Aflatoxin G_1 Metabolism by *Rhizopus* Species

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Rhizopus stolonifera, *R. arrhyzus*, *R. oryzae*, and *R. sp.* degraded aflatoxin G_1. An intermediate in the biological degradation was isolated and identified as a previously reported metabolite of *A. flavus* (aflatoxin B_3) and *A. parasiticus* (parasiticol). Radioisotope data conclusively demonstrated that this metabolite was derived from aflatoxin G_1 degradation by the *Rhizopus spp.*

Biological degradation of the aflaxtoxins has aroused considerable interest because of implications in detoxification of feeds and foodstuffs. A large number of microorganisms have been screened for biological degradation of the aflatoxins. Aflatoxin B_1 was degraded by *Tetrahymena pyriformis* W (Teunisson and Robertson, 1967) and a steroid-hydroxylating microorganism, *Dactylium dendroides* (Detroy and Hesseltine, 1968). The latter investigators identified a metabolite of aflatoxin B_1 degradation as a reduced form of the toxin that was $^1/_{18}$ as toxic as aflatoxin B_1.

During routine analyses for aflatoxins B and G in peanut samples (in our laboratory), one sample was found to contain a fluorescent metabolite (AF-1) that had an R_f on tlc slightly lower than aflatoxin G_2 (R_f 0.19 compared to 0.25 for aflatoxin G_2). Two species of *Aspergillus* were among the fungi isolated from these peanut samples. These two species were cultured on shredded wheat and replicates were extracted with $CHCl_3$ at intervals of 4 days up to 28 days. Small amounts of AF-1 were detected in the mold cultures 3 weeks after inoculation. The compound was present in higher concentration in a culture contaminated with a species of *Rhizopus*. The *Rhizopus sp.* was isolated and subsequently cultured in aflatoxin-supplemented and non-aflatoxin-supplemented media. AF-1 was produced in older cultures of the *Aspergillus sp.* and also from aflatoxin G_1 metabolism by the *Rhizopus sp.*

Rhizopus species are commonly found associated with Georgia peanuts, particularly *R. stolonifera*, *R. arrhyzus*, and *R. oryzae* (Jackson and Bell, 1969). This paper reports our investigation of the metabolism of aflatoxin G_1 (I) by the *Rhizopus sp.* isolated in our laboratory and three known *Rhizopus* species and the isolation and identification of the metabolite AF-1.

EXPERIMENTAL

Rhizopus Culture. The *Rhizopus sp.* isolated in our laboratory, *Rhizopus stolonifera* (NRRL 1477), *Rhizopus arrhyzus* (NRRL 2582), and *Rhizopus oryzae* (NRRL 395) were cultured in 500-ml Erlenmeyer flasks containing 25 g of shredded wheat coated with approximately 10 mg of aflatoxin G_1 plus 60 ml of Difco potato dextrose (PDA) broth (pH 5.1). Purified aflatoxin G_1 was coated onto the dry shredded wheat from chloroform solution. All traces of the chloroform were removed prior to the addition of the PDA broth. Uninoculated flasks containing aflatoxin G_1 and *Rhizopus* cultures not supplemented with aflatoxin G_1 but otherwise treated the same were used as controls. The mold cultures were incubated at 27° C for 3–7 weeks prior to extraction with chloroform.

^{14}C-Labeled aflatoxin G_1 (sp. act. 100 cpm/μg) was supplemented to some *Rhizopus* cultures to conclusively establish that AF-1 was derived from the degradation of aflatoxin G_1.

Isolation of Metabolite. After incubation, *Rhizopus* cultures were extracted three times with chloroform in a Waring Blendor. The extracts were filtered through anhydrous sodium sulfate and concentrated under vacuum. The chloroform extract was chromatographed on a column (15 cm × 2 cm i.d.) containing Merck silica gel (0.05–0.20 mm) (Brinkmann Instruments, Inc., Westbury, N.Y.). AF-1 was eluted from the column with anhydrous ethyl ether, while the aflatoxin G_1 remained on the column. Aflatoxin G_1 was subsequently eluted from the column with chloroform. The fractions containing AF-1 (monitored by tlc) were combined and concentrated under vacuum. The compound was further purified by chromatography on a silica gel column (70 × 2 cm i.d.) with 10% *n*-hexane in chloroform as the eluting solvent. The fractions containing AF-1 were concentrated under vacuum and crystallized from chloroform/*n*-hexane solution to produce colorless crystals with a melting point of 239–241° C.

