3-Hydroxyaflatoxin B_1: a New Metabolite of *in vitro* Aflatoxin B_1 Metabolism by Vervet Monkey (*Cercopithecus aethiops*) Liver

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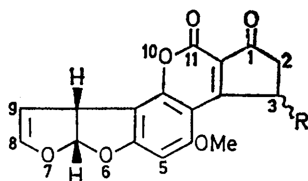
In vitro metabolism of aflatoxin B_1 by Vervet monkey liver yielded a new metabolite which was characterised as 3-hydroxyaflatoxin B_1 (2,3,6a,9a-tetrahydro-3-hydroxy-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*]-benzopyran-1,11-dione).

THE aflatoxins comprise a group of acutely toxic and highly hepatocarcinogenic metabolites elaborated by the ubiquitous mould *Aspergillus flavus*. Aflatoxin B_1 (1) is the most potent of these toxins and occurs frequently in nature.[1] In studies on the transformation of aflatoxin B_1 by the liver tissues of various animals, four identifiable metabolites were obtained, *viz.* aflatoxin M_1 (benzylic hydroxylation),[2,3] aflatoxin P_1 (*O*-demethylation),[4] aflatoxin B_{2a} (hydration),[5] and aflatoxicol (reduction of the cyclopentenone system).[6]

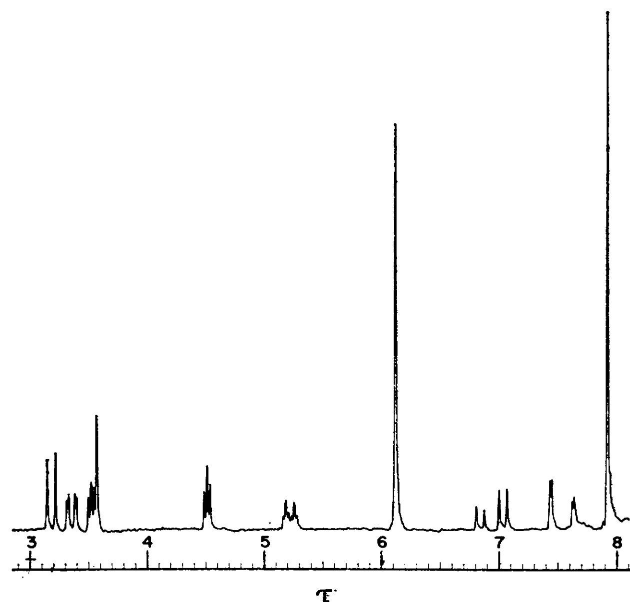
In our subsequent investigations on the metabolism of aflatoxin B_1 in mammalian systems, its transformation by the liver of a non-human primate, the Vervet monkey, was studied. This investigation led to the isolation of a new metabolite of aflatoxin B_1.

This product is formulated as 3-hydroxyaflatoxin B_1 (2) on the basis of the following evidence. High resolution mass spectroscopy established the molecular composition ($C_{17}H_{12}O_7$). The mass spectrum showed the molecular ion (base peak) at *m/e* 328 and a prominent fragment at *m/e* 299 (18%) due to the loss of CHO, a fragmentation characteristic of the bisdihydrofuran system.[7] The u.v. and i.r. spectra were virtually identical with those of aflatoxin B_1. The above evidence and n.m.r. data (see later) show that this compound contains one oxygen atom more than aflatoxin B_1; a direct comparison with aflatoxin M_1 established their non-identity.



(1) R = H
(2) R = OH
(3) R = OAc

Acetylation of compound (2) with acetic anhydride-pyridine (conditions which left aflatoxin B_1 unchanged) gave the monoacetate (3). The chromophoric system was unaffected by acetylation, and since 3-hydroxyaflatoxin B_1 (2) did not react with ethereal diazomethane, the hydroxy-group must be non-phenolic. The n.m.r.



Fourier-transform proton n.m.r. spectrum of 3-acetoxyaflatoxin B_1 (3): 7539 transients at 4 s intervals

spectrum of (3) (250 μg), recorded by the pulse Fourier-transform technique with accumulation of 7539 transients (Figure), showed the following features. A one-proton singlet at τ 3·59 was assigned to the aromatic proton, and the two three-proton singlets at τ 6·15 and 7·95 were attributed to the methoxy- and acetoxy-groups, respectively. The chemical shifts and multiplicities of the signals assigned to the protons of the bisdihydrofuran system correspond closely to those reported for aflatoxin B_1,[8] *viz.* one-proton peaks at τ 3·20 (d, *J* 7·0 Hz, 6a-H), 3·52 (t, *J* 2·3 Hz, 8-H), 4·54 (t, *J* 2·3 Hz, 9-H), and 5·23 (dt, *J* 2·3 and 7·0 Hz, 9a-H). A distinct ABX pattern was assigned to the protons of the cyclopentenone system: signals for H_A and H_B appeared at τ 6·98 and 7·54,

