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Biosynthetic Relationship among Aflatoxins B_1, B_2, G_1, and G_2

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***Aspergillus parasiticus* NIAH-26, a UV-irradiated mutant of *A. parasiticus* SYS-4 (NRRL 2999), produces neither aflatoxins nor precursors. When sterigmatocystin (ST) or *O*-methylsterigmatocystin was fed to this mutant in YES medium, aflatoxins B_1 (AFB_1) and G_1 (AFG_1) were produced. When dihydrosterigmatocystin (DHST) or dihydro-*O*-methylsterigmatocystin was fed to this mold, aflatoxins B_2 (AFB_2) and G_2 (AFG_2) were produced. The reactions from ST to AFB_1 and DHST to AFB_2 were also observed in the cell-free system and were catalyzed stepwise by the methyltransferase and oxidoreductase enzymes. In the feeding experiments of strain NIAH-26, the convertibility from ST to AFB_1-AFG_1 was found to be remarkably suppressed by the coexistence of DHST in the medium, and the convertibility from DHST to AFB_2-AFG_2 was also suppressed by the presence of ST. When some other mutants which endogenously produce a small amount of aflatoxins (mainly AFB_1 and AFG_1) were cultured with DHST, the amounts of AFB_1 and AFG_1 produced were significantly decreased, whereas AFB_2 and AFG_2 were newly produced. In similar feeding experiments in which 27 kinds of mutants including these mutants were used, most of the mutants which were able to convert exogenous ST to AFB_1-AFG_1 were also found to convert exogenous DHST to AFB_2-AFG_2. These results suggest that the same enzymes may be involved in the both biosynthetic pathways from ST to AFB_1-AFG_1 and DHST to AFB_2-AFG_2. The reactions described herein were not observed when the molds had been cultured in the YEP medium.**

Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) are toxic secondary metabolities produced by certain strains of the common molds *Aspergillus flavus* and *A. parasiticus* (4, 22). The biosynthetic pathway of AFB_1 has been extensively studied (4, 5, 22, 24), and it has been suggested that AFB_1 is produced from sterigmatocystin (ST) and *O*-methylsterigmatocystin (OMST); also, some enzyme activities relating to several steps in the biosynthetic pathway have been reported (2, 4, 26–28). However, there are many conflicting hypotheses concerning the biosynthetic pathways of other aflatoxins (5, 6, 13–16, 20, 23), and the precise relationship between AFB_1 and other aflatoxins is still unclear. Maggon and Venkitasubramanian suggested that AFB_2, AFG_1, and AFG_2 may be metabolically related to AFB_1 by a direct interconversion process (23). In contrast, Dutton et al. suggested that AFB_1 and AFB_2 may arise independently via a branched pathway (13).

Dihydrosterigmatocystin (DHST) and dihydro-*O*-methylsterigmatocystin (DHOMST) are fungal secondary metabolites that have been isolated from *A. Versicolor* (Vuill.) Tiraboshi (10, 17) and *A. flavus* (10, 11), respectively. These compounds are dihydroderivatives of ST and OMST and seemed to be precursors of AFB_2, because AFB_2 is a dihydro derivative of AFB_1. In this study, the biosynthetic relationship among four kinds of aflatoxins (AFB_1, AFG_1, AFB_2 and AFG_2) has been examined by both feeding experiments and a cell-free system.

MATERIALS AND METHODS

Microorganisms. The strains used in this study were an aflatoxin-producing strain of *A. parasiticus* SYS-4 (NRRL 2999) and 27 UV-irradiated mutants of SYS-4 in which aflatoxin production was lost or was less than 10 ng of total aflatoxins per mg of mycelia (wet weight) (30). NIAH-204 is an averantin-accumulating mutant; NIAH-26 does not accumulate aflatoxins or precursors.

