Evidence for the Probable Final Steps in Aflatoxin Biosynthesis

Moneesh Chatterjee and Craig A. Townsend*

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

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The final steps in the biosynthesis of the potent environmental carcinogen aflatoxin B_1 (**8**) are believed to involve the oxidative cleavage and rearrangement of *O*-methylsterigmatocystin (**7**) with loss of a C_1-unit. The means by which this overall transformation occurs is not known and has been addressed using cell-free conversions of samples of radiolabeled **7** that were obtained by the incorporation of either [1-^{14}C]- or [2-^{14}C]acetate. The proportion of radioisotope detected in aflatoxin B_1 relative to that of the C_1-unit liberated (formaldehyde, formic acid, or carbon dioxide) was tested. [^{14}C]Carbon dioxide alone was isolated in the proper stoichiometry to limit the possible mechanisms that can be acting at the conclusion of this biosynthetic pathway.

Introduction

The aflatoxins have been recognized for 30 years as fungal contaminants of foodstuffs. The recent discovery of remarkably site-selective reaction of metabolically activated aflatoxin B_1 (**8**, AFB$_1$) with the p53 tumor suppressor gene has forged a direct chemical link to the incidence of liver and kidney carcinomas in humans.[1] The biosynthesis of the mycotoxin **8** proceeds in a complex yet chemically efficient manner to convert the simple aromatic polyketide norsolorinic acid (**1**)[2,3] to averufin (**2**)[4–6] and versicolorin B (**3**).[7] The subsequent biosynthetic enzymes can utilize **3** to give ultimately aflatoxin B_2, the tetrahydrobisfuran analogous to **8**,[7,8] or it can undergo a critical desaturation reaction to versicolorin A (**4**).[7,9] Once the dihydrobisfuran is in place, the compounds of this series become increasingly carcinogenic as the xanthone (**5–7**) and, finally, coumarin (**8**) nuclei are created (Scheme 1).

The identities of the anthraquinone intermediates in aflatoxin biosynthesis are now securely established, and the probable conversions that carry one to the next can be rationalized.[10] By comparison presumed oxidative cleavage of the anthraquinone nucleus of **4**, aromatic deoxygenation, cyclization, and decarboxylation to give xanthone **5** are not well understood biosynthetic transformations.[10,11] This dihydroxyxanthone, however, is sequentially methylated to give *O*-methylsterigmatocystin (**7**, OMST).[12–14] While cell-free conversions of sterigmatocystin (**6**, ST) and OMST to AFB$_1$ have been reported,[12,13,15,16] these important final stages of the biosynthesis have not been determined despite being the subject of several mechanistic proposals.[11,17,18] Described in this paper are expreiments that examine the oxidative cleavage of the xanthone ring system of OMST and define the location and oxidation state of the C_1-fragment lost in the conversion to AFB$_1$. These findings and other evidence place strict limits on the intermediates likely to be involved in the rearrangement process and the nature of the reactions themselves.

Results and Discussion

Sites of isotopic enrichment from a prior incorporation experiment with A-ring [^{13}C]-labeled averufin (**2**)[19] and the pattern of doubly [^{13}C]-labeled acetate utilization in ST[20] and AFB$_1$[21] have made it possible to assign unambiguously the anthraquinone carbons lost in the formation of aflatoxin. Thus, in the cleavage of the anthraquinone and reclosure to the xanthone, the C-10 carbonyl in **2** (hence **4**) was lost. Similarly C-5 in **2**/**4** is also lost. It can be deduced that this carbon corresponds to C-10 in xanthones **5–7**.[19]

