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Purification and Analysis of Versiconal Hemiacetal Acetate and Versicolorin A Using Low-Pressure Liquid Chromatography and High-Performance Liquid Chromatography

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To elucidate the enzymatic conversion of versiconal hemiacetal acetate (VHA) in the biosynthesis of aflatoxin B_1 (AFB) by *Aspergillus parasiticus*, methods were adapted for the purification and analysis of this and the related intermediate versicolorin A (VA). Following production of these biosynthetic intermediates, VHA and VA were purified on a low-pressure silica gel column developed with dichloromethane-based solvent systems. Both compounds were pure as determined by thin-layer chromatography in several solvent systems. VHA was quantitated with molar extinction coefficients established in an acidified ethanol solution. Using reversed-phase high-performance liquid chromatography (HPLC), two separation systems were developed. An isocratic HPLC solvent system consisting of methanol/acetonitrile/0.053 N glacial acetic acid in water (42.5:42.5:15) (q.s.) was successful in separating VHA, VA, and related compounds. The retention times for VHA and VA were 2.31 and 3.26 min, respectively. An isocratic solvent system of acetonitrile/0.053 N glacial acetic acid in water (55:45) was required to separate a product of the enzyme-catalyzed reaction, tentatively identified as versicolorin C (VC), from VA. With this solvent system, the retention times for VHA, VC, and VA were 3.72, 7.12, and 7.82 min, respectively. This HPLC system offers a rapid and sensitive assay for VHA conversion in an *A. parasiticus* cell-free extract.

Versicolorin A (VA), sterigmatocystin (ST), and aflatoxin B_1 (AFB) are biogenetically related mycotoxins formed by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Singh and Hsieh, 1977). They have been found carcinogenic in laboratory animals (Hendricks et al., 1980; Wogan et al., 1974), and AFB is implicated in the etiology of human liver cancer in some African and Asian countries (Van Rensburg et al., 1985). The health significance of AFB has prompted active investigation into its biosynthesis in the toxigenic fungi for possible control of the production of these mycotoxins in foodstuffs such as grains and nuts (Bennett and Christensen, 1983). With use of radiotracer techniques in conjunction with mutants and metabolic inhibitors, and also cell-free studies, it has been established that the sequence of biosynthesis for known intermediates is acetate → norsolorinic acid → averantin → averufanin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin → *O*-methylsterigmatocystin → aflatoxin B_1 (Anderson and Dutton, 1980; Bennett et al., 1980; Bhatnagar et al., 1987; Cleveland et al., 1987; Hsieh et al., 1973, 1976b; Hsieh and Mateles, 1970; Lee et al., 1976; Lin et al., 1973; McCormick et al., 1987; Singh and Hsieh, 1976, 1977; Wan and Hsieh, 1980) (Figure 1). Of particular interest in this pathway are the enzymatic reactions involved in the conversion of VHA to VA (Wan and Hsieh, 1980; Anderson and Dutton, 1980), thereby creating the bisfuran portion of the molecule (Hsieh et al., 1976a) and converting a compound with negligible mutagenicity to one with substantial mutagenicity (Wong et al., 1977). As part of the effort to monitor and characterize these enzyme reactions, methods for the efficient production, purification, and analysis of these compounds were developed.

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MATERIALS AND METHODS

Organisms. Both an aflatoxin-producing strain, *A. parasiticus* ATCC 15517, and a versicolorin A accumulating mutant, *A. parasiticus* ATCC 36537 (Lee et al., 1975), were used in this study. The latter was suppled by Dr. J. W. Bennett of Tulane University, New Orleans, LA. Fungal conidia were harvested from well-sporulated cultures on potato dextrose agar (Difco Laboratories, Detroit, MI) and stored at 4 °C in a 0.01% sodium lauryl sulfate solution for up to 3 months.

Chemicals and Solvents. The dichloromethane, hexane, and methanol used for low-pressure liquid chromatography were Baker Resi-Analyzed grade (J. T. Baker Chemical Co., Phillipsburgh, NJ). The formic acid was ACS reagent grade supplied by Allied Chemical (Morristown, NJ). Acetic acid was ACS reagent grade obtained from Fisher Scientic Co. (Fair Lawn, NJ). The water and methanol used for HPLC analysis were HPLC grade from Fisher, and HPLC-grade acetonitrile was obtained from J. T. Baker. Dichlorovos was obtained from Chem Service (West Chester, PA).

