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Naphtho-γ-Pyrone Production by *Aspergillus niger* Isolated from Stored Cottonseed

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***Aspergillus niger* was found to be the predominant fungal contaminant of stored cottonseed. Seven strains were isolated and grown on rice. The hexane-insoluble material from methylene chloride extracts of 2-week-old cultures contained components toxic to mice. Based on high-pressure thin-layer and liquid chromatographic analyses, the major components in the mixture were eight different naphtho-γ-pyrones. Of these, the hydrated dimeric naphthopyrones aurasperones B and C occurred in higher yield than aurasperones A, iso-A, and D and the monomeric naphthopyrones flavasperone and rubrofusarin, all of which were present in the mixture. In addition, fonsecin monomethyl ether was isolated. This metabolite may be a precursor in the biosynthesis of the hydrated aurasperones; it has not been identified previously as a metabolite of *A. niger*. The relative amounts of the different naphthopyrones were dependent on both the growth substrate and the fungal isolate.**

Tanaka and co-workers (8–10) have isolated from the mycelium of *Aspergillus niger* several yellow pigments which have the structure of dimeric linear naphtho-γ-pyrones (Fig. 1). Besides isolating the dimeric naphtho-γ-pyrones aurasperone A (IVa), aurasperone B (Va), and aurasperone C (Vb), Tanaka et al. (9) also have found the monomeric naphthopyrones rubrofusarin (II) and flavasperone (I). Recently, Ghosal and co-workers (5) found several new types of aurasperones (aurasperones D [IVc] and E [Vc] and isoaurasperone A [IVb]) and showed that the aurasperones are acutely toxic to mice and rats, acting mainly as central nervous system depressants. They also observed that the presence of mango in the culture medium induced naphtho-γ-pyrone production by their particular strain of *A. niger*. Gorst-Allman et al. (7) have found that a strain of *A. niger* isolated from Mozambican ground nuts produces dimeric naphthopyrones (nigerone [VIa] and nigerone methyl ether [VIb]) having a C-10–C-10′ linkage rather than the C-10–C-7′ linkage found in the aurasperones.

Since *A. niger* is one of the most common fungal contaminants of stored foods (3, 6), the presence of high levels of toxic naphtho-γ-pyrones could present a serious health hazard to animals and people who might consume contaminated foodstuffs. In the present study, we report the development of thin-layer and high-pressure liquid chromatographic methods for the examination of naphtho-γ-pyrones produced by different *A. niger* isolates. In addition, we have isolated fonsecin monomethyl ether (IIIb) as a natural metabolite of *A. niger* and suggest this metabolite is a precursor to aurasperones B (Va) and C (Vb).

MATERIALS AND METHODS

Isolation of *A. niger* from cottonseed. Cottonseed was stored for at least 1 year (3). The seed was surface sterilized by immersion in a 2% sodium hypochlorite solution (bleach) for 2 min, followed by rinsing several times with sterile, distilled water. The surface-sterilized seeds were then transferred to petri plates containing potato glucose agar and cultured at 27°C for periods of up to 7 days. The predominant fungus isolated was *A. niger*. Autoclaved seeds showed no fungal outgrowth, indicating that the *A. niger* was from internal contamination of the seeds and did not arise from laboratory contamination after surface sterilization. The *A. niger* cultures isolated were maintained on slants of potato glucose agar kept at 5°C. Cultures were also maintained in lyophil as part of the Southern Regional Research Center (SRRC) culture collection.

Isolation of naphtho-γ-pyrones. For isolation of naphtho-γ-pyrones the *A. niger* isolates were subcultured on five different media: rice, mango extract in Richards medium (5), corn, cottonseed, or yeast extract-sucrose (YES) medium. The rice, corn, and cottonseed (300 g per Fernbach flask) were autoclaved with 150, 110, and 110 ml of water, respectively, before addition of approximately 10^8 spores (hydrated with 5 ml of 0.1% Tween 20) of *A. niger* from a single agar slant. The cultures were incubated for 14 days at 27°C before extraction. Mango extract medium was prepared as described by Ghosal et al. (5), using 150 g of fresh mango per liter of Richards medium (potassium nitrate, 10 g; potassium monobasic phosphate, 5 g; magnesium sulfate, 2.5 g; ferric chloride, 0.02 g; and sucrose, 50 g [per liter]). YES medium contained 40 g of sucrose and 20 g of yeast extract per liter. All cultures were incubated without shaking.

