Incorporation of Molecular Oxygen in Aflatoxin B_1 Biosynthesis

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The final steps in the biosynthesis of the potent environmental carcinogen aflatoxin B_1 (**7**) involve the oxidative cleavage of the xanthone *O*-methylsterigmatocystin (**6**, R = Me) and rearrangement to the substituted coumarin skeleton of the mycotoxin itself. This process has been determined to require loss of a xanthone nuclear carbon at the oxidation state of carbon dioxide and argues for the intervention of at least two oxidative reactions in this poorly understood transformation. The incorporation of ^{18}O-labeled molecular oxygen in the biosynthesis of aflatoxin B_1 is reported and allows a minimal mechanism to be proposed where a monooxygenase activation of the xanthone is followed by a dioxygenase-mediated aryl cleavage to initiate the final rearrangement and decarboxylation to aflatoxin B_1. Similarly, two other sites of heavy oxygen incorporation are consistent with proposed Baeyer−Villiger-like reactions taking place earlier in the pathway.

Introduction

The biosynthesis of the potent mycotoxin aflatoxin B_1 (**7**) is one of the most complex among aromatic polyketides. An unusual hexanoyl starter unit[1−4] is believed to be homologated by the successive addition of seven malonyl CoA molecules without reduction to give the C_{20}-tetrahydroxyanthraquinone norsolorinic acid (**1**). Important genetic and biochemical evidence has been gathered recently to support the roles of two specialized fatty acid synthases (FAS) apparently responsible for formation of this C_6-primer and a separately transcribed polyketide synthase (PKS).[5] An efficient sequence of reduction and oxidation steps leads through a series of now known intermediates to versicolorin A (**3**), a C_{18}-anthraquinone containing the characteristic dihydrobisfuran and entailing loss of two carbons as acetate.[6,7]

The post-bisfuran portion of the biosynthetic pathway is significantly less well-understood, but is thought to be initiated by a cryptic reductive/oxidative scission of the anthraquinone nucleus to yield ultimately the C_{17}-xanthone **5** (R = H) accompanied by loss of the C-6 hydroxyl of **3** and a C_1-unit derived from the anthraquinone carbonyl (C-10), presumably as carbon dioxide.[8−10] A Baeyer−Villiger-like oxidation (to **4**) has been proposed to play a role in this ring cleavage reaction and would be predicted[11] to introduce oxygen (*O) in the central aromatic ring (at C-5 in **5**) and raise the oxidation state of C-10 to a carboxylate.[10,12] Transmethylation reactions have been established to occur successively at the C-5 and C-7 phenols to give *O*-methylsterigmatocystin **6** (R = Me).[13]

The final transformations of the pathway involve a further oxidative cleavage of the first aryl ring and rearrangement to afford the substituted C_{16} coumarin nucleus of aflatoxin B_1 (**7**) with loss of a second C_1-unit. Based on the regiospecificity of earlier ^{13}C-incorporation experiments,[14] it was possible to deduce unequivocally that C-10 of **6** (R = Me) is the source of this carbon fragment. Subsequent radiochemical experiments established that loss of the C_1-unit takes place as carbon dioxide.[15] These and other data[16−18] can be taken to support the view that at least two oxidative transformations are required, the first likely being a monooxygenase step.[15] In this paper we demonstrate the incorporation of heavy isotope from $^{18}O_2$ (*O) at C-16, C-8, and C-1 of aflatoxin B_1 (**7**). The role of these oxidative transformations in the biosynthesis of **7** is discussed and, in particular, places limits on the final mechanistic steps leading to the cleavage of xanthone **6**.

Results and Discussion

The pattern of deuterium incorporation from [2-$^2H_3$]-acetate into sterigmatocystin (**8**, R = H)[17] and aflatoxin B_1 (**15**)[16] shown in Scheme 2 is consistent with the action of a monooxygenase (*i.e.* *O from $^{18}O_2$) to give epoxide **9** and "NIH shift" to yield 10-hydroxyl-*O*-methylsterigmatocystin (**10**) as the first oxidative step in xanthone

