Identification of Averantin as an Aflatoxin B$_1$ Precursor: Place-ment in the Biosynthetic Pathway

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A new blocked mutant of Aspergillus parasiticus produces no detectable aflatoxin B$_1$, but accumulates several polyhydroxyanthraquinones. One of these pigments was identified as averantin. This is the first report of its formation by A. parasiticus. Radiotracer studies with [¹⁴C]averantin showed that 15.3% of label from averantin was incorporated into aflatoxin B$_1$. This incorporation was blocked by dichlorvos. With radiotracers and other mutants, averantin was placed after norsolorinic acid and before averufin in the biosynthetic pathway in which the general steps are norsolorinic acid → averantin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin → aflatoxin B$_1$.

Aflatoxins are a family of toxic, carcinogenic secondary metabolites produced by certain strains of Aspergillus flavus and A. parasiticus. Aflatoxin biosynthesis proceeds through a polyketide pathway in which the general steps are acetate → anthraquinones → xanthones → aflatoxin B$_1$. There are several recent reviews of aflatoxin biosynthesis (1a, 10, 19, 20).

¹⁴C radiotracer studies have been used to identify five compounds as aflatoxin precursors. These intermediates are norsolorinic acid (12), averufin (17, 18), versiconal hemiacetal acetate (3, 8, 24), versicolorin A (14), and sterigmatocystin (11). The structures of these compounds and of aflatoxin B$_1$ are presented in Fig. 1. Norsolorinic acid, averufin, versiconal hemiacetal acetate, and versicolorin A are substituted polyhydroxyanthraquinones; sterigmatocystin is a xanthone. Norsolorinic acid (4, 16), averufin (6), and versicolorin A (15) are accumulated by blocked mutants of A. parasiticus. Versiconal hemiacetal acetate accumulates during dichlorvos treatment of aflatoxicogenic strains of A. parasiticus (8, 24). Sterigmatocystin, which shows the highest incorporation of [¹⁴C]label into aflatoxin B$_1$ of the known precursors, is a natural metabolite of A. versicolor (9, 13) and a number of other fungal species (21). There is one published report of sterigmatocystin from an aflatoxicogenic species (22).

We have isolated a new mutant strain of A. parasiticus that is blocked in aflatoxin production and that accumulates a number of polyhydroxyanthraquinones. We here identify one of these polyhydroxyanthraquinones. Radiotracer studies with [¹⁴C] indicated that label from this compound was incorporated in aflatoxin B$_1$ by resting cells of wild-type A. parasiticus. Experiments with dichlorvos, other blocked mutant strains, and known aflatoxin precursors placed the new intermediate in the aflatoxin biosynthetic pathway.

MATERIALS AND METHODS

Strains. The wild-type strain was an aflatoxigenic isolate of A. parasiticus designated SU-1 (NRRL-5862). The blocked mutant strains were A. parasiticus ATCC 24690, which accumulates norsolorinic acid (NOR-1); A. parasiticus ATCC 15517, which accumulates averufin (AV-1); and a new blocked mutant, which we have designated ver-mu-39. Strain ver-mu-39 was isolated from a versicolorin A-accumulating strain of A. parasiticus (ATCC 36537) by nitrosoguanidine treatments by the method of Donkersloot and Matales (5). A preliminary report of the isolation of ver-mu-39 was presented elsewhere (J. W. Bennett, F. Kronberg, and G. Gougis, Abstr. Annu. Meet. Ann. Soc. Microbiol. 1976, 06, p. 181).

Production and isolation of averantin. Strain ver-mu-39 was cultured in liquid defined medium (1) by the procedure described by Lee et al. (15). Wet mycelia were extracted with acetone until colorless, and water was added to make a 30% acetone solution. The acetone solution was washed with hexane, and the pigment was then partitioned from the acetone into chloroform. The chloroform fraction was evaporated to dryness and then further purified by dry column chromatography (7) on Silica Gel GH-R (Brinkman) with chloroform-acetone (95:5) as the developing solvent. The isolated pigment was recrystallized twice from chloroform. Purity of the recrystallized pigment was determined by the appearance of only one spot after thin-layer chromatography on adsorbosil-1 (Applied Science, Inc.) plates developed in chloroform-acetone (95:5) and on GH-R plates developed in benzene-acetic acid (95:5).

