Parasiticol: A New Metabolite from *Aspergillus parasiticus*

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Parasiticol, a metabolite structurally related to the aflatoxins, has been isolated and characterized. The compound is elaborated by several strains of the *Aspergillus flavus* series previously shown to be aflatoxin producers. The metabolite was separated and purified by chromatography on three columns (silicic acid, silica gel, and alumina) followed by recrystallization from chloroform-hexane. Parasiticol is differentiated from aflatoxins B_1, B_2, G_1, G_2, M_1, M_2, B_{2a}, G_{2a} and aspertoxin by thin-layer chromatography. Ultraviolet and infrared absorption spectra, nuclear magnetic resonance, and mass spectrometry studies were used to elucidate the structure. Parasiticol is as acutely toxic to ducklings as aflatoxin B_1, but it is only $^1/_{100}$ as toxic as B_1 in chick embryo studies.

Since the discovery of the aflatoxins in Great Britain in 1960 (Allcroft and Carnaghan, 1963), other metabolites produced by strains in the *Aspergillus flavus* series have been isolated and characterized. Among these are aflatoxins M_1 and M_2 (Holzapfel *et al.*, 1966) and B_{2a} and G_{2a} (Dutton and Heathcote, 1966), as well as the sterigmatocystins, aspertoxin (Rodricks *et al.*, 1968), and *O*-methylsterigmatocystin (Burkhart and Forgacs, 1968).

We have been preparing quantities of aflatoxins for animal feeding tests, biological assays, microbial transformations, and detoxification studies. While separating aflatoxins B_1, B_2, G_1, G_2, and M on columns, a fluorescing substance was observed that eluted with G_2 and before M. We were interested in this specific compound because it appeared to be present in amounts greater than some of the other fluorescent compounds produced on solid substrates by *Aspergillus parasiticus* and because a column fraction containing the compound was toxic to ducklings. This paper describes the isolation and characterization of this toxic metabolite, parasiticol, a name derived from one of the producing organisms and the presence of a hydroxyl group in the molecule.

EXPERIMENTAL PROCEDURES

Production and Isolation. Parasiticol was produced by two strains of *A. parasiticus* NRRL 2999 grown on rice (6 kg) and NRRL 3145 grown on wheat (8 kg), according to the methods previously reported for aflatoxin production (Shotwell *et al.*, 1966; Stubblefield *et al.*, 1967). Other strains of the *A. flavus* series have shown the ability to produce the metabolite, but yields by all organisms investigated were low: 0.1 to 3 μg per g substrate.

Portions (1 to 3 kg) of molded grain were steeped in chloroform, the extracts concentrated, and the concentrates added to hexane to precipitate aflatoxins (Shotwell *et al.*, 1966). The precipitates contained parasiticol along with aflatoxins B_1, B_2, G_1, G_2, and small amounts of M. Crude and column products mentioned in this work were assayed for toxin content on thin-layer plates made of Adsorbosil-1 and developed with acetone:chloroform:water (12:88:1.5 v./v./v.) (Stubblefield *et al.*, 1969).

Crude precipitates (2 to 3 g per column) were purified and aflatoxins were partially separated on a number of silicic acid columns (Shotwell *et al.*, 1966). Elution of each column with ethanol:chloroform (1:99 v./v.) resulted in a fraction containing free B_1 and one containing B_1, B_2, G_1, and G_2. Parasiticol and the remaining G_2 were removed with ethanol:chloroform (5:95 v./v.). The fractions from all the silicic acid columns containing a total of 50 mg of parasiticol were combined and concentrated. The concentrate was placed on a Silica Gel G column and eluted with acetone:ethanol:chloroform (2.0:0.75:97.3 v./v./v.) (Stubblefield *et al.*, 1968). Most of the aflatoxin G_2 and all the trace amounts of B_1, B_2, and G_1 were separated from the desired compound.

Product containing parasiticol (44 mg) from Silica Gel G treatment was chromatographed on a column (0.9 × 30 cm) of alumina (Woelm, Act. I, Alupharm Chem., New Orleans, La.), which had been packed as a slurry. Chloroform (with 0.75% ethanol added as preservative) was used to form the slurry and to elute the column. The flow rate was adjusted to 18 ml per hour, and 5-ml fractions were collected and monitored by the use of thin-layer chromatography (tlc). Fractions containing parasiticol were combined before being concentrated *in vacuo*, and then the compound was precipitated in hexane (10 vol). Aflatoxin G_2 remained on the column.

