Synthesis of Versicolorin A by a Mutant Strain of *Aspergillus parasiticus* Deficient in Aflatoxin Production

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A mutant strain of *Aspergillus parasiticus* lost its aflatoxin producing ability but produced four yellow pigments. The major one represented 0.13% of the mycelial mass and was identified as versicolorin A by comparison of its physical and chemical properties with those reported for an authentic compound. A trihydroxyanthraquinone, versicolorin A contains the unique difurano group present in aflatoxins and has been proposed as an intermediate in the biosynthesis of these compounds. The isolation and identification of the pigment from mutant *A. parasiticus* corroborate this theory.

Certain strains of the mold *Aspergillus parasiticus* produce secondary metabolites known as aflatoxins. One aspect of our research effort has been the study of genetic inheritance of aflatoxin producing ability by mutants of toxic mold strains. Bennett and Goldblatt (1973) reported 43 mutant strains with altered fluorescence on de Vogel agar. A number of these mutants selected on the basis of aberrant aflatoxin production also had aberrant mycelial pigmentation. The mycelia of one mutant was deep yellow and contained no detectable aflatoxin but did contain four yellow pigments which seemed closely related. We report the isolation and identification of the major pigment from this mutant.

PROCEDURE AND RESULTS

Mutant Strain. The mutant strain 1-11-105wh-1 (Bennett and Goldblatt, 1973) was cultured on liquid medium (Adye and Mateles, 1964) in Fernbach flasks for 7 days at 30°C without shaking. The mycelia from 20 flasks were collected separately on cheesecloth by pouring the contents of the flasks through the cloth. Each mycelia mass was then washed twice with distilled water, and the water wash combined with the medium. A 500-ml aliquot of the medium and 50 g of mycelia were extracted, the latter in a Waring Blendor, each with two 100-ml portions of chloroform. When the extracts were chromatographed on thin layer to determine aflatoxins (Pons et al., 1968), none was detectable. As all of the yellow pigments were contained in the nonsporulating mycelia, the medium was discarded, and the wet, washed mycelia extracted until colorless with acetone in a Waring Blendor.

Each Fernbach flask contained about 60 g of wet mycelia which required 1000 ml of acetone for extraction. The combined extracts from about 5 Fernbach flasks were filtered through sodium sulfate to remove water, then evaporated to dryness. Batches of this residue were washed with petroleum ether. Each batch was then dissolved in 500 ml of 30% acetone in water, transferred to a 1-l. separatory funnel, and extracted with 100-ml portions of hexane until the hexane extracts were colorless. Most of the major pigment, along with two other yellow ones, was partitioned into hexane; yield, 640 mg of crude pigments from about 1.2 kg of wet mycelia. Calculations made on a dry weight basis indicate that the crude pigments represented 0.4% of the mycelial mass. Batches (100 mg) of the pigments were chromatographed on silica gel columns (60 g of activated Whatman Chromedia 32, 100–200 mesh). The gel was slurried in benzene and packed in the columns each of which was eluted with 250 ml of benzene and then with 500 ml of benzene–acetic acid (95:5, v/v). The major pigment was eluted by the benzene–acetic acid and fractions richest in the pigment were combined and further purified by preparative thin-layer chromatography (TLC) on Adsorbisil 1 plates (500 mm thick); benzene–acetic acid (95:5, v/v) was the developing solvent. The major pigmented zone was scraped from each plate, and the pigment eluted with acetone and recrystallized from hexane. The yield of fine yellow-orange needles was about 30 mg or 0.13% of the dried mycelia.