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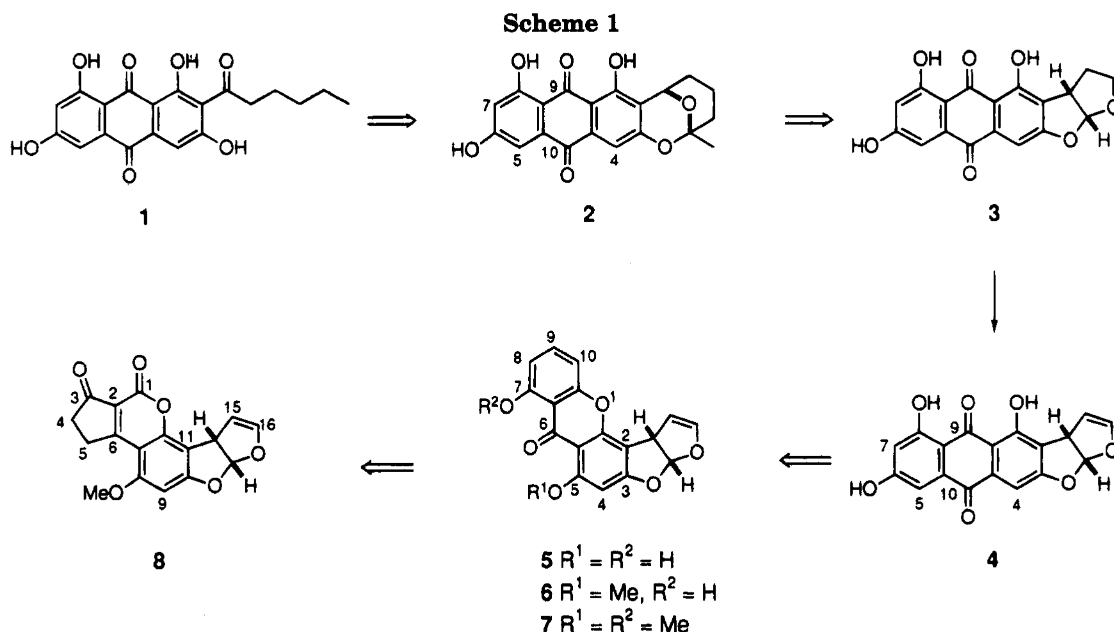
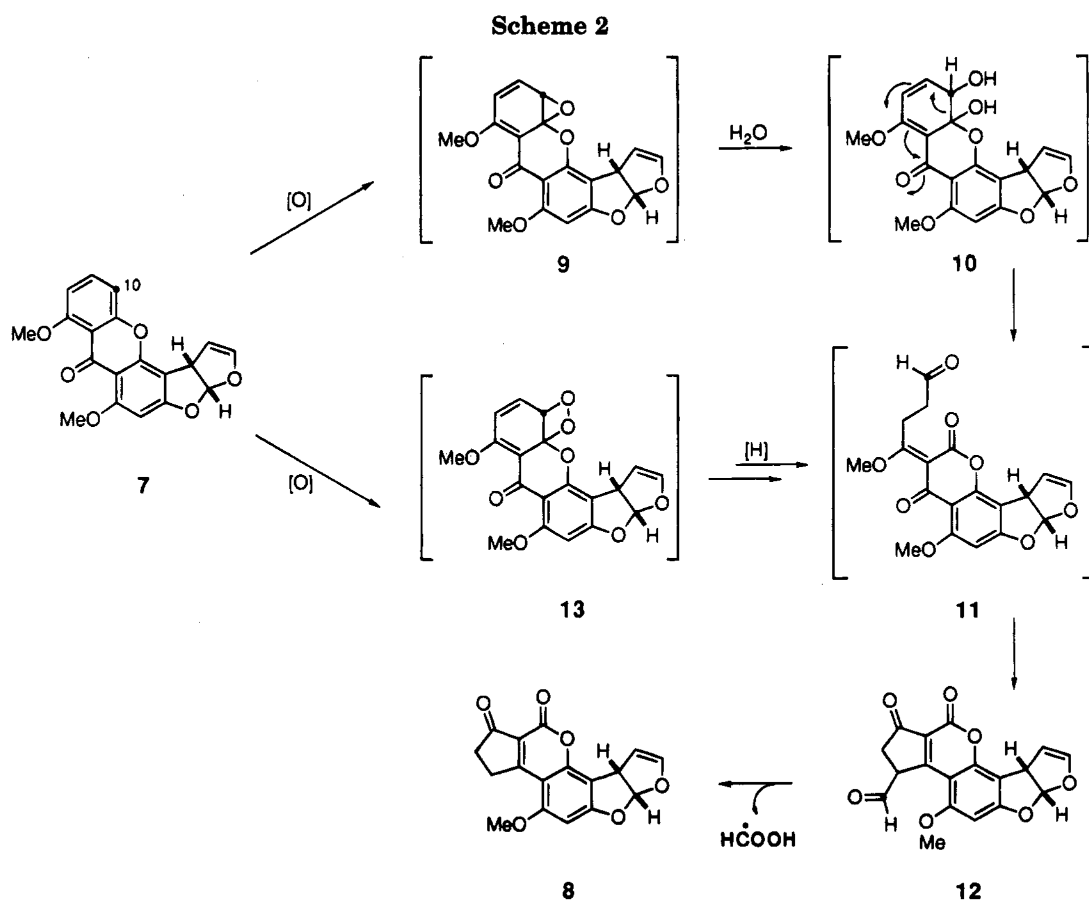
Scheme 1



At least partial purification of an apparent oxidoreductase capable of carrying out the transformation of OMST to AFB_1 has been reported[22] suggesting that perhaps a single enzyme alone is required to execute this seemingly multistep reaction. With this idea in mind, the hypothesis outlined in Scheme 2 was conceived to account for a single oxidation initiating this transformation. In this proposal OMST (**7**) undergoes a monooxygenase reaction in keeping with the observed NADPH requirement of this overall conversion.[16,23] The reactive aryl epoxide **9** can be visualized to readily hydrate (to **10**) and cleave to thestable anion of β-keto lactone **11**. It is worthy of note that the latter cleavage process is advantaged by the presence of the methoxyl at C-7. Aldol condensation would afford **12**, which upon favorable retroaldol reaction would be predicted to release C-10 of **7** as formic acid in the generation of AFB_1 (**8**). The same net transformation could be achieved by a dioxygenase. Decomposition of the presumed dioxetane intermediate **13** would require a subsequent side chain reduction step to give aldehyde **11**.

