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DEMONSTRATION OF BAEYER-VILLIGER OXIDATION AND THE COURSE OF CYCLIZATION IN BISFURAN RING FORMATION DURING AFLATOXIN B_1 BIOSYNTHESIS

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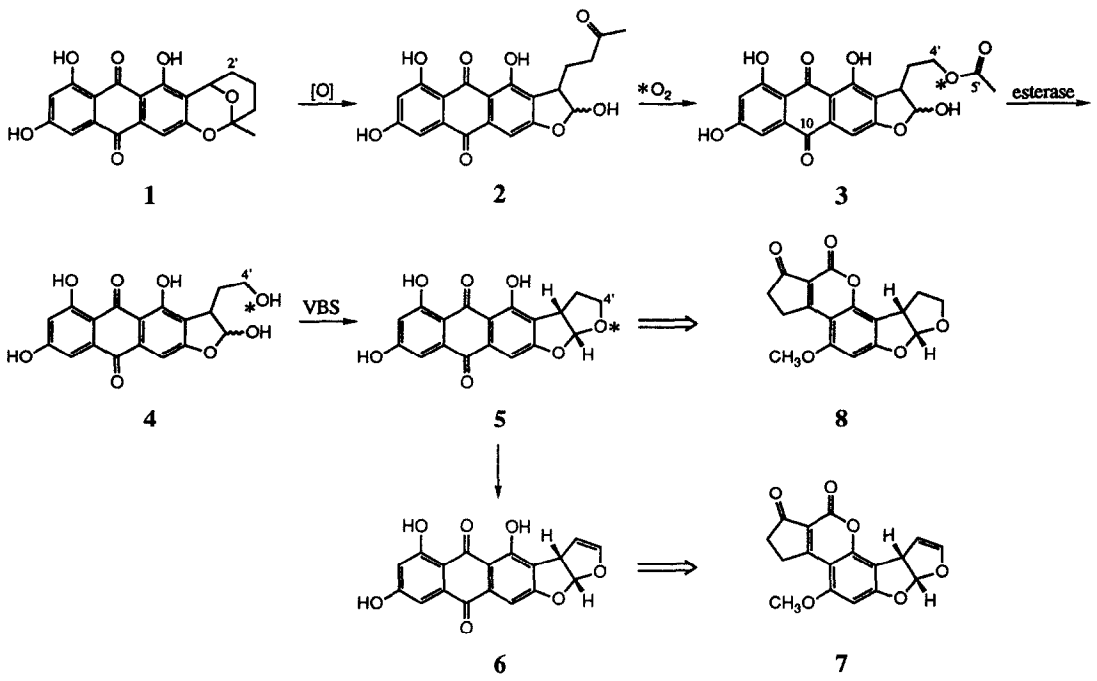
Abstract: The mechanism of tetrahydrobisfuran formation in aflatoxin B_1 biosynthesis is defined in experiments that monitor the incorporation of molecular oxygen into an anthraquinone intermediate of the pathway in conjunction with cell-free reactions that complete the synthesis of the tetrahydrobisfuran present in versicolorin B wherein the second ring bears the labeled oxygen.

The dihydrobisfuran ring system is generally recognized as the structural component responsible for the extreme carcinogenicity of aflatoxin B_1 (**7**) and its congeners. This metabolite of the molds *Aspergillus flavus* and *Aspergillus parasiticus* is a wide-spread contaminant of foodstuffs whose facile and remarkably site-selective reaction with the p53 tumor-suppressor gene has been linked directly to the occurence of hepatocellular carcinomas in humans.[1-4] Versicolorin B synthase (VBS) catalyzes the cyclization reaction in which the second furan ring is first generated in **5**. This protein has been purified to homogeneity[5-8] for mechanistic study and the design of irreversible inhibitors.[9] Clearly inactivation of aflatoxin biosynthesis would be most desirable *prior* to formation of the dihydrobisfuran ring system present in the subsequent intermediate, versicolorin A (**6**), the first significant mutagen in the pathway. Reported here are ^{18}O-incorporation experiments (*O, Scheme I) that, first, support the apparent Baeyer-Villiger oxidation of **2** to **3** and, second, define the course of dehydrative closure to the tetrahydrobisfuran catalyzed by VBS. These experiments demonstrate the incorporation and utilization of labeled oxygen at the specified sites in **3** and **5** shown in Scheme I. These findings constrain the possible mechanisms that may be acting in these processes as an unambiguous prelude to further studies of the enzymology and mechanism.

Among polyketide-derived natural products, the bisfuran is unique to this family of environmental toxins and is known to be synthesized *in vivo* in an intriguing and efficient set of reactions from averufin (**1**, Scheme I). Oxidative rearrangement of **1** is initiated at C-2' and proceeds to give the first furan ring in hydroxyversicolorone (**2**).[10-12] It would appear that the methyl ketone of the latter undergoes a Baeyer-Villiger-like reaction to introduce an atom from molecular oxygen (*O) into the carbon chain. The resulting versiconal acetate (**3**) is cleaved by an esterase[5,6,13,14] to versiconal (**4**). VBS carries this racemic substance[5,6,13] to the optically active versicolorin B (**5**). This compound can partition metabolically[13,15,16] toward generally minor metabolites containing the less toxic tetrahydrobisfuran, *e.g.* aflatoxin B_2 (**8**), or, more importantly, be oxidatively desaturated[13,17] to versicolorin A (**6**) with formation of the dihydrobisfuran ring system. This key intermediate can be further transformed to the increasingly carcinogenic intermediates leading to aflatoxin B_1 (**7**).

