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Aflatoxin Biosynthesis: Detection of Transient, Acetate-Dependent Intermediates in *Aspergillus* by Kinetic Pulse-Labeling

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A simple technique was developed for the detection of intermediary metabolites of *Aspergillus versicolor* that are putative precursors of aflatoxin. Minicolony populations were allowed to metabolize [1,2-^{14}C]acetate over various time intervals. The biosynthetic reactions were quenched by quick-freezing the minicolonies, the cells were disrupted, and the metabolites were extracted into acetone. Small silica thin-layer chromatographic plates were then used to separate any radioactive metabolites present. Elution in two or three different directions was often necessary. Radioautography of the thin-layer chromatography plates provided a sensitive assay for the appearance of the various intermediates in a timing pattern which implicated the sequence of formation. Transient intermediates were distinguished from dead-end metabolites by the rapid formation and disappearance of the former. At least five unknown precursors of versicolorin A, a dead-end metabolite, were recognized. The kinetic pulse-labeling technique should be generally applicable to other fungal species whenever the entrapment of intermediary metabolites in the mycelium poses a technical problem.

An ideal sequence of approaches (Zamir, *in* P. Steyn, ed., *Biosynthesis of Mycotoxins*, in press) for the elucidation of a biosynthetic pathway includes: (i) kinetic pulse-labeling procedures, (ii) whole-cell feeding experiments, (iii) in vitro proof of each step at the enzymatic level, and (iv) in vivo proof by the study of appropriate mutants.

(i) Kinetic pulse-labeling. This technique was originally developed by Calvin and co-workers (2, 21, 22) in the formidable task of elucidating the path of carbon in photosynthesis. Despite the early statement of Calvin (22) that "it is worth emphasizing that these techniques are not limited to photosynthesis, but also have broad application to many in vivo biological processes," the method has remained relatively unexploited, fungal secondary metabolism being an especially good example. In plant systems, some time course studies have been carried out on the sequential appearance of terpenoids (1) in *Tanacetum vulgare L.* and of alkaloids (18) in young seedlings of *Vinca rosea*. The tracing of $^{14}CO_2$ has been somewhat more studied (3, 4, 13, 16, 17). The sole application of the kinetic pulse-labeling technique in fungi, apart from the present study, has been the work of Forrester and Gaucher (12) on the metabolites of *Penicillium patulum*. The method consists of following the metabolism of plausible radioactive precursors as a function of time, thus revealing an ordered sequence of metabolite appearance. If successful, it is an excellent and rapid qualitative probe which can suggest a preliminary outline of a given metabolic pathway.

(ii) Whole-cell feeding. Suspected precursors are synthesized and tagged with one or more radioactive isotopes (^{14}C, ^{3}H) or stable isotopes (^{13}C, ^{2}H, ^{18}O) and supplied in vivo to the appropriate organism during growth. Subsequent isolation, purification, and analysis of the product implicates the precursor-product relationships involved. One can therefore deduce an overall scheme from kinetic pulse-labeling and confirm each step of this scheme by whole-cell feeding.

(iii) Enzymological confirmation. The in vitro conversion of one intermediate to another by a pure enzyme, together with information obtained by the preceding two approaches, constitutes unambiguous proof of the enzymatic reaction. This enzymological approach is the most difficult, as exemplified by the relatively few pathways of secondary metabolism that have been defined step by step at the enzymatic level.

(iv) Study of mutants. To establish rigorously that the enzymatic reactions proven in enzymological confirmation experiments are indeed biosynthetic reactions in vivo, mutants blocked at the various enzymatic steps should be isolated. Application of kinetic pulse-labeling and whole-cell-feeding approaches to the isolated mutants confirms the identity of the various precursors under study.

A challenging problem for application of these approaches is the biosynthesis of aflatoxins. The problem of aflatoxin biosynthesis is conveniently subdivided (20) into three multistep sequences: biosynthesis of bisfurano-anthraquinones, biosynthesis of bisfurano-xanthones, and biosynthesis of aflatoxins. This study deals with the first metabolic segment, the biosynthetic steps leading to bisfurano-anthraquinones.

