Carbon-13 Nuclear Magnetic Resonance Assignments and Biosynthesis of Versicolorin A in *Aspergillus parasiticus*

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The ^{13}C n.m.r. spectrum of versicolorin A has 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 versicolorin A derived from singly and doubly labelled [^{13}C]acetate. The biosynthesis of versicolorin A in relation to that of previously determined C_{20} anthraquinones and aflatoxin B_1 is discussed. The absolute configuration of versicolorin A is deduced.

THE biosynthesis of aflatoxin B_1 (1) has been comprehensively studied.[1] A pathway for the biosynthesis of aflatoxin B_1 was proposed to involve the intermediates: acetate→averufin (2)→[versiconal acetate]→versicolorin A (3)→sterigmatocystin (5)→aflatoxin B_1.[2,3] Versicolorin A is, therefore, an important intermediate in the conversion of averufin into sterigmatocystin. A detailed knowledge of the folding pattern of acetate units in versicolorin A was essential to substantiate this hypothesis and to study the rearrangement which leads to the unusual head-to-head linkage of the two acetate units for coupling the bisdihydrofuran ring and the aromatic system, as found in versicolorin A (see later), sterigmatocystin,[1] and the aflatoxins.[1] We now report a study with [1-^{13}C]acetate and [1,2-^{13}C]acetate undertaken to clarify this biosynthetic problem.

The yellow-orange pigment versicolorin A is elaborated by *Aspergillus parasiticus*[4] and *A. versicolor*.[5] A mutant of *A. parasiticus*, 1–11–105 wh–1, was used; it accumulates versicolorin A 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 4 to day 7 with [1-^{13}C]acetate or [1,2-^{13}C]acetate, and was harvested after 8 days.

^{13}C *N.m.r. Assignments of Versicolorin A* (3).—The assignment of the ^{13}C n.m.r. spectrum of versicolorin A is presented in the Table. No previous study of the ^{13}C n.m.r. spectrum of versicolorin A has been reported, although two groups have investigated versicolorin C,[6,7] the racemic 3',4'-dihydro-derivative of versicolorin A. The data reported herein have been obtained for $(CD_3)_2SO$ solutions at 95 °C, because of the low solubility of these metabolites. Versicolorin A is still sparingly soluble at this temperature and only 30 mg dissolves in 1.5 ml of $(CD_3)_2SO$. Elevated temperatures have distinct disadvantages on the sensitivity in pulsed F.T. ^{13}C n.m.r. spectroscopy. The temperature at the sample can vary by ±5 °C (according to the instruction manual of the V-6040 temperature controller) for a 12 mm sample tube configuration on the Varian XL-100-15 spectrometer. This effect gives rise to line broadening with spectral accumulation and a subsequent decrease in the signal-to-noise ratio. Furthermore, the dipole–dipole interaction, which is normally the dominant relaxation mechanism in large organic molecules,[8] decreases with increasing temperature.[9a] The relaxation time (T_1) of the carbon atoms will, therefore, increase. In averufin (2) the T_1 values of the corresponding quaternary carbon atoms at room temperature are in the order of 10—15 s. These two temperature effects have a negative effect on the observation of the quaternary carbon resonances of versicolorin A.

The majority of the anthraquinonoid ^{13}C signals were assigned by comparison with averufin.[3] The signals of atoms C(5)—C(12) show a maximum deviation of 0.3 p.p.m. between averufin and versicolorin A, since changes in the non-aromatic region have only a small effect on the resonance positions of these carbon atoms.[3] The signals of the two carbonyl carbon atoms C(9) and C(10) at δ 188.7 and 180.4 are characteristic for carbonyl

