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Averufanin Is an Aflatoxin B_1 Precursor between Averantin and Averufin in the Biosynthetic Pathway

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Wild-type *Aspergillus parasiticus* produces, in addition to the colorless aflatoxins, a number of pigmented secondary metabolites. Examination of these pigments demonstrated that a major component was an anthraquinone, averufanin. Radiolabeling studies with [^{14}C]averufanin showed that 23% of the label was incorporated into aflatoxin B_1 by the wild type and that 31% of the label was incorporated into *O*-methylsterigmatocystin by a non-aflatoxin-producing isolate. In similar studies with blocked mutants of *A. parasiticus* the ^{14}C label from averufanin was accumulated in averufin (72%) and versicolorin A (54%) but not averantin. The results demonstrate that averufanin is a biosynthetic precursor of aflatoxin B_1 between averantin and averufin.

Aflatoxins are toxic and carcinogenic secondary metabolites of *Aspergillus flavus* Link ex. Fries and *Aspergillus parasiticus* Spear. ^{14}C radiotracer studies and mutants of *A. parasiticus* deficient in toxin formation that accumulate norsolorinic acid (NOR) (13, 16), averantin (AVN) (5), averufin (AVR) (9, 17, 18), or versicolorin A (VERA) (14, 15) have been used to elucidate the aflatoxin biosynthetic pathway. No mutant accumulates versiconal hemiacetal acetate, but this pigment has been shown to be an aflatoxin B_1 precursor (19). The accepted scheme of these precursors in aflatoxin biosynthesis is NOR → AVN → AVR → versiconal hemiacetal acetate → VERA → sterigmatocystin → aflatoxin B_1 (3). A recent report of a non-aflatoxin-producing isolate of *A. parasiticus* identified a xanthone, *O*-methylsterigmatocystin (OMST), that is an intermediate between sterigmatocystin and aflatoxin B_1 (7).

Wild-type *A. parasiticus*, in addition to the aflatoxins, forms a deep yellow pigmentation when grown in static culture. Two of the known aflatoxin precursors, AVR and VERA, have been reported as components of this yellow pigmentation (4). Since anthraquinone precursors have been found in fermentation of the wild type, it suggested that other pigments produced by the fungus are also precursors. The current study was carried out to characterize one such pigment produced by *A. parasiticus* and identify its role in aflatoxin biosynthesis.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type strain used in this study was an aflatoxigenic isolate of *A. parasiticus* designated SU-1 (NRRL 5862). The blocked mutant strains used in feeding studies were *A. parasiticus* SRRC 165 (AV-1) which accumulates AVR, *A. parasiticus* SRRC 163 (AVN-1) which accumulates both VERA and AVN, and *A. parasiticus* SRRC 164B (VER-1) which accumulates VERA. The non-aflatoxin-producing isolate used in feeding studies was *A. parasiticus* SRRC 2043 which accumulates OMST. Growth medium, replacement medium, and low-sugar replacement medium were prepared by the methods of Adye and Mateles (1).

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Production, isolation, and characterization of pigment. *A. parasiticus* SU-1 was grown in surface culture on growth medium at 28°C for 5 to 7 days. Mycelial mats were harvested by filtration and extracted with acetone until colorless. The combined extracts were concentrated to dryness over a steam bath. The residue was redissolved in methanol and separated by Sephadex LH-20 column chromatography into two fractions eluted from the column with methanol. A blue fluorescent aflatoxin-containing fraction was eluted first (visualized with a long-wavelength UV lamp), followed by a yellow anthraquinone-containing fraction. The anthraquinone fraction was rechromatographed on a Sephadex LH-20 column (12 by 1 in. [30 by 2.5 cm]) with acetone as the eluting solvent. The major pigment eluted in the first 100-ml fraction; this pigment was further purified by preparative silica gel thin-layer chromatography (TLC) with toluene-ethyl acetate-acetic acid (50:30:4, vol/vol/vol) (TEA) as the developing solvent. The compound with $R_f = 0.74$ was repurified with acetone on a Sephadex LH-20 column before spectral analysis. UV and visual spectra of the isolated pigment were recorded in methanol and methanol with aluminum chloride on a Shimadzu UV-160 spectrophotometer. Mass spectra were recorded on a Finnigan 4000 MAT with a ballistic probe. The melting point of the pigment was characterized with a Thomas Scientific apparatus. The colors of the pigment on silica gel TLC were observed in visible

