

## Precursor Recognition by Kinetic Pulse-Labeling in a Toxigenic Aflatoxin B<sub>1</sub>-Producing Strain of *Aspergillus*

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Kinetic pulse-labeling of aflatoxin pathway compounds was carried out in *Aspergillus parasiticus*, beginning with radioactive acetate. Norsolorinic acid, averufin, versicolorin A, and sterigmatocystin (all known as compounds which can be incorporated into the aflatoxin molecule) were radiotraced to follow their order of appearance. Aflatoxin species B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> were included. Norsolorinic acid and averufin appeared as early transient intermediates followed in order by versicolorin A, aflatoxins, and sterigmatocystin. To date, a mutually confirming array of results has been obtained with established precursors in wild-type strains of *A. parasiticus* and *A. versicolor* (as well as with an aflatoxin pathway mutant of *A. parasiticus*), which together establish a practical methodology for recognition of new pathway intermediates. The kinetic of pulse-labeling for sterigmatocystin in relation to aflatoxins suggests that dual branchlets may exist to aflatoxins; i.e., sterigmatocystin may not be an obligatory aflatoxin precursor.

Aflatoxins are ubiquitous in the food supply, being highly toxic and among the most potent of the carcinogens. In this context, it is important to appreciate the details of the biochemical steps employed in the synthesis of these compounds. The fragmentary outlines of the biosynthetic steps for aflatoxins were described in a recent review by Steyn et al. (24). The incomplete sequence of precursors illustrated in Fig. 1 is widely accepted, although absolute proof awaits completion of rigorous biochemical-genetic analyses. Norsolorinic acid (Nsl), averufin (Avf), versicolorin A (VsA), and sterigmatocystin (Stm) have been detected in wild-type strains of *Aspergillus parasiticus* (Fig. 1). Versiconal hemiacetal acetate (Vla) is, however, accumulated by wild type in the presence of an insecticide, dichlorvos (7, 20, 23, 24, 26, 27). An aflatoxin-negative mutant (ATCC 36357) obtained from *A. parasiticus* ATCC 15517 by nitrosoguanidine mutagenesis (13) contains a block between VsA and Stm. Thus, the mutant does not produce Stm or aflatoxins, accumulates Avf and VsA behind the block, and is able to convert exogenous Stm to aflatoxin (21).

Another wild-type species of *Aspergillus*, *A. versicolor* is less toxigenic since it fails to produce aflatoxins. Although *A. versicolor* and *A. parasiticus* are different species, they produce similar secondary metabolites. *A. versicolor* accumulates Avf, VsA, versicolorin B (VsB), and

Stm. Recently, the technique of kinetic pulse-labeling, enormously successful in tracing metabolite sequence in biochemical pathways of plants (3-5) and bacteria (11, 18, 19) was adapted to the aflatoxin system in *Aspergillus*. *A. versicolor* (NRRL 5219) was chosen as the simplest preliminary system to verify the utility and validity of kinetic pulse-labeling for identifying the sequence in which established aflatoxin precursors are formed. Minicolony populations were allowed to metabolize [1-<sup>14</sup>C]acetate over various time intervals. The biosynthetic reactions were quenched by the quick-freezing of minicolonies, disruption of the cells, and extraction of the various metabolites in acetone. Small silica thin-layer chromatographic (TLC) plates were then used to separate radioactive metabolites; elution in two or three different directions was often necessary. Radioautography of the TLC plates provided a sensitive assay for the appearance of the various intermediates in a timing pattern which implicated the sequence of precursor formation in *A. versicolor*. Transient intermediates were distinguished from dead-end metabolites by the rapid formation and disappearance of the former.