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respectively (J_{AB} −19·1, J_{AX} 6·7, J_{BX} 1·5 Hz; 2-H_2), and H_X (5-H) was represented by a pair of doublets at τ 3·38 (J_{AX} 6·7, J_{BX} 1·5 Hz). The large geminal coupling (J −19·1 Hz) for the methylene protons is consistent with the reported values for the δ-protons of cyclopentenones.[9] The location of the secondary hydroxy-group at the allylic 3-position of (2) is supported by the negative colour tests observed with both Tetrazolium Blue and 2,3,5-triphenyltetrazolium chloride. These tests are very sensitive for the presence of an α-ketol group.

In the metabolic studies of aflatoxin B_1 with the microsomal fraction of the liver of Vervet monkey traces of aflatoxin B_{2a} and a number of minute blue fluorescent metabolites were produced, but no aflatoxin P_1 was detected.

We hope to obtain sufficient 3-hydroxyaflatoxin B_1 (2) for toxicological evaluation in order to establish whether the hydroxylation represents a detoxification or an activation step. 3-Hydroxyaflatoxin B_1 has also been produced by the microsomal fraction of the liver of the baboon (*Papio ursinus*), another non-human primate.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. U.v. absorptions were measured for solutions in methanol with a Unicam SP 800 spectrometer. I.r. spectra were recorded with a Perkin-Elmer 237 spectrometer. Mass spectra were taken with an A.E.I. MS9 double-focusing spectrometer. The Fourier transform proton n.m.r. spectrum (7539 transients at 4·0 s intervals) was recorded with a Varian XL-100 spectrometer. T.l.c. was carried out on Merck precoated silica plates (thickness 0·25 mm).

Incubation of Aflatoxin B_1.—The preparation of the microsomal fraction[10] was carried out at 5°. Chilled Vervet monkey liver tissue (50 g) was homogenized in 0·25M-sucrose–0·01M-Tris–3mM-magnesium chloride (pH 7·8) (200 ml). The homogenate was filtered through cheesecloth and the filtrate centrifuged for 30 min at 13,000 g. The supernatant was filtered through glass wool and centrifuged for 60 min at 105,000 g to produce the microsomal pellet. It was suspended in 0·01M-Tris–3mM-magnesium chloride buffer (pH 7·8) (50 ml) to yield the enzyme source.

On a small scale, this enzyme fraction (3 ml), aflatoxin B_1 [0·4 mg in methanol (0·4 ml)], NADP (0·5 mg), glucose 6-phosphate (20 mg), and glucose 6-phosphate dehydrogenase (20 units) were diluted to 5 ml with the above buffer (pH 7·8). The mixture was incubated for 16 h at 37° in a water-bath equipped with a shaker, and extracted with chloroform. T.l.c. with chloroform–methanol (97 : 3 v/v) showed the presence of aflatoxin B_1 (R_F 0·42) and a new bright green fluorescent metabolite (R_F 0·30) in the ratio 5 : 1.

Isolation of 3-Hydroxyaflatoxin B_1.—The chloroform extract was purified by column chromatography on oxalic acid-impregnated aluminium oxide. Gradient elution with hexane–chloroform gave a fraction containing the new metabolite. Lipid material was removed by partition between hexane and 90% methanol. The methanolic layer containing the green fluorescent spot was applied to silica thin-layer plates; multiple development with chloroform–methanol (98 : 2 v/v) gave a band from which *3-hydroxyaflatoxin B_1* (2) was eluted by methanol. Several preparations afforded sufficient material (300 μg) for structure analysis; m.p. 280° (decomp.) (from chloroform); $\lambda_{max.}$ 223, 266, and 367 nm (ε 17,000, 9850, and 15,800); $\nu_{max.}$ (KBr) 1758, 1690, 1628, and 1595 cm^{-1} (Found: M^+, 328·0587. $C_{17}H_{12}O_7$ requires M, 328·0583).

Acetylation of (2) (260 μg) with acetic anhydride–pyridine gave 3-acetoxyaflatoxin B_1 (3) (250 μg), m.p. 305° (decomp.) (from chloroform); $\lambda_{max.}$ 224, 266, and 367 nm; $\nu_{max.}$ ($CHCl_3$) (CO) 1768, 1745, and 1695 cm^{-1}, M^+, 370 (Calc. for $C_{19}H_{14}O_8$: M, 370).

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