Both *Aspergillus parasiticus* and *A. versicolor* produce C_6-anthraquinones, bisfurano-anthraquinones, and bisfurano-xanthones in common. However, *A. parasiticus* produces aflatoxins, whereas *A. versicolor* does not. Since the metabolites produced in common by these closely related species undoubtedly arise via similar biogenetic pathways, *A. versicolor* is the most convenient species for the study of the early steps of aflatoxin biosynthesis. Sterigmatocystin (a bisfurano-xanthone) is the last intermediate of aflatoxin biosynthesis known to be common to both species (14). Previous kinetic pulse-labeling techniques (12) were unsatisfactory since the metabolites of *A. versicolor* consistently became trapped in the mycelium, thereby preventing release into the growth medium. A new method having potential application to the identification of any fungal metabolites which may also be trapped in the mycelium is described in this paper.

MATERIALS AND METHODS

Strain and cultivation. *A. versicolor* NRRL 5219 was kindly supplied by the late Dorothy Fennel. Stock cultures were maintained on 2% agar (Difco) slants of Czapek Dox medium (15) containing (in grams per liter): sucrose, 30; $NaNO_3$, 3; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; and $FeSO_4 \cdot 7H_2O$, 0.01. The organism was grown and maintained at 28°C.

Biochemicals and chemicals. Averufin, versicolorins A and B, and sterigmatocystin were isolated from *A. versicolor* NRRL 5219 and identified by comparing their spectroscopic and thin-layer chromatographic (TLC) properties with authentic samples which were kindly supplied by N. Terashima (Nagoya University). Norsolorinic acid and the "unknown" compound were isolated and kindly provided by J. Bennett (Tulane University). All other biochemicals and chemical reagents were of the best quality available from commercial sources.

Single minicolonies. To ensure genetic homogeneity, we prepared a spore suspension from a single colony of *A. versicolor* NRRL 5219. Serial dilutions from the suspension were plated on soft agar medium (modified Czapek Dox medium with 1% sucrose and 0.5% agar). Separate minicolonies formed two days after inoculation. The soft agar medium was chosen to enable easy transplantation of minicolonies. Colonies of uniform size were selected with the aid of a dissecting microscope (2-mm diameter; ×25 magnification) and were transferred to 0.5-dram (ca. 0.70-g) culture vials containing 0.5 ml of liquid Czapek Dox medium and 1% sucrose. Three minicolonies were transplanted into each vial with the aid of an inoculating loop. It was important to place the colonies carefully into the vials so that the aerial conidiophores were not submerged. At this stage, the white colonies were not yet producing secondary metabolites. Production of the pigments was easily visualized since the lower surface of the colonies sequentially became yellow and then orange-red. The colored pigments are produced after 8 days; at about half-production, i.e., 3 to 4 days after inoculation, radiolabeled precursors were introduced.

Radioactive feeding. The $^{14}CH_3{}^{14}CO_2Na$ (New England Nuclear Corp.) employed in all of the experiments was of high specific activity (54.0 mCi/mmol) (8). Sterile water was added to samples of an appropriate volume of this radioactive ethanolic solution, and the radioactive solution was sterilized by passage through a 0.22-μm membrane filter (type GSWP02500; Millipore Corp., Bedford, Mass.). The same amount of this sterile $^{14}CH_3{}^{14}CO_2Na$ solution was simultaneously added to all of the vials (0.29 μCi/vial; each vial contained three minicolonies) at 3 to 4 days after inoculation.

Reaction quenching. At chosen intervals, the biosynthetic reaction was quenched by freezing. For each time interval, five vials were quenched simultaneously, and each vial was analyzed independently to check the reproducibility of the method. The quenching of the reaction in a vial was accomplished in the following manner. The liquid medium was withdrawn with a Pasteur pipette and kept for monitoring the uptake of acetate, and the minicolonies were frozen by immersing the vial in liquid nitrogen.

