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Yellow pigments used in rapid identification of aflatoxin-producing *Aspergillus* strains are anthraquinones associated with the aflatoxin biosynthetic pathway

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Abstract

Studies on biological control of aflatoxin production in crops by pre-infection with non-toxigenic *Aspergillus flavus* strains have created a need for improved methods to screen isolates for aflatoxigenicity. We have evaluated two empirical aflatoxigenicity tests: (i) yellow pigment production, and (ii) the appearance of a plum-red color in colonies exposed to ammonium hydroxide vapor. Yellow pigments from aflatoxigenic *A. flavus* were shown to function as pH indicator dyes. Seven pigments representing most of the pigmentation in extracts have been isolated using color changes when chromatography spots were exposed to ammonium hydroxide vapor to guide fractionation. Their structures have been shown to be norsolorinic acid, averantin, averufin, versicolorin C, versicolorin A, versicolorin A hemiacetal and nidurufin, all of which are known anthraquinone pigments on, or associated with, the aflatoxin biosynthetic pathway in *Aspergillus* spp. Thus, the basis of both empirical tests for aflatoxigenicity is detecting production of excess aflatoxin biosynthetic intermediates.

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1. Introduction

Aflatoxins were the first mycotoxins discovered, and the most commonly found form, aflatoxin B_1, is the most potent natural mutagen and carcinogen known [1]. The most important producing organism is *Aspergillus flavus* Link (producing aflatoxins B_1 and B_2), but *Aspergillus parasiticus* Speare (aflatoxins B_1, B_2, G_1 and G_2) and *Aspergillus nomius* also produce aflatoxins [2]. Aflatoxins are economically important contaminants of agricultural products including corn (maize, *Zea mays* L.), peanuts (ground nuts, *Arachis hypogaea* L.), cottonseed (*Gossypium hirsutum* L.), copra (*Cocos nucifera* L.) and various other tree nuts [3]. Aflatoxin levels are regulated by many governments, and the aflatoxin levels which they mandate are often below those routinely encountered when corn is grown under hot, dry conditions [4,5]. Monitoring of aflatoxin contamination in foods and feeds is usually done in developed countries with commercially available enzyme-linked immunosorbant (ELISA) assay kits, but the expense of the kits often precludes their use in developing countries [3]. Among the less-expensive alternative monitoring techniques under consideration are cultural screens for aflatoxigenic *A. flavus* [6]. Some potentially useful cultural screening methods are empirical. Doubts about their validity may have contributed to resistance to their adoption.

Various approaches are being studied to reduce aflatoxin contamination of crops before harvest [7]. Observations that *A. flavus* isolates from plants have lower frequencies of toxigenicity than isolates from the soil reservoir [6,8], suggest that aflatoxin production is more important to help *A. flavus* compete successfully in soil, than to colonize and propagate itself in plants. These observations provide a rationale for the use of non-toxigenic *A. flavus* strains as biological control agents which would be applied to crops before they become colonized with aflatoxigenic *A. flavus* from reservoirs in the soil. Initial efforts at evaluating this approach indicate it is very promising [7,9–11]. Exploring this approach has created an additional need for rapid, convenient, reliable methods to screen *A. flavus* isolates to identify non-aflatoxigenic strains, which may subsequently be evaluated for other properties important in a successful biological control strain (e.g., growth in culture, infectivity when applied, stability in storage). While cultural methods for aflatoxigenicity testing based on detection of aflatoxin blue fluorescence under UV light have been described [12,13], more sensitive, convenient assays were sought.

In the present study, we have investigated the biochemical basis underlying two rapid, convenient empirical cultural methods for predicting aflatoxigenicity in *A. flavus*, the yellow pigment method developed by Lin and Dianese [14] and the ammonium hydroxide vapor color change method developed by Saito and Machida [15]. In the yellow pigment method of Lin and Dianese aflatoxigenic *A. flavus* colonies grown about five days on potato dextrose agar or other suitable solid agar culture media produce yellow pigment most visible on the underside of the colonies viewed through the bottom of the dish. The pigment is absent from non-aflatoxigenic colonies, which are off-white or grey. In the Saito and Machida method [15], a culture is prepared in the same manner, then the lid is inverted, a few drops (0.2–0.5 ml) of concentrated ammonium hydroxide solution (25–27%

w/v NH_4OH) are placed in it and the bottom of the dish inverted over it, so the agar surface is exposed to vapors from the ammonium hydroxide. Aflatoxigenic *A. flavus*, particularly the bottom side of colonies, quickly turns a plum-red color. There is no noticeable color change when non-aflatoxigenic *A. flavus* colonies are treated in the same manner.

These two cultural methods have been extensively validated [6,14,15] by using an established analytical method (e.g., ELISA, TLC, HPLC) to quantify aflatoxin production or the lack of it by the colonies when they are grown in pure culture on a variety of media. However, widespread acceptance of these two methods appears to have been limited by their empirical nature. In the present study, we have attempted to identify the molecular basis of these assays in order to provide a rationale for their effectiveness and to better define the limits of their reliability. The approach taken was to: (i) purify pigment(s) present in aflatoxigenic *A. flavus* cultures, but absent from non-aflatoxigenic *A. flavus* cultures, (ii) determine their chemical structures before and after ammonium hydroxide treatment, and (iii) attempt to rationalize their link to aflatoxin production.