Our next objective, accomplished in this paper, was the extension of this procedure to *A. parasiticus*, the toxigenic strain. The results confirm the general validity of this approach and provide a rigorous basis for recognition of pos-

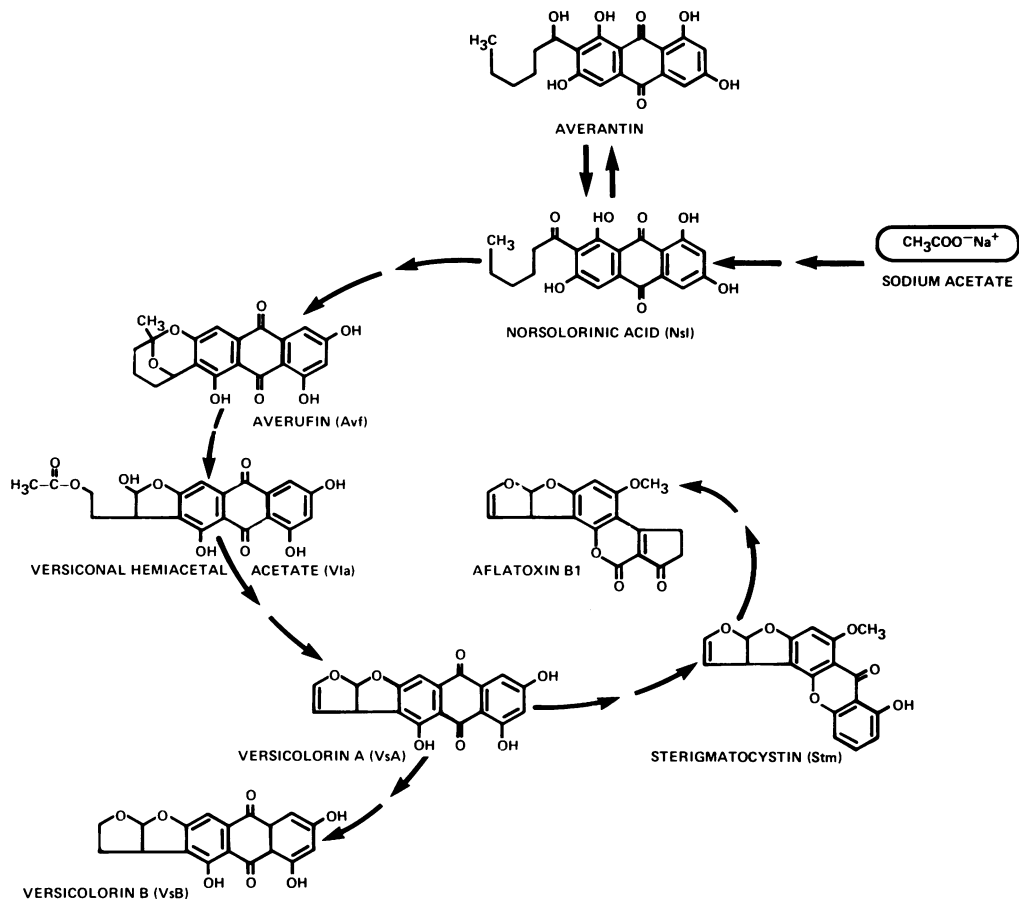


FIG. 1. Fragmentary sequence of known structures deduced to be aflatoxin B<sub>1</sub> precursors based upon their incorporation into aflatoxin. Arrows are not intended to denote the absolute number of steps intervening between structures shown. VsB is thought to be a shunt metabolite derived from VsA by reduction of the double bond in the bisfuranomoiety.

sible new precursors which may then be isolated for structure determination and characterization.

#### MATERIALS AND METHODS

**Methodology.** Most details of the kinetic pulse-labeling technique used in this study were described with *A. versicolor* (28). The following procedural improvement was made. A sterile cellophane paper was placed on the surface of the agar culture medium to facilitate the transplantation of individual micocolonies and to eliminate the tedium of micocolony washing to separate agar particles from mycelium. In addition, the presence of 0.08% sodium desoxycholate (16) in the medium was necessary with *A. parasiticus* (unlike *A. versicolor*) to discourage confluent growth so that individual micocolonies could be isolated. In every experiment small amounts of the known compounds indicated were added as carriers.

