Structure and Carbon-13 Nuclear Magnetic Resonance Assignments of Versiconal Acetate, Versiconol Acetate, and Versiconol, Metabolites from Cultures of *Aspergillus parasiticus* treated with Dichlorvos

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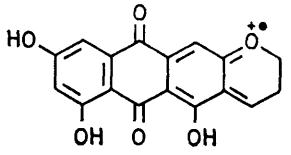
The structure of versiconal acetate, a metabolite elaborated by cultures of *Aspergillus parasiticus* treated with dichlorvos was investigated. ^1H and ^{13}C n.m.r. studies of versiconal acetate and its 3,6-*OO*-dimethyl derivative indicated that versiconal acetate exists in polar solvents such as $(CD_3)_2SO$ as an equilibrium mixture of the isomers (6a—c). In $(CD_3)_2CO$ solution the angular hemiacetal form (6c) was absent. Similarly, 3,6-*OO*-dimethylversiconal acetate exists in $(CD_3)_2SO$ solution as the angular hemiacetal (7b) whereas in $CDCl_3$ solution the compound is present as the branched benzylic aldehyde (7a). Two minor metabolites were identified as versiconol acetate (11) and versiconol (12).

THE testing of isotopically labelled potential precursors for incorporation into aflatoxin B_1 (1), a metabolite of *Aspergillus flavus* and *Aspergillus parasiticus*, established its anomalous acetate–polymalonate origin.[1,2] Treatment of cultures of *A. parasiticus* with dichlorvos [*OO*-dimethyl-*O*-(2,2-dichlorovinyl)phosphate] resulted in reduced aflatoxigenicity and the formation of an orange-red pigment, versiconal acetate.[3] Knowledge of the structure of versiconal acetate and related compounds, might clarify the intriguing rearrangement in the biotransformation of the C_6 side-chain of averufin (2a) into the C_4 bisdihydrofuran grouping of versicolorin A (3) and of sterigmatocystin (4).

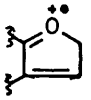
Schroeder *et al.*[4] formulated versiconal acetate as a branched benzylic aldehyde (5) on some physicochemical data. However, Cox *et al.*[5] concluded mainly on the basis of ^{13}C n.m.r. data that in $(CD_3)_2SO$ solution an equilibrium mixture of (6a and c) was established. The authors[5] based their structural assignments on the reputed presence of a ^{13}C signal at δ 113.0 p.p.m., attributed to a five-membered hemiacetal carbon atom as well as the absence of a signal due to an aldehyde carbon atom. However, the lack of any discernible ^{13}C resonance between δ 120.0 and 110.2 p.p.m. in our recordings of versiconal acetate in solutions of $(CD_3)_2SO$, $(CD_3)_2CO$, or a 1 : 1 $CDCl_3$–$(CD_3)_2SO$ prompted us to initiate a new structural investigation.

An aflatoxigenic strain of *A. parasiticus* (ATCC 15517) was grown on a yeast extract–sucrose (YES) medium and treated with dichlorvos. Three pigments were isolated from the mycelium and identified as versiconal acetate (6a—c), versiconol acetate (11), and versiconol (12). Versiconal acetate and versiconol were identified by direct comparison with authentic samples. Versiconol acetate is a new metabolite.

Versiconal Acetate.—Versiconal acetate, $C_{20}H_{16}O_9$, m.p. 234—236°, had u.v. and i.r. data which indicated the 1,3,6,8-tetrahydroxyanthraquinone structure. The electron impact mass spectrum of versiconal acetate lacked the expected M^+, but a fragment was found at *m/e* 382 ($M^+ - H_2O$) due to the ready loss of water from the molecular ion. In addition, strong peaks were observed at *m/e* 340 ($M^+ - CH_3COOH$, $C_{18}H_{12}O_7$), 322, 312, 311, and 297. The peaks at *m/e* 311 and 297 were attributed to fragments *a* and *b*, which also feature



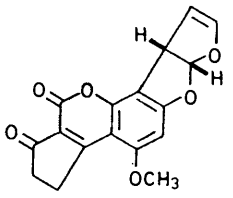
a
m/e 311



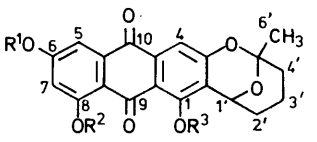
b
m/e 297

prominently in the mass spectrum of versicolorin C (≡ racemic dihydroversicolorin A). Chemical ionization mass spectroscopy of versiconal acetate gave a distinct peak at *m/e* 401 ($M^+ + H$).

The ^1H n.m.r. spectrum of versiconal acetate in $(CD_3)_2CO$ had the following characteristics. An AX system [δ 7.36 and 6.76 (^3J 2.2 Hz) due to 5- and 7-H, respectively] and a singlet at δ 7.30 (4-H) represented the aromatic protons. A two-proton triplet at δ 4.34 (J 6.4 Hz) was assigned to 4′-H_2. 1′-H, 2′-H, and 3′-H_2 appeared as multiplets at δ 6.47, 3.36, and 2.35, respectively. The three-proton singlet at δ 2.11 was assigned to the 6′-H_3. Upon decoupling of 1′-H, the multiplet at δ 3.36 collapsed to a doublet of doublets (J 9 and 4 Hz) whereas the resonances due to the 4′-H_2 and 2′-H were affected by decoupling of 3′-H_2. The presence of the isomeric hemiacetal (6c) was precluded by the presence and chemical shift of only two intramolecular hydrogen-bonded hydroxy-groups (δ 12.63 and 12.29). Although the foregoing data attest to the existence of versiconal acetate in $(CD_3)_2CO$ solution as the hemiacetal (6a), the presence of the open form (6b) cannot be excluded. However, the ^1H n.m.r. spectrum of versiconal acetate in $(CD_3)_2SO$ showed three peaks due to the protons of intramolecular hydrogen-bonded hydroxy-groups at δ 13.32, 12.33, and 11.93 with widths at half-height ($\Delta\nu_{\frac{1}{2}}$) of 1.5, 8.0, and 8.0 Hz, respectively. The resonance at δ 13.32 was assigned to the proton of the 8-hydroxy-group of the angular hemiacetal (6c). This assignment was confirmed by the chemical shifts of

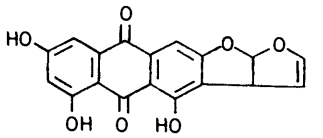


(1)

