NEW ANTIBIOTICS FROM THE FUNGUS *EPICOCCUM NIGRUM*

I. FERMENTATION, ISOLATION AND ANTIBACTERIAL PROPERTIES

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An atmosphere isolate of the fungus *Epicoccum nigrum* was found to exhibit an activity against *Staphylococcus aureus*. A more active, non-sporulating variant of this strain was selected. From its fermentation broth, two novel compounds, epicorazines A and B, were isolated by preparative TLC and tested against a series of bacteria.

In the course of a screening program for new antibiotics, a strain of the fungus *Epicoccum nigrum* (Hyphomycetes, Tuberculariaceae, Dictyosporae) was found to exhibit an antibacterial activity against *Staphylococcus aureus*. This report deals with some features of this strain, fermentation studies and antibacterial properties of its purified metabolites.

1. The Producing Organism

The producing organism is a variant of strain 751–5 of *Epicoccum nigrum*, which was isolated from atmosphere in 1958 at the Centre de Recherches de Bioclimatologie of Pau (France). This fungus grows usually saprophytically on various decaying plants. In the southwest of France, corn appears to be a specially favourable substratum and *Epicoccum* anemophilous spores are quite abundant in the atmosphere in autumn (GOURMEL *et al.*[1]). Contrary to the numerous *Epicoccum* strains which were isolated in Pau and Bordeaux, strain 751–5 exhibited an antibiotic activity against *Staphylococcus aureus*.

About 60 "species" had been described by various authors in the genus *Epicoccum* LINK, according to such characters as shape and size of spores and sporodochia, or colour of the pigment produced by the mycelium. In 1959, a taxonomic study was made by SCHOL-SCHWARZ[2] at the Centraalbureau voor Schimmelcultures. Seventy isolates of *Epicoccum* were examined, 47 of which sporulated, and also 19 type specimens and 96 other herbarium specimens. This study led the author to the opinion that the development and the structure of the spore are the only constant factors in every strain and that none of the other characters can be considered as taxonomic characters. Therefore the only logical course seems to be to class these strains as one species, *Epicoccum nigrum* LINK***. However, the appearance of cultures of *Epicoccum*, even when not sporulating, is so typical that, in spite of the

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*** According to SCHOL-SCHWARZ, the genus *Cerebella*, established by CESATI in 1851, should be regarded as a synonym of *Epicoccum*; its only species (*C. andropogonis*) becomes therefore the second species of *Epicoccum* as *E. andropogonis* (CES.) comb. nov.

divergency in colour, they can be recognized as *Epicoccum*. Such a variability among the strains excludes a taxonomic division of the species into any sub-species or varieties.

The initial isolate of strain 751–5 contrasted with the majority of our usual *E. nigrum* cultures by its abundant sporulation on agar media, which showed numerous sporodochia. However, after several years, sterile areas appeared in the subcultures; the mycelium from those areas could be isolated and proved morphologically stable on prolonged cultivation. Moreover, culture broths of this variant were five times more active against *S. aureus* than those of the mother strain.

This variant grows slowly on CZAPEK's agar or on yeast extract-glucose agar; 15 days-old colonies grown at 25°C in Petri dishes are only 30~35 mm in diameter. Growing is equally slow on malt agar, but a few spores are produced on sparsely disseminated sporodochia.

2. Fermentative Production

Seed medium consisted of glucose 2% and yeast extract 0.5% in distilled water. Composition of fermentation medium was: corn-steep 2% (0.4 g N/liter), sucrose 3%, K_2HPO_4 0.1%, $MgSO_4$ 0.05% in distilled water. All media were sterilized by autoclaving at 120°C for 15 minutes.

An aqueous suspension of mycelial fragments was obtained by scraping stock cultures on agar slopes in the presence of sterile water. It was used to inoculate 250 ml flasks containing 50 ml of seed medium; incubation was carried out at 25°C for 3 days on a rotatory shaker (150 rev./min.). Then 4% by volume of this culture was transferred to a 20-liter Biolafitte fermentor containing 15 liters of the fermentation medium.