**Microbiological aspects.** Stock cultures of *A. parasiticus* (ATCC 15517) were maintained on 2% (wt/

vol) agar (Difco Laboratories) slants of yeast extract-sucrose medium containing (per liter) 200 g of sucrose and 20 g of yeast extract (Difco). For preparation of single micocolonies, serial dilutions of ATCC 15517 were prepared, and the 10<sup>-5</sup> dilution was used for inoculation: 0.1 ml of the 10<sup>-5</sup> dilution was plated on 40 petri dishes containing yeast extract, sucrose medium, and 3% sodium desoxycholate. Before inoculation, the agar was covered with a 7-cm-diameter circle of cellophane (Dupont 215-PD 62). Growth was allowed to proceed (approximately 2 days) until the colonies were 2 to 3 mm in diameter (magnification ×20). Two colonies were transferred aseptically into 0.5-dram vials containing 0.7 ml of Anderson and Smith medium (2). At this stage the white micocolonies do not yet produce pigments.

*A. parasiticus* ATCC 36537, kindly supplied by J. Bennett, was cultured as above except that the Anderson-Smith medium was replaced by a modified yeast extract-sucrose medium (50 g of sucrose, 20 g of yeast extract, and 20 g of agar per liter).

**Radioactive feeding, reaction quenching, and extraction procedure.** [ $^{14}\text{C}$ ]sodium acetate (1 mCi in 1 ml of ethanol [New England Nuclear Corp.] of high specific activity [56.2 mCi/mmol]) was used in all of the experiments. A 0.12-ml portion of this solution was added to 10 ml of water. The solution was sterilized by passage through a 0.45- $\mu\text{m}$  membrane filter (Millipore Corp.; SXHA 013-OS) into a presterilized vial. A 25- $\mu\text{l}$  portion of this solution (678,539.7 dpm) was added aseptically to individual vials, each containing two minicolonies. Additions were made with the aid of a 25- $\mu\text{l}$  microbipipette (Schwarz/Mann) assembled with sterilized pipette tips. At chosen time intervals the biosynthetic reactions were quenched by freezing according to previous procedure (28).

Extraction involved sonication of the minicolonies followed by solubilization of small molecules in acetone (28). After multiple extraction with acetone, the solvent was evaporated in a rotary evaporator, and the residue was spotted on silica TLC plates.

**Linear TLC procedure.** TLC plates (10 by 10 cm) were used (0.25 mm precoated silica G-25 HR, Brinkmann Instruments Inc.). The entire extract containing authentic metabolites as additions (Nsl, Avf, VsA, VsB, Stm, and all four molecular species of aflatoxin) was spotted at the origin. Elution was done in two directions. The plates were developed three times in solvent 1 (benzene/cyclohexane/acetone [88:7:5; vol/vol]). Solvent 2 was benzene/acetic acid (95:5; vol/vol).

**Radial TLC.** The radial chromatographic apparatus used was adapted from that of G. L. Litt and R. G. Johl (15). The bottom of a 9-cm-diameter glass petri dish was used as a well, and TLC plates (10 by 10 cm) were used. Fifty milliliters of solvent allows for quick equilibration of the vapor phase. The wick assembly was prepared by folding a plastic strip (2.5 by 4.0 cm) in half and perforating the middle of the crease with a small hole to accommodate a double strand of common string. The middle of the TLC plate was marked with a pencil to facilitate proper positioning of the wick. A small circle of 1-cm radius was drawn around the wick site, and the application origins of various samples were then distributed along the circle. Chloroform/acetone (9:1; vol/vol) was employed to distinguish between aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, whereas benzene/acetic acid (95:5; vol/vol) was the solvent used for the separation of all other metabolites.

**Identification of labeled metabolites of *A. parasiticus*.** Authentic unlabeled reference metabolites were added as markers which were readily monitored by the measurement of fluorescence under long-wavelength ultraviolet light. The positions of the fluorescent compounds in the two-directional linear TLC runs were matched to the darkened areas on the corresponding autoradiograms. The elution positions of the different authentic metabolites were scraped from the silica gel G-25 HR TLC plates (10 by 10 cm) and eluted five times with 1 ml of acetone. The solvent was evaporated to dryness. The residue was dissolved in 100  $\mu\text{l}$  of acetone and spotted on a silica gel plate (10 by 10 cm) for radial TLC. Three elutions in benzene/acetic acid (95:5; vol/vol) yielded excellent resolutions of Nsl, Avf, Stm, VsA, and VsB. The aflatoxins, which on the linear TLC remained near the origin,

were scraped and resolved by radial TLC with chloroform/acetone (9:1; vol/vol). Authentic aflatoxin standards were added to the extract. Autoradiograms were then obtained from these TLC plates by exposing them with an X-ray film in the dark for 5 days. The X-ray films were developed by the same procedure used previously (28).