Scheme I



The insecticide Dichlorvos inhibits the esterase that deacylates versiconal acetate (**3**) leading to the accumulation of this metabolite.[18,19] The method of analysis chosen to monitor the course of ^{18}O (*O) incorporation and utilization in these experiments was the isotope-induced chemical shift method developed notably by Vederas.[20] Wild-type *Aspergillus parasiticus* (ATCC 15517) was incubated for 48 hours at 28 °C in two liters of Adye and Mateles medium[21] until the onset of aflatoxin biosynthesis.[22] The mycelia were isolated and resuspended in a high glucose (36 g/L) replacement medium[23] containing Dichlorvos (10 μL/L). The cell suspensions were then connected to a closed-atmosphere system designed by Vederas,[24] and as used by Townsend.[25] An approximately equal ratio of $^{16}O_2$:$^{18}O_2$ was maintained throughout the subsequent 48 hours of incubation at 28 °C. Extraction of the bright orange mycelia and media with acetone and 1:1 ethyl acetate:chloroform, respectively, was followed by purification by silica gel chromatography (60:25:15 hexanes:acetone:ethyl acetate with 0.1% acetic acid) to afford 94 mg of versiconal acetate (**3**).

Analysis of the sample by proton-decoupled ^{13}C-NMR (10% d_6-DMSO in $CDCl_3$) using a narrow spectral window (350 Hz) revealed an ^{18}O isotope-induced shift at the C-5' ester carbon (δ = 181.6 ppm). The magnitude of the obtained shift was $\Delta\delta$ 0.042 ppm as would be expected for an ester heavy oxygen bound to an sp^2 hybridized center.[26,27] The ratio of ^{16}O to ^{18}O was measured by integration of the two peaks and was found to be 61:39. Basing the degree of incorporation on analysis of C-5' rather than C-4' is warranted since the carbonyl oxygen, formed from the hydrolysis of the intramolecular ketal of averufin, can possess no label from molecular oxygen.[12] Moreover, no second shifted peak corresponding to labeled ester carbonyl oxygen was evident. Mass

spectral analysis of the product averaged from ten isotopic abundance chemical ionization scans (CI, NH_3 as reagent gas) indicated double incorporation of label as compared to a natural abundance standard, in a ratio of 36:46:18, unlabeled as compared to singly labeled and to doubly labeled material. The second oxygen is presumably incorporated at C-10 of the anthraquinone.[28] These data indicate that the oxidation of **2** to **3** does proceed as a Baeyer-Villiger-like reaction and requires molecular oxygen for the process.

Cell-free extract capable of converting **3** to **5**[13] was prepared from 60 h cultures of wild-type *A. parasiticus* (ATCC 15517) by homogenization with 0.2 M KH_2PO_4 buffer (pH 7.5; 15% glycerol) and glass powder. The ^{18}O-labeled versiconal acetate (**3**; 26 mg) was then incubated with 150 mL of this solution at 28 °C for four hours. Extraction of the cell-free incubation mixture with 1:1 acetonitrile:ethyl acetate was followed by purification of the versicolorin B obtained by silica gel chromatography (50:35:15 hexanes:acetone:ethyl acetate) to afford 18 mg of material. $\{^1H\}^{13}C$-NMR analysis of this anthraquinone ($CDCl_3$), again employing a narrow spectral width (350 Hz), indicated an ^{18}O-induced chemical shift acting on the C-4' signal (δ = 67.3 ppm), with a magnitude of $\Delta\delta$ 0.028 ppm as expected for this sp^3 hybridized center.[26,27] The ratio of ^{16}O to ^{18}O was again estimated by integration of the two peaks and found to be 58:42 representing a net incorporation rate of 105 ± 3% from versiconal acetate (**3**). Isotopic abundance CI mass spectral analysis as above indicated incorporation of two atoms of ^{18}O in a ratio of 39:46:15 unlabeled: singly labeled: doubly labeled material, as compared to a natural abundance standard. This measurement indicates a 93 ± 5% incorporation of total ^{18}O in both positions. Variations in the two rates can be understood in the inherent error of the NMR integration as well as the potential ability for the labeled anthraquinone oxygen to exchange slowly with the medium.

In conclusion, the isotope-induced chemical shift method has been used to show the clear incorporation of molecular oxygen into versiconal acetate (**3**). The appearance of heavy isotope in the expected ^{16}O:^{18}O ratio at C-4'/C-5' is fully in keeping with the proposed intervention of a Baeyer-Villiger-like reaction. This step adroitly introduces oxygen in the midst of a carbon chain and which is liberated next by an esterase as versiconal (**4**) where the labeled oxygen is retained as a potentially nucleophilic primary alcohol. The undiminished proportion of labeled oxygen incorporated into the second furan ring of versicolorin B (**5**) characterizes the key chemical step catalyzed by VBS as attack on the electrophilic hemiacetal center of **5** by the C-4' hydroxyl. This reaction, therefore, is an intramolecular analogue of the broader class of glycosidase reactions central to polysaccharide biosynthesis and provides a point of entry into the rational design of irreversble inhibitors of VBS.

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