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Note

Separation of aflatoxin biosynthetic intermediates by high-performance liquid chromatography

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During the investigation of the biosynthesis of aflatoxin B_1 (AF) by cell-free extracts of *Aspergillus flavus*, various putative precursors and their products had to be separated and quantified. The precursors include the anthraquinones norsolorinic acid (NA), averufin (AVF), versiconal hemiacetal acetate (VHA), versicolorin A (VA), and the xanthone derivative sterigmatocystin (ST). The structures of these compounds are shown in Fig. 1.

Previous studies[1–6] had used thin-layer chromatography for the separation of these metabolites, but even using two-dimensional systems it is not possible to separate all of them; in particular the AVF–VA pair is difficult to resolve. For this reason a rapid and effective separation was sought which could also afford a quantification of the amounts present. These criteria were ideally met by high-performance liquid chromatography (HPLC), and a method of separation was developed which is described here.

EXPERIMENTAL

Materials and reagents

Standards were obtained by purifying metabolites from various strains and mutants of *Aspergillus* species[7,8] and aflatoxin B_1 was obtained from Sigma (St. Louis, MO, U.S.A.).

All organic solvents were of analytical reagent grade, and HPLC-grade water was obtained by passing water previously purified by a Millipore R/Q system (Millipore, Bedford, MA, U.S.A.) through a Millipore Norganic cartrige.

Apparatus

A Varian Model 5000 liquid chromatograph Aerograph Operations, Walnut Creek, CA, U.S.A.) fitted with a column oven and a Varian Vari-Chrom UV–Visible detector (operated at 325 nm and 0.1 a.u.f.s.), was used. A Hewlett-Packard HP3390A plotting integrator (Palo Alto, CA, U.S.A.) was used to calculate peak areas and component concentrations. The column was a Spherisorb S5ODS1 5-μm reversed-phase column (25 cm × 4.5 mm I.D. stainless steel) (Phase Separations, Clwyd, U.K.), and was preceded by a guard column dry-packed with 40-μm Vydac RP (4 cm × 4 mm I.D.) (Varian). Samples (0.5–10.0 μl) were injected onto the column by means of a six-port loop injector.

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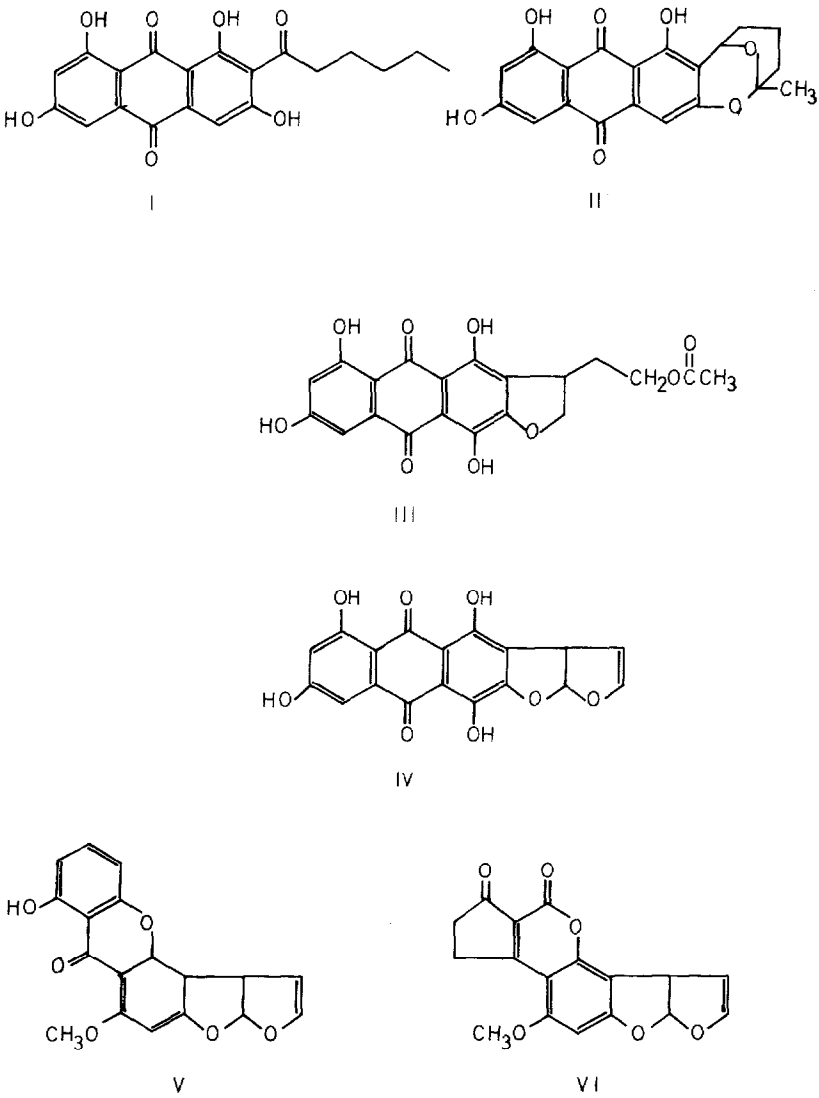