Apparatus. Infrared spectra were obtained with a Model 257 Perkin-Elmer infrared spectrophotometer equipped with a 6X Beam condenser. The sample was coated onto KBr blocks as a thin film.

Ultraviolet spectra were obtained with a Beckman Model DBG recording spectrophotometer. Low-resolution mass spectra were obtained with an LKB 9000 GC-mass spectrometer. High-resolution mass spectral analyses were made with an MS-9 (A.E.I. Inst. Co., Manchester, England) by the Department of Chemistry, Florida State University, Tallahassee, Fla. Samples were introduced into the mass spectrometer by the direct-probe method, and ionization was effected by electron-impact at 70 eV.

Melting points were determined with a Kofler micro-melting point apparatus.

Thin-layer chromatography (tlc) was done on glass plates (20 × 20 cm or 20 × 10 cm) coated with 0.25 mm layers of Silica Gel GHR. The developing solvent was chloroform-acetone, 93:7 v/v.

Hydrogenations were performed in an Ogg-Cooper micro-hydrogenation apparatus (A. H. Thomas Co.) with 5% palladium on carbon in ethyl acetate at room temperature and

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atmospheric pressure. The acetate derivative was prepared with acetic anhydride and pyridine at 70° C under a nitrogen atmosphere.

^{14}C-Labeled aflatoxin was prepared by incubation of *Aspergillus parasiticus* in D-glucose-UL-^{14}C followed by purification (Robertson *et al.*, 1967) to yield crystalline ^{14}C-labeled aflatoxin G_1. Radiological purity was monitored by the distribution of radiation on tlc and recrystallization to a constant sp. act. Radioactivity was measured in a Packard Tri-carb liquid scintillation spectrometer.

Nuclear magnetic resonance spectra (nmr) were obtained with a Varian A-60A. The spectrum of AF-1 was taken in deuteropyridine. Spectra of AF-1 acetate derivative were obtained in deuteropyridine and deuterochloroform. All nmr spectra were obtained at ambient temperature.

RESULTS AND DISCUSSION

AF-1 appeared in extracts from cultures of the three known *Rhizopus* species and the *Rhizopus sp.* isolate (identified as intermediate between *Rhizopus arrhyzus* Fischer group and *Rhizopus microsporus* van Teigh group) when they were cultured in media supplemented with aflatoxin G_1 (I). AF-1 was not present in extracts from *Rhizopus* cultures not supplemented with aflatoxin G_1, or in cultures supplemented with aflatoxin G_1 but not inoculated with *Rhizopus sp.* *R. oryzae* metabolized aflatoxin G_1 and accumulated AF-1 more rapidly than the other species studied. AF-1 was detected in cultures of *R. oryzae* 1 week after inoculation and biodegradation was complete approximately 4 weeks after inoculation. Degradation of aflatoxin G_1 and accumulation of AF-1 was most pronounced between 2 and 3 weeks after inoculation with *R. oryzae*.

It was conclusively demonstrated that AF-1 was derived from the degradation of aflatoxin G_1 with the use of highly purified ^{14}C-labeled aflatoxin G_1, since 45% of the radioactivity in extracts of *R. oryzae* cultures incubated with ^{14}C-labeled aflatoxin G_1 was associated with AF-1.

AF-1 was not detected in cultures supplemented with the other three aflatoxins (B_1, B_2, or G_2). However, a compound with an R_f on tlc slightly lower than AF-1 was detected in cultures supplemented with aflatoxin G_2. This was presumed to be the dihydro derivative of AF-1, since its R_f on tlc was identical to the synthetic dihydro derivative of AF-1.

