ISOLATION OF AVERUFIN FROM A MUTANT OF ASPERGILLUS PARASITICUS IMPAIRED IN AFLATOXIN BIOSYNTHESIS

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SUMMARY: A mutant of Aspergillus parasiticus impaired in aflatoxin biosynthesis was shown to accumulate large amounts of the polyhydroxyanthraquinone averufin ($C_{20}H_{16}O_7$). The possible relationship between averufin and aflatoxin biosynthesis is discussed.

The aflatoxins (1) are a group of related secondary metabolites produced by certain members of the Aspergillus flavus group and some other fungi. These compounds are toxic as well as carcinogenic and have been implicated in animal (2) and human (3,4) food poisonings. Studies on the biosynthesis of aflatoxin B_1, the major metabolite, have shown that the basic skeleton of the molecule is derived from acetate units and that methionine contributes the methoxy-methyl group (5, Figure 1a). A possible scheme consistent with these findings has been proposed for the conversion of a hypothetical C_{18}-polyketide derived polyhydroxynaphthacene via a bisfuranoanthraquinone into the aflatoxins (5).

In order to investigate further the biochemical events prior to the final elaboration of the aflatoxins, a search was conducted for mutants impaired in aflatoxin biosynthesis. We now wish to report the production of

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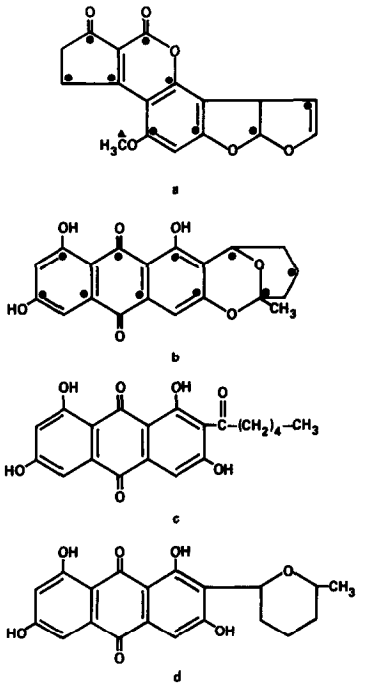

Fig. 1a. Structure and labeling pattern of aflatoxin B_1 with $CH_3\overset{\bullet}{C}OOH$ and $\overset{\blacktriangle}{C}H_3S(CH_2)_2CH(NH_2)COOH$ as precursors.

b. Structure and hypothetical labeling pattern of averufin ($C_{20}H_{16}O_7$) with $CH_3\overset{\bullet}{C}OOH$ as precursor.

c. Structure of norsolorinic acid ($C_{20}H_{18}O_7$).

d. Structure of a minor anthraquinoid pigment ($C_{20}H_{18}O_7$) from A. flavus.

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major amounts of a pigment by one of the mutants isolated. Analysis of this pigment and its triacetate identify it as averufin, a compound that has been previously detected as a minor metabolite in A. versicolor (6-8). Inspection of its structure (Figure 1b) and experimental evidence suggest that it is derived from a C_{20}-polyketide intermediate.

Mutations were induced by treatment of conidia of the parent strain, A. parasiticus ATCC 15517, with N-methyl-N'-nitro-N-nitrosoguanidine (9). After segregation and phenotypic expression, single colony isolates were tested for aflatoxin production on a yeast extract-sucrose medium in surface culture (10). One mutant, W49, was found in which production of aflatoxins

was reduced to less than 2% of that of the parent, as measured by the absorbance of chloroform extracts of the broth at 362 nm. In addition, the reverse of the mycelial mat became orange pigmented. Thin-layer chromatography of acetone extracts of the mycelium on silicagel (Eastman Chromagram 6060) with chloroform-acetone-acetic acid 97:2:1 as solvent showed one major compound with an R_f of 0.5.