TABLE 1. Efficiency of conversion of averufanin to other secondary metabolites in the aflatoxin biosynthetic pathway and to aflatoxin[a]

Fungal strain used	Metabolite analyzed	dpm recovered[b]	Incorporation efficiency (%)
AVN-1	AVN	402	0
	VER A	3.76×10^5	51.2
AV-1	AVR	5.3×10^5[c]	71.7[c]
VER-1	VER A	3.97×10^5[c]	53.9[c]
SRRC 2043	OMST	2.35×10^5	32
SU-1	Aflatoxin B_1	1.69×10^5	23

[a] The amount of averufanin added was 7.35×10^5 dpm (0.334 μCi) from a stock averufanin solution of 1.67 μCi/ml in acetone.

[b] Corrected for recovery (83 ± 4%) of added radioactivity in control experiments with autoclaved mycelia.

[c] Corrected for radioactivity recovered in B_1.

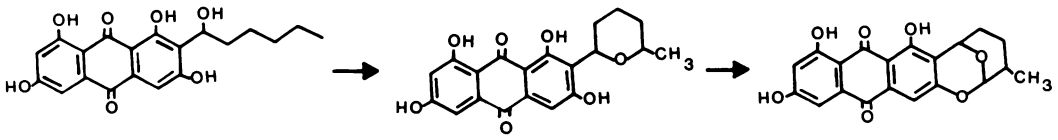

FIG. 1. Structures of AVN, averufanin, and AVR.

light, long-wavelength UV, and after spraying with a 1% solution of Naturstoffreagenz A (NA) (β-aminodiethylester of diphenylboric acid; Sigma Chemical Co., St. Louis, Mo.) in methanol.

Radiolabeling studies. The media, culture conditions, and procedures were identical to those used by Lee et al. (14) for the preparation of radiolabeled VERA. A total of 1 mCi of [1-^{14}C]acetate was added to the fermentation medium. In the present study, the mycelial pellets were harvested by vacuum filtration before extraction with acetone. The extract was separated by preparative TLC with the TEA solvent for development. The band at R_f = 0.74 was scraped from the plate, and the pigment was eluted with acetone.

The isolated ^{14}C-labeled pigment was fed to resting cell cultures of SU-1, AVN-1, AV-1, VER-1, and SRRC 2043 by the procedure described by Lee et al. (14). After 20 h the cultures were extracted by standard procedures (10, 14) and assayed for radioactivity in aflatoxin B_1 (SU-1), OMST (SRRC 2043), VERA (VER-1), AVN and VERA (AVN-1), or AVR (AV-1). These metabolites were identified by cochromatography with standard compounds on silica gel TLC with TEA. Kodak I scintillation solvent and a Beckman LS1800 liquid scintillation counter were used for the ^{14}C assay.