Radioactive counting. A three-channel Nuclear Chicago liquid scintillation counter (Mark II) was used. Samples from the liquid media were counted in 15 ml of Aquasol.

Extraction and separation of metabolites. Acetone (0.5 ml/vial) was added to the frozen colonies, and the mixture was sonicated for 15 min to disrupt the cells. It was not necessary to wash the cells since residual label (sodium acetate) was completely metabolized at this time, as seen by the absence of labeled sodium acetate at chromatogram origins, where it would remain with the solvent systems employed. After extensive extraction with acetone, the solvent was evaporated, and the residue was spotted on TLC plates (2.5 by 2.5 inches [ca. 6.35 by 6.35 cm]; Silica Baker-Flex 1B-F; 0.25-mm thickness) impregnated with silver nitrate. The impregnation of the TLC plates was performed by dipping them into 10% $AgNO_3$ in CH_3OH-water (1:1) and allowing them to dry at room temperature in darkness before use. The TLC plates were developed in two dimensions: solvent 1 was $CHCl_3$-benzene (7:3) and solvent 2 was ether-pyridine (9:1).

Identification of metabolites. Fluorescent materials were detected under long-wavelength UV light, and those whose positions matched darkened areas on the autoradiograms were assumed to be metabolites. Unlabeled authentic metabolites were added to determine the coincidence of migration position for unknown and known metabolites on the basis of both fluorescence and radiolabel.

Autoradiograms. The autoradiograms were prepared by placing X-ray film (Kodak X-Omat R film XR-5; 8 by 10 inches [ca. 20.3 by 25.4 cm]; folder wrapped) against a TLC plate and keeping it lightproof by leaving it in a Kodak X-ray exposure holder (9 by 10 inches [ca. 22.9 by 25.4 cm]) in a darkroom (10,000 dpm of ^{14}C can be detected in one spot of about 3-mm diameter after approximately 4 days). The time required for detection depends on the amount of radioactivity on the plate and the number of spots. The X-ray film was developed and fixed by using the standard Kodak X-ray developer and fixer (Kodak 146-5327 and Kodak 166-6106, respectively).

RESULTS

Two- and three-dimensional chromatographic analysis of the metabolites of *A. versicolor*. Since very small samples (three minicolonies per vial) were used, 2.5- by 2.5-inch silica plates were satisfactory for good resolution. Plates were eluted by ascending chromatography. Two or three developments in alternate directions were used for separation (Fig. 1). The solvents employed were as follows: solvent 1, chloroform-benzene (7:3); solvent 2, ether-pyridine (9:1); and solvent 3, benzene-cyclohexane-acetone (88:7:5).

Uptake of the radioactive acetate by the three minicolonies. The uptake of acetate was monitored by counting samples of the growth media transferred from the vials at the different quenching times. After 15 h (Fig. 2), most of the acetate was taken up. It is interesting to note that some of the secondary intermediate metabolites are formed before the completion of acetate uptake (7). On the other hand, other secondary intermediate metabolites are synthesized after 15 h.

Sequential appearance of the metabolites. For each time interval, five vials (three minicolonies per vial) were examined independently. Although in all of the experiments described in this work at least four vials per time interval showed reproducible results, the major merit of this approach was that it allowed the qualitative determination of the sequence of intermediates and metabolites formed with time (Fig. 3).