Identification of AF-1. The uv spectrum of AF-1, λ_{max}^{MeOH} 330, 264, and 256 nm indicated that part of the uv chromophore of G_1 was altered. This uv spectrum was similar to that of tetrahydrodesoxoaflatoxin B_1 (Asao *et al.*, 1965), which has λ_{max}^{MeOH} at 332, 264, and 255 nm and indicates that the dilactone system of aflatoxin G_1 (I) was absent in AF-1. This was further supported by the infrared spectrum of AF-1, which lacked absorption at 1760 and 1695 cm^{-1} but did exhibit absorption at 1735 cm^{-1} for the monolactone. The ir spectrum of AF-1 exhibited absorption at 3520 cm^{-1}, indicating that the compound contained an OH group. This was further substantiated when the monoester of AF-1 was prepared (*m/e* 344, R_f 0.71, uv λ_{max}^{MeOH} 330, 263, 256 nm, m.p. 190–192° C).

High-resolution mass spectroscopy of AF-1 showed *m/e* 302.0780 with an empirical formula of $C_{16}H_{14}O_6$. The presence of a fragment ion peak (molecular ion minus 18) at *m/e* 284 supported the presence of an OH group.

Nmr analysis showed that the dihydro difurano portion of G_1 (I) was present in AF-1 (triplet, 1 proton, δ6.54, J = 2.5 Hz; triplet, 1 proton, δ5.55, J = 2.5 Hz; doublet, 1 proton, δ6.86, J = 7 Hz; doublet of triplets, 1 proton, δ4.86, J = 2.5 and 7 Hz). Further evidence for the presence of the dihydro difurano portion was provided when the tetrahydro difurano derivative was prepared (*m/e* 304, R_f 0.15, m.p. 220–221° C, uv λ_{max}^{MeOH} 330, 264, and 255 nm). Nmr also showed that the aromatic proton (singlet, δ6.50) and the OCH_3 (singlet, δ3.92) were present. Chemical shifts that differed from aflatoxin G_1 were singlet, δ6.08, 1 proton, and a coupled pair of protons, triplet, δ3.25, J = 6 Hz and triplet, δ4.40, J = 6 Hz.

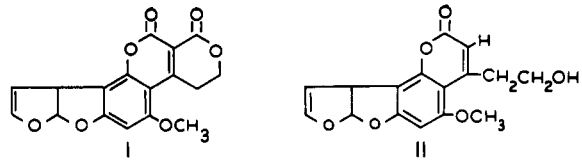

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Figure 1. Structures of aflatoxin G_1 and aflatoxin B_3 (parasiticol)

These data agree with those reported for aflatoxin B_3 (II) from *A. flavus* (Heathcote and Dutton, 1969) and more recently with parasiticol (II) from *A. parasiticus* (Stubblefield *et al.*, 1970). Heathcote and Dutton (1969) suggest that this compound may be the first step in the biological degradation of aflatoxin G_1. Stubblefield *et al.* (1970) speculate that parasiticol may be formed in lieu of aflatoxin formation from the same precursor. The *Rhizopus spp.* studied do not biosynthesize the aflatoxins and it has been conclusively demonstrated that AF-1 is the result of biological degradation of aflatoxin G_1 by these fungi. Therefore, our data strongly support the hypothesis presented by Heathcote and Dutton (1969) that AF-1 (aflatoxin B_3 or parasiticol) is an intermediate in the biological degradation of aflatoxin G_1 in *A. flavus* or *A. parasiticus*. However, this compound cannot be discounted as a possible precursor to aflatoxin biosynthesis, since many biosynthetic and biodegradative pathways are merely reverse processes affected by the enzyme kinetics.

AF-1 did not accumulate in significant amounts in either the *A. parasiticus* or *Rhizopus spp.* cultures until considerable time had elapsed and vegetative growth had apparently slowed or ceased. This indicated that the compound was formed as a result of changes in the culture medium, which were, in turn, a result of fungal growth or the action of enzymes produced during later periods of the growth cycle. This further supports the hypothesis that AF-1 results from degradation of aflatoxin G_1 in *A. parasiticus* and *A. flavus*.

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