Carbon-13 Nuclear Magnetic Resonance Assignments of Some Fungal C_{20} Anthraquinones; their Biosynthesis in Relation to that of Aflatoxin B_1

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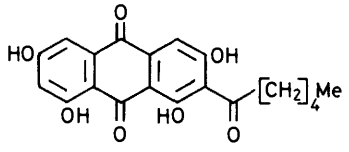
The ^{13}C n.m.r. spectra of four related secondary fungal metabolites, averufin, averufanin, averantin, and 1'-methoxyaverantin, have been assigned. The distributions of ^{13}C label and of the intact acetate units have been determined from the ^{13}C n.m.r. spectra of averufin derived from single and doubly labelled [^{13}C]acetate. These data enabled a prediction of the mode of folding of the single chain acetate–polymalonate-derived polyketide precursor of these C_{20} anthraquinones. A modified pathway which involves an epoxide intermediate for the conversion of averufin into the versicolorins, and, therefore, into the aflatoxins is proposed.

THE enormous biosynthetic potential of the poly-β-ketide route is evidenced by the wide range of fungal aromatic compounds which originate in this way. A survey of these metabolites has revealed that few decaketides are produced; furthermore a sharp cut-out occurs at this C_{20} level.[1]

Studies with *Aspergillus parasiticus* and some of its mutants have established that the anthraquinones norsolorinic acid (1),[2] averufin (2),[3] versiconal acetate,[4] and versicolorin A (3)[5] and the xanthone sterigmatocystin (4)[6] can be converted efficiently into aflatoxin B_1 (5) and are, therefore, implicated as intermediates in aflatoxin biosynthesis. Averufin, an orange pigment is elaborated by *A. versicolor*,[7] *A. ustus*,[8] and *A. parasiticus*.[9] A detailed knowledge of the assembly pattern of acetate units in averufin is a prerequisite for verifying its role as an early precursor in aflatoxin B_1 biosynthesis.[10] From such data, the mode of biogenesis of related decaketide congeners,[11] *e.g.* averantin (6a), averythrin (7), norsolorinic acid (1), averufanin (8), and hydroxyaverufin, can be predicted readily.



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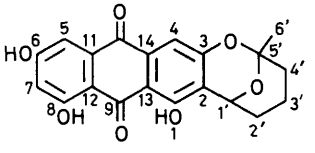

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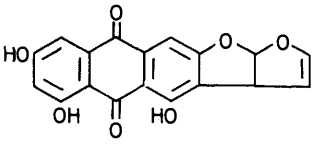

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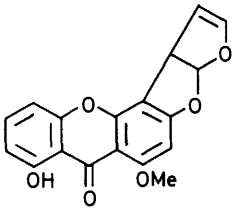

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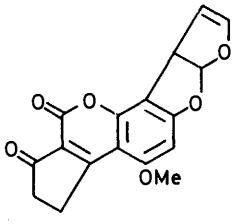

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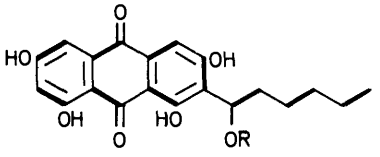
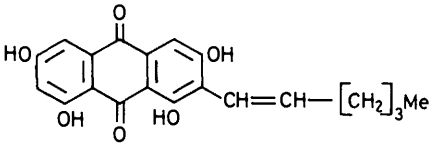


(6) a; R = H b; R = Me



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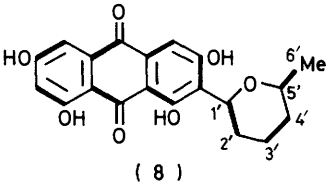

(8)

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The biosynthesis of the anthraquinones is usually discussed in relation to the acetate–polymalonate pathway (*e.g.* for islandicin [12]). The C_{21} anthraquinones rutilantonone and daunomycin,[13] however, contain a nonaketide linked to a propionate fragment. Anthraquinones are frequently transformed into xanthones, *e.g.* ravenelin [14] and tajixanthone.[15] In aflatoxin biosynthesis, averufin is converted *via* a versicolorin and sterigmatocystin into the final product. This conversion involves the loss from averufin of two acetate-derived *methyl* carbon atoms as well as two side-chain carbon atoms. The mode of folding of the decaketide, the putative intermediate, to generate the fused carbocyclic architecture of averufin was deduced by us to be that shown in Figure 1(a) from the pattern of acetate

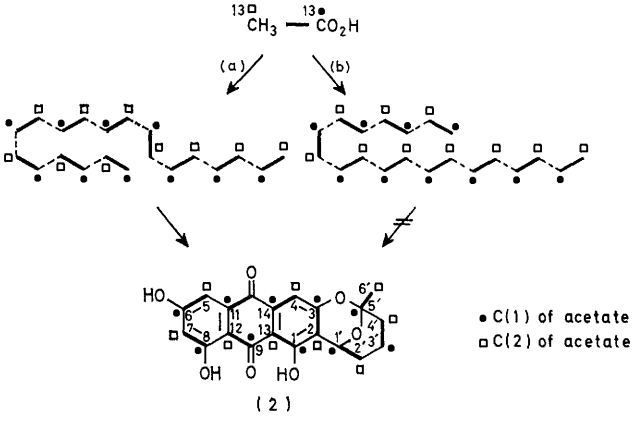

FIGURE 1 Alternative arrangements of acetate units in the biosynthesis of averufin

units in aflatoxin B_1.[16] In contrast, Seto *et al.*[17] predicted the alternative arrangement (b) on the basis of a ^{13}C n.m.r. study of the related sterigmatocystin. Some of the crucial signal assignments were, however, interchanged in their study. Turner [11] favoured arrangement (b) for averufin and the related C_{20} anthraquinones. We now report a study with [1,2-^{13}C]acetate undertaken to clarify this biosynthetic ambiguity.