After 14 days of incubation, the *A. niger* culture (in the case of liquid culture, the mycelial mat) was soaked twice overnight in 1.5 liters of methylene chloride per flask. The methylene chloride was removed on a Büchi rotary evaporator, and the residual red paste was treated with 9 volumes (vol/wt) of cold hexane and kept at 5°C overnight. The red precipitate was collected, dissolved in 20 ml of methylene chloride, and filtered. Samples were examined by high-pressure thin-layer chromatography (HPTLC). For high-pressure liquid chromatography (HPLC) analysis, the methylene chloride was removed and a portion was redissolved in acetonitrile. In rice cultures the yield of hexane-insoluble material was 0.65 g/kg, of which almost 85% was a mixture of naphtho-γ-pyrones.

The individual naphtho-γ-pyrones were isolated and purified by HPTLC (see Fig. 2) and HPLC (see Fig. 3) and were identified by their melting points, migration and staining behavior on thin-layer chromatography (TLC) (9), and infrared, UV, nuclear magnetic resonance, and mass spectra. The analytical data agreed with reported values for the pertinent naphthopyrones. Toxicity tests were performed as described previously (3).

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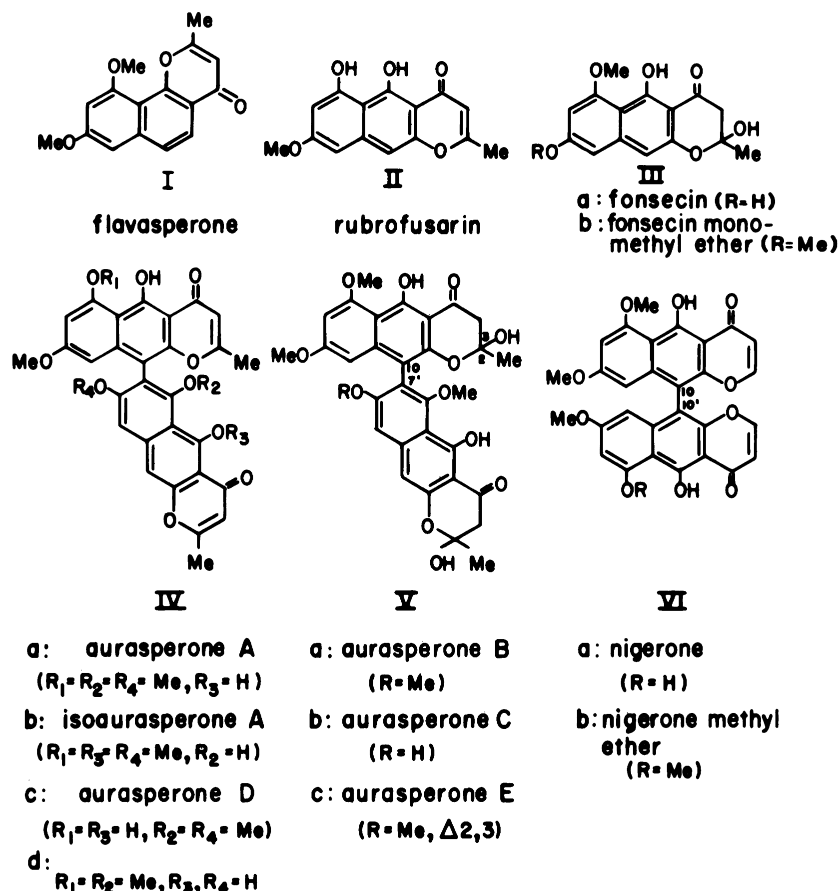

FIG. 1. Chemical structures of naphthopyrones from *A. niger*.

