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Inhibition of Aflatoxin Production and Tentative Identification of an Aflatoxin Intermediate "Versiconal Acetate" from Treatment with Dichlorvos

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In general, aflatoxin production by *Aspergillus flavus* and *A. parasiticus* was greatly reduced in vitro in the presence of the insecticide dichlorvos. Reduction in yield of the toxins was accompanied by the appearance of a previously unidentified orange pigment. Spectral analyses of the pigment and of its methylated and acetylated derivatives indicated the compound to be versiconal acetate (IV). The data suggest that IV is an intermediate in the metabolic cycle that may terminate in the production of aflatoxin or of the versicolorins, or both. Dichlorvos apparently inhibits biosynthesis of the difurano ring structure common to the aflatoxins and the versicolorins.

The highly toxic and carcinogenic properties of the aflatoxins and the omniprescence of the fungi producing these toxins in food and feed crops have encouraged much basic and applied research (3). Although detection, prevention, and elimination of aflatoxin contamination have had priority, investigations of the basic metabolic functions of the causative organisms and of the effectiveness of various natural and artificial stimuli have not been neglected.

Rao and Harein (9) investigated the insecticide dichlorvos as an inhibitor of aflatoxin production when applied to rice, corn, wheat, and peanuts. They found that dichlorvos, at 5 and 10 μg/ml, reduced aflatoxin production by averages of 62 and 59%, respectively. At 20 μg/ml, aflatoxin production was reduced to levels too low for analysis by ultraviolet (UV) spectrophotometry. Hsieh (6) recently reported that actively synthesizing cultures, supplemented with 10 μg of dichlorvos per ml and [*1*-^{14}C] acetate, produced a reduced amount of aflatoxin which contained almost no label from the acetate, suggesting inhibition of an early step in the pathway leading to the biosynthesis of the aflatoxins.

In a series of elegant degradative experiments, Biollaz, Buchi, and Milne (1) developed a biosynthetic scheme that suggests an interrelation of many of the bisfurano compounds of fungal origin. Of specific interest to our study, they suggested that a precursor was common to the aflatoxins and to the versicolorins. Lin and Hsieh (7) reported evidence that averufin is a precursor of aflatoxin B_1 and suggested that a pathway different from the scheme proposed by Biollaz, Buchi, and Milne may actually be used.

We have confirmed that, in general, dichlorvos significantly reduced aflatoxin production by toxin-producing strains of *Aspergillus flavus* Link and *A. parasiticus* Speare and provided evidence of the mode of interference in the biosynthetic pathway.

MATERIALS AND METHODS

Cultures of fungi and production of metabolites. Two high-aflatoxin-producing strains, one low-producing strain of *A. flavus*, and one high-producing strain of *A. parasiticus* were selected for these experiments. Strain designations were P-70-51i, CS-70-111a, P-70-40a, and P-69-3b, respectively. Another strain of *A. flavus*, P-70-41b, was included. This latter strain produced no aflatoxin but did produce *O*-methylsterigmatocystin and dihydro-*O*-methylsterigmatocystin (2).

Cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of water and 3 g of ground Spanish peanut. Volume of water was adjusted to allow for addition of the dichlorvos suspension. Flasks were stoppered with cotton plugs and autoclaved for 20 min at 121.1 C. After sterilization, dichlorvos in sterile water was added to concentrations of 0, 5, 10, 15, and 20 μg/ml (w/v basis).

Each flask was inoculated with a loop of spores of a 7- to 10-day culture of the respective strain from a Czapek agar slant. The spores were dispersed throughout the medium, and the flasks were incubated for 7 days at 25 C. Aflatoxins and other

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secondary metabolites were then extracted by adding acetone to the culture and then proceeding with the aqueous acetone method of Pons and Goldblatt (8). Aflatoxins were quantified by visual comparison with known standards on thin-layer chromatography (TLC).

An unknown orange pigment accumulated in all cultures treated with dichlorvos. To obtain the compound in sufficient quantity for identification, fermentations with *A. flavus* isolate P-70-51i were made in 2.8-liter Fernbach flasks. Each flask contained 30 g of ground peanut and 500 ml of water with dichlorvos added to a concentration of 10 μg/ml. The orange pigment was separated from aqueous acetone extracts of 18 flasks by column chromatography on silica gel (Baker 3405). The column was packed as a slurry with chloroform. The aflatoxins were eluted with chloroform, and the orange pigment was then eluted with 15% acetone in chloroform. It was necessary to rechromatograph the pigment to obtain a separation of sufficient purity for crystallization. After recrystallization from 15% acetone in chloroform, the compound appeared pure by TLC (Adsorbosil-1, 0.5 mm thick, developed with 15% acetone in chloroform). The combined yield of 18 flasks was about 70 mg of pure compound. Identification was then undertaken by chemical and physical methods.

