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Isolation and Structure of Red Pigments from *Aspergillus flavus* and Related Species, Grown on a Differential Medium

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A group of 25 strains of *Aspergillus* spp., most of them known for producing aflatoxins or other mycotoxins, was cultivated on *Aspergillus* differential medium, containing ferric ions. Two red pigments produced by the test-sensitive strains have been isolated and identified as ferriaspergillin (1) and ferrineoaspergillin (2). The formation of the yellow pigmentation on ADM is related to the production of aspergillic acid (3) or neoaspergillic acid (4).

Among the causal agents of toxicoses by foodstuffs, fungi of the genera *Aspergillus* and *Penicillium* play a major role. They are widespread and are common in stored products such as grains, field crops, and harvested forage, which are generally used for feeding domestic animals and man (Moreau, 1979).

Therefore, simple and rapid methods for the detection of mycotoxins in deteriorated substrates or for identification of the species responsible for the damages are of utmost importance.

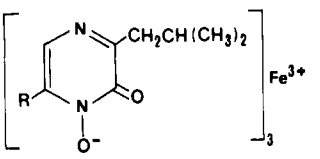
Bothast and Fennell (1974) proposed a diagnostic method for the identification of *Aspergillus flavus* and closely related species, which are often connected with the presence of aflatoxins. The method is based on the development of a characteristic yellow pigmentation, when the mold is cultivated on a particular medium (*Aspergillus* differential medium, ADM), containing iron citrate. Only a few species other than *A. flavus* give the same pigmentation.

In order to check whether there was any correlation between the yellow pigment and fungal metabolites, we isolated the pigments produced by test-sensitive strains. This paper reports their structural elucidation.

RESULTS AND DISCUSSION

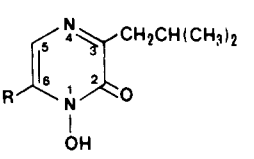
A group of 25 strains of *Aspergillus* spp., most of them the same ones that were examined by Bothast and Fennell (1974), were cultivated on Petri plates of ADM, and the crude EtOAc extracts were compared by TLC (hexane–AcOEt, 9:1) on silica gel plates. Two red pigments 1 and 2 were produced by most of the strains examined. Table I reports the results of this screening.

Scheme I



1 R = $CH_3CH_2CH(CH_3)-$

2 R = $(CH_3)_2CHCH_2-$



3 R = $CH_3CH_2CH(CH_3)-$

4 R = $(CH_3)_2CHCH_2-$

The strains *A. flavus* IPV-F 16L and *Aspergillus melleus* CBS 546.65 were used for large-scale cultivation, in order to obtain a sufficient quantity for structural elucidation of 1 and 2, respectively (see Scheme I for structures). The pigments were again isolated by chromatography of the hexane extract of the dried mycelia, grown on stationary liquid culture.

Although the pigment 1 appears as an oil, of low polarity, its color, its absence in cultures grown on iron-deprived media, and the disturbance of the NMR spectrum by paramagnetic atoms led us to suspect that it contained iron.

Alkali hydrolysis of the pigment 1 gave $Fe(OH)_3$ and a single, pure compound. Examination of the spectral (UV, mass, ^1H NMR, and ^{13}C NMR; see Experimental Section) data indicated that this compound is (+)-aspergillic acid (3), mp and optical rotation also being consistent with literature data (Wilson, 1971). Aspergillic acid is a known metabolite of various isolates and substrains of *A. flavus* and related *Aspergilli*. From the value of the molecular ion in the mass spectrum of 1, it can be inferred that the pigment is a compound which contains three molecules of aspergillic acid and one atom of iron, which can coordinate

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Table I. Screening for Yellow Pigmentation of the Reverse of the Colony on ADM and Related Pigments Isolated

strain no.	organism	pigmentation of the reverse of the colony	pigment isolated by TLC
	A. flavus Group		
CBS 109.45	*A. flavus* Link ex Fr.	+	1
CBS 113.32	*A. flavus*	+	1
CBS 573.65	*A. flavus*	+	1
IPV-F 16L	*A. flavus*	+	1
IPV-F 21L	*A. flavus*	+	1
IPV-F 30L	*A. flavus*	+	1
IPV-F 58L	*A. flavus*	+	1
CBS 485.65	*A. flavus* var. columnaris Raper & Fennel	+	1
CBS 133.52	*A. oryzae* (Ahlburg) Cohn	−	none
CBS 102.22	*A. oryzae* var. effusus Tiraboschi (Ohara)	−	none
CBS 574.65	*A. oryzae* var. effusus	−	none
CBS 921.70	*A. parasiticus* Speare	+	1
CBS 126.59	*A. parasiticus*	+	2
CBS 501.65	*A. subolivaceus* Raper & Fennell	+	1
	A. ochraceus Group		
CBS 467.65	*A. auricomus* (Guèguen) Saitô	+	2
CBS 546.65	*A. melleus* Yukawa	+	2
CBS 108.08	*A. ochraceus* Wilhelm	+	2
CBS 549.65	*A. sclerotiorum* Huber	+	2
CBS 550.65	*A. sulphureus* (Fres.) Thom & Church	+	2
	A. wentii Group		
CBS 120.51	*A. thomii* G. Smith	−	none
CBS 121.32	*A. wentii* Wehmer	−	none
CBS 118.34	*A. wentii*	−	none
CBS 123.35	*A. wentii*	−	none
CBS 229.67	*A. wentii*	−	none
CBS 104.07	*A. wentii*	−	none

the hydroxamic acid moiety of the organic molecules.