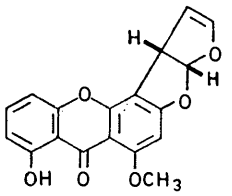


(2)

a ; $R^1 = R^2 = R^3 = H$
b ; $R^1 = CH_3, R^2 = R^3 = H$
c ; $R^1 = R^3 = CH_3, R^2 = H$
d ; $R^1 = R^2 = CH_3, R^3 = H$



(3)



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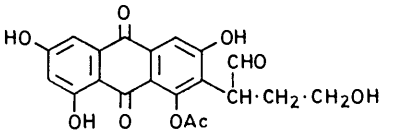
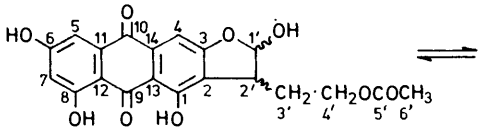
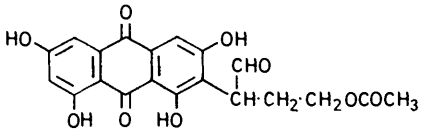


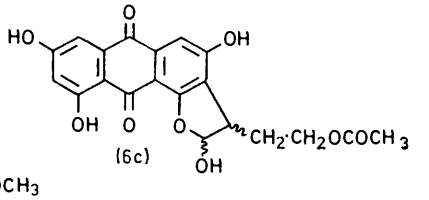
(5)



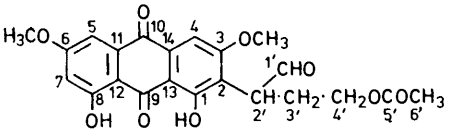
(6a)



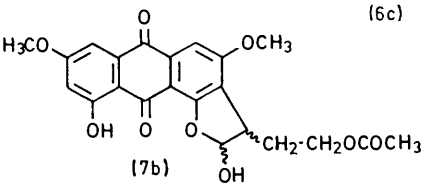
(6b)



(6c)



(7a)



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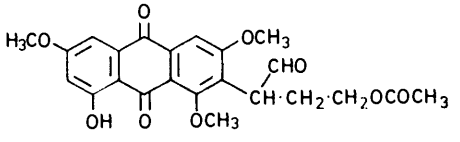

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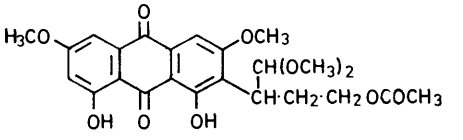

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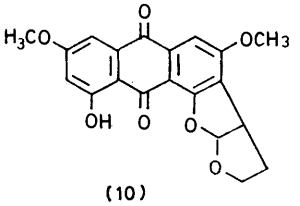
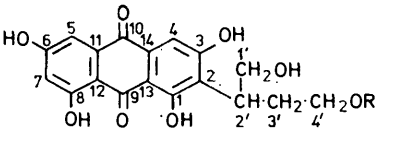


(10)



(11) R = $COCH_3$
(12) R = H

the 8-hydroxy-proton in (2c) (δ 13.32) and the 1-hydroxy-proton in (2d) (δ 13.52) (see Table 1). Additional evidence for the presence of the angular isomer (6c) in $(CD_3)_2SO$ solution was the doubling of the resonance of 7-H in the ratio 7 : 3 [(6a, b) δ 6.23 (^3J 2 Hz); (6c) δ 6.29 (^3J 2 Hz)]. An intense broad peak at δ 6.95 was assigned to 4-H of (6a, b) and 5-H of (6a—c). A sharp singlet at δ 7.23 was attributed to 4-H of the minor angular isomer (6c). The broad signal at δ 6.01 had the same integrated intensity as the signal at δ 7.23 and was assigned to 1'-H of (6c). No doubling of the 2'-, 3'-, 4'-, and 6'-protons was observed although the individual peaks in these multiplets were broader than the individual aromatic resonances. Therefore in $(CD_3)_2SO$ solution versiconal acetate exists as an equilibrium mixture of the isomers (6a—c) of which (6c) constitutes 30%. In contrast to our findings, Cox *et al.*[5] obtained from their ^1H n.m.r. data no indication of a mixture of isomers for versiconal acetate.

To control the formation in solution of the isomeric hemiacetals (6a and c) it was deemed necessary to protect the 3-hydroxy-group in versiconal acetate. Conditions for the selective methylation of a 1,6,8-trihydroxyanthraquinone were investigated on averufin (2a). Treatment of (2a) for a prolonged period with an excess of ethereal diazomethane gave 6-*O*-methyl- (2b), 1,6-*OO*-dimethyl- (2c), and 6,8-*OO*-dimethyl-averufin (2d). ^1H N.m.r. data of structural relevance for averufin (2a) and its derivatives are given in Table 1. The *peri*-*O*-methyl group in the major di-*O*-methyl derivative (2c) must be located at C(1), in accordance with the downfield shift of 4-H (0.27 p.p.m.). Similar downfield shifts were observed for the chemical shift of 4-H upon methylation of the 1-hydroxy-group of 6,8-*OO*-dimethylnidurufin (0.18 p.p.m.) [6,7] and aversin (0.36 p.p.m.).[8,9] In contrast methylation of the 8-hydroxy-group has a minimal effect on the chemical shift of 5-H. From the relative yields of the di-*O*-methyl products it was apparent that the 1-hydroxy-group reacted more readily than the 8-hydroxy-group with diazomethane. It is important to note that alkylation of the 1-hydroxy-group in (2b) caused a strong hypsochromic shift (20 nm) in the long wavelength absorption of (2c). Virtually exclusive monomethylation of the 6-hydroxy-group in (2a) was achieved by treatment of the compound with excess of ethereal diazomethane for 5 min.

Controlled treatment of versiconal acetate with ethereal diazomethane gave 3,6-*OO*-dimethylversiconal acetate (7a and b) and 1,3,6-*OOO*-trimethylversiconal acetate (8) as the major and minor product, respectively. Analysis of the reaction course did not indicate any monomethylation. The 3- and 6-hydroxy-groups were therefore methylated with the same facility. Furthermore, the 1-hydroxy-group of versiconal acetate reacted more readily with diazomethane than that of averufin (2a). The location of the *peri*-*O*-methyl group at C(1) in (8) was determined from the downfield shift (0.40 p.p.m.) of 4-H upon methylation of the 1-hydroxy-group of 3,6-*OO*-dimethylversiconal acetate as well as u.v. data (see Experimental section).