Fig. 1. Structures of aflatoxin biosynthetic intermediates. I = Norsolorinic acid; II = averufin; III = versiconal hemiacetal acetate; IV = versicolorin A; V = sterigmatocystin; VI = aflatoxin B_1.

RESULTS AND DISCUSSION

Of several solvent systems investigated, a ternary solvent system consisting of methanol–tetrahydrofuran (2:1, Solvent A) and water (Solvent B) at a flow-rate of 2.0 ml/min and a temperature of 50°C with a linear gradient from 45% B to 25% B over 6 min was found to effect the separation.

A typical chromatogram is shown in Fig. 2. Retention times (min) were: aflatoxin B_1, 1.92 ± 0.04; VHA, 2.67 ± 0.24; ST, 3.89 ± 0.09; VA, 5.93 ± 0.22; AVF,

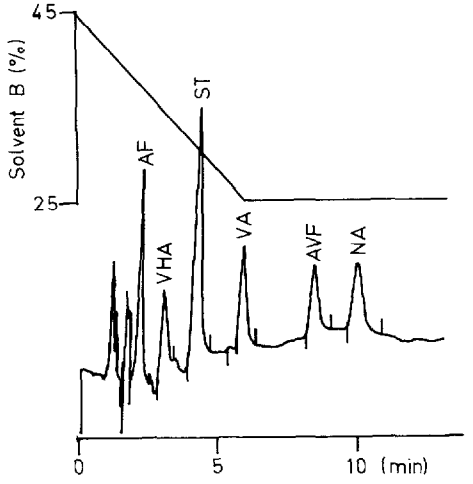

Fig. 2. Chromatogram of aflatoxin biosynthetic intermediates: aflatoxin B_1 (AF), versiconal hemiacetal acetate (VHA), sterigmatocystin (ST), versicolorin A (VA), averufin (AVF), norsolorinic acid (NA). Solvent A = methanol–tetrahydrofuran (2:1); solvent B = water. Gradient, 45% B to 25% B over 6 min; flow-rate, 2 cm^3 min^{-1}; temperature, 50°C.

8.52 ± 0.21; NA, 10.05 ± 0.31. These times are the means of 12 runs over a period of 30 days; their reproducibility was excellent, and quantification was reproducible to 2% at the 5–20-μg level.

Although this solvent system gave a good separation of all of the components, it was occasionally desirable to separate only some of them. In particular, we were interested in separating AVF from VA and VHA, and ST from aflatoxin B_1. In the former case, an isocratic solvent consisting of acetonitrile–tetrahydrofuran–water (25:20:55) gave a good separation, with the components eluting in the following times (min): VHA, 2.12; VA, 2.98; AVF, 5.07. It should be noted that aflatoxin B_1 coelutes with VA under these conditions.