For the structure determination, two mycelial mats grown on 100 ml yeast extract-sucrose medium at 30°C for 8 days were exhaustively extracted with acetone and the filtered and concentrated extract subjected to silicagel column chromatography with chloroform as eluant. The pigment fraction was dried and triturated with petroleum ether until the supernatant became colorless. The light orange residue was recrystallized from acetone to give 120 mg of averufin with a melting point of 283-289°C (dec.); a mass spectrum (70 eV) with peaks at m/e (rel. intensity) 368 (82, $M^+ = C_{20}H_{16}O_7$), 350 (23, M - H_2O), 325 (87, M - CH_3CO), 311 (37, M - CH_3COCH_2), 310 (100, M - CH_3COCH_3) and 297 (59, M - $CH_3COCH_2CH_2$); λ_{max} (in ethanol) 223, 256 (sh), 265, 286 (sh), 294, 319 and 453 nm ($10^{-3}\varepsilon$ respectively 33, 16.5, 18.5, 24.8, 30.8, 12.5 and 10.5); ν_{max} (in "Nujol") 3400, 1677, 1622, 1569, 1270, 1210 and 1025 cm^{-1}. For confirmation, the tri-O-acetyl derivative was prepared (6) and recrystallized twice from ethanol to give yellow needles (73% overall yield) with a melting point of 207-211°C; an $[\alpha]_D^{22}$ of -15° (5 g/l in $CHCl_3$); a mass spectrum (70 eV) with peaks at m/e (rel. intensity) 494 (14, $M^+ = C_{26}H_{22}O_{10}$), 451 (69), 410 (100), 368 (50), 352 (84), 340 (26), 325 (61), 310 (75) and 297 (45); λ_{max} (in ethanol) 244, 248, 281, 282, 335 and 370 nm ($10^{-3}\varepsilon$ respectively 18.3, 18.3, 45, 45, 4.9 and 4.25); ν_{max} (in $CHCl_3$) 1778, 1768, 1678 and 1666 cm^{-1}; τ (in $CDCl_3$) 8.6-7.8 (unresolved m, 9H including a sharp peak at 8.40 (s, 3H)), 7.63 (s, 3H), 7.52 (s, 6H), 4.77 (broad t, 1H), 2.75 (d, 1H, J = 2.5 Hz), 2.37 (s, 1H) and 2.03 (d, 1H, J = 2.5 Hz); elemental analysis C, 63.22, H, 4.77% ($C_{26}H_{22}O_{10}$ requires C, 63.16; H, 4.48%). It was estimated from the absorbance (453 nm) of the acetone extract that averufin

comprised about 7% of the W49 mycelium (on a dry weight basis), compared to 0.01% in A. versicolor (6).

The most striking observation with respect to aflatoxin biogenesis is that averufin appears to be derived from a C_{20}-polyketide. This is supported by the finding that acetate is incorporated efficiently into averufin (D.P.H. Hsieh, personal communication). Recently (11), another mutant of A. parasiticus producing about 20% as much aflatoxin as the parent has been reported to produce norsolorinic acid ($C_{20}H_{18}O_7$, Figure 1c), a metabolite that is structurally related to averufin. The formation of these compounds might suggest a C_{20} rather than a C_{18} intermediate in aflatoxin biosynthesis. Another possibility is that since one of the steps in aflatoxin synthesis has been blocked in the averufin-producing mutant, an additional acetyl group is added before the polyketide is released from the multi-enzyme complex. Finally, the possibility that averufin arises by a completely independent pathway which is greatly enhanced in the mutant relative to the aflatoxin pathway should be considered. In this respect the finding of minor amounts of the $C_{20}H_{18}O_7$ tetrahydroxyanthraquinone (Figure 1d) in the mycelium of a wild-type strain of A. flavus should be mentioned (12). The original averufin-producing strain of A. versicolor has also been mutated to produce this metabolite (7). Experiments are presently conducted to differentiate among these three alternatives.

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