RESULTS

Physical and spectral characterization of pigment from SU-1. A yellow pigment was isolated from *A. parasiticus* at an R_f of 0.74 on silica gel TLC in TEA (0.05% of dry mycelial weight). Spraying the plate with NA caused the spot to turn pink (visible light). Comparison with standard compounds showed that anthraquinones with a side chain at the 2-position (e.g., NOR and AVN) turn purple with NA spray reagent and that anthraquinones with a cyclized group (e.g., AVR, VERA, and VERC) turn pink. The isolated pigment had major absorbances in the UV and visible spectra at 460, 312, 290, 263, and 219 nm, similar to the versicolorin-type anthraquinones (8). A bathochromic shift of 60 nm, to 520 nm, after the addition of aluminum chloride was compatible with the shift observed with an AVR standard. The mass spectrum of the isolated anthraquinone exhibited a molecular ion at *m/z* 370, suggesting a structure similar to NOR (M^+ = 370), AVN (M^+ = 372), or AVR (M^+ = 368). Although both the isolated pigment and NOR have molecular ions at M^+ = 370, these two compounds can be clearly distinguished since NOR becomes purple with NA spray, while the isolated pigment turns pink with this reagent. The mass spectrum of NOR has a base peak at *m/z* 299, a relatively small molecular ion (20% base peak), and fragments at *m/z* 327, 314, and 272. The isolated pigment has base peaks at *m/z* 370 and 286 and major fragments in the mass spectrum at *m/z* 352, 327, 325, 313, 311, 299, 297, and 272. The major fragments of the isolated pigment are identical to those reported for averufanin (2, 6). The compound had a melting point of 257 to 259°C (reported melting point for averufanin is 260°C [6]). All the data are compatible with the identification of the pigment as averufanin.

Conversion of [1-^{14}C]acetate to [^{14}C]averufanin. Averufanin was extracted from the mycelial pellets from shake cultures of SU-1 incubated on replacement medium with 1,000 μCi of [1-^{14}C]acetate; the isolated pigment contained 1.67 μCi of radioactivity or 0.167% of the initial radioactivity was incorporated into averufanin. Therefore, based on the accumulation of between 0.4 to 0.6 mg of averufanin per g of mycelial dry weight, the specific activity of the isolated averufanin was 17.7 μCi/mol.

Conversion of [^{14}C]averufanin to aflatoxin B_1 and aflatoxin precursors. Feeding ^{14}C-labeled averufanin to resting SU-1 cells resulted in conversion of 23% of the label into aflatoxin B_1. The 23% conversion is intermediate between the reported 15.3% conversion of AVN into B_1 (5) and the 49.4% conversion of AVR into B_1 (19). The results suggest placement of averufanin in the pathway between AVN and AVR.

[^{14}C]averufanin was fed to *A. parasiticus* mutants to identify the efficiency of conversion to other intermediates in the aflatoxin pathway. ^{14}C label from averufanin was incorporated into AVR by strain AV-1, VERA by strains VER-1 and AVN-1, and OMST by strain SRRC 2043 (Table 1). Since no label from averufanin was incorporated into AVN by strain AVN-1, averufanin appears to follow AVN in the aflatoxin biosynthetic pathway.

Blocked mutants AV-1 and VER-1 are leaky (9, 15) and produce a small amount of aflatoxin (5 to 10% of strain SU-1 levels). In feeding studies with the mutants, some of the radioactivity from [^{14}C]averufanin was detected in aflatoxin B_1 (0.52×10^5 dpm in AV-1 and 0.37×10^5 dpm in VER-1). To obtain the correct incorporation efficiency (Table 1) for [^{14}C]averufanin into AVR or VERA, the loss of disintegrations per minute owing to the conversion of [^{14}C]AVR into B_1 was taken into account. A correction for disintegrations per minute recovered in these secondary metabolites was made based on the efficiency of [^{14}C]averufanin-to-B_1 conversion of 23%.

DISCUSSION

A major anthraquinone component of wild type *A. parasiticus* was isolated and characterized as averufanin. The metabolite has been isolated previously from *A. flavus* (11), *Aspergillus versicolor* (2, 6), *Bipolaris* sp. (2), and a UV-induced mutant of *A. versicolor* (12). Although averufanin had been isolated in earlier studies, it had not been proposed as a biosynthetic precursor of aflatoxin. Feeding experiments in the current study showed that averufanin is converted into AVR, VERA, OMST, and aflatoxin B_1 but not AVN, placing it after AVN and before AVR in the biosynthetic pathway (Fig. 1).

The proposed aflatoxin biosynthetic pathway now consists of the following steps: acetate → NOR → AVN → averufanin → AVR → versiconal hemiacetal acetate → VERA → sterigmatocystin → OMST → aflatoxin B_1.

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