Polyketide-derived Fungal Metabolites from *Bipolaris sorokiniana* and their Significance in the Biosynthesis of Sterigmatocystin and Aflatoxin B_1

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The metabolites averufin, versiconol, versiconal acetate, versiconol acetate, versicolorin C, sterigmatocystin, and a new metabolite, 3,8-dihydroxy-4-(2,3-dihydroxy-1-hydroxymethylpropyl)-1-methoxyxanthone were isolated from cultures of *Bipolaris sorokiniana*. Evidence is presented for the reformulation of bipolarin as versiconol. The biosynthetic implications of the natural occurrence of versiconal acetate and of versiconol acetate are discussed.

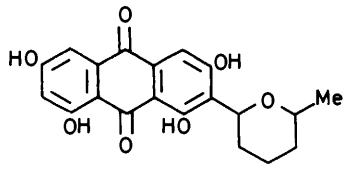
The biosynthesis of the hepatocarcinogens, sterigmatocystin, and aflatoxin B_1, and their congeners has formed the substance of many detailed studies, involving the use of blocked mutants, enzyme inhibitors, and the conversion of primitive and advanced precursors into end products.[1-4] The postulated biosynthetic sequence comprises the following key intermediates: acetate-polymalonate ⟶ polyhydroxyanthraquinones (averantin, norsolorinic acid, averufin, versiconal acetate, and the versicolorins) ⟶ xanthones (sterigmatocystin) ⟶ coumarins (aflatoxin). Several unique biosynthetic steps take place, *e.g.* the conversion of the unbranched C_6 side-chain of averufin into the branched-chain aldehyde, versiconal acetate,[5,6] by migration of an anthraquinone unit from the α- to the β-position of the side-chain; the Baeyer–Villiger oxidation of the terminal acetyl group,[7,8] and subsequently the oxidative transformation of the anthraquinones into xanthones (sterigmatocystin).

Aucamp and Holzapfel[9] earlier reported the characterization of averufanin (**1**), versicolorin C (**2**), bipolarin (**3**), and sterigmatocystin (**4**) from maize cultures of *Bipolaris sorokiniana*. However, the structure proposed for bipolarin (**3**) was not consistent with the currently accepted biosynthetic pathway leading to sterigmatocystin,[1,4] and prompted our present study of metabolites produced by *B. sorokiniana* on solid and liquid media. This paper relates the characterization of averufin (**5**), versicolorin C (**2**), versiconol (**6**), versiconol acetate (**7**), versiconal acetate (**8a**), sterigmatocystin (**4**), and 3,8-dihydroxy-4-(2,3-dihydroxy-1-hydroxymethylpropyl)-1-methoxyxanthone (**9**) from cultures of *B. sorokiniana*, grown on a chemically defined medium; the novel dioxopiperazine, bipolaramide,[10] was also produced under these conditions. Cultivation of the fungus on solid maize also leads to the formation of versiconol.

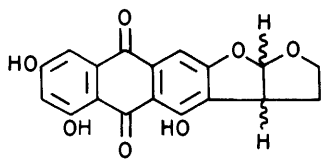
The strain of *B. sorokiniana* MRC 93 used in this study is the same as that previously used by Aucamp and Holzapfel,[9] then called I.M.I. 115076. *B. sorokiniana* was grown in stationary culture on a concentrated potato-dextrose broth; the isolation of the pigments involved separation on silica gel using column and thin layer chromatography. Compounds (**2**), (**4**)—(**7**), and (**8a**) were characterized by direct comparison with available reference samples. Evidence for the structural assignment of (**9**) is presented below.

The complete analysis of the ^{1}H n.m.r. spectra of the aliphatic side-chain of compounds (**6**) and (**7**) and of 3,6-*O*,*O*-dimethylversiconal acetate (**8b**) has previously failed owing to inadequate resolution at 100 MHz.[5] The analysis at 500 MHz of these deceptively simple spin systems is given below.

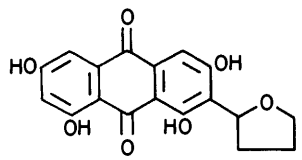
Versiconol (**6**) had m.p. 263—264 °C and u.v. spectral data, $\lambda_{max.}$ (MeOH) 223, 265, 293, 320, and 455 nm (log ε 4.56, 4.26, 4.50, 4.08, and 3.96) which were similar to those reported by



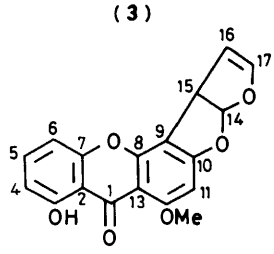
(**1**)