## RESULTS

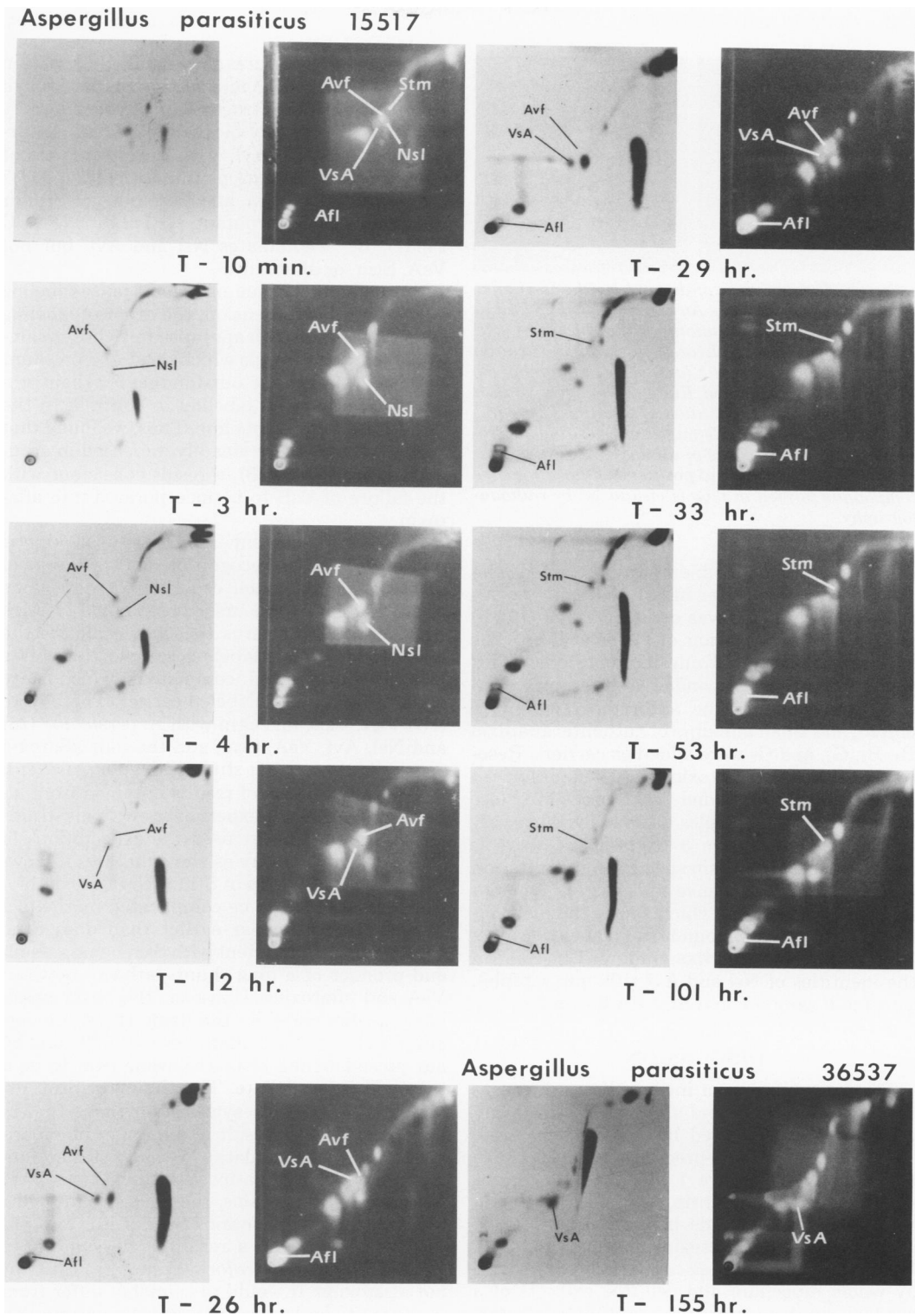
**Ordered appearance of known aflatoxin precursors.** Pulse-labeling of aflatoxin precursors was initiated through the administration of [ $^{14}\text{C}$ ]acetate. Minicolonies were sacrificed at various times to obtain samples for detection of transiently labeled intermediates. The absolute timing of precursor appearance is a highly variable function of culture and nutritional history. The relative timing of sequential appearance of compounds is reliable, however. Figure 2 reveals an order of metabolite appearance that is generally consistent with expectations suggested by the pathway order given in Fig. 1.