Optimal conditions for antibiotic production were obtained when cultivating the fungus for 72 hours; at 25°C during first 36 hours, then at 20°C for the remainder of the fermentation; agitation was 200 rpm throughout the cultivation and aeration was regulated to obtain a concentration of 0.25 mmole of dissolved O_2 by liter. Foaming was controlled by automatic addition of chemical antifoam (Rhodorsil antimousse 426 R). The pH of the broth remained nearly constant (about 4.6) from the beginning to the end of the fermentation.

3. Isolation of the Antibiotic Compounds

Liquid fermentation broth was separated from mycelium in a Westfallia centrifuge. The 15-liter liquid was extracted with 10 liters of $CHCl_3$. The chloroformic extract was dried on Na_2SO_4 and concentrated under reduced pressure to 250 ml. After standing at 4° C for 24 hours, a precipitate appeared which proved to be the previously obtained 3,6-dibenzyl-2,5-dioxopiperazine (BAUTE *et al.*[3]). This compound was removed by filtration and the filtrate was concentrated again to 10 ml (F).

This liquid was subjected to a thin-layer chromatography on silica gel with a mixture of $CHCl_3$-CH_3OH (94: 6) as eluent. On comparison with a similar chromatogram obtained from a non-inoculated medium, three spots could be regarded as fungal metabolites; they were detected by UV absorption at 254 nm and by the iodine-azide spray reagent[4]. The antibiochromatogram technique revealed that the two main spots, A (Rf 0.7) and B (Rf 0.5), were active against *Staphylococcus aureus*. As further structural studies (DEFFIEUX *et al.*[5,6]) showed that A and B were novel diketopiperazine compounds, they were given the trivial names epicorazines A and B.

Attempts to isolate these metabolites by column chromatography were unsuccessful, but their separation could be performed on preparative thick-layer (0.5 mm) of silica gel (Merck 7747) previously

washed by migrating a mixture of CH_3OH - HCl (90: 10). Each plate (200×200 mm) received 0.8 ml of filtrate F as a 190-mm long streak and was developed by a system of $CHCl_3$ - CH_3OH (95: 5). Streaks of epicorazines A and B were detected by UV at 254 nm and eluted with $CHCl_3$ - CH_3OH (90: 10). Homologous eluates were pooled and evaporated to dryness; recrystallizations in a mixture of hexane - ether - acetone (10: 10: 10) yielded pure epicorazine A (150 mg) as colourless prisms, m.p. 195~198°C, $[\alpha]_D^{22}$ −293° (*c* 1.7 mg/ml, $CHCl_3$) and epicorazine B (10 mg) as amber-coloured prisms, m.p. 192°C, $[\alpha]_D^{22}$ −320° (*c* 5 mg/ml, CH_3OH).

4. Antibacterial Properties of Epicorazines A and B

Table 1. Antibacterial spectra of epicorazines A and B

Test organisms	Minimal inhibitory concentrations (mcg/ml)	
	Epicorazine A	Epicorazine B
Escherichia coli	>100	>100
Pseudomonas aeruginosa	>100	>100
Salmonella typhi	>100	>100
Proteus mirabilis	>100	>100
Staphylococcus aureus		
IP 678	20	30
IP A238	25	30
IP 52148	50	75
IP 6454	50	75
IP 6455	50	75
Enterobacter sp.	>100	>100
Streptococcus (A)	50	75
— (D)	50	75

Table 1 summarizes the antibacterial activities of epicorazines A and B, which were determined by the agar dilution method. The metabolites, being poorly soluble in water, were dissolved and diluted in dimethylformamide prior to incorporation into MUELLER-HINTON blood agar plates. The plates were inoculated according to the ICS agar dilution procedure and incubated at 37°C for 18 hours. Gram-positive bacteria seem to be more sensible to both compounds than Gram-negative ones.

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