to NMR. No trace of the other diastereomer could be detected, so we assumed that the reduction of **7a** would yield enantiomerically pure isoxazoline **8** (R = Me). The reduction product of **7a** (10.71 mg) was dissolved in dry ethanol and diluted to 5 mL (2.14 g/mL ethanol). In a 10-cm cell this solution gave α -0.480°, which gives $[\alpha]^{25}_D$ -224.1°. The cycloadducts produced in entry 5 were separated into two fractions of regioisomers by flash chromatography. The major fraction contained the diasteromeric pair **6a + 6b**, and no trace of the other regioisomer could be detected by NMR. The reduction product of this fraction gave $[\alpha]^{25}_D$ -139.7° (2.46 mg/mL ethanol), corresponding to an ee of 62.3% assuming a linear relationship. The optical purity of the reduction product of **5a + 5b** was established in the same way; $[\alpha]^{25}_D$ -18.6° (1.88 mg/mL ethanol) gave an ee of 8.3%.

X-ray Crystallography of 7a. Crystals were obtained from a 1:1 ether/pentane solution, and a crystal with dimensions 0.36 × 0.16 × 0.13 mm was used for data collection on an Enraf-Nonius CAD4F-11 diffractometer. The angular settings of 25 reflections were measured to calculate the lattice parameters. Intensity data for reflections with $\theta < 60°$ were collected by the $\theta/2\theta$ scan method using monochromated Cu Kα radiation. Three intensity control reflections, which were measured every 2 h, indicated a slight decay (4%) of the crystal. The measured intensities were rescaled to account for this decay. A total of 1682 unique reflections were recorded and, of these, 1503 reflections with $I > 3\sigma(I)$ were considered observed. All intensities were corrected for Lorentz and polarization effects, but not for absorption or extinction. Crystal data: $C_{26}H_{31}NO_3$, M = 405.54, trigonal, space group $P3_2$, $a = b$ = 11.692 (3) Å, c = 14.357 (5) Å, V = 1699.8 Å^3, d_{calcd} = 1.188 g cm^{-3}, Z = 3.

The structure was solved with the program MITHRIL,[22] which provided the non-hydrogen atom positions (Table II, Supplementary Material). Methyl hydrogen positions were determined from Fourier difference synthesis maps, and the remaining hydrogen atoms were included at expected positions. Refinement was carried out by the full-matrix least-squares method using anisotropic temperature factors for the non-hydrogen atoms. The hydrogen atoms were assigned a temperature factor equal to the U_{eq} value of their parent atoms. The hydrogen atom parameters were not refined.

After refinement the R and R_w values were 0.058 and 0.085, respectively. The weighting scheme used in the later part of the refinement was $w = 1/(1 + ((|F_{ob}| - 7)/5)^2)$. The form factors used were those given by Cromer and Mann.[23] All calculations were performed on a DEC system-10 computer mainly using the X-RAY 72 program system.[24]

Supplementary Material Available: Atom-numbering scheme for **7a** (Figure 4), positional and thermal parameters for **7a** (Tables II and III), and bond lengths and bond angles for **7a** (Table IV) (4 pages). Ordering information is given on any current masthead page. The structure factor table is available from the Department of Structural Chemistry.

(22) Gilmore, C. J. *J. Appl. Crystallogr.* **1984**, *17*, 42.
(23) Cromer, D. T.; Mann, J. B. *Acta Crystallogr., Sect. A* **1968**, *A24*, 321.
(24) Stewart, J. M.; Kruger, G. J.; Ammon, H. L.; Dickinson, C.; Hall, S. R. "The X-ray system" (version of June 1972), Technical Report TR-192, Computer Science Center, University of Maryland, College Park, MD, 1972.

Hydroxyversicolorone: Isolation and Characterization of a Potential Intermediate in Aflatoxin Biosynthesis

Craig A. Townsend,* Kathleen A. Plavcan, Kollol Pal, and Susan W. Brobst

Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

Michael S. Irish, E. Wesley Ely, Jr., and Joan W. Bennett

Department of Biology, Tulane University, New Orleans, Louisiana 70118

Received June 17, 1987

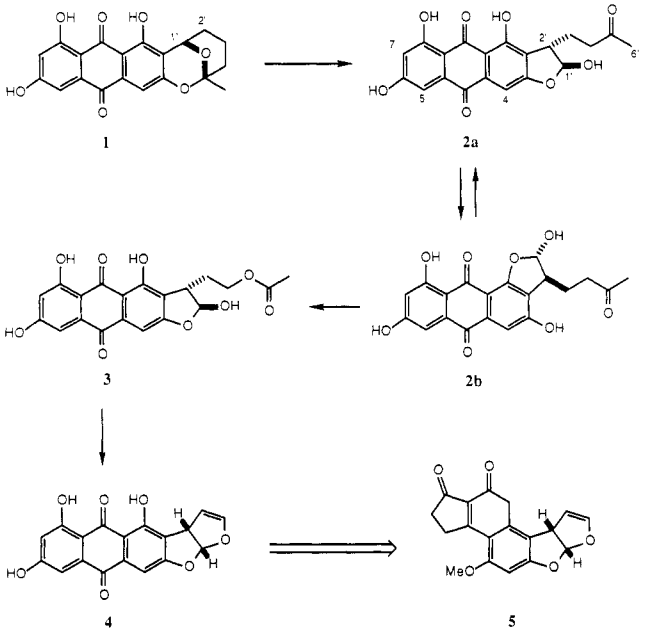
Mutagenesis of aflatoxigenic, wild-type *Aspergillus parasiticus* (SU-7) is described to afford a new mutant that accumulates two polar anthraquinone metabolites in a 20:1 ratio. These pigments were isolated and characterized as 1'-hydroxyversicolorone (**2**) and versicolorone (**10**), respectively, by spectroscopic and chemical means. The potential role of hydroxyversicolorone as the product of the first oxidative step of three in the conversion of the side chain of averufin (**1**) to the dihydrobisfuran of versicolorin A (**4**) and aflatoxin B_1 (**5**) is discussed.