2. Materials and methods

2.1. Materials

Deuterated solvents were obtained from Cambridge Isotope Laboratories, Cambridge, MA. Preparative thin layer chromatography (TLC) sheets (silica gel G, 20×20 cm, 500 μm) were obtained from Analtech, Newark, DE. Analytical TLC sheets (40×80 mm Polygram Sil/UV_{254}, 0.2 mm silica gel on plastic) were obtained from Macherey-Nagel, Duren, Germany. Octadecyl functionalized (C_{18}) silica gel was obtained from Aldrich Chemical, Milwaukee, WI. Organic solvents were obtained from Fisher Scientific, Pittsburgh, PA, and used without purification.

2.2. Aspergillus flavus cultures

The two *A. flavus* cultures used in this study (an aflatoxin-producing isolate, F3W4, and a non-aflatoxigenic isolate, CT3) were isolated from the soil of a corn field in Mississippi in the summer of 2001 [6] by plating samples on modified dichloronitroanaline rose Bengal agar according to Horn and Dorner [16]. Aflatoxigenicity and non-aflatoxigenicity in the strains were determined by the methods of Abbas et al. [6,17]. Cultures were maintained on Czapak agar slants and on silica gel [16,18]. Aflatoxin production was measured in cultures growing on potato dextrose agar (PDA) in 9-cm petri dishes for 5 days at 28–30 °C with either a 12 h dark/12 h light photoperiod or continuous darkness. The fungal biomass (mycelia, conidia heads, conidia) on the PDA plates was sampled by cutting ten 2 mm discs with a cork borer and transferring them to 20-ml plastic scintillation vials. Fresh weights were recorded (typically 0.5–1 g). Methanol:water (70:30, v/v) was added (10:1 ratio, v/w) to vials, and the vials were shaken for 30 min at high speed using a reciprocal shaker. An aliquot of extract was removed and centrifuged (12,000*g*, 10 min) in a microcentrifuge (MicroSpin 12sp, DuPont Sorvall Instruments). The supernatant was assayed for the presence of aflatoxins using ELISA kits ("Vertox", Neogen, Lansing, MI) used according to the manufacturer's instructions and confirmed by LC/MS according to the method of Abbas et al. [17]. All experiments used at least ten culture plates and were conducted at least twice.

2.3. Yellow pigment production

PDA in 9-cm petri dishes was inoculated with a 2 mm disc of *A. flavus* culture and incubated at 28–30 °C in an incubator (Precision Scientific, Chicago, IL) for five days, at which time fungal mycelium covered the agar surface. Attempts to extract yellow pigments directly from the agar with organic solvents (methanol, dimethylsulphoxide) were unsuccessful because agar gel pieces retained pigments. However, pigments were readily extracted from lyophilized aflatoxigenic *A. flavus* cultures (mycelium plus agar medium) with methanol. Lyophilized cultures of an aflatoxigenic isolate of *A. flavus* (F3W4, 146 g dry weight), and a non-aflatoxigenic isolate of *A. flavus* (CT3, 90 g dry weight) on PDA medium were placed in sintered glass filter funnels and eluted with methanol until the effluent was no longer yellow. The extracts from each fungal isolate were combined, evaporated to dryness under reduced pressure, and the residues weighed (F3W4, 28.5 g; CT3, 24.4 g). The extracted mycelium plus agar medium was discarded.

2.4. pH-Dependent color changes in yellow pigments

The yellow to plum-red color change observed in the Saito and Machida test was replicated with extracted crude yellow pigment dissolved in methanol by adding 1–2 drops of concentrated ammonium hydroxide solution, or a solution of any other alkaline material tested (saturated aqueous sodium bicarbonate, saturated aqueous sodium carbonate, 1 M sodium hydroxide, 1 M potassium hydroxide). The alkali-induced color change was reversed (i.e., red was turned to yellow) by adding an excess (5–10 drops) of any acid tested (1 N hydrochloric acid, 5% acetic acid). A second round of color change was initiated by second addition of alkali in excess of the added acid (e.g., 10–15 drops of 1 N sodium hydroxide).

Color cycling was confirmed under conditions of the Saito and Machida test by inducing the yellow to plum-red color change with concentrated ammonium hydroxide solution in the dish lid of an inverted culture of aflatoxigenic *A. flavus* (F3W4), then reverting the color from plum-red back to yellow by wiping the concentrated ammonium hydroxide solution from the dish lid, replacing it with a few drops of glacial acetic acid, and replacing the inverted culture over it.

