NEW ANTIBIOTIC PIGMENTS RELATED TO FUSARUBIN FROM *FUSARIUM SOLANI* (MART.) SACC.

I. FERMENTATION, ISOLATION, AND ANTIMICROBIAL ACTIVITIES

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A cholesterol-decomposing fungus, *Fusarium solani* (MART.) SACC. strain PP 96, was found to produce several different naphthaquinone pigments in a glycerol-mineral salts medium. Three novel compounds structurally related to fusarubin were isolated by chloroform extraction followed by silicic acid column chromatography and preparative thin-layer chromatography. The purified compounds were found to have relatively low activity against bacteria, yeasts and filamentous fungi.

Recently a cholesterol-attacking fungus, identified as *Fusarium solani* (MART.) SACC., was isolated in our laboratory from soil. It has been found to produce several pigments, one of which has been identified as fusarubin. This naphthaquinone pigment was previously reported to be produced by *Fusarium javanicum*[1], *F. martii*[2], *F. solani*[2], and an unidentified *Fusarium* sp.[2]. Fusarubin has been reported to have antimicrobial[3], phytotoxic[2], and antitumor[4] properties.

Since the other pigments in the fusarubin complex had not been reported before, we decided to purify them and determine their properties. This paper describes the production, isolation, and purification as well as the antimicrobial properties of the four major members of the complex. Identification and chemical structures of these compounds are given in the accompanying paper[5].

Materials and Methods

Isolation of Culture

The cholesterol-attacking strain PP 96 was isolated from a moistened clay soil sample collected at Busch Campus, Rutgers University, Piscataway, New Jersey, U.S.A., previously enriched with cholesterol and kept at room temperature for one month. It was transferred onto agar plates containing the same mineral salts medium to which 0.15% cholesterol was added as the sole carbon source. After several transfers on agar plates, the organism was transferred into shake flasks containing the same mineral salts-cholesterol medium.

Taxonomy

Identification of the culture as *F. solani* (MART.) SACC. was done by both Dr. P. E. NELSON of Pennsylvania State University and Dr. A. A. SAMSON of Centraalbureau voor Schimmel-cultures (Netherlands).

Preservation of Culture

Long term preservation of *F. solani* (MART.) SACC. PP 96 was carried out in liquid nitrogen[6]. Working stock cultures were maintained on agar slants containing the same ingredients as the production medium and were stored at 5°C.

Media

(1) Production medium: The production medium contained (in g) NH_4NO_3 1, K_2HPO_4 0.25,

$MgSO_4 \cdot 7H_2O$ 0.25, NaCl 0.05, $FeSO_4 \cdot 7H_2O$ 0.0001, glycerol 10 ml, tap water 1 liter.

(2) Inoculum development medium: This was the same as the production medium except that the glycerol percentage was 10%.

Production Methods

(1) Shake flasks: The fungus was grown in 250 ml Erlenmeyer flasks containing 100 ml of the glycerol-mineral salts medium. The pH was about 7.0 before autoclaving. The flasks were incubated at 28°C for 5 days on a Gyrotory shaker (New Brunswick Scientific Co., Edison, N. J.) running at 325 rpm.

(2) Fermentor: Production of pigments was carried out in a 50-liter fermentor (Fermacell Model F-50, New Brunswick Scientific Co.) containing 30 liters of medium. The agitation system consisted of two 5-inch diameter four-bladed impellers rotating at 350 rpm. The air flow was 15 liters per minute. Fermentation was carried out at 28°C for 5 days.

(3) Inoculum development: One liter quantities of inoculum were grown in 2-liter Erlenmeyer flasks. The cultures were incubated at 28°C on a shaker running at 250 rpm for 2~3 days. One liter of inoculum was used for each 30-liter batch. Five ml of antifoam (Dow Corning C) were used per batch.

Isolation and Purification of Pigments

After 5 days, 30 liters of whole broth were adjusted to pH 2 with HCl and centrifuged to separate mycelium from beer. The beer was evaporated to 2 liters in a vacuum concentrator (Turba-Film evaporator, Rodney-Hunt Machine Co., Orange, Mass.). Both mycelium and concentrated beer were extracted 3 times by shaking with chloroform. After 3 water washes the combined extracts were concentrated to 50 ml.

The concentrate was then passed through a column containing 700 g of silicic acid (SilicAR CC-4, Mallinckrodt Chemical Works, St. Louis, Mo.) which was previously washed with redistilled chloroform. Pigments were eluted from the column with chloroform. Similar fractions were combined and concentrated to minimal volumes in a rotary vacuum evaporator. The concentrated combined fractions were purified further by passing each one through another column containing 300 g of SilicAR CC-4. Purity was checked by thin-layer chromatography (TLC) using precoated plastic sheets (5 × 20 cm-Polygram sil G, UV 254-Brinkman Instruments, Inc., Westbury, N. Y.) with ethyl acetate as the solvent.