The second pigment, 2, has a mass spectrum similar to that of 1, with an identical molecular ion. Hydrolysis of 2 again gave $Fe(OH)_3$ and neoaspergillic acid (4) previously isolated from *Aspergillus sclerotiorum* (Micetich and McDonald, 1964), which is an isomer of aspergillic acid. It is therefore identical with ferrineoaspergillin, a pigment recently isolated from *Aspergillus ochraceus* Wihl. (Maebayashi et al., 1978), which is a complex of three neoaspergillic acid molecules and one atom of iron.

The pigments 1 and 2 were quantitatively obtained when methanol solutions of aspergillic and neoaspergillic acid, respectively, were treated with $FeCl_3$.

From the results here reported, it appears that the yellow pigmentation on ADM can be produced by any mold which is able to produce aspergillic acid, neoaspergillic acid, or any of their congeners which contain the chelating hydroxamic acid moiety (Middleton et al., 1978). This property is not restricted to fungi of the genus *Aspergillus*, as is shown by the isolation of pulcherrimin from *Candida pulcherrima* (Kluyver et al., 1953). Other complexes of this series probably exist, (as it is known that the amino acid composition of the culture medium can favor the biosynthesis of a particular pyrazinedione (Ueno et al., 1977), and ADM is a medium which is rich in amino acids. It can also be noted that attempts to obtain aspergillic acid from cultures of *A. flavus* IPV-F 16 L grown on ADM, when sodium citrate was substituted for iron citrate, were negative. Thus iron ions may have a favorable influence on the production of this class of metabolites, possibly via formation of the complex.

These coordination compounds can be ascribed to the class of siderophores, compounds which are increasingly attracting interest, also on account of their potential therapeutic effect (Carrano et al., 1978).

EXPERIMENTAL SECTION

Microorganisms. Most of the strains examined were supplied by the Centraalbureau voor Schimmelcultures (CBS), Baarn. Four *A. flavus* strains were isolated from corn maize at the Istituto di Patologia Vegetale (IPV) of Milan University. The strains were grown in Roux flasks, each containing 80 mL of liquid ADM medium, for 10 days at 24 °C.

Isolation of Pigments. The mycelium was filtered, dried, crushed, and extracted in a Soxhlet apparatus with hexane. The residue obtained by evaporation of the solvent was chromatographed on preparative Merck plates with hexane–EtOAc (9:1) as the eluant. Hexane extraction of mother liquors gave no pigments.

The strains *A. flavus* IPV-F 16 L and *A. melleus* CBS 546.65 were used for the structure determination of the two red pigments 1 (8 mg/flask) and 2 (6 mg/flask), respectively. The purification of the two pigments was performed as described above. *Pigment 1*: oil; R_f 0.13; MS, molecular ion at m/e 725, fragment ions at m/e 681, 502, 223; IR ν_{max} (neat) 2950, 2920, 2860, 1580 cm^{-1}; UV λ_{max} (EtOH) 310 nm (ϵ 20900), 410 (4200). *Pigment 2*: mp 130–131 °C [lit. mp 135–136 °C (Maebayashi et al., 1978)]; R_f 0.08; MS, molecular ion at m/e 725, fragment ions at m/e 681, 502, 223; IR ν_{max} (KBr) 3430, 2955, 2860, 1525, 1490 cm^{-1}; UV λ_{max} (EtOH) 226.5 nm (ϵ 52500), 310 (20000), 410 (4500).

Hydrolysis of Pigment 1. A solution of 50 mg of 1 in MeOH (5 mL) was stirred with 2 N KOH (10 mL) at room temperature for 0.5 h; the mixture was centrifuged to separate $Fe(OH)_3$ and the supernatant was acidified and extracted with ether. After evaporation of the solvent, the residue was crystallized from hexane–ether to give aspergillic acid (3): mp 93–94 °C [lit. mp 97–99 °C (Wilson, 1971)]; MS, molecular ion peak at m/e 224 (23), fragment ions at m/e 207 (76), 193 (29), 182 (100), 166 (79), 153 (34), 137 (79); ^1H NMR ($CDCl_3$, 90 MHz) δ 0.1–1.1 [9 H, $(C\text{-}H_3)_2CH$– and CH_3CH_2–], 1.37 (3 H, d, J = 6 Hz, CH_3CH–), 1.78 (2 H, m, CH_3CH_2CH–), 2.25 [1 H, m, $(CH_3)_2CHCH_2$–], 2.76 [2 H, d, J = 6 Hz, $(CH_3)_2CHCH_2$–], 3.18 (1 H, m, CH_3CHCH_2–), 7.35 (1 H, s, H-5), 10.05 (1 H, s, –NOH); ^{13}C NMR ($CDCl_3$) δ 152.2, 151.7, and 140.1 (s, C-2, C-3,

and C-6), 121.7 (d, C-5), 41.8, and 27.4 (t, $-CH_2-$), 34.1 and 27.0 (d, –CH–), 22.5, 17.9, and 11.6 (q, CH_3-).