The ^1H n.m.r. data for solutions of 3,6-*OO*-dimethylversiconal acetate in $CDCl_3$ (7a) and $(CD_3)_2SO$ (7b) and for the trimethyl derivative (8) are given in Table 2. In $CDCl_3$ solution, the one-proton singlet at δ 9.68 in the two compounds (7a) and (8) was assigned to the aldehyde proton; the lack of coupling between the 1'- and 2'-protons indicates a dihedral relationship of *ca.* 90°.[10] In this solvent the 3,6-*OO*-dimethyl derivative is present in the branched benzylic aldehyde form (7a). However, in $(CD_3)_2SO$ solution the intramolecular hydrogen bonding is reduced and the compound is present in the angular hemiacetal form (7b), as is evident from the multiplet at δ 6.00 assigned to the hemiacetal proton and the presence of only one intramolecular hydrogen-bonded hydroxy-proton (δ 13.16). In $CDCl_3$ the 1- and 8-hydroxy-groups share the proton acceptor (9-carbonyl group); the absence of hemiacetal formation must be due to the strong intramolecular hydrogen bonding which holds these two hydroxy-groups in a fixed orientation in six-membered chelated rings. *X*-Ray crystallography of averufin (2a) [11] and versicolorin C[12] gave values of 1.78 Å for the O(9) · · · H(1) and 1.81 Å for the O(9) · · · H(8) intramolecular hydrogen bond length.

Versiconal acetate was readily converted into a single product *viz.* versicolorin C by treatment with anhydrous acidic methanol. However, treatment of 3,6-*OO*-dimethylversiconal acetate under these conditions gave as major product the dimethyl acetal (9) and in poor yield, 3,6-*OO*-dimethylisoversicolorin C (10). The ^1H n.m.r. spectrum of (9) indicated the presence of two aromatic (δ 3.90 and 3.97), and two aliphatic methoxy-groups (δ 3.15 and 3.41). Reaction of 3,6-*OO*-dimethylversiconal acetate with aqueous acidic methanol gave (10) in better yield. The differences observed in the

TABLE 1

Chemical shifts (δ) and multiplicities (J/Hz) in the ^1H n.m.r. spectra of averufin and derivatives (in $CDCl_3$)

Compound	4-H	5-H	7-H	1-OR	6-OR	8-OR	1'-H	6'-H
Averufin (2a)	7.28	7.24 (d, J 2.5)	6.65 (d, J 2.5)	R = H 12.51 *		R = H 12.32 *	5.38	1.58
6-*O*-Methylaverufin (2b)	7.25	7.33 (d, J 2.5)	6.65 (d, J 2.5)	R = H 12.48 *	R = Me 3.9	R = H 12.31 *	5.35	1.56
1,6-*OO*-Dimethylaverufin (2c)	7.52	7.28 (d, J 2.5)	6.68 (d, J 2.5)	R = Me 3.96 *	R = Me 3.90 *	R = H 13.32	5.39	1.58
6,8-*OO*-Dimethylaverufin (2d)	7.18	7.40 (d, J 2.5)	6.74 (d, J 2.5)	R = H 13.52	R = Me 3.92 *	R = Me 3.98 *	5.36	1.56

* May be interchanged.

ease of formation of the bisfuran systems in versiconal acetate and 3,6-*OO*-dimethylversiconal acetate can be attributed to the difference in reactivity of the 1- and 3-hydroxy-groups.

In the assignment of the signals of the ^{13}C n.m.r. spectra of versiconal acetate and its derivatives use was made of chemical shift values of related substances [2,13] as well as C–H coupling constants over one and more bonds. In aromatic systems these couplings are usually in the order $^1J \gg {}^3J > {}^2J > {}^4J$.[14] In addition deuterium isotope shifts [13,15] and the technique of heteronuclear selective population inversion (s.p.i.) [16] proved an invaluable aid in the assignment of quaternary carbon atoms. In a heteronuclear ^{13}C-{H} s.p.i. experiment a selective radiofrequency π-pulse applied at a proton transition will invert the populations of the upper and lower energy levels. A strong non-selective radiofrequency $\pi/2$ pulse is then immediately applied in the usual way to the ^{13}C nuclei. The intensities of the ^{13}C transitions progressively connected to these energy levels will increase while the regressively connected transitions will appear as negative peaks (see Figure 1c). The technique is advantageous in the assignment of quaternary carbon atoms with relatively long relaxation times.[17] In a repetitively pulsed s.p.i. ^{13}C n.m.r. experiment, the repetition rate is determined by the relaxation time of the proton thus resulting in a large increase in the signal-to-noise ratio or a substantial time-saving in obtaining the same signal-to-noise ratio.[18]

The assigned proton noise decoupled (p.n.d.) natural abundance 25.2 MHz ^{13}C n.m.r. spectrum of 3,6-*OO*-dimethylversiconal acetate in $CDCl_3$ (7a) is shown in Figure 1b. The ^{13}C n.m.r. data for (7a) and related compounds, obtained from p.n.d. and n.O.e. enhanced single frequency spectra, are given in Table 3.

The multiplicities observed in the n.O.e. enhanced single frequency spectrum of (7a) were used to determine the resonances which arose from methyl (three), methylene (two), methine (five), and quaternary (twelve) carbon atoms. Chemical shifts and ^1J(C,H) values distinguished between the 6'-methyl carbon {δ 20.8 [^1J(C,H) 129.3 Hz]} and the two *O*-methyl carbons which resonated at δ 56.4 [3-OCH_3, ^1J(C,H) 146.0 Hz] and 56.1 [6-OCH_3, ^1J(C,H) 145.5 Hz] as well as between C(3') {δ 26.5 [^1J(C,H) 129.1 Hz]} and C(4') {δ 62.3 [^1J(C,H) 147.4 Hz]}.