Standard samples of metabolites. The structures of the substances used in this study are shown in Fig. 1. ST was isolated from mycelia of *Aspergillus versicolor* and purified by recrystallization from acetone (18). OMST was prepared by methylation of ST with methyl iodide and sodium carbonate in acetone (19). DHST and DHOMST were prepared by hydrogenation of ST or OMST, respectively, with 5% palladium charcoal (12). The concentration of the precursors was determined based on UV absorption spectra by using molar absorption coefficients (10).

A standard kit (Makor) was used for the analysis of AFB_1, AFB_2, AFG_1, and AFG_2.

Feeding experiment. The tip culture method (30) was used for the feeding experiments. A spore suspension (5 μl) was inoculated into 250 μl of YES medium (2% yeast extract and 20% sucrose) containing various precursors of aflatoxin biosynthesis. When specified, YEP medium (containing 20% peptone instead of sucrose) was used. After 4 days of incubation, the culture media were separated from the mycelia by filtration and centrifugation, and the aflatoxins contained in the media and the precursors accumulated in the mycelia were examined by silica gel thin-layer chromatography (TLC) with a developing solution composed of chloroform–ethyl acetate–90% formic acid (6:3:1, vol/vol/vol). Aflatoxins were densitometrically quantified by TLC (30). The mycelial wet weight was always measured to confirm that the results obtained were not due to the growth inhibition of molds with added precursors.

Cell-free extract. Cell-free extracts were prepared by the method of Cleveland et al. (9), with some modifications. Mutant NIAH-26 was grown in surface culture on 2 ml of YES medium in glass tubes (1.7 cm in diameter) at 28°C for 4 days. The resultant mats of mycelia were collected, washed, and ground with sea sand in a mortar and pestle in the solution containing 0.1 M potassium phosphate buffer

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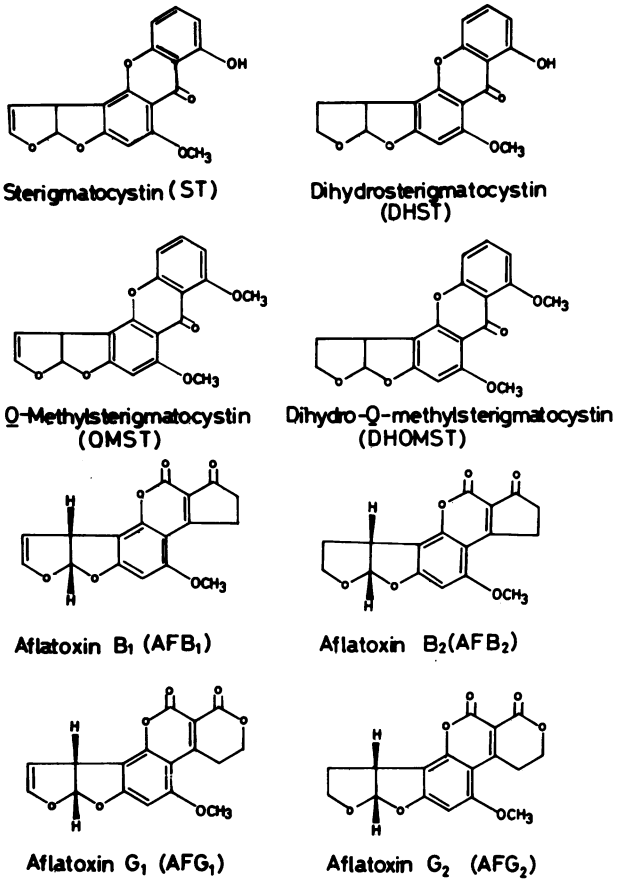

FIG. 1. Structures of the substances used in this study.

(pH 7.5) and 0.2 mg of phenylmethylsulfonyl fluoride (Nakarai Chemicals Ltd.) per ml. The homogenate was centrifuged at 900 × *g* for 2 min and then at 20,000 × *g* for 15 min. The 20,000 × *g* supernatant was used for enzyme assays. Protein concentration was determined by the method of Bradford (7).