Analytical methods. Mass spectra were obtained at 70 eV on a Finnegan MAT (Cincinnati, Ohio) model 4000 gas chromatograph-mass spectrometer. Samples were introduced by direct inlet probe at room temperature and then heated to 250°C at a rate of 15°/min (ballistic mode). HPTLC was carried out on plates (LHP-KF; 10 by 10 cm) obtained from Whatman, Inc., Westbury, N.Y., and developed with benzene-ethyl acetate-formic acid (10:4:1). Components were identified by their color, fluorescence under long-wavelength UV light (Mineralight), quenching of UV absorption when visualized under short-wavelength UV light, and color when plates were stained with the Gibbs reagent spray (8, 9). HPLC was on a LiChrosorb RP 18 column (10-μm particle size, 0.5 by 25 cm; Brownlee Labs, Santa Clara, Calif.) protected by a Whatman Guard column (0.5 by 3 cm) containing Perisorb RP 18 silica (30 to 40 μm; Merck & Co., Inc., Rahway, N.J.). The components were eluted at ambient temperature at 1.5 ml/min, using a linear gradient (curve 6 of the Waters model 2000 solvent programmer) of 0.1 M acetic acid-acetonitrile (6:4) to 0.1 M acetic acid-acetonitrile (4:6) in 10 min. Solvents were pumped by two Waters 6000 A pumps interfaced with the solvent programmer. The eluent was continuously monitored with a Waters 440 UV detector at either 340 or 405 nm. The maximum sensitivity used was 0.05 absorbance unit full scale. The UV detector was interfaced with a Hewlett-Packard HP1000 computer programmed to calculate peak areas and retention times. The 0.1 M acetic acid solvent was filtered through a 0.45-μm nitrocellulose filter (Millipore Corp., Bedford, Mass.) before use. Samples in acetonitrile were either filtered through a 0.45-μm Teflon filter (Millipore) or centrifuged at 3,000 × *g* for 10 min before being loaded into the Waters U6K injector. Samples loaded were typically 10 to 25 μl containing 10 to 1,000 ng of material.

TABLE 1. Toxicity of crude naphtho-γ-pyrone material

Sample[a]	Approx LD_{50}[b] (mg/kg)	Approx time to death after injection (days)
YES medium	50	1
Rice	112	1
Mango	110	2
Cottonseed	55	14

[a] Samples were the hexane-insoluble material from a methylene chloride extract of *A. niger* SRRC 3583 grown on the different substrates for 14 days. Crude extract was dissolved in dimethyl sulfoxide for intraperitoneal injection into 20-g male ICR albino mice. Injection of an equivalent amount of dimethyl sulfoxide gave no toxicity.

[b] LD_{50}, 50% lethal dose. Five mice were used for each dose.

RESULTS AND DISCUSSION

The hexane-insoluble residue from methylene chloride extraction of the *A. niger* cultures contained a mixture of yellow pigments which were acutely toxic to mice at doses ranging from 50 to 100 mg/kg of body weight (Table 1). This mixture may contain malformins previously shown to be highly toxic metabolic products of *A. niger* (1). Analysis by