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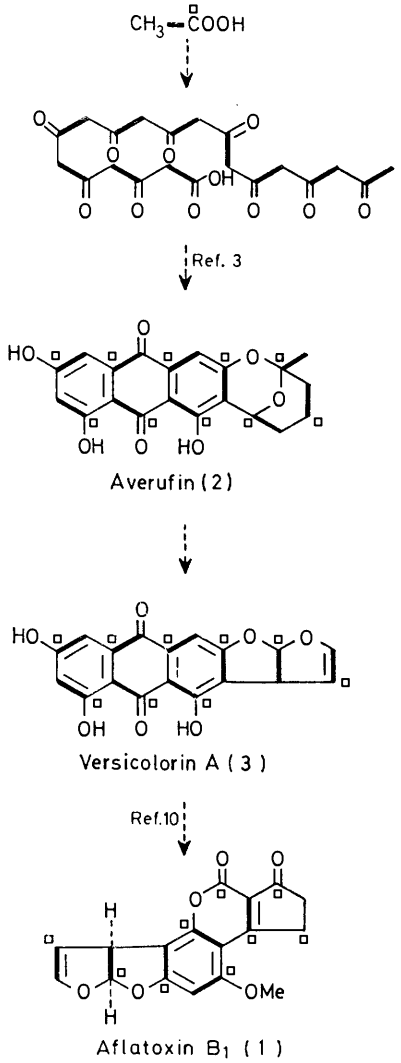
moieties of this type, the corresponding values of averufin being δ 188.7 and 180.7.

The oxygen-bearing aromatic carbon atoms [C(1), C(3), C(6), and C(8)] resonated between δ 158 and 165, and could be assigned as follows. The values for C(6) and C(8) are expected to be identical to those of averufin



SCHEME Biosynthetic scheme

and were assigned to the signals at δ 165.0 and 164.0, respectively. In the previously assigned anthraquinones, including the two studies on versicolorin C,[6,7] C(1) resonates at the highest field amongst this group of oxygen-bearing aromatic carbon atoms. Therefore, the signals at δ 158.2 and 163.4 were assigned to C(1) and C(3), respectively. These two resonances have been unambiguously assigned in the related C_{20} anthraquinones, *e.g.* averufin [3] by addition of D_2O–H_2O (1 : 1) to solutions of these compounds. Upon addition of this mixture to averufin the resonances at δ 158.1 and 164.1 appeared as doublets due to the deuterium isotope effect. These signals were assigned to C(1) and C(8) since C(1)–OH and C(8)–OH can form strong intramolecular hydrogen bonds to the C(9) carbonyl.

The assignments for the signals of C(5), C(7), and C(12) were obtained from the close similarity of these resonances to their counterparts in averufin, and attributed to the signals at δ 108.8, 107.8, and 108.4, respectively. The lower intensity observed during rapid pulsing (pulse delay = 3 s, pulse angle = 90°) of the resonance at δ 108.4 (less than one third of the proton-bearing carbon atoms at δ 107.8 and 108.8) confirmed that this signal represented a quaternary carbon atom.

The three remaining carbon atoms *ortho* to aryloxy-substituents, *viz.* C(2), C(4), and C(13) could be distinguished as follows. Both C(2) and C(13) are α to two oxygen-bearing carbon atoms, whereas C(4) is α to only one such carbon atom. Therefore, the signal due to C(4) should appear at the highest field, *viz.* either at δ 101.6 or at 101.2 (see later). In versicolorin C, [6,7] C(2) resonates at δ 119.7, while C(13) resonates at δ 110.4. In the related anthraquinones,[3] C(2) resonates between δ 115 and 122, consequently the signal at δ 120.4 could be assigned to C(2) and that at δ 111.3 to C(13).

The remaining two anthraquinonoid carbon atoms,

^{13}C N.m.r. data of versicolorin A (3) and 6-*O*-methylversicolorin A (4)