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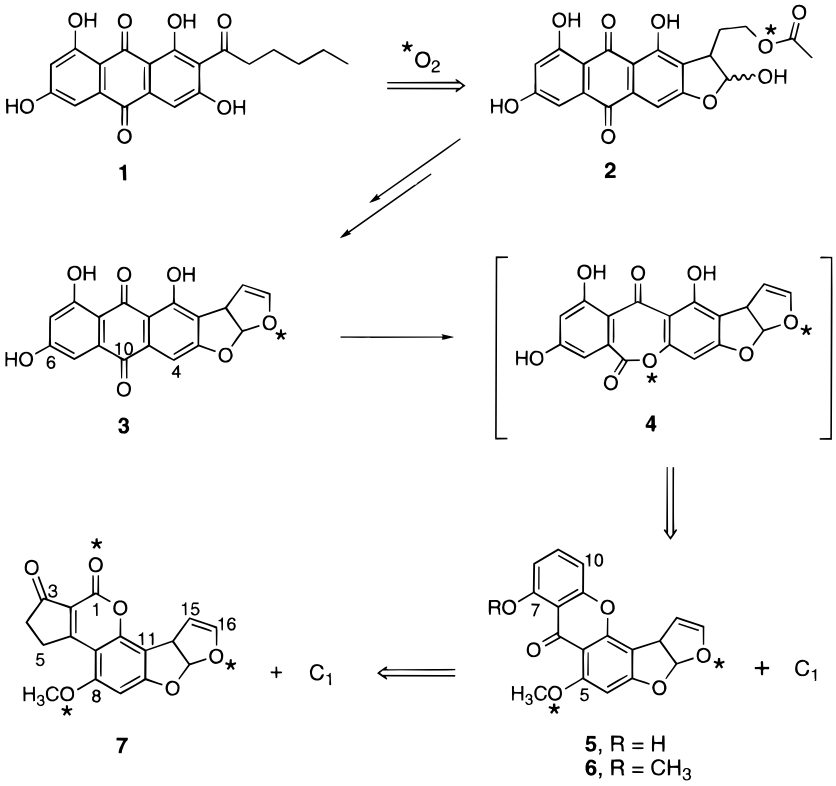
Scheme 1



cleavage.[15,16] While the dimethoxyphenol **10** is unknown as a natural product and untested as an intermediate, its role in the overall cleavage process is additionally consistent with the observed NADPH-dependence of cell-free reactions capable of the transformation of **6** (R = Me) to **7** (or **8**, R = Me to **13**/**15**).[15,18,19] Release of C-10 as carbon dioxide,[15] however, implies a further elevation of oxidation state to achieve both aryl cleavage and appropriate loss of C-10. While several mechanistic variations on the second process can be visualized, they may be broadly classified as either (path A, Scheme 2) a second monooxygenase step schematically represented as **11**, or (path B) as a dioxygenase-mediated reaction symbolized as **14**. The incorporation of $^{18}O_2$ (*O) at C-1 in **7**/**15** strongly supports the latter of these two possibilities as elaborated below.

To examine the uptake of dioxygen during the course of aflatoxin biosynthesis, the wild-type *Aspergillus parasiticus* strain SU-1 (ATCC 56775) was grown in a closed system described by Vederas[20] to allow replacement of consumed oxygen with a mixture of $^{18}O_2$ and $^{16}O_2$. SU-1 mycelia were initally grown on Adye and Mateles medium under normal atmospheric conditions until aflatoxin B_1 production had begun, at *ca.* 30 h of growth. A fixed weight of the harvested cells was then transferred into a replacement medium (RM) and the closed-system oxygen incorporation apparatus was attached. The cell suspensions were charged with an approximately equal ratio of ^{18}O:^{16}O gas, which was maintained throughout the subsequent 33 h of growth.

Extraction and purification of aflatoxin B_1 from the SU-1 mycelia and media afforded approximately 18 mg of purified toxin. The sample was analyzed by proton-decoupled ^{13}C-NMR spectroscopy as isotopic substitution with ^{18}O gives rise to an upfield shift in the position of resonances of directly bound carbon atoms.[21] The ^{13}C-{^1H}-NMR spectrum of ^{18}O:^{16}O-labeled aflatoxin B_1 (**7**) revealed that three of the six oxygen atoms were derived from molecular oxygen corresponding to those at carbon centers C-16, C-8, and C-1. Examination of the ratio of ^{18}O to ^{16}O as judged by the integrated peak areas showed a 39 ± 2% incorporation of ^{18}O-label at each position. The presence of ^{18}O at C-16 (δ = 145.2 ppm), the second furan ring oxygen, was expected and served as an internal standard. A prior experiment carried out in this laboratory had shown ^{18}O-enrichment (*O) specifically into the ester oxygen of versiconal acetate (**2**).[22] Subsequently hydrolysis and cyclization/dehydration of labeled **2** in a cell-free system completed the formation of the labeled tetrahydrobisfuran of optically active versicolorin B.[22] The magnitude of the isotopic shift was $\Delta\delta$ 0.018 ppm as is commonly observed for singly bonded oxygen atoms.[21] Importantly, the methoxy group of aflatoxin B_1 (**7**, C-17) was also clearly shown to contain ^{18}O-label (δ = 56.5 ppm, $\Delta\delta$ 0.030 ppm). Such a result is in keeping with the proposed Baeyer−Villiger oxidation of versicolorin A (**3** to **4**) to ultimately effect cleavage of the central ring and initiate the rearrangement to form the xanthone nucleus of sterigmatocystin (**5**). Finally, ^{18}O-incorporation was also observed at the carbonyl oxygen (C-1) of the coumarin nucleus of aflatoxin B_1 (**7**, δ = 155.2 ppm) which points to the involvement of a monooxygenase followed by a dioxygenase-catalyzed step in the oxidative ring cleavage of *O*-methylsterigmatocystin (**6**). The magnitude of the isotopic shift was $\Delta\delta$ 0.034 ppm, as would be expected for this sp^2 hybridized center.[21] The 39 ± 2% enrichment of ^{18}O uniformly observed at all

