TWO NEW METABOLITES OF *ASPERGILLUS FLAVUS* (LINK)

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1. Introduction

About a dozen different fluorescent substances have been isolated from cultures of *Aspergillus flavus* (Link) in natural and synthetic media by Smith and McKernam [1] and by Asao, Buchi et al. [2]. Only the aflatoxins B_1, B_2, G_1, G_2 and three related quinones have been reported as being toxic. We therefore decided to investigate the nature and toxic properties of two substances previously referred to as fractions 10 and 11 by Smith and McKernam [1]. It is shown in this report that these substances may be purified as crystalline, unsaturated fatty acids. The substance B_o which gives a blue fluorescence is as toxic to chick embryo as aflatoxin B_1 while that which gives a green fluorescence in ultraviolet light (G_o) is as toxic as aflatoxin G_2.

2. Methods and results

A local strain of *Aspergillus flavus* was cultured at room temperature for a week in sterilized palm sap obtained from a variety of *Elaeis guineensis*. The active substances were extracted with chloroform from both the mycelial felt and the liquid broth. The combined chloroform extract was filtered through alumina and columns of cellite–545, concentrated, and chromatographed on a kieselgel G thin layer plate. Of the twelve fluorescent bands thus visualized, a blue one and a green one near the base line were scraped off and eluted with chloroform in the dark. The eluates were further purified by thin layer chromatography in kieselgel G layers 0.25 mm thick and 50:2 v/v chloroform in methanol developer. The substances designated B_o and G_o ran near the origin and had R_f values of 0.20 and 0.13, respectively (fig. 1). They were crystallized

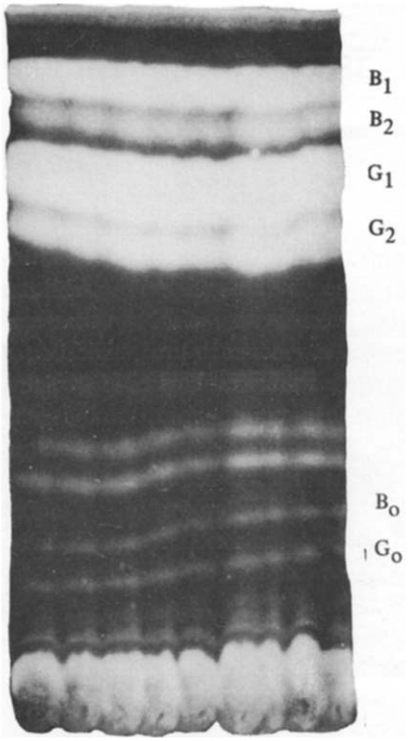


Fig. 1. Thin layer chromatogram of fluorescent metabolites purified on 0.25 mm thick kieselgel G layer with 50:2 v/v chloroform in methanol developer.

by the addition of *n*-hexane to the concentrated chloroform extract and cooling overnight at −20°C.

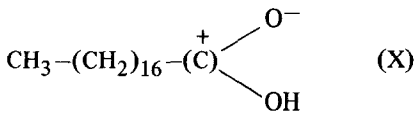
Determination of the molar extinction coefficient (E) of B_o and G_o was done. The infrared analyses were by the "Nujol-Mull" technique. The ultraviolet spectra and the nuclear magnetic resonance of B_o and G_o were also obtained. So was the mass spectro-

metry with solid samples of B_o and G_o. The iodine number of B_o and G_o were determined as described by Bassir [3]. The carbon and hydrogen contents were determined, since the preliminary analyses showed B_o and G_o to contain only the elements C, H, and O. The value of O was thus obtained by difference.

The results of these analyses are summarized in table 1.

These data lead to the conclusion that B_o and G_o are non-aromatic but probably spatial isomers of hydroxy-conjugated fatty acids. B_o and G_o are probably diene acids. This is supported by the fact that the maximum absorption observed (λ obs.) was 217 mμ for each compound and their molar extinction coefficients (E) were 19500 ± 10.70 and 21965 ± 15.83, respectively. These agree fairly well with data published by Scott [4] for the chromophore having λ obs. = 217 mμ and E = 21000.

In order to locate the double bonds and finally the structure, oxidative ozonolysis of B_o and G_o was carried out. The reaction products were resolved by gas-liquid chromatography and identified by mass spectrometry. This technique was very similar to that applied by Bergstrom [5] in the elu :idation of the structure of the prostaglandins. The molecular peak in each case was obtained at m/c = 284. This corresponds to the carbonium ion



(X)

The carbonium peak at m/c = 284 suggests that the first double bond, numbering from the methyl group, is after the last carbon atom in the original intact molecule. Since the negatively charged oxygen moiety was introduced as a result of the oxidation and hydrolysis, the resultant m/c value for the cleaved substance is (284–16), i.e., 268. So, X really has an m/c value of 268. The m/c value for the original molecule was 380 in each case. The material lost during ozonolysis in each case was (380–268) = 112. This figure represents the molecular weight of either a fragmented or an intact molecule which may be represented as

$$= CH–CH=CH\,(CH_2)_2.COOH \qquad (Y)$$

This gives the double bond linkage for moiety (X). Combining X and Y one now has

Table 1
Analyses of substances B_o and G_o.

Properties		B_o	G_o
Melting point (°C)		52–53	51–52
Specific rotation		+ 6.30	+ 6.10
Analysis (%)	C	76.19 ± 1.55	76.23 ± 1.40
	H	11.11 ± 1.21	11.05 ± 1.12
	O	12.70	12.72
Mol. wt. (mass spec.)		380	380
Molecular formula		$C_{24}H_{42}O_3$ 44	$C_{24}H_{42}O_3$ 44
Iodine number		15.90 ± 0.35	15.46 ± 0.42

$$CH_3–(CH_2)_{16}–\underset{OH}{C}=\underset{H}{C}–CH=CH–(CH_2)_2–COOH \quad (Z)$$

Toxicity titration of 6-day old White Rock chick embryos with solutions of B_o, G_o, and aflatoxins B_1, B_2, G_1, and G_2 in propylene glycol was performed by the method of Verret et al. [6]. The dosage was 0.3 μg/egg delivered through 0.05 ml of propylene glycol while the untreated consisted of eggs drilled at the air sac end and sealed with cellophane. All eggs were incubated at 37°C. The results in table 2 show B_o to be as toxic as aflatoxin B_1 while G_o is as toxic as aflatoxin G_2.

3. Discussion

Extracted uninoculated palmsap does not reveal these fluorescent substances when investigated by thin layer techniques. Unsaturation, therefore, might be biosynthetic, or degradative of some complex compounds such as the aflatoxins.

Elementary analysis gave the empirical formula $C_{24}H_{42}O_3$. The small amount of available substance made the determination of structure by chemical means very difficult, and resort was made to mass spectrometry. The mass ion peak of each compound was 380. This led to the correction of the empirical formula from $C_{24}H_{42}O_3$ to $C_{24}H_{44}O_3$.

Table 2
Percentage mortalities of the embryos.

Substance	Afl. B_1	Afl. B_2	Afl. G_1	Afl. G_2	B_o	G_o	Control	Untreated
Percent mortality	92	53	68	36	90	27	2	1

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