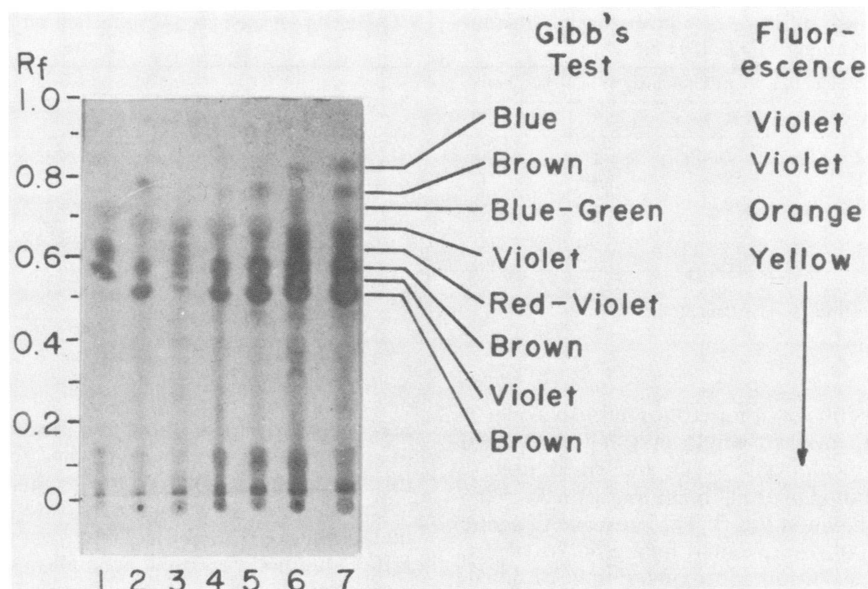

FIG. 2. Separation of naphthopyrones by HPTLC. Naphthopyrones were visualized with the Gibbs reagent spray. The samples used were the mixtures obtained after recovery of hexane-insoluble material from the *A. niger* methylene chloride extracts. Lanes 1 through 7 contained extracts from *A. niger* SRRC 3586, SRRC 3513, SRRC 3514, SRRC 3581, SRRC 3583, SRRC 397, and SRRC 393, respectively. Quantitative data for some of these samples are given in Table 4, and R_f values for purified components are given in Table 2.

HPTLC showed that the mixture contained more than 18 components (Fig. 2). Based on its UV spectra and staining properties, only the material migrating at R_f = 0.5 to 0.8 contained naphtho-γ-pyrones. At least eight naphthopyrone components could be distinguished by HPTLC. Those components that stained brown in the Gibbs test had UV spectra similar to that of fonsecin (IIIa), with absorptions at λ_{max} = 405, 335, 322, 270, and 230 nm (4). Except for flavasperone (I) (2), which migrated at R_f = 0.81, the components had UV spectra more characteristic of linear naphtho-γ-pyrones such as rubrofusarin (II) (5). The material migrating at R_f = 0.76 was isolated as a crystalline yellow solid (mp = 171 to 174°C; that reported for IIIb = 176°C [4]) with a molecular ion at M^+ = 304 (73%) and fragment ions at *m/e* = 286 (22%), 262 (14%), 247 (74%), 246 (100%), 232 (22%), 220 (49%), and 190 (30%). The compound also had infrared, nuclear magnetic resonance, and UV spectra identical to those reported for fonsecin monomethyl ether (IIIb) (4).

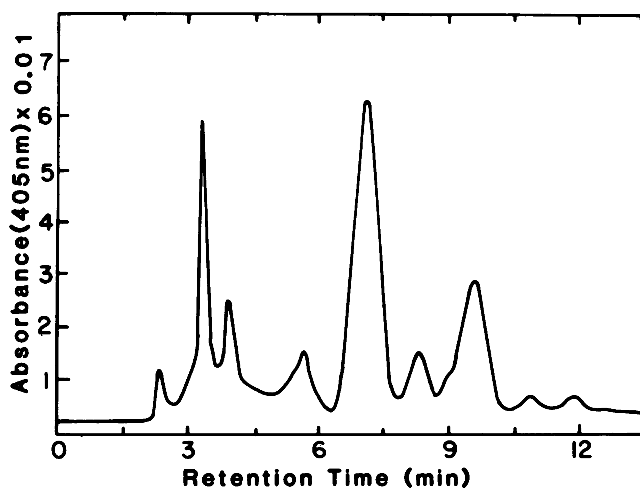

FIG. 3. HPLC profile of naphthopyrones from *A. niger* SRRC 3583 grown on rice. Pooled material recovered from R_f = 0.5 to 0.8 after HPTLC of the hexane-insoluble material was dissolved in acetonitrile and analyzed as described in the text. The *k'* values for individual components of the mixture are given in Table 2.

The presence of fonsecin monomethyl ether (IIIb) from *A. niger* has not been reported previously. This compound was isolated by Galmarini and Stodola as a synthetic compound from the methylation of fonsecin (IIIa), a metabolite of *Aspergillus fonsecaeus* (4). Also, the only monomeric linear naphthopyrones from *A. niger* reported previously were rubrofusarin (II) (5) and its monomethyl derivative (7). Since the monomeric forms presumably are precursors of the dimers, the isolation of IIIb suggests that hydration can precede dimerization in the biosynthesis of aurasperones B and C.