Pigmented mutants generated from wild-type aflatoxigenic strains of *Aspergillus parasiticus* have played an essential role in investigations of the aflatoxin biosynthetic pathway.[1-4] In particular, tetrahydroxyanthraquinone derivatives isolated from these mutants in radioactive form have been shown not only to label aflatoxin B_1 (**5**) but also to incorporate radiolabel in a fixed sequence among themselves.[4-7] Central among these is averufin (**1**), which in specifically labeled forms has given intact incorporations of marker into aflatoxin and, in turn, has allowed the complete mapping of the carbon skeleton from this central intermediate into the highly rearranged coumarin nucleus of **5** bearing fused cyclopentenone and dihydrobisfuran rings.[8]

(1) Donkersloot, J. A.; Mateles, R. I.; Yang, S. S. *Biochem. Biophys. Res. Commun.* **1972**, *47*, 1051-1055.
(2) Lee, L. S.; Bennett, J. W.; Goldblatt, L. A.; Lundin, R. E. *J. Am. Oil Chem. Soc.* **1971**, *48*, 93-94. Detroy, R. W.; Fieser, S.; Ciegler, A. *Can. J. Microbiol.* **1973**, *19*, 1373-1378.
(3) Lee, L. S.; Bennett, J. W.; Cucullu, A. F.; Stanley, J. B. *J. Agric. Food Chem.* **1975**, *23*, 1132-1134.
(4) Bennett, J. W.; Lee, L. S.; Shoss, S. M.; Boudreaux, G. H. *Appl. Environ. Microbiol.* **1980**, *39*, 835-839.
(5) Singh, R.; Hsieh, D. P. H. *Arch. Biochem. Biophys.* **1977**, *178*, 285-292. Hsieh, D. P. H.; Singh, R.; Yao, R. C.; Bennett, J. W. *Appl. Environ. Microbiol.* **1978**, *35*, 980-982 and references cited.
(6) Hsieh, D. P. H.; Lin, M. T.; Yao, R. C.; Singh, R. *J. Agric. Food Chem.* **1976**, *24*, 1170-1174.
(7) For reviews, see: Bennett, J. W.; Christensen, S. B. *Adv. Appl. Microbiol.* **1983**, *29*, 53-92. Heathcote, J. G.; Hibbert, J. R. *Developments in Food Science, Vol. 1, Aflatoxins: Chemical and Biological Aspects*; Elsevier: Amsterdam, 1978; pp 151-172.

0022-3263/88/1953-2472$01.50/0

Scheme I



On the basis of the results of experiments examining the mode of bisfuran formation from the six-carbon side chain of averufin (**1**), we proposed,[9] but could not directly prove, an oxidative migration of the anthraquinone nucleus from C-1′ to C-2′ to give hydroxyversicolorone (**2**), a previously unknown compound (Scheme I). The latter was suggested to undergo Baeyer–Villiger-like oxidation to afford versiconal hemiacetal acetate (**3**), a pigment known to accumulate in the wild-type strain in the presence of dichlorvos.[10] A third oxidative transformation then would carry **3** to versicolorin A (**4**), apparently the first dihydrobisfuran-containing intermediate of the pathway. In this paper we describe the isolation and characterization of hydroxyversicolorone (**2**) from a new mutant of *A. parasiticus* (WE-47), which we have designated *hvn-1*.

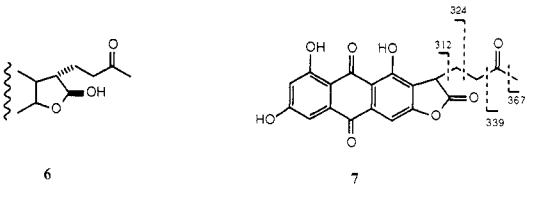
Spores of wild-type *A. parasiticus* (SU-7) were mutagenized by UV irradiation in four experiments where the fraction of survivors ranged from 1% to 10%.[11] Presumptive mutants (122) were selected and screened on the basis of altered fluorescence when grown on an aflatoxin-producing-ability medium.[12] Of these, eight exhibited unusual chromatographic profiles when crude extracts were examined by thin-layer chromatography in a standard battery of solvent systems. Two of the presumptive mutants produced an orange fluorescent spot that did not correspond in mobility to anthraquinones previously associated with the aflatoxin biosynthetic pathway. One of these (WE-47, *hvn-1*) was grown in stationary cultures of liquid defined medium[13] to obtain 153 g (wet weight) of nonsporulating mycelia.

The filtered mycelia were extracted with acetone using a blender. The crude extract was concentrated and extracted with pentane. Flash chromatography of the remaining orange solid afforded fractions containing two polar compounds: components A (5%) and B (95%). The major component was further purified by recrystallization. The characterization of this compound is now described.

Component B, an orange solid, gave ultraviolet and infrared spectra consistent with a 1,3,6,8-tetrahydroxyanthraquinone structure.[14] High-resolution mass spectral data indicated the molecular formula $C_{20}H_{16}O_8$. The 400-MHz ^1H NMR spectrum of B showed three intramolecularly hydrogen-bonded hydroxyl groups at δ 13.38, 12.53, and 12.20 with widths at half-height ($\Delta\nu_{1/2}$) of 1.9, 17.1, and 13.8 Hz respectively. Three additional hydroxyl groups appeared as a very broad signal (δ 11.09). A doublet at δ 7.73, exchangeable with deuterium oxide, indicated a secondary hydroxyl group. Irradiation of this resonance caused the collapse of a signal at δ 5.89, assigning the methine proton of the secondary hydroxyl group at a chemical shift consistent with that expected for a hemiacetal function. Decoupling of this methine proton caused sharpening of the δ 3.09 resonance. Further irradiations attributed the δ 2.56, 1.94, and 1.68 multiplets and the δ 2.07 methyl group to a 2-butanone fragment. These data suggested the partial structure **6**.

The remaining aromatic resonances were assigned to the three hydrogens of the anthraquinone nucleus. It was evident from both the integration and number (six) of these peaks, and the six phenolic hydroxyl groups, that component B consisted of a 1:1 mixture of two anthraquinones. In fact, it appeared likely that the natural product is a mixture of linear and angular hemiacetal forms (**2a** and **2b** respectively), consistent with the presence of three hydrogen-bonded hydroxyl groups. The aromatic resonance at δ 7.26 (1 H, s) was attributed to H-4 of the angular isomer (**2b**) which, owing to the 1-*O*-substitution pattern, exhibits a characteristic downfield shift relative to H-4 of the linear isomer, **2a** (δ 7.11).[15] Distinct resonances were seen for H-5 and H-7 representing the linear and angular forms of **2**. These were assigned on the basis of chemical shift comparisons and with reference to acetal **8** (see below). These pairs of assignments were confirmed by homonuclear decoupling experiments.

