Biosynthesis of Versiconal Acetate, Versiconol Acetate, and Versiconol, Metabolites from Cultures of *Aspergillus parasiticus* treated with Dichlorvos. The Role of Versiconal Acetate in Aflatoxin Biosynthesis

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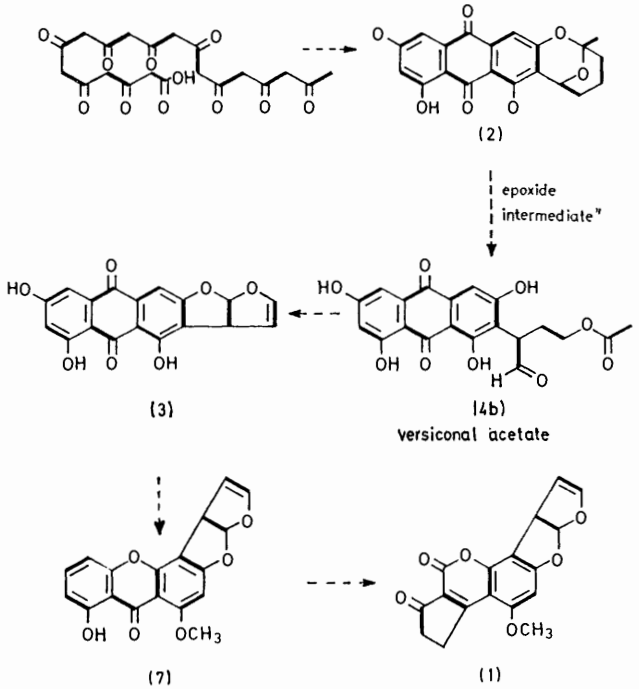
The biosynthesis of versiconal acetate, versiconol acetate, and versiconol, metabolites isolated from cultures of *Aspergillus parasiticus* which had been treated with the insecticide dichlorvos, was studied by ^{13}C n.m.r. spectroscopy. The distribution of ^{13}C label and the arrangement of intact acetate units in the three metabolites derived from [1-^{13}C]- and [1,2-^{13}C]-acetate, respectively is in accord with their origin from a single C_{20} polyketide precursor. The significance of versiconal acetate in the biosynthesis of aflatoxin B_1 is outlined.

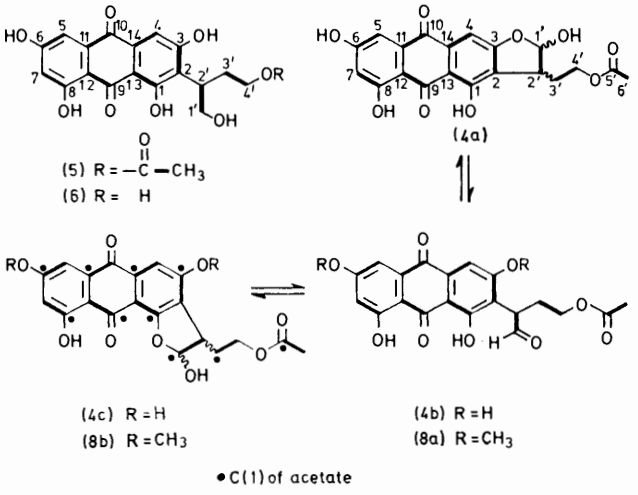
AFLATOXIN B_1 (1), a metabolite from the ubiquitous mould *Aspergillus flavus* as well as *A. parasiticus*, was first characterized[1] in 1963 as the culmination of a search for the causative factor of a mycotoxicosis amongst poultry, identified as Turkey-X disease. The biosynthesis of aflatoxin B_1 has been studied in some detail[2,3] and much conflicting speculation has been cleared up.[4] The use of mutants blocked in aflatoxin B_1 production has helped to identify intermediates in the biosynthesis of the metabolite. During fermentation the intermediates may never leave the enzyme surface and are converted into the end products; in a mutant, however, the obligatory intermediate(s) preceding the deleted enzyme(s) in a pathway may accumulate in sufficient quantity to be characterized. Investigations with mutants of *A. parasiticus* impaired in aflatoxin B_1 production, showed that norsolorinic acid,[5] averufin (2),[6]



SCHEME Proposed biogenetic pathway

and versicolorin A (3)[7] accumulated instead of (1), thus indicating the sequence of biochemical events prior to the final elaboration of aflatoxin B_1.