Assay of enzyme activity. The cell-free extract (2 mg of protein per ml) was incubated in a reaction mixture containing 90 mM of phosphate buffer (pH 7.5), 10% glycerol, and either 98 μM ST, 98 μM DHST, 20 μM OMST, or 20 μM DHOMST. When specified, *S*-adenosylmethionine (SAM) (0.2 mg/ml, no. A-4377; Sigma Chemical Co.) or NADPH (2 mM; Sigma) was added to the reaction mixture. The total volume was 50 μl in a microtube (1.5 ml). After incubation at 37°C for 30 min, the reaction was terminated by adding 80 μl of water-saturated chloroform and mixing with a Vortex mixer. After centrifugation at 10,000 × *g* for 1 s, 50 μl of the lower layer (chloroform layer) was spotted on the silica gel plate with micropipettes, and the reaction products were analyzed by TLC as described above.

Analysis of reaction products. The reaction products (OMST, DHOMST, AFB_1, AFB_2, AFG_1, AFG_2) were identified by comparing the TLC chromatogram, fluorescence excitation spectra, and the effect of trifluoroacetic acid treatment with the corresponding data of the standard samples. Metabolites of both culture and cell-free systems were separated by silica gel TLC with two kinds of developing solutions, benzene-ethyl acetate (5:5, vol/vol) and chloroform–ethyl acetate–90% formic acid (6:3:1, vol/vol/vol). After development, the plates were inspected under long-wavelength (365 nm) UV light, and fluorescence photographs were taken (30). Although the standard samples and reaction products of OMST and DHOMST produced a pale blue fluoresence when the former developing solution was used, they appeared as yellow fluorescent spots when the latter solution was used. The resultant spots of OMST, DHOMST, AFB_1, and AFB_2 in the cell-free system were identified by measuring the fluorescence excitation spectra with a Shimadzu TLC densitometer CS-930 with fluorescence attachment and by comparing with the values obtained in the standard samples. The R_f values of AFB_1, AFG_1, and OMST on the TLC plate changed after treatment with trifluoroacetic acid (3), unlike that of the other compounds (AFB_2, AFG_2, and DHOMST). These sensitive tests were also used for identification of metabolites.

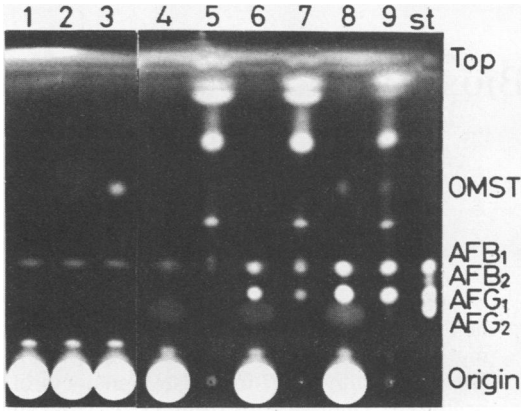

FIG. 2. Production of AFB_1 and AFG_1 from ST or OMST in feeding experiments. Mutant *A. parasiticus* NIAH-26, which does not accumulate aflatoxins and precursors, was cultured in three media: YES (lanes 1, 4, and 5), YES to which 16 μM ST had been added (lanes 2, 6, and 7), and YES to which 13 μM OMST had been added (lanes 3, 8, and 9). After 4 days of culture, 10 μl of each culture medium (lanes 4, 6, and 8) and 50 μl of the mycelial extract (lanes 5, 7, and 9) were analyzed by TLC with chloroform–ethyl acetate–90% formic acid (6:3:1, vol/vol/vol). Lanes 1 through 3 contained medium only. Fluorescence photographs are shown.

RESULTS

Production of aflatoxins in feeding experiments. When *A. parasiticus* NIAH-26 was cultured in the YES medium to which ST or OMST had been added, two fluorescent spots (one blue and one green) formed which had R_f values corresponding to those of authentic AFB_1 and AFG_1 (Fig. 2). Their chromatographic behavior after treatment with trifluoroacetic acid confirmed that these products were AFB_1 and AFG_1. It should be noted that no other spots were observed, suggesting that AFB_1 and AFG_1 are the final products. Growth inhibition by these substances was not detected (data not shown).