Carbon atom	Versicolorin A (3) δ [a]	Versicolorin A (3) 1-^{13}C Enrichment [b]	Versicolorin A (3) $^1J_{(CC)}$	6-*O*-Methylversicolorin A (4) δ [a]	Δδ *	Averufin (2) δ [a]
1	158.2	1.24	62.7	158.2		158.1
2	120.4	1.06	62.6	120.5		115.8
3	163.4	1.48	~61.5	163.6		159.9
4	101.6	0.99	63.9	101.7		107.6
5	108.8	0.88	62.4	107.5	−1.3	109.1
6	165.0	1.55	62.7	165.7	+0.7	164.9
7	107.8	0.98	69.8	106.4	−1.4	108.0
8	164.0	1.38	69.6	163.9	−0.1	164.1
9	188.7	1.72	58.5	188.8		188.7
10	180.4	*c*	54.6	180.1	−0.3	180.7
11	134.6	0.92	53.8	134.4	−0.2	134.6
12	108.4	0.73	58.8	*c*		108.6
13	111.3	0.89	~61.0	111.3		108.2
14	135.1	2.14	63.1	135.1		132.9
1′	112.8	1.95	33.2	112.9		
2′	47.2	0.71	33.0	47.2		
3′	101.2	2.36	75.0	101.2		
4′	145.3	1.00	74.9	145.4		
OMe				56.0		

[a] Relative to internal $(CH_3)_4Si$. Measured from internal $(CD_3)_2SO$ and corrected by using the expression $\delta[(CH_3)_4Si] = \delta[(CD_3)_2SO] + 39.7$ (J. T. Clerc, E. Pretsch, and S. Sternhell, '^{13}C Kernresonanzspektroskopie,' Akademische Verlagsgesellschaft, Frankfurt am Main, 1973). [b] Ratios between peak heights of the observed resonances of ^{13}C enriched and natural abundance versicolorin A recorded under identical conditions. Pulse spacing = 3 s. [c] Not observed.

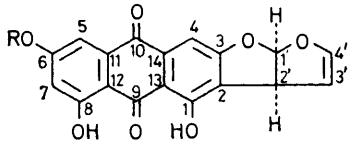
* $\Delta\delta = \delta(4) - \delta(3)$.

C(11) and C(14), resonate at δ 134.6 and 135.1. These signals were assigned by comparison with 6-*O*-methylversicolorin A(4). Methylation of phenol results in an upfield shift for the non-oxygen-bearing carbon atoms.[9b] In the spectrum of (4) (see Table), the signals of C(5), C(7) (*ortho* to the methoxy group), C(8), C(11) (*meta* to the methoxy group), and C(10) were shifted to higher field. The resonance at δ 135.1 was unaffected and assigned to C(14).

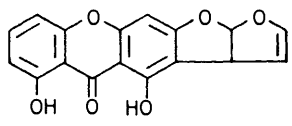
Three of the carbon signals of the bisdihydrofuran

moiety [C(1′), C(2′), and C(4′)] were unambiguously assigned by comparison with the corresponding ^{13}C signals of aflatoxin B_1 and sterigmatocystin.[1] However, C(3′) and C(4) resonate very closely at δ 101.6 and 101.2. Since, in versicolorin A obtained from [1,2-^{13}C]acetate the signals at δ 101.2 and 101.6 have directly bonded carbon–carbon coupling constants of 75 and 63.9 Hz, the carbon atom which resonates at δ 101.2 couples to C(4′). The signals at δ 101.2 and 101.6 were thus assigned to C(3′) and C(4), respectively.

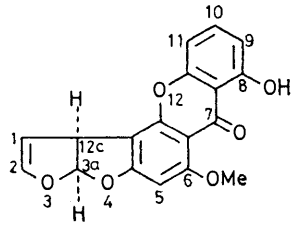
Biosynthetic Conclusions.—The rapidly pulsed (pulse delay 3 s, pulse angle 90°) p.n.d. spectrum of versicolorin



(3) R = H
(4) R = Me



(6)



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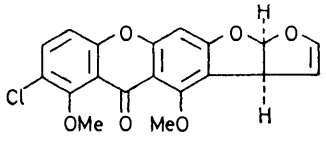

(7)

A derived from [1-^{13}C]acetate showed eight enhanced carbon signals representing C(1), C(3), C(6), C(8), C(9), C(14), C(1′), and C(3′). An acetate–polymalonate pathway with the labels occupying alternating positions in the anthraquinone part of the molecule requires the above eight carbon atoms together with C(11) to be enriched by [1-^{13}C]acetate. Similar problems in the enrichment ratios were encountered during the study of averufin,[3] in this case the problem was circumvented by recording the natural abundance spectrum of averufin and of averufin derived from [1-^{13}C]- and [2-^{13}C]-acetate, respectively with a long pulse delay (40 s). This procedure was, however, impracticable for versicolorin A because of its low solubility, lower enrichment ratio, and the high temperature required for the recordings. The results obtained from versicolorin A derived from [1,2-^{13}C]acetate established that C(11), in fact, was derived from acetate. The anomalous enrichment ratios obtained, especially that for C(11), are due mainly to the long relaxation time of quaternary carbon atoms.