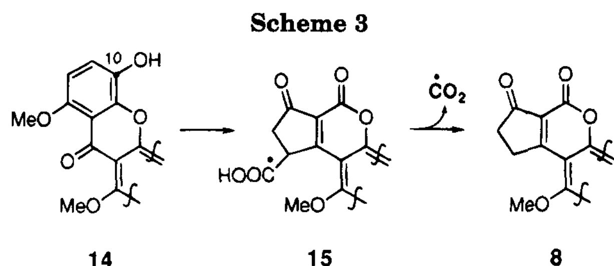
Alternative proposals[15,18,24] have invoked the intermediacy of 10-hydroxy-*O*-methylsterigmatocystin (**14**), not a known natural product so far as we are aware, but 10-

Scheme 2



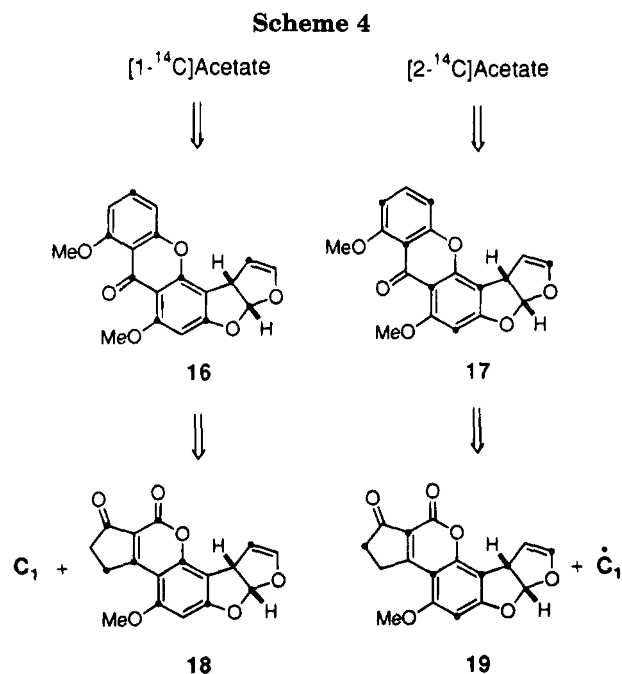
methoxysterigmatocystin is known.[24] Therefore, an analogous process can be advanced involving two net oxidative steps and consequent loss of C-10 as carbon dioxide as summarized in Scheme 3.

Scheme 3



The oxidation state of the C_1-fragment lost reflects, therefore, the minimum number of oxidative transformations that must occur in the overall conversion of OMST (**7**) to AFB_1 (**8**). To examine this question experimentally, [^{14}C]sterigmatocystin was obtained by incorporation[25] of radiolabeled acetate by a mutant of *Aspergillus versicolor* (ATCC 28286). The labeled sterigmatocystin samples so obtained were separately diluted with carrier and methylated to give OMST **16** and **17**. [1-^{4}C]- and [2-^{14}C]-acetate label **6**/**7** as shown in Scheme 4. AFB_1 derived from the latter would be predicted to liberate a radioactive C_1-fragment while the former, acting as a control, should not. By selective processing and trapping from cell-free incubations, it is possible to determine the fraction of radioactivity lost as formaldehyde, formic acid, and/or carbon dioxide.[26] By monitoring the amount and specific radioactivity of the aflatoxin simultaneously produced, the ratio of total radioisotope partitioned between it and the C_1-fragment can be quantitated and should theoretically be 7:1 (see Scheme 4).

Scheme 4



Sterigmatocystin (**6**) production in five media[27] was assayed by thin layer chromatography but found to be most successful in Czapek.[27a] After systematic variation of the culture conditions, addition of trace minerals, and resuspension of the mycelia in a replacement medium[25] at a density of 15 g/100 mL gave good incorporations of radiolabeled acetate (see Experimental Section). The labeled samples of sterigmatocystin were isolated and purified, and a portion (2–4 mg) was diluted with ca. 10 mg of radioinactive carrier and treated with dimethyl sulfate in acetone[28] to afford OMST **16** and **17** in greater than 95% yield.

