Conversion of Aflatoxin B_1 to Isomeric Hydroxy Compounds by *Rhizopus* spp.

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An investigation of aflatoxin B_1 degradation by a *Rhizopus arrhizus* isolate from Georgia peanuts and three known *Rhizopus* species revealed that two fluorescent metabolites of aflatoxin B_1 accumulated during degradation. They were identified by physical, chemical, and spectroscopic data as hydroxylated stereo isomers derived from reduction of the ketone function on the cyclopentane ring of aflatoxin B_1. It was conclusively shown with ^{14}C-labeled aflatoxin B_1 that these metabolites were derived from aflatoxin B_1. Two additional fluorescent metabolites appeared during purification of the hydroxy isomers. These were identified as ethyl ether derivatives of the hydroxylated compounds and apparently were formed spontaneously from either one or both hydroxy isomers.

Many microorganisms have been screened for biological degradation of the aflatoxins. Teunisson and Robertson (1967) and Robertson *et al.* (1970) reported that the ketone carbonyl on the cyclopentane ring of aflatoxin B_1 was biologically reduced by *Tetrahymena pyriformis* W. Detroy and Hesseltine (1968, 1970) reported that the same biologically reduced metabolite was produced from aflatoxin B_1 by *Dactylium dendroides*. They also detected another blue fluorescent metabolite produced by *D. dendroides*, but no chemical structure was proposed.

A previous study (Cole and Kirksey, 1971) reported that aflatoxin G_1 was degraded by *Rhizopus* spp. The present paper reports the degradation of aflatoxin B_1 by *R. arrhizus* isolated from Georgia peanuts, and by three other *Rhizopus* species, *R. stolonifer* (NRRL 1477), *R. arrhizus* (NRRL 2582), and *R. oryzae* (NRRL 395).

EXPERIMENTAL SECTION

Rhizopus Culture. *Rhizopus* spp. were cultured in 500-ml Erlenmeyer flasks containing 25 g of crushed shredded wheat coated with 10 mg of purified aflatoxin B_1 plus 60 ml of Difco potato dextrose broth (pH 5.1). Some *Rhizopus* cultures were supplemented with ^{14}C-labeled aflatoxin B_1. The mold cultures were incubated at 27° for up to 10 days prior to extraction.

Isolation of B_1 Metabolites. *Rhizopus* cultures were initially extracted with hot chloroform at 24-hr intervals from 1 to 10 days. Subsequent to this experiment all cultures were extracted after 7 days' growth. Extracts were filtered through anhydrous sodium sulfate and concentrated under vacuum. The crude extracts analyzed on thin-layer chromatography (tlc) contained blue fluorescent spots at R_f 0.30 (I) and 0.26 (II), as compared to aflatoxin B_1 with R_f 0.33 (Figure 1).

Initial attempts to separate the metabolites from aflatoxin B_1 utilized Merck silica gel (0.05–0.20 mm) (Brinkmann Instruments, Inc., Westbury, N.Y.) column chromatography (70 × 2.2 cm o.d.) with 10% *n*-hexane in chloroform as the eluting solvent. The two B_1 metabolites were unstable under these chromatographic conditions, since they decreased in concentration with the concomitant appearance of two new nonpolar blue fluorescent compounds at R_f 0.81 (III) and 0.73 (IV) (Figure 1). A second silica gel column chromatographic system packed in a slurry of toluene and eluted with a linear gradient from toluene to ethyl acetate was found to be more satisfactory. This chromatographic system completely separated I and II and III and IV from aflatoxin B_1 without loss of I and II. Compound I was separated from II by repetitive column chromatography using this same chromatographic system. Compound III was separated from IV by repetitive silica gel column chromatography using a linear gradient elution from toluene to toluene–ethyl acetate, 1:1 v/v. Compounds I and II were crystallized from benzene–*n*-hexane solution to yield colorless crystals. Compound I melted (dec.) at 224–226°; II decomposed over a broad range, starting at 233°. Compounds III and IV were crystallized from ethanol solution to yield colorless crystals with melting points of 198–200 and 194–196°, respectively.