A mutant of *A. parasiticus*, ATCC 24551, was used; it is impaired in aflatoxin production and accumulates large quantities of averufin in the mycelium. Conidia of this strain were inoculated into the low salts medium and incubated without shaking as surface cultures at 27 °C. In enrichment experiments, the growing organism was treated every 24 h from day 3 to day 6 with [1-^{13}C]acetate, [2-^{13}C]acetate, or [1,2-^{13}C]acetate, and harvested after 10 days. In preliminary experiments on the effect of sodium acetate on the production of averufin by this mutant, we noted a mildly stimulatory effect. Furthermore, averufin was still produced at a level of 1 g of sodium acetate per 100 ml of medium. At this concentration, aflatoxin production was completely inhibited in the parent strain, ATCC 15517.[18]

^{13}C N.m.r. Signal Assignments of Averufin (2), *Averufanin* (8), *Averantin* (6a), *and 1′-Methoxyaverantin* (6b).—^{13}C N.m.r. spectroscopy is particularly useful for distinguishing between different foldings [16,19] of a progenitor poly-β-ketide and for the elucidation of anomalous routes [20-22] which involve molecular rearrangements and fission of the β-ketide or of an intermediate. Its successful application, however, depends upon the correct assignment of ^{13}C n.m.r. signals. We, therefore, present a complete assignment of the ^{13}C n.m.r. spectrum of averufin. The spectra of averufin biosynthesised from singly labelled acetate confirmed the expected distribution of ^{13}C in averufin and provided an independent check on the assignment of the ^{13}C resonances. In addition, the assignments of the related anthraquinones averufanin (8),[23] averantin (6a),[24] and 1′-methoxyaverantin (6b) are presented.

Two previous ^{13}C n.m.r. studies of averufin have been published. Fitzell *et al.*[25] only described a partial assignment, and the assignments of Berger and Jadot [26,27] differ from ours on certain crucial points. In both reported studies, $(CD_3)_2SO$ was used as solvent. We preferred $CDCl_3$–$(CD_3)_2SO$ (1 : 1); this may account for some differences in chemical shifts. Figure 2 shows the proton noise decoupled (p.n.d.) spectrum of averufin obtained at 25.2 MHz. The Table summarises the ^{13}C n.m.r. chemical shifts obtained from this spectrum as well as the coupling constants from an n.O.e. enhanced single frequency spectrum of a sample in which all the hydroxy-protons were replaced by deuterium.

The carbon signal(s) of the methyl group [C(6′), δ 27.5], the three methylene groups (δ 15.6, 27.1, and 35.5), and the single methine group [C(1′), δ 66.3] could be distinguished from their multiplicity in an off-resonance proton decoupled ^{13}C n.m.r. spectrum. By correlating the residual splittings [16,28,29] observed in a

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^{13}C N.m.r. data

Carbon atom	Averufin (2) δ_C [a]	$^1J(^{13}C,H)$	$>^1J(^{13}C,H)$	$^1J(^{13}C,^{13}C)$	Enrichment factors [c] Rapid pulsing [d] 1-^{13}C	Rapid pulsing [d] 2-^{13}C	Long delay [e] 1-^{13}C	Long delay [e] 2-^{13}C	Averufanin (8) δ_C [b]	Averantin (6a) δ_C [b]	1'-Methoxy-averantin (6b) δ_C [b]
1	158.1 Sd		1.8	64.1	1.80	0.80	1.50	0.94	160.7	160.4	162.5
2	115.8 Sq		4.9	65.3	0.74	2.42	1.19	4.41	119.4	121.7	118.9
3	159.9 St		2.7	65.2	2.08	0.92	1.58	0.92	162.5	163.3	163.2
4	107.6 D	168.4		65.1	1.00	4.82	1.00	5.36	109.2	109.1 †	108.9 †
5	109.1 Dd	166.4	4.9	62.1	0.96	5.00	(0.75)	4.73	109.0	108.8 †	108.7 †
6	164.9 St		2.3	63.3	1.78	1.15	1.89	1.02	165.1	164.9	165.2
7	108.0 Dd	162.1	4.3	70.0	0.96	4.57	0.89	4.50	108.0	107.9	108.0
8	164.1 Sd		3.4	70.1	1.56	0.70	1.94	0.94	164.3	164.3	164.3
9	188.7 S			57.7	1.61	1.31	2.00	1.06	188.7	188.7	188.7
10	180.7 St		5.0	53.6	0.38	2.08	1.16	3.98	180.8	180.8	181.0
11	134.6 S			53.8	1.06	1.00	1.77	0.95	134.7	134.6	134.6
12	108.6 St		6.0	58.3	0.57	2.29	(0.52)	3.53	108.5	108.5	108.4
13	108.2 Sd		6.3	64.2	0.47	3.00	(0.65)	3.26	108.3	108.2	108.1
14	132.9 S			65.0	0.76	0.88	1.86	1.06	133.1	132.8	133.2
1′	66.3 Dm	155.9		34.8	3.06	1.13	2.65	1.36	74.6	67.8	76.0
2′	27.1 Tm	128.2		34.6	1.06	5.25	1.06	5.69	29.2	35.7	33.0
3′	15.6 Tm	130.4		31.6	3.29	1.13	3.00	1.32	23.1	24.7	25.1
4′	35.5 Tm	127.1		31.8	1.00	4.77	0.86	4.41	32.5	31.2	31.2
5′	100.9 Sm			48.8	2.10	0.89	2.13	1.11	75.1	22.2	22.0
6′	27.5 Q	128.0		49.0	0.95	5.13	0.79	4.46	21.9	13.9	13.8
OCH_3											56.7