(i) Quenching time = 15 min. At 15 min

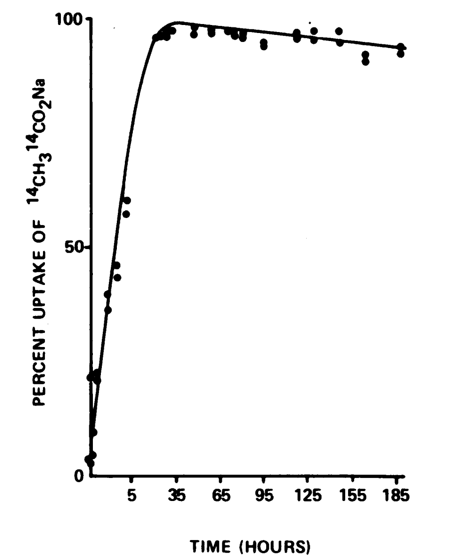

FIG. 2. *Uptake of acetate by minicolonies of A. versicolor.*

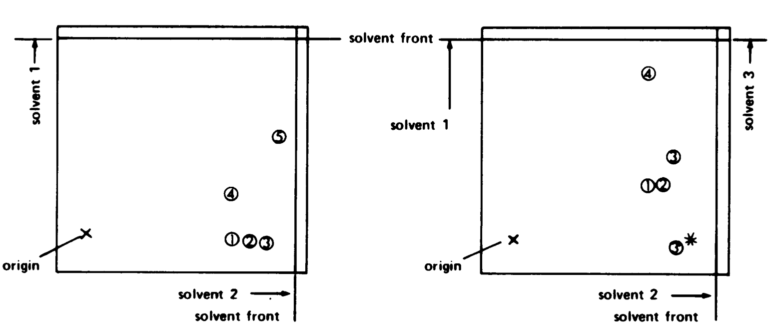

FIG. 1. *Two- and three-dimensional chromatographic analysis of transient metabolites from A. versicolor. Compound 1, versicolorin B (orange fluorescence under long-wavelength UV light); compound 2, norsolorinic acid (maroon purple fluorescence under long-wavelength UV light; kindly supplied by J. Bennett); compound 3, averufin and versicolorin A (orange fluorescence under long-wavelength UV light); compound 3*, unknown (isolated from an A. parasiticus mutant by J. Bennett; this unknown was separated from averufin only after elution in three directions); compound 4, sterigmatocystin (dark fluorescence under long-wavelength UV light); compound 5, demethylsterigmatocystin (brown spot under long-wavelength UV light).*