Nonmutant Strain. The nonmutant parent strain, SU-1(NRRL A-16,462), was cultured, and about 130 g of washed mycelia extracted as described for the mycelia of the mutant strain. The yield of crude pigments was much less than that from the mutant strain (0.02% for the parent strain, compared to 0.4% of the mycelial mass for the mutant strain). The hexane residue was separated by preparative TLC with benzene–acetic acid (9:1, v/v), and the pigmented zones were eluted from the developed plates with acetone. When the pigments were rechromatographed in two solvents, benzene–acetic acid (95:5, v/v) and benzene–ethyl acetate–formic acid (75:21:4, v/v) one moved at the same rate as that of the major pigment from the mutant strain. The molecular weight (mass spectrometry) of this pigment was 338.

Identification of Pigment as Versicolorin A. The recrystallized pigment from the mutant strain had the following properties: mp 287–288°C dec; mol wt 338 (mass spectrometry, CED-21-110-B spectrometer); infrared absorptions at 1625, 1679, and 3340 cm^{-1}; and ultraviolet absorption maxima in EtOH, nm (ϵ), at: 222 (31488), 254 (15227), 265 (17756), 290 (26547), 321 (12118), and 453 (8166). The pigment was soluble in hexane, acetone, ethanol, methanol, and chloroform; it was insoluble in water and in sodium bicarbonate. It turned solutions of sodium carbonate and sodium hydroxide purple, a positive test for hydroxyanthraquinones.

To form the methyl ether derivative, the pigment (20 mg), dimethyl sulfate (0.7 ml), dry acetone (25 ml), and anhydrous potassium carbonate were heated at reflux (3.5 g) for 6 hr. The reaction mixture was poured into boiling water and cooled overnight. The bright yellow derivative was extracted from the water with chloroform, and the extract purified by preparatory TLC with chloroform–acetone (9:1, v/v) as the developing solvent. Recrystallization of the derivative from ethanol yielded fine yellow needles, mp 240°C, mol wt 380 (mass spectrometry, Figure 1, V).

The nuclear magnetic resonance spectrum (NMR) of the methyl ether derivative (using a Varian Model A-60A spectrometer) was entirely consistent with the presence of an anthraquinone nucleus having three hydroxyls in the 1, 6, and 8 positions and a difurano side group. Presence of the difuran moiety was further evidenced by the increased molecular weight of the methyl ether derivative, 380 to 440

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Table I. Ultraviolet and Infrared Absorption of Versicolorin A from Two Mold Sources

A. parasiticus			*A. versicolor*[a]	
λ[b]	ε	Log ε	λ	Log ε
222	31488	4.49	222	4.45
254	15227	4.18	255	4.13
265	17756	4.25	267	4.26
290	26547	4.42	290	4.40
321	12118	4.08	326	3.83
453	8166	3.91	450	3.85
cm^{-1}[c]			cm^{-1}	
3340			3340 (hydroxyl)	
1679			1679 (nonchelated quinone carbonyl)	
1625			1625 (chelated quinone carbonyl)	

[a] Hamasaki et al. (1967). [b] Scanning DK-2A spectrophotometer. [c] KBr disk.

Table II. Nuclear Magnetic Resonance Spectra of Trimethyl Ether Derivatives of Versicolorin A from Two Mold Sources

Chemical shift (τ)		
A. parasiticus	*A. versicolor*[a]	Proton assignments
2.56[b]	2.57 (s, 1 H)	4 H[c]
2.70	2.70 (d, J = 2.5 Hz, 1 H)	5 H
3.25	3.25 (d, J = 2.5 Hz, 1 H)	7 H
3.28	3.28 (d, J = 7 Hz, 1 H)	H_c
3.56	3.52 (t, J = 2.5 Hz, 1 H)	H_b
4.66	4.66 (t, J = 2.5 Hz, 1 H)	H_a
5.23	5.23 (t of a d, J = 2.5 and 7.0 Hz, 1 H)	H_d
	5.98	
6.07 (overlapping singlets)	6.06 6.08 (s, 3 × OCH_3)	3 × OCH_3

[a] Hamasaki et al. (1967). [b] In $CDCl_3$, relative to Me_4Si as internal standard. [c] Figure 1, V.