Conversions of OMST to AFB_1 were carried out in cell-free extracts (CFE) of the *A. parasiticus* mutant AVN-1 (ATCC 56774).[2] This is a doubly blocked mutant that accumulates averantin, an early anthraquinone intermediate in the biosynthetic pathway, and only a minimal background amount of aflatoxin. This strain is, nonetheless, unimpaired in its catalysis of the later steps of aflatoxin biosynthesis when supplied with an intermediate as **6** or **7**. The CFE was prepared by breaking the mycelia in phosphate buffer containing 10% glycerol and other protein stabilizers.[22] The cell debris were removed, and the supernatant after centrifugation was used as the CFE. No background of the highly fluorescent AFB_1 could be detected in this partially purified CFE by thin layer chromatography when visualized by long wavelength UV light. In preliminary trials with radioinactive OMST and added NADPH, aflatoxin production was monitored by reversed-phase HPLC fitted with an integrator recorder and diode array detector. Aflatoxin B_1 was observed at 360 nm while OMST and ST were followed at 310 and 329 nm, respectively. Fewer than 100 ng of aflatoxin could be quantitated by this method. Mycelia harvested between 65 and 96 h were assayed, and ca. 84 h was found to be optimal for production of **8**.

At this point the way was clear to react [^{14}C]OMST **16** and **17** in parallel incubations with the CFE to determine the identity of the C_1-fragment released. In a typical experiment, 150 nmol of unlabeled OMST **7**, [1-^{14}C]OMST **16**, and [2-^{14}C]OMST **17** were separately incubated with 5 mL of CFE and NADPH for 3–7 h. Incubation with unlabeled OMST was performed in order to determine the extent of conversion to AFB_1 by HPLC assay (see Experimental Section). Formaldehyde and formic acid could be detected by standard methods[26] and, if radiolabeled, separately derivatized as their crystalline dimedone and substituted phenacyl esters, respectively. An accurately measured excess of formaldehyde and sodium formate (100 mg) was added to each reaction mixture after incubation for the appropriate amount of time, the mixture was acidified, and the volatile components were distilled to a liquid nitrogen-cooled trap. No radioactivity was detectable from either [^{14}C]OMST **16** or **17** (experiments 1 and 2, Table 1). Given the extent of conversion

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of substrate to aflatoxin (1.9–5.9%), less than 1% of the expected radioactivity could have been readily detected. As a control, [^{14}C]formaldehyde and sodium formate (ca. 1000 dpm) were separately incubated under analogous conditions and gave the expected appearance of radioactivity in the volatile fraction (experiments 3 and 4, Table 1).

Table 1. Summary of Results for Conversion of Radiolabeled OMST to AFB_1

expt	[^{14}C] substrate	% convn to AFB_1	theoretical dpm in C_1 unit: HCHO/HCOONa	theoretical dpm in C_1 unit: CO_2^{a}	obsd dpm^b	% of theoretical dpm in C_1 unit
1	OMST **16**	5.9	519		9	2
2	OMST **17**	5.9	754		37	5
3	$H^{14}CHO$		1093		982	90
4	$H^{14}CO_2Na$		999		937	94
5	OMST **16**	1.9–5.9		217^c	1^c	−3 to 6
6	OMST **17**	1.9–5.9		323^c	245^c	70–88
7	$H^{14}CHO$			1093	9	1
8	$H^{14}CO_2Na$			999	37	4

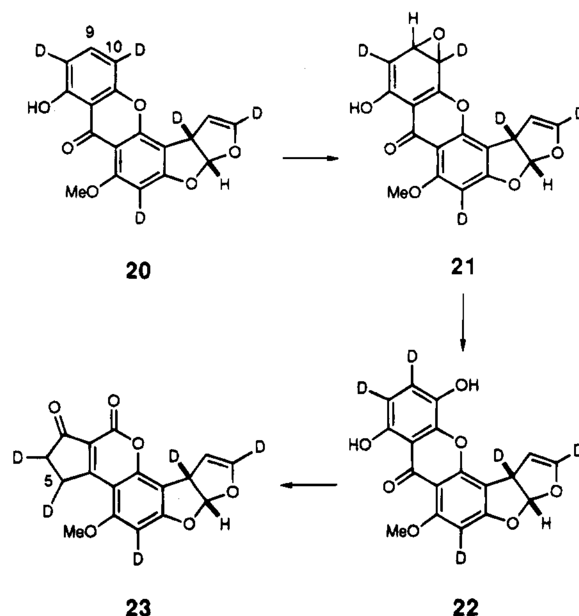
^{a} Corrected for efficiency of CO_2 trapping. ^{b} Corrected for background dpm. ^{c} Average of five runs.