The measured carbon–carbon coupling constants are given in the Table, and proved that versicolorin A is derived from the following intact units: C(4′)–C(3′), 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 carbon–carbon coupling constants fit well with the exception for those of C(1)–C(13) and C(2)–C(3). The observed discrepancy between the values for the pairs of C(1)–C(13) and C(2)–C(3) is likely due to the fact that for C(3) and C(13), one of the legs of the satellites, resulting from the carbon–carbon coupling, appeared as shoulders on the natural abundance peaks of C(6) and C(1′), respectively. This leads to only one arrangement of the nine intact acetate units in versicolorin A on the basis of its polyketide origin. These results confirm our previous conclusions based on the studies of aflatoxin B_1 [1] and averufin.[3] These compounds and versicolorin A are apparently formed from a common linear polyketide progenitor which then cyclizes as shown in the Scheme.

In this biosynthetic sequence versicolorin A is the first member which contains the unusual head-to-head linkage for the coupling of the bisdihydrofuran ring and the aromatic system. This unique linkage can be adequately explained by an epoxide intermediate.[3] These results represent additional proof for the biosynthetic relationship of versicolorin A→sterigmatocystin→aflatoxin B_1. The role of versicolorin A in the aflatoxin biosynthesis was furthermore evidenced by its ready conversion into aflatoxin B_1 by a resting cell culture of wild type *A. parasiticus*.[10]

The recognized intermediacy of versicolorin A in the biogenesis of sterigmatocystin requires that the compounds will have the same absolute configuration. The *cis*-ring fusion of the bisdihydrofuran unit in these compounds is well established. The absolute configuration of the chiral centres in versicolorin A has not yet been determined. The co-existence of a number of structurally and biogenetically related bisdihydrofuran compounds with similar optical rotation in cultures of *A. versicolor*, *viz.* the linear xanthone, sterigmatin (6), $[\alpha]_D$ −377°, [11] the angular xanthone, sterigmatocystin (5), $[\alpha]_D$ −387°, [12] and the linear polyhydroxyanthraquinone, versicolorin A (3), $[\alpha]_D$ −354° [5] suggests that these substances have the same absolute configuration.

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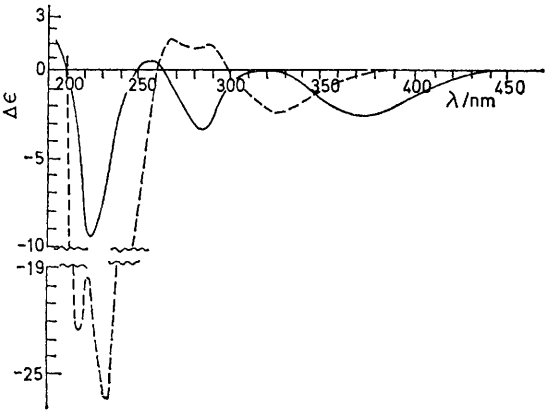
[11] T. Hamasaki, K. Matsui, K. Isono, and Y. Hatsuda, *Agric. Biol. Chem.*, 1973, **37**, 1769.

[12] J. E. Davies, D. Kirkaldy, and J. C. Roberts, *J. Chem. Soc.*, 1960, 2169.