Production and Extraction of VHA and VA. VHA is accumulated in the mycelium of *A. parasiticus* when the aflatoxin-producing cultures are treated with the pesticide and cholinesterase inhibitor dichlorovos (phosphoric acid, 2,2-dichloroethenyl dimethyl ester) (Yao and Hsieh, 1974). To produce VHA, 1×10^7 *A. parasiticus* ATCC 15517 conidia were added to 100 mL of minimum mineral (MM) medium (Adye and Mateles, 1964) in 500-mL baffled flasks (Bellco Glass Inc., Vineland, NJ) and incubated at 30 °C, 100 rpm for 24 h and then at 200 rpm for 24 h. The mycelium was then transferred to 100 mL of

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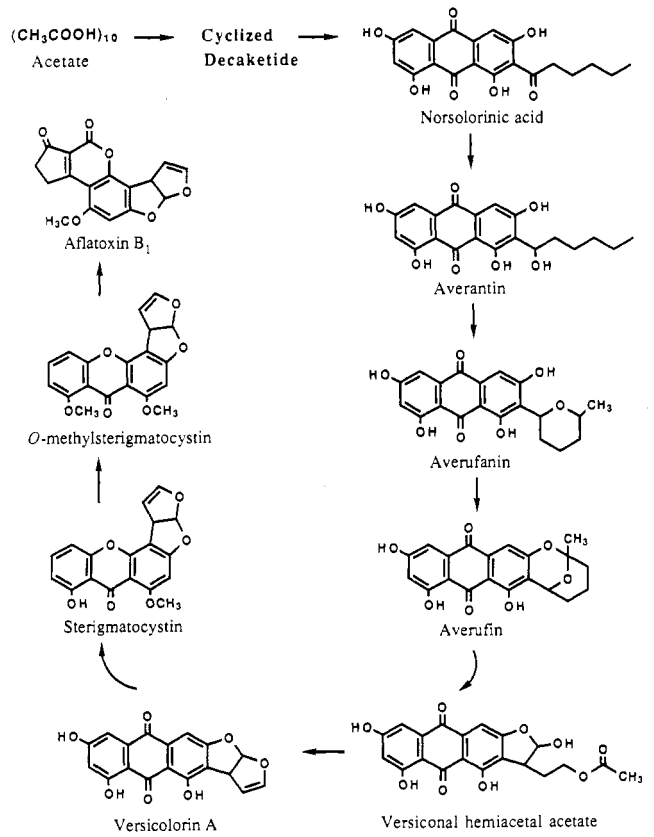

Figure 1. Currently accepted pathway of aflatoxin biosynthesis.

modified nitrogen-free replacement medium (RM) containing 36 g/L glucose rather than the 15 g/L called for in the original medium (Adye and Mateles, 1964), again in the baffled flasks. The replacement medium also contained 20 ppm (20 μg/mL) dichlorovos and 15 g of cells (wet weight)/100 mL of medium, with a procedure modified from Yao and Hsieh (1974). The dichlorovos stock was prepared as a stock solution of 2000 ppm dichlorovos (2000 μg/mL) in 95% ethanol, and 1 mL of the stock was added to 100 mL of RM. The cultures were further incubated at 30 °C, 200 rpm, for 48 h.

Production of VA was accomplished by adding 1×10^7 *A. parasiticus* ATCC 36537 conidia to 100 mL of YES medium (Davis et al., 1966), consisting of 20% sucrose in 2% yeast extract, to 500-mL flasks. The fungus was cultured statically at room temperature, and the mycelia were harvested in 10 days, after a confluent mat of dark orange pigmented mycelia was obtained.

The mycelia were harvested by vacuum filtration, pooled and exhaustively extracted with acetone. To this acetone extract was added water to 66% of the total volume of the mixture. The pigments in the acetone/water mixture were partitioned into chloroform. The chloroform extract was dried by passage through anhydrous sodium sulfate and concentrated under vacuum.

