Bisfuran Formation in Aflatoxin Biosynthesis: The Role of Versiconal Acetate

Craig A. Townsend,*† Siegfried B. Christensen, and Steven G. Davis

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

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Having securely established the origin of the aflatoxin B_1 (**1**, Chart I) bisfuran carbon skeleton using specifically labeled samples of averufin (**2**),[1] we sought to examine the process by which the two terminal carbons of the linear C_6 side chain of the latter are lost. Reincorporation and [^{13}C]acetate studies with mutants of *Aspergillus parasiticus* suggest that averufin (**2**)[2-4] and versicolorin A (**3**)[3,5,6] serve as sequential intermediates in the biosynthesis of this potent mycotoxin. Treatment of *A. parasiticus* cultures (wild-type) with dichlorvos (dimethyl(2,2-dichlorovinyl) phosphate), an insecticide, causes marked inhibition of aflatoxin production and concomitant accumulation of versiconal acetate (**5**)[7] together with lesser amounts[8] of versiconol (**6**, R = H) and versiconol acetate (**6**, R = $COCH_3$). In uninhibited cultures of the fungus ^{14}C-labeled versiconal acetate, like averufin, is transformed into radiolabeled aflatoxin B_1.[3,9] Administration of [1,2-$^{13}C_2$]acetate to chemically blocked fermentations yields pigments **5** and **6** displaying the distribution of intact acetate units shown[10] (indicated by heavy lines, dot representing C-1). In sum, these results appear to place versiconal acetate (**5**) between averufin (**2**) and versicolorin A (**3**) in the pathway to aflatoxin. Circumstantial structural evidence suggests that loss of the terminal pair of carbons as acetate may occur by way of a Baeyer-Villiger-like oxidation acting on the methyl ketone derived from opening of the averufin side chain. However, under the conditions of the [^{13}C]acetate-labeling experiments it is equally possible that the *O*-acetyl unit of **5** or **6** (R = $COCH_3$) may arise not by intramolecular rearrangement but by trivial acylation by endogenous acetylCoA. We demonstrate herein that the former situation prevails.

[1′-^{2}H,^{13}C]Averufin, prepared as described in the accompanying paper,[1] was treated with a standard solution[11] of deuterium chloride in tetrahydrofuran–deuterium oxide at reflux for 17 days under nitrogen and isolated in essentially quantitative yield. At 300 MHz[12] the diastereotopic hydrogens of the 4′-methylene and

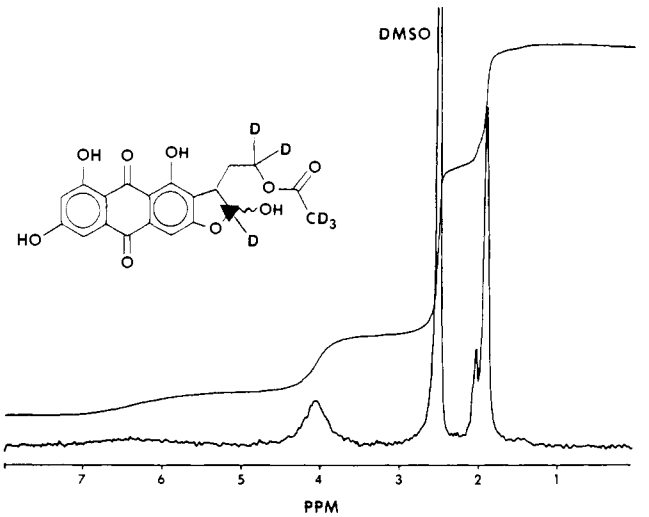
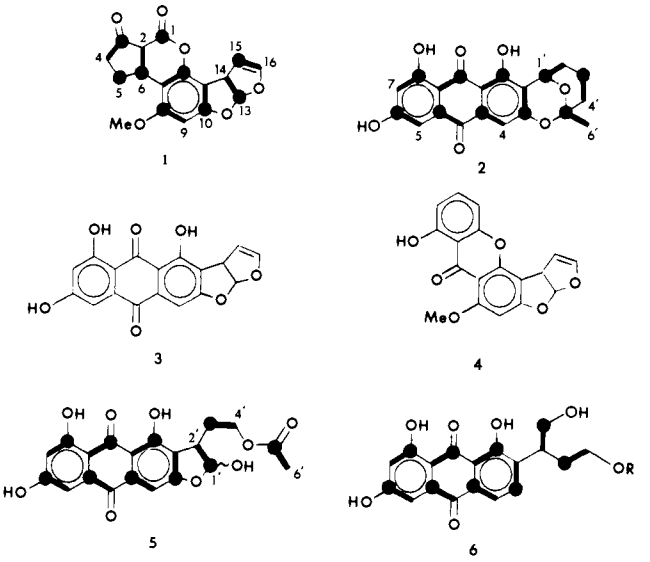
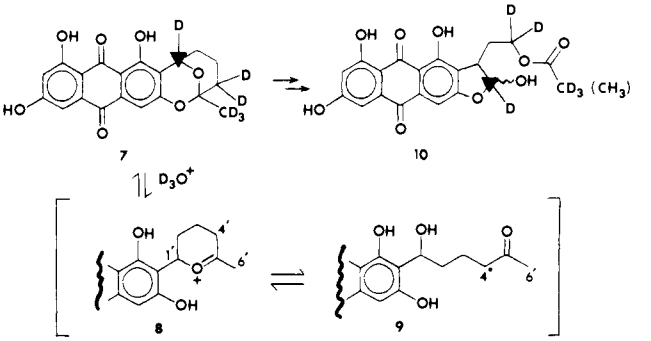