When either DHST or DHOMST, which are dihydro derivatives of ST or OMST, respectively, was added to the medium, two fluorescent spots (one blue and one green) were produced (Fig. 3), which were identified as AFB_2 and AFG_2.

These results indicate that both ST and OMST are precursors in the biosynthesis of AFB_1-AFG_1, and both DHST and DHOMST are precursors of AFB_2-AFG_2. No aflatoxins, however, could be produced when the molds were cultured in the YEP medium (data not shown).

Production of aflatoxins in the cell-free system. When the cell free extract of NIAH-26 mycelia which had been cul-

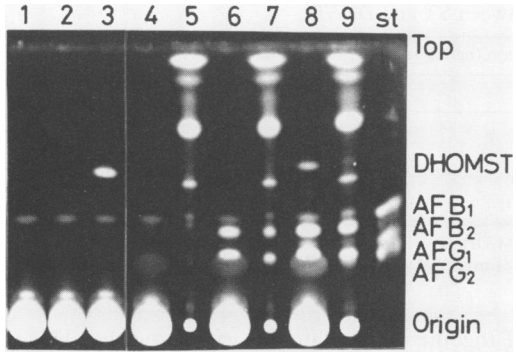

FIG. 3. Production of AFB_2 and AFG_2 from DHST or DHOMST in feeding experiments. *A. parasiticus* mutant NIAH-26 was cultured in three media: YES medium (lanes 1, 4, and 5), YES to which 16 μM DHST had been added (lanes 2, 6, and 7), and YES to which 16 μM DHOMST had been added (lanes 3, 8, and 9). After 4 days of culture, 10 μl of each culture medium (lanes 4, 6, and 8) and 50 μl of the mycelial extract (lanes 5, 7, and 9) were analyzed as described in the legend to Fig. 2. Lanes 1 through 3 contained medium only.

tured in YES was incubated with ST in the presence of SAM and NADPH, two new spots were observed (Fig. 4). An upper yellow fluorescent spot appeared only in the presence of SAM and was identified as OMST by comparison with the standard sample. Although the cell-free extract could produce a small amount of OMST without the addition of SAM, the addition of SAM to the reaction mixture always enhanced the production of OMST. The lower blue fluorescent spot which appeared in the presence of both SAM and NADPH was confirmed to be AFB_1 by treatment with trifluoroacetic acid.

Two spots were also observed when the cell-free extract was incubated with DHST in the presence of SAM and NADPH. The upper yellow fluorescent spot had an R_f value slightly lower from that of OMST and was identified as DHOMST by comparison with an authentic standard. The lower blue fluorescent spot was determined to be AFB_2.

Subsequently, a cell-free extract was incubated with OMST or DHOMST to investigate the relationship between OMST and AFB_1 and between DHOMST and AFB_2. AFB_1 and AFB_2 were produced from OMST and DHOMST, respectively (Fig. 4). Both reactions required only NADPH irrespective of the presence of SAM.

These results indicate that AFB_1 is produced from ST through the formation of OMST and that AFB_2 is produced from DHST through the formation of DHOMST. Moreover, based on the requirement for SAM or NADPH, it is assumed that the methyltransferase and oxidoreductase enzymes are involved in both reactions. All enzymatic reactions found here did not occur when the extracts were heat treated (data not shown).

The enzymatic reactions described above were not observed when the cell-free extract of the molds that had been cultured in the YEP medium was used (data not shown).