Treatment of cultures of *A. parasiticus* with dichlorvos [*OO*-dimethyl-*O*-(2,2-dichlorovinyl)phosphate],



an insecticide, caused a serious impairment in the production of aflatoxin B_1 and the concomitant formation of initially a single orange pigment, versiconal acetate (4a—c).[8] Two additional orange pigments, versiconol acetate (5) and versiconol (6) were also isolated.[9] Furthermore, dichlorvos inhibited the conversion of versiconal acetate into aflatoxin B_1, but not that of versicolorin A (3) and sterigmatocystin (7).[10] These findings indicated that (3) and (7) are beyond the enzymatic step inhibited by dichlorvos. Versiconal acetate (4a—c) is therefore a pivotal intermediate in the biosynthetic sequence.

With the knowledge of the structure of versiconal acetate and its two co-metabolites at hand it was decided to undertake a biosynthetic study using [1-^{13}C]- and [1,2-^{13}C]-acetate in order to clarify the intriguing rearrangement in the biotransformation of the C_6 sidechain of averufin (2) into the C_4 bisdihydrofuran moiety of *e.g.* (3).[11,12]

A yeast extract–sucrose (YES) medium was inoculated with spores of *A. parasiticus* (ATCC 15517) and incubated without shaking as surface cultures at 27°. After 24 h dichlorvos was added to the medium to give a concentration of 20 p.p.m. In enrichment experiments the growing organism was pulsed every 24 h, starting 24 h

after inoculation, with the requisite precursor and harvested on day 7. The metabolites were isolated from the mycelium in good yield in the usual way (*cf.* Experimental section).

^{13}C N.m.r. spectroscopy is particularly useful for distinguishing between different foldings[4,11,13] of the progenitor poly-β-ketide and for the elucidation of anomalous routes[14-16] which involve molecular rearrangements and fission of the β-ketide or of an intermediate.

The complete assignment of the ^{13}C n.m.r. spectra of versiconal acetate and its derivatives as well as the two co-metabolites has been accomplished (see preceding paper).[9] From both the ^1H and ^{13}C n.m.r. data it was clear that versiconal acetate exists in solution as an equilibrium mixture of the three isomers (4a—c); the equilibrium is solvent dependent.[9] This phenomenon would lead to an increase in the number and complexity of the signals in the ^{13}C n.m.r. spectrum especially in the case of the metabolite derived from doubly labelled precursor (see below). To surmount this problem it was deemed necessary to protect the 3-hydroxy-group in versiconal acetate derived from singly and doubly labelled ^{13}C acetate. Methylation with an ethereal solution of diazomethane gave 3,6-*OO*-dimethylversiconal acetate which is present in $CDCl_3$ solution as the branched benzylic aldehyde (8a) and in $(CD_3)_2SO$ solution as the angular hemiacetal (8b).[9]

The proton-noise-decoupled (p.n.d.) 25.2 MHz ^{13}C n.m.r. spectrum of 3,6-*OO*-dimethylversiconal acetate (8a), derived from [1-^{13}C]acetate was recorded in $CDCl_3$. The presence of ten enhanced carbon signals (enriched 3.0 fold above the natural abundance level as determined by mass spectrometry) *viz.* C(1), C(3), C(6), C(8), C(9), C(11), C(14), C(1′), C(3′), and C(5′) supported an acetate–polymalonate pathway for the biosynthesis of versiconal acetate.