Product from the alumina column (39 mg, 100% pure by tlc) was treated with decolorizing carbon (Stubblefield *et al.*, 1968) and crystallized twice from chloroform-hexane. White, crystalline parasiticol (20 mg) had the following characteristics: m.p. 233.4° to 234.1° C (decomp.) (Mettler FP-1); λ_{max}^{MeOH} 217, 225 (sh), 253, 262, and 325 mμ (ϵ 17,300, 12,600, 6800, 7400, and 9700, respectively); γ_{max} 3350 (broad), 1728, 1625, and 1605 cm.$^{-1}$ (obtained by evaporating methanol from solvent on KRS-5 plate from Wilks).

Found: C, 64.21; H, 4.65. Required ($C_{16}H_{14}O_6$): C, 63.60; H, 4.67.

Deuterated parasiticol used in the mass spectrometry studies was prepared by dissolving the crystalline product (1 mg) in deuterated methanol (CH_3OD) (1.2 ml) and then adding deuterium oxide (D_2O) (0.8 ml) until cloudy. After the crystals settled, excess solvent was removed by capillary

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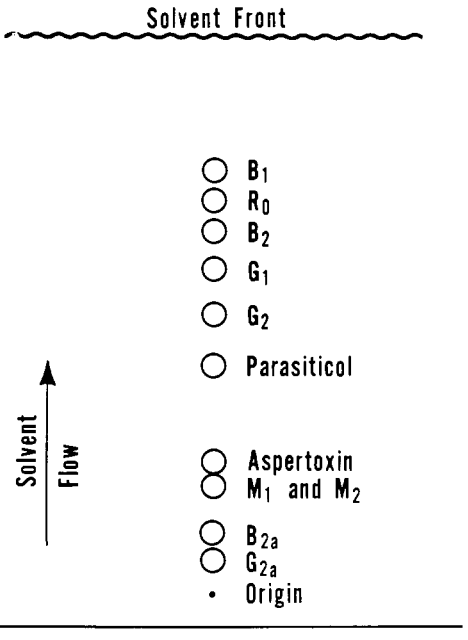

Figure 1. Thin-layer chromatography of metabolites formed by aflatoxin-producing fungi in acetone: chloroform:water (12:88:1.5, v./v./v.)

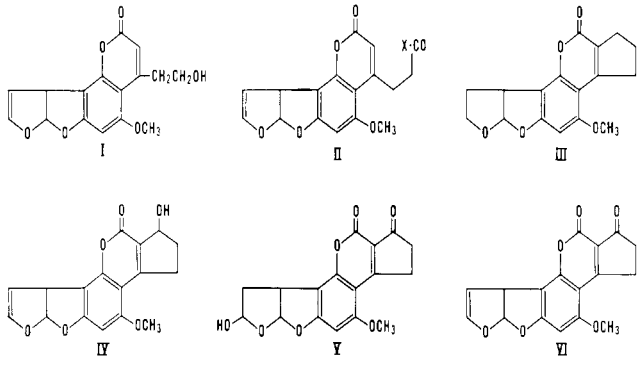

Figure 2. Structures: (I) parasiticol, (II) synthetic intermediate, (III) tetrahydrodeoxoaflatoxin B_1 (IV) aflatoxin R_0, (V) aflatoxin B_{2a}, and (VI) aflatoxin B_1

pipet. Final traces of solvent were removed under a stream of nitrogen, and the deuterated product (1 mg) was dried 3 hr in an Abderhalden drier.

RESULTS

Parasiticol was first observed in fractions from silicic acid columns. Its mobility on thin-layer plates served to differentiate it from other metabolites produced by *A. flavus* strains (Figure 1). The compound moves between G_2 and aspertoxin on tlc plates developed in acetone:chloroform:water (12:88:1.5 v./v./v.) and gives a bright blue fluorescence under long-wave ultraviolet light (366 mμ).