The pH value for the yellow-to-red color transition of extracted pigments was determined by dissolving a sufficient amount (1–2 mg) of crude extract or a purified pigment (e.g., averufin) in 40 ml water in a glass beaker to give a readily visible yellow color. A pH meter probe was placed in the liquid and it was stirred magnetically while 0.01 M NaOH was added in small increments from a burette. After addition of each aliquot of base up to pH 8, the pH of the solution was recorded and the color noted.

2.5. Thin layer chromatography assay for yellow pigments

During purification of yellow pigments, spots corresponding to the desired pigments were identified on aluminum-back silica gel TLC sheets by noting the color of the spots before and after placing the TLC sheet in an enclosed chamber containing concentrated ammonium hydroxide solution. Under these conditions most anthraquinone pigments change from yellow to pink or red, but ones with extended conjugation (norsolorinic acid) change from red to purple. The R_f values were calculated as the ratio of the distance traveled by the center of the spot to the distance traveled by the solvent front.

2.6. Fractionation of *A. flavus* culture extracts

Crude methanol extracts from lyophilized cultures of an aflatoxigenic *A. flavus* isolate (F3W4, 28.5 g) and non-aflatoxigenic *A. flavus* isolate (CT3, 24.4 g) were dissolved in water and passed through columns of 100 g C_{18}-silica. Similar results were obtained with columns of Amberlite XAD-2 resin. The column was washed with 1000 ml distilled water, and the combined effluent and water were evaporated under reduced pressure at 45 °C to give the unbound fraction, F0 (F3W4, 25.7 g; CT3, 24.0 g), representing almost all of the extract, which was presumably mostly unutilized dextrose from the culture medium. The column was successively eluted with 10, 20, 50 and 100% methanol in water and evaporated in the same manner to give fractions F10 (F3W4, 2.23 g; CT3, 0.22 g), F20 (F3W4, 48 mg; CT3, 32 mg), F50 (F3W4, 190 mg; CT3, 78 mg), and F100 (F3W4, 350 mg; CT3, 88 mg), respectively. Each fraction was analyzed by normal phase TLC, and exposure to ammonium hydroxide vapor indicated that fractions F20, F50, and F100 contained yellow pigments exhibiting pH-dependent color change. These fractions were subjected to repeated preparative normal phase TLC on silica gel developed with various solvent systems until they were sufficiently pure to enable unambiguous structure identification.

2.7. Characterization of purified anthraquinone pigments

UV–Vis absorption spectra were obtained on a Beckman DU-7000 instrument (Beckman, Fullerton, CA). Negative mode electrospray ionization mass spectrometry (ESI-MS) was performed on a ThermoFinnigan LCQ Classic Ion-trap instrument (Finnigan MAT/Thermoquest, San Jose, CA). Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Varian Inova either 500 or 600 MHz instrument (Varian, Palo Alto, CA). HPLC analyses of column fractions and purified yellow pigments were carried out essentially by the method of McCormick et al. [19] on a Waters HPLC system consisting of two model 510 pumps and a model 991 photodiode array detector. A 4.6 mm × 250 mm Phenomenex Aqua 5 μ C_{18} column with a guard column (Phenomenex, Torrance, CA) was used. The solvent system used was a linear gradient consisting of A (0.1 M acetic acid) and B (methanol:tetrahydrofuran 2:1, v/v) going from 33% A to 21% A in 30 min, then to 0% A in 10 min, held at 0% A for 10 min, then returned to 33% A in 3 min. The flow rate was 1 ml/min, and detection was by UV absorbance at 290 nm.

3. Results

3.1. Extraction of yellow pigments

Yellow pigments were readily and efficiently extracted with methanol from lyophilized cultures of aflatoxigenic *A. flavus* grown on PDA. All solvents and extraction conditions that were tested without first removing the water from the agar resulted in unacceptably low extraction efficiencies.

3.2. Molecular mechanism of ammonium hydroxide-induced color change

The crude yellow pigment extract dissolved in methanol replicated the color change of the Saito and Machida [15] test from yellow to plum-red upon addition of a few drops

(0.2–0.5 ml) of concentrated ammonium hydroxide. The color change was replicated with all bases examined (sodium bicarbonate, sodium carbonate, sodium hydroxide, potassium hydroxide) and addition of an excess of any acid tested (acetic acid, hydrochloric acid) restored the yellow color. Addition of an excess of base initiated a second cycle of color change. Addition of a dilute solution of base (0.01 M NaOH) from a burette, while monitoring pH with a pH meter, demonstrated that the color transition occurred between pH 5.5 and 6.5. The pH-reversible color change is consistent with yellow pigments acting as pH indicator dyes, in which a functional group dissociates in a pH-dependent manner, altering the net visible light absorption wavelength optimum by altering the extent of conjugation. The absorption spectra of anthraquinone pigments contain the same series of peaks with the same λ_{max} values on both sides of the pH transition; the color transition results from relative differences in extinction coefficients for some of the peaks. No evidence was obtained for ammonia reacting with a yellow pigment to give a red pigment with altered covalent structure.