Relationship between the biosynthesis of AFB_1-AFG_1 and AFB_2-AFG_2. After *A. parasiticus* NIAH-26 was cultured with either or both ST and DHST, the amount of each aflatoxin produced was measured (Table 1). When only ST was added in the culture medium, approximately 10% of ST added was converted to AFB_1-AFG_1. When only DHST was added in the medium, about 40% of DHST added was converted to AFB_2-AFG_2. On the other hand, the production of AFB_1-AFG_1 from exogenous ST decreased to about 50% in the presence of DHST, whereas AFB_2-AFG_2 was

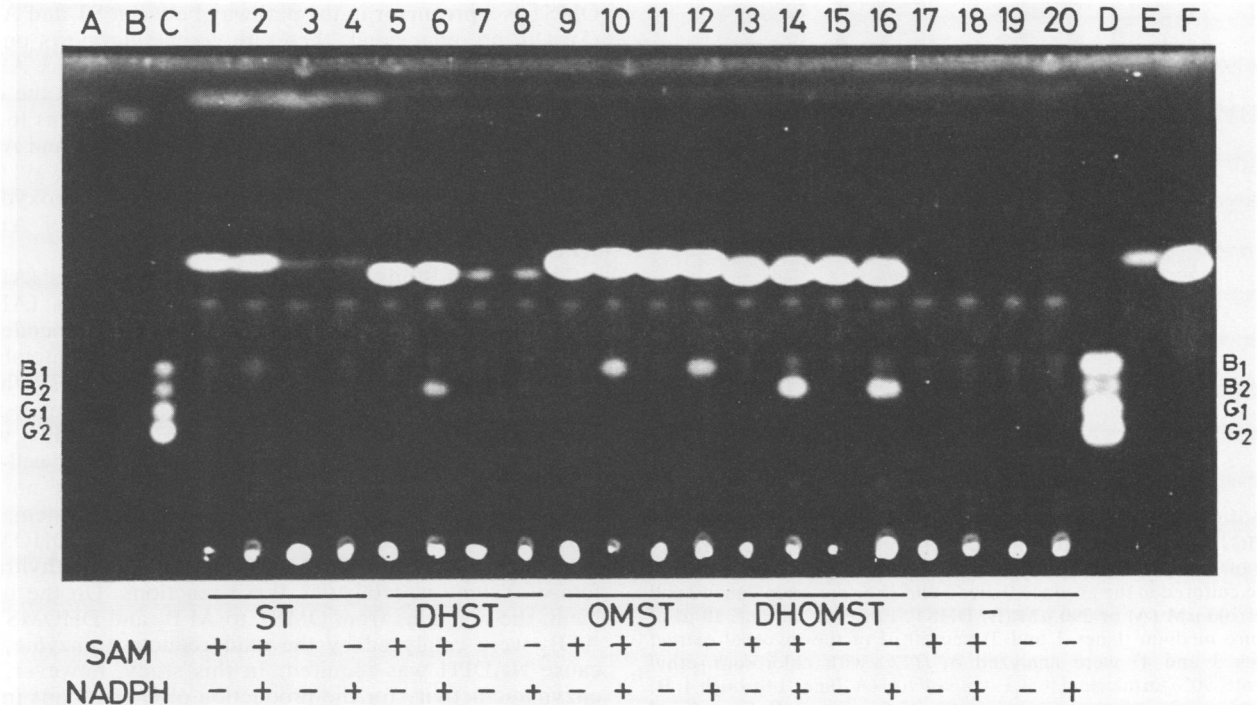

FIG. 4. Production of AFB_1 and AFG_1 in cell-free systems. Cell-free extract was incubated with ST (lanes 1 through 4), DHST (lanes 5 through 8), OMST (lanes 9 through 12), or DHOMST (lanes 13 through 16). SAM and NADPH were also added where specified. After incubation, the reaction products were analyzed by TLC, and fluorescence photographs were taken. Lanes 17 through 20 contained no substrate. Lanes A through F contained the following standard samples: A, ST; B, DHST; C and D, AFB_1, AFB_2, AFG_1, and AFG_2; E, OMST; F, DHOMST.

