APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2804–2807
0099-2240/87/122804-04$02.00/0

Vol. 53, No. 12

Conversion of a New Metabolite to Aflatoxin B_2 by *Aspergillus parasiticus*

THOMAS E. CLEVELAND,* DEEPAK BHATNAGAR, CHRISTOPHER J. FOELL, AND SUSAN P. McCORMICK

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70179

Received 18 June 1987/Accepted 9 September 1987

A new metabolite which could be converted to aflatoxin (AF) B_2 was detected during cofermentation analysis of two nonaflatoxigenic strains (SRRC 2043 and SRRC 163) of *Aspergillus parasiticus*. SRRC 2043, which accumulates the xanthone *O*-methylsterigmatocystin (OMST), a late precursor in the AFB_1 pathway, was observed to accumulate another chemically related compound (HOMST; molecular weight, 356); SRRC 163 is blocked early in the pathway and accumulates averantin. During cofermentation of the two strains, levels of OMST and HOMST were observed to be greatly reduced in the culture, with simultaneous production of AFB_1, AFB_2, and AFG_1. Intact cells of SRRC 163 were able to convert pure OMST or its precursor, sterigmatocystin, to AFB_1 and AFG_1 without AFB_2 accumulation; the same cells converted isolated HOMST to AFB_2 with no AFB_1 or AFG_1 production. The results indicate that AFB_2 is produced from a separate branch in the AF biosynthetic pathway than are AFB_1 and AFG_1; AFB_2 arises from HOMST, and AFB_1 and AFG_1 arise from sterigmatocystin and OMST.

Certain aspects of the biosynthetic relationship among aflatoxins (AFs) B_1, B_2, G_1, and G_2, which are produced by *Aspergillus parasiticus*, remain unclear. Results of some studies (6, 12, 18) have suggested that AFB_1 is the progenitor of the other three toxins, whereas others (9) have postulated that AFB_1 and AFB_2 are produced by separate pathways. In a recent study (11) no interconversion of AFB_1, AFB_2, AFG_1, or AFG_2 by intact cells of blocked mutants of *A. parasiticus* was observed, indicating that AFB_2 or the AFG toxins might not arise from AFB_1. In the proposed metabolic grid for the branched AFB_1 and AFB_2 biosynthetic pathway, the biosyntheses diverge, beginning at the metabolic precursor versiconal hemiacetal acetate (9, 17). The chemical intermediates placed in the AFB_1 pathway after the versiconal hemiacetal acetate branch have been demonstrated to be in the following sequence: versicolorin A (15) → sterigmatocystin (ST) (13) → *O*-methylsterigmatocystin (OMST) (5) → AFB_1. The sequence of biosynthetic steps resulting in AFB_2 formation remains largely hypothetical. It has been speculated, however, that versicolorin C (9) and 5-hydroxydihydrosterigmatocystin (10) may be AFB_2 precursors.

In this study a unique secondary metabolite (designated HOMST) was identified in cultures of *A. parasiticus*. HOMST was demonstrated to be an intermediate in the AFB_2 pathway but not in the AFB_1 pathway. This was achieved by cofermentation of two *A. parasiticus* mutants (SRRC 2043 and SRRC 163) which are blocked at different steps in the AF biosynthetic pathway. SRRC 2043 is impaired near the terminus of the AF pathway and accumulates OMST (5) with no production of AFs, while the SRRC 163 mutant is blocked early in the AF pathway (4) but has the enzymes required for the conversion of intermediates to AFs in the late stages of the pathway (after versicolorin A synthesis) (7, 13, 14).

* Corresponding author.

MATERIALS AND METHODS

Cultures. Cultures of *A. parasiticus* SRRC 2043 and SRRC 163 were maintained as described for a previous investigation (7). Single or cofermentations of the two strains were conducted by inoculating 100 ml of liquid growth medium (1) in 250-ml flasks with about 2×10^5 viable spores of each strain in 1 ml of deionized water and incubating at 28°C with shaking on a Psycrotherm incubator shaker (New Brunswick Scientific Co., Inc.) (150 rpm) for 4 days.