Apparatus. Ultraviolet spectra (uv) were obtained with a Beckman Model DBG recording spectrophotometer. Infrared spectra (ir) were obtained with a Model 257 Perkin-Elmer infrared spectrophotometer equipped with a 4X beam condenser. Samples were coated onto KBr blocks as thin films. High-resolution (hrp) and low-resolution mass spectral analyses (lrp) were made with an MS-9 (A.E.I. Instrument Co., Manchester, England) mass spectrometer. 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. Nuclear magnetic resonance spectra (nmr) were obtained with a Jeolco PS-100 nmr spectrometer (100 MHz) and a Minimar 60 nmr spectrometer (60 MHz). Spectra of I and II were analyzed in dimethylsulfoxide-d_6, while spectra of III and IV were analyzed in deuteriochloroform. All nmr spectra were obtained at room temperature.

^{14}C-Labeled aflatoxin B_1 was prepared by incubating an aflatoxin B_1-producing strain of *Aspergillus flavus* in D-glucose-UL-^{14}C followed by purification (Cole and Kirksey, 1971) to yield crystalline ^{14}C-labeled aflatoxin B_1. Radiological purity was monitored by the distribution of radiation on tlc and recrystallization to a constant specific activity. The specific activity of the ^{14}C-labeled aflatoxin B_1 was adjusted to 150 cpm/μg prior to addition to fungal cultures. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Tlc was done on glass plates (20 × 20 cm or 20 × 10 cm) coated with 0.25-mm layers of silica gel G-HR. The developing solvent was chloroform–acetone, 93:7 v/v. Spots were visualized under long- and shortwave uv light.

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

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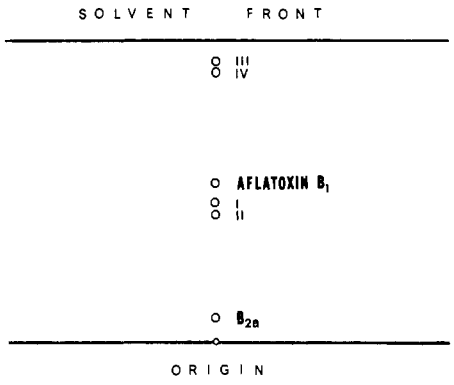


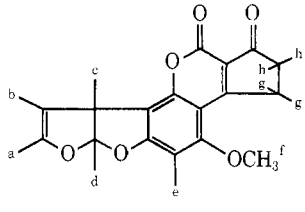
Figure 1. Thin-layer chromatogram of aflatoxin B_1 and related compounds

RESULTS AND DISCUSSION

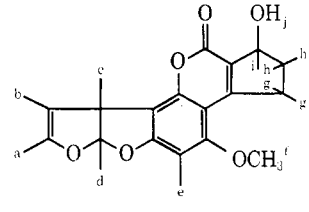
Aflatoxin B_1 metabolites I and II appeared in extracts from cultures of all *Rhizopus* fungi. Compound I appeared in *Rhizopus* extracts after the first day of incubation with aflatoxin B_1, while II did not appear until 3 days after inoculation.

It was conclusively established with radioisotope experiments that I and II were derived from supplemented ^{14}C-labeled aflatoxin B_1, since 31% of the relative radioactivity recovered from tlc plates was associated with I and 27% of the relative radioactivity was associated with II. This demonstrated that 42% of the relative radioactivity remained as unaltered aflatoxin B_1 after 1 week of incubation.