(**2**)

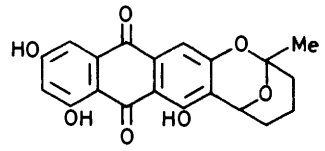


(**3**)

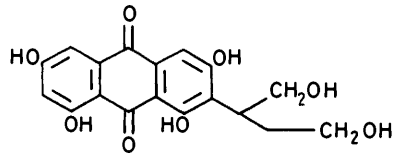


(**4**)

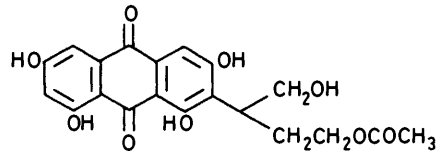
Aucamp and Holzapfel for bipolarin.[9] These authors[9] reported a molecular ion at *m/z* 342 for bipolarin based on electron impact mass spectral studies. Versiconol in fact gives a similar apparent molecular ion at *m/z* 342 ($M^+ - 18$) under these conditions and mass spectral fragmentations virtually identical with those reported for bipolarin. The true molecular weight (M^+ 360, $C_{18}H_{16}O_8$) of versiconol was only obtained by field desorption ionisation techniques. Aucamp and Holzapfel[9] furthermore based their structural postulate on the observation that only four protons exchanged for deuterium as determined by mass spectrometry. This observation, however, can be explained by the facile loss of water under these conditions leading to a tetrahydrofuranyl derivative (*m/z* 342) containing only four exchangeable protons. The analysed high field ^{1}H n.m.r. spectrum of versiconol is shown in Figure 1.



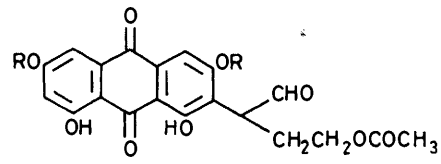
(5)



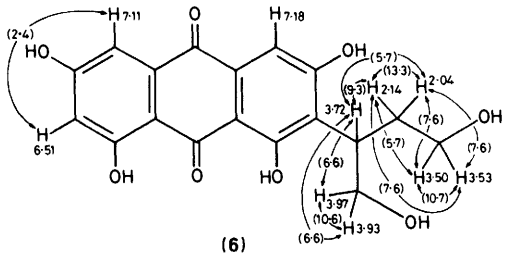
(6)



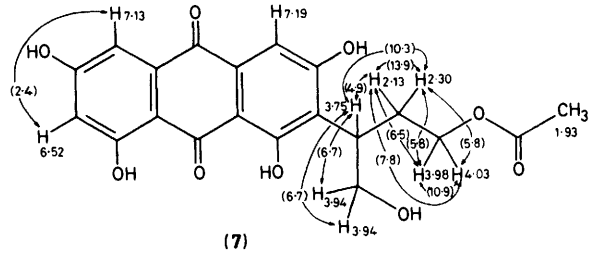
(7)



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



(6)



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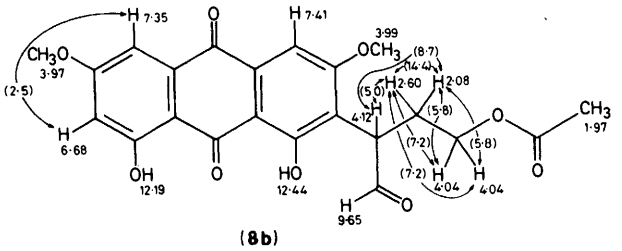


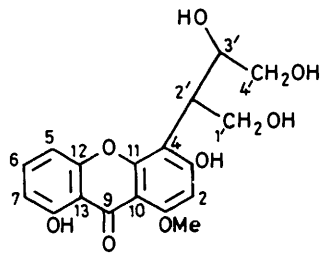
(8b)

Figure 1. The ^{1}H n.m.r. spectral data and proton-proton coupling constants of versiconol (**6**), versiconol acetate (**7**), and 3,6-*O*,*O*-dimethylversiconal acetate (**8b**)

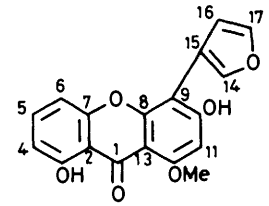
Maize cultures of *B. sorokiniana* were subsequently extracted and the pigment-containing fraction scrutinized for substances with characteristics to be expected for bipolarin; a reference sample of this metabolite was not available for direct comparison. A major pigment with the same i.r. spectrum, m.p. (mixed m.p. 264 °C), and mobility on several silica thin layer chromatographic systems as versiconol was obtained. No other substance resembling bipolarin was obtained. The foregoing information proves that the pigment called bipolarin was incorrectly formulated by Aucamp and Holzapfel[9] and that it was identical with the subsequently discovered versiconol (**6**).[5,11]