[a] Relative to internal Me_4Si. Capital letters refer to the pattern resulting from directly-bonded protons and small letters to C,H-couplings over more than one bond: S = singlet, D or d = doublet, T or t = triplet, Q or q = quartet, m = multiplet. [b] Relative to internal Me_4Si. Measured from internal $(CD_3)_2SO$ and corrected by using the expression $\delta(Me_4Si) = \delta[(CD_3)_2SO] + 39.7$.[34] [c] Ratios between peak heights of the observed resonances of ^{13}C-enriched and natural-abundance averufin recorded under identical conditions. [d] Pulse spacing 1.6 s. [e] Pulse spacing 40.8 s, normalized (see text).

† May be interchanged.

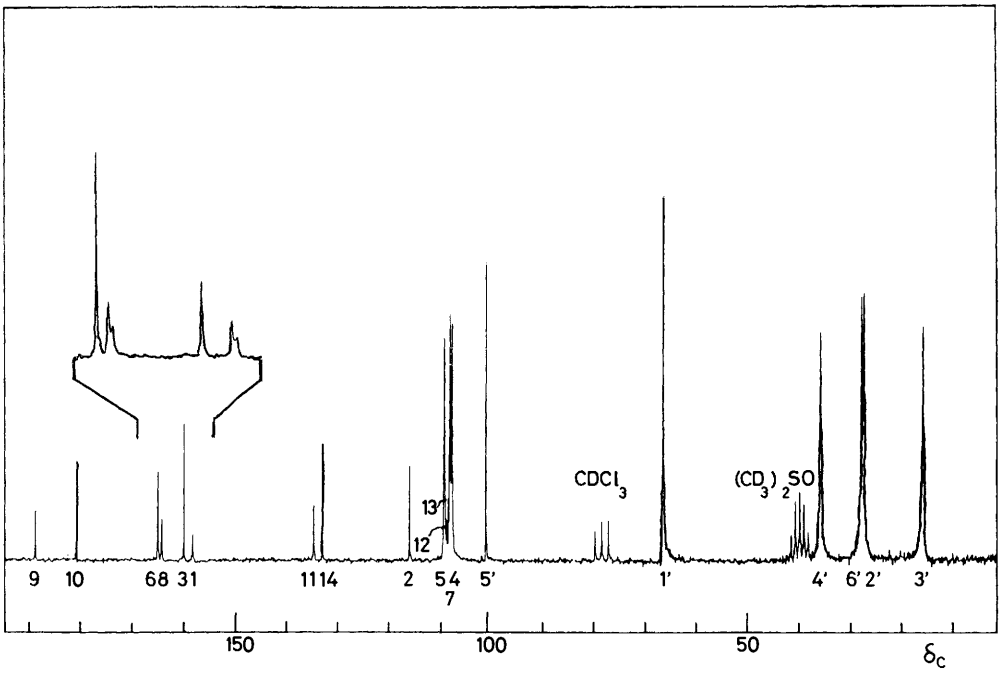

FIGURE 2 Natural-abundance proton noise decoupled 25.2 MHz ^{13}C n.m.r. spectrum of averufin (2); spectral width 5 000 Hz, 90° r. pulse (50 μs); transients 25 K. Insert: after addition of D_2O–H_2O (1 : 1)

series of off-resonance decoupled ^{13}C n.m.r. spectra with the known chemical shifts of the aromatic resonances [23,30] the peak at δ 108.0 was assigned to C(7) and the resonances at δ 107.6 and 109.1 were attributed to C(4) and C(5). The latter resonances were assigned to individual carbon atoms from the splitting patterns observed in n.O.e. enhanced single frequency spectra. The C(5) signal was a doublet of doublets centred at δ 109.1 {^1J[C(5),H(5)] 166.4, ^3J[C(5),H(7)] 4.9 Hz}, and that of C(4) was a doublet at δ 107.6 {^1J[C(4),H(4)] 168.4 Hz}. The C(7) signal appeared as a doublet of doublets, revealing the three-bond carbon–proton coupling to H(5) (^3J 4.3 Hz) in D_2O-exchanged samples. Before deuterium exchange of the hydroxy-protons, a further coupling to the hydrogen-bonded hydroxy-group was in operation, resulting in a broad triplet fine structure of the two legs of the doublet [C(7)] arising from directly bonded carbon–hydrogen coupling. This is in agreement with earlier observations that coupling to hydroxy-protons is only observed when the hydrogen exchange rate is slow, as in hydrogen-bonded species.[29,31-33]

The resonances of the three methylene carbon atoms were assigned on the basis of chemical shift considerations. Shift increments, determined for methyl, methoxy, and phenyl groups in monosubstituted cyclohexanes [34] were added to the appropriate shifts for the carbon atoms in tetrahydropyran to give the following values: C(2′) δ 33.5, C(3′) δ 20.9, C(4′) δ 39.9. These values, though consistently higher than the experimental results, suggested the sequence given in the Table.