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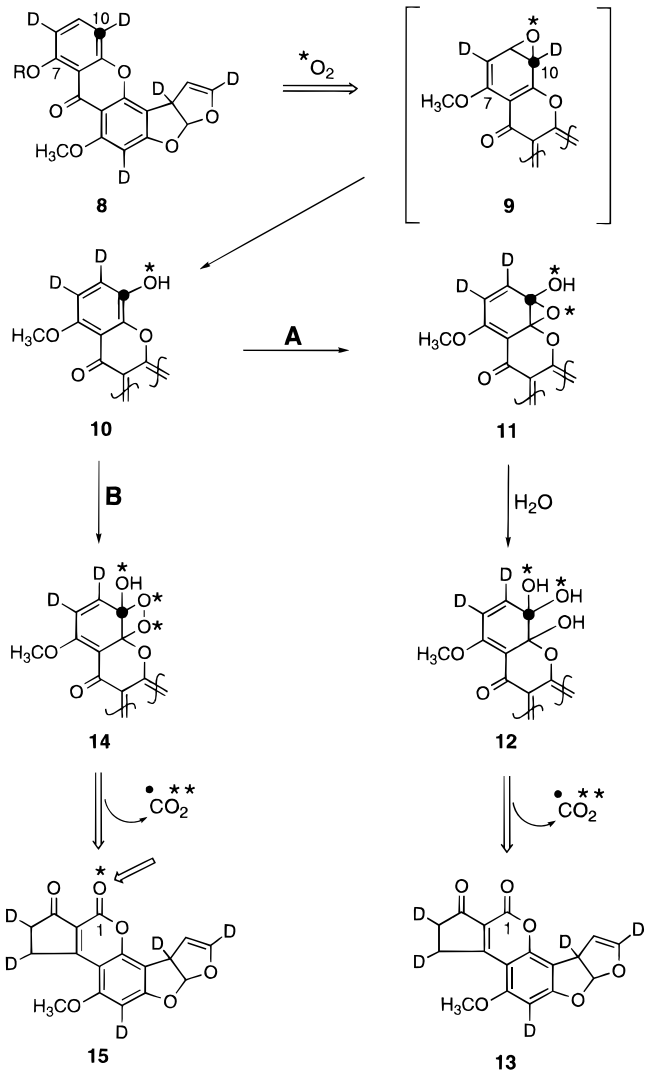
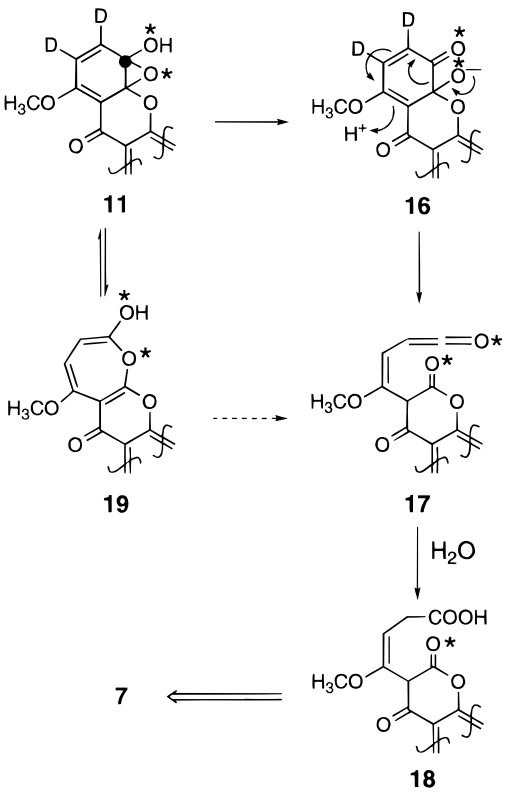
Scheme 2



Scheme 3



three of the positions removes the possibility of exchange at any intermediate stage of the biosynthesis.

