to higher field (because of isotope effects) compared with the ^{13}C signal due to the corresponding ^{13}C–^{1}H species in p.n.d. ^{13}C n.m.r. spectra; the latter signal exhibits a concomitant decrease in intensity.[27–29] In a parallel experiment [2-^{13}C]- and [2-^{13}C, 2-$^{2}H_3$]-acetate were separately administered to cultures of *C. arbuscula*. A comparison of the p.n.d. ^{13}C n.m.r. spectra of aurovertin B obtained from each precursor showed no significant difference in the enhancement of the signals of the enriched carbon atoms, except for the intensity of the C(24) methyl signal which was decreased by 85% in the spectrum of the doubly labelled compound (Figure 2d). The corresponding ^{13}C–^{2}H signal, which is obscured by the C(2) resonance, must be a complex multiplet (a heptet for $^{13}C^{2}H_3$ together with a quintet for any $^{13}C^{2}H_2{}^{1}H$ species). ^{2}H N.m.r. spectroscopy is a powerful tool for examining the fate of hydrogen atoms during biosynthesis and gave a direct indication of the presence of deuterium in aurovertin B derived from [2-^{13}C, 2-$^{2}H_3$]-acetate. The ^{2}H n.m.r. spectrum showed a doublet [J(^{13}C, ^{2}H) 19.8 Hz] centred at δ 2.09 which is assigned to the ^{13}C-^{2}H species at C(24). A singlet at δ 2.11 corresponds to the C(24) ^{12}C–^{2}H species.

The evidence outlined above is consistent with the biosynthesis of aurovertin B (and D) *via* pathway 3: a C_{20}-polyketide formed from an acetate-starter unit and nine malonate units is methylated at C_{18}, followed by the loss of the starter-acetate unit, C_{19}–C_{20}, through a retro-Claisen cleavage. The direct derivation of the *O*-acetate moiety from acetyl–CoA would explain the disparity in the enrichment of these carbon atoms compared with the other enriched skeletal carbon atoms in the aurovertins B and D derived from the different ^{13}C-labelled acetates. The low enrichment observed for the same carbon atoms in the [2-^{13}C]malonate-derived compounds is explicable by a limited malonyl–CoA ⟶ acetyl–CoA conversion and is in keeping with a previous observation that malonate does not enrich the carbon atoms of acetoxy-substituents.[24,25]

An alternative explanation, which would also account for the different enrichment levels of the *O*-acetate moiety, is the participation of an oxygen function at C_{15} of the C_{20}-polyketide in the above deacylation; the acetate function at C(5) thus originates as the starter-acetate unit of the C_{20}-polyketide.

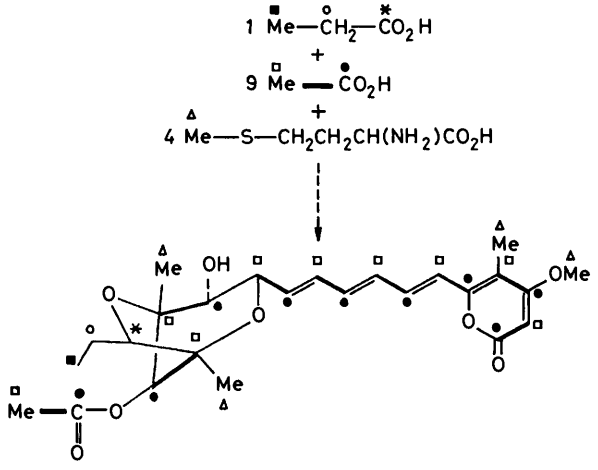
The loss of the starter-acetate unit probably also occurs in the biosynthesis of barnol [7] and stellatin [8] and precludes the necessity of involving a mechanistically undesirable C-alkylation of the terminal methyl group of a polyketide.

An investigation of the one remaining biosynthetic hypothesis, as outlined in pathway 4, required a study of the incorporation of appropriately labelled propionate precursors.

Administration of [1-^{14}C]propionate (400 mg, specific activity 12.0 μCi mmol^{-1}) to growing cultures of *C. arbuscula* gave, in good yield, the aurovertins B (specific activity 2.38 μCi mmol^{-1}) and D (specific activity 1.53 μCi mmol^{-1}). The precursor role of propionate was confirmed by the absolute incorporation value (1.2%). The low dilution values (assuming one labelled position) for the aurovertins B (4.2) and D (7.85) indicate that high enrichment can be obtained in studies with ^{13}C-labelled precursors.

