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NEW METABOLITES OF *ASPERGILLUS FLAVUS*

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Abstract—Two pigments have been isolated from a yeast culture of *A. flavus*. One of these is identical with a tetrahydroxy-anthraquinone previously isolated from *A. versicolor*, the other being versicolorin C. The culture medium also yielded small amounts of aflatoxins M_1 and GM_1 and also a new compound, designated aflatoxin B_3, and for which a tentative structure is put forward.

DURING our investigation of the various aflatoxins produced by a strain of *A. flavus*[1] (C.M.I. 91019b), several coloured metabolites were also observed in extracts from a culture of the mould. In order to investigate these metabolites further, the mould was cultured on an aqueous medium containing yeast extract and glucose. The mycelium obtained from this culture was carefully dried, ground to a powder, and soxhlet-extracted with chloroform acetone (9:1 v/v) until the extracting solvent was colourless. The extract was evaporated to a suitable volume *in vacuo*, and subjected to column chromatography on kieselgel.

New pigments

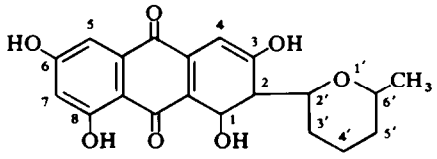
The pigments were separated from the aflatoxins by elution with diethyl ether, the fraction so obtained was evaporated to a small volume, and then examined by TLC using kieselgel G and chloroform as the solvent. A number of orange spots was observed on the chromatoplate, the main one occurring at an R_f value of about 0·34, (this was at a position a little lower than aflatoxin G_2 in the fraction containing the aflatoxins). This orange spot was resolved into two compounds Or_1 and Or_2, at R_f values of 0·46 and 0·39 respectively, by preparative TLC using kieselgel G and ethyl acetate–benzene (1:9 v/v) as the solvent.

Compound Or_1 (from chloroform) had m.p. 271°, λ_{max} (MeOH) 223 mμ, 263 mμ, 292 mμ, 312 mμ and 452 mμ, (ε in order 20,180; 14,500; 17,880; 9940 and 8650), and was shown by mass spectrometry to have a mol wt of 370, and a mol formula of $C_{20}H_{18}O_7$. On full methylation it yielded a tetramethoxy derivative. The properties of Or_1 indicated that it was a derivative of a tetrahydroxy-anthraquinone and this was further confirmed by the NMR spectrum* of its tetramethoxy derivative. The spectrum was, in fact, consistent with that of a 1,3,6,8-tetrahydroxy-2,2′-(6′-methyl-tetrahydropyran) anthraquinone (I) and a compound with this structure was recently isolated from a culture of *A. versicolor* by Holker *et al.*[2] That structure I was the correct one for our compound was verified by comparing its properties with those of an authentic sample of material obtained from Dr. J. S. E. Holker of Liverpool University. The two compounds were also shown to have identical NMR spectra.

Versicolorin C

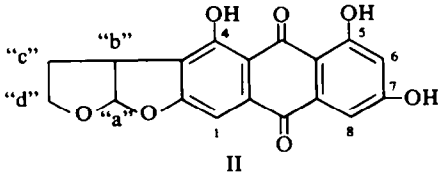
Compound Or_2 (from $CHCl_3$) had m.p. 350°, $[\alpha]_D = 0$, ($c = 0{\cdot}1$ in $CHCl_3$), λ_{max}

* Carried out on a Varian HA-100 instrument and using deuterochloroform as the solvent.



I

(MeOH) 222 mμ, 265 mμ, 290 mμ, 312 mμ and 450 mμ, (ε in order 28,100; 16,600; 22,900; 11,120 and 6920), and was shown by mass spectrometry to have a mol wt of 340, and a mol formula of $C_{18}H_{12}O_7$. On full methylation it yielded a trimethoxyl derivative the properties of which indicated that Or_2 was a trihydroxy-anthraquinone. The NMR spectrum* of Or_2 was identical with that published by Hamasaki *et al.*[3] for dihydroversicolorin A, which is also the same for the natural products, Versicolorins B and C (II), which the same authors isolated from a culture of *A. versicolor*.[3] Our compound seems to correspond to Versicolorin C, which was claimed to be the racemic form of Versicolorin B[3], as it has no optical properties and its other properties were identical with those of an authentic sample of Versicolorin C kindly donated by Dr. Y. Hatsuda, Tottori University.



II

A discrepancy noted in the NMR spectrum was the assignment by Hamasaki *et al.* of the signal at 5·95 τ to the protons at "d" and at 6·37 τ to the proton at "b". In our spectrum the multiplet at 5·95 τ was shown to be coupled to the doublet at 3·62 τ, (proton associated with this signal assigned to position "a") by irradiation of the multiplet at 2572 cycles/sec, thus the multiplet at 5·95 τ must be assigned to the proton at position "b", and hence it follows that the signal at 6·37 τ must be assigned to the proton at position "d". Our assignments seem to agree with those in the literature for similar systems, e.g. Aversin.[4]

The isolation of these two pigments from a toxin-producing strain of *A. flavus* shows the close biogenetic link between this species and *A. versicolor*, a point made by other workers[5] and thus adds further weight to the view that the aflatoxins and sterigmatocystin are derived from the same or similar precursors.[6] The isolation of Versicolorin C from both species would indicate that this compound is a common precursor as suggested by Thomas.[7]