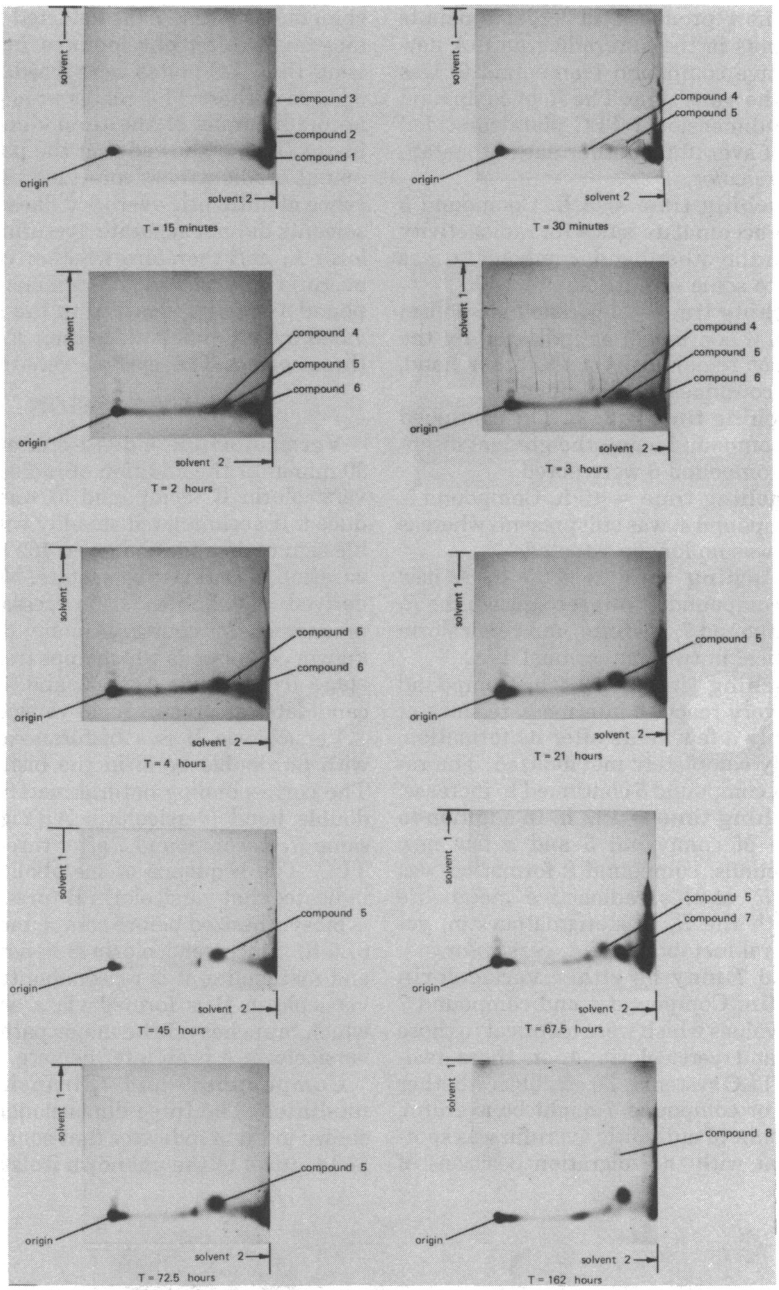

FIG. 3. *Autoradiograms showing the sequential appearance of the metabolites. Quenching times (T) ranged between 15 min (upper left) and 162 h (lower right).*

after the feeding of radioactive acetate, three radioactive spots moving with the solvent front (solvent 2) were formed (compounds 1, 2, and 3). Their identity remains unknown since none of the autoradiograms coincided with the TLC profiles of known compounds from *A. versicolor*. Radioactive acetate remained at the origin on these silica plates with the elution solvents used.

(ii) Quenching time = 30 min. The next metabolites to appear were compounds 4 and 5, seen as very faint spots (not very radioactive) in the autoradiogram. Compound 4 is unknown, whereas the R_f of compound 5 in these two solvents was identical to the R_f of versicolorin B.

(iii) Quenching time = 2 h. Compounds 4 and 5 were now produced in larger amounts (very dark spots in the autoradiogram). A new very radioactive compound (compound 6) was produced at the same time. The R_f of compound 6 in this two-dimensional TLC plate coincided with the R_f of averufin, another natural metabolite of *A. versicolor*.

(IV) Quenching time = 3 h. Compound 5 continued to accumulate since its radioactivity increased. On the other hand, compound 6 was metabolized to some extent.

(v) Quenching time = 4 h. The metabolism of compound 6 continued, as indicated by the very faint spot remaining. On the other hand, compound 5 continued to accumulate.

(vi) Quenching time = 21 h. The continued presence of compound 5 and the gradual disappearance of compound 6 were noted.

(vii) Quenching time = 45 h. Compound 5, as well as compound 4, was still present, whereas compound 6 was no longer detected.

(viii) Quenching time = 67.5 h. A new metabolite (compound 7) was recognized; the R_f values of compound 7, averufin, and versicolorin A were identical in two-dimensional TLC.

(ix) Quenching time = 72.5 h. Compound 7 must be a very reactive intermediate since at this stage, only a few hours after its formation, it was already completely metabolized. The radioactivity of compound 5 continued to increase.

(x) Quenching time = 162 h. In addition to the presence of compound 5 and a few new minor compounds, compound 8 formation was noted. The R_f of this radioactive metabolite coincided with the R_f of sterigmatocystin, yet another natural metabolite of *A. versicolor*.