To assign the quaternary carbon signals extensive use was made of carbon–hydrogen couplings over more than one bond and of selective proton decoupling experiments. The only aliphatic quaternary carbon atom, C(5′), resonated at δ 100.9 in accordance with chemical shifts of other carbon atoms in similar environments.[16] Irradiation at the frequency of the methyl protons changed the pattern of the resonance at δ 100.9, thus confirming this assignment. The remaining quaternary carbon atoms can be divided into four different groups: (i) the carbonyl carbon atoms [C(9) and C(10); δ 180.7 and 188.7], (ii) the oxygen-bearing aromatic carbon atoms [C(1), C(3), C(6), and C(8); δ 158—165], (iii) the carbon atoms *ortho* to aryloxy-substituents [C(2), C(12), and C(13); δ 108.2, 108.6, and 115.8], and (iv) the carbon atoms [C(11) and C(14)] which resonate at δ 132.9 and 134.6; these two values are similar to that of benzene.

The resonance at δ 180.7 had a triplet fine structure which changed upon irradiation at the frequency of either H(4) or H(5). It was, therefore, assigned to C(10) and the resonance at δ 188.7 to C(9).

In aromatic systems, the carbon–hydrogen coupling constants over more than one bond are usually in the order $^3J > {}^2J > {}^4J$, with values of 7—10, 1—4, and ≤1 Hz, respectively.[34] The resonances of the carbon atoms in group (ii) are, therefore, expected to show fine structure due to two-bond couplings to H(4), H(5), and H(7) and three-bond couplings to H(1′). Two of the resonances appeared as triplets at δ 159.9 and 164.9 and the others as doublets (δ 158.1 and 164.1). Decoupling of the proton H(1′) caused collapse of the resonance at δ 158.1 to a singlet and changed the triplet at δ 159.9 to a doublet. The latter signal also changed to a doublet upon decoupling of H(4). The peaks at δ 158.1 and 159.9 were, therefore, assigned to C(1) and C(3), respectively. The triplet at δ 164.9 with two-bond couplings to H(5) and H(7) was then assigned to C(6) and the doublet at δ 164.1 to C(8). Decoupling of H(7) changed the former signal to a doublet and caused collapse of that at δ 164.1 to a singlet, thus confirming the assignment.

Deuterium-induced isotope shifts have been used to assign various carbon atom resonances.[29,35,36] Amide carbonyl carbon atoms or carbon atoms α to the amide nitrogen experience an isotope shift of 0.1 p.p.m. upon exchange of the amide proton(s) for deuterium.[35] Wehrli [33] has similarly reported an α isotope shift of 0.39 p.p.m. for C(5) in 5-hydroxyflavanones. Addition of H_2O–D_2O (1 : 1) to a compound with exchangeable protons will result in a doubling of specific ^{13}C resonances due to isotope shifts large enough to be resolved (≥0.1 p.p.m.), provided that the hydrogen–deuterium exchange is sufficiently slow. The insert in Figure 2 shows the resonances of the four oxygen-bearing carbon atoms of averufin after addition of H_2O–D_2O (1 : 1). The two resonances assigned to C(1) and C(8) (see before) appeared as doublets with deuterium shifts of 0.25 and 0.21 p.p.m., respectively. The signals of C(6) and C(3) were unaffected. These results confirm our earlier assignments and show that the technique used by Feeney *et al.*[36] and Newmark and Hill [35] can facilitate the assigning of carbon atoms bearing hydrogen-bonded hydroxy-groups and distinguishing them from carbon atoms bearing free hydroxy-groups.

The signal at δ 115.8 changed from a quartet to a triplet upon decoupling of H(1′) and was, therefore, assigned to C(2), the only carbon atom of group (iii) less than four bonds away from H(1′). The triplet at δ 108.6 and the doublet at δ 108.2 showed splittings of the expected magnitude for three-bond carbon–hydrogen couplings in aromatic systems (*ca.* 6 Hz) and were, therefore, assigned to C(12) and C(13), respectively. The assignment was confirmed by selective decoupling of the proton at C(7) which changed the C(12) resonance into a doublet.

The two resonances at δ 134.6 and 132.9 due to

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C(11) and C(14) could not be individually assigned from the natural abundance spectrum of averufin. The ^{13}C n.m.r. spectra of biosynthetic averufin derived from [1,2-^{13}C]acetate provided an independent assignment of these two resonances, *viz.* δ 134.6 and 132.9, to C(11) and C(14), respectively.

The ^{13}C assignments of the carbon atoms of the anthraquinone moieties of averufanin (8), averantin (6a), and 1'-methoxyaverantin (6b) (see Table) are based mainly on a comparison with the assignments of the corresponding carbon atoms in averufin. With the exception of C(1)—C(4) a maximum variation of only 0.4 p.p.m. was observed for the signals of the anthraquinonoid carbon atoms of the four compounds. The assignment of the C(4) and C(5) signals was confirmed by the splitting pattern observed in the n.O.e. enhanced single frequency ^{13}C n.m.r. spectrum of (8). The C(4) signal appeared as a doublet [^1J(C,H) 166.4 Hz] and that of C(5) as a doublet of doublets [^1J(C,H) 167, ^3J(C,H) 3.9 Hz]. Addition of H_2O–D_2O (1 : 1) to a solution of averantin (6a) gave isotope shifts of 0.21 and 0.17 p.p.m., respectively, for the peaks at δ 160.4 [C(1)] and 164.3 [C(8)], respectively. This proved that no interchange of the C(1) and C(3) resonances occurred.