In the p.n.d. ^{13}C n.m.r. spectrum of aurovertin B derived from [1-^{13}C]propionate (Figure 4b), only the signal assigned to C(3) (δ 85.4) was enhanced (enrichment factor 14.1). Mass spectrometry indicated an enrichment of 13.0%. Incorporation of [3-^{13}C]-propionate is more complex: although C(1) is preferentially labelled (enrichment factor of 11.4 for the signal at δ 11.7 significant enrichment is also observed for the [2-^{13}C]acetate-derived carbons (average enrichment factor 1.5) (Figure 4c). The result is explained by the fact that the organism converts (*ca.* 12%) [3-^{13}C]propionate *via* [3-^{13}C]pyruvate into [2-^{13}C]acetate through the loss of the C(1) of propionate. The process, however, does not proceed *via* a symmetrical intermediate (*e.g.* succinate) as the carbon atoms in aurovertin B derived from C(1) of acetate are not enriched.

The results indicate that aurovertin B (and D) is formed from a propionate-starter unit and eight malonate units (see Scheme 2). The occurrence of a pathway involving propionate as a starter unit is unique amongst



SCHEME 2 Biosynthesis of aurovertin B *via* the propionate–polymalonate route.

fungal metabolites. A single report on this role of propionate in the biosynthesis of a fungal metabolite, homo-orsellinic acid, has appeared in the literature, but from the reported evidence it is apparent that propionyl–CoA is substituting for acetyl–CoA as the normal metabolic product is orsellinic acid.[10]

The formation of aurovertin B by two biosynthetic pathways raises the interesting question as to whether both pathways operate simultaneously. This aspect of the biosynthesis was approached as follows. In a parallel experiment, a mixture of (2*S*)-methionine and sodium propionate admixed either with (2*S*)-[*methyl*-^{14}C]methionine or [1-^{14}C]propionate was added to cultures of *C. arbuscula* in order to obtain an indication of the dilution values for ^{13}C-labelled precursor experiments (see Table 2). An interesting aspect is the fact that the dilution values obtained for aurovertin D are consistently higher than those for aurovertin B. The p.n.d. ^{13}C n.m.r. spectrum of aurovertin B obtained when a mixture of (2*S*)-[*methyl*-^{13}C]methionine and [1-^{13}C]propionate was administered to the culture medium (Figure 4d) proved that C(3) (enrichment factor 9.3), derived from propionate, as well as C(1), C(20), C(21), C(22), and C(23) (enrichment factors 10.7, 13.5, 14.0, 14.4, and 13.8, respectively), derived from methionine, were enriched. The above results and especially the significant lower enrichment of C(1) prove that both biosynthetic pathways operate independently. Furthermore, whereas the aurovertins B and D can be formed *via* two pathways, it would appear that aurovertin C(3) is derived only *via* a single pathway using the acetate–polymalonate–methionine route.

TABLE 2

Feeding experiments with ^{14}C-labelled precursors [a]

	Precursor		Product: Aurovertin B			Product: Aurovertin D		
	Amount (mg)	Specific activity (μCi mmol^{-1})	Yield (mg)	Specific activity (μCi mmol^{-1})	Dilution	Yield (mg)	Specific activity (μCi mmol^{-1})	Dilution
Sodium [1-^{14}C]acetate	500	41.00	37	26.13	15.7	40	22.76	18.0
(2*S*)-[*methyl*-^{14}C]Methionine	150	12.42	76	3.52	17.6			
Sodium [1-^{14}C]propionate	400	12.00	61	2.83	4.2	62	1.53	7.8
(2*S*)-[*methyl*-^{14}C]Methionine [b]	150	49.73	46	34.08	7.3	36	18.13	13.7
Sodium [1-^{14}C]propionate [c]	200	24.00	46	3.19	7.5	40	1.87	12.8

[a] Values relative to 0.4 l of culture medium. [b] Admixed with 200 mg sodium propionate. [c] Admixed with 150 mg (2*S*)-methionine