TABLE 1. Competition experiment between ST and DHST[a]

ST	DHST	Aflatoxin production (ng/mg [wet wt] of mycelia) (%)[b]			
		AFB_1	AFB_2	AFG_1	AFG_2
−	−				
+	−	7.5 ± 0.2 (100)		7.2 ± 0.1 (100)	
+	+	4.4 ± 0.03 (59)	14.4 ± 1.4 (47)	4.1 ± 0.6 (57)	8.5 ± 0.7 (31)
−	+		30.5 ± 2.9 (100)		27.6 ± 0.7 (100)

[a] *A. parasiticus* NIAH-26 was cultured as indicated with or without 32 μM ST or 32 μM DHST or both by the tip culture method.
[b] Values represent the means and differences of duplicate experiments. The mean value of mycerial wet weight was 19.1 mg per 250 μl of the culture medium.

newly produced. In a similar manner, the amount of AFB_2-AFG_2 produced from DHST was also decreased depending on the addition of ST.

To examine the site of competition of these conversion reactions, the mutants NIAH-25 and NIAH-204, which produce only a small amount of AFB_1 and AFG_1, were incubated with a high concentration of DHST. The endogenous formation of AFB_1-AFG_1 was found decreased, whereas AFB_2 and AFG_2 were newly produced (Fig. 5). This indicates that the competition occurred during the steps involved in the intracellular biosynthesis but not at the entry site(s) of exogenous precursors into the cells. The production of OMST was also decreased by the addition of DHST, which led to the production of DHOMST, although these mutants endogenously accumulated a slight amount of

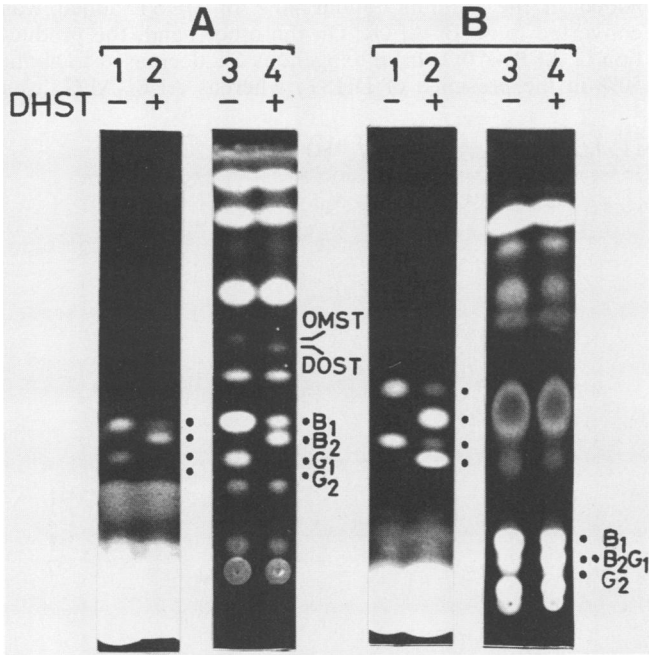

FIG. 5. Effect of exogenous DHST on the endogenous production of AFB_1 and AFG_1. Mutants NIAH-25 (A) and NIAH-204 (B) were cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 200 μM (A) or 290 μM (B) DHST. For each mutant, 10 μl of culture medium (lanes 1 and 2) and 50 μl of the mycelial extract (lanes 3 and 4) were analyzed by TLC with chloroform–ethyl acetate–90% formic acid (6:3:1, vol/vol/vol) (A, lanes 1 through 4; B, lanes 1 and 2) or benzene-ethyl acetate (5:5, vol/vol) (B, lanes 3 and 4). No significant difference in the mycelial wet weight was found between the paired experiments. Fluorescence photographs are shown. The four dots indicate the positions of standard samples of AFB_1, AFB_2, AFG_1, and AFG_2. The positions of standard samples OMST and DHOMST (shown as DOST) are also indicated.

OMST together with aflatoxins in the mycelia. This suggests that the same methyltransferase enzyme would catalize the methylation of both ST and DHST. Furthermore, the common enzyme(s), whose activity has not been detected, may catalyze the formation of both AFG_1 and AFG_2, because a reduction of the amount of G aflatoxins was observed as in the case of B aflatoxins in the competition experiments (Table 1, Fig. 5).