The absence of radioactivity released as formaldehyde or formate led to examination of the CO_2 liberated. Carbon dioxide evolved during each incubation was trapped by hyamine hydroxide solution contained in a plastic well suspended in each reaction vessel. Reactions were quenched by acidification to pH 2, which also converted dissolved bicarbonate to CO_2 for efficient trapping. As the amounts of CO_2 in question were very low in these experiments, the efficiency of trapping by hyamine hydroxide was calibrated in each incubation. This was achieved by treating the CFE with a known quantity of $NaH^{14}CO_3$ and trapping the $^{14}CO_2$ released. In five trials, the actual amount of $^{14}CO_2$ generated from labeled OMST **16** and **17** was compared to the theoretical amount of $^{14}CO_2$ expected based on the extent of conversion to AFB_1 as determined by HPLC (Scheme 4). [^{14}C]-OMST **16** labeled from [1-^{14}C]acetate gave no radiolabel in CO_2, as anticipated, while **17** derived from [2-^{14}C]-acetate gave 70–88% of the predicted value (experiments 5 and 6, Table 1). It could be argued that loss of C-10 in **17** could have occurred as formaldehyde or formate only to be oxidized in the CFE to CO_2. To test this possibility [^{14}C]formaldehyde and [^{14}C]sodium formate were individually incubated with the CFE. In neither of these control reactions could $^{14}CO_2$ be detected (experiments 7 and 8, Table 1). It should be borne in mind that the predicted amount of radioactivity in the C_1-unit requires dividing the total activity present in AFB_1, as determined by HPLC assay, and the specific activity of **17** by eight and presumes a homogeneous level of radiolabeled acetate incorporation in **17**. Errors in the these calculations, however, are probably greater than in the trapping experiment itself and account for the range in values observed in these experiments. Nonetheless, three oxidation states of C_1-loss have been examined in controlled experiments where only radiolabeled CO_2 was observed from **17**.

Conclusion

The outcome of these experiments is, therefore, remarkably clean with C-10 loss from OMST in its conversion to aflatoxin B_1 occurring specifically as carbon dioxide. While not a proven intermediate, this observation points strongly to 10-hydroxyl-OMST (**14**) being involved in the oxidative rearrangement process. An important finding made by Simpson[29] addresses this point further. The incorporation of [2-$^2H_3$]acetate into AFB_1 (**23**, Scheme 5) gave the interesting observation of deuterium incorporation at C-5, an acetate carboxyl-derived center (cf. **18**) suggesting a deuterium migration in the course of the biosynthesis. A similar experiment by Sankawa[30] gave ST labeled with deuterium in the expected manner shown in **20** (Scheme 5). In accord with the interpretation of the former observation,[29] it is proposed that the first oxidation in the overall conversion of OMST (**7**) to aflatoxin B_1 (**8**) involves aryl epoxide formation at C-9/10 (**21**) and NIH shift[31] to give 10-hydroxyl-OMST (**22**). Subsequent cleavage, either through the action of a monooxygenase or dioxygenase, and reclosure with loss of CO_2 could be envisioned as proposed in Scheme 3. Such a process accomplishes in two oxidative steps first hydroxylation and then aryl cleavage to give, after closure, demethylation and decarboxylation, alfatoxin B_1 (**8**). Whether all of these steps can be carried out sequentially by a single enzyme or several is not known.

Scheme 5



More complex mechanisms of xanthone cleavage can be visualized. Hydroxylation at C-10 and subsequent demethylation may be invoked to give **24**.[18,32] Tautomerization then to diketone **25** has been proposed as has oxidation/demethylation to give quinonoid compound **26**.[18] The latter is suggested then to be reduced to **25**, which presumably must undergo Baeyer–Villiger oxidation to initiate aryl cleavage followed by cyclopentenone fusion and decarboxylation. In view of the mechanistic economy of all previous steps in the biosynthesis, it does not seem necessary to invoke these or other more complex processes where a simple one will suffice. However, definition of the final steps in the biosynthesis of aflatoxin

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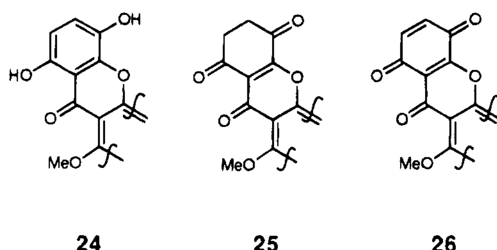
Scheme 6



B_1 can be addressed in direct incorporation experiments with, *e.g.*, **11**, **24**, and **26** and by the isolation, purification, and study of the enzyme(s) involved. Similarly, the proposed role of 10-hydroxydihydrosterigmatocystin in aflatoxin B_2 biosynthesis suggested in earlier experiments can be resolved.[33]