Although an equilibrium exists between the linear hemiacetal (4a) and the branched benzylic aldehyde (4b) in both $(CD_3)_2CO$ solution and in $CDCl_3$–$(CD_3)_2SO$ (4 : 1) the ^{13}C resonances were relatively sharp. However, even in these solvents the signals due to the anthraquinone carbon atoms C(1)–C(4), C(13), and C(14) and the aliphatic carbon atoms C(2′) and C(3′) were broadened while the resonance due to C(1′) was not detected. The measured directly bonded carbon–carbon coupling constants, ^1J(C,C) obtained from the p.n.d. ^{13}C spectrum of versiconal acetate derived from [1,2-^{13}C]acetate in the latter two solvents are given in the Table. Some of the ^1J(C,C) values which could not be accurately determined due to broadening of the appropriate signals are enclosed by brackets in the Table. The data show unambiguously that C(5)–C(6), C(7)–C(8), C(9)–C(12), C(10)–C(11), and C(5′)–C(6′) are derived from intact acetate units. Neither the folding pattern of the original β-ketide nor a biosynthetic pathway could be formulated on the basis of the above results.

The p.n.d. ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived 3,6-*OO*-dimethylversiconal acetate (8a) in $CDCl_3$ is shown in the Figure. The measured ^1J(C,C) values are given in the Table and prove unambiguously that the compound (and thus versiconal acetate) is derived

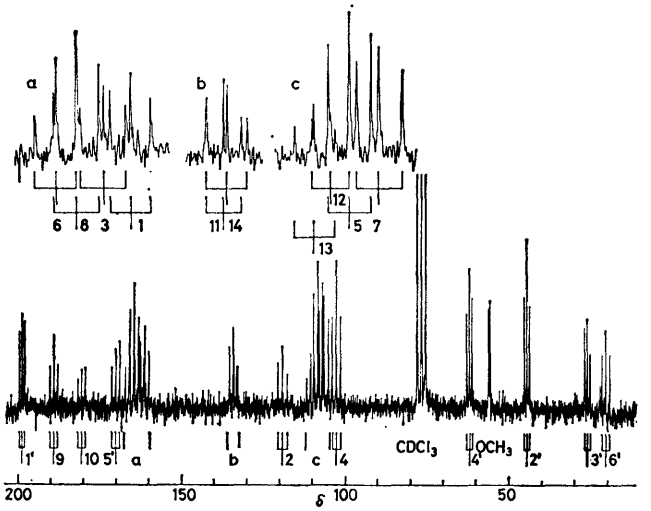
^{13}C Chemical shifts [δ (p.p.m.)] and directly-bonded (C,C) coupling constants [^1J(C,C)/Hz] of [1,2-^{13}C]acetate-derived versiconal acetate (4a—c), 3,6-*OO*-dimethyl versiconal acetate (8a, b), versiconol acetate (5), and versiconol (6)

	Versiconal acetate				3,6-*OO*-Dimethylversiconal acetate					Versiconol (6)		Versiconol acetate (5)	
	$CDCl_3$–$(CD_3)_2SO$ (4 : 1)		$(CD_3)_2CO$		(8a) $CDCl_3$		[1-^{13}C]-acetate ¶	(8b) $(CD_3)_2SO$		$CDCl_3$–$(CD_3)_2SO$ (1 : 1)		$CDCl_3$–$(CD_3)_2SO$ 1 : 1	
Carbon atom	δ †	^1J(C,C)	δ ‡	^1J(C,C)	δ ‡	^1J(C,C)		δ †	^1J(C,C)	δ †	^1J(C,C)	δ †	^1J(C,C)
1	159.9	(63)§	161.1	id.	161.7 *	62.6	2.67	160.1	70.0	162.9 *	62.2	163.0 *	61.5
2	120.2	(62)	121.9	(61)	119.5	71.1	0.71	123.9	(77)	122.9	68.5	121.4	68.7
3	164.1	(62)	165.8	(63)	163.4 *	70.6	2.17	160.1	76.3	163.1 *	68.7	163.3 *	68.9
4	103.2	(63)	103.8	(65)	103.0	64.3	1.10	103.8	66.4	108.9	63.0	108.6	63.0
5	109.2	62.5	109.6	63.0	108.6	66.0	0.91	106.5	66.0	108.6	62.4	108.6	63.0
6	165.0	63.4	165.9	63.1	166.2 *	65.3	2.57	164.8	66.3	164.7 *	62.9	164.8 *	62.9
7	108.0	70.1	108.9	70.2	106.0	70.6	0.92	106.5	70.2	107.9	70.2	107.9	70.1
8	164.5	70.0	165.8	70.5	165.0 *	70.0	1.76	164.2	69.5	164.2 *	69.6	164.2 *	69.8
9	189.2	58.7	190.9	58.1	189.6 *	58.0	2.56	184.9	(57)	188.8 *	58.6	188.8 *	58.5
10	181.0	53.7	181.6	53.9	181.0	54.8	0.99	180.9	53.9	181.0	53.0	181.0	53.4
11	134.5	53.7	136.3	53.9	134.5 *	54.4	2.00	134.0	53.8	134.7 *	53.6	134.6 *	53.5
12	108.4	59.0	n.o.	n.o.	109.7	57.4	1.21	110.1	57.2	108.6	58.0	108.6	58.6
13	110.2	id.	110.1	id.	110.7	62.6	0.51	110.4	(70)	108.1	61.7	108.1	62.0
14	135.1	(64)	136.7	(65)	134.3 *	63.9	1.41	135.0	65.8	132.1 *	64.0	132.2 *	64.1
1′	n.o.	n.o.	n.o.	n.o.	199.6 *	38.5	2.41	108.2	(42)	63.2 *	38.4	62.9 *	37.7
2′	44.1	(35)	45.5	(41)	44.8	38.7	1.15	44.0	(40)	34.9	37.2	35.0	37.6
3′	28.2	id.	~28	obs.	26.5 *	39.1	2.96	29.1	(37)	32.5 *	37.8	27.8 *	38.9
4′	61.8	38.7	62.7	38.7	62.3	38.8	0.92	61.7	38.5	60.1	37.6	62.9	37.7
5′	170.1	58.8	170.7	59.3	170.6 *	60.0	2.31	170.2	58.9			169.8 *	59.6
6′	20.6	59.8	20.8	59.5	20.8	59.5	1.02	20.8	59.0			20.8	59.8
3-OCH_3					56.4		0.88	56.3					
6-OCH_3					56.1		1.15	56.1					