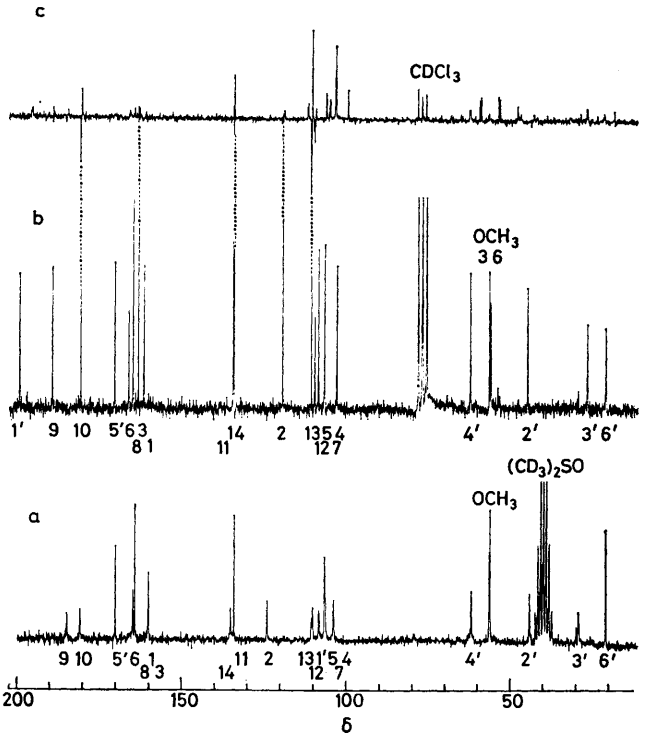


FIGURE 1 The natural abundance 25.2 MHz ^{13}C n.m.r. spectra of 3,6-*OO*-dimethylversiconal acetate (a) in $(CD_3)_2SO$ (proton-noise-decoupled, spectral width 5 000 Hz, 50° radiofrequency pulse of 30 μs duration; pulse delay 4 s, transients 1 331); (b) in $CDCl_3$ (proton-noise-decoupled, spectral width 5 000 Hz, 90° radiofrequency pulse of 16 μs duration; pulse delay 60 s; transients 256); and (c) after a low-field 4-H transition has been selectively inverted (spectral width 5 000 Hz; 90° radiofrequency pulse of 52 μs duration; pulse delay 1 s; π pulse 0.1 s; transients 4 K)

The signal at δ 199.6 [^1J(C,H) 178.6 Hz] is characteristic of an aldehyde carbon atom [19] and was assigned to

TABLE 2

Chemical shifts (δ) and multiplicities (J/Hz) in the ^1H n.m.r. spectra (in $CDCl_3$) of derivatives of versiconal acetate

Compound	4-H	5-H	7-H	1-OR	3-OR	6-OR	8-OR	1'-H	2'-H	3'-H	4'-H	6'-H
(7a)	7.26 (s)	7.27 (d, *J* 2.4)	6.63 (d, *J* 2.4)	R = H 12.60 (s) *	R = Me 3.97 (s)	R = Me 3.91 (s)	R = H 12.12 (s) *	9.68 (s)	*ca.* 3.9	2.63 (m)	4.06 (t, *J* 7)	1.99 (s)
(7b) †	7.21 (s)	6.93 (d, *J* 2.4)	6.63 (d, *J* 2.4)		R = Me 3.93 (s)	R = Me 3.82 (s)	R = H 13.16 (s)	6.00 (s, br)	Obscured		4.09 (t, *J* 7)	1.99 (s)
(8)	7.66 (s)	7.31 (d, *J* 2.4)	6.72 (d, *J* 2.4)	R = Me 3.93 (s)	R = Me 3.99	R = Me 3.91 (s)	R = H 13.20 (s)	9.68 (s)	Obscured	2.63 (m)	4.11 (t, *J* 7)	1.99 (s)
(9) ‡	7.38 (s)	7.32 (d, *J* 2.4)	6.64 (d, *J* 2.4)	R = H 12.71 (s) *	R = Me 3.97 (s)	R = Me 3.90 (s)	R = H 12.24 (s) *	5.10 (d, *J* 9 Hz)	4.3 (m)	2.2 (m)	*ca.* 3.9	1.94 (s)
(11) §	7.37 (s)	7.23 (d, *J* 2.3)	6.61 (d, *J* 2.3)	R = H 13.02 (s) *	R = H	R = H	R = H 12.31 (s) *	3.83 (m)	Obscured	2.17 (m)	4.03 (t, *J* 7)	2.00 (s)
(12) †	7.18 (s)	7.23 (d, *J* 2.3)	6.54 (d, *J* 2.3)	R = H 12.79 (s) *	R = H	R = H	R = H 12.15 (s) *	3.0—4.0	3.0—4.0	1.95 (m)	3.0—4.0	

* May be interchanged. † In $(CD_3)_2SO$. ‡ The protons of the 1'-methoxy-groups appear as singlets at δ 3.15 and 3.41. § In $CDCl_3$–$(CD_3)_2SO$.

C(1'). The only aliphatic methine carbon atom, C(2'), resonated at δ 44.8. Although no proton–proton coupling constant was observed for 1'- and 2'-H (see before) the ^2J(C,H) value of 24.0 Hz, typical for a

coupling between an aldehyde proton and an aliphatic carbon atom,[20] proved the relationship between C(1′) and C(2′) in (7a). The remaining three methine carbon resonances with chemical shifts characteristic of aromatic carbon atoms, were assigned from the splitting pattern observed in the n.O.e. enhanced single frequency spectrum. The C(5) atom gave rise to a doublet of doublets centred at δ 108.6 [^1J(C,H) 167.5, ^3J(C,H) 5.0 Hz] while C(7) was observed as a doublet of triplets at δ 106.0 [^1J(C,H) 162.7, ^3J(C,H) 4.7, ^3J(C,OH) 7.7 Hz] which changed to a doublet of doublets after replacement of the exchangeable protium atoms with inverted is shown in Figure 1c. The singlet at δ 189.6 was assigned to C(9) [21] and the multiplet at δ 170.6 to C(5′).

Deuterium induced isotope shifts were used to assign various carbon resonances.[13,15] We reported previously [13] that addition of a mixture of H_2O and D_2O (1 : 1) to a compound with exchangeable protons will result in a doubling of a specific ^{13}C resonance due to isotope shifts large enough to be resolved (≥0.1 p.p.m.), provided the protium–deuterium exchange rates are sufficiently slow. Addition of this mixture to the $CDCl_3$ solution of 3,6-*OO*-dimethylversiconal acetate (7a) resulted in a

TABLE 3

^{13}C N.m.r. data of versiconal acetate (6a—c), 3,6-*OO*-dimethylversiconal acetate (7a, b), 3,6-*OO*-dimethylversiconal acetate dimethyl acetal (9), 3,6-*OO*-dimethylisoversicolorin C (10), versiconol acetate (11), and versiconol (12)