Purification of VHA and VA. (*a*) *Equipment.* The low-pressure liquid chromatography system used to purify VHA and VA was essentially that described by Hsieh et al. (1986) for the purification of aflatoxin M_1 and consisted of a Lab Data Control Division Minipump (Milton Roy Co., Riviera Beach, FL), a Fluid Metering Inc. Model PD-60-LF pulse dampener (Oyster Bay, NY), a Rheodyne Model 5041 injector (Cotati, CA) with a 5-mL sample loop, a prepacked silica gel 60 Lobar column (310 mm × 25 mm (i.d.)) (EM Industries Inc., Cherry Hill, NJ), and a hand-held Model UVL-21 Blak-Ray lamp at 366 nm (San Gabriel, CA) for detection. Before injection, the column was conditioned with two to three column volumes of running solvent. After each use, the column was reconditioned by passing two bed volumes of methanol through the column.

(*b*) *Purification of VHA.* The running solvent for VHA purification consisted of dichloromethane/methanol/formic acid (99:2:0.15). For each purification, the extract was loaded onto the system in a 5-mL volume of running solvent, containing ca. 1 mg of VHA. At a flow rate of 4 mL/min, VHA retention time was ca. 60 min. The VHA was detected by its yellow/orange fluorescence under ultraviolet light. The VHA fraction was collected and the volume reduced under vacuum.

(*c*) *Purification of VA.* The running solvent for VA purification consisted of dichloromethane/hexane/methanol/acetic acid (80:20:0.4:3). The extract was loaded onto the system in a 5-mL volume, containing 0.3 mg of VA. At a flow rate of 4 mL/min, VA retention time is ca. 50 min. The compound was detected by its orange/yellow fluorescence under ultraviolet light. The VA fraction was collected and the volume reduced under vacuum.

Table I. Ultraviolet/Visible Absorption Maxima and Molar Extinction Coefficients for VHA under Acidic, Neutral, and Basic Conditions, Determined As Described under Materials and Methods

basic solution		neutral solution		acidic solution	
max, nm	Σ max	max, nm	Σ max	max, nm	Σ max
550	4650	510	4130	453	5740
400	4470	316	18600	325	5490
325	33100	262	12500	291	20900
264	9360	252 (sh)	10200	268 (sh)	11400
232	15200	221	18800	255 (sh)	9820
				223	21600

Spectrophotometric Data for VHA. The ultraviolet/visible spectrum of VHA was obtained under basic (1×10^{-3} M sodium hydroxide in 95% ethanol), neutral (compound dissolved in 95% ethanol, nothing else added), and acidic (1×10^{-3} M hydrochloric acid in 95% ethanol) conditions on a Cary recording ultraviolet-visible spectrophotometer, Model 219 (Varian Instrument Division, Palo Alto, CA). To determine the extinction coefficients for the compound under these three conditions, VHA was weighed on a Cahn Model 4700 automatic electrobalance (Cahn Instruments, Cerritos, CA).

Analysis of VHA and VA by HPLC. Samples were analyzed on a Waters Associates liquid chromatograph (Milford, MA), consisting of two Model 510 pumps, a Model 680 automated gradient controller, a Model 481 Lambda-Max variable-wavelength UV–vis detector, and a U6K septumless injector. The components were quantified on a Hewlett-Packard HP3390C plotting integrator (Palo Alto, CA). The column was an EM Science Hibar EC 5-μm C_{18} reversed-phase column (25 cm × 4 mm (i.d.) stainless steel) (EM Industries Inc., Cherry Hill, NJ) and the guard column a Brownlee Labs NewGuard holder containing a Brownlee Labs Aquapore ODS 7-μm C_{18} spherical cartridge (1.5 cm × 3.2 mm (i.d.)) (Brownlee Labs, Santa Clara, CA). Two isocratic solvent systems were used during the course of the investigation. The first was capable of separating AFB, VHA, ST, and VA, consisting of methanol/acetonitrile/0.053 N glacial acetic acid in water (42.5:42.5:15) (q.s.). A second solvent system was required to separate a compound tentatively identified as versicolorin C (VC) from VA, consisting of acetonitrile/0.053 N glacial acetic acid in water (55:45). The detector was operated at 222 or 288 nm depending on sample composition. Attenuation was set through the recorder/integrator at 8–16 mV maximum signal voltage at full-scale deflection, depending on the sensitivity required. This is approximately equivalent to 0.01–0.02 AUFS.