Compound 7 may be either versicolorin A or averufin. Compound 4 and compound 7 exhibited R_f values which were identical to those of averufin and versicolorin A on these two-dimensional TLC systems. To establish whether compound 4 or compound 7 might be averufin, a known amount of authentic averufin was spotted coincident with the migration positions of compounds 4 and 7 (as detected in the autoradiograms). After development in the third solvent, the TLC plates were dried, and autoradiograms of these TLC plates were prepared. The autoradiograms of the three-dimensional TLC plates (Fig. 4) showed that the position of compound 7 radioactivity coincided with the fluorescence of authentic averufin. These three elution solvents did not separate averufin from versicolorin A, and therefore whether compound 7 is averufin or versicolorin A remains at issue. Compound 4 may be identical to the unknown provided by J. Bennett since their R_f values in this three-solvent TLC system were the same.

DISCUSSION

Versicolorin B, a dead-end metabolite. At 30 min after the addition of radioactive acetate, versicolorin B (compound 5) was already produced. It accumulated steadily with no detectable sign of degradation, even 162 h after administration of radioactive acetate. Not all acetate-derived metabolites are necessarily aflatoxin precursors, of course. Among these, the unknown compounds which appeared at an earlier stage (compounds 1, 2, 3, and 4) are feasible candidates as precursors of versicolorin B.

Versicolorin B is a bisfurano-anthraquinone with no double bond in the bisfurano moiety. The corresponding natural metabolite with the double bond (versicolorin A [Fig. 5]) has the same R_f as compound 7 after three-dimensional TLC. The sequence of metabolite appearance indicates that versicolorin B (present at 30 min) is biosynthesized before compound 7 (present at 67.5 h). Since versicolorin B behaves as a dead-end metabolite, it is reasonable to assume that versicolorin B is formed via a shunt pathway which branches off the major pathway of which versicolorin A is an intermediate.

Compounds 6 and 7, transient biointermediates. The three-dimensional TLC system shown in Fig. 4 indicates that compound 6 might be identical to the unknown isolated by J. Ben-

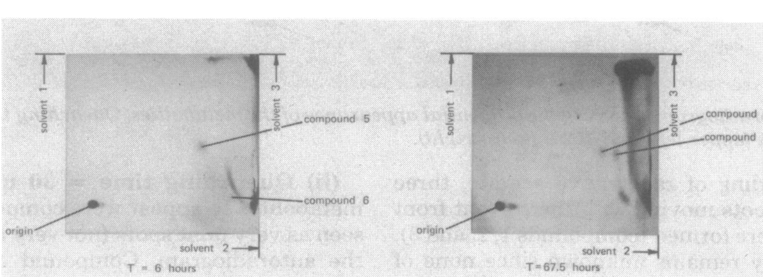

FIG. 4. *Identification of the averufin spot. Autoradiograms of three-dimensional TLC preparations indicated that compound 7 (and not compound 6) is probably averufin. On the right plate the position of compound 7 coincided with that of fluorescent authentic averufin.*