Physical and chemical analyses. The orange pigment and authentic samples of versicolorin B and C were spotted on a Silicar TLC-7G silica gel plate, 0.25 mm thick, and developed with toluene-ethyl acetate (27:12, v/v).

UV spectra were taken in 95% ETOH with a recording spectrophotometer (Beckman model DB-G). A holmium oxide standard (A. H. Thomas, Co.) was used to calibrate the instrument. Infrared (IR) spectra were taken with an IR spectrophotometer (Perkin-Elmer model 257) equipped with a 4× beam condenser. Samples to be analyzed were coated on KBr blocks as a thin film. Nuclear magnetic resonance (NMR) spectra were performed with a Jeolco spectrometer (Minimar-60). Samples of the orange pigment were analyzed in dimethylsulfoxide-d_6 solution; its methylated derivative was analyzed in chloroform-d solution. Tetramethylsilane was used as the internal standard.

Mass spectra were obtained on a high-resolution mass spectrometer (Dupont/CEC 21-110B). All samples were introduced with a specially designed glass probe at 190 to 300 C. The ion-source temperature was varied from 250 to 305 C. Spectra were recorded oscillographically, and high-resolution measurements were made by peak matching.

An acetylation reaction was performed on the orange pigment by dissolving it in 1 ml of tetrahydrofuran followed by the addition of 0.5 ml of acetic anhydride. The solution was then placed in an acylation tube at 130 C for about 12 h. After removal of volatile products with an N_2 stream at 60 C, a portion of the sample was prepared for spectrometric analysis.

The remainder of the sample from the acetylation reaction was then hydrolyzed in 2 ml of *n*-butanol containing 1.25 N HCl. To accelerate the reaction, the solution was placed in an acylation tube at 90 C for 2 h. A mass spectrum was run on the residue after removal of the volatile products.

Versicolorin C (4) and the orange pigment were methylated with dimethylsulfate and anhydrous potassium carbonate in dry acetone under reflux for 6 h. After filtration and concentration of the reaction mixture, the methylated compounds were crystallized from acetone solution.

RESULTS

Effect of dichlorvos on yields. Dichlorvos at 5, 10, 15, and 20 μg/ml inhibited aflatoxin B_1 production by three isolates of *A. flavus* and one isolate of *A. parasiticus* (Table 1). With two isolates of *A. flavus* and the one *A. parasiticus* (isolates nos. P-70-51i, CS-70-111a, and P-69-3b), dichlorvos at 5 μg/ml reduced aflatoxin production ca. 90%. Increasing the concentration of dichlorvos tended to decrease aflatoxin yields further, but at a sharply reduced rate. Aflatoxin B_2 yields were decreased proportionately. Yields of aflatoxin G_1 and G_2 by *A. parasiticus* (isolate P-69-3b) were also decreased in a similar manner by the addition of dichlorvos (Table 1).

TABLE 1. *Reduction of aflatoxin production by the incorporation of the insecticide dichlorvos into a liquid medium*

Isolate	Concn of dichlorvos (μg/ml)	Aflatoxins produced			
		Untreated		Treated	
		B_1 (μg/flask)	G_1 (μg/flask)	B_1 (% control)	G_1 (% control)
Aspergillus flavus P-70-51i	0	3,300			
	5			10	
	10			10	
	15			9	
	20			8	
A. flavus CS-70-111a	0	1,250			
	5			8	
	10			5	
	15			4	
	20			3	
A. flavus P-70-40a	0	100			
	5			150	
	10			83	
	15			68	
	20			68	
A. parasiticus P-69-3b	0	1,200	310		
	5			14	15
	10			8	5
	15			6	5
	20			6	5

With *A. flavus* isolate P-70-40a, a low-yielding producer of aflatoxin B_1 and B_2, dichlorvos at 5 μg/ml appeared to stimulate aflatoxin production and to increase the yield by 50% over that of the untreated control. At higher concentrations, dichlorvos reduced yields of toxins but not to the extent noted with other strains. At 20 μg/ml of the insecticide, yields were reduced only 32% compared to 92% for isolate P-70-51i and 97% for isolate CS-70-111a.

Dichlorvos (by visual observation) did affect growth of all strains of the fungus. With increasing concentrations of the insecticide, sporulation was delayed but no differences were observed between the untreated control and cultures treated at 5 μg/ml.