The postulated structures were supported by the mass spectral fragmentation pattern, which showed a weak parent ion at m/z 384 and a base peak corresponding to loss of water (m/z 366). The remaining major fragments arise from an $(M - 2)^+$ peak (m/z 382), presumably due to formation of lactone **7**, with the m/z 324 and 312 peaks derived from McLafferty rearrangements. These fragmentations are shown in structure **7**.



It was anticipated that derivatization would simplify the spectral data and firmly establish the proposed structures. Treatment of the regioisomeric mixture (**2a** and **2b**) in methanol with camphorsulfonic acid afforded a single product as evidenced by simplification of the NMR

(8) Townsend, C. A.; Christensen, S. B.; Davis, S. G. *J. Am. Chem. Soc.* **1982**, *104*, 6152–6153. Simpson, T. J.; de Jesus, A. E.; Steyn, P. S.; Vleggaar, R. *J. Chem. Soc., Chem. Commun.* **1982**, 631–634. Townsend, C. A.; Davis, S. G. *J. Chem. Soc., Chem. Commun.* **1983**, 1420–1422.

(9) Townsend, C. A.; Christensen, S. B. *Tetrahedron* **1983**, *39*, 3575–3582. Townsend, C. A. *Pure Appl. Chem.* **1986**, *58*, 227–238.

(10) Cox, R. H.; Churchill, F.; Cole, R. J.; Dorner, J. W. *J. Am. Chem. Soc.* **1977**, *99*, 3159–3161. Fitzell, D. L.; Singh, R.; Hsieh, D. P. H.; Motell, E. L. *J. Agric. Food Chem.* **1977**, *25*, 1193–1197 and references cited.

(11) Bennett, J. W.; Goldblatt, L. A. *Sabouraudia* **1973**, *11*, 235–241.

(12) Hara, S.; Fennell, D. I.; Heseltine, C. W. *Appl. Microbiol.* **1974**, *27*, 1118–1123.

(13) Adye, J.; Mateles, R. I. *Biochim. Biophys. Acta* **1964**, *86*, 418–420.

(14) Aucamp, P. J.; Holzapfel, C. W. *J. S. Afr. Chem. Inst.* **1970**, *23*, 40–56. Bullock, E.; Kirkaldy, D.; Roberts, J. C.; Underwood, J. G. *J. Chem. Soc.* **1963**, 829–835.

(15) Townsend, C. A.; Davis, S. G.; Christensen, S. B.; Link, J. C.; Lewis, C. P. *J. Am. Chem. Soc.* **1981**, *103*, 6885–6888.

Table I. Carbon Chemical Shift Assignments of 2a, 2b, and 8

carbon atom[a]	8[b]	2a[c]	2b[c]	3 (linear)[d]	3 (angular)[d]
5′	207.5	207.7	–	170.0	–
9	189.3	189.0	184.7	188.8	184.6
10	180.8	180.8	181.5	180.5	181.5
6	(165.2)	(164.9, 164.2, 164.1, 164.0)		164.9	(163.9)
8	(164.25)			164.1	–
3	(164.1)	(163.6)	160.8	163.7	160.8
1	159.2	159.1[f]	159.0	159.6	158.9
14	(135.5)	(135.2,[f] 134.7, 134.5, 134.4)		134.8	134.4
11	(134.8)			134.6	(134.7)
2	121.5	121.5[f]	122.7	120.3	122.3
4	113.4	108.7	108.8	103.1	103.6
13	111.1	110.5[f]	109.4	110.0	109.6
5	108.9	108.7	107.1	108.7	107.1
12	108.5	108.4	–	108.3	–
7	108.0	107.9	–	107.8	–
1′	102.5	102.4[f]	107.7	n.o.[g]	–
OMe	55.9				
2′	45.1	46.2[f]	45.4	43.7	–
6′	29.7	29.8	–	20.6	–
3′	23.9[e]	24.1[e]	–	27.7	–
4′	23.9[e]	24.1[e]	–	61.7	–

[a] All chemical shift values are for DMSO-d_6 solutions using the central resonance of the solvent as reference: δ 39.5. [b] Concentration ca. 25 mg/3 mL. [c] Concentration ca. 100 mg/3 mL. [d] Taken from Steyn et al. ref 16, corrected as noted in *a*. [e] Signals are isochronous. [f] This resonance is broad at this concentration but becomes sharp at lower concentration. [g] Not observed.

spectrum. Formation of the methyl acetal was confirmed by the appearance of a three-proton singlet at δ 3.48 and the absence of the 1′-OH resonance in the ^{1}H NMR spectrum. Moreover, only one set of anthraquinone methine resonances (δ 6.60, 7.13, and 7.17 for H-7, H-5, and H-4, respectively) and three phenolic hydroxyl groups (δ 12.10, 12.43, both intramolecularly hydrogen bonded, and 11.36) were evident. The chemical shift of the singlet attributed to H-4 (δ 7.17) corresponds to the H-4 resonance of the linear hemiacetal isomer. The H-1′ methine (δ 5.67) showed a clean doublet (J = 2.0 Hz), indicating a trans disposition of the 1′-methoxyl group and the butanone side chain. Homonuclear decoupling as above unambiguously supported the presence of the 2-butanone side chain bound at C-2′. Inter alia irradiation of H-2′ (δ 3.29) collapsed the H-1′ doublet to a singlet and each of the well-separated multiplets for the diastereotopic H-3′ methylene hydrogens to doublets of triplets.

The mass spectral fragmentation pattern lacked a parent ion but showed a peak corresponding to the loss of methanol (m/z 366). The other major fragments were attributed to loss of acetyl (m/z 323) and subsequent methylene loss (m/z 309) from the m/z 366 peak. All of these data are consistent with structure 8.