(mass spectrometry) after reaction with acetic anhydride and concentrated hydrochloric acid. The reaction, which adds an acetyl group to the double bond of a terminal furan ring (Figure 1, VI), was proposed by Pohland et al. (1970) as a confirmatory test for aflatoxin B_1.

The data reported by Hamasaki et al. (1967) on versicolorin A from *A. versicolor* indicate that the pigment isolated from this mutant strain of *A. parasiticus* had physical and chemical properties consistent with their report for versicolorin A. Melting points were 287–288°C (sealed tube) for the pigment isolated from the *A. parasiticus* mutant and 289°C reported for versicolorin A by Hamasaki et al. (1967). Molecular weights were determined by osmometer for versicolorin A (Hamasaki et al.) and 338 determined by mass spectrometry for the pigment isolated in this study. The theoretical molecular weight for $C_{18}H_{10}O_7$ is 338. The melting points and molecular weights of the methyl ether derivatives agreed closely: 241°C and 384 (rast), respectively, for the derivative of versicolorin A (Hamasaki et al.) vs. 240°C and 380 (mass spectrometer) for the derivative of the pigment isolated from the mutant *A. parasiticus*. Comparisons of absorption data for the pigments from the two mold sources (Table I) and of the NMR spectra of the trimethyl ether derivatives (Table II) further corroborated that the two pigments are identical.

DISCUSSION

The discovery of aflatoxins has aroused much interest over their biogenesis, and many of the biogenetic pathways

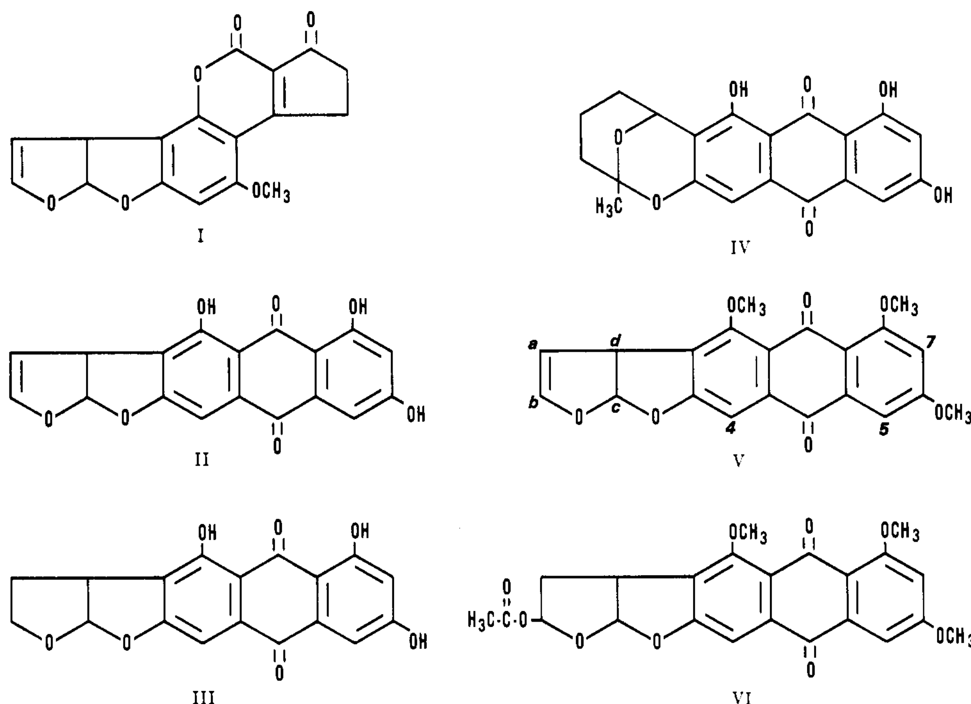

Figure 1. I, aflatoxin B_1, mol wt 312; II, versicolorin A, mol wt 338; III, versicolorin C, mol wt 340; IV, averufin, mol wt 368; V, versicolorin A trimethyl ether, mol wt 380; VI, acetal derivative of V, mol wt 440.

hypothesized include hydroxyanthraquinones as intermediates (Thomas, 1965; Aucamp and Holzapfel, 1970). Recently Heathcote et al. (1973) proposed versicolorin A (Figure 1, II) as a precursor of aflatoxin B_1 (Figure 1, I).