The first clue to the structure of parasiticol (I) (Figure 2) came from its ultraviolet absorption spectrum, which was similar to those of three compounds reported in the literature. One was a synthetic intermediate (II) reported by Roberts *et al.* (1968), another was tetrahydrodeoxoaflatoxin B_1 (III) formed by reduction of B_1 (Roberts *et al.*, 1968), and the other was aflatoxin R_0 (IV) that was produced by microbial transformation of aflatoxin B_1 (VI) (Detroy and Hesseltine, in press). The structures of these compounds with similar spectra suggested that parasiticol was a difurocoumarin but that it lacked unsaturation conjugated with the coumarin ring.

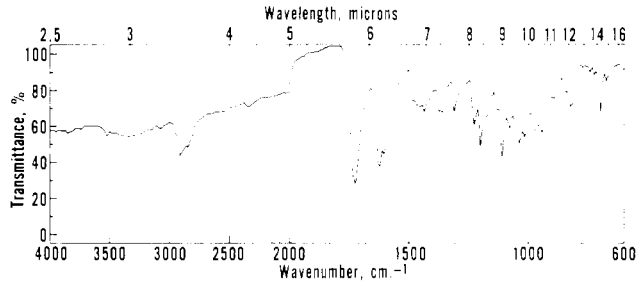

Figure 3. Infrared spectrum of parasiticol

Chemical Shifts (τ)

H_a	H_b	H_c	H_d	H_e	H_f	H_g	H_h	H_i
3.25(d)	5.25 (d of t)	4.54(t)	3.58(t)	3.61(s)	6.14(s)	6.11(t)	6.83(t)	3.98(s)
(J = 7)	(J = 2.5,7)	(J = 2.5)	(J = 2.5)			(J = 6)	(J = 6)	

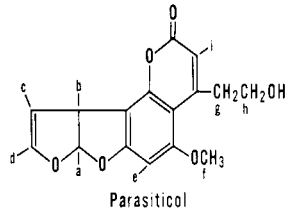

Figure 4. Nuclear magnetic resonance data and structure of parasiticol

Measured in deuterochloroform ($CDCl_3$) on Varian HA-100: s = singlet; d = doublet; t = triplet

Evidence that the parasiticol structure was closely related to aflatoxins was provided by infrared studies (Figure 3). Bands at 1728, 1625, and 1605 cm^{-1} are typical of the coumarin ring present in aflatoxins. A band at 1540 to 1558 cm^{-1} in the spectra of B_1 and G_1 due to the ring carbonyl (keto and lactone) was missing in the spectra of parasiticol. A very broad band in the region of 3350 cm^{-1} indicated a hydroxyl group could be present in the molecule, but the evidence was not conclusive.

Nuclear magnetic resonance (nmr) studies suggested the complete structure of parasiticol as depicted with protons labeled in Figure 4. Chemical shifts due to protons (a) through (d) correspond to those reported by Rodricks (1969) for aflatoxins B_1 and G_1. The data confirm the unsaturation in our compound at the 2,3 positions of the difuro ring. Singlets at τ3.61 and τ6.14 also present in B_1 and G_1 are attributed to aryl hydrogen (e) and methoxyl group (f), respectively (Roberts *et al.*, 1968). The singlet at τ3.98 (i) is typical of an isolated proton and corresponds to the coumarin hydrogen of the synthetic intermediate (II) shown previously. This chemical shift requires the proton to be at the 3 position of the coumarin ring. Triplets at τ6.11 (g) and τ6.83 (h) are evidence for methylene groups with the structure XCH_2CH_2Y where X and Y are neither hydrogen nor the same functional groups. This evidence led to the postulation that X was the coumarin ring at the 4 position and Y was a hydroxyl group. Nmr was not used to establish the presence of the hydroxyl first suggested by infrared studies because of the scarcity of material.

Final structure proof was provided by mass spectrometry. The parent peak of parasiticol was recorded at mass 302.0811, as compared to the theoretical molecular weight for the proposed structure of 302.0790. Detection of a fragment at

Table I. Production of Parasiticol by Organisms of the *Aspergillus flavus* Series on Wheat and Rice[a]

Organism, Taxon	Substrate	Yield, μg/g of Substrate[b]
NRRL 2999	Wheat	3.4
(*A. parasiticus*)	Rice	1.0
NRRL 3145	Wheat	1.1
(*A. parasiticus*)	Rice	2.0
NRRL 3251	Wheat	0.9
(New)	Rice	0.6
NRRL 3161	Wheat	0.1
(Intermediate between *A. flavus* and *A. parasiticus*)	Rice	0.9
NRRL 3517	Wheat	N.D.[c]
(*A. flavus*)	Rice	N.D.
NRRL 3525	Wheat	N.D.
(*A. flavus*)	Rice	N.D.