At the earliest sampling time of 5 min, five major radiolabeled compounds were seen (same as the more intense spots visualized at 10 min, Fig. 1). None of these compounds coincided with the positions of authentic known compounds shown (on the right) as fluorescent spots under ultraviolet illumination. The next sample (100 min) contained both Nsl and Avf. Consideration of their structures provides strong evidence that Nsl preceded Avf. This is consistent with the sequence of samples shown between 3 and 12 h where Nsl disappeared before Avf. At 12 h the appearance of VsA was clearly discerned. At a quench time of 26 h Avf was no longer labeled, VsA accumulation was greater, and radioactive aflatoxins were now detectable.

Surprisingly, Stm was not located before the 33-h sample, well after the first detection time for aflatoxins. Stm showed progressive accumulation through 53 h. After this time, Stm declined, whereas aflatoxins steadily increased through final sampling (about 100 h).

**Tracer analysis of the mutant strain.** The mutant strain (*A. parasiticus* ATCC 36537) showed a similar progression of pulse pattern with respect to Nsl, Avf, and VsA. The dead-end accumulation of VsA behind the mutant block is apparent (see lower right panel of Fig. 2). In the mutant, VsA levels remained high long after the total disappearance of VsA in corresponding samples taken from wild-type *A. parasiticus*.

**Confirmation of identities of aflatoxin pathway compounds formed de novo.** A rigorous analysis was carried out to establish the identities of Nsl, Avf, VsA, Stm, and the four molecular species of aflatoxin. This is illustrated in Fig. 3 with the aflatoxin compounds. Two



**FIG. 2.** Sequential appearance of aflatoxin intermediates as revealed by paired autoradiograms (left) and the corresponding TLC chromatograms (right) in wild-type *A. parasiticus*. Times of quenching ranged from 10 min to 101 h. Also shown is the dead-end accumulation of VsA (after 155 h) in the mutant strain of *A. parasiticus* ATCC 36537.

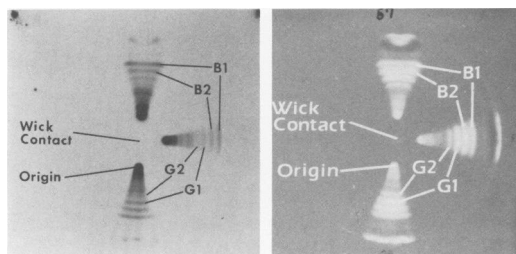


FIG. 3. Confirmation of de novo synthesis of aflatoxins  $B_1$ ,  $B_2$ ,  $G_1$ , and  $G_2$ . An extract was prepared at 32 h (top origins) and another at 194 h (right and bottom origins are duplicates) after administration of [ $^{14}\text{C}$ ]acetate. Authentic (unlabeled) aflatoxins  $B_1$ ,  $B_2$ ,  $G_1$ , and  $G_2$  were added. Right, a photograph taken under ultraviolet illumination of a radial TLC chromatogram which reveals migration positions corresponding to each species of aflatoxin. Left, the coincidence of the latter band positions with radioactive compounds formed in vivo is visualized by radioautography.