* Carbon atoms derived from [1-^{13}C]acetate. † Relative to internal $(CH_3)_4Si$. Measured from internal $(CD_3)_2SO$ and corrected by using the expression $\delta[(CH_3)_4Si] = \delta[(CD_3)_2SO + 39.7]$. ‡ Relative to internal $(CH_3)_4Si$. § The values in parentheses were obtained from broad peaks. ¶ Enrichment factors, pulse spacing 40 s, normalized. n.o. = not observed, obs. = obscured, id. = indeterminable.

from ten intact acetate units, *viz.* C(1)–C(13), C(2)–C(3), C(4)–C(14), C(5)–C(6), C(7)–C(8), C(9)–C(12), C(10)–C(11), C(1′)–C(2′), C(3′)–C(4′), and C(5′)–C(6′). In $(CD_3)_2SO$ solution the compound is present as the angular hemiacetal (8b). Some of the resonances are broadened with the result that less accurate ^1J(C,C) values were measured (see values in brackets in Table). The observed ^1J(C,C) values however, still attest to the presence of ten intact acetate units in (8b). The ^1J(C,C) values for C(1)–C(13), C(2)–C(3), C(4)–C(14), and C(10)–C(11) were also instrumental in the assignment of the carbon atoms C(1) (δ 160.1), C(3) (δ 160.1), C(14) (δ 135.0), and C(11) (δ 134.0) in unlabelled 3,6-*OO*-dimethylversiconal acetate (8b) in $(CD_3)_2SO$ solution.[9]

It is of interest to note that although the resonance in the p.n.d. ^{13}C n.m.r. spectrum of versiconal acetate due



Proton-noise-decoupled 25.2 MHz ^{13}C n.m.r. spectrum of [1,2-^{13}C]acetate-derived 3,6-*OO*-dimethylversiconal acetate (8a) in $CDCl_3$ (spectral width 5 000 Hz; 70° radiofrequency pulse of 40 μs duration, pulse delay 10 s, transients 3113). Inserts: scale × 5

to the C(1′) atom of the intact C(1′)–C(2′) acetate unit is never observed, a directly bonded (C,C) coupling is present for C(2′) (see Table).