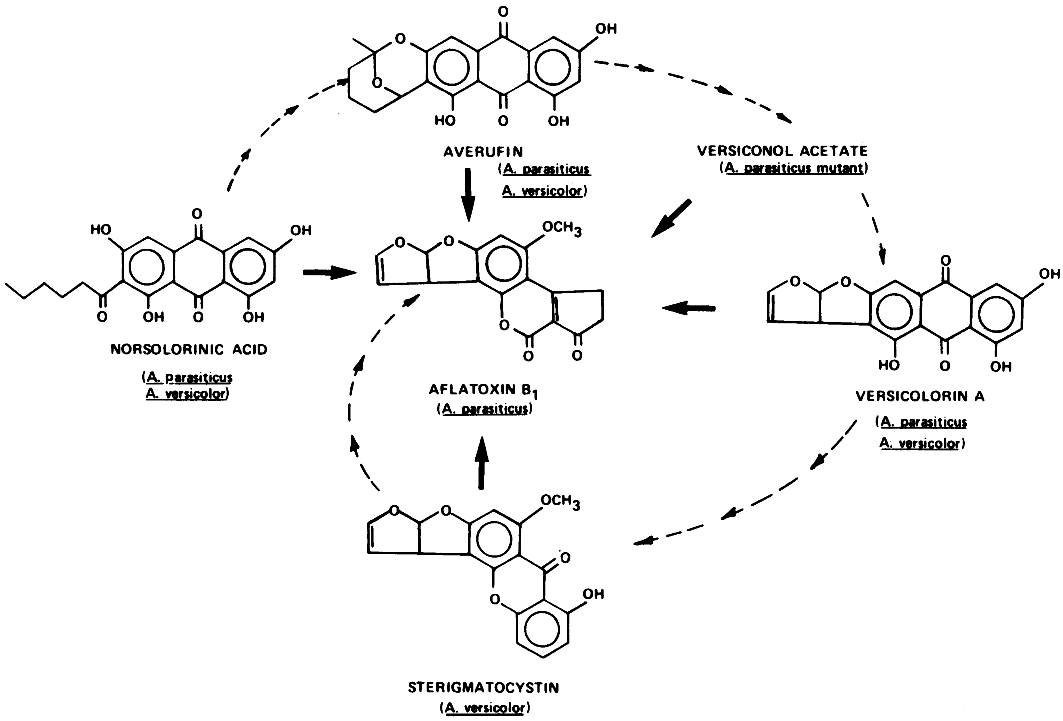

FIG. 5. *Precursors to aflatoxin B_1. Heavy solid arrows represent the results of whole-cell feeding experiments that established labeled norsolorinic acid, averufin, versicolorin A, and sterigmatocystin as precursors of aflatoxin B_1. Addition of dichlorvos to a mutant strain of A. parasiticus induced the accumulation of a new compound, versiconol acetate, which was also efficiently incorporated into aflatoxin B_1. The species known to produce each metabolite are listed in parentheses. Dotted arrows indicate a plausible pathway to aflatoxin B_1 and also emphasize that many intermediates are yet to be found among these known metabolites.*

nett (unpublished data) from an *A. parasiticus* mutant. The identity of compound 7 is still uncertain since its R_f coincided with that of fluorescent authentic averufin, but authentic versicolorin A also had the same R_f as compound 7 in the three-dimensional TLC system. It seems unlikely that, by coincidence, averufin and versicolorin A are produced and metabolized simultaneously. Therefore, averufin or versicolorin A, or both, must have been produced between 45 and 67.5 h and were readily metabolized. In either case, compound 7 must be a reactive transient intermediate.

Compound 6 is also a transient biointermediate. It is interesting to note that this kinetic pulse-labeling experiment could detect compound 6, which is not usually found in mature *A. versicolor* strains but which may be produced by mutants of *A. parasiticus* (personal communication, J. Bennett). Compound 6 is probably a major intermediate in aflatoxin biosynthesis.

Unknown transient intermediates in *A. versicolor*. Despite the simplicity of this method, it is possible to identify various new biointermediates as well as already acknowledged natural metabolites. Before this study, the only precursors to versicolorin A postulated were norsolorinic acid, averufin, and versiconol acetate (19). It now appears that the biosynthesis of the bisfurano-anthraquinones involves more unknown intermediates than anticipated.

Norsolorinic acid, averufin, and versicolorin A are established metabolites of both *A. parasiticus* and *A. versicolor* (14). Versiconol acetate was only produced when the insecticide dichlorvos was added to the cultures of *A. parasiticus* (9). In our study, norsolorinic acid was not detected, although at least five unknown intermediates appear as transient metabolites. Four of these unknowns occur before the formation of versicolorin A. One of the transient compounds (compound 6) appears to be very similar to the unknown compound isolated by J. Bennett from an *A. parasiticus* mutant, judging from R_f patterns established by TLC. The biosynthesis of the bisfurano-anthraquinones (versicolorins) appears, therefore, to be very complex, necessitating the elucidation of the structures of many intermediates.

Application of this new kinetic pulse-labeling technique to various mutants of *A. versicolor* and *A. parasiticus* is an approach that merits additional effort.

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