This conclusion was supported by the fact that the main Cotton effects in the c.d. spectrum of (3a*R*, 12c*S*) sterigmatocystin [13] are similar to those of versicolorin A (3) (see Figure) and those of the linear xanthone austocystin A (7), $[\alpha]_D$ −301°.[14] The observed differences in the Cotton effects can be attributed to the dissimilar electronic transitions of anthraquinones and xanthones, *e.g.* versicolorin A (3) had $\lambda_{max.}$(EtOH) 222, 255, 267, 290, 326, and 450 nm (log ε 4.45, 4.13, 4.26, 4.40, 3.83, and



C.d. absorptions for versicolorin A (——) and sterigmatocystin (– – – –) in MeOH at 20 °C

3.85) [5] while sterigmatocystin (5) had $\lambda_{max.}$(EtOH) 205, 233, 246, and 325 nm (log ε 4.40, 4.49, 4.53, and 4.21).[12] In versicolorin A (3) and sterigmatocystin (5) the chiral units have the same orientation to the chromophores, as these compounds are planar except for the dihydrofuran ring which protrudes from this plane; the bond angle C(2)–C(2′)–C(3′) is 113.8° for sterigmatin [15] and that of sterigmatocystin [16] is 114.2°.

Versicolorin A is, therefore, the important precursor of sterigmatocystin although versicolorin C (optically inactive) frequently co-occurs in fungal cultures with the optically active austocystins, sterigmatocystin, and aflatoxins. The biosynthetic pathway leading to versicolorin C apparently diverges at an early stage from the main route leading to sterigmatocystin and the aflatoxins.

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[14] P. S. Steyn and R. Vleggaar, *J.C.S. Perkin I*, 1974, 2250.
[15] K. Fukuyama, T. Tsukihara, Y. Katsube, T. Hamasaki, Y. Hatsuda, N. Tanaka, T. Ashida, and M. Kakudo, *Bull. Chem. Soc. Japan*, 1975, **48**, 1639.

EXPERIMENTAL

For n.m.r. instrumental data see ref. 1. U.v. absorptions were measured for solutions in methanol (Unicam SP 800 spectrometer). I.r. spectra were recorded on a Perkin-Elmer 237 spectrometer for solutions in chloroform. Mass spectra were taken on an A.E.I. MS9 double-focusing spectrometer.

Incorporations of Sodium [1-^{13}C]- *and* [1,2-^{13}C]-*Acetate.*—Preliminary experiments on cultures of *A. parasiticus* (1–11–105 wh–1) grown in static culture on the low salts medium [17] showed that versicolorin A production reached a maximum on the eighth day after inoculation.

To each of ten 500-ml Erlenmeyer flasks containing the 4-day old growth of *A. paraciticus* on the low salts medium (100 ml) was added [1-^{13}C]acetate (250 mg, 90% enriched) every 24 h from day 4 to day 7. The mycelium was harvested after a further 24 h. The pigment was obtained by chloroform–methanol (1 : 1 v/v) extraction of the mycelium in a Soxhlet apparatus, followed by solvent partition (hexane–90% methanol). The 90% methanol layer contained versicolorin A and was separated by chromatography on Silica gel H (250 g) under pressure (1 kg/cm^2). The column was developed with chloroform–methanol (97 : 3). Crystallization from chloroform–methanol gave versicolorin A, m.p. 303—306 °C; yield 80 mg. In a separate experiment [1,2-^{13}C]acetate (250 mg, 90% enriched) and sodium acetate (500 mg) was added as above to yield versicolorin A (40 mg).

6-O-*Methylversicolorin A* (4).—A solution of versicolorin A (50 mg) in chloroform–acetone (1 : 1 v/v) (30 ml) at 20 °C was treated with an excess of ethereal diazomethane in ether (20 ml), and stirred for 10 min. Acetic acid (2 ml) was added and the solvent removed under reduced pressure. The yellow-orange solid was dissolved in chloroform, washed twice with water, dried, and purified by chromatography on SiO_2 gel as above; yield 45 mg; m.p. (MeOH) 231—235 °C, $\lambda_{max.}$(MeOH) 224, 287, 316, and 442 nm (log ε 3.88, 3.86, 3.32, and 3.33, respectively); $\nu_{max.}$($CHCl_3$) 1 616, 1 380, 1 308, and 1 288 cm^{-1} (Found: *m/e* 352.064. $C_{19}H_{12}O_7$ requires M^+ 352.058).

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