Experimental Section

General Methods. ^1H and ^{13}C NMR were recorded on a Bruker AMX 300 spectrophotometer (operating at 300 MHz for ^1H and 75 MHz for ^{13}C). Chemical shifts for ^1H and ^{13}C NMR spectra are reported in ppm downfield from TMS. UV spectra were recorded on a Beckman DU-70 spectrophotometer. Flash silica column chromatography was performed using 200–240-mesh Merck silica gel. NADPH, *S*-adenosylmethionine, phenylmethanesulfonyl fluoride, and standards of *O*-methylsterigmatocystin, aflatoxin B_1, and sterigmatocystin were obtained from Sigma. Anhydrous potassium carbonate, anhydrous acetone, hyamine hydroxide, β-mercaptoethanol, and dimethyl sulfate were purchased from Aldrich. [1-^{14}C]- and [2-^{14}C]acetate were purchased from Amersham. Permafluor V scintillation cocktail was purchased from Packard Instrument Co. Detection of radioactivity was performed using a Beckman LS 5801 scintillation counter. All chemicals used were of the highest purity unless otherwise stated.

A Varian 5020 microprocessor controlled liquid chromatograph equipped with a 250- × 4.6-mm Adsorbosphere HS 5 C-18 (Alltech) reversed phase column was used for HPLC assays. Column effluent was monitored by UV at 280 nm on an ABI 1000S diode array detector interfaced with a Waters 745 data module recorder/integrator. All solvents were HPLC grade and filtered through a 0.45-μm filter prior to use.

Organisms. Fungi were purchased from ATCC. *Aspergillus versicolor* M1101 (ATCC 28286) was used for the production of sterigmatocystin (**6**). The *A. parasiticus* mutant strain AVN-1 (ATCC 56774) was used to study the conversion of **6** to **8**.

Media. The basal medium (Czapek medium) used to grow *A. versicolor* contained per liter: $NaNO_3$, 3 g; KH_2PO_4, 1 g; anhydrous $MgSO_4$, 0.25 g; KCl, 0.5 g; $FeSO_4$, 0.01 g; sucrose, 30 g. For the ^{14}C-labeled acetate feeding experiments, the Czapek basal medium was supplemented with trace metals (2 mL).[27b] The resting medium (RM) used to prepare the resting cultures of *A. versicolor* contained per liter: K_2HPO_4, 5 g; anhydrous $MgSO_4$, 0.25 g; KCl, 0.5 g; and trace metals, 2 mL.

The basal medium used to grow AVN-1 contained per liter: glucose, 50 g; $(NH_4)_2SO_4$, 3 g; K_2HPO_4, 10 g; anhydrous $MgSO_4$, 1 g; and trace metals, 2 mL.

Typically, 1 L of the growth medium was autoclaved (140 °C, 20 psi) for 25 min in a 4-L Erlenmeyer flask and cooled to rt prior to inoculation.

Culture Techniques. The fungi were grown on potato agar plates for 3–6 days. The agar plates contained per liter: yeast extract, 5 g; Bactoagar, 5 g; potato dextrose agar, 19 g. The spores were suspended in a solution of Tween 80 (0.05% v/v) in NaCl (0.85 % w/v, 20 mL). A portion (1 mL) of the culture spore suspension was then transferred to 1 L of the desired growth medium in 4-L Erlenmeyer flasks, and the flasks were incubated on a rotary shaker at 175 rpm at 27–30 °C in the dark.

Biosynthesis of Sterigmatocystin (6).[27c] *A. versicolor* M1101 was grown on Czapek medium (1 L) as described above. Sterigmatocystin production was evident by TLC (silica/chloroform:methanol 98:2) on the sixth day of incubation. Sterigmatocystin production was estimated by UV spectrophotometry at 329 nm using a molar extinction coefficient of 13 100.[34] After ST production had tapered off (24 days), the mycelia were vacuum filtered and ST was extracted from the aqueous phase with chloroform. The solid mycelia were then suspended in acetone (500 mL) and sonicated to break open the cells. The broken cell suspension was then heated to about 50 °C for 4–5 h to extract ST from the cells. The acetone was filtered and evaporated to yield a yellow brown solid, which was then combined with the organic phase, dried over magnesium sulfate, and purified by flash silica column chromatography (CHCl.MeOH 99:1) to yield 140 mg of ST, mp 246–247 °C (lit.[34] mp 246 °C). ^1H NMR (300 MHz; $CDCl_3$): 7.47 (dd, J = 8.3, 8.3 Hz, 1H); 6.80 (d, J = 7.2 Hz, 1H); 6.78 (dd, J = 8.3, 0.9 Hz, 1H); 6.72 (dd, J = 8.3, 0.9 Hz, 1H); 6.48 (dd, J = 2.8, 2.1 Hz, 1H); 6.39 (s, 1H); 5.42 (dd, J = 2.6, 2.6 Hz, 1H); 4.76 (ddd, J = 7.2, 2.16, 2.14 Hz, 1H), 3.97 (s, 3H). ^{13}C NMR (300 MHz; $CDCl_3$): 181.3; 164.5; 163.2; 162.2; 154.0; 153.0; 145.3; 135.6; 113.2; 111.2 (2C); 108.8; 106.5; 105.8; 102.5; 90.4; 56.7; 48.0.