The 500 MHz ^{1}H n.m.r. spectral data of versiconol acetate (**7**) are shown in Figure 1. The parameters are very similar to those of versiconol (**6**), except for 3'-H which shows the expected downfield shift ($\Delta\delta$ −0.49 p.p.m.). Versiconal acetate (**8a**) was converted into 3,6-*O*,*O*-dimethylversiconal acetate (**8b**) by treatment with ethereal diazomethane in order to suppress acetal formation involving the C-1 and C-3 hydroxy groups. In deuteriochloroform (Figure 1), no hemiacetal formation was observed; the same was found in our earlier ^{13}C n.m.r. studies.[5] The lack of (^{1}H, ^{1}H) coupling between the benzylic proton and the aldehydic proton indicates a dihedral relationship of close to 90°.[12] A poorly resolved ^{1}H n.m.r. spectrum of (**8b**) was obtained in [$^2H_6$]dimethyl sulphoxide. The resonance due to the aldehyde proton was absent and the hemiacetal proton appeared as a broad hump around δ_H 6.1.

A new xanthone (**9**), $C_{18}H_{18}O_8$ (M^+ 362), was obtained in very low yield from the mycelial mat of *B. sorokiniana*. The light yellow substance had m.p. 235 °C, $\lambda_{max.}$ (MeOH) 230, 247, and 325 nm (log ε 4.43, 4.45, and 4.16, respectively), and strong i.r. absorptions at $\nu_{max.}$ 1 645, 1 607, and 1 580 cm^{-1}. These spectral properties and the co-occurrence of (**9**) with sterigmatocystin



(9)



(10)

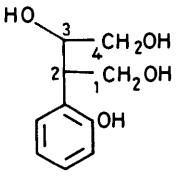
(**4**) indicate their close structural relationship. Its ^{1}H n.m.r. spectrum supports the presence of the xanthone part of the molecule, *viz.* δ_H 7.54 (6-H, t, *J* 8.2 Hz), 6.92 (5-H, d, *J* 8.1 Hz), 6.68 (7-H, d, *J* 8.1 Hz), 6.43 (2-H, s), and 3.90 (1-OMe, s). The aliphatic part of the molecule was not well resolved in the ^{1}H n.m.r. spectrum and was partially obscured by the methanol resonances.

The lack of sufficient quantities of the xanthone (**9**) precluded the unambiguous assignment of its ^{13}C n.m.r. signals. The assignments of the xanthonyl part of the molecule are based on its broad-band proton-decoupled and off-resonance proton-decoupled data and a comparison with the ^{13}C resonances of sterigmatocystin (**4**)[13] and isosterigmatocystin (**10**) (see Table). The assignments of the ^{13}C signals of isosterigmatocystin are based on the known chemical shifts for this group of compounds,[1,13] on deuterium isotope shifts obtained in the broad-band proton-decoupled ^{13}C n.m.r. spectrum when the exchangeable protons had been partially exchanged with deuterium,[14] and by selective population inversion (S.P.I.) experiments.[15]

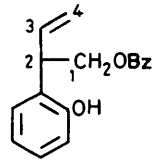
Table. 125.76 MHz ^{13}C N.m.r. data for sterigmatocystin (**4**), isosterigmatocystin (**10**), and 3,8-dihydroxy-4-(2,3-dihydroxy-1-hydroxymethylpropyl)-1-methoxyxanthone (**9**)

Carbon atom	(4) δ_C[a]	(10) δ_C[b]	(10) J(C,H)(Hz)	(9) δ_C[c]
1	181.08s	180.38s		183.21s
2	108.87s	107.88s		109.53s[d]
3	162.22s	161.05s		165.63s[e]
4	111.10d	109.99d	163.1	111.26d
5	135.48d	135.61d	161.2	136.89d
6	105.75d	105.95d	166.6	107.36d
7	154.83s	154.34s		159.31s[f]
8	153.87s	155.41s		156.39s[f]
9	106.46s	100.10s		106.46s[d]
10	164.45s	162.05s		162.85s[e]
11	90.43d	95.54d	162.0	97.86d
12	163.17s	159.86s		162.22s[e]
13	105.85s	103.79s		105.67s[d]
14	113.22d	142.12d	207.4	62.30t
15	47.98d	115.13s		42.03d
16	102.43d	112.24d	177.1	72.67d
17	145.25d	141.48d	203.4	66.02t
18	56.67q	55.74q	145.2	56.46q

[a] δ_C in p.p.m. relative to Me_4Si; solvent $CDCl_3$. Letters refer to the pattern resulting from one-bond (C,H) couplings; s = singlet, d = doublet, t = triplet, and q = quartet. [b] Solvent $(CD_3)_2SO$. [c] Solvent CD_3OD. The numbering system differs from that used for the name [shown in structure (**9**)] for ease of comparison with compounds (**4**) and (**10**). [d-f] Signals may be interchanged.