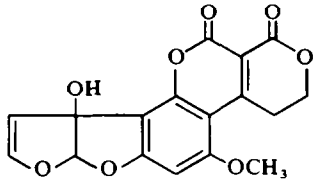
New aflatoxin metabolites

Two aflatoxin-containing fractions were also obtained from these culture media by extraction with chloroform and column chromatography as described previously.[1] The first fraction from the column contained the aflatoxins B_1, B_2, G_1 and G_2, and the second, three metabolites, which were separated by preparative TLC using Kieselgel G, and chloroform as the solvent. Two of these compounds behaved

* Obtained on a Varian HA-100 instrument using deuterochloroform as the solvent.

similarly (on TLC) to aflatoxins B_{2a} and G_{2a}, one of R_f value 0·18 fluorescing blue under UV light, and the other of R_f value, 0·15 fluorescing green. The third compound gave a blue fluorescent spot with an R_f value of 0·28, i.e. somewhere between the positions of aflatoxins M_1 and G_2 in the same TLC system. On further examination of the compounds thought to be aflatoxins B_{2a} and G_{2a}, it became apparent that they were in fact aflatoxin M_1, (by comparison with an authentic sample of this compound kindly donated by Dr. I. F. M. Purchase, N.N.R.I. South Africa), and a compound which appeared to be the G analogue of aflatoxin M_1 (designated as aflatoxin GM_1). This compound, which has the structure (III), has been tentatively reported elsewhere (Nabney *et al.*,[8] Purchase *et al.*[9]).

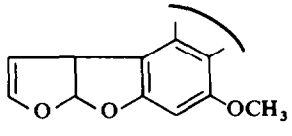
It has (from $CHCl_3$) m.p. 276°, λ_{max} (MeOH) 235 mμ, 262 mμ and 358 mμ (ε in order 21,200; 16,300 and 12,000). Mol wt, 344,* mol formula, $C_{17}H_{12}O_8$.* It forms a mono-acetyl derivative (m.p. 280°) on treatment with acetic anhydride/pyridine. Due to the lack of material its structure could not be definitely assigned, but the properties so far determined were consistent with the tentative structure. (III).



III

Aflatoxin B_3

The third compound isolated was named aflatoxin B_3 because of its blue fluorescence, although it became apparent later that it was closer in structure to the aflatoxin G series. It has (from $CHCl_3$): m.p. 217°, λ_{max} (MeOH) 229 mμ, 253 mμ, 262 mμ and 326 mμ; ε in order 10,000; 7300, 7550 and 9350; Mol wt, 302,* Mol formula, $C_{16}H_{14}O_6$;* IR spectrum (main bands) 3400, 3040, 2900, 1725, 1622 and 1070 cm^{-1}. Negative reactions were obtained with Gibbs reagent, neutral ferric chloride and 2,4-dinitrophenyl hydrazine reagent. A mono-acetyl derivative was formed on treatment with acetic anhydride/pyridine. The NMR spectrum† of aflatoxin B_3 showed that the bisdihydrofuran system, which is found in aflatoxins B_1 and G_1, was present in the molecule together with the same aromatic nucleus and OMe group, accounting for eight protons, see IV: i.e. Triplets at 3·40 τ (1 proton); and 4·58 τ (1 proton), J = 1·5 c/s; Multiplet at 5·32 τ (1 proton); doublet at 3·14 τ (1 proton), J = 3·0; singlets at 6·40 τ (3 protons), 3·54 τ (1 proton) cf. the spectra of aflatoxins B_1 and G_1.[10]



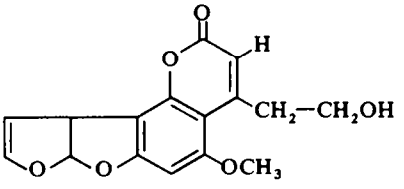
IV

* Determined by mass spectrometry.

† Determined on a Varian HA-100 instrument using deuteropyridine.

Of the remaining 6 protons, four were observed as a pair of coupled triplets at 6·75 τ, and 5·97 τ, $J = 3{\cdot}0$, (cf. aflatoxin G_1 triplets at 6·84 τ and 5·73 τ, $J = 3{\cdot}0$), and another as a singlet at 3·73 τ. The remaining proton was not detected even after treatment with deuterium oxide and was, therefore, assumed to be present at an OH group giving rise to a very broad signal. The presence of an alcohol group was supported by the band in the IR spectrum at 3400 cm^{-1}, by the formation of a mono-acetyl derivative, and the absence of phenolic-type reactions. In spite of the similarities of the NMR spectra of aflatoxin G_1 and B_3, the IR spectrum of B_3 showed that the dilactone system was lacking in this compound (G_1 giving rise to bands at 1770 cm^{-1}, and 1670 cm^{-1})[4] although a strong band at 1725 cm^{-1} indicated the presence of a lactone group.

All these facts can be reconciled in structure V, the olefinic proton giving rise to the singlet at 3·73 τ, and the 4 protons on the aliphatic side-chain producing the coupled pair of triplets at 6·75 τ and 5·97 τ.



V

It can be seen from the above structure that aflatoxin B_3 can easily be obtained from aflatoxin G_1 by a simple hydrolytic process followed by decarboxylation (another point in favour of Structure III). This process occurs in the mould culture itself, as no aflatoxin B_3 was obtained when quantities of pure aflatoxin B_1 or G_1 were subjected to the same extraction procedures.

This decarboxylation process may very well be the first step in the microbial breakdown and disappearance of the aflatoxins from mould cultures, observed by many workers, including ourselves and, e.g. Ciegler *et al.*[11]

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