Assignments of the carbon NMR spectra of acetal 8 and the linear and angular forms of hydroxyversicolorone, **2a** and **2b**, are shown in Table I. These were made with reference to the extensive and careful work of Steyn et al.[16] on versiconal acetate (also shown in Table I) and related structures in this series. As the methyl acetal 8 existed in one regioisomeric form, an APT (Attached Proton Test[17]) spectrum was run first and led easily to assignment of the methoxyl, the side-chain carbons C-2′–C-6′, and the four midfield resonances for C-1′, C-4, C-5, and C-7. The remainder of the carbons in 8 have no directly bound hydrogen and are more difficult to assign. However, the South African group has observed[16] that the positions of C-5–C-12 are substantially invariant as a function of alterations in the side chain across the range of known *Aspergillus* 2-substituted tetrahydroxyanthraquinone metabolites. Comparisons, therefore, to versiconal acetate (**3**, Table I) led to the further assignments shown for 8 in Table I with the only ambiguities being the cluster of phenolic carbons at 164–165 ppm (C-3, C-6, and C-8) and the pair of peaks at δ 134.8 and 135.5 attributable to C-11 and C-14.

Turning to the 1:1 mixture of regioisomeric forms of hydroxyversicolorone (**2a** and **2b**), an APT spectrum[17] and a proton/carbon correlation[18] led directly to assignments of the side-chain carbons C-2′–C-6′ and to designation of the linear and angular forms of C-1′, C-4, C-5, and C-7. These agreed remarkably well with the corresponding chemical shift assignments for the linear and angular forms of versiconal acetate (**3**, Table I) made by Steyn et al.[16] and further confirmed the assignments made for 8. No doubling of most of the side-chain-carbon resonances (C-3′–C-6′) was detected, paralleling earlier observations with versiconal acetate. However, unlike the reported data[16] for **3**, resonances were readily seen for C-1′ in both regioisomeric forms of **2**. For reasons that are not clear, the signal for the linear form was broad (δ 102.4), but sharpened at lower concentration. Further assignments of **2a** and **2b** were made by comparison to 8 and **3** as above. The concentration-dependent peak broadening noted for C-1′ of **2a** was also observed for five other resonances, those assigned to C-1, C-2, C-2′, C-13, and probably C-14 (δ 135.2), all of the linear isomer. As was the case for 8, some uncertainty remains in assigning the phenolic carbons (C-3, C-6, and C-8), where five lines fall in a tight cluster, and similarly for C-11 and C-14.

Dehydration of the natural hemiacetal mixture (**2a** and **2b**) gave a single product. The ^{1}H NMR spectrum of this compound also exhibited only three phenolic hydroxyl groups (δ 11.40, 12.15, and 13.21), and a single set of anthraquinone resonances was observed (δ 6.62, 7.18, and 7.85). This region lacked the H-4 resonance of the angular anthraquinone isomer. A single vinyl ether resonance appeared as a singlet at δ 8.02 while the resonances corresponding to H-1′ and H-2′ of the starting material were no longer evident. The side-chain resonances (H-3′ and H-4′) simplified to first-order triplets [δ 2.91 (2 H, J = 7.1

(16) Compare: Steyn, P. S.; Vleggaar, R.; Wessels, P. L.; Cole, R. J.; Scott, D. B. *J. Chem. Soc., Perkin Trans. 1* **1979**, 451–459.

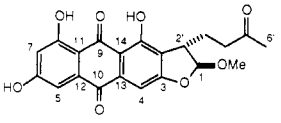
(17) Patt, S. L.; Shoolery, J. N. *J. Magn. Reson.* **1982**, *46*, 535–539 and references cited.

(18) Bax, A.; Morris, G. A. *J. Magn. Reson.* **1981**, *42*, 501–505. Bax, A. *J. Magn. Reson.* **1983**, *53*, 517–520.

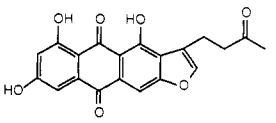
Hz) and 2.99 (2 H, J = 7.1 Hz)] owing to destruction of the chiral centers at C-1′ and C-2′.

A parent ion at m/z 366 in the electron-impact mass spectrum and the absence of McLafferty rearrangements supported structure **9**. High mass fragments were derived from this ion by loss of acetyl (C_2H_3O) and subsequent cleavage of a methylene unit (m/z 323 and 309, respectively).

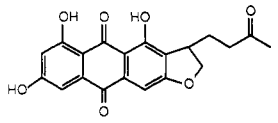
We propose the name hydroxyversicolorone for the natural mixture of compounds **2a** and **2b** by analogy to versicolorone (**10**), previously reported as a minor metabolite from *A. versicolor*.[19] It is interesting to note that both hydroxyversicolorone (**2**) and versiconal acetate (**3**) exist as an approximately 1:1 mixture of isomeric hemiacetals while the two derivatives **8** and **9** derived from **2** were isolated and observed by NMR spectroscopy as single isomers, like averufin (**1**). The strong hydrogen bond between the 1-OH and the anthraquinone carbonyl presumably accounts for the thermodynamic stability of the linear form.



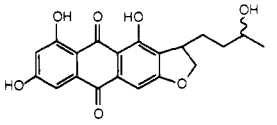
8



9



10



11

In order to characterize the minor component produced by this mutant, component A, we carried out a large-scale fermentation of *hvn-1*. After the usual isolation procedure, the crude acetone extract was chromatographed and the fraction containing hydroxyversicolorone (**2**) and component A was fractionally recrystallized from ethyl acetate/hexane to remove most of the hydroxyversicolorone. The component A enriched supernatant was purified further by using a Chromatotron and then recrystallized from acetone/water to give an orange powder.

^{1}H NMR data indicated three phenolic hydroxyl groups, two of which were hydrogen bonded (δ 12.82 and 12.18). The singlet (δ 7.19) and two mutually coupled doublets (δ 7.09 and 6.57) indicated the characteristic 1,3,6,8-tetrahydroxyanthraquinone system. A methyl ketone was apparent from a resonance at δ 2.06. Decoupling of the upfield resonances provided information concerning the remainder of the structure. Irradiation of the δ 3.38 resonance caused each of the doublets of doublets at δ 3.75 and 3.68 to collapse to doublets (J = 10.0 Hz), indicating two geminally coupled hydrogens. The chemical shift of these signals is characteristic of a methylene group adjacent to oxygen, and the 10-Hz geminal coupling constant is consistent with a methylene group adjacent to a hetero atom in a five- or six-membered ring. It was difficult to determine if this irradiation affected the δ 1.94 signal, and no change in either the δ 2.34 or 2.20 peaks was apparent. Irradiation of the δ 1.94 multiplet simplified each of the two doublets of triplets at δ 2.34 and 2.20 to doublets (J = 17.6 Hz). This result demonstrated the geminal relationship of the hydrogens that gave rise to the collapsed signals, which, in conjunction with their chemical shifts, indicated a methylene group adjacent to a carbonyl. The δ 1.94 two-proton multiplet was assigned, therefore, to the C-3′ methylene group.