Extraction of fungal metabolites and purification of an AFB_2 precursor. Extraction of metabolites from liquid cultures was conducted exactly as described previously (15). The metabolite of interest was purified by dissolving the dried, crude extraction product (from two 100-ml cultures of SRRC 2043) in about 0.5 ml of acetone and streaking the solution onto a thin-layer chromatographic (TLC) plate (0.25 mm by 20 cm^2) coated with silica gel G (Merck & Co., Inc., Piscataway, N.J.). The plate was developed in an ether-methanol-water (EMW; 96:3:1; vol/vol/vol) solvent system. A blue-green fluorescent band (under longwave UV light) containing the metabolite was scraped from the silica gel plate and eluted from the silica with acetone. The metabolite was rechromatographed several times until a single band for the metabolite was observed. Finally, the acetone eluate was dried under nitrogen, and the dried residue was dissolved in about 100 μl of acetone for use in biosynthetic conversion studies with intact cells.

The AFs or OMST produced in the cultures was developed, identified, and quantitated by one-dimensional TLC by established procedures (7). HOMST from the fungal extracts was quantitated spectrophotometrically relative to an analytical HOMST standard by using the same fluorometric method as that used for OMST quantitation (5).

Conversion of metabolites to AFs. For biosynthetic conversion experiments with intact cells, the incubation medium was prepared by adding 25 μl of acetone containing ST (5 μg), OMST (5 μg), or HOMST (5 or 10 μg) to 10 ml of low-sugar replacement medium (16). Three-day-old mycelia

of SRRC 163 were harvested by vacuum filtration and washed extensively with low-sugar replacement medium. The mycelia (0.5 g [wet weight]) were added to each flask containing the incubation medium, and the reactions were carried out as described previously (5, 7).

Synthesis and characterization of the OMST analog. OMST was chemically derivatized by a trifluoroacetic acid (TFA) treatment procedure (2). Larger-scale TFA treatment of OMST to produce its analog, HOMST, was conducted by streaking 0.1 ml of acetone (containing 0.1 mg of OMST) onto the TLC plate and treating the streaked spot with approximately 50 μl of TFA. The plate was dried under a warm airstream to remove excess TFA (2), and the HOMST was separated by TLC and purified as described above.

The UV absorption spectrum and molar extinction coefficients at 209, 238, and 317 nm were determined for HOMST in methanol by using a spectrophotometer (model UV 160; Shimadzu).

Analysis of metabolites. Identities of the metabolites and the AFs were established by chromatographic behavior on TLC and by mass spectrometry with a spectrometer (4000; Finnigan) by using analytical standards of ST, OMST, and AFB_1, AFB_2, AFG_1, and AFG_2 (Sigma Chemical Co., St. Louis, Mo.). TLC was performed with three solvent systems: (i) EMW, (ii) toluene-ethyl acetate-acetone, (iii) toluene-ethyl acetate-acetic acid. The R_f values of the metabolite standards have been reported previously (5). The fluorescence of the metabolites on TLC plates was visualized under longwave UV illumination before and after the plates were sprayed with 20% (vol/vol) aluminum chloride in ethanol and heated for a few minutes in an oven at 120°C (8, 14).

Preparation of [^{14}C]HOMST. Radiolabeled HOMST was produced by adding 10 ml of an aqueous solution containing 1 mCi of [1-^{14}C]acetate (59 mCi mmol^{-1}; Moravek Biochemicals Inc.) to 30 g of wet pellets of 2-day-old mycelia of SRRC 2043 in 100 ml of replacement medium by established procedures (5). The [^{14}C]HOMST was isolated and purified from the culture as described above, and its specific activity was determined as described previously (5).