[a] Wheat or rice (300 g) incubated at 28° C on a rotary shaker, 200 rpm for 6 days. [b] As determined by thin-layer chromatography and densitometry. [c] Not detected. Sensitivity is 100 ppb.

mass 31 is presumptive evidence for the presence of a primary alcohol and a molecular ion peak at 284 is due to elimination of water (P-18). The hydroxyl group was confirmed by preparing the deuterated derivative and investigating its mass spectrum. The parent peak shifted to mass 303.0908, as compared to the calculated mass 303.0868 required for deuterated parasiticol, and a fragment at mass 284.067 indicated the loss of deuterated water (284.068 calc).

Duckling tests conducted by the Wisconsin Alumni Research Foundation (WARF) revealed that parasiticol has approximately the same acute toxicity as aflatoxin B_1, as both caused death at 50 μg per total dose. However, the two do not possess the same tendency to cause biliary hyperplasia. Parasiticol caused slight hyperplasia, but the effect did not depend on dose level.

Chick embryo toxicity studies showed the LD_{50} of parasiticol is 5 to 10 μg per egg. The LD_{50} of aflatoxin B_1 is 0.05 μg per egg or 100 to 200 times more toxic than parasiticol. Both metabolites caused death of embryos during the 21-day incubation period.

DISCUSSION

Even though we have not made a thorough survey of strains from the *A. flavus* series, we have found strains in three taxons that produced parasiticol on two different substrates (Table I). All these organisms produce aflatoxins B_1, B_2, G_1, G_2, and M (Hesseltine *et al.*, in press) on wheat and rice except NRRL 3251, NRRL 3517, and NRRL 3525, which do not produce G_1 or G_2. Parasiticol was not detected in the two cultures of *A. flavus* (NRRL 3517 and NRRL 3525) that were tried. The existence of a compound with the structure of parasiticol may indicate which of several postulated metabolic pathways is most probable for the elaboration of flatoxins (Biollaz *et al.*, 1968). It could be that the aflatoxins have a common precursor and that the elimination of one or more carbon atoms from this precursor leads to the formation of parasiticol rather than B_1 or G_1.

Duckling tests have been conducted by WARF on a number of compounds related to the aflatoxins, isolated or prepared in our laboratory. These are aflatoxin B_{2a} (V) (Figure 2), the hemiacetal formed by the action of an acid on B_1 (VI) (Ciegler and Peterson, 1968), aflatoxin R_0 (IV), and parasiticol. Acid treatment might be very effective in detoxification of aflatoxins because B_{2a} is $^1/_{200}$ as active as B_1 in acute toxicity and tendency to cause liver lesions in ducklings (Lillehoj and Ciegler, 1969). Aflatoxin R_0 has $^1/_{18}$ the activity of B_1 in causing bile duct hyperplasia in ducklings (Detroy and Hesseltine, 1968). Parasiticol has the same acute toxicity as B_1, but has little tendency to cause biliary hyperplasia. Comparison of the biological activity of these compounds will lead to a more complete picture of the toxicity of aflatoxins.

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Note.—During the preparation of this manuscript, a recent publication [Heathcote, J. G., Dutton, M. F., *Tetrahedron* **25,** 1497 (1969)] was called to our attention which reported the isolation of a new metabolite, aflatoxin B_3, that is identical with parasiticol. The culture (*A. flavus*, C.M.I. 91019b) that produced aflatoxin B_3 is the same as one of the *A. parasiticus* cultures (NRRL 2999) that produced parasiticol. Differences were noted in the observed melting points and in the nmr data of the two compounds. However, Dr. Heathcote recently discovered the sweep width of his nmr spectrum was twice that used in his original calculations. When the doubled value is applied, the nmr data of aflatoxin B_3 and parasiticol agree.

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