The mass spectrum of component A fully supported its structural assignment as **10**. A parent ion was observed at m/z 368, and prominent fragments were evident at m/z 325 ($M^+ - CH_3CO$), 310 (McLafferty rearrangement), and 297 ($M^+ - CH_3CO - 2 \times CH_2$).

A compound characterized earlier as **10** has been isolated[19] from *A. versicolor* and named versicolorone (from which the name hydroxyversicolorone for component B was derived). While the UV and mass spectra reported by Berger[19] for **10** are largely in accord with those obtained for component A, there are discrepancies between the results obtained above and the NMR data reported by the Belgian group and their interpretation.[20] Notably, H-4 is reported to resonate at δ 7.88, an anomalously low field value in this structural series,[21] whereas this hydrogen was observed in the present study at δ 7.19. For reasons that are unclear, the signals we attribute to H-1′ and H-2′ have been reversed, as have the assignments of the C-3′ and C-4′ methylene resonances. Despite these differences in interpretation and small differences in observed chemical shifts, it seems likely that component A and versicolorone isolated by Berger are indeed the same.

To unambiguously secure the structure of component A as **10**, a sample of hydroxyversicolorone (**2**) was treated with sodium borohydride in water. A sample of versicolorone (**10**) was obtained in addition to small amounts of starting material and the overreduced product **11**. Purification by silica gel chromatography and crystallization from acetone/water gave a red-orange crystalline compound whose physical and spectral properties were identical with those of natural versicolorone (**10**, component A).

In conclusion, a new mutant (WE-47, *hvn-1*) has been generated from an aflatoxigenic strain of *A. parasiticus*. The principal polar anthraquinone metabolites have been characterized as 1′-hydroxyversicolorone (**2**) and versicolorone (**10**). The biosynthetic conversion of averufin (**1**) to aflatoxin B_1 (**5**) is held to involve the intermediacy of versicolorin A (**4**)[5-7] in which the anthraquinone remains intact but formation of the bisfuran is complete. The present isolation of **2** supports the proposed order[9] of stable intermediates in bisfuran formation as shown in Scheme I, each related to its immediate predecessor by an oxidative transformation. In separate studies, hydroxyversicolorone has been obtained by independent synthesis and found to be identical in all respects with the natural material. Experiments to determine its role as the first oxidative step in bisfuran formation beyond averufin will be reported elsewhere.[22]

Experimental Section

Infrared (IR) spectra were obtained on a Perkin-Elmer Model 599B spectrophotometer, and absorbances are reported relative to polystyrene. Ultraviolet (UV) spectra were measured on a Cary 219 double-beam spectrophotometer in ethanol. Nuclear magnetic resonance (NMR) spectra were recorded in dimethyl sulfoxide-d_6, except as noted, with a Varian XL-400 spectrometer; chemical shifts are reported relative to tetramethylsilane. Mass spectra (MS) were provided by the MIT mass spectrometry facility using

(19) Berger-Deguee, M.; Berger, Y. *Phytochemistry* **1982**, *21*, 1449–1451.

(20) Dr. Berger kindly responded to our request for a comparison sample or copies of the original 250-MHz ^{1}H NMR spectra, but unfortunately, he has moved from the University of Liege and the reference material and spectra are no longer available.

(21) Townsend, C. A.; Christensen, S. B.; Davis, S. G. *J. Chem. Soc., Perkin Trans 1*, in press.

(22) Townsend, C. A.; Whittamore, P. R. O.; Brobst, S. W. *J. Chem. Soc., Chem. Commun.*, in press.

a Varian MAT 731 high-resolution mass spectrometer, equipped with a combined EI/FI/FD source, or were obtained on a VG Micromass 70-S mass spectrometer at the Johns Hopkins University. Peaks greater than 10% are reported in the EI mass spectra. High-resolution fast-atom-bombardment mass spectra (HRFABMS) were obtained by using DMF and peak matching to 3-nitrobenzyl alcohol. Chemical-ionization spectra (CIMS) utilized ammonia as the ionizing gas.

High-pressure liquid chromatography (HPLC) was performed with a Perkin-Elmer Series 4 liquid chromatograph, a Perkin-Elmer LC85B spectrophotometric detector, and a Rheodyne injection system. Analyses were conducted with a Regis Val-U-Pak octadecylsilyl column (5 μm, 25 cm × 4.6 mm i.d.) eluted with water/acetonitrile (30:70). Preparative HPLC was done on a Regis octadecylsilyl column (10 μm, 25 cm × 21.1 mm i.d.) eluted with water/acetonitrile (60:40).

Melting points were measured on a Mel-Temp apparatus and are uncorrected. Elemental analyses were obtained from Galbraith Laboratories. Optical rotations were recorded on a Perkin-Elmer Model 141 polarimeter. All solvents were either spectral grade or distilled prior to use.