In the HPLC studies, gradient elution was necessary to

TABLE 2. Migration of naphthopyrones on HPTLC and HPLC

Naphthopyrone[a]	HPTLC (R_f)[b]	HPLC (k')[c]	Yield (mg/kg)[d]
Flavasperone	0.81	1.64	19
Fonsecin monomethyl ether	0.76	1.47	56
Rubrofusarin	0.72	2.47	23
Aurasperone A	0.67	4.81	8
Isoaurasperone A	0.61	5.27	6
Aurasperone B	0.56	3.18	291
Aurasperone D	0.53	3.68	35
Aurasperone C	0.49	4.25	124

[a] The naphthopyrones were purified as described in the text and identified by comparison of spectral properties with those previously reported.
[b] With benzene-ethyl acetate-formic acid (100:40:10, vol/vol) on Whatman LHP-KF.
[c] Gradient elution, 40 to 60% CH_3CN in 0.1 M acetic acid, 10 min.
[d] *A. niger* SRRC 3583 was cultured on rice for 14 days at 27°C. The total hexane-insoluble material from 1 kg of rice fermented was 0.65 g.

TABLE 3. Effect of growth substrate on aurasperone production by *A. niger* SRRC 3583

Substrate	Production (% of total area) of aurasperone:			
	A[a]	B	C[b]	D
Rice	2	52	22	6
Mango	1	58	14	6
Cottonseed	12	36	30	8
Corn	3	65	2	5
YES medium	3	43	38	4

[a] Mixed with isoaurasperone A.
[b] May contain some aurasperone E (less than 10%).

TABLE 4. Aurasperone production on rice by different *A. niger* isolates

SRRC isolate	Production (% of total area) of aurasperone:			
	A[a]	B	C[b]	D
396	1	51	29	4
397	1	55	19	6
3514	13	23	36	15
3572	8	29	32	15
3581	1	53	22	6
3583	2	52	22	6
393	1	51	23	8

[a] Mixed with isoaurasperone A.
[b] May contain some aurasperone E (less than 10%).

elute the less polar dimeric naphthopyrones, aurasperones A and isoaurasperone A, in less than 15 min and to prevent peak broadening. A typical profile for an unpurified mixture of naphthopyrones (pooled material migrating with $R_f = 0.5$ to 0.8 on HPTLC) is shown in Fig. 3. The presence of acetic acid in the solvent mixture prevented tailing of the peaks. Under the conditions used, the approximate limit of detection for aurasperone B (with monitoring at 405 nm) was 10 ng per injection, which gave a peak height of 1 cm at a detector setting of 0.005 absorbance unit full scale. Partially purified naphtho-γ-pyrone fractions were obtained from HPTLC plates and further purified by HPLC. The k' values on HPLC and the R_f values on TLC were compared for the individual naphthopyrones (Table 2); yields are corrected for differences in molar absorptivities at 405 nm (5). The order of elution of the naphthopyrones on TLC corresponds to that previously reported (5, 9). However, on reversed-phase HPLC the order of elution only partly corresponded to the polarity of the individual compounds. The monomeric naphthopyrones eluted before the dimeric ones. Otherwise, the individual naphthopyrones eluted in reverse order of their polarity. The hydrated aurasperones B and C (Va and Vb) occurred in significantly greater amounts than the other substances. For aurasperones B and C, the molecular ions were not obtained in mass spectra. Rather, the largest molecular ions were at m/e = 570 and 556 for B and C, respectively. However, other properties (UV and nuclear magnetic resonance spectra) were in accord with the identity of these molecules as the hydrated forms rather than as the corresponding unsaturated forms, IVa and IVd (Fig. 1). Dehydration probably occurred under the conditions used for obtaining the mass spectra.

The variation of aurasperone production for *A. niger* SRRC 3583 grown on different substrates is shown in Table 3. Relatively more aurasperone C was formed when *A. niger* SRRC 3583 was cultured on YES medium or cottonseed than when it was cultured on rice, mango, or corn. Significant amounts of aurasperone A were observed only when this *A. niger* isolate was cultured on cottonseed.

Aurasperone production, besides varying slightly with the substrate, varied with the *A. niger* isolate (Table 4). Yields of aurasperone B were less from *A. niger* SRRC 3514 and SRRC 3572 than from the other isolates. Also, HPTLC comparison of naphthopyrones from different strains of *A. niger* (Fig. 2) demonstrated that the monomeric species (fast-migrating components) are formed in much lower amounts (or are absent) in some of the strains.

Our results show that naphtho-γ-pyrones are common metabolites of *A. niger* isolated from stored cottonseed. The HPLC method reported here allows rapid separation and quantitation of the four main aurasperones found in extracts of *A. niger* and could be useful for monitoring the extent of contamination of a commodity infected with *A. niger*.

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