The color cycling observation was shown not to be an artifact of extraction by inducing a similar color cycling in viable cultures of aflatoxigenic *A. flavus* under the conditions of the Saito and Machida test [15]. After inducing the color change from yellow to plum-red with concentrated ammonium hydroxide to the inverted petri dish lid, the concentrated ammonium hydroxide was wiped away, a few drops of glacial acetic acid were added and the reddened *A. flavus* culture inverted over it again. The original yellow color was quickly restored. It was possible to replicate the multiple color reversal observed with extracted pigments by alternating inverted *A. flavus* cultures between lids to which concentrated ammonium hydroxide or glacial acetic acid were added.

3.3. Isolation of yellow pigments

The methanol extract of yellow pigments was fractionated according to the scheme shown in Fig. 1. Each step of the purification was monitored by demonstrating a color change on exposure to concentrated ammonium hydroxide, either by adding it drop-wise to samples in solution, or by exposing spots on thin layer chromatograms to ammonium hydroxide vapors in an enclosed chamber. The extract was initially fractionated by reverse phase chromatography on a C_{18} silica open column eluted with a step gradient of methanol in water. Yellow pigments undergoing color change on exposure to ammonium hydroxide were found in fractions eluted with 20, 50, and 100% methanol in water. Each of these fractions was further fractionated by repeated preparative normal phase thin layer chromatography on silica gel. Preparative TLC on silica gel was used to isolate the following anthraquinone pigments [20] from the 20% methanol fraction: versicolorin C, using the solvent system chloroform/acetone/acetic acid: 320/16/1; and versicolorin A hemiacetal using the solvent system toluene/ethyl acetate/acetic acid: 80/16/1; and nidurufin and four unknown pigments, **8**–**11**, using the solvent system toluene/ethyl acetate/acetic acid: 200/40/1. From the 50% methanol fraction preparative TLC on silica gel was used to isolate the following: averufin using the solvent system chloroform/acetone/acetic acid: 400/50/1; and unknowns **12** and **13** using toluene/ethyl acetate/acetic acid: 80/16/1. From the 100% methanol fraction preparative TLC on silica gel was used to isolate the following: versicolorin A and norsolorinic acid using the solvent system toluene/ethyl acetate: 5/1; and averantin using toluene/ethyl acetate/acetic acid: 200/40/1.

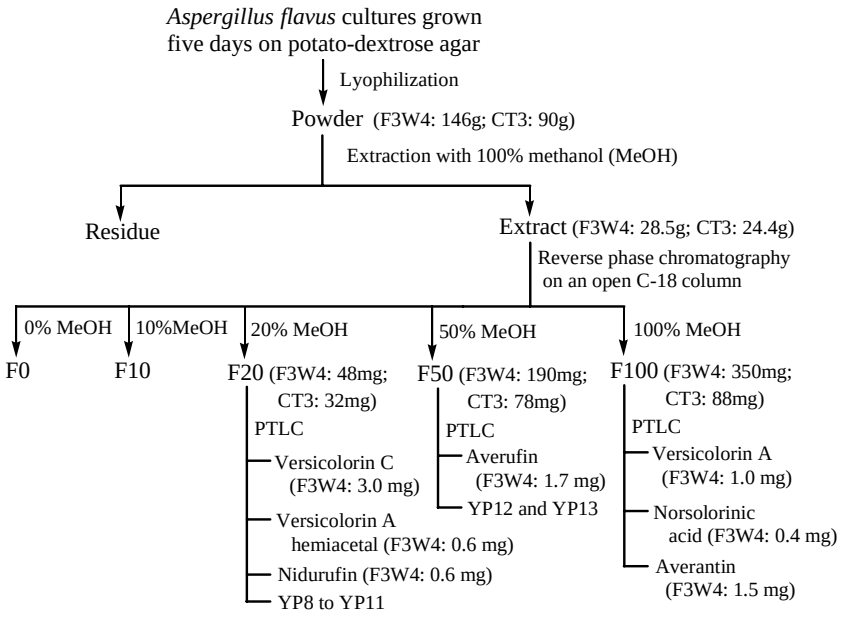

Fig. 1. The scheme used to fractionate culture medium from an aflatoxigenic isolate of *A. flavus* (F3W4) and a non-aflatoxigenic isolate (CT3). Isolated yields of fractions and yellow pigments are given in brackets. No yellow pigments were isolated from reverse phase chromatography fractions of the non-aflatoxigenic isolate, CT3, or detected by HPLC according to the method of McCormick et al. [19]. *Abbreviations*: MeOH, methanol; PTLC, preparative normal phase thin layer chromatography.

3.4. Characterization of yellow pigments

The assignment of structures to seven yellow pigments described below was based on spectral analysis, particularly ultraviolet/visible absorption spectroscopy, negative ion electrospray ionization mass spectrometry (ESI-MS/MS) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR), and on comparison of chromatographic properties with published values.