In averantin (6a), the resonances due to the carbon atoms of the side chain were assigned with the aid of the reported chemical shifts of heptan-1-ol.[37] The observed upfield shift (2.7 p.p.m.) upon methylation, as for the C(2') resonance in 1-methoxyaverantin (6b), confirmed the assignment. In the assignment of the side-chain carbon atoms of averufanin (8) the residual splittings [16] in the off-resonance proton decoupled spectrum were correlated with the proton chemical shifts {δ[H(1')] 5.1, δ[H(5')] 3.7}; the data furnished the assignment for these carbon atoms as given in the Table. The high-field resonance observed for averufanin was assigned to C(3') in analogy to the reported chemical shift of δ 24.3 for 2-methyltetrahydropyran.[38] The tentative assignment of the signal at δ 29.2 to C(2') is based on the steric compression effect of the proximate anthraquinone moiety.

Biosynthetic Conclusions.—The p.n.d. spectrum of [1-^{13}C]acetate-derived averufin [Figure 3(b)] showed ten enhanced carbon signals (*ca.* 4% ^{13}C above the natural abundance), *viz.* C(5'), C(3'), C(1'), C(3), C(14), C(11), C(6), C(8), C(9), and C(1). Similarly the spectrum [Figure 3(c)] of the [2-^{13}C]acetate-derived averufin showed that the remaining ten carbon atoms were enriched. These data support an acetate–polymalonate pathway with the labels occupying alternating positions as shown in Figure 1.

The enrichment factors obtained from the ^{13}C n.m.r. spectra of ^{13}C-enriched compounds are frequently quoted in biosynthetic studies [12,14,15] and have been used in proposing a unique biosynthetic pathway for ravenelin.[14] Simpson [39] proposed the recording of the ^{13}C n.m.r. spectra of natural abundance and of ^{13}C-enriched compounds under identical conditions as the only reliable method for obtaining the enrichment factors. In our present study on averufin, we found different enrichment factors when spectra of the natural abundance and specifically enriched compounds were recorded under identical concentration and experimental conditions with either a short pulse spacing (1.6 s) or a long pulse spacing (40.8 s); the latter being equal to three times the longest $T_{1,C}$. The enrichment factors were obtained by dividing the computer printed signal height in the spectra of the enriched compounds by the corresponding signal height in those of natural abundance averufin. The enrichment factors obtained from the spectra shown in Figure 2 were normalized to obtain an average unit ratio for the unenriched carbon atoms. The intensity ratios of the values in parentheses were not used in the normalization. A much wider scatter of enrichment factors was obtained under the conditions of short pulsing. A sound reason for the wide spread in enrichment factors is not apparent; these have no biosynthetic implications in the case of averufin. Enrichment factors, particularly those obtained from rapid pulsing experiments, must be viewed with great caution as these can lead to erroneous conclusions, *e.g.* C(11) and C(14) in averufin did not exhibit the expected enrichment during rapid pulsing.

The p.n.d. spectrum of averufin derived from [1,2-^{13}C]-acetate (Figure 4) had satellite peaks due to ^{13}C–^{13}C spin–spin coupling on all carbon signals. In this spectrum all the carbon signals except those from C(6') showed in addition very small symmetrically placed satellite pairs due to multiply labelled species. Measurement of the couplings to C(12) and C(13) in the normal spectrum was prevented by the low intensities of these peaks and the overlap with resonances from C(4), C(5), and C(7). A ^{13}C n.m.r. spectrum was, therefore, recorded with low power noise decoupling of the protons [40] and using an inversion recovery pulse sequence in which the delay time between the 180 and 90° pulses was such that the proton-bearing aromatic carbon atoms C(4), C(5), and C(7) had no *z*-components just before the 90° pulse was applied. The result is shown as an insert in Figure 4. The measured carbon–carbon couplings are given in the Table. The data confirm all the assignments of the carbon signals for averufin (2). The resonance assigned to C(1') showed a coupling of 34.8 Hz which is also observed at δ 27.1 [C(2')]. The two unassigned carbon resonances at δ 134.6 and 132.9 had C,C-couplings of 53.8 and 65.0 Hz, respectively, whereas C(10) and C(4) showed couplings of 53.6 and 65.1 Hz, respectively. This led to the assignment of C(11) and C(14) signals. The carbon–carbon couplings proved that averufin is derived from the following sequence of intact units: C(6')–C(5'), C(4')–C(3'),

[37] E. Breitmaier and W. Voelter, '^{13}C NMR Spectroscopy,' Verlag Chemie, Weinheim, 1974, p. 138.
[38] A. J. de Hoog, *Org. Magnetic Resonance*, 1974, **6**, 233.
[39] T. J. Simpson, *Chem. Soc. Rev.*, 1975, **4**, 497.
[40] E. Oldfield, R. S. Morton, and A. Allerhand, *J. Biol. Chem.*, 1975, **250**, 6368.

C(2′)–C(1′), C(2)–C(3), C(4)–C(14), C(10)–C(11), C(5)–C(6), C(7)–C(8), C(12)–C(9), and C(13)–C(1).