Reductions in the production of aflatoxins were accompanied by the appearance of an orange pigment. By direct visual comparison, the concentration of orange pigment was in direct proportion to reduction in aflatoxin production. Thus, production of the orange pigment was greatest by *A. flavus*, isolate P-70-51i, at a dichlorvos concentration of 20 μg/ml. The yield of the orange pigment also increased with increasing concentration of the insecticide in the fermentation by the other aflatoxin-producing cultures.

Fermentations by *A. flavus*, isolate P-70-41b, were also affected by dichlorvos. Although not quantified, visual comparison on TLC clearly showed a decrease of the yield of *O*-methylsterigmatocystin that was related to increasing concentrations of the insecticide. As with the aflatoxin-producing isolates, this decrease was inversely related to the production of the orange pigment.

Physical and chemical properties of pigment. The R_f values × 100 on a Silicar TLC-7G plate developed with toluene-ethyl acetate (27:12, v/v) were as follows: unknown orange pigment, R_f = 32; versicolorin B, R_f = 67; versicolorin C, R_f = 67. With chloroform-acetone (85:15, v/v), the R_f values × 100 were 33, 79, and 79, respectively.

The metabolite was crystallized from chloroform-acetone solution to yield orange needles, melting point 216 to 220 C. The UV spectrum of the orange pigment showed λ_{max}^{etoh} 225, 267, 298, 323, and 480 nm (ϵ_{max} 23,800; 14,000; 23,000; 11,300; and 7,260 respectively). Comparisons of this UV spectrum with spectra of the anthraquinones, emodin (λ_{max}^{etoh} 224, 256, 268, 293, and 442 nm), versicolorin B (λ_{max}^{etoh} 227, 268, 298, 323, and 478 nm), and versicolorin C (λ_{max}^{etoh} 226, 268, 297, 323, and 468 nm) demonstrated that the orange pigment contained an anthraquinone-type structure. The IR spectrum showed hydroxyl absorptions at 3,540 and 3,250 cm^{-1}, quinone carbonyl absorptions at 1,670 w (nonchelated), 1,630 (chelated), and 1,619 cm^{-1}, and additional carbonyl absorptions at 1,715 and 1,725 cm^{-1}.

The NMR spectrum of the orange pigment showed two one-proton chemical shifts at δ 7.04 (J = 4.0 Hz) and δ 6.48 (J = 4.0 Hz) with coupling constants corresponding to two meta-positioned aromatic protons (H_2 and H_3). A third aromatic proton, although not clearly evident, appeared to be overlapped with the signal at δ 7.04 as a singlet (H_1). Other chemical shifts were a broad triplet centered at δ 4.08 which integrated for two protons and a four- to five-proton signal positioned at δ 1.98.

The NMR spectrum of the methylated derivative of the orange pigment (Fig. 1) showed chemical shifts for aromatic protons at δ 7.54 (1H, singlet), δ 6.75 (1H, doublet; J = 4.0 Hz), and δ 7.32 (1H, doublet; J = 4.0 Hz). Other chemical shifts were singlet (1H) at δ 9.69, a 4- to 5-proton signal including a probable 3-proton singlet at δ 1.96, and complex signals in the region of δ 3.85 to 4.05 integrating for about 15 protons. The latter signal contains protons for approximately four methoxy groups.

The mass spectrum of the orange pigment showed principal high-mass ions at m/e 340, 322, 312, 311, and 297. Other ions of significance were noted at 382, 354, and 325. Significant low-mass ions were observed at 43, 45, and 60 showing the presence of acetic acid. This probably originated from an acetate group on the molecule through thermal decomposition at the high run temperature (200 to 210 C). The peaks at 340, 325, 311, and 297 had intensities similar to those in the versicolorin spectrum, except that 325 appeared to be of lower intensity and 311 appeared to be more intense. A high-resolution measurement showed that the 340 peak had an elemental composition of $C_{18}H_{12}O_7$, the same as for the molecular ion of versicolorins B and C.

The mass spectrum of the acetylated sample showed an apparent molecular ion at m/e 466 and even-mass fragments differing by 42 and 60 mass units down to m/e 322. High-resolution measurements showed that the 466 and 322 peaks had elemental compositions of $C_{24}H_{18}O_{10}$ and $C_{18}H_{10}O_6$, respectively.

The hydrolysis product obtained after acetylation of the orange pigment produced a mass spectrum having principal high-mass ions at 340, 325, 311, and 297. The pattern was virtually identical to that of versicolorin C.