Separation of the yellow pigments was carried out by a preparative TLC procedure. One ml of each purified concentrated pigment mixture was loaded onto a 1000 μ TLC plate (Uniplate, Silica Gel G-Analtech, Inc., Newark, Delaware). Each plate was developed with ethyl acetate (acid free). After development the plate was scanned under U. V. light. Since the yellow pigment exhibited strong fluorescence it was easily outlined by means of a pointed needle. Pigments were eluted from the plate with ethyl acetate.

Determination of Bioactivity

(1) Diffusion assay: Detection of antimicrobial activity in column eluates was done by a disc-plate method (ODEN *et al.*[7]). Small volumes of eluate were dried and the residues dissolved in dimethylsulfoxide (DMSO). To each disc 25 μl of solution containing 25 μg of residue were added. The yeast was grown on Antibiotic Medium #12 (Difco) and the bacterium on Nutrient Agar (Difco). For both the inoculum and the assay stage the yeast was incubated at 28°C for 2 days and the bacterium at 37°C for 1 day. Controls were run using DMSO only.

(2) Dilution assay: Minimum inhibitory concentrations (M.I.C.) were determined by the method of LECHEVALIER *et al.*[8]. Assay plates were incubated at 28°C for 2 days for yeasts and filamentous fungi and 37°C for 1 day for bacteria.

Results

Fermentation

The organism grows well in shake flasks on a large variety of nutrient media. Glycerol-mineral salts medium was found to be the best for growth and antibiotic production. One peculiarity of this

organism is its ability to grow in cholesterol-mineral salts medium, a non-permissive substrate for most Fusaria. Replacement of cholesterol by glycerol resulted in the production of a blood-red pigment in both mycelium and broth which exhibited antimicrobial activity. Yeast extract (0.5%) inhibits pigment production.

Isolation and Separation of Components

Seven major fractions were obtained from the initial chromatography. Two main fractions, "fast" (#2) and "slow" (#5), were the most active against bacteria and yeast using the diffusion assay method (Table 1). Both of the main fractions were purified again separately by column chromatography. The "slow" pigment mixture was located in fractions 102~180 with an orange to distinct red color and the "fast" pigment mixture was located in fractions 126~150 with a very distinct orange color. Similar fractions were combined, concentrated and tested for purity by TLC. Each pigment mixture was found to be composed of 2 different compounds. One of each was yellow and the other orange.

Table 1. Antimicrobial activities of combined fractions (combined by color) from chromatography of the original concentrated chloroform extract.

Fraction No.	Tube No.	Color	Activity* against	
			Sarcina lutea (mm)	*S. cerevisiae* (mm)
1	54~90	Magenta	17	<6.4
2**	91~140	Orange	20	21.5
3	141~178	Pink purple	18	<6.4
4	179~192	Crimson	31	<6.4
5***	193~340	Peach to orange	33.5	23.5
6	341~400	Faint purple	26	<6.4
7	401~500	Light pink	18	<6.4

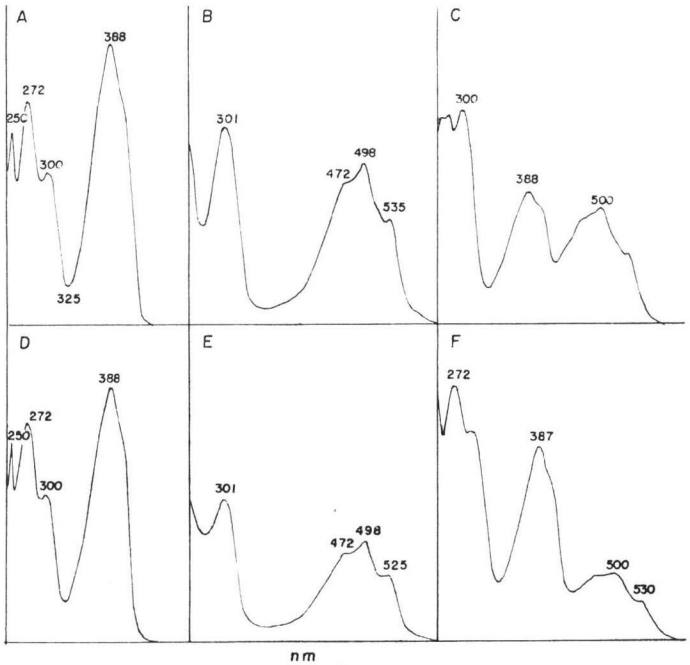
* Activity was tested using the agar diffusion method with 25 μg of residue (dissolved in DMSO) per disc. The numbers refer to the diameter of the clear zone. The discs were 6.4 mm in diameter.

** "Fast" fraction mixture.

*** "Slow" fraction mixture.

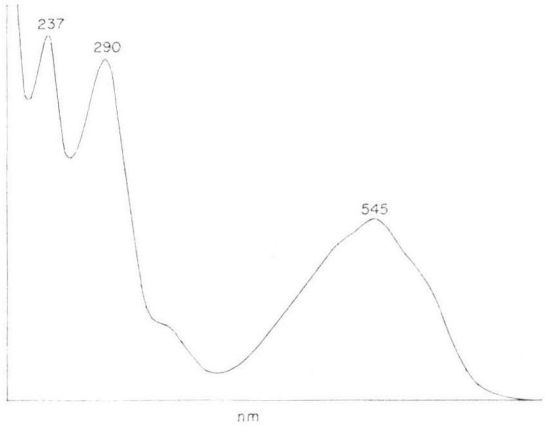
Fig. 1. Ultraviolet-visible absorption spectra.