The isolation of versicolorin A from the mutant strain and chromatographic and mass spectral evidence of its presence in the nonmutant strain as well as reports of other hydroxyanthraquinones as secondary metabolites of *A. parasiticus* (Heathcote and Dutton, 1969; Lee et al., 1971) add credence to these theories.

However, little experimental evidence for the involvement of specific hydroxyanthraquinones had been obtained until 1973 when Lin et al. reported that *A. parasiticus* incorporated [^{14}C]averufin (Figure 1, IV) into aflatoxin B_1. Similar labeling experiments are now underway in this laboratory; we are using the yellow pigmented mutant and the nonmutant *A. parasiticus* to determine whether versicolorin A is also an intermediate in the biosynthesis of aflatoxin B_1.

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Rapid Screening Method for Aflatoxin in a Number of Products

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An improved qualitative minicolumn procedure for screening a number of different types of products for aflatoxin is presented. High-speed blending of the sample with aqueous methanol followed by purification with zinc acetate and re-extraction with benzene before subjecting to minicolumn chromatography is a simple, economical, and rapid method for aflatoxin detection. Sensitivities of 2 ppb can be achieved and the use of disposable plastic and glass items makes the method practical for field or in-plant application.

Since the introduction of the qualitative minicolumn technique by Holaday (1968), there has been a continuing interest in improving the range of commodities that can be analyzed with this technique and in minimizing the investment in time and equipment. During the past few years, a number of minicolumn methods have been proposed (Velasco, 1972; Pons et al., 1973; Holaday and Barnes, 1973; Shannon et al., 1975; McKinney, 1975) and the method described here utilizes some of the features of several of these methods. The purpose of this work was to develop a method that could screen a wide range of commodities and to provide a simpler and more rapid procedure which utilizes a number of plastic and glass disposable items that reduces the amount of maintenance and glassware cleanup, keeps the cost per determination at a low level, and decreases the danger of aflatoxin carry-over to a subsequent sample.

The simplicity of the method lends itself to field or in-plant use where laboratory facilities are limited or nonexistent. A subsequent report will describe a self-contained field unit which will require neither outside power nor running water.

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EXPERIMENTAL SECTION

Equipment used included a Chromatovue chamber equipped with long-wave uv, Ultra-Violet Products, Inc. (San Gabriel, Calif.); a Waring Blendor; vacuum source, either a water aspirator or small vacuum pump is satisfactory.

Supplies used included minicolumns packed with ca. 15 mm of Florisil (100–200 mesh) on bottom and ca. 15 mm of neutral alumina (100–200 mesh) on top (see Figure 1). For best results, the interface of the two materials should be as straight as possible. The alumina should have an activity grade of V. Some brands of alumina have a slight fluorescence which can cause interference. Two which do not fluoresce and which give excellent results are E. Merck and Woelm brands. The glass tubing is 5.5 mm i.d. and 160 mm long. Packing to hold the Florisil and alumina in place is made from paper pulp which provides a tight seal. This material is available from most chemical supply houses. Columns may be purchased from Tudor Scientific Glass Co. (Belvedere, S.C.).

Disposable items used included: culture tubes, 18 × 150 mm; plastic tube closures, 16 mm; pipets, 1 ml; plastic funnels, 2.25 in. top diameter.

Reagents used were: benzene; methanol–water solution (80:20, v/v); salt solution (600 g of sodium chloride, 600 g of zinc acetate, and 15 ml of glacial acetic acid dissolved in 4000 ml of distilled water); hexane–acetone solution (80:20, v/v). All reagents should be ACS grade.