Figure 1. ^{2}H{^{1}H} NMR spectrum of versiconal acetate **10** (15 mg in 2.5 mL of Me_2SO, 8000 transients) recorded under the following conditions: Bruker WM-300 instrument, 46.1 MHz, spectral width 2000 Hz, 4K points, acquisition time 1.024 s, 90° pulse.

Chart I



Scheme I



the 6′-methyl hydrogens are discernable, the former exchanging significantly more rapidly than the latter. Presumably the exchange process proceeds initially by opening of the ketal side chain to an oxonium species **8** (Scheme I) which will exist principally

† Research Fellow of the Alfred P. Sloan Foundation 1982-1984.

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(12) ^{1}H NMR analyses of unlabeled and variously ^{2}H- and/or ^{13}C-labeled samples of averufin at 500 MHz in Me_2SO-d_6 in tandem with extensive homonuclear decoupling experiments allowed complete assignment of the averufin side-chain hydrogens and established, in keeping with the X-ray structure,[13] a chair conformation for the E-ring in solution.

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as the 4′,5′- or 5′,6′-vinyl ether or which may open further to the hydroxy ketone **9** to allow exchange in a more conventional fashion.[14]

It was anticipated from experiments described in the accompanying paper[1] that in a dichlorovos-inhibited culture of *A. parasiticus*, multiply labeled averufin **7** would suffer conversion to versiconal acetate **10** having both ^{13}C and ^{2}H isotopes at C-1′, the hemiacetal carbon. For the sake of the envisioned experiment, a *deuterium* NMR spectrum[15] of this multiply labeled product was expected to provide the following: First, the 1′-D, which had been introduced in synthetic averufin[1] at a known level of enrichment (ca. 85%), would serve as an internal reference for integration of the other labeled centers. Second, if an intramolecular Baeyer–Villiger oxidation were operative, the integrated intensities of deuterium label at C-1′:4′:6′ ought to be 0.85:2:3. Whereas, if the terminal *O*-acetyl unit were derived trivially by intermolecular reaction with endogenous acetylCoA, an acetyl methyl signal of very low if detectable relative intensity would be expected at a clearly distinguishable chemical shift.

Averufin (**7**, 63 mg) was apportioned equally among 21 250-mL Erlenmeyer flasks containing 10 g of wet 48 h-old mycelial pellets of *A. parasiticus* (ATCC 15517) in 100 mL of a replacement medium[9a] and 10 ppm of dichlorovos and incubated for 40 h. In the $^{2}H\{^{1}H\}$ spectrum of the isolated versiconal acetate (Figure 1) the hemiacetal carbon 1′-D appears as a very broad resonance between δ 5.5 and 7. The breadth of this signal arises from several effects: low rotational mobility, ^{13}C coupling, and the fact that versiconal acetate in Me_2SO exists as an equilibrating mixture[7,8] of the hemiacetal shown in **10** and that formed to the anthraquinone 1-OH as well as a small amount of open form. A two-deuterium signal for C-4′ appears at δ 4.1, sharper owing to greater local molecular motion. Last, at about δ 1.9 a three-deuterium singlet is observed (with small acetone impurity to lower field), corresponding to retention of the trideuteriomethyl.