The foregoing results indicate a single polyketide chain as the most likely progenitor. The labelling pattern obtained from doubly labelled acetate distinguishes clearly between the alternative modes of cyclization and supports our predicted folding mode (a) [16] (Figure 1). The data support the precursor–product relationship of averufin and aflatoxin and provide evidence against the intermediacy of a formal C_4 unit linked to a C_{14} anthraquinone [41] or the involvement of a C_{18} naphthacene precursor.[42] The latter

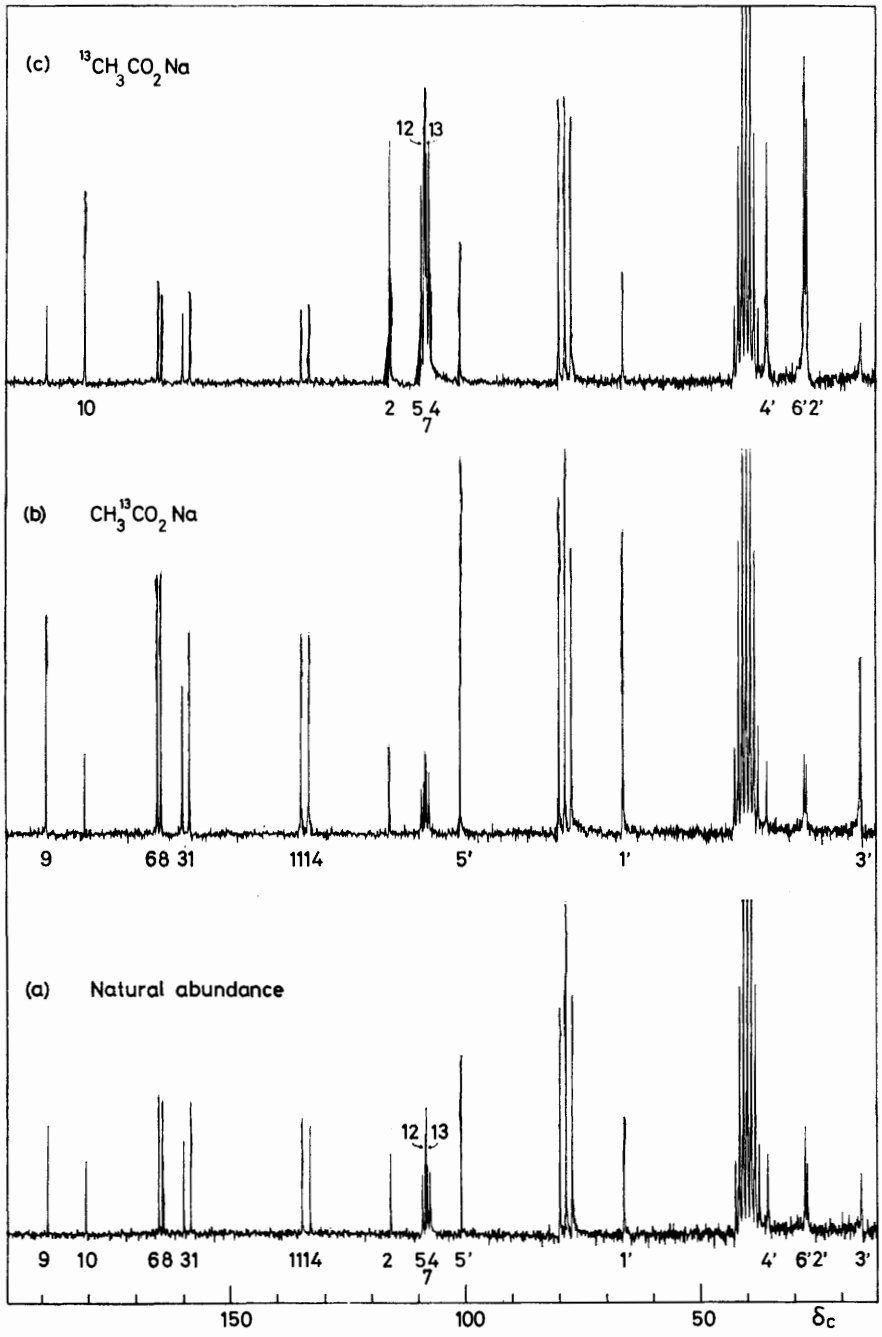


FIGURE 3 Proton noise decoupled 25.2 MHz ^{13}C n.m.r. spectra of averufin (2); spectral width 5 000 Hz; pulse delay 40 s; 90° r.f. pulse (18 μs); transients 1 024: (a) natural abundance, (b) derived from [1-^{13}C]acetate, (c) derived from [2-^{13}C]acetate

[41] J. G. Heathcote, M. F. Dutton, and J. R. Hibbert, *Chem. and Ind.*, 1973, 1027.

[42] M. Biollaz, G. Büchi, and G. N. Milne, *J. Amer. Chem. Soc.*, 1970, **92**, 1035.

mechanism was shown to be unlikely by the very low incorporation of naphthacenequinones and benzanthrones into aflatoxin B_1.[2]

Our biosynthetic studies on aflatoxin B_1 (5), sterigmatocystin (4),[16] and averufin furnish vital information on the acetate–polymalonate pathway of the related C_{20} anthraquinones, *e.g.* norsolorinic acid (1),[20] averantin (6a), 1′-methoxyaverantin (6b), averythrin, hydroxyaverufin, averufanin (8), and nidurufin. The arrangements of intact acetate units in these compounds will be the same as for averufin and are indicated in the drawings of averantin and averufanin. The folding of the suggested polyketide precursor of these compounds as proposed by Roberts [43] is incorrect.

