MYCOVERSILIN, A NEW ANTIFUNGAL ANTIBIOTIC

I. FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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A new antifungal antibiotic, mycoversilin, was isolated from the culture filtrate of *Aspergillus versicolor* $(N_5)_{17}$ by repeated column chromatography and recrystallized from ethyl acetate as homogeneous fine needles. Maximum production took place in a medium containing 4% glucose and 1% peptone at pH 3.5, temperature 28°C after 8~9 days of incubation under stationary condition. Mycoversilin is a narrow spectrum antibiotic with activity against filamentous fungi, particularly *Trichophyton rubrum* (MIC 15 μg/ml).

Aspergillus versicolor is noted for the production of complex xanthones[1], anthraquinones[2~4] and a number of toxins[5] but very little information is known about production of antibiotics by this organism except antibiotic MM 4086[6] and versimide[7].

In our search for antibiotics that preferentially inhibit fungal growth, a new antifungal antibiotic, versicolin[8~11], was isolated from *A. versicolor*. In subsequent work, this organism progressively lost its productivity; it was therefore mutagenised to give a derivative, *A. versicolor* $(N_5)^{12}$, which yielded as much versicolin as the parent. The mutant producer again became completely inactive in course of time; the inactive mutant was further mutagenised[13] and from the surviving population eighteen antibiotically active mutants[14] were isolated and tested. One mutant, *A. versicolor* $(N_5)_{17}$, was arbitrarily selected for further studies. The antibiotic obtained therefrom was found to be different from versicolin and subsequently named mycoversilin[15].

In the present paper the fermentation, isolation, purification and antimicrobial properties of the antibiotic are described.

Materials and Methods

Organism

The antagonist was maintained at 4°C in Czapek-Dox agar slants containing 0.1% each of peptone, yeast extract and beef extract.

The test organism used for the assay was a strain of *Trichophyton rubrum* obtained from the School of Tropical Medicine, Calcutta, India; it was incubated for 7 days at 30°C on Sabouraud agar slants and kept at 4°C for maintenance.

Fermentation

Fermentations were carried out under stationary culture conditions in 250-ml Erlenmeyer flasks in a medium containing (g/liter): glucose (E. Merck, India) 40.0, peptone (BDH, India) 10.0 and distilled water to 1