***O*-Methylsterigmatocystin (7).** Synthesis of *O*-methylsterigmatocystin was carried out by the method of Davies[28] using sterigmatocystin (30 mg, 92.5 μmol) in dry acetone (12 mL), anhydrous K_2CO_3 (500 mg, 3.6 mmol), and dimethyl sulfate (354 μL, 3.8 mmol). The reaction mixture was heated to reflux until no starting material was evident. The acetone was evaporated, the resulting solid was washed with water, and the product was extracted into chloroform. The chloroform layer was then dried over anhydrous $MgSO_4$ and purified via flash silica column chromatography to yield 35 mg of partially purified OMST. The crude product was recrystallized from ethanol to yield 24 mg of pure OMST (77% yield), mp 265–266 °C (lit.[34] mp 265–267 °C). ^1H NMR (300 MHz; $CDCl_3$): 7.49 (dd, J = 8.3, 8.3 Hz, 1H); 6.92 (dd, J = 8.3, 0.9 Hz, 1H); 6.78 (d, J = 7.2 Hz, 1H); 6.76 (dd, J = 8.3, 0.9 Hz, 1H); 6.48 (dd, J = 2.7, 2.1 Hz, 1H); 6.38 (s, 1H); 5.43 (dd, J = 2.7, 2.5 Hz, 1H); 4.78 (ddd, J = 7.2, 2.21, 2.15 Hz, 1H), 3.96 (s, 3H); 3.92 (s, 3H). ^{13}C NMR (300 MHz; $CDCl_3$): 174.6; 162.8 (2C); 160.5; 156.6; 152.9; 145.1; 133.5; 112.9; 108.9; 106.1 (2C); 105.5; 102.6 (2C); 90.3; 56.7 (2C); 48.1.

Production of [^{14}C]Sterigmatocystin. *A. versicolor* M1101 was grown in two 4-L triply baffled Erlenmeyer flasks (Bellco) in Czapek medium that had been supplemented with trace metals. The flasks were incubated at 28 °C at 175 rpm. Once ST production had started (fifth day of incubation), the mycelia were vacuum filtered and washed extensively with resting medium (ca. 2 L) to yield about 161 g of cells (wet wt). Eight 500-mL Erlenmeyer flasks each containing 100 mL of resting medium that had been supplemented with trace metals were autoclaved and then cooled to rt. The mycelia (ca. 16 g wet wt) were then resuspended in each of the flasks. To each flask was then added 25 mg of glucose (sterile filter) and 250 μCi (specific activity 55 Ci/mol) of the appropriate [^{14}C]sodium acetate. One set of four flasks contained mycelia fed [1-^{14}C]-acetate while the other set of four was fed [2-^{14}C]acetate. The flasks were then connected in series to an air tight system. Each set of the flasks was isolated by a KOH (3 M) trap to ensure no contamination of label between the two sets of flasks. Compressed air was circulated through the fermentation setup by a peristaltic pump. The flasks were then incubated at 28 °C at 175 rpm in an incubator shaker in the dark. After 24 h a further 250 μCi of the appropriately labeled [^{14}C]acetate was added to each of the eight flasks, and the mycelia were incubated for another 5 days. The mycelia were filtered and steeped in acetone (ca. 300 mL) overnight. The acetone extract was filtered and evaporated to half its volume,

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and the mycotoxins were extracted into chloroform. The chloroform layer was dried over anhydrous $MgSO_4$, evaporated, and purified by flash silica column chromatography ($CHCl_3$:MeOH 99:1) to yield radiolabeled sterigmatocystins (8.54 mg, specific activity 0.86 Ci/mol, 1.13% total incorporation isolated) and (5.45 mg, specific activity 1.90 Ci/mol, 1.59% total incorporation isolated), respectively, from the uptake of [1-^{14}C] and [2-^{14}C]acetate.