Conclusion

Incorporation of molecular oxygen as $^{18}O_2$ into aflatoxin B_1 (**7**/**15**) was monitored by the isotope-induced chemical shift method.[21] ^{18}O-Labeling was found to occur with equal efficiency at three carbon positions: C-16, C-8, and C-1. The appearance of heavy isotope (*O) at C-16 was fully in accord with earlier cell-free experiments where a Baeyer−Villiger-like insertion of molecular oxygen was demonstrated to ultimately label the second furan ring of versicolorin B (hence of **3**).[22] Similarly, the present experiment shows the incorporation of molecular oxygen at the methoxy position, C-8, in aflatoxin (**7**) to support the putative Baeyer−Villiger-like cleavage of the anthaquinone nucleus, presumably by way of an intermediate as **4**.[23]

The similarly efficient incorporation of ^{18}O at C-1 in aflatoxin allows the more complex issue of the final oxidative steps in the biosynthesis to be addressed. The salient features of this minimally two-step process are illustrated in Scheme 2.[15] The pattern of deuterium incorporation from labeled acetate into sterigmatocystin (**8**, R = H)[17] and aflatoxin B_1 (**15**)[16] coupled with the loss of C-10 in **8** (R = Me) as carbon dioxide[15] support monooxygenase reaction to **10** invoking a "NIH shift" in the course of phenol formation.[15,16] Further oxidation leading finally to cleavage of the A-ring could be visualized to involve (path A) a second monooxygenase-catalyzed step to give, for example, the reactive intermediate **11**. This species could be visualized to rapidly hydrate in the regiochemical sense indicated in **12** to afford aflatoxin **13** bearing *no* heavy isotope at C-1. Alternatively (path B, Scheme 2), dioxygenase-catalyzed addition of molecular oxygen, illustrated here as the dioxetane **14**,[24] followed by cleavage, cyclization, and decarboxylation can be visualized by any of several routes to lead to aflatoxin **15** bearing ^{18}O at C-1. The experiments here clearly support the latter course of events in keeping with earlier implications from cell-free studies[18,19] to promote aryl cleavage and generate the coumarin nucleus of the mycotoxin.

Other more elaborate mechanisms of xanthone cleavage can be envisioned (Scheme 3). Among these a second monooxygenase-mediated reaction (path A, Scheme 2) could be directed to give aflatoxin B_1 (**7**) having ^{18}O label at C-1, contrary to the general conclusions of Scheme 2. For example, the aryl epoxide **11** could cleave to **16** and subsequently rearrange to give the ketene **17**. Hydration of this proposed intermediate could give acid **18** prior to closure, demethylation, and decarboxylation to aflatoxin B_1 (**7**).[15] Alternatively, valence tautomerization of **11** could also be imagined to give the seven-membered

(23) Insertion of molecular oxygen between the C-10/12 can be excluded in this step as such a ring scission would yield a chemically symmetrical intermediate. Randomization of paired labels from [1,2-$^{13}C_2$]acetate is not observed.[14]

(24) Alternative mechanisms for both the monooxygenase/"NIH shift" and dioxygenase steps may be considered in light of recent reports: Vannelli, T.; Hooper, A. B. *Biochemistry* **1995**, *34*, 11743−11749 and Sanvoisin, J.; Langley, G. J.; Bugg, T. D. H. *J. Am. Chem. Soc.* **1995**, *117*, 7836−7837.

oxepin **19** from which the ketene **17** could also be derived for further reaction to aflatoxin. However, while mechanisms such as these can be visualized to play a role in the biosynthesis, they are fundamentally higher energy processes and less probable on chemical grounds. Nonetheless, final mechanistic resolution of these remarkable oxidative cleavage and rearrangement reactions will soon become accessible in experiments at the enzymic and genetic levels.[5]

Experimental Section

General Methods. ^1H and ^{13}C NMR spectra were recorded on a Varian Unity^{plus} 500 spectrophotometer. Chemical shifts for ^1H and ^{13}C NMR spectra are reported in ppm referenced to $CHCl_3$ (7.26 ppm and 77.00 ppm). Flash chromatography was performed using 200−240 mesh Merck silica gel. ^{18}O gas (97.3 atom % ^{18}O) was purchased from Isotec, Inc. (Miamisburg, OH). Molecular oxygen was supplied to the mycelia utilizing a closed-atmosphere system described by Vederas.[20]

Organisms. Fungi were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The *Aspergillus parasiticus* wild-type strain SU-1 (ATCC 56775) was used for the production of aflatoxin B_1.