Carbon atom	(6a, b) δ *	(6c) δ *	(7b) δ *	(7a) δ †	^1J(C,H)/ Hz	$^{>1}J$(C,H)/ Hz	(9) δ †	(10) δ †	(11) δ *	(12) δ *
1	159.8	159.1	160.1	161.7 (St)		4.8	162.4	159.8	163.0	162.9
2	120.5	122.5	123.9	119.5 (Sm)			122.7	122.5	121.4	122.9
3	163.9	161.0	160.1	163.4 (Sm)			164.0	161.1	163.3	163.1
4	103.3	103.8	103.8	103.0 (D)	166.2		103.4	104.0	108.6	108.9
5	108.9	107.3	106.5	108.6 (Dd)	167.5	5.0	108.3	107.0	108.6	108.6
6	165.1	(164.1)	164.8	166.2 (Sm)			166.1	165.2	164.8	164.7
7	108.0		106.5	106.0 (Ddd)	162.7	7.7; 4.6	106.8	107.0	107.9	107.9
8	164.3		164.2	165.0 (St)		4.3	165.0	165.0	164.2	164.2
9	189.0	184.8	184.9	189.6 (S)			190.1	185.4	188.8	188.8
10	180.7	181.7	180.9	181.0 (St)		4.6	181.5	182.0	181.0	181.0
11	134.8	(134.9)	134.0	134.5 (S)			134.9	134.4	134.6	134.7
12	108.5		110.1	109.7 (Sq)		5.7	110.1	n.o.	108.6	108.6
13	110.2	109.6	110.4	110.7 (Sdd)		6.3; 4.6	110.6	110.8	108.1	108.1
14	135.0	134.6	135.0	134.3 (Sd)		2.2	133.5	135.9	132.2	132.1
1′	n.o.		108.2	199.6 (Ddt)	178.6	7.4; 3.7	104.3	113.7	62.9	63.2
2′	43.9		44.0	44.8 (Ddqn)	125.1	24.0; 4.0	35.8	43.7	35.0	34.9
3′	27.9	29.1	29.1	26.5 (Tm)	129.1		28.1	31.0	27.8	32.5
4′	61.9		61.7	62.3 (Tq)	147.4	3.9	63.1	67.4	62.9	60.1
5′	170.2		170.2	170.6 (Sqt)		7.1; 2.9	170.8		169.8	
6′	20.8		20.8	20.8 (Q)	129.3		20.9		20.8	
3-OCH_3			56.3	56.4 (Q)	146.0		56.3	56.1		
6-OCH_3			56.1	56.1 (Q)	145.5		56.1	55.8		
OCH_3							53.7			
OCH_3							52.5			
Solvent	$(CD_3)_2SO$	$(CD_3)_2SO$	$(CD_3)_2SO$	$CDCl_3$			$CDCl_3$	$CDCl_3$	$CDCl_3$–$(CD_3)_2SO$ 1 : 1	$CDCl_3$–$(CD_3)_2SO$ 1 : 1

* Relative to internal Me_4Si. Measured from internal $(CD_3)_2SO$ and corrected by using the expression δ[Me_4Si] = S[$(CD_3)_2SO$ +39.7]. † Relative to internal Me_4Si. Capital letters refer to the pattern resulting from directly bonded (C,H) couplings and small letters to that from (C,H) couplings over more than one bond. S = singlet, D or d = doublet, T or t = triplet, Q or q = quartet, qn = quintet, m = multiplet. n.o. = not observed.

deuterium. The C(4) signal appeared as a doublet at δ 103.0 [^1J(C,H) 166.2 Hz]. Identical splitting patterns were observed for the corresponding carbon atoms of averufin (2a).[13]

The quaternary carbon atoms of 3,6-*OO*-dimethylversiconal acetate (7a) can be divided into four different groups: (i) the carbonyl carbon atoms [C(9), C(10), and C(5′)] at δ 189.6, 181.0, and 170.6, (ii) the oxygen-bearing carbon atoms [C(1), C(3), C(6), and C(8)] between δ 161 and 167, (iii) the carbon atoms *ortho* to aryloxy substituents [C(2), C(12), and C(13)] at δ 109.7, 110.7, and 119.5, and (iv) C(11) and C(14) which resonate at δ 134.5 and 134.3.

The resonance at δ 181.0 has a triplet fine structure in the n.O.e. enhanced single frequency spectrum. The selective inversion of either a 4- or 5-H transition affected this signal, assigning it to C(10). The spectrum obtained with the low-field transition of 4-H selectively doubling of the resonances at δ 165.0 (Δδ 0.22 p.p.m.) and 161.7 (Δδ 0.25 p.p.m.). These two resonances must therefore be assigned to C(1) and C(8). Selective inversion of a 2′-H transition changed the intensities of the individual peaks of the resonances at δ 161.7 [C(1)] and 163.4 [C(3)]. These results proved unambiguously the assignment of the group (ii) carbon atoms as given in Table 3 (see also Figure 1c).

After replacing the exchangeable protons with deuterium the group (iii) carbon atom resonances appeared as a multiplet (δ 119.5), a doublet {δ 110.7, [$^{>1}J$(C,H) 6.3 Hz]}, and a triplet (δ 109.7), respectively, in the n.O.e. enhanced single frequency spectrum. Selective population inversion of the low-field 4-H transition changed the intensities of the peaks at δ 119.5 and 110.7 (see Figure 1c). These signals must, therefore, be due to C(2) and C(13). The resonance at δ 119.5 was assigned to C(2) as this signal exhibits additional

splitting due to coupling to protons other than 4-H. The triplet at δ 109.7 and the doublet at δ 110.7 were, therefore, assigned to C(12) and C(13), respectively. The assignment was confirmed by the selective inversion of a 5-H transition with a subsequent effect on the C(12) resonances.

The resonance at δ 134.3 appeared as a doublet with $^{>1}J$ (C,H) 2.2 Hz. Selective inversion of a 4-H transition affected this doublet. The resonances at δ 134.5 and 134.3 were therefore, assigned to C(11) and C(14), respectively, because 4-H is only two bonds removed from C(14) against four bonds from C(11). The ^{2}J(C,H) coupling from 4-H to C(14) was not observed in (2a).[13]

Using the selectivity which can be obtained with the s.p.i. technique[22] it was possible to correlate specific proton and ^{13}C resonances with individual methoxy-groups. Application of a selective pulse at δ_H 4.70 [ν_H(OCH_3, low-field) + $\frac{1}{2}^{1}J$(C,H) (OCH$_3$)] affected only the low-field methoxy-carbon doublet centred at δ 56.4. Similarly, placing the selective pulse with a power of *ca.* 5 Hz at δ_H 3.86 [ν_H(OCH_3, high-field) − 5 Hz] changed the intensities of the C(6) resonances at δ 164.8. Therefore the signal of the protons of the high-field methoxy-group (δ_H 3.91) and the high-field carbon resonance at δ 56.1 were due to the 6-methoxy-group.

The ^{1}H n.m.r. data of 3,6-*OO*-dimethylversiconal acetate indicated that in $(CD_3)_2SO$ solution the compound exists as the hemiacetal (7b) (see before). Its p.n.d. ^{13}C n.m.r. spectrum is shown in Figure 1a. The majority of the ^{13}C signals of this isomer (7b) was assigned by comparison with the resonances of the corresponding carbon atoms observed in (7a).