(**11**)



(**12**)

The ^{13}C n.m.r. signals of the aliphatic side-chain of (**9**) were assigned by comparison with the signals of (**11**), a diastereoisomeric mixture obtained from the osmium tetraoxide oxidation of (**12**) and a subsequent debenzylation reaction.[16] Two sets of peaks in the ratio 2:1 were obtained for the carbon atoms. From the chemical shift values of the ^{13}C resonances of the carbon atoms of the side-chain, *viz.* δ_C 70.78 (73.26) [C-3], 64.25 (64.60) [C-4], 61.33 (62.10) [C-1], 44.89 (44.03) p.p.m. [C-2], it was evident that the chemical shifts of the minor diastereoisomer, indicated in brackets, agree fairly closely with those of the natural product (**9**) (see Table).

The high resolution mass spectrum of compound (**9**) was quite informative. Major peaks were obtained at (i) *m/z* 362.101 (M^+. Calc. for $C_{18}H_{18}O_8$: 362.100), (ii) 301.071 (Calc. for $C_{16}H_{13}O_6$: 301.071), (iii) 283.060 (Calc. for $C_{16}H_{11}O_5$: 283.060), and (iv) 255.060 (Calc. for $C_{15}H_{11}O_4$: 255.065). The fragments (ii)—(iv) support the proposed structure of (**9**) (see Figure 2). Compound (**9**) seems to be derived from the enzymatic degradation of sterigmatocystin (**4**).

Aflatoxin biosynthesis is restricted to the moulds *Aspergillus flavus* and *Aspergillus parasiticus* only. Versiconal acetate (**8a**) and versiconol acetate (**7**) were previously obtained only upon treatment of the cultures of these fungi with the enzyme inhibitor, dichlorvos [*O,O*-dimethyl-*O*-(2,2-dichlorovinyl) phosphate].[5] This first report on their production under natural conditions strongly supports their intermediacy in sterigmatocystin and aflatoxin biosynthesis. The nature and mechanism of the conversion of averufin into aflatoxin *via* versiconal acetate was recently studied in detail.[7,8,17,18] The simultaneous production of averufin, versiconal acetate, versiconol acetate, versicolorin C, and sterigmatocystin emphasises their role in sterigmatocystin and aflatoxin biosynthesis. This is consonant with the observed distribution of acetate-derived labels in these substances, a finding which indicates a common polyketide folding pattern, and by their conversion into aflatoxin B_1 using wild-type strains of *A. parasiticus*.

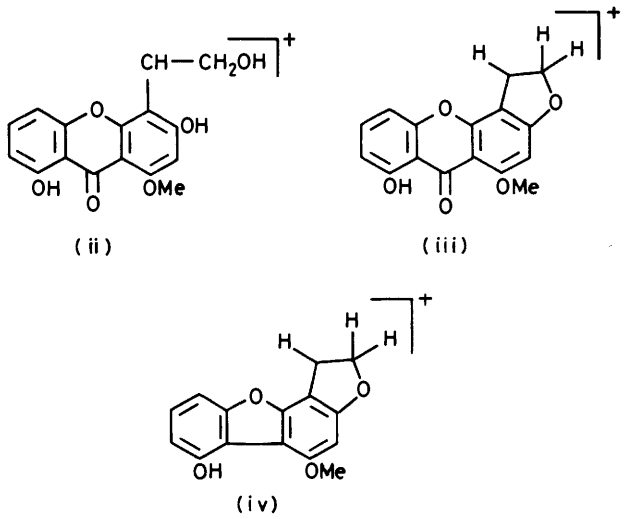


Figure 2. Major mass spectral fragments of compound (**9**)

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 8-100 spectrophotometer. I.r. spectra were recorded on a Perkin-Elmer 237 spectrometer. Mass spectra were recorded on a Varian MAT 212 spectrometer. ^1H and ^{13}C N.m.r. spectra were recorded on a Bruker WM-500 spectrometer. Merck silica gel (0.063—0.200 mm) was used for column chromatography, and Merck silica gel t.l.c. plates ($60F_{254}$, 0.25-mm thickness) were used for t.l.c. purifications.