Generation and Fermentation of *Aspergillus parasiticus* Mutant WE-47 (*hvn-1*). The wild-type aflatoxigenic strain of *A. parasiticus* (SU-7) was a transfer of ATCC 15517. Throughout, mutant and wild-type cultures were grown at 27 °C in the dark. For mutagenesis, spore suspensions were prepared and irradiated with UV light as described earlier.[11] Prior to UV treatment, appropriately diluted spore suspensions were plated onto potato dextrose agar (Difco) supplemented with 0.5% yeast extract (PDA+YE); appropriately diluted aliquots of the irradiated spore suspension were plated onto "aflatoxin-producing-ability medium" (APA).[12] Viable counts on PDA+YE were recorded after 3–4 days, and mutagenized colonies were scored for fluorescence on APA after 9–12 days by scanning inverted Petri plates under long-wave UV. Presumptive mutants (122) were selected on the basis of the absence or alteration of fluorescent intensity as compared to the wild type. Presumptive mutants were subcultured onto PDA+YE and then tested by growth in 10 mL of the defined medium formulated by Adye and Mateles (AM)[13] for 7 days. The resultant mycelial pad was removed, blotted dry, and transferred to chloroform (2 mL) for 24 h to yield a crude mycelial extract. These extracts were spotted against authentic standards of aflatoxins B_1, B_2, G_1, G_2, norsolorinic acid, averufin, versicolorin A, versicolorin hemiacetal acetate (versiconal acetate), and sterigmatocystin on silica gel plates, and developed in two solvent systems (ether/methanol/water, 96:3:1, v/v/v, and benzene/acetic acid, 95:5, v/v). After developing, the plates were sprayed with 20% aluminum chloride in ethanol (wt/v) and heated for 5 min at 120 °C to detect the characteristic yellow color of sterigmatocystin.[23]

Eight mutants presented unusual chromatographic profiles and were selected for further analysis. Each was grown in AM medium (100 mL) for 7 days, and the mycelia were filtered through cheesecloth, washed with deionized water, resuspended in 500 mL of acetone, and sonicated for 10 min. The resultant mycelial slurry was filtered through a Büchner funnel and washed with acetone until colorless. The extract was reduced to approximately 300 mL in a rotary evaporator and then mixed with deionized water (100 mL) and dichloromethane/ether (400 mL, 4:1, v/v) in a separatory funnel. After shaking, the lower, pigmented layer was drawn off, the extraction was repeated twice, and the pigmented extracts were pooled and evaporated to dryness. The resultant residue was resuspended in dichloromethane/ether (4:1, v/v) and spotted for thin-layer chromatography against authentic standards as above. Plates were developed in five solvent systems: benzene/methanol/acetic acid (95:5:5, v/v/v), toluene/ethyl acetate/acetic acid (50:30:40, v/v/v), ether/methanol/water (96:3:1, v/v/v), chloroform/2-propanol (99:1, v/v), and chloroform/acetone/water (88:12:1.5 v/v/v). Two mutants (WE-11 and WE-47) yielded an orange fluorescent spot of unique chromatographic mobility. Bulk batches of mycelia of WE-47 grown for 7 days in stationary culture in AM medium (500 mL) were used for extraction of metabolites as described below.

(23) van Egmond, H. P.; Palsch, W. E.; Dejill, E.; Schuler, P. L. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 110–114.

Extraction of Mutant WE-47 (*hvn-1*). The medium was filtered, and the yellow mycelia were washed thoroughly with water. The wet mycelia (153.0 g) were pulverized in acetone by using a Waring blender until the extract obtained was nearly colorless and the cell debris was off-white. The mixture was filtered and the supernatant concentrated under reduced pressure and extracted with pentane to give a viscous orange pentane extract (2.15 g). The remaining orange solid was preadsorbed on silica gel and fractionated by column chromatography eluting with an ethyl acetate/hexane gradient (30–100% ethyl acetate, in increments of 25%). Elution with 50–100% ethyl acetate/hexane gave a mixture (168.8 mg) containing about 95% of component B (**2a/2b**) and 5% of minor component A. The column fractions were concentrated, and the residues were recrystallized from acetonitrile to yield hydroxyversicolorone (**2a/2b**) (92.5 mg, 0.1% of wet weight).

Hydroxyversicolorone (2a/2b). Hydroxyversicolorone was obtained as an orange powder from acetone: mp 247–249 °C; TLC (95:5 $CHCl_3/CH_3OH$) R_f 0.1; HPLC t_R (30:70 H_2O/CH_3CN) 7.0 min; $[\alpha]^{25}_D$ 0° (*c* 0.006, $CHCl_3$); IR ($CHCl_3$) 3390, 1705, 1600 (s), 1440, 1320, 970 cm^{-1}; UV λ_{max} 223 (ε 24 000), 265 (20 300), 293 (19 500), 316 (22 600), 476 (5300), 528 nm (4000); ^1H NMR (asterisk denotes angular isomer **2b**) δ 1.68 (1 H, m, H-3′), 1.94 (1 H, m, H-3′), 2.06 (3 H, s, H-6′), 2.56 (2 H, t, *J* = 6.6 Hz, H-4′), 3.09 (1 H, dt, *J* = 1.5, 6.3 Hz, H-2′), 5.89 (1 H, br dd, *J* = 5.9, 1.5 Hz, H-1′), 6.56 ($^1/_2$H, d, *J* = 2.2 Hz, *H-7), 6.58 ($^1/_2$H, d, *J* = 2.2 Hz, H-7), 7.06 ($^1/_2$H, d, *J* = 2.2 Hz, *H-5), 7.10 ($^1/_2$H, d, *J* = 2.2 Hz, H-5), 7.11 ($^1/_2$H, s, H-4), 7.26 ($^1/_2$H, s, *H-4), 7.73 (<1 H, br d, *J* = 5.9 Hz, 1′-OH), 11.09 ($^3/_2$H, br s, 3 (OH)), 12.20 ($^1/_2$H, br s, $\Delta\upsilon_{1/2}$ = 13.8 Hz, OH), 12.53 ($^1/_2$H, br s, $\Delta\upsilon_{1/2}$ = 17.1 Hz, OH), 13.38 ($^1/_2$H, s, $\Delta\upsilon_{1/2}$ = 17.1 Hz, OH), 13.38 ($^1/_2$H, s, $\Delta\upsilon_{1/2}$ = 1.9 Hz, 8-OH); ^{13}C NMR see Table I; EIMS, *m/z* (relative intensity) 384 (2), 382 (22), 368 (28), 367 (60), 366 (100), 347 (21), 340 (26), 339 (32), 324 (97), 313 (34), 312 (48), 311 (92), 310 (86), 309 (62), 299 (46), 298 (76), 297 (86), 296 (78), 285 (46), 270 (26), 269 (22), 245 (20), 155 (28), 70 (35), 61 (47); CIMS (NH_3), *m/z* (relative intensity) 385 ($(M + 1)^+$, 16), 369 (100); HRFABMS obsd *m/z* 385.0927 ($(M + 1)^+$), $C_{20}H_{17}O_8$ requires 384.0923.