Hydrolysis of Pigment 2. Working in the same way as described for pigment 1, neoaspergillic acid (4) was isolated: MS, molecular ion peak at *m/e* 224 (8), fragment ions at *m/e* 207 (33), 193 (20), 182 (42), 166 (80), 153 (20), 123 (100); ^1H NMR ($CDCl_3$, 100 MHz) δ 0.9–1.1 [12 H, $(CH_3)_2CH-$], 2.22 [2 H, m, $(CH_3)_2CH-$], 2.72 (4 H, m, $-CH_2CH-$), 7.35 (1 H, br s, H-5), 9.92 (1 H, –NOH).

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An Investigation into the Possible Presence of Volatile *N*-Nitrosamines in Cooking Oils, Margarine, and Butter

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Following a report of the occurrence of volatile nitrosamines in various vegetable oils and margarines in Germany, a study was carried out to determine the nitrosamine levels in such products sold in retail outlets in Canada. Thirty-eight samples of various vegetable oils, ten of butter, fourteen of margarine, and six of lard were analyzed. All the oils and lard were negative. Only one butter and five margarine samples contained trace levels (0.2–3.8 ppb) of *N*-nitrosodimethylamine and/or *N*-nitrosomorpholine. Further investigations at the plant level failed to uncover any definite source of the nitrosamine contamination detected in some of the margarine samples. Recent samples of margarine from these plants were, however, either negative or contained insignificant levels of volatile nitrosamines. It was concluded that nitrosamine levels in these products are either negative or negligible and, therefore, should not be a matter of concern.

Studies carried out during the last 10 years have shown that traces of certain volatile *N*-nitrosamines (simple called nitrosamines), which are potent carcinogens, are present in various foods and beverages such as cured meats, fish, cheese, fried bacon, instant skim milk powder, malt, beer, and whiskies (Fazio et al., 1979; Goff and Fine, 1979; Pensabene et al., 1974; Spiegelhalder et al., 1979; Hotchkiss et al., 1979; Sen et al., 1979). The nitrosamines in the above-mentioned products are believed to be formed by the interaction of amines and added nitrite or gaseous nitrogen oxides in hot flue gas used for the commercial processing of these items.

Recently, Hedler et al. (1979) of Germany reported the occurrence of fairly high levels (up to 27.8 ppb) of *N*-nitrosodimethylamine (NDMA) and/or *N*-nitrosodiethylamine (NDEA) in various vegatable oils such as olive oil, soybean oil, plant germ oil, sunflower oil, and a few unspecified vegetable oils. Of particular interest is the fact that a high percentage of the olive (8/16), soybean (5/5), and plant germ (6/6) oils analyzed were found to be positive for nitrosamines. Other oils were found to contain these nitrosamines only occasionally. To our knowledge, this appears to be the only documented evidence for the presence of nitrosamines in these products. The source of the contamination or the mechanism of formation of the two nitrosamines is, however, unclear. In addition, the same workers noted the presence of NDMA (up to 5.8 ppb) and/or NDEA (up to 7.5 ppb) in 37/107 margarine samples examined.

The Canadian Health Protection Branch has been monitoring the situation since 1974. A brief summary of our up-to-date findings is presented in this report.

EXPERIMENTAL SECTION

Samples. Most of the samples were purchased in the Ottawa area and analyzed within 1–2 weeks. Some of the samples of margarine, butter, boiler additives, and miscellaneous ingredients of margarine were directly obtained from manufacturing plants located in various parts of Canada.

Nitrosamine Analysis. Since the study was carried out in two stages (1974 and 1980), the techniques used for nitrosamine analysis varied considerably between the two studies. These are described as follows.

(*a*) *1974 Study.* A mixture consisting of a 50-g aliquot of the oil and 350 mL of 3 N KOH was boiled under a reflux condenser for 6 h, and the mixture was then distilled (at atmospheric pressure) until ~200 mL of distillate was collected. The distillate was made alkaline and extracted with dichloromethane (DCM), and the DCM extract cleaned up on a basic alumina column as described previously (Sen, 1978). The purified extract was carefully concentrated (by using a Kuderna-Danish type concentrator) to 1.0 mL, and a 5–10-μL aliquot was analyzed by

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