Radiolabel experiments. The media, culture con-
dions, and procedures described by Lee et al. (14) were used to prepare \[^{14}C\] precursors to test percent conversion. Strain ver-mu-39 and NOR-1 were incubated in resting cell media with [\(^{14}C\)]acetate to prepare \[^{14}C\] averantin and \[^{14}C\] norsolorinic acid. The resultant radiolabeled averantin was added to resting cell cultures of NOR-1, AV-I, and SU-1 to test incorporation of averantin into norsolorinic acid, averufin, and aflatoxin B\(_1\), respectively. The radiolabeled norsolorinic acid was added to resting cell cultures of ver-mu-39 to test incorporation of norsolorinic acid into averufin. In addition, SU-1 was incubated with \[^{14}C\] averantin with and without 20 \(\mu\)g of dichlorvos (dimethyl 1,2-dichlorovinyl phosphate) per ml to test the effect of this inhibitor on the incorporation of the pigment into aflatoxin B\(_1\).

Physical and chemical analysis. The melting point was determined by a sealed tube on a Thomas 4 looker apparatus. High resolution mass spectrum was obtained with a CED-21-110B spectrometer. A Fourier Transform Infrared was used to obtain the infrared spectrum. The ultraviolet spectrum was determined on a Beckman DK2-A recording spectrophotometer. The nuclear magnetic resonance spectrum was measured with a Varian CFT-20 spectrometer with a proton probe at 80 MHz. The solvent was deuterated dimethyl sulfoxide with tetramethyldsilane as the reference.

RESULTS

Characterization of averantin. The purified, recrystallized pigment from ver-mu-39 represented 0.22\% of the dried mycelia. The compound melted at 232°C. The mass spectrum shown in Fig. 2 exhibited a molecular ion at 372 \(m/e\) and a large peak at 354 \(m/e\), indicative of the loss of water. The melting point, infrared absorption (Fig. 3), and ultraviolet absorption maxima in ethanol [in nanometers \((\alpha)\)] at 222 (26,784), 262 (15,810), 298 (20,386), 315 (22,022) and 453 (6,658) are compatible with melting point and absorption reported for a pigment isolated from \(A\). versicolor and identified as 1,3,6,8-tetrahydroxy-2-[1-hydroxyhexyl] anthraquinone or averantin (2).

A comparison of our nuclear magnetic resonance data with the data reported by Birkinshaw et al. (2) is shown in Table 1. These data are consistent with their proposed structure for averantin. Birkinshaw et al. used D\(_2\)O–NaOD and were not able to report a hydroxyl shift. With deuterated dimethyl sulfoxide, we showed the different hydroxyl signals. The hydroxyl sig-

![Fig. 1. Structures of known aflatoxin precursors and aflatoxin B\(_1\). I, Norsolorinic acid, \(C_{20}H_{18}O_7\), molecular weight 370. II, Averantin, \(C_{20}H_{19}O_8\), molecular weight 372. III, Averufin, \(C_{20}H_{19}O_8\), molecular weight 368. IV, Versiconal hemiacetal acetate, \(C_{20}H_{19}O_8\), molecular weight 400. V, Versicolorin A, \(C_{18}H_{16}O_3\), molecular weight 338. VI, Sterigmatocystin, \(C_{18}H_{17}O_7\), molecular weight 324. VII, Aflatoxin B\(_1\), \(C_{17}H_{15}O_8\), molecular weight 312.](http://aem.asm.org/graphics/FIG-1.jpg)
nals of the methine carbon tend to be in exchange with the two hydroxyls in the adjacent rings, giving rise to a lower proton integration.

The structure of averantin is shown in Fig. 1 (II), along with the structures of the other known precursors of aflatoxin B₁. The thin-layer chromatographic mobility of averantin was compared with the mobility of norsolorinic acid (Fig. 1, I), averufin (Fig. 1, III), versicolorin A (Fig. 1, V) and versicolinal hemiacetal acetate (Fig. 1, IV) in two solvent systems (Table 2). Averantin has a different mobility from any of the known pigmented precursors in both solvent systems.