The n.O.e. enhanced single frequency spectrum of (7b) confirmed the assignments of the aromatic and aliphatic methine carbon atoms. The resonance at δ 108.2 [C(1′)] exhibited a directly bonded C–H coupling of 175.1 Hz whereas the resonances at δ 106.5 [C(5) and C(7)] and 103.8 [C(4)] appeared as doublets, due to directly bonded C–H couplings, with values typical of substituted aromatic systems.[19] The assignment of C(11), C(14), C(1), and C(3) was based on the observed directly bonded (C,C) coupling constants in the spectrum of [1,2-^{13}C]acetate-derived 3,6-*OO*-dimethylversiconal acetate in $(CD_3)_2SO$ solution.[23]

The ^{13}C chemical shift values of the dimethyl acetal (9) and 3,6-*OO*-dimethylisoversicolorin C(10) are given in Table 3. The assignment of the resonances of these two compounds is based on comparisons with the assignments of the corresponding carbon atoms in 3,6-*OO*-dimethylversiconal acetate in $CDCl_3$ (7a) and in $(CD_3)_2SO$ (7b) solution.

From the results in Table 3 the following conclusions were reached about the relationship of the chemical shift values of the anthraquinone moiety and the structures of the currently studied compounds and of averufin (2a)[13] and versicolorin A(3).[24] The chemical shift of the anthraquinone- 9- and 10-carbonyl carbon atoms is independent of the nature of the side-chain. The observed values are δ 188.7—190.1 for C(1) and 180.1—181.5 for C(10). Chelation of the 9-carbonyl oxygen with only one phenolic proton resulted in an upfield shift of *ca.* 5 p.p.m. for the C(9) resonance whereas the C(10) resonance shifted to lower field by *ca.* 1 p.p.m. This result is in agreement with previous observations[5,21] which indicated that chelation of the carbonyl group with a single phenolic hydroxy-group resulted in a downfield shift of *ca.* 5 p.p.m. whereas chelation with two phenolic hydroxy-groups caused a downfield shift of *ca.* 10 p.p.m. of the carbonyl carbon resonance. The chemical shifts of C(5)—C(12) are very little affected by the nature of the side-chain. Methylation of the 6-hydroxy-group resulted in the following shifts: *ca.* 1 for C(6); *ca.* −2 for C(7); *ca.* 1 for C(8); *ca.* 1 p.p.m. for C(12). The chemical shifts of the remaining anthraquinone carbon atoms C(1)—C(5), C(13), and C(14), however depend

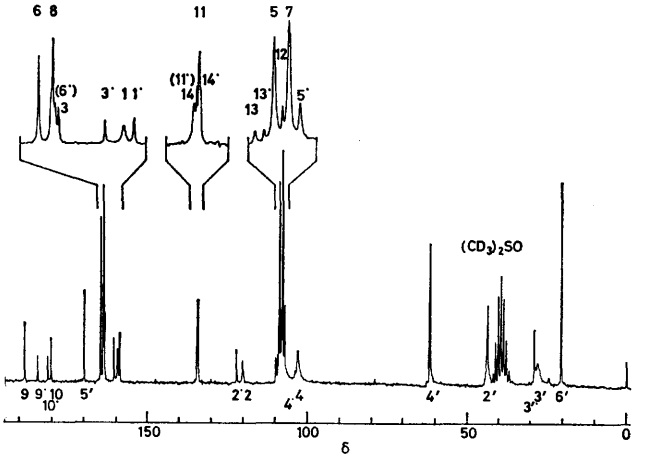


FIGURE 2 The proton-noise-decoupled natural abundance 25.2 MHz ^{13}C n.m.r. spectrum of versiconal acetate in $(CD_3)_2SO$. The carbon resonances marked ● are attributed to structure (6c). The assignment of the carbon resonances in parentheses is only tentative, see text (spectral width 5 000 Hz, 50° radio-frequency pulse of 30 μs duration; transients 60 K)

critically on the nature of the side-chain. Upfield shifts were observed for the resonances of C(1), C(3), C(5), and C(6) when the 1-hydroxy-group was used in ring closure as in (7b) and (10), whereas downfield shifts of *ca.* 3 for C(2), and of *ca.* 1 p.p.m. for C(4) and C(14), were observed. The chemical shift value of the C(1′) atom is characteristic of the nature of the ring system attached to the C(2) and C(3) atoms of the anthraquinone moiety. C(1′) resonated between δ 112.8 and 113.6 in the case of a bisdihydrofuran[2,24] or a bistetrahydrofuran system[25] and at δ 108.2 for a five-membered hemiacetal ring as in (7b).

The assigned p.n.d. spectrum of versiconal acetate (6a—c) in $(CD_3)_2SO$ solution is reproduced in Figure 2. In the solvent, the number of resonances were accounted for by an equilibrium mixture of the isomers (6a—c). The ^{13}C n.m.r. spectrum of versiconal acetate in $(CD_3)CO$ or in a mixture of $(CD_3)_2SO$ and $CDCl_3$ showed only 19 carbon resonances, corresponding to the values given for the isomers (6a, b) in Table 3. The latter solvent system contained only enough $(CD_3)_2SO$ to keep versiconal

acetate (70 mg) in solution in $CDCl_3$ (1.4 ml). Taking the previous evidence into consideration, the additional peaks observed in the ^{13}C spectrum of versiconal acetate in $(CD_3)_2SO$ could be ascribed to the angular hemiacetal (6c). The resonances attributed to C(6) and C(11) of (6c) (in brackets in Table 3 and Figure 2) are only tentative. The resonance at δ 107.3 was assigned to the C(5) atom of (6c) on the basis of an s.p.i. experiment.[22] When a selective π-pulse of 10 Hz was applied at 83 Hz downfield from the broad proton resonance at δ_H 6.95 the ^{13}C resonances centred at δ 108.9 and 107.3 both appeared as a doublet of doublets with directly bonded (C,H) couplings of 166.2 and 166.7 Hz and vicinal (C,H) coupling constants of 4.9 and 4.0 Hz, respectively. The C(4) resonances of (6a, b) were also affected in this experiment. Assuming that the spin–lattice relaxation times of these two C(5) resonances are identical a ratio of 7 : 3 for (6a, b) and (6c) was calculated from the peak intensities. It is pertinent to note that no peak was observed in the region δ 120—110.2 in any of the solvents or solvent mixtures employed in this study. Cox *et al.*[5] assigned a peak at δ 113.0 to C(1′) whereas Fitzell *et al.*[26] preferred a signal at δ 116.2 for C(1′).