An isocratic solvent system of acetonitrile–water (1:1) gave a convenient separation of ST (retention time 5.87 min) and aflatoxin B_1 (2.50 min). Under these conditions VA eluted in 3.87 min.

If it is necessary to separate the major aflatoxins, this can be achieved by using an isocratic system consisting of acetonitrile–methanol (3:2, v/v) (45%) and water (55%). The following retention times (min) were obtained: aflatoxin G_2, 3.00; aflatoxin G_1, 3.22; aflatoxin B_2, 3.61; aflatoxin B_1, 4.00.

In order to demonstrate the effectiveness of the system reported here, chromatographs of crude extracts from *A. parasiticus* strain W49 (Donkersloot), which accumulates AVF, and strain 1-11-105 Wh1 (Bennett), which accumulates VA are shown in Figs. 3 and 4, respectively.

Separation of the major aflatoxins was achieved by using an isocratic system consisting of acetonitrile–methanol (3:2) (45%) and water (55%). The following retention times (min) were obtained: aflatoxin G_2, 3.00; aflatoxin G_1, 3.27; aflatoxin B_2, 3.61; and aflatoxin B_1, 4.00.

The metabolites O-methylsterigmatocystin (OMS), dihydrosterigmatocystin

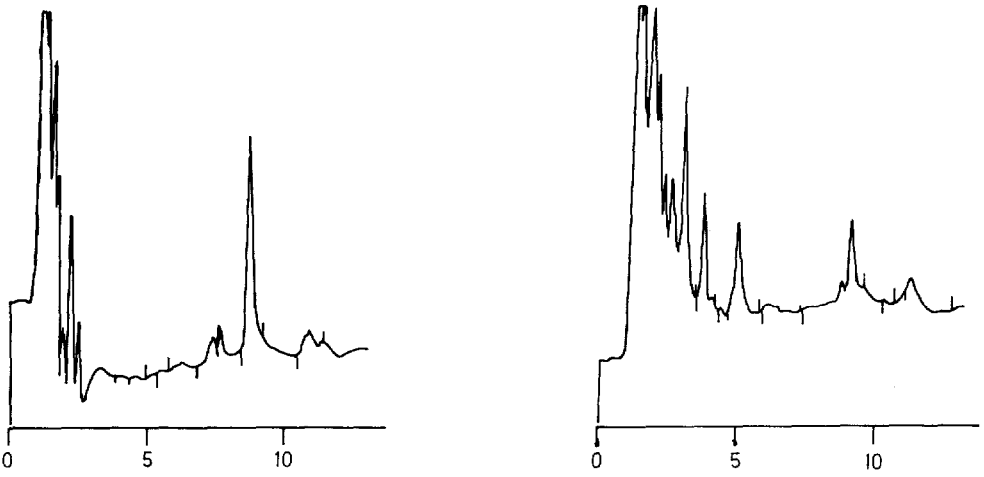

Fig. 3. Chromatogram of a crude extract from *A. parasiticus* strain W49. Chromatographic conditions as for Fig. 2.

Fig. 4. Chromatogram of a crude extract from *A. parasiticus* strain 1-11-105 Wh 1. Chromatographic conditions as for Fig. 2.

(H_2-ST), averantin and versicolorin C were tested on the methanol–tetrahydrofuran system, and it was found that a good separation of OMS, H_2-ST and ST was obtained, with retention times (min) of 2.57, 3.63 and 4.03, respectively. Averantin could be resolved from VA and AVF, with retention times (min) of 7.95, 5.95 and 8.26 respectively. Versicolorin C, however, could not be resolved from VA using this solvent system.

These solvent systems have therefore proved useful as a means of investigating aflatoxin biosynthetic intermediates quickly and accurately.

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