Radiolabel incorporation into AFB_2. The [^{14}C]HOMST (3.9 nmol; specific activity, 5.4 mCi mmol^{-1}) was added to 1 g (wet weight) of 3-day-old SRRC 163 mycelia in 10 ml of low-sugar replacement medium (as described above) to test for the conversion of [^{14}C]HOMST to AFB_2. An equal quantity of labeled HOMST was similarly added to autoclaved SRRC 163 mycelia as a control. After 20 h of incubation at 28°C with constant shaking on a Psycrotherm incubator shaker (150 rpm), the reaction products were chromatographed by TLC (EMW solvent system), and the radioactivity in the specific zones of the TLC plate containing HOMST and AFB_2 were examined as described previously (5).

Statistical method. Experiments to determine AF levels produced by fungal cultures, supplied with the various combinations of OMST, ST, or HOMST, were conducted three times with two to three replicate cultures per experiment. An analysis of variance was conducted as a test of statistical significance ($P \leq 0.05$). Confidence limits were used to test for statistical differences between individual treatments.

RESULTS

The metabolites OMST, AFB_1, AFB_2, AFG_1, and AFG_2 were produced during cofermentation of the nonaflatoxigenic strains SRRC 2043 and SRRC 163, as determined by their relative mobilities in three solvent systems (EMW, toluene-ethyl acetate-acetic acid, and toluene-ethyl acetate-acetone) which yielded R_f values similar to those reported previously for these metabolites (5). OMST and another previously undescribed metabolite, designated HOMST, were observed in fermentations containing SRRC 2043 mycelia alone. The relative mobilities of HOMST were 0.25 in EMW, 0.12 in toluene-ethyl acetate-acetic acid, and 0.08 in toluene-ethyl acetate-acetone solvent systems. These R_f values were different from those of OMST and AFB_1, AFB_2, AFG_1, and AFG_2 in these solvent systems (5). OMST was produced in 10- to 15-fold higher concentrations (262.6 μg/g [dry weight] of mycelia) than was HOMST (20.5 μg/g [dry weight] of mycelia) under the culture conditions described above. OMST levels in cultures were observed to be greatly reduced (from 262.6 to 34.7 μg/g [dry weight] of mycelia), and no HOMST was detected during the cofermentation of strains SRRC 2043 and SRRC 163; however, the production of 110.9 μg of AFB_1, 2.2 μg of AFB_2, 5.6 μg of AFG_1, and traces of AFG_2 (<0.01 μg) per g (dry weight) of mycelia was detected during cofermentation of the two strains. SRRC 163 cultures, by themselves, produced no detectable levels of any of the metabolites (OMST, HOMST, or AFs) under the culture conditions described above.

HOMST exhibited a blue-green fluorescence under longwave UV illumination, and the fluorescence of HOMST changed to yellow-green on the TLC plate after the compounds were sprayed with $AlCl_3$; both of these are properties that are similar to those of OMST (5, 8, 14). Mass spectral analysis of HOMST (Fig. 1A) demonstrated a molecular ion at *m/z* 356 and major fragments at *m/z* 327 and 299. The UV absorption spectrum for HOMST in methanol (Fig. 1B) displayed three peaks at 209, 238, and 317 nm, with molar extinction coefficients of 21,766, 25,711, and 11,867, respectively.

About 0.1% of the radioactive label from the originally added [^{14}C]acetate was incorporated into [^{14}C]HOMST (yield, about 20 nmol), resulting in a specific activity of 5.4 mCi mmol^{-1} for [^{14}C]HOMST.