Isolation of the Metabolites.—Bipolaris sorokiniana (MRC 93) was grown in stationary culture (25 °C) in Erlenmeyer flasks (350 × 500 ml) on potato dextrose broth (100 ml). After 14 days the mycelial mats were extracted with acetone and the solvent evaporated. The residue (42 g) was partitioned between hexane and 90% methanol, and the 90% methanol residue was partitioned between chloroform and water. The chloroform layer gave a residue (12 g). Extraction of the water layer with ethyl acetate gave a residue (1.3 g). The above material from the chloroform extraction was separated by column chromatography on silica gel (1 kg) using chloroform with an increasing percentage of methanol. This procedure yielded a mixture of sterigmatocystin and bipolaramide and a number of pigments. The mixture of sterigmatocystin and bipolaramide was separated by column chromatography on silica gel using benzene–acetone (4:1 v/v); crystallization from acetone gave sterigmatocystin, m.p. 244 °C (lit.,[19] 246 °C) (323 mg) and bipolaramide, m.p. 296 °C (lit.,[10] 296—297 °C) (180 mg).

The pigments were purified by thin layer chromatography

using mixtures of chloroform and methanol, and crystallized from methanol–chloroform to give: averufin, m.p. 282 °C (lit.,[9] 280 °C) (60 mg); versicolorin C, m.p. 305 °C (decomp.) (lit.,[9] 320 °C) (27 mg); versiconol acetate, m.p. 225—228 °C (lit.,[5] glass) (45 mg); versiconal acetate, an amorphous solid (lit.,[5] m.p. 234—236 °C) (110 mg); and versiconol, m.p. 264 °C (lit.,[9] 261 °C and [5] 257—259 °C) (83 mg). All the above compounds were also characterized by direct comparison with available reference substances.

The material (1.3 g) obtained from the above ethyl acetate extraction was separated on silica gel (200 g) in mixtures of chloroform and methanol. The new compound, 3,8-*dihydroxy*-4-(2,3-*dihydroxy*-1-*hydroxymethylpropyl*)-1-*methoxyxanthone* was crystallized from methanol–chloroform to give light yellow crystals (9 mg), m.p. 235 °C. (Found: M^+, 362.102. $C_{18}H_{18}O_8$ requires M, 362.100); $\lambda_{max.}$ 325, 247, and 230 nm (log ε 4.16, 4.45, and 4.43); $\nu_{max.}$ 1 645, 1 607, 1 580, 1 465, 1 272, 1 238, 1 210, 1 102, 1 064, 1 058, and 823 cm^{-1}.

Extraction of Versiconol from Maize.—*Bipolaris sorokiniana* (MRC 93) was grown in bulk on wet sterilized maize meal for 20 days. The dried, milled, mouldy maize (2 kg) was extracted with chloroform–methanol (1 : 1 v/v) for 24 h. The crude extract (170 g) was partitioned between hexane and 90% methanol and the 90% methanol residue was separated between chloroform and water. The chloroform residue (9.5 g) was fractionated by column chromatography on silica gel (1 kg) using chloroform with an increasing percentage of methanol. This procedure yielded bipolaramide, sterigmatocystin, averufin, and versiconol. The versiconol-containing fraction was dissolved in chloroform (100 ml) and extracted twice with 0.5M-Na_2CO_3 (40 ml). The aqueous Na_2CO_3 layer was acidified to pH 3 with dilute hydrogen chloride and extracted with chloroform. This organic material was further purified by preparative thin layer chromatography and crystallized from methanol–chloroform to give versiconol (12 mg), m.p. 264 °C. A mixture of crystals of versiconol obtained from the solid medium and reference material (versiconol) gave a mixed m.p. of 264 °C.

Isosterigmatocystin.—Sterigmatocystin (300 mg) in 15% ethanolic KOH (100 ml) was heated under reflux (120 °C) for 8 h. Work-up [20] gave light yellow crystals of isosterigmatocystin (255 mg), m.p. 233—235 °C (lit.,[20] 233—234 °C).

Methylation of Versiconal Acetate with Diazomethane.—Versiconal acetate (100 mg) in acetone (20 ml) was treated with an excess of cold (0 °C) ethereal diazomethane for 5 min. Work-up [5] gave a mixture of products which was purified by column chromatography on silica gel (100 g) using chloroform. The major product was further purified by thin layer chromatography using chloroform–methanol (99:1 v/v). Crystallization from methanol–chloroform gave 3,6-*O*,*O*-dimethylversiconal acetate (65 mg), m.p. 105 °C (lit.,[5] 96—98 °C).

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