TABLE 3

Feeding experiments with ^{13}C-labelled precursors

Precursor	Atom % ^{13}C	Amount (mg)	Yield (mg): Aurovertin B	Yield (mg): Aurovertin D
Sodium [1-^{13}C]acetate	90.0	400	31	62
Sodium [1-^{13}C]acetate	90.0	400	19	
Sodium [2-^{13}C]acetate	91.3	500	12	
Sodium [2-^{13}C]acetate	90.6	400	16	
Sodium [1,2-^{13}C]acetate	C(1) 93.1; C(2) 92.0	500 [c]	12	
Sodium [1,2-^{13}C]acetate	C(1) 93.1; C(2) 92.0	500 [c]	27	43
(2*S*)-[*methyl*-^{13}C]Methionine	90.0	150	9	
(2*S*)-[*methyl*-^{13}C]Methionine	90.0	150	21	20
[2-^{13}C]Malonic acid [a]	90.6	250	35	42
Sodium [2-^{13}C, 2-$^{2}H_3$]acetate [b]	93.0	400	17	
Sodium [2-^{13}C, 2-$^{2}H_3$]acetate [b]	93.0	400	32	37
Sodium [1-^{13}C]propionate	93.2	400	17	
Sodium [3-^{13}C]propionate	92.4	200	39	57
Sodium [1-^{13}C]propionate + (2*S*)-[*methyl*-^{13}C] Methionine	93.2 90.0	200 150	36	24

[a] Admixed with 400 mg sodium acetate. [b] 99 atom % ^{2}H. [c] Admixed with 500 mg sodium acetate.

The simultaneous independent operation of the two enumerated pathways for the biosynthesis of the aurovertins B and D has, to our knowledge, not been observed for fungal metabolites.

EXPERIMENTAL

Mass spectra were recorded on an A.E.I. MS 9 double-focusing spectrometer. ^{1}H N.m.r. spectra were recorded on a Varian EM-390 continuous-wave instrument (90 MHz) or a Varian XL-200 Fourier-transform (F.T.) spectrometer (200 MHz) (Me_4Si as lock signal and internal reference where applicable). ^{2}H N.m.r. spectra were recorded (for solutions in $CHCl_3$) on a Varian XL-200 F.T. instrument operating at 30.7 MHz. ^{13}C N.m.r. spectra (25.2 MHz) were recorded on a Varian XL-100-15 F.T. spectrometer equipped with a 16K Varian 620i computer and a gated gyrocode decoupler.

Isolation of the Metabolites.—Conical flasks (50 × 500 ml) containing an F14 medium (100 ml) at pH 6.2 [16] were inoculated with a small piece of mycelium of *Calcarisporium arbuscula* NRRL 3705, pre-grown on Czapek agar slopes for 14 d. The mould was grown in a stationary culture at 25 °C in the dark. After 18 d the cultures were filtered and the mycelium macerated in a Waring blender with acetone. The acetone solution was evaporated to dryness.

The chloroform extract of the culture filtrates was concentrated and the residue combined with that obtained from the acetone solution. The residues were partitioned between n-hexane and 90% methanol. The 90% methanol solution was concentrated and the residue partitioned between chloroform and water. The chloroform solution was dried (Na_2SO_4), filtered, and evaporated to dryness. The crude aurovertins were purified by both (i) pressure column chromatography (1 kg cm^{-2}) on silica gel (Merck Type H for t.l.c.) using ethyl acetate as eluant and (ii) preparative t.l.c. on silica gel using ethyl acetate to give the aurovertins B (1) (325 mg) and D (2) (680 mg).[3]

Incorporation of Labelled Precursors.—Preliminary experiments on cultures of *C. arbuscula* showed that aurovertin production commenced on day 7 and reached a maximum 14 d after inoculation of the medium.

To each of four 500-ml flasks containing the 7-day old growth of *C. arbuscula* was added the requisite, labelled precursor every 12 h from day 7 to day 13. The cultures were harvested on day 14 as described. The labelled aurovertins B and D were separated and purified by preparative t.l.c. using ethyl acetate.

A summary of the yields of the aurovertins B and D for the differently labelled precursors is given in Tables 2 and 3.

We thank Dr. C. W. Hesseltine, U.S. Department of Agriculture, Peoria for cultures of *C. arbuscula*, Dr. P. E. Linnett, Shell Research Ltd., Sittingbourne for an authentic sample of aurovertin B, Dr. T. Wirthlin, Varian A.G., Zug for recording ^{2}H n.m.r. spectra, and Dr. A. E. de Jesus for microbiological assistance.