Incorporation of [14C]averantin into aflatoxin B₁. The conversions of [14C]averantin and of [1-14C]acetate into aflatoxin B₁ are compared in Table 3. Averantin showed an incorporation efficiency of 15.3%, and acetate showed an incorporation efficiency of 0.72%. Also presented in Table 3 are the specific activities of the precursors and the product (aflatoxin B₁) and the relative specific activities. Specific activity is a measure of the amount of radioactivity in a compound. The specific activities of the precursors were different, because the sodium acetate was not diluted with unlabeled carrier and had a high specific activity (58.5 μCi/mol), whereas the specific activity of the randomly labeled averantin was low (0.243 μCi/mol). However, the specific activity of the product formed from the two sources did not show such a wide difference. The specific activity of the aflatoxin B₁ formed from [14C]averantin was 0.061, and the specific activity of the aflatoxin B₁ formed with [1-14C]acetate was 0.431. The relative specific activity reflects the precursor-product relationship. The relative specific activity for averantin was 0.251 and that for acetate was 0.007. These results indicate that averantin serves as an effective precursor to aflatoxin B₁ synthesis by resting cells of A. parasiticus and that averantin was converted into aflatoxin B₁ without intermediary degradation into acetate.

Placement of averantin in biosynthetic pathway. When [14C]averantin was added to resting cells of A. parasiticus in the presence of 20 μg of dichlorvos per ml, the incorporation efficiency was 3.5%, indicating that dichlorvos blocks the incorporation of label from [14C]averantin into aflatoxin B₁. Because dichlorvos
blocks a step in the aflatoxin pathway after averufin (23, 24), additional labeling experiments with [14C]averufin and resting cells of the blocked mutants that accumulate norsolorinic acid and averufin were performed. These results are presented in Table 4. Although averufin formed by resting cells of AV-1 retained 31.1% of the label from averantin, there was no detectable radioactivity in the norsolorinic acid of resting cells of NOR-I. These results indicate that averantin is before averufin and after norsolorinic acid in the biosynthetic pathway. The efficient transformation of [14C]-labeled norsolorinic acid into averufin (26.8%) by resting cells of ver-mu-39 places norsolorinic acid before averantin in the aflatoxin pathway (see Table 4).

**DISCUSSION**

The use of blocked mutants, metabolic inhibitors, radioactive labeling experiments, and cellfree systems are the major experimental approaches for identifying an intermediate in a biosynthetic pathway. Our experiments fulfill three of these criteria for the demonstration of averantin as a new aflatoxin B1 precursor. First, averantin is produced by a blocked mutant of A.

**TABLE 3. Incorporation of [14C]averufin and [1.14C]acetate into aflatoxin B1**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Compound</th>
<th>µCi</th>
<th>µmol</th>
<th>dpm (×10^6)</th>
<th>Sp act (Ci/mmol)</th>
<th>Relative sp act*</th>
<th>% Conversion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor added</td>
<td>Averufin</td>
<td>0.1</td>
<td>0.411</td>
<td>22.2</td>
<td>0.243</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>0.1</td>
<td>0.170</td>
<td>2.164</td>
<td>58.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product (aflatoxin B1)</td>
<td>Averufin</td>
<td>0.015</td>
<td>0.251</td>
<td>3.4</td>
<td>0.061</td>
<td>0.251</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>0.107</td>
<td>0.248</td>
<td>15.6</td>
<td>0.431</td>
<td>0.007</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* Specific activity of product/specific activity of precursor.

* Disintegrations per minute for product/disintegrations per minute for precursor.
parasiticus (ver-mu-39) which makes no detectable aflatoxin but which accumulates several anthraquinone pigments, including averantin. Second, radiostrainer experiments show that label from [14C]averantin is efficiently incorporated into aflatoxin B1 with little or no intermediary degradation into acetate. Finally, dichlorvos, an organophosphate insecticide which specifically inhibits aflatoxin B1 synthesis after averufin and before versicolonal hemiacetal acetate, also inhibits the conversion of [14C]averantin into aflatoxin B1.

Experiments that demonstrate the transformation of label from [14C]averantin into averufin but not into norsolorinic acid, and the transformation of [14C]norsolorinic acid into averantin, place averantin early in the aflatoxin pathway. On the basis of these results and previous studies (11, 12, 14, 17, 23, 24), we add a new step to the biosynthetic pathway to aflatoxin B1 in which the general sequence is acetate → hypothetical polyketide intermediate → norsolorinic acid (Fig. 1, I) → averantin (Fig. 1, II) → averufin (Fig. 1, III) → versicolonal hemiacetal acetate (Fig. 1, IV) → versicolorin A (Fig. 1, V) → sterigmatocystin (Fig. 1, VI) → aflatoxin B1 (Fig. 1, VII).

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LITERATURE CITED