When the convertibilities from ST, OMST, DHST, or DHOMST to aflatoxins were examined in feeding experiments for 27 kinds of mutants including NIAH-25, NIAH-26, and NIAH-204, most of the mutants that were able to convert one of these precursors to aflatoxins could also convert all the other precursors to aflatoxins (Table 2), suggesting that not only methyltransferase but also oxidoreductase would be commonly used in the both pathways from ST to AFB_1-AFG_1 and from DHST to AFB_2-AFG_2.

DISCUSSION

With respect to the biosynthetic pathway of AFB_1, it has been reported that AFB_1 is formed from ST (4) and that OMST is a precursor in the pathway between ST and AFB_1 (5). On the other hand, there are conflicting views on the biosynthetic pathways of other aflatoxins such as AFB_2, AFG_1, and AFG_2. Several workers have reported the conversion of labeled AFB_1 into AFB_2 and G aflatoxins (6, 20, 23). However, Dutton et al. suggested that AFB_1 and AFB_2 could arise independently via a branched pathway (13). Elsworthy et al. (14) also have reported that 5-hydroxydihydrosterigmatocystin may be a direct precursor or AFB_2-AFG_2 based on tracer experiments.

The present study demonstrated that aflatoxins (AFB_1, AFG_1) containing dihydrobisfuran and aflatoxins (AFB_2, AFG_2) containing tetrahydrobisfuran were independently produced from ST and DHST, respectively. The metabolic scheme proposed for the late stages of aflatoxin biosynthesis is shown in Fig. 6. It should be noted that interconversions between AFB_1 and AFB_2 or between AFG_1 and AFG_2 were not observed in either the feeding experiments or cell-free system, indicating that these interconversions are not the main biosynthetic pathways. In the cell-free system, the reactions from ST to OMST and from DHST to DHOMST were dependent on SAM, indicating that the methyltransferase enzyme is catalyzing these reactions. On the other hand, the reactions from OMST to AFB_1 and DHOMST to AFB_2 were catalyzed by the oxidoreductase enzyme, because NADPH was required. In this study, however, the enzymatic activity for the production of G aflatoxins in the cell-free system could not be obtained.

Also, based on the similarity of the pathways from ST to AFB_1-AFG_1 and from DHST to AFB_2-AFG_2, both reactions seemed to be catalyzed by the same enzyme systems. In fact, the competition experiments showed that the enzy-

TABLE 2. Conversion to aflatoxins in feeding experiments[a]

Mutant[b]	Aflatoxin production[c]	ST → AFB_1-AFG_1[d]	OMST → AFB_1-AFG_1[e]	DHST → AFB_2-AFG_2[f]	DHOMST → AFB_2-AFG_2[g]
NIAH-1	−	−	−	−	−
NIAH-3	−	−	−	−	−
NIAH-4	−	−	−	−	−
NIAH-9	−	++	++	++	++
NIAH-10	−	−	−	−	−
NIAH-12	−	−	−	−	−
NIAH-13	−	−	−	−	−
NIAH-14	−	−	−	−	−
NIAH-23	−	−	−	−	−
NIAH-26	−	++	++	++	++
NIAH-31	−	−	−	−	−
NIAH-35	−	−	−	−	−
NIAH-37	−	−	−	−	−
NIAH-212	−	−	−	−	−
NIAH-215	−	−	−	−	−
NIAH-221	−	−	−	−	−
NIAH-222	−	−	−	−	−
NIAH-16	+	++	++	++	++
NIAH-22	±	−	−	−	−
NIAH-25	±	+	+	+	+
NIAH-201	±	−	++	+	++
NIAH-202	+	+	+	++	++
NIAH-203	+	−	++	++	++
NIAH-204	++	+	+	++	++
NIAH-205	±	−	−	−	+
NIAH-210	±	+	++	++	++
NIAH-213	±	+	+	+	+