*3.5. Averufin (**1**)*

Pigment **1** (isolated yield: 1.65 mg from 28.5 g methanolic extract of aflatoxigenic *A. flavus*) was yellow on silica gel and turned red on exposure to ammonium hydroxide. Pigment **1** exhibited the following properties: R_f value of 0.89 on TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v); retention time 32.09 min in HPLC similar to the method of McCormick et al. [19]; UV λ_{max} in ethanol (nm): 224 (29,800), 257 (14,100), 267 (15,500), 294 (26,700), 323 (7,800), 451 (8,600); ^{1}H NMR (acetone-d_6) δ ppm: 7.14 (d, $J = 2.4$ Hz, 1H, H-5), 7.09 (s, 1H, H-4), 6.41 (d, $J = 2.4$ Hz, 1H, H-7), 5.28 (dd, $J = 1.8$, 4.2 Hz, 1H, H-11), 1.54-1.95 (m, 6H, H-12, 13, 14), 1.52 (s, 3H, H-16); MS (negative ESI-MS, relative intensity): *m/z* 367 ($M - H^+$, 100), MS/MS of *m/z* 367: *m/z* 367 (90), 324 (2), 310 (10), 297 (14), 295 (30), 284 (100), 283 (12), corresponding to a molecular weight of 368 and the formula $C_{20}H_{16}O_7$. Thus, **1** is an anthraquinone pigment to which the structure of averufin was assigned based on comparison with reported values [20,21].

3.6. Norsolorinic acid (**2**)

Pigment **2** (isolated yield: 0.44 mg from 28.5 g methanolic extract of aflatoxigenic *A. flavus*) was red on silica gel and turned purple on exposure to ammonium hydroxide. Pigment **2** exhibited the following properties: R_f value of 0.90 on TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v); retention time 43.14 min in HPLC similar to the method of McCormick et al. [19]; UV λ_{max} in ethanol (nm): 237 (34,900), 271 (25,500), 284 (25,900), 315 (30,000), 473 (9500); ^{1}H NMR (acetone-d_6) δ ppm: 7.20 (s, 1H, H-4), 7.06 (br, 1H, H-5), 6.61 (br, 1H, H-7), 3.18 (t, $J = 7.2$ Hz, 2H, H-12), 2.28 (m, 2H, H-13), 1.71 (m, 2H, H-15), 1.58 (m, 2H, H-14), 0.91 (t, $J = 7.2$ Hz, 3H, H-16); MS (negative ESI-MS, relative intensity): *m/z* 369 ($M - H^+$, 100), MS/MS of *m/z* 369: *m/z* 369 (100), 351 ($M - H_2O - H^+$, 60), 341 (10), 325 (12), 313(3), 308 (5), 297 ($M - C_5H_{11}^+$, 15), 284 (3), 271 (5), 270 ($M - COC_5H_{11}^+$, 12), corresponding to a molecular weight of 370 and the formula $C_{20}H_{18}O_7$. Thus, **2** is an anthraquinone pigment to which the structure of norsolorinic acid was assigned based on comparison with reported values [20].

3.7. Averantin (**3**)

Pigment **3** (isolated yield: 1.48 mg from 28.5 g methanolic extract of aflatoxigenic *A. flavus*) was yellow on silica gel and turned red on exposure to ammonium hydroxide. Pigment **3** exhibited the following properties: R_f value of 0.80 on TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v); retention time 33.04 min in HPLC similar to the method of McCormick et al. [19]; UV λ_{max} in ethanol [nm (ε)]: 224 (7915), 261 (3242), 294 (3839), 315 (2365), 453 (954); MS (negative ESI-MS, relative intensity): *m/z* 371 ($M - H^+$, 100), 353 ($M - H_2O - H^+$, 40), 311 (13), MS/MS of *m/z* 371: *m/z* 353 (100), MS/MS of *m/z* 353: *m/z* 353 (68), 335 (6), 324 (9), 310 (31), 295 (29), 284 (100), 256 (30), corresponding to a molecular weight of 372 and the formula $C_{20}H_{20}O_7$. Thus, **3** is an anthraquinone pigment to which the structure of averantin was tentatively assigned based on comparison with reported values [22].

3.8. Versicolorin C (**4**)

Pigment **4** was present in the largest amount of any pigment in methanolic extracts of aflatoxigenic *A. flavus*, yielding 3.01 mg purified pigment isolated from 28.5 g methanolic extract. Pigment **4** was orange-red on silica gel, and turned pure red on exposure to ammonium hydroxide. It exhibited the following properties: R_f value of 0.82 on TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v); retention time 17.22 min in HPLC similar to the method of McCormick et al. [19]; UV λ_{max} in ethanol (nm): 224 (40,900), 255 (19,200), 267 (22,000), 292 (37,700), 327 (11,200) and 451 (10,900); ^{1}H NMR (acetone-d_6) δ ppm: 7.16 (br, 1H, H-5), 7.08 (s, 1H, H-4), 6.48 (d, $J = 6.0$ Hz, 1H, H-14), 6.43 (br, 1H, H-7), 4.17 (t, $J = 7.2$ Hz, 1H, H-13a), 4.10 (t, $J = 7.8$ Hz, 1H, H-13b), 3.60 (m, 1H, H-11), 2.26 (m, 2H, H-12); MS (negative ESI-MS, relative intensity): *m/z* 339 ($M - H^+$, 100), MS/MS of *m/z* 339: *m/z* 339 ($M - H^+$, 55), 324 (3), 311 (100), 310 (10), 309 (5), 297 (2), 283 (13), corresponding to a molecular weight of 340 and the formula $C_{18}H_{12}O_7$. Thus, **4** is an anthraquinone pigment to which the structure of versicolorin C was assigned based on comparison with reported values [20,21].