The mass spectrum of the methylation product of versicolorin C showed that the compound

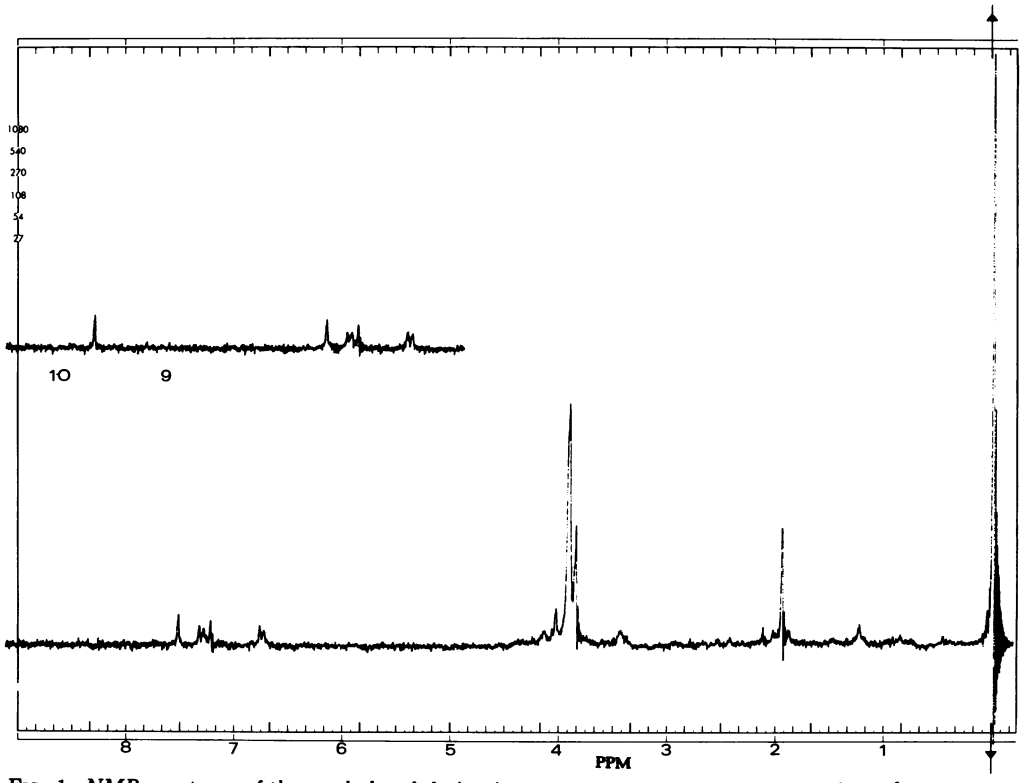
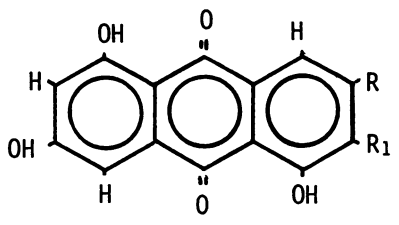


FIG. 1. *NMR spectrum of the methylated derivative of the orange pigment in chloroform-d.*

methylated exactly as expected, i.e., the three phenolic OH groups were converted to methyl ethers. On the other hand, the orange pigment methylated to produce a compound having an apparent molecular ion at 456 and an M-31 peak at 425. From a high-resolution measurement, the 456 peak gave an elemental composition of $C_{24}H_{24}O_9$. Thus, two more oxygens were present in the orange pigment than in versicolorin.

DISCUSSION

The data indicate that the orange pigment has an anthraquinone structure with an aromatic substitution pattern identical to versicolorins B and C (I). The NMR spectrum of the orange pigment and its methylated derivative also provide strong evidence for the absence of a tetrahydro-difurano ring system. Chemical shifts for the tetrahydro-difurano protons H_a, H_b, and H_c (II) could be difficult to detect due to splitting; however, the well-defined chemical shift for the doublet at approximately δ 6.40 (H_d in II) is lacking in the NMR spectrum of the orange pigment.