RESULTS

VHA and VA Production and Purification. From 100 mL of nitrogen-free resting cell medium (containing 15 g of cells, wet weight) 5 mg of VHA was obtained. Approximately 16 mg of VA was produced/100 mL of YES medium, using the culture technique described above.

Spectrophotometric Data for VHA. The absorption spectrum of VHA is most reproducible when the compound is dissolved in a solvent with a hydrogen ion concentration of 10^{-2}–10^{-3} M. The extinction coefficients obtained for VHA under basic, neutral, and acidic conditions are shown in Table I. The data for VHA in basic solution are not reproducible as the compound is not stable under oxidative conditions and should not be used for quantitation.

Analysis of VHA and VA by HPLC. The isocratic solvent system consisting of methanol/acetonitrile/0.053

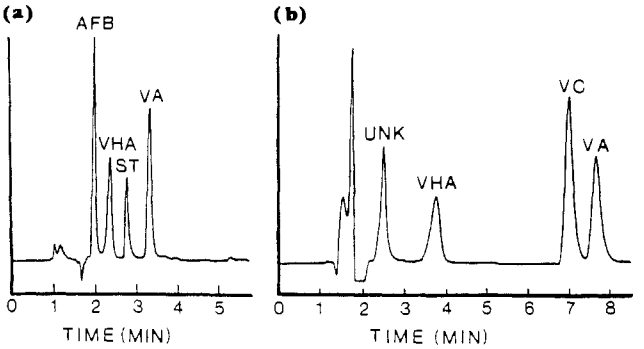

Figure 2. Chromatogram of AFB and intermediates of interest: (a) reference standard mixture containing 10 ng of AFB, VHA, ST, and VA each; (b) mixture containing 20 ng of VHA and VA along with the two enzymatically formed compounds, one of unknown structure and one tentatively identified as VC. Conditions are given under Materials and Methods.

N glacial acetic acid in water (42.5:42.5:15) (q.s.) was effective in separating the components of interest. A small amount of acetic acid in the running solvent improved the peak shapes of VHA and VA evidently by suppressing ionization of these acidic polyhydroxyanthraquinones (Kingston and Chen, 1976). At a solvent flow rate of 1.5 mL/min, the retention times (min) of the metabolites of interest were as follows: AFB, 1.92: VHA, 2.31; ST, 2.72; VA, 3.26. Figure 2a shows a typical chromatogram with detection by absorbance at 222 nm. Separation of a compound formed upon addition of VHA to an *A. parasiticus* cell-free extract and extracted under acidic conditions, tentatively identified as VC by its mass spectrum (Cole and Cox, 1981), required a different separation method as it was found to cochromatograph with VA under the previous conditions. For this separation the acetonitrile-/0.053 N glacial acetic acid in water (55:45) solvent system was used, again at a flow rate of 1.5 mL/min. The retention times (min) were as follows: VHA, 3.72; VC, 7.12; VA, 7.82. Detection sensitivity for VHA, VC, and VA was enhanced by monitoring absorbance at 288 nm rather than 222 nm. This separation is shown in Figure 2b. In addition to the compound tentatively identified as VC, also seen in the chromatogram is an unknown compound with a retention time of 2.63 min, formed during the enzymatic reaction. This compound is thought to be a polyhydroxyanthraquinone that is converted to VC under acidic conditions. The precise enzymatic conditions that lead to the formation of these two compounds from VHA will be discussed in a forthcoming paper.