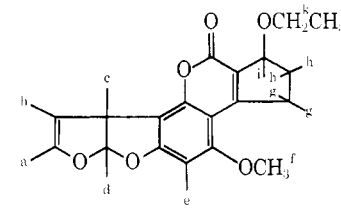
Identification of B_1 Metabolites. The uv spectra of all four compounds were nearly identical: I, λ_{max}^{EtOH} 331, 261, and 255 nm (ϵ_{max} 13,780, 10,000, and 8,790); II, λ_{max}^{EtOH} 332, 261, and 255 nm (ϵ_{max} 25,200, 19,000, and 17,000); III, λ_{max}^{EtOH} 332, 261, and 255 nm (ϵ_{max} 14,200, 9660, and 8830); IV, λ_{max}^{EtOH} 331, 261, and 255 nm (ϵ_{max} 15,750, 12,280, and 11,170). These spectra were similar in shape to the uv spectra of two previously reported compounds, parasiticol or aflatoxin B_3 (λ_{max}^{EtOH} 332, 264, and 255 nm) (Cole and Kirksey, 1971; Detroy and Hesseltine, 1968, 1970; Heathcote and Dutton, 1969) and tetrahydroxyaflatoxin B_1 (λ_{max}^{MeOH} 332, 264, and 255 nm) (Asao *et al.*, 1965). This demonstrated that the ketone carbonyl in the cyclopentane ring of aflatoxin B_1 was not present as part of the uv chromophore in the four B_1-derived compounds. This was supported by the ir spectra of all four compounds, which lacked absorption at 1760 and 1685 cm^{-1}, but exhibited absorption at 1735 cm^{-1} (internal ester not conjugated with ketone). The ir spectra of compounds I and II exhibited absorption at 3560 cm^{-1}, suggesting a hydroxyl group. Additional evidence for a hydroxyl group was provided when I and II acetates were prepared with acetic anhydride and pyridine. Also, the base peak in lrp and hrp mass spectral analyses for both compounds was at *m/e* 296 (molecular ion minus 18), which supported the presence of an OH group in I and II. The lrp and hrp mass spectral analyses of I and II were identical and demonstrated molecular ion peaks at *m/e* 314 and molecular formulas of $C_{17}H_{14}O_6$ for both compounds. The fragmentation patterns of I and II were identical and both readily lost H from the molecular ion. Mass spectral analyses of III and IV showed both metabolites contained molecular ion peaks at *m/e* 342 and both had molecular formulas of $C_{19}H_{18}O_6$. The fragmentation patterns of III and IV were identical. Major fragments were *m/e* 313 (loss of C_2H_5 from molecular ion), *m/e* 296 (loss of OC_2H_5 from molecular ion), *m/e* 269 (loss of CO_2 from *m/e* 313), and *m/e* 253 (loss of CO from *m/e* 313). Major fragments that were common to all four compounds were *m/e* 313, 296, 269 and 253. Fragmentation patterns of III and IV were identical to I and II after loss of C_2H_5 and OC_2H_5, which suggested that III and IV were the ethyl ether derivatives of I and II. This was confirmed when the nmr spectra of III and IV showed the typical chemical shifts and splitting patterns for CH_3CH_2O (3-proton triplet at δ 1.22 (J = 7 Hz) coupled with a 2-proton quartet at δ 3.64 (J = 7 Hz) (Figure 2). A comparison of the nmr spectrum of aflatoxin B_1 with all four metabolites in conjunction with all other data established that the differences were in the cyclopentane ring system. Compounds I and II contained an OH



Aflatoxin B_1



Compounds I and II



Compounds III and IV

Chemical shifts, δ

	Ha[3]		Hb[3]		Hc[5]		Hd[2]		He[1]		Hf[1]	
	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz
Aflatoxin B_1	6.54	2.5	5.52	2.5	4.82	2.5, 7.0	6.86	7.0	6.50		4.00	
Compound I	6.67	2.5	5.35	2.5	4.70	2.5, 7.0	6.85	7.0	6.57		3.79	
Compound II	6.66	2.5	5.33	2.5	4.68	2.5, 7.0	6.85	7.0	6.54		3.77	
Compound III	6.50	2.5	5.50	2.5	4.82	2.5, 7.0	6.82	7.0	6.40		3.91	
Compound IV	6.48	2.5	5.52	2.5	4.79	2.5, 7.0	6.79	7.0	6.38		3.87	

	Hg[5]		Hh[5]		Hi[5]		Hj[1]		Hk[4]		Hl[3]	
	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz	δ, ppm	J = Hz
Aflatoxin B_1	3.45	*	2.68	*								
Compound I	3.10	*	2.48	*	4.93	*	4.95					
Compound II	3.10	*	2.47	*	4.95	*	4.96					
Compound III	3.35	*	2.20	*	4.96	*			3.65	7.0	1.24	7.0
Compound IV	3.33	*	2.16	*	4.94	*			3.64	7.0	1.21	7.0

1 = singlet; 2 = doublet; 3 = triplet; 4 = quartet; 5 = multiplet; * = complex

Figure 2. Nuclear magnetic resonance data and structures of aflatoxin B_1 and related compounds

Table I. Molecular Weights of Various Aflatoxin B_1-Derived Compounds

Compound	Mol wt
Aflatoxin B_{2a}	330
O-Ethyl B_{2a}	358
Hydroxycyclopentane B_{2a}	332
Hydroxycyclopentane-*O*-ethyl B_{2a}	360
Compound I	314
Compound II	314
Compound III	342
Compound IV	342

Table II. Effect of Incubation Period on pH and Aflatoxin B_1 Degradation by *Rhizopus arrhizus*