The measured directly bonded (C,C) coupling constants observed in the p.n.d. ^{13}C n.m.r. spectra of both versiconol acetate (5) and versiconol (6) (Table) derived from [1,2-^{13}C]acetate were compatible with their biogenetic origin from ten and nine intact acetate units, respectively arranged in a similar fashion as for versiconal acetate.

The ^1J(C,C) values for the anthraquinonoid carbon atoms C(5)—(12) as found in averufin (2), versicolorin A (3), versiconal acetate (4a—c), versiconol acetate (5), and versiconol (6) are consistent. Methylation of the 6-hydroxy-group of (4a—c) resulted in a *ca.* 3 Hz increase in ^1J[C(5),C(6)]; a similar effect was observed for ^1J[C(5),C(2)] in the acetylation of 1-hydroxynaphthalene.[17] These changes in ^1J(C,C) values can be employed to distinguish between similar couplings in a specific compound.

In a preliminary study on the production of versiconal acetate by cultures of *A. parasiticus* we found that initially only versiconal acetate is formed and only after four days after dichlorvos addition did the production of first versiconol acetate and then versiconol commence. The three metabolites were all isolated as the racemates as none exhibited any c.d. activity or optical rotation. Optically active (—)-versiconol has, however, been isolated from cultures of *A. versicolor*.[18]

The above study on the [1,2-^{13}C]acetate-derived versiconal acetate and its two co-metabolites together with our previous biosynthetic studies on averufin (2),[11] versicolorin A (3),[12] as well as sterigmatocystin (7) and aflatoxin B_1 (1) [4] furnish vital information regarding the biosynthetic conversion of the original C_{20} polyketide precursor into aflatoxin B_1 and its congeners (see Scheme). The product–precursor relationship between aflatoxin B_1 and norsolorinic acid,[19] averufin (2),[20] versicolorin A (3),[21] and sterigmatocystin (7),[22] respectively has been demonstrated.

Of major importance in the biosynthetic pathway is the intriguing rearrangement of the C_6 side-chain of *e.g.* averufin (2) into the C_4 bisdihydrofuran moiety with a head-to-head linkage to the aromatic system as in *e.g.* versicolorin A (3). We proposed [11] a mechanism which involves the ring-opening of averufin, followed sequentially by dehydration and epoxidation. Rearrangement of the epoxide [23] affords the branched benzylic aldehyde. The terminal acetyl group can be removed by a Baeyer–Villiger oxidation, an intramolecular process, thus leading to versiconal acetate a known intermediate in aflatoxin biosynthesis.[8] Our explicit results on the structure of versiconal acetate and the rearrangement of intact acetate units in this compound are consistent with the proposed mechanism. The presence of the intact C(5′)–C(6′) acetate unit in versiconal acetate together with the enrichment factor(s) (calculated as for averufin [11]) obtained for C(5′) in the [1-^{13}C]acetate-derived 3,6-*OO*-dimethylversiconal acetate (see Table) and for C(5′) and C(6′) in [1,2-^{13}C]acetate-derived (8a) (see Figure) are in agreement with the origin of the *O*-acetate group as outlined.

EXPERIMENTAL

For instrumental data see ref. 9.

Incorporations of Sodium [1-^{13}C]- *and* [1,2-^{13}C]-*Acetate.*—Conical flasks (15 × 500 ml) containing YES medium (2% yeast extract–20% sucrose; 100 ml) were inoculated with a spore suspension of *A. parasiticus* (ATCC 15517) (prepared from oatmeal–agar slopes) in sterile water. The mould was grown in stationary culture at 27°. After 24 h an ethanol solution of dichlorvos (0.2%, 1 ml) was added to each flask. At the same time additions of either [1-^{13}C]acetate (1 g, 90% enriched) or [1,2-^{13}C]acetate [1.0 g (90% enriched) diluted with 2.0 g of ' cold ' sodium acetate] were commenced and repeated three times every 24 h.

On day 7 the cultures were filtered and the mycelium treated as described previously [9] to give either [1-^{13}C]- or [1,2-^{13}C]-acetate-derived *versiconal acetate* (140 mg; 133

mg), *versiconol acetate* (35 mg; 40 mg), and *versiconol* (26 mg; 17 mg).

We thank E. Kwant for microbiological assistance.

[7/2232 *Received*, 20*th December*, 1977]

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