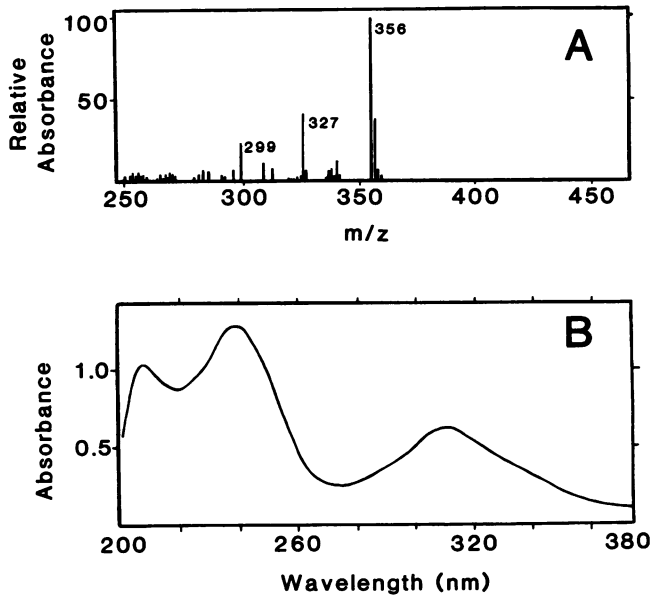

FIG. 1. Mass spectrum (A) and UV absorption spectrum (B) of HOMST.

TABLE 1. Metabolites produced by SRRC 163 intact cells when fed HOMST, ST, or OMST

Added metabolite(s)	Metabolite(s) (μg) produced by intact cells (0.5 g [wet wt])[a]		
	AFB_1	AFB_2	AFG_1
No metabolite	0	0	0
ST	2.07	0	0.16
OMST	2.25	0	0.24
HOMST	0	0.28	0
ST + OMST	4.31	0	0.41
ST + HOMST	1.67	0.36	0.14
OMST + HOMST	2.54	0.37	0.23

[a] Metabolites were produced by SRRC 163 cells in a low-sugar replacement medium, as described in the text. Values represent the mean of three experiments, with two replicate cultures per experiment. Statistical comparisons within columns were conducted as described in the text.

Intact cells of SRRC 163 converted labeled or unlabeled HOMST to AFB_2 (Tables 1 and 2). The results which support the observation that the conversion product in the HOMST-supplied cells was indeed AFB_2 were as follows: (i) the product yielded the same mass spectrum as that of an analytical standard of AFB_2 (8); (ii) the product did not react with TFA; and (iii) the product comigrated with AFB_2 by TLC in the three solvent systems used. Only about 25% of the 3.9 nmol of [^{14}C]HOMST supplied to heat-treated mycelia was recovered by the extraction and TLC procedure in control experiments (Table 2). After a correction was made for this nonenzymatic loss of the metabolite, a conversion efficiency of about 40% (mol/mol) was calculated for the HOMST to AFB_2 conversion reaction. However, the specific activity of the labeled AFB_2 product was nearly identical to that of the parent precursor (Table 2), demonstrating that there is no dilution or pooling of the labeled product with unlabeled AFB_2 from another reaction. No significant radioactive label was found associated with OMST, AFB_1, AFG_1, or AFG_2 during the labeling experiments in which HOMST was used as a precursor.

The addition of ST or OMST to SRRC 163 mycelia in resting media resulted in the formation of AFB_1 and AFG_1 without any detectable AFB_2 or AFG_2 production (Table 1); results indicated that slightly more AFB_1 and AFG_1 were obtained when OMST was supplied to the SRRC 163 mycelia as compared with when ST was supplied to the same mycelia. Cofeeding of unlabeled HOMST with either OMST or ST to SRRC 163 mycelia did not result in significant ($P \leq 0.05$) increases in the AFB_1, AFB_2, or AFG_1 products as compared with quantities of metabolites obtained when the compounds were fed individually to the same cultures.

TABLE 2. Conversion of [^{14}C]HOMST to AFB_2 by intact cells of SRRC 163

Mycelia	Total nCi[a]		Total nmol[b]		Sp act (nCi/nmol)	
	HOMST	AFB_2	HOMST	AFB_2	HOMST	AFB_2
Heat-treated cells	5.4[c]	tr	1.0	0	5.4	ND[d]
Intact cells	1.2	2.3	0	0.4	ND	5.8

[a] Radiolabeled HOMST (21 nCi; 3.9 nmol) was fed to both intact cells and heat-treated cells of SRRC 163, and the radioactivity in HOMST and AFB_2 was determined as described in the text. tr, Trace.

[b] Metabolites were quantitated fluorometrically, as described in the text.

[c] Mean values represent the average of two determinations from two separate experiments. Individual determinations did not vary more than 20% from the mean.

[d] ND, Not determined.