Media. The Adye and Mateles medium[25] used to initially culture the SU-1 strain contained per liter the following: sucrose, 50 g; potassium phosphate monobasic, 10 g; ammonium sulfate, 3 g; magnesium sulfate (anhydrous), 1 g; and trace metals, 2 mL. The replacement medium (RM)[25] contained per liter: glucose, 36 g; potassium phosphate monobasic, 5 g; potassium chloride, 0.5 g, magnesium sulfate (anhydrous), 0.25 g; and trace metals, 2 mL.

The growth medium (1 L, in 4 L Erlenmeyer flasks) was autoclaved (140 °C, 20 psi) for 20 min and cooled to room temperature prior to inoculation.

Culture Techniques. The fungi were grown on potato agar plates for 3−7 days. The agar plates contained the following: yeast extract, 2.5 g; bacto-agar, 2.5 g; potato dextrose agar, 19 g (Difco; Detroit, MI); and distilled water, 500 mL (20 plates). The spores were suspended in a solution of Tween 80 (0.05% V/V), 9 mL, which was subsequently diluted in NaCl (0.85% w/v), 9 mL. A fraction of the SU-1 cultured spore suspension (3 mL) was then transferred to 1 L of Adye and Mateles medium in 4 L Erlenmeyer flasks. The flasks were incubated at 28−30 °C in the dark at 220 rpm for the requisite amount of time.

Biosynthesis of ^{16}O,^{18}O-Labeled Aflatoxin B_1 (4). SU-1 was initially cultured in two 4 L flasks each containing 1 L of Adye and Mateles medium as described above. AFB_1 production was apparent by 30 h of growth, as observed by TLC (R_f = 0.56; 6:3:1, chloroform:ethyl acetate:formic acid). At the onset of AFB_1 production, the SU-1 mycelia were vacuum filtered, rinsed thoroughly with RM medium (supplemented with 36 g of glucose), and transferred into two 4 L flasks each containing 1 L of RM medium. The oxygen apparatus was then attached and equipped with a homogenizer to keep the circulating oxygen gas moist and 1.5 L of KOH to trap CO_2 released by the fungi. Initially *ca.* 1.3 L of $^{18}O_2$ was supplied to compensate for the amount of $^{16}O_2$ already present within the system. Once consumed, the oxygen was replenished with an approximately equal ratio of $^{18}O_2$:$^{16}O_2$. The mycelia were cultured for an additional 33 h under these conditions. The SU-1 cells, 63.9 g, were harvested by vacuum filtration, flash frozen in liquid nitrogen, and steeped in 250 mL of acetone overnight. The SU-1 medium was extracted three times with chloroform and the latter were consolidated with that extracted directly from the SU-1 mycelium. The organic extracts were dried over magnesium sulfate and concentrated *in vacuo*. Partial purification of AFB_1 was achieved by flash silica column chromatography (5:4.8:0.2, chloroform:ethyl acetate: acetic acid). AFB_1 was subsequently recrystallized in acetone to give 18 mg of purified material.

^1H NMR (400 MHz, $CDCl_3$) δ: 6.82−6.80 (d, J = 7.2 Hz, 1H, H-13), 6.46 (t, J = 2.6 Hz, 1H, H-16), 6.42 (s, 1H, H-9), 5.49−5.48 (t, J = 5.6 Hz, 1H, H-15), 4.78 (dt, J = 7.2 Hz, 2.5 Hz, 1H, H-14), 3.95 (s, 3H, H-17), 3.39−2.62 (m, 4H, H-4/H-5).

^{13}C NMR (500 MHz, $CDCl_3$)(^{18}O-labeled aflatoxin B_1, **15**) ^{13}C/^{16}O shifts at δ: 177.0 (s, 1C, C-6), 165.6 (s, 1C, C-10), 161.4 (s, 1C, C-8), 155.2 (s, 1C, C-1), 152.8 (s, 1C, C-12), 145.2 (s, 1C, C-16), 117.3 (s, 1C, C-2), 113.4 (s, 1C, C-13), 107.7 (s, 1C, C-11), 103.9 (s, 1C, C-7), 102.6 (s, 1C, C-15), 90.7 (s, 1C, C-9), 56.5 (s, 1C, C-17), 47.8 (s, 1C, C-14), 35.0 (s, 1C, C-4), 29.0 (s, 1C, C-5). ^{18}O-Shifted resonances at δ: 155.1 (s, 1C, C-1), 145.2 (s, 1C, C-16), 56.4 (s, 1C, C-17).

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