[a] Results are qualitative as follows: − < ± < + < ++.
[b] Mutants were isolated by UV irradiation (30).
[c] Aflatoxin production of all mutants was less than 10 ng of total aflatoxins per mg of wet weight of mycelia. − < ± < + < ++.
[d] Mutants were incubated with and without 8 μM ST for 4 days. The difference in production of AFB_1-AFG_1 between the incubation with and without ST was visually estimated by TLC.
[e] OMST concentration was 141 μM.
[f] DHST concentration was 290 μM.
[g] DHOMST concentration was 140 μM.

matic reactions from exogenous ST to AFB_1-AFG_1 and from exogenous DHST to AFB_2-AFG_2 were mutually affected (Table 1). Interestingly, although *A. parasiticus* SYS-4 (29), a wild-type strain, naturally produces a large amount of AFB_1-AFG_1 and a very small amount of AFB_2-AFG_2, the mutant NIAH-26, which does not produce aflatoxins and was obtained from SYS-4, exhibited a remarkable convertibility from DHST to AFB_2-AFG_2.

The addition of exogenous DHST inhibited the endogenous production of AFB_1 and AFG_1 (Fig.5), indicating that such inhibition may occur during the step(s) involved in the intracellular biosynthesis. By using the leaky mutants capable of producing a small amount of aflatoxins for the competition experiments of aflatoxin production, a remarkable inhibition of AFB_1-AFG_1 production could be observed.

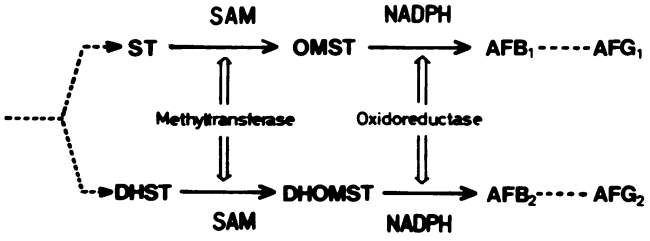

FIG. 6. Metabolic scheme for the late stages of aflatoxin biosynthesis. Solid lines indicate the reactions confirmed in this paper, and the dotted lines indicate hypothetical reactions.

Moreover, among the 27 kinds of mutants used in the present study, most of the mutants which were able to convert exogeneous ST to AFB_1-AFG_1 could also convert exogenous DHST to AFB_2-AFG2, and most of the mutants able to convert exogenous OMST to AFB_1-AFG_1 could also convert exogenous DHOMST to AFB_2-AFG_2.

The results described above strongly suggest that the same enzymes such as methyltransferase and oxidoreductase may be involved in both pathways from ST to AFB_1-AFG_1 and DHST to AFB_2-AFG_2. The determination of the ratio of AFB_1 and AFG_1 to AFB_2 and AFG_2 can be obtained by the determination of the ratio of ST of DHST, which may also be regulated in any of the step(s) preceding the formation of these compounds (Fig. 6). Although some molds are known to produce M aflatoxins (10, 21), the enzymes described herein may be related to the formation of these aflatoxins. In fact, aspertoxin (3-hydroxy-6,7-dimethoxydifuroxanthone), which is structurally comparable to OMST or DHOMST and seems to be a precursor of aflatoxins M_1 and GM_1, has been isolated from *A. flavus* (25).

In this study, the reactions of ST → OMST → AFB_1-AFG_1 and DHST → DHOMST → AFB_2-AFG_2 were not detected in either the feeding or cell-free experiments when the YEP culture medium was used. This finding suggests that the enzymes which catalyze these late stages of the biosynthetic pathways of aflatoxins (at least methyltransferase and oxidoreductase) are not induced by the carbon sources that are not related to the formation of aflatoxins (1).

After submission of this manuscript, Cleveland et al. (8)

showed that AFB_2 arises from a new metabolite, designated HOMST and that AFB_1 and AFG_1 arise from ST and OMST; they also presented evidence for the absence of a relationship between HOMST and either of the other precursors (ST and OMST). The biosynthetic relationship among HOMST and DHST and DHOMST, which latter two substances have been demonstrated to be precursors of AFB_2-AFG_2 in this paper, remains unclear.

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