DISCUSSION

While the yield of VHA by the replacement culture method is not exceptionally high, the crude extract was free of pigments that can interfere with VHA purification. Additionally, only small amounts of AFB are present in the acetone extract of the mycelium, rendering the purification safer and less tedious. Until recently, thin-layer chromatography (TLC) has been the most commonly used method for purification of these compounds; however, it is limited by the capacity of the plates and the time and problems associated with recovering carcinogens such as VA, ST, and AFB from the plate. HPLC is another method used with some frequency, although it is limited by the initial expense of the system and low capacities, unless semipreparative or preparative systems are used. The low-pressure LC system for the purification of VHA and VA established a practical and inexpensive method for the preparation of milligram quantities of these aflatoxin biosynthetic intermediates. The components for this low-pressure LC system are relatively inexpensive and therefore offer an economical solution when investigating a method for the purification of these compounds. As the elution times using this system are approximately 1 h, it is possible to complete several purifications per day. Additionally, the system is versatile, as the solvent composition can be modified for the purification of other aflatoxin biosynthetic intermediates.

It was observed during the purification of VHA that the molar extinction coefficients cited for VHA in the literature could not be reproduced, although the compound was pure and its identity was confirmed by mass spectrometry. Also, the cited values in the literature do not agree among themselves (Steyn et al., 1979; Yao and Hsieh., 1974; Schroeder et al., 1974; Anderson and Dutton, 1980). The values cited by Yao and Hsieh (1974) and Schroeder et al. (1974), both determined in ethanol, agree well, assuming a typographical error in one of the absorbance maximum wavelengths cited by Yao and Hsieh (1974). However, these values do not agree with those determined by Steyn et al. (1979) where it is stated "Schroeder et al. ... reported the long wavelength absorption erroneously as 480 nm (log Σ 3.86)". Steyn et al. (1979) cite the long-wavelength absorption as 452 nm, which compares favorably with the absorption maximum of 453 nm determined by us in acidic solution. Steyn et al. (1979) also reports an absorbance at 315 nm with an Σ value of 10 200 in methanol, whereas Yao and Hsieh (1974) report an absorbance at 312 nm with an Σ of 17 100 in methanol. Anderson and Dutton (1980) determined an absorbance at 450 nm and an Σ of 7400 but did not state the nature of the solvent. The extinction coefficients and absorbance maxima we determined in acidic solvent agree reasonably well with all of the values cited, except that determined by Yao and Hsieh (1974). In this case the absorbance maximum at 312 nm (Σ 17 100) agrees well with the absorbance at 316 nm (Σ 18 600) determined by us using neutral ethanol as the solvent. As both VHA and VA are usually purified using solvent systems containing 3–5% of a relatively nonvolatile acid such as acetic acid for ion suppression and improved chromatography, the absorption spectrum of these pigments may be unknowingly determined under acidic, rather than neutral, conditions. This could explain the disparity in the extinction coefficient values cited in the literature. We have observed that the absorption spectrum of VHA changes quite abruptly with small changes in the hydrogen ion concentration of the solvent. However, reproducible results can be obtained if the spectrum is always obtained under mildly acidic conditions, as the absorption spectrum does not change with a hydrogen ion concentration of 10^{-3} M or greater, within reasonable limits.

The HPLC system used in the present study is a convenient, rapid, and sensitive method for assaying for the enzymatic conversion of VHA in cell-free extracts of *A. parasiticus*. Previously cited methods for the separation of various aflatoxins and their biosynthetic intermediates (Kingston and Chen, 1976; Berry et al., 1984) either require gradient capabilities and temperature control or result in unacceptably long retention times. During the preparation of this manuscript, a paper by McCormick et al. (1988) appeared which describes an HPLC procedure for determining the chromatographic profile of aflatoxin precursors. This procedure, using a binary solvent gradient system, would have been able to separate VHA, VA, and VC. The separation systems described in the present communication are isocratic and are conducted at room temperature. The methods we have developed will facilitate further investigation into the enzymology of the conversion of

VHA to VA. This is the step in the pathway of aflatoxin biosynthesis where formation of the bisfuran ring system, shown to be required for the mutagenicity and carcinogenicity of the bisfuranoid compounds, occurs.

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Registry No. VHA, 62886-00-4; VA, 6807-96-1; AFB, 1162-65-8; VC, 16049-49-3.

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