3.9. Nidurufin (**5**)

Pigment **5** (isolated yield: 0.61 mg from 28.5 g methanolic extract of aflatoxigenic *A. flavus*) was yellow on silica gel and turned red on exposure to ammonium hydroxide. Pigment **5** exhibited the following properties: R_f value of 0.58 on TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v); retention time 12.56 min in HPLC similar to the method of McCormick et al. [19]; UV λ_{max} in ethanol (nm): 224 (26,800), 254 (12,600), 267 (13,800), 293 (23,500), 323 (6900), 452 (7700); ^{1}H NMR (acetone-d_6) δ ppm: 7.08 (br, 1H, H-5), 7.07 (s, 1H, H-4), 6.31 (br, 1H, H-7), 5.13 (br, 1H, H-11), 3.92 (m, 1H, H-12), 1.64-2.28 (m, 4H, H-13, 14), 1.54 (s, 3H, H-16); MS (negative ESI-MS, relative intensity): *m/z* 383 ($M - H^+$, 100), MS/MS of *m/z* 383: *m/z* 383 ($M - H^+$, 3), 365 ($M - H_2O - H^+$,100), 284 (5), 270 (5), corresponding to a molecular weight of 384 and the formula $C_{20}H_{16}O_8$. Thus, **5** is an anthraquinone pigment to which the structure of nidurufin was assigned based on comparison with reported values [20,21].

3.10. Versicolorin A hemiacetal (**6**)

Pigment **6** (isolated yield: 0.64 mg from 28.5 g methanolic extract of aflatoxigenic *A. flavus*) was orange-red on silica gel and turned pure red on exposure to ammonium hydroxide. Pigment **6** exhibited the following properties: R_f value of 0.64 on TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v); retention time 9.82 min in HPLC similar to the method of McCormick et al. [19]; UV λ_{max} in ethanol (nm): 224 (36,900), 255 (19,200), 267 (21,200), 293 (32,400), 323 (15,300) and 451 (10,300); ^{1}H NMR (acetone-d_6) δ ppm: 7.23 (br, 1H, H-5), 7.09 (s, 1H, H-4), 6.62 (br, 1H, H-7), 6.52 (d, $J = 6.0$ Hz, 1H, H-14), 5.72 (br, 1H, H-13), 4.15 (m, 1H, H-11), 2.33 (m, 2H, H-12); MS (negative ESI-MS, relative intensity): *m/z* 355 ($M - H^+$, 100), MS/MS of *m/z* 355: *m/z* 355 ($M - H^+$, 58), 337 ($M - H_2O - H^+$, 60), 327 (100), 311 (26), 309 (18), 299 (6), 284 (36), 271 (48), corresponding to a molecular weight of 356 and the formula $C_{18}H_{12}O_8$. Thus, **6** is an anthraquinone pigment to which the structure of versicolorin A hemiacetal was assigned based on comparison with reported values [23,24].

3.11. Versicolorin A (**7**)

Pigment **7** (isolated yield: 1.01 mg from 28.5 g methanolic extract of aflatoxigenic *A. flavus*) was orange-red on silica gel and turned pure red on exposure to ammonium hydroxide. Pigment **7** exhibited the following properties: R_f value of 0.87 on TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v); retention time 19.22 min in HPLC similar to the method of McCormick et al. [19]; UV λ_{max} in ethanol (nm): 224 (30,800), 254 (16,100), 266 (17,900), 292 (25,300), 320 (12,700) and 451 (7700); ^{1}H NMR (acetone-d_6) δ ppm: 7.23 (d, $J = 2.5$ Hz, 1H, H-5), 7.21 (s, 1H, H-4), 6.92 (d, $J = 7.0$ Hz, 1H, H-14), 6.64 (d, $J = 2.5$ Hz, 1H, H-7), 5.43 (t, $J = 2.5$ Hz, 1H, H-13), 4.89 (br, 1H, H-11), 4.81 (dd, $J = 2.0$, 7.0 Hz, 1H, H-12); MS (negative ESI-MS, relative intensity): *m/z* 337 ($M - H^+$, 100), 309 (6), MS/MS of *m/z* 337: *m/z* 337 ($M - H^+$, 60), 309 (100), 308 (51), 293 (12), corresponding to a molecular weight of 338 and the formula $C_{18}H_{12}O_7$. Thus, **7** is an anthraquinone pigment to which the structure of versicolorin A was assigned based on comparison with reported values [20].