Treatment	pH	B_1 degraded
Uninoculated	6.75[a]	None
	6.74[b]	None
First day	6.72	Some I present
	6.73	Some I present
Second day	6.55	B_1 and I about equal concns
	6.52	No II present
Third day	6.18	Appearance of II
	6.40	Appearance of II
Fourth day	5.05	Equal amount of I and II
	6.15	B_1 approximately 50–60% degraded
Fifth day	5.95	Same as fourth day
	5.65	Same as fourth day

[a] Extracted first day. [b] Extracted fifth day.

group in place of the ketone carbonyl and III and IV contained OC_2H_5 at this position (Figure 2). The ethyl ether derivatives of I and II had formed spontaneously on silica gel columns using 10% *n*-hexane in chloroform as the eluting solvent. The ethanol preservative in the chloroform (0.75%) may have been the source of the C_2H_5 group. III and IV could have formed from I and/or II *via* a dehydration reaction on the chromatographic column.

Comparisons with data of Detroy and Hesseltine (1968) and Robertson *et al.* (1970) show that compound I is probably identical to their B_1 reduction product. Compound II was not reported to be formed by *Tetrahymena pyriformis* W., but a blue fluorescent compound that Detroy and Hesseltine referred to as "R_1" may be identical to compound II. These investigators presented no structure for "R_1."

The nmr spectrum of I was identical to the nmr spectrum of II (Figure 2). This demonstrated that I and II are stereoisomers that apparently are coincidentally equivalent when analyzed *via* nmr. The conformation of the OH group in II differs enough from the OH group in I to permit complete separation of these compounds on tlc. Since the R_f of II is less than the R_f of I, the OH group in II is apparently positioned further out of the plane of the cyclopentane ring than the OH group in I. Compound III apparently differed from compound IV in the same fashion, since they were also equivalent in nmr but were completely separated on tlc (Figure 1).

The high degree of reactivity of the double bond in the terminal furan ring of the aflatoxins (Dutton and Heathcote, 1969) required strong justification for rejecting the possibility that I, II, III, and IV were aflatoxin B_{2a} or *O*-ethyl B_{2a}-type compounds. This possibility was carefully considered but rejected for the following reasons. (1) The 4-dihydrodifuran ring protons (H_a, H_b, H_c, H_d) were present on all four compounds, as shown by comparison of the chemical shifts and coupling constants in their nmr spectra with the nmr spectrum of aflatoxin B_1 (Figure 2). (2) The absence of an additional complex 2-proton signal at δ 2.15–2.25 typical of the CH_2 present in the terminal furan ring of B_{2a} and B_{2a} acetate (Rodricks, 1969). (3) The molecular weights of all the possible aflatoxin B_{2a} and *O*-ethyl B_{2a}-type compounds did not correspond to the molecular weights observed for I, II, III, or IV (Table I). This is due to the additional oxygen atom required for the B_{2a} type compounds. The presence of the dihydrodifuran ring system in all four B_1 derived compounds was further demonstrated when the tetrahydrofuran derivatives were prepared (I and II, *m/e* 316; III and IV, *m/e* 344).

Additional evidence to discount the possibility that B_{2a} type compounds were formed by *Rhizopus* spp. is presented in Table II, which shows the effect of fungal growth on pH and aflatoxin B_1 degradation. Compounds I and II were formed in the culture medium at pH levels above 5.5 (Table II). The formation of B_{2a} type compounds in the medium is most unlikely at pH levels above 5.5 (Heathcote, 1972).

Also the R_f values of I and II are considerably different than aflatoxin B_{2a} in our chromatographic system (Figure 1).

The most logical position of chemical alteration, especially ethylation, would be in the highly reactive terminal furan ring system. However, this is apparently not the case with I and II or III and IV, in which chemical alteration has occurred in the cyclopentane ring system.

The biological alteration of aflatoxin B_1 differed from the biological alteration of aflatoxin G_1 by *Rhizopus* spp. (Cole and Kirksey, 1971). Metabolism of aflatoxin B_1 was incomplete (about 60%) and occurred within the first week of culture. No further metabolism of aflatoxin B_1 occurred after the first week of fungal growth. The greatest breakdown of aflatoxin G_1 by *Rhizopus* spp. occurred between the second and third week in culture and degradation was more complete than degradation of aflatoxin B_1 by *Rhizopus* spp. (Cole and Kirksey, 1971).

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