The obtained ^1H and ^{13}C n.m.r. spectral data of versiconal acetate could be rationalized in the following manner. Versiconal acetate exists as an equilibrium mixture of (6a and b) in solvents like $(CD_3)_2CO$, CD_3OD, and $CDCl_3$–$(CD_3)_2SO$ where the intramolecular hydrogen-bonding between the 9-carbonyl group and the 1-hydroxy-proton is stronger than the intermolecular hydrogen-bonding between the substrate and solvent. The conversion between (6a and b) must be at a rate where coalescence or near coalescence of the peaks of the C(1′) atom in the two forms occurs.[19] At this exchange rate all the ^1H and ^{13}C resonances with much smaller chemical shift differences should appear as sharp peaks. 1′-H resonance observed at δ 6.47 in $(CD_3)_2CO$ indicated that the equilibrium is in favour of the hemiacetal form (6a) in this solvent. However, the observed broadness of some of the peaks in $(CD_3)_2CO$ could be explained by the existence of (6a) as a number of stereoisomers. In $(CD_3)_2SO$ intra- and inter-molecular hydrogen bonding compete with each other making the 1-hydroxy-group available for ring closure to form the angular hemiacetal form (6c). The resonances assigned to the angular hemiacetal (6c) in Figure 2 were in general narrower than those attributed to (6a, b). The ratio of isomers (6a) : (6b) is unknown, while the isomer (6c) contributes *ca.* 30% to the isomeric population. In ^1H and ^{13}C n.m.r. spectra neither 1′-H nor the C(1′) atom of (6a and b) is detected; this evidence together with the relative broadness of the peaks assigned to (6a and b) can be rationalized on the supposition that the activation energy for the transition between (6a and b) is very small. Although (6c) must have a higher enthalpy (ΔH) than (6a and b), its narrower ^{13}C n.m.r. signals indicate that its lifetime must be longer than that of either (6a and b).

Versiconol Acetate (11) *and Versiconol* (12).—Two minor metabolites, versiconol acetate (11), and versiconol (12),[27] both with u.v. characteristics virtually identical to those of versiconal acetate (6a—c) were obtained from the mycelium of cultures of *A. parasiticus* which had been treated with dichlorvos. The ^1H n.m.r. assignments for the two compounds are summarized in Table 2.

The mass spectrum of versiconol acetate (11), $C_{20}H_{18}O_9$, showed a very weak M^+ peak at 402. The loss of the elements of acetic acid from the M^+ ion gave the fragment ion at *m/e* 342 ($C_{18}H_{14}O_7$). Peaks at *m/e* 311 and 297 due to fragments a and b, respectively featured prominently in the spectrum. The mass spectrum of versiconol (12) lacked the expected M^+ but a prominent ($M^+ - H_2O$) ion at *m/e* 342 ($C_{18}H_{14}O_7$) was again observed. Strong peaks at *m/e* 311 and 297 were also present.

The chemical shifts of the carbon atoms of versiconol acetate (11) and versiconol (12) (see Table 3) were assigned by comparison of their resonances in the p.n.d. and n.O.e. enhanced single frequency spectra with those of versiconal acetate (6a—c) and its derivatives. The carbon of the 1′-methylene group in (12) appeared at δ 63.2 while the signal at δ 60.1 was assigned to C(4′). In versiconol acetate the C(4′) resonance has shifted to low field and merged with the resonance due to C(1′) at δ 62.9.

The biosynthesis of versiconal acetate from [1-^{13}C]- and [1,2-^{13}C]-acetate and its pivotal role in the biosynthesis of aflatoxin B_1 forms the subject of a separate paper.[23]

EXPERIMENTAL

M.p.s were determined on a Kofler hot-stage apparatus. U.v. absorptions were measured (for solutions in methanol) on a Unicam SP 800 spectrometer, i.r. spectra on a Perkin-Elmer 237 spectrometer, and mass spectra on an A.E.I. MS9 double-focusing spectrometer. ^1H N.m.r. spectra were recorded with a Varian HA-100 spectrometer (100 MHz; Me_4Si as lock signal and internal reference) or a Varian XL-100-15 Fourier transform spectrometer (100.1 MHz). 25.2 MHz ^{13}C N.m.r. spectra were recorded with a Varian XL-100-15 Fourier transform spectrometer equipped with a 16 K Varian 620i computer and a gated gyrocode decoupler.

Isolation of the Metabolites.—Conical flasks (80 × 500 ml) containing YES medium (2% yeast extract, 20% sucrose; 100 ml) were inoculated with a spore suspension of *Aspergillus 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 [*OO*-dimethyl-*O*-(2,2-dichlorovinyl)phosphate] (0.2%, 1 ml) was added to each flask to give a final concentration of 20 p.p.m. dichlorvos and 1% ethanol.

After an additional 6 days the cultures were filtered and the mycelium was extracted for 24 h with chloroform–methanol (1 : 1 v/v) in a Soxhlet apparatus. The combined extracts were concentrated and the residue was partitioned between 90% methanol (1 l) and n-hexane. The aqueous methanol solution was concentrated under reduced pressure and the residue was partitioned between ethyl acetate (1 l) and water.

The crude products obtained from the ethyl acetate solution were separated and purified by pressure column chromatography (1 kg cm^{-2}) on silica gel (Merck type H for t.l.c.) using chloroform–methanol (9 : 1 v/v) as eluant; fractions (10 ml) were collected and appropriate fractions (t.l.c.) were combined to give the following three metabolites: versiconal acetate (6a—c) (607 mg), m.p. 234—236° (from acetone); $\lambda_{max.}$ 223, 265, 291, 315, and 452 nm * (log ε 4.41, 4.16, 3.39, 4.01, and 3.85); $\nu_{max.}$(KBr) 2 920, 1 710sh, 1 600, 1 385, 1 280br, 1 190, and 1 165 cm^{-1} [Found: C, 57.7; H, 4.15%; *m/e* 382.071 2. Calc. for $C_{20}H_{16}O_9,H_2O$: C, 57.4; H, 4.3%. Calc. for $C_{20}H_{14}O_8$: (*M* − H_2O), 382.068 8]; *versiconol acetate* (11) (120 mg), a glass; $\lambda_{max.}$ 225, 265sh, 294, 315, and 453 nm (log ε 4.49, 4.20, 4.41, 4.11, and 3.93); $\nu_{max.}$(KBr) 1 700sh, 1 620br, and 1 600 cm^{-1} (Found: *m/e*, 342.076 5. $C_{18}H_{14}O_7$ requires *m/e*, 342.073 9; this fragment corresponds to $M^+ - CH_3CO_2H$); and versiconol (12) (100 mg), m.p. 257—259° (acetone) (lit.,[27] 265°); $\lambda_{max.}$ 224, 266, 294, 315, and 455 nm (log ε 4.48, 4.15, 4.38, 4.09, and 3.87) (Found: *m/e*, 342.074 9. $C_{18}H_{14}O_7$ requires 342.073 9; this fragment corresponds to $M^+ - H_2O$).