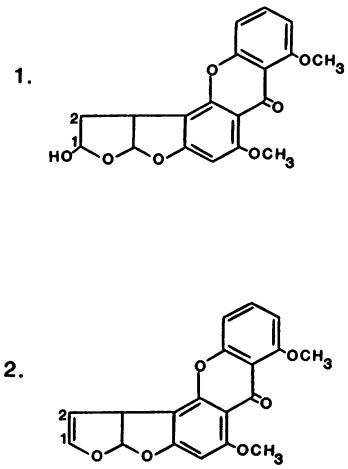

FIG. 2. Structures of the postulated hydrated HOMST (compound 1) and OMST (compound 2).

However, when ST and OMST were fed together to the intact cells of SRRC 163, additive increases in AFB_1 and AFG_1 without any production of AFB_2 resulted; the cofeeding of HOMST with either ST or OMST did not result in any additive production of AFB_2.

Treatment of OMST with TFA resulted in the production of a single blue-green fluorescent compound which comigrated with HOMST by TLC in the three solvent systems that were used and exhibited the same extinction coefficient values and mass spectrum as those of HOMST. This compound was also converted to AFB_2 with equal efficiency (about 40%) as that with HOMST by intact cells of SRRC 163. These results suggest that this chemically derived compound from OMST by the TFA treatment is identical to the HOMST isolated from SRRC 2043 mycelia.

DISCUSSION

The cofermentation technique used in the present study, which was similar to that used to study the biosynthetic pathway of an antibiotic (3), provided the initial evidence for the presence of an as yet unidentified metabolite in cultures of SRRC 2043 which was determined to be a precursor of AFB_2. The new compound, designated HOMST, was specifically converted to AFB_2 because [^{14}C]HOMST was converted to [^{14}C]AFB_2 without any production of AFB_1 or AFG_1.

HOMST was found to be very similar, chemically, to another aflatoxin pathway intermediate, OMST, as judged by mass spectrometry and UV absorbance spectra (8), as well as by the visual determination of color and fluorescence under UV illumination before and after treatment with $AlCl_3$ (5, 8, 14). HOMST was different from OMST, however, in that its molecular weight was 356, which is 18 units higher than that of OMST (molecular weight, 338). In addition, HOMST exhibited considerably different R_f values during TLC than did OMST (5) and had different extinction coefficient values (8). HOMST could be synthesized by the derivatization of OMST with TFA. The nature of the derivatization reaction (2) suggested that the AFB_2 precursor HOMST may have a chemical structure similar to that shown in Fig. 2 (compound 1). Hydration of the vinyl ether double bond between C_1 and C_2 of OMST (Fig. 2, compound 2) by the TFA treatment is consistent with the observed

mass difference of 18 between HOMST and OMST. A similar reaction has been demonstrated by a TFA derivatization reaction (2) that resulted in the hydration of the bisfuran of AFB_1 to give AFB_{2a}. Further extensive chemical characterization by optical rotatory dispersion, nuclear magnetic resonance, and infrared spectrometry will be required to determine the chemical structure and stereochemistry of HOMST.

The experimental results obtained in this investigation indicate that AFB_2 and AFB_1 and AFG_1 arise from separate branches (shown below) in the aflatoxin biosynthetic pathway, with a bifurcation in the pathway before the ST or OMST intermediates:

$$//\rightarrow ST \rightarrow OMST \rightarrow AFB_1/AFG_1$$
$$//\rightarrow HOMST \rightarrow AFB_2$$

The following lines of evidence developed during this study support this hypothesis. (i) HOMST, an OMST derivative, is converted to AFB_2 with no production of AFB_1 or AFG_1, whereas under identical conditions, OMST or ST is converted to AFB_1 and AFG_1 with no detectable AFB_2 synthesis; and (ii) no additive increases or decreases in AFB_1, AFG_1, or AFB_2 production were observed during the cofeeding of HOMST with either ST or OMST, indicating an absence of an interrelationship between HOMST and either of the other precursors (ST and OMST) in the AF biosynthetic scheme.

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