Acetal 8. (1*R*)-(−)-10-Camphorsulfonic acid (10.0 mg, 0.04 mmol) was added to a slurry of hydroxyversicolorone (**2a/2b**) (42.5 mg, 0.11 mmol) in methanol (2 mL) with powdered 4-Å molecular sieves. After being stirred at 25 °C for 0.5 h, the mixture became homogeneous. Stirring was continued for 20 h, then the reaction mixture was filtered through cotton, and the methanol was removed under reduced pressure. The residue was partitioned between ethyl acetate (10 mL) and water (10 mL). The aqueous layer was extracted with ethyl acetate (2 × 10 mL), and the combined organic extracts were washed with water (10 mL) and brine (10 mL) and dried over anhydrous magnesium sulfate. Filtration and removal of the solvent afforded a crude product, which was purified by silica gel chromatography (20% ethyl acetate/hexane) and recrystallization (ether/pentane) to give an orange solid (35.6 mg, 81%): mp 230–232 °C; TLC (97:3 $CHCl_3/CH_3OH$) R_f 0.24; IR ($CHCl_3$) 1705, 1620, 1600 (s), 1355, 1310, 1260, 895 cm^{-1}; UV λ_{max} 222 (ε 20 900), 264 (15 100), 293 (14 500), 317 (17 700), 475 (4800), 529 nm (3300); ^1H NMR δ 1.74 (1 H, dddd, *J* = 7.7, 7.7, 8.0, 13.6 Hz, H-3′), 1.97 (1 H, dddd, *J* = 5.5, 7.7, 7.7, 13.6 Hz, H-3′), 2.50 (3 H, s, H-6′), 2.58 (2 H, t, *J* = 7.7 Hz, H-4′), 3.29 (1 H, ddd, *J* = 2.0, 5.5, 8.0 Hz, H-2′), 3.48 (3 H, s, 1′-OCH_3), 5.67 (1 H, d, *J* = 2.0 Hz, H-1′), 6.60 (1 H, d, *J* = 2.7 Hz, H-7), 7.13 (1 H, d, *J* = 2.7 Hz, H-5), 7.17 (1 H, s, H-4), 11.36 (1 H, s), 12.10 (1 H, s), 12.43 (1 H, s). Anal. Calcd for $C_{21}H_{18}O_8$: C, 63.31; H, 4.55. Found: C, 62.89; H, 4.97. The corresponding cis-fused isomer accounted for 10% of the material as evidenced by the resonance at δ 5.84 (d, *J* = 6.6 Hz, H-1′): ^{13}C NMR see Table I; EIMS, *m/z* (relative intensity) 368 (7), 367 (18), 366 (65), 324 (29), 323 (100), 309 (18), 297 (13), 163 (13), 97 (18), 95 (15), 91 (17), 85 (13), 84 (10), 83 (24), 81 (19), 79 (11), 73 (14), 71 (22), 69 (34), 67 (18), 60 (13), 57 (44), 56 (15), 55 (46), 45 (34), 43 (94), 41 (44), 39 (13); CIMS (NH_3), *m/z* (relative intensity) 399 ($(M + 1)^+$, 100), 385 (9), 367 (7).

Benzofuran 9. To a slurry of hydroxyversicolorone (**2**) (40.9 mg, 0.11 mmol) in benzene (1 mL) at 0 °C were added thionyl chloride (0.42 mmol) and pyridine (0.09 mL, 1.1 mmol), producing a brown heterogeneous mixture. The mixture was warmed to 25 °C, stirred for 3 h, poured into 10% aqueous hydrochloric acid

(10 mL), and extracted with ethyl acetate (3 × 10 mL). The organic layer was washed with water (3 × 10 mL) and brine (2 × 10 mL), dried over anhydrous magnesium sulfate, and filtered and the solvent removed in vacuo. The crude product was purified by silica gel chromatography (20% ethyl acetate/hexane) and recrystallization (ether/pentane) to yield an orange solid (12.8 mg, 33%): mp 271–273 °C; TLC (97:3 $CHCl_3/CH_3OH$) R_f 0.20; IR ($CHCl_3$) 1705, 1600 (s), 1330, 970 cm^{-1}; UV λ_{max} 268 (ϵ 20 100), 278 (20 700), 282 (22 100), 449 nm (7800); ^1H NMR δ 2.14 (3 H, s, H-6′), 2.91 (2 H, t, J = 7.1 Hz, H-3′ or H-4′), 2.99 (2 H, t, J = 7.1 Hz, H-4′ or H-3′), 6.62 (1 H, d, J = 2.5 Hz, H-7), 7.18 (1 H, s, H-4), 7.85 (1 H, d, J = 2.5 Hz, H-5), 8.02 (1 H, s, H-1′), 11.40 (1 H, s), 12.15 (1 H, s), 13.21 (1 H, s); DCI (no ionizing gas), m/z (relative intensity) 367 (9), 366 (37), 324 (26), 323 (98), 310 (10), 309 (9), 73 (18), 69 (10), 61 (11), 57 (10), 55 (10), 45 (13), 43 (100), 41 (8); HRMS obsd m/z 366.0749, $C_{20}H_{14}O_7$ requires 366.0740.

Synthetic Versicolorone. To a 500-mL flask equipped with a dropping funnel and reflux condenser were added hydroxyversicolorone (216 mg, 0.56 mmol) and enough tetrahydrofuran (100 mL) to dissolve the material. Water (200 mL) was added, and the mixture was cooled to 0 °C. An aqueous solution of sodium borohydride (21.7 mg/50 mL, 0.57 mmol) was added dropwise, giving a red solution upon addition. After 0.5 h, more sodium borohydride (12 mg, 0.31 mmol) was added, and the reaction mixture was stirred for an additional 1.5 h. The reaction was quenched with 10% aqueous hydrochloric acid and partitioned between ethyl acetate and water. The aqueous layer was reextracted with ethyl acetate, the combined organic extracts were washed with brine, dried over anhydrous magnesium sulfate, and filtered, and the solvent was removed under reduced pressure.