It may be concluded that in the formation of versiconal acetate (**5**) from averufin (**2**), an intramolecular Baeyer–Villiger-like oxidation[17] takes place to lead to the ultimate loss of the two terminal carbons of the averufin side chain as acetate in the course of bisfuran formation. Whether this oxidative cleavage occurs before or after the chain branching process where the anthraquinone nucleus migrates from C-1′ to C-2′ cannot be determined at present. However, given the constraint of deuterium retention at C-1′ from **2** to **5** and to C-13 in **1**, both the nature and sequence of the biosynthetic events may be explored in biochemical and stereochemical experiments with potential intermediates accessible by extension of the synthetic methods developed for this program.[18]

Acknowledgment. Professor J. W. Bennett (Tulane) is warmly thanked for assistance with microbiological aspects of this work. The National Institutes of Health are gratefully acknowledged for financial support (Grant ES 01670) and for providing partial funding to acquire the Bruker WM-300 instrument used (Grant GM 27512). We are pleased to note the essential services of NSF-supported instrument facilities at Yale University (Northeast Regional NMR Facility; 500 MHz) and The Johns Hopkins University (Middle Atlantic Mass Spectrometry Laboratory).

Registry No. (±)-**2**, 79896-28-9; (±)-[1-^{2}H,^{13}C]-**2**, 83152-86-7; **5**, 62886-00-4; (±)-**7**, 83199-79-5.

(14) Monitoring exchange by ^{1}H NMR at 300 MHz revealed complete loss of proton intensity at C-4′ and C-6′. Similarly, mass spectral analysis gave a strong M + 7 for the major molecular species. Interestingly, exchange at the aryl positions is minimal within the limits of detection by ^{1}H or ^{2}H NMR.

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(17) It is noted that such an oxidation may occur to carry the B-series aflatoxins to the G series. Mass spectral analysis of the latter was carried out to complete the proof that the side chain oxidative rearrangement of **7** to **10** is strictly intramolecular. An in-beam electron impact spectrum was obtained at 210 °C that failed to give a good molecular ion, but strong fragments[8] were observed for unlabeled versiconal acetate at *m/z* 382 ($M^+ - H_2O$) and 340 (M^+ – HOAc). Prominant peaks for the analogous fragments of **10** were seen at *m/z* 389 and 344, from whose relative intensities minimal specific incorporations of **7** were correspondingly computed to be 21% and 20%, respectively.

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Complexes of the New Ligand Tetracyanobiimidazole

P. G. Rasmussen,* R. L. Hough, J. E. Anderson, O. H. Bailey, and J. C. Bayón

Department of Chemistry, University of Michigan Ann Arbor, Michigan 48109

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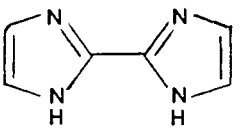
The search for planar, conjugated molecules, capable of forming stacked complexes, led to our previous investigations of 2,2′-biimidazole (H_2biim), **1**, which revealed a variety of structural



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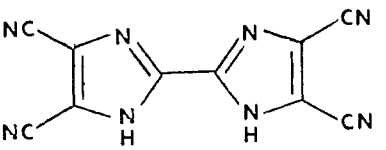
possibilities.[1-3] Recently, in an effort to increase the polarizability and acceptor properties of our compounds, we have synthesized a remarkable new species, 4,4′,5,5′-tetracyano-2,2′biimidazole (H_2Tcbiim), **2**.



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Whereas H_2Biim has been known for many years[4] and is readily synthesized from the bisulfite addition salt of glyoxal reacting with ammonia,[5] similar methods using diaminomaleonitrile (DAMN) and a variety of coupling reagents failed to produce H_2Tcbiim.[6] The preparation of H_2Tcbiim proceeds by a ring coupling reaction of 4,5-dicyanoimidazole with 2-diazo-4,5-dicyanoimidazole.[7] These reagents were prepared by the methods of the du Pont group.[8,9]

H_2Tcbiim is a colorless, high-melting, air-stable solid. It is somewhat more soluble in most solvents than H_2Biim and is far more acidic, $pK_1 \approx 2.1$, $pK_2 \approx 5.5$ measured in 40% (v/v) acetonitrile–0.1 M TEAP. The mass spectrum shows, in addition to a large parent ion peak at *m/e* 234.1, principally decomposition by loss of HCN and $(CN)_2$. Three ^{13}C NMR peaks appear at 140.8, 118.0, and 110.3 ppm relative to Me_4Si. Extended Hückel

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(7) In a typical reaction, 0.01 mmol of 2-diazo-4,5-dicyanomidazole is prepared and added moist to 0.01 mmol of 4,5-dicyanoimidazole suspended in 30 mL of CCl_4. The slow evolution of nitrogen provides a convenient monitoring of the coupling reaction, which proceeds smoothly at 45 °C. **Caution**: The zwitterion 2-diazo-4,5-dicyanoimidazole decomposes explosively at 150 °C, as one might expect, given its unusual stoichiometry (C_5N_6).

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