Table 1
Properties of some unidentified yellow pigments isolated from aflatoxigenic *A. flavus* cultures

Pigment	Molecular weight	Isolated yield (mg)[a]	R_f value on TLC[b]	Retention time in HPLC (min)[c]	λ_{max}, in UV-visible spectrum (nm)[d]	Mass spectrometry (negative ion ESI-MS, relative intensity)
8	428	0.62	0.54[e], 0.26[f]	12.04	224, 254, 267, 292, 325, 451	*m/z* 427 ($M-H^+$, 100), 355 (62), MS/MS of *m/z* 427: *m/z* 427 ($M-H^+$, 93), 399 (13), 355 (9), 337 (30), 311 (100), 309 (6), 283 (29), 270 (5)
9	428	0.42	0.69[e]	20.70	224, 266, 293, 324, 453	*m/z* 427 ($M-H^+$, 100), 355 (12), 311 (11), 285 (87)
10	428	0.53	0.57[e]	12.04	221, 254, 266, 291, 323, 451	*m/z* 427 ($M-H^+$, 100), 355 (11), 311 (7), MS/MS of *m/z* 427: *m/z* 427 ($M-H^+$, 2), 355 (100), 337 (27), 311 (2)
11	368	1.67	0.55[e]	8.04	224, 256, 267, 292, 322, 451	*m/z* 367 ($M-H^+$, 100), 177 (18), MS/MS of *m/z* 367: *m/z* 367($M-H^+$, 2), 177 (100)
12	368	0.65	0.81[e]	33.04	254, 266, 293, 325, 452	*m/z* 367 ($M-H^+$, 100), 177 (8), MS/MS of *m/z* 367: *m/z* 177 (100)
13	368	1.01	0.59[e]	12.56	254, 266, 293, 325, 451	*m/z* 367 ($M-H^+$, 100), 177 (5), MS/MS of *m/z* 367: 367 ($M-H^+$, 4), 177 (100)

[a] Yield from 28.5 g of methanolic extract of aflatoxigenic *A. flavus* culture.
[b] Ratio of the distance migrated in TLC on silica gel in the indicated solvent system to the distance migrated by the solvent front.
[c] Retention time for HPLC carried out similar to the method of McCormick et al. [19].
[d] Extinction coefficients not determined.
[e] TLC on silica gel in the solvent system toluene/ethyl acetate/acetic acid: 50/30/4 (v/v/v).
[f] TLC on silica gel in the solvent system methylene chloride/acetone/acetic acid: 85/15/1 (v/v/v).

3.12. Other yellow pigments

The seven pigments with structures identified above represented the majority of the yellow/orange pigmentation extracted from aflatoxigenic *A. flavus* cultures. The methanolic extracts also contained small amounts numerous other pigments with yellow spots on TLC, which turned red on exposure to ammonium hydroxide. Some properties of six purified metabolites, **8**–**13**, are given in Table 1. For each, the UV spectrum indicates it is an anthraquinone pigment, but there was insufficient additional information to assign a structure.

3.13. Comparison of pigment production in aflatoxigenic and non-aflatoxigenic A. flavus

Yellow pigments were readily detectable by HPLC analysis [19] of C_{18} reverse phase column fractions of extracts from aflatoxigenic *A. flavus* cultures, although there was substantial batch-to-batch variability in the ratios of the various pigments to versicolorin C, the most abundant anthraquinone pigment. However, when cultures of non-aflatoxigenic

A. flavus were extracted and processed in the same way, no versicolorin C or any other anthraquinone pigment could be detected in any C_{18} reverse phase column fractions [limit of detection of versicolorin C is 2.5 pmol (0.85 ng) with a signal-to-noise ratio of 3].

4. Discussion

HPLC analysis of extracts from aflatoxigenic and non-aflatoxigenic *A. flavus* cultures indicated that the seven anthraquinone pigments for which the structures have been identified constitute a substantial majority of the yellow color present in aflatoxigenic extracts. Thus, they play the dominant role in forming the basis for the two widely used empirical tests for aflatoxigenecity in *A. flavus* being studied, the yellow pigment test of Lin and Dianese [14] and the ammonium hydroxide vapor color change test of Saito and Machida [15]. All the pigments identified are known anthraquinone derivatives associated with the aflatoxin biosynthetic pathway in *Aspergillus* species (Fig. 2) as either biosynthetic intermediates (norsolorinic acid, averantin, averufin, versicolorin A), or branch pathway or side-reaction/decomposition products of intermediates (nidurufin, versicolorin C, versicolorin A hemiacetal) [25,26]. Based on their UV–Vis absorption spectral properties, the six additional pigments partially described in Table 1 also appear to be anthraquinones, possibly dimethyl-derivatives of versiconal hemiacetal acetate (**8**–**10**) and versicolorin C (**11**–**13**). In summary, the results of this study indicate that the color change associated with