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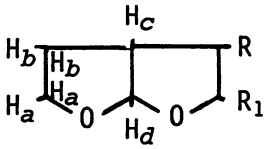
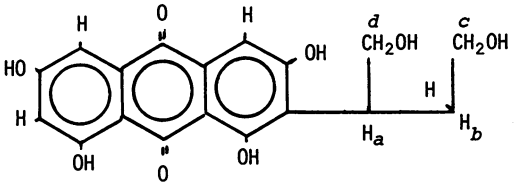


II

The NMR spectrum of the methylated derivative of the orange pigment (Fig. 1) clearly showed the same aromatic substitution pattern as the methylated derivative of versiconol as reported (5) by Hatsuda et al., (one aromatic proton doublet at δ 6.91, J = 2.5 Hz, coupled meta to another proton doublet at δ 7.34, J = 2.5 Hz and an additional one-proton singlet at δ

7.58). In comparison, the methylated orange pigment showed one aromatic proton doublet at δ 6.75, J = 4.0 Hz coupled to another proton doublet at δ 7.32, J = 4.0 Hz and an additional one-proton singlet at δ 7.54. Additional signals were one-proton singlet 9.69 (aldehyde) and three-proton singlet at δ 1.96 (methyl protons of acetate or methyl ketone). Complex signals were superimposed on the three-proton singlet (δ 1.96) and the methoxy region (δ 3.85 to 4.05). In the NMR spectrum of versiconol tetramethyl ether and the diacetate of versiconol tetramethyl ether, these same regions contained signals for the protons H_a, H_b, H_c, and H_d (III).



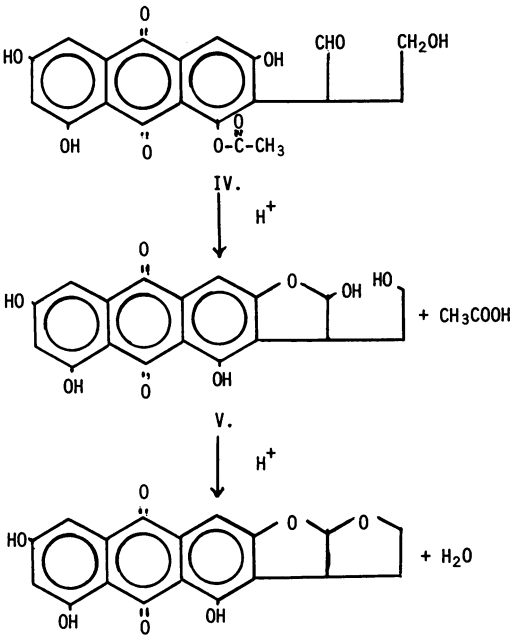
Versiconol (III)

A signal comparable to that of H_c was present in the spectrum of the orange pigment (two-proton triplet at δ 4.08 compared to signal at about δ 4.00 in the spectrum of the diacetate of versiconol tetramethyl ether). The protons comparable to the H_b protons could have been superimposed on the signal at δ 1.96 since it integrated for four or five protons instead of three protons. The protons comparable to H_d appeared to be absent in the spectrum of the orange pigment and its methylated derivative. The H_a protons were not visible in the spectrum of versiconol tetramethyl ether or the diacetate derivative of versiconol tetramethyl ether. This could presumably be the case for the comparable protons in the orange pigment.

The IR, UV, and mass spectra of the hydrolysis product obtained after acetylation of the orange pigment and the NMR of its methylated derivative identify the hydrolysis product as versicolorin C. Additional confirmation is provided by an R_f value for this hydrolysis product on TLC identical to authentic versicolorin C. Thus, the acetylated orange pigment was converted to versicolorin C under acid conditions.

The acetylated product gives a mass spectrum consistent with that expected for the acetate of versicolorin C. On the other hand, the methylation product contains two additional oxygens. The NMR spectra of the methylated product show the presence of $CH_3C{=}O$ (81.98) and CHO (89.69) groups attached to the molecule and the absence of the tetrahydro-difurano ring system of versicolorin.

The conversion of the orange pigment to versicolorin C under acid conditions is consistent with an acetal reaction for structure IV in the following pathway.



VI. Versicolorin C

Acetylation conditions are probably sufficient to convert IV to versicolorin acetate. Thus IV is suggested as the probable structure of the orange pigment with the possible exception that the acetyl group may be located on one of the other phenolic OH groups or on the alcohol group. By analogy to the structure for versiconol (10), the orange pigment can be tentatively designated as "versiconal acetate."

Biollaz, Buchi, and Milne (1) reported that aflatoxins can be derived from naphthacene derivatives and proposed a scheme that derives sterigmatocystin, aflatoxins, and the versicolorins from a common precursor. Our data indicate that the elaboration of the anthraquinone precursor proceeds normally in the presence of dichlorvos but that further development into aflatoxin or sterigmatocystin is greatly reduced, leaving the anthraquinone precursor to accumulate. Thus dichlorvos partially blocks the elaboration of sterigmatocystin and aflatoxin at that point in the metabolic pathway. This theory is further substantiated by the reduction in the production of *O*-methylsterigmatocystin by *A. flavus*, isolate P-70-41b, and the corresponding accumulation of the same anthraquinone precursor.

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