At least three hypothetical mechanisms have been proposed to explain the conversion of averufin into aflatoxin B_1 and the rearrangement which leads to the unique head-to-head linkage in aflatoxin B_1 for the coupling of the dihydrofuran ring and the aromatic system. Thomas [44] proposed a Baeyer–Villiger reaction of an acetylfuran intermediate (9), whereas Kingston *et al.*[45] suggested that the key rearrangement step involves a pinacol-type rearrangement of the open-chain form of nidurufin (10). Tanabe *et al.*[46] recently favoured a Favorskii rearrangement which involved a cyclopropanone intermediate (11). However, we propose the involvement of an epoxide intermediate for this rearrangement (see Scheme). This mechanism consists of ring opening of averufin, followed sequentially by dehydration (as found for averythrin), and epoxidation. Rearrangement of the epoxide [47] will afford the branched benzylic aldehyde. The terminal acetyl group can be removed by a Baeyer–Villiger oxidation as shown, thus

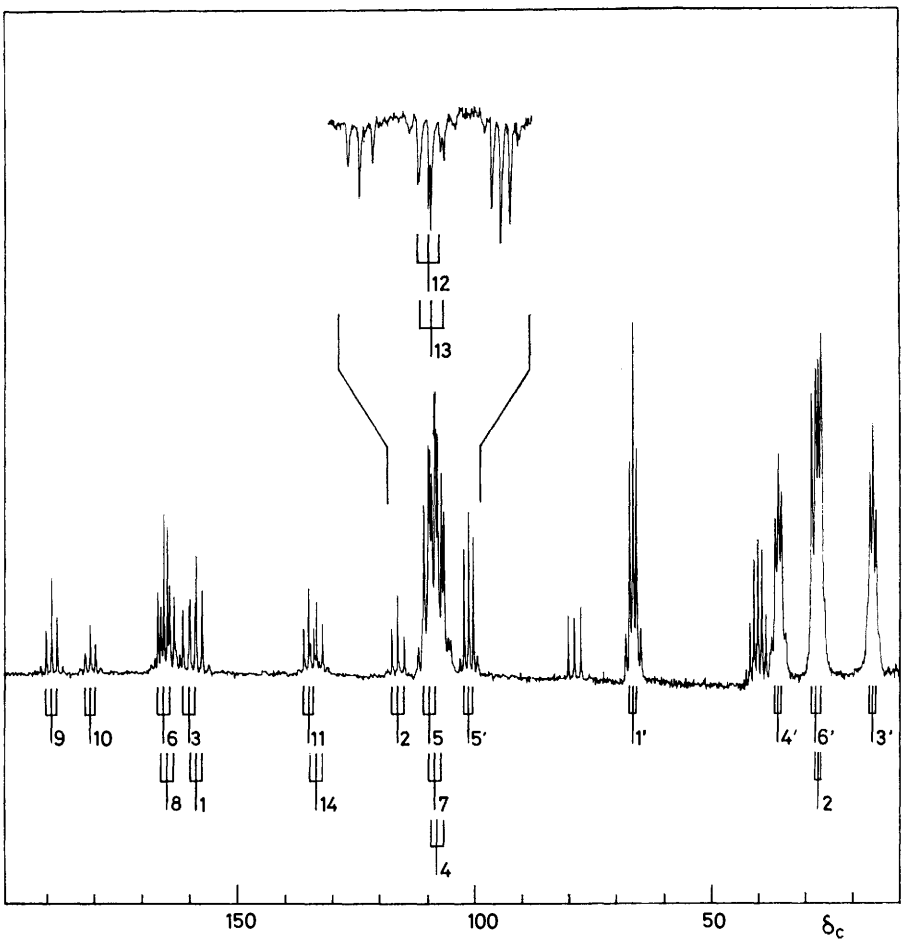
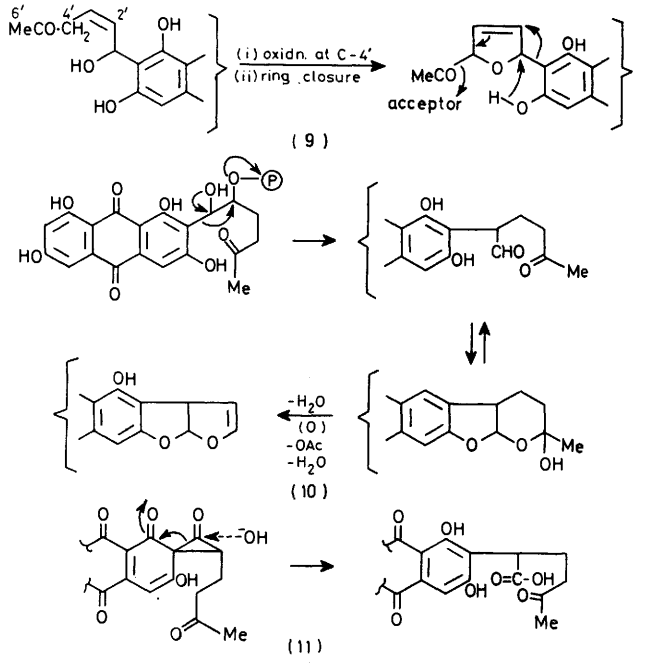


FIGURE 4 Proton noise decoupled 25.2 MHz ^{13}C n.m.r. spectrum of averufin (2) derived from [1,2-^{13}C]acetate; spectral width 5 000 Hz, pulse-delay 1 s; 90° r.f. pulse (50 μs); transients 20 K. Insert: lower power off-resonance proton decoupled partially relaxed spectrum

[43] J. C. Roberts, *Fortschr. Chem. org. Naturstoffe*, 1973, **31**, 119.
[44] R. Thomas, personal communication to M. O. Moss in ' Phytochemical Ecology,' ed. J. B. Harborne, Academic Press, London, 1972, p. 140.
[45] D. G. I. Kingston, P. N. Chen, and J. R. Vercellotti, *Phytochemistry*, 1976, **15**, 1037.
[46] M. Tanabe, M. Uramoto, T. Hamasaki, and L. Cary, *Heterocycles*, 1976, **5**, 355.
[47] A. Rosowsky in ' Heterocyclic Compounds with Three- and Four-membered Rings,' ed. A. Weisberger, Interscience, New York, 1964, p. 246.

leading to a lactol as in versiconal acetate,[4,48] a known intermediate in aflatoxin biosynthesis. This aspect is being investigated.