[0/1330 *Received*, 27*th August*, 1980]

REFERENCES

[1] For a preliminary account of part of the early work, see P. S. Steyn, R. Vleggaar, and P. L. Wessels, *J. Chem. Soc., Chem. Commun.*, 1979, 1041.
[2] M. D. Osselton, H. Baum, and R. B. Beechey, *Biochem. Soc. Trans.*, 1974, **2**, 200.
[3] L. J. Mulheirn, R. B. Beechey, D. P. Leworthy, and M. D. Osselton, *J. Chem. Soc., Chem. Commun.*, 1974, 874.
[4] H. A. Lardy, J. L. Connelly, and D. Johnson, *Biochemistry*, 1964, **3**, 1961; A. M. Robertson, R. B. Beechey, C. T. Holloway, and I. G. Knight, *Biochem. J.*, 1967, **104**, 54C; P. E. Linnett, A. D. Mitchel, M. D. Osselton, L. J. Mulheirn, and R. B. Beechey, *ibid.*, 1978, **170**, 503.
[5] G. J. Kruger, P. S. Steyn, R. Vleggaar, and C. J. Rabie, *J. Chem. Soc., Chem. Commun.*, 1979, 441.
[6] M. Satre, personal communication.
[7] J. Better and S. Gatenbeck, *Acta Chem. Scand., Ser. B*, 1977, **31**, 391.
[8] T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1978, 627.
[9] M. Uramoto, L. W. Cary, and M. Tanabe in ' Abstract of the Annual Meeting of the Agricultural Chemical Society of Japan,' Yokohama, 1977, p. 153.
[10] K. Mosbach, *Acta Chem. Scand.*, 1964, **18**, 1591.
[11] T. G. Dekker, K. G. R. Pachler, and P. L. Wessels, *Org. Magn. Reson.*, 1976, **8** 530.
[12] K. G. R. Pachler and P. L. Wessels, *J. Magn. Reson.*, 1973, **12**, 337; *ibid.*, 1977, **28**, 53.
[13] K. G. R. Pachler, P. L. Wessels, J. Dekker, J. J. Dekker, and T. G. Dekker, *Tetrahedron Lett.*, 1976, 3059.
[14] V. A. Chertkov and N. M. Sergeyev, *J. Am. Chem. Soc.*, 1977, **99**, 6750; H. Seel, R. Aydin, and H. Günther, *Z. Naturforsch., Teil B*, 1978, **33**, 353.
[15] J. T. Clerc, E. Pretsch, and S. Sternhell, ' ^{13}C-Kernresonanzspektroskopie,' Akademische Verlagsgesellschaft, Frankfurt am Main, 1973.
[16] C. L. Baldwin, L. C. Weaver, R. N. Brooker, T. N. Jacobsen, C. E. Osborne, and H. A. Nash, *Lloydia*, 1964, **27**, 88.
[17] P. E. Linnett, personal communication.
[18] R. J. Abraham, ' The Analysis of High Resolution NMR Spectra,' Elsevier, Amsterdam, 1971, p. 46.
[19] J. Jokisaari, *Org. Magn. Reson.*, 1978, **11**, 157.
[20] T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1976, 258; *J. Chem. Soc., Perkin Trans. 1*, 1979, 1233.
[21] M. Yamazaki, Y. Maebayashi, and T. Tokoroyama, *Tetrahedron Lett.*, 1977, 489.
[22] A. J. Birch and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 816.
[23] H.-J. Bauch, R. P. Labadie, and E. Leistner, *J. Chem. Soc., Perkin Trans. 1*, 1975, 689.
[24] R. J. White, E. Martinelli, G. G. Gallo, G. Lancini, and P. Beynon, *Nature*, 1973, **243**, 273; A. Karlsson, G. Sartori, and R. J. White, *Eur. J. Biochem.*, 1974, **47**, 251.
[25] R. H. White, *Biochemistry*, 1980, **19**, 9.
[26] A. G. McInnes, J. A. Walter, and J. L. C. Wright, *Tetrahedron Lett.*, 1979, 3245.
[27] Y. Sato, T. Oda, and H. Saitô, *Tetrahedron Lett.*, 1976, 2695.
[28] Y. Sato, T. Oda, E. Miyata, and H. Saitô, *FEBS Lett.*, 1979, **98**, 271.
[29] U. Sankawa, H. Shimada, and K. Yamasaki, *Tetrahedron Lett.*, 1978, 3375.