samples were analyzed; one was quenched relatively soon (32 h) after initial aflatoxin appearance, and the other was quenched long (194 h) after the administration of [ $^{14}\text{C}$ ]acetate. The spot areas excised from bidimensional TLC plates (which corresponded to appropriate radioactive spots on the autoradiograms) were eluted, and small amounts of authentic aflatoxin  $B_1$ ,  $B_2$ ,  $G_1$ , and  $G_2$  were added as carriers. Resolution of the four aflatoxins was then performed by application of radial TLC procedure (see above). The two samples analyzed yielded very similar results. Figure 3 (right panel) reveals each separated aflatoxin species on the criterion of ultraviolet fluorescence and is an exact match for Fig. 3 (left panel) which shows the distribution of aflatoxin compounds radiolabeled in vivo.

The same analysis was employed to confirm the identities of Nsl and Avf (105-min sample), VsA (26-h sample), and Stm (53-h sample).

## DISCUSSION

The large number of intermediates intervening between acetate and aflatoxin  $B_1$  constitute a long and complicated biochemical pathway. Relatively few of these precursor molecules have been identified. Figure 1 illustrates the latter structures: Nsl, averantin, Avf, Vla, VsA, and Stm. These compounds have all shown to be incorporated into aflatoxin molecules (6, 9-15, 17). In addition, Vla was converted to VsA both by whole mycelium and cell-free extracts of a versicolorin A-accumulating mutant of *A. parasiticus* (1). The origin of the oxygen atoms in averufin was investigated with oxygen-18 gas and [ $^{13}\text{C}$ ,  $^{18}\text{O}_2$ ]acetate (25). The sequential ar-

rangement of structures shown in Fig. 1 has been deduced from reasonable guesses made through examination of the structures and from a variety of apparent pathway blocks (22). Thus, *A. versicolor* forms Nsl, Avf, VsA, and Stm, but not aflatoxins. *A. parasiticus* mutant ATCC 36537 accumulates Avf, Vla, and VsA, but not Stm or aflatoxins. Another mutant, NRRL 6109 (ATCC 24551) (8), accumulates Nsl and Avf, but not VsA, Stm, or aflatoxins.

Since the technique of kinetic pulse-labeling has a temporal dimension, the order of labeling can be discerned with appropriately chosen sample times. Compounds which dead-end on shunt branches will also be outstanding for their progressive increase in labeling in contrast to the usual transiency of labeling. Thus, we found that VsB in *A. versicolor* steadily increased in accumulation of label (28), a result consistent with the failure of VsB to be incorporated into aflatoxin.

Before our current application of kinetic pulse-labeling for recognition and isolation of new precursors, initial experiments were done with established precursor compounds to work out the timing patterns that would dictate the best choice of postlabeling sample times. We followed only those compounds which were available to us as unlabeled carrier. These were Avf, VsA, VsB, and Stm with *A. versicolor* (28) and Nsl, Avf, VsA, Stm, and the four aflatoxin species in the current study with *A. parasiticus*.

The combination of results obtained from kinetic pulse-labeling experiments with all strains and mutants thus far used fits expectations of the aflatoxin pathway as shown in Fig. 1. However, the relationship of Stm with aflatoxin biosynthesis appears to be complicated by the fact that aflatoxins appear earlier than does Stm. This would be consistent with Stm being a dead-end product of a long shunt pathway between VsA and aflatoxins. However, the latter possibility is eliminated by the demonstrated incorporation of Stm into aflatoxins (9, 17, 21) and by our pulse-labeling results showing Stm to be a transient intermediate. The timing of Stm formation after aflatoxin synthesis has nevertheless been a repeatable result in a number of experiments (unpublished data). We suggest therefore that the aflatoxin pathway bifurcates to form two routes to aflatoxins, the longer route being one that uniquely contains Stm. If this hypothesis is correct, then a resultant dilemma is to explain why *A. versicolor* accumulates Stm but not aflatoxins. It would presumably differ from *A. parasiticus* by two mutations (one in each branchlet leading to aflatoxins), or perhaps a deficient enzyme operates in a similar step of each branchlet (mutation thereby interrupting

both branchlets). Plausible mechanistic schemes exist in support of the postulated pathway arrangement.

#### ACKNOWLEDGMENT

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