Synthesis of [^{14}C]-*O*-Methylsterigmatocystin (16/17). Unlabeled **6** (10.46 mg) was mixed with radiolabeled sterigmatocystin from incorporation of [1-^{14}C]acetate (4.07 mg, specific activity 0.86 Ci/mol) and methylated with dimethyl sulfate using a procedure similar to that described above to give **16** in 98% yield (15.01 mg, specific activity 0.24 Ci/mol). Similarly, unlabeled **6** (11.00 mg) was mixed with radiolabeled sterigmatocystin from incorporation of [2-^{14}C]acetate (2.15 mg, specific activity 1.90 Ci/mol) and methylated to afford **17** in 96% yield (13.12 mg, specific activity 0.31 Ci/mol).

Preparation of Cell-Free Extracts.[22] AVN-1 was grown on the growth medium described above, and after the desired incubation time (usually 84 h) the mycelial pellets were harvested on cheese cloth by filtration and washed extensively with buffer (0.05 M potassium phosphate, pH 7.5; 10% glycerol; 2 mM β-mercaptoethanol and 100 mM phenylmethanesulfonyl fluoride). The wet mycelia (ca. 25 g wet wt) were then frozen in liquid nitrogen and broken open under liquid nitrogen with an Oster blender using 15-s cycles for 1−2 min. Alternatively, the frozen cells were lyophilized and the dried cells were then pulverized using a mortar and pestle. The powdered mycelia were suspended in buffer (ca. 200 mL) and the mixture was stirred at 4 °C for 30 min. The mixture was first centrifuged at 5000*g* for 5 min to remove most of the cell debris. The supernatant was further centrifuged at 20000*g* for 15 min to remove the remaining cell debris. The supernatant thus obtained was then stirred with Cell Debris Remover (CDR, 15 g/100 mL; Whatman) for 30 min at 4 °C and then centrifuged at 5000*g* for 5 min. This partially purified cell free extract was used to assay for enzyme activity.

Assay for Enzyme Activity. To the cell-free extract (5 mL) was added a solution of OMST in acetone (20 μL, 3 mM) and NADPH (40 μL, 1.6 mg/mL). The reaction mixture was kept at 37 °C. One mL fractions of the reaction mixture were assayed over time. The reaction mixture was quenched with chloroform (1 mL), and the mycotoxins were extracted into the organic layer. The organic layer was concentrated and applied to TLC plates, eluted ($CHCl_3$:EtOAc:90% HCOOH 6:3:1), and compared to a standard sample of AFB_1. Alternatively, the organic layer was analyzed by HPLC (250 × 4.6 mm C-18, Adsorbosphere column) using a gradient of 30−60% acetonitrile in 30 min. AFB_1 and OMST eluted at 14.7 and 21.7 min, respectively. Quantitation was achieved by digital integration. The integrated peak areas were compared to standard curves to ascertain the amount of OMST remaining as well as the amount of AFB_1 produced and were used to determine the extent of conversion.

Detection of [^{14}C]-Labeled Formate, Formaldehyde, and CO_2. To CFE (5 mL) in a 15-mL test tube was added NADPH (500 μM). The test tube was sealed with a rubber septum which was equipped with a plastic well (Kontes). In the plastic well was placed a piece of filter paper that had been soaked with a methanolic solution of hyamine hydroxide (40 mL, 1 M). The reaction was initiated by the addition of a solution (in acetone) of either [1-^{14}C]- or [2-^{14}C]acetate-labeled OMST (150 nmol; 30 mM) by syringe. A control reaction with unlabeled OMST was carried out in parallel in order to determine the extent of conversion to AFB_1 (by HPLC using conditions described above). Another control reaction containing no OMST but a known quantity of $NaH^{14}CO_3$ (ca. 1000 dpm) was also carried out in parallel to calibrate the hyamine hydroxide uptake of CO_2. This was necessary since the trapping efficiency of the methanolic solution of hyamine hydroxide decreased with time. The reaction was left standing at rt for 3−7 h. Trifluoroacetic acid or phosphoric acid (100 μL) was added to pH 2, and the reaction mixture was then incubated at 37 °C for 90 min. The plastic well containing the filter paper was removed and counted for ^{14}C radioactivity in 20 mL of Permafluor V. Results obtained for five experiments are tabulated below.

Table 2. Conversion of OMST 17 to Aflatoxin B_1 19 and CO_2

no.	% convn to AFB_1	CO_2 trapping efficiency (%)	expected counts (dpm)	obsd counts (dpm)	% $^{14}CO_2$ of theoretical
1	5.9	63	475	418	88
2	5.8	63	457	343	75
3	3.8	38	182	134	74
4	5.6	37	305	265	74
5	1.9	82	196	137	70

The acidified reaction mixture was centrifuged to remove precipitated protein. Sodium formate (100.0 mg) and formaldehyde (100.0 mg) were added, and the acid volatiles were distilled over and trapped in liquid nitrogen. The acid volatiles were then tested for radioactivity to determine the presence of [^{14}C]formate or [^{14}C]formaldehyde.

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