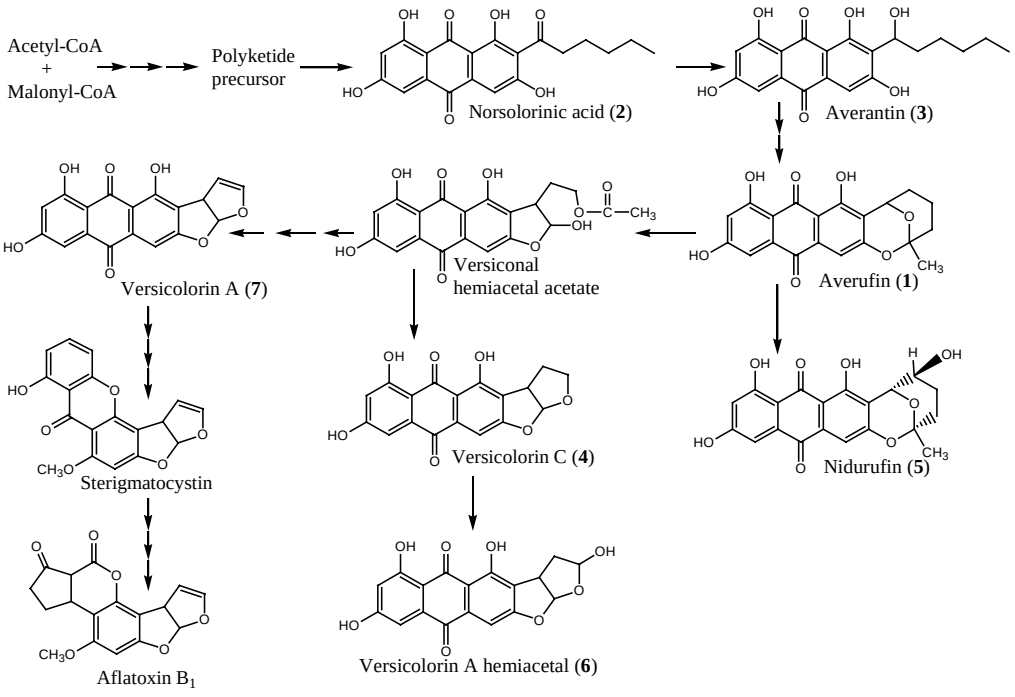

Fig. 2. Some anthraquinone pigments on the aflatoxin biosynthetic pathway and on some presumed branch pathways. The polyketide initial phase and the final finishing phase are shown in abbreviated form. The seven yellow pigments isolated and identified in this study are indicated by assigned numbers.

ammonium hydroxide vapor exposure in the Saito and Machida [15] test is due to anthraquinone pigments behaving as pH indicator dyes. No evidence was found for color generation by pigments undergoing a chemical reaction such as decomposition or reacting with ammonia. pH indicator dyes change color because protonation or deprotonation of functional group(s) at the transitional pH changes the extent of conjugation in a chromophore in the molecule. The chromophore in the anthraquinone pigments associated with the aflatoxin biosynthetic pathway meets this structural requirement.

The recognition that the pigments which form the basis of the empirical aflatoxigenicity tests of Lin and Dianese [14] and Saito and Machida [15] are associated with the aflatoxin biosynthetic pathway provides a simple biochemical rationale for the predictive powers of the two tests. Aflatoxigenic *A. flavus* colonies are yellow and turn red at higher pH values in the Saito and Machida [15] test because they contain readily visible amounts of yellow anthraquinone pigments that are either biosynthetic intermediates needed to make aflatoxins, or unutilized branch pathway products. There are plausible explanations for why biosynthetic intermediates would be present in aflatoxigenic *A. flavus* colonies. The anthraquinone pigments may be held in storage for subsequent rapid conversion to aflatoxin, or excess intermediates may be needed to drive an unfavorable biosynthetic step(s) by mass action. Recognizing the molecular basis of these tests makes it possible to predict that they will give few, if any, false positives. False positives would result from mutations in the small number of enzymes that catalyze the final steps of the aflatoxin biosynthetic pathway after the anthraquinone ring system has been converted to a different chromophore [25,26]. This prediction has been confirmed in a large study of *A. flavus* isolates from the Mississippi Delta [6], which compared cultural methods for aflatoxigenicity assessment to ELISA and TLC methods which detect aflatoxin production in culture. Among 517 isolates of *A. flavus* there were no (0%) false positives for aflatoxigenicity with either the Lin and Dianese [14] or Saito and Machida [15] cultural tests [6]. Knowing the molecular basis of these aflatoxigenicity tests also makes it possible to predict that false negatives could occur when yellow pigment levels are too low to be detected with the naked eye, but high enough to support synthesis of aflatoxins at levels detectable with sensitive fluorescence-based assays. In the study of 517 Mississippi *A. flavus* isolates [6] a substantial false negative rate of 19% was observed. However, most of these false negatives appear to have occurred by a mechanism other than sensitivity of the eye for yellow pigment being lower than the sensitivity of ELISA for aflatoxin, because the false negative rate was almost as high in isolates producing large amounts of aflatoxin (>10,000 ng/g) (29% false negatives) as it was in isolates producing low levels of aflatoxin (20–10,000 ng/g) (31% false negatives).

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