The structural assignment of averufin has been a topic of much conflicting speculation. Our chemical shift data (Table) together with the established arrangement of intact acetate units in averufin clearly differentiate between the two previously proposed structures.[7,49] These results support the structure proposed



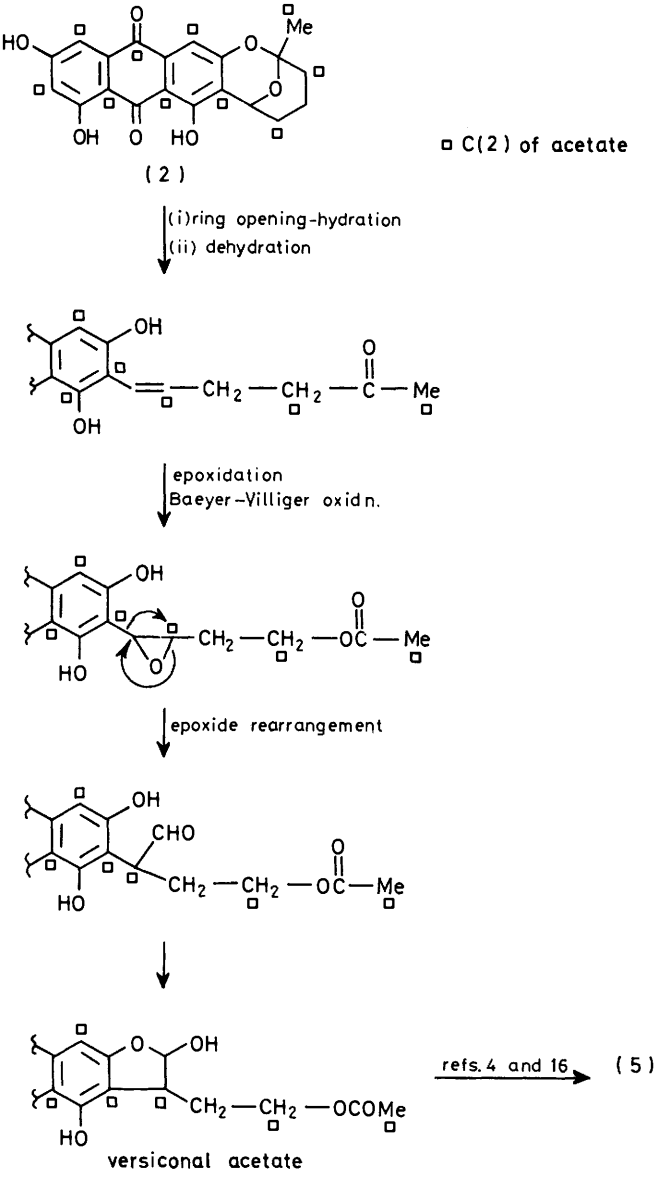
by Holker *et al.*[49] and confirmed by synthesis[30] and *X*-ray crystallography.[50]

EXPERIMENTAL

For instrumental data see ref. 16.

Incorporations of Sodium [1-^{13}C]-, [2-^{13}C]-, *and* [1,2-^{13}C]-*Acetate.*—Preliminary experiments on cultures of *A. parasiticus* (ATCC 24551) grown in static culture on the low salts medium[51] showed that averufin production commenced on the third day after inoculation and reached a maximum on the twelfth day.

To each of fifty 50 ml Erlenmeyer flasks containing the 3-day old growth of *A. parasiticus* on the low salts medium (10 ml) was added [1-^{13}C]-, [2-^{13}C]-, or [1,2-^{13}C]-acetate (250 mg; 90% enriched) every 24 h from day 3 to day 6. The mycelium was harvested after a further 6 days. The pigment was obtained by extraction of the mycelium with chloroform (Soxhlet) followed by solvent partition (hexane–90% methanol). The 90% methanol layer contained the averufin, which was separated by chromatography on silica gel H (500 g) under pressure (0.66 kg cm^{-2}). The column was developed with chloroform–methanol (98 : 2).



SCHEME Proposed structural rearrangement of averufin into aflatoxin B_1

Recrystallization from chloroform–methanol gave averufin, m.p. 290—295° (*ca.* 350 mg).

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[48] R. Cox, F. Churchill, R. J. Cole, and J. W. Dorner, *J. Amer. Chem. Soc.*, 1977, **99**, 3159.
[49] J. S. E. Holker, S. A. Kagal, L. J. Mulheirn, and P. M. White, *Chem. Comm.*, 1966, 911.
[50] Y. Katsube, T. Tsuhikara, N. Tanaka, T. Hamasaki, and Y. Hatsuda, *Bull. Chem. Soc. Japan*, 1972, **45**, 2091.
[51] D. P. H. Hsieh and S. L. Yang, *Appl. Microbiol.*, 1975, **29**, 17.