Reaction of Averufin with Diazomethane.—Averufin (2a) (500 mg) in chloroform–methanol (1 : 1 v/v) (50 ml) was treated with an excess of an ethereal diazomethane solution. After 5 h formic acid (2 ml) was added to the reaction mixture and the solvent removed under reduced pressure. The residue was separated by column chromatography on silica gel (Merck type H for t.l.c.) (150 g) under pressure (1 kg cm^{-2}). The column was developed with chloroform–methanol (99.5 : 0.5 v/v). The appropriate fractions were combined to give after crystallization three products: 6-O-*methylaverufin* (2b) (110 mg), m.p. 196—198° (acetone); $\lambda_{max.}$ 223, 265, 291, 320, and 448 nm (log ε 4.52, 4.19, 4.44, 3.82, and 3.98); $\nu_{max.}$(KBr) 2 920, 1 590, and 1 560sh cm^{-1} (Found: C, 66.05; H, 4.9%; *m/e*, 382. $C_{21}H_{18}O_7$ requires C, 66.0; H, 4.7%; *M*, 382); 1,6-OO-*dimethylaverufin* (2c) (205 mg), m.p. 170—172° (acetone); $\lambda_{max.}$ 223, 252, 272, 288, 313, and 428 nm (log ε 4.48, 4.08, 4.38, 4.56, 3.96, and 3.83); $\nu_{max.}$(KBr) 2 940, 1 625, and 1 585 cm^{-1} (Found: C, 66.85; H, 5.1%; *m/e*, 396. $C_{22}H_{20}O_7$ requires C, 66.7; H, 5.05%; *M*, 396); and 6,8-OO-*dimethylaverufin* (2d) (115 mg), m.p. 208—209° (acetone); $\lambda_{max.}$ 225, 250, 287, 313, and 439 nm (log ε 4.57, 4.16, 4.50, 3.92, and 3.90); $\nu_{max.}$(KBr) 2 940, 1 620, and 1 595 cm^{-1} (Found: C, 66.5; H, 5.15%; *m/e* 396. $C_{22}H_{20}O_7$ requires C, 66.7; H, 5.05%; *M*, 396).

Reaction of Versiconal Acetate with Diazomethane.—Versiconal acetate (6a—c) (300 mg) in acetone (30 ml) was treated with an excess of cold (0 °C) ethereal diazomethane for 5 min. After addition of acetic acid (2 ml) the ethereal solution was washed with water and dried (Na_2SO_4). The crude mixture was separated and purified by pressure column chromatography (1 kg cm^{-2}) on silica gel (Merck type H for t.l.c.; 170 g) using chloroform–n-hexane–acetone (70 : 22 : 8 v/v/v) as eluant. Combination of the appropriate fractions gave: 1,3,6-OOO-*trimethylversiconal acetate* (8) (5 mg), m.p. 143—145° (acetone); $\lambda_{max.}$ 218, 270, 285, 315, and 430 nm (log ε 4.90, 4.38, 4.50, 3.96, and 3.80); $\nu_{max.}$(KBr) 1 715, 1 665, and 1 615 cm^{-1} (Found: *m/e*, 442.124 6. $C_{23}H_{22}O_9$ requires *M*, 442.126 4), and 3,6-OO-*dimethylversiconal acetate* (7a,b) (185 mg), m.p. 96—98° (from methanol); $\lambda_{max.}$ 225, 265, 284, 321, and 442 nm (log ε 4.60, 4.34, 4.52, 4.04, and 4.07); $\nu_{max.}$(KBr) 2 940, 1 730, 1 675w, 1 630, 1 605, and 1 280 cm^{-1} (Found: C, 61.4; H, 4.8. $C_{22}H_{20}O_9$ requires C, 61.7; H, 4.7%).

Preparation of Versicolorin C.—A solution of versiconal acetate (6a—c) (60 mg) in saturated methanolic hydrochloric acid (10 ml) was stirred at room temperature. The solvent was evaporated and the residue crystallized from acetone to give versicolorin C (38 mg), m.p. >320° (decomp.) (lit.,[28] >310°), identical with an authentic sample.

Treatment of 3,6-OO-*Dimethylversiconal Acetate* (7a,b) *with Acid.—Method A.* A solution of (7a,b) (110 mg) in saturated anhydrous methanolic hydrogen chloride (10 ml) was stirred at room temperature. After 5 min the solvent was evaporated under reduced pressure and the residue purified by p.l.c. on silica gel. The plates were developed three times with dichloromethane to give two products.

The *dimethyl acetal* (9) (50 mg) had m.p. 151—153° (from acetone–methanol); $\lambda_{max.}$ 224, 254, 266, 285, 322, and 447 nm (log ε 4.64, 4.22, 4.34, 4.53, 3.97, and 4.03); $\nu_{max.}$(KBr) 2 940, 1 725, 1 620sh, 1 600, and 1 570 cm^{-1} (Found: C, 60.95; H, 5.4. $C_{24}H_{26}O_{10}$ requires C, 60.75; H, 5.5%). 3,6-OO-*Dimethylisoversicolorin C* (10) (12 mg) had m.p. 245° (acetone); $\lambda_{max.}$ 217, 265, 274, 315, and 430 nm (log ε 4.87, 4.30, 4.45, 3.96, and 3.92); $\nu_{max.}$(KBr) 2 940, 1 625, 1 600, and 1 575 cm^{-1} (Found: *m/e*, 368.088 9. $C_{20}H_{16}O_7$ requires *M*, 368.089 6).

Method B. A solution of (7a,b) (50 mg) in methanol (50 ml) was treated with 6N-hydrochloric acid (10 ml) for 30 min. The solvent was removed under reduced pressure and the residue purified by p.l.c. on silica gel using chloroform–hexane–acetone (70 : 20 : 10). An orange band was eluted which yielded 3,6-OO-*dimethylisoversicolorin C* (10) (30 mg).

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* Schroeder *et al.*[4] reported the long wavelength absorption erroneously as 480 nm (log ε 3.86).

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