After purification by preparative TLC (40% acetone/hexane) and Chromatotron (50% ethyl acetate/hexane), the product was recrystallized (five times from acetone/water) and dried under vacuum: mp 208–210 °C; UV (EtOH) λ_{max} 222 (ϵ 26 700), 252 (13 300), 264 (16 500), 298 (17 700), 318 (26 400), 468 (6000), 483 nm (6400); IR ($CHCl_3$) 3020, 1700, 1600, 1185, 800, 670 cm^{-1}; ^1H NMR δ 1.94 (2 H, m, H-3′), 2.06 (3 H, s, H-6′), 2.20 (1 H, dt, J = 7.4, 17.6 Hz, H-4′), 2.34 (1 H, dt, J = 7.8, 17.6 Hz, H-4′), 3.38 (1 H, m, H-2′), 3.68 (1 H, dd, J = 6.6, 10.0 Hz, H-1′), 3.75 (1 H, dd, J = 7.6, 10.0 Hz, H-1′), 6.57 (1 H, d, J = 2.6 Hz, H-7), 7.09 (1 H, d, J = 2.6 Hz, H-5), 7.19 (1 H, s, H-4), 11.21 (1 H, br s, 6-OH), 12.18 (1 H, s, 8-OH), 12.82 (1 H, s, 1-OH); MS, m/z (relative intensity) 368 (28), 325 (6), 310 (100), 297 (19), 285 (6); HRMS obsd m/z 368.0905, $C_{20}H_{17}O_7$ requires 368.0896.

Natural Versicolorone (10). A large growth (16 L of medium) of mutant WE-47 was grown as standing cultures for 1 week. The mycelia were filtered, washed well with water, and pulverized in a blender with acetone. The residual cells were extracted with acetone until colorless. The solvent was removed, and the material was extracted with pentane. The residue was preadsorbed and chromatographed on silica gel eluted with 97:3 chloroform/methanol. The fraction containing mainly hydroxyversicolorone was recrystallized several times from ethyl acetate/hexane to afford pure hydroxyversicolorone and a supernatant that was enriched with component A. This material was purified further by using a Chromatotron and was eluted with 50:50 ethyl acetate/hexane. The component A fraction was recrystallized from acetone/water several times to afford an orange powder, which was dried under vacuum for 12 h. Mobility on TLC, MS fragmentation pattern, and the ^1H NMR data were indistinguishable from those of the synthetic material above: mp 209–211 °C (lit.[19] mp 210 °C); $[\alpha]^{25}_D$ 0°.

Alcohol 11. Alcohol **11** was obtained from sodium borohydride reduction of **2** in water (0 °C, 5 min) and isolated by Chromatotron (70:30 ethyl acetate/hexane), dried under vacuum at refluxing toluene temperature for 2 h: mp 245–248 °C; UV λ_{max} (ethanol) 222 (ϵ 24 100), 266 (17 100), 297 (18 300), 316 (21 400), 479 (5500), 545 nm (3000); ^1H NMR (acetone-d_6) δ 1.10 (3 H, d, J = 6.7 Hz, H-6′), 1.4 (2 H, m, H-4′), 1.85 (2 H, m, H-3′), 3.70 (2 H, m, H-2′, H-5′), 3.93 (1 H, dd, J = 11.0, 4.0 Hz, H-1′), 4.14 (1 H, dd, J = 11.0, 5.3 Hz, H-1′), 6.60 (1 H, d, J = 2.3 Hz, H-7), 7.18 (1 H, d, J = 2.3 Hz, H-5), 7.20 (1 H, s, H-4); MS m/z (relative intensity) 370 (8), 368 (11), 358 (13), 339 (20), 310 (43), 297 (23), 285 (21); HRMS calcd 370.1053, obsd 370.1057.

Acknowledgment. The National Institutes of Health (ES 01670) and the U.S. Department of Agriculture (Cooperative Agreement 58-7B30-566) are acknowledged for financial support of this research, and Sigma Xi is thanked for an Undergraduate Research Award to M.S.I. Major analytical instrumentation at JHU was obtained through grants from the NIH (RR 01934 and RR 02318) and the NSF (PCM 83-03176). The mass spectral facility at the Massachusetts Institute of Technology, supported in part by the NIH (RR 00317), is thanked for providing analyses in early phases of this work and Dr. J. L. Kachinski (JHU) for subsequent analyses. Prof. J. R. Hwu (JHU) is thanked for providing access to his Chromatotron.

Registry No. **1**, 14016-29-6; **2a**, 111975-78-1; **2b**, 113794-77-7; **4**, 6807-96-1; **5**, 1162-65-8; **10**, 84062-31-7.

Synthesis of 4-Substituted-3-alkoxy-3-cyclobutene-1,2-diones

Michael W. Reed, Daniel J. Pollart, Steven T. Perri, Lafayette D. Foland, and Harold W. Moore*

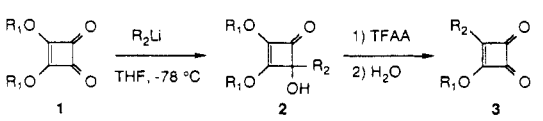
Department of Chemistry, University of California, Irvine, California 92717

Received December 11, 1987

4-Substituted-3-alkoxycyclobutenediones **3** were obtained from dialkoxycyclobutenediones (dialkyl squarates) **1** by the addition of organolithium reagents followed by hydrolysis of the resulting hydroxycyclobutenone **2**. A particularly useful one-pot procedure is described which involves treatment of **1** with the organolithium reagent at −78 °C in THF followed by addition of trifluoroacetic anhydride (TFAA) and an aqueous workup.

Reported here is a general and useful method for the synthesis of a variety of 4-substituted-3-alkoxycyclobutenediones **3** starting from 3,4-dialkoxycyclobutenediones **1**.[1,2] Such compounds are of interest since they can function as starting materials for highly substituted annulated hydroquinones and benzoquinones from the thermolysis of respectively 4-aryl-4-hydroxy- and 4-alkynyl-4-hydroxy(or trialkylsiloxy or allyloxy)cyclobutenones.[3-5] In addition, they are of further interest since

Scheme I



(1) For an independent report of this synthetic methodology, see: Liebeskind, L. S.; Fengl, R. W.; Wirtz, K. R.; Shawe, T. T. *J. Org. Chem.*, following article in this issue.

(2) Diethoxycyclobutenedione (diethyl squarate) is now commercially available from Aldrich Chemical Co.

0022-3263/88/1953-2477$01.50/0