A, "fast yellow"; B, "fast orange"; C, "fast" pigment mixture; D, "slow yellow"; E, "slow orange"; and F, "slow" pigment mixture.



The yellow pigments exhibited very strong fluorescence under UV light and this helped in their separation after preparative TLC. The best test for purity was by UV-visible spectrum analysis since the yellow pigments exhibited maximum peaks at 388 nm, the orange pigments at 500. The original extracts which contained both had peaks at 388 and at 500 nm. UV-visible spectra of the "fast yellow", "fast orange", "slow yellow", "slow orange" (fusarubin)[5] and the mixtures are shown in Fig. 1. Treatment of the "slow orange" at 160°C for 30 minutes resulted in the formation of a purple material "anhydrofusarubin"[5]. Its UV spectrum is shown in Fig. 2.

Fig. 2. Ultraviolet-visible absorption spectrum of anhydrofusarubin (purple).



Antimicrobial Activity

Minimal inhibitory concentrations (M.I.C.) of the four pure pigments and anhydrofusarubin against different groups of bacteria and against yeast and fungi are recorded in Table 2. The activity increased according to the following sequence (M.I.C. in μg/ml): filamentous fungi (30~150), yeasts (6~125) and Gram-negative bacteria (50~100), Gram-positive bacteria (10~75), and acid-fast bacteria (3~40). An exception to this sequence was that the M.I.C. with *Sarcina lutea* UC 3383 were high with both "slow orange" (150 μg/ml) and "anhydrofusarubin" (125 μg/ml). It is obvious that the new "fast yellow" pigment was the most active of the four pigments since it showed M.I.C. of 6~20 (acid-fast bacteria), 10~50 (Gram-positive bacteria), 75 (Gram-negative bacteria), 6~50 (yeasts) and 40~125 (fungi).

Table 2. Minimum inhibitory concentrations of the four separated and purified pigments and their anhydro form against bacteria, yeasts and fungi.

Test organism	M.I.C. (μg/ml)*				
	Fast		Slow		Anhydro-fusarubin
	Orange	Yellow	Orange	Yellow	
Acid-fast bacteria:					
Mycobacterium smegmatis IMRU 24**	30	06	10	10	03
Mycobacterium rhodochrous IMRU 21	40	20	30	40	40
Gram-positive bacteria:					
Sarcina lutea IMRU 14	20	20	20	20	30
Sarcina lutea UC 3383***	75	50	150	75	125
Bacillus megaterium IMRU 10	50	10	20	30	30
Bacillus subtilis ATCC 7972	75	50	40	40	40
Staphylococcus aureus ATCC 6538P	75	50	50	40	50
Gram-negative bacteria:					
Escherichia coli B PP 01****	75	75	75	50	75
Serratia marcescens IMRU 70	100	75	100	100	100

(continued)

Table 2. (continued)

Test organism	M.I.C. (μg/ml)*				
	Fast		Slow		Anhydro-fusarubin
	Orange	Yellow	Orange	Yellow	
Yeasts:					
Candida albicans ATCC 18527	75	30	30	100	100
Candida lipolytica PP 47	75	40	75	40	50
Candida utilis NRRL Y-900	100	06	75	100	100
Saccharomyces cerevisiae ATCC 9763	75	50	100	75	125
Fungi:					
Penicillium chrysogenum ATCC 12690	75	100	100	75	100
Penicillium terrestre PP 84	75	50	30	40	50
Trichoderma viride PP 24	75	100	100	75	100
Mucor rouxii IMRU 80	100	125	100	100	125
Aspergillus wentii PP 06	150	125	150	100	125
Aspergillus sulfureus PP 17	150	125	125	100	125
Aspergillus niger PP 29	150	125	100	100	125
Aspergillus foetidus ATCC 10254	75	40	50	50	75

* Duplicate assays were run. The values given are averages.
** IMRU. Institute of Microbiology, Rutgers University Culture Collection.
*** UC. Upjohn Company Culture Collection.
**** PP. Pilot Plant Culture Collection, Rutgers University.

Discussion

The results of this investigation show that in addition to fusarubin at least three other antimicrobial pigments are produced by *F. solani* strain PP 96.

The four pigments can be interconverted under various acidic and basic conditions which are indicated in the accompanying paper[5]. However, the conditions for interconversion are much harsher than those used in the isolation procedures. The initial TLC (two distinct orange spots) and spectrophotometric (λ_{max} at 388 and 500 nm, see Fig. 1 C and F) examinations of crude chloroform extracts gave evidence for all four pigments being true secondary metabolites, not artifacts produced during purification.

The production and purification methods which were developed have made possible the preparation of purified samples of each of the four compounds for identification and structural studies[5].

The four compounds were shown to have antibacterial and antifungal activities but at relatively low levels. The reported antitumor activity of fusarubin[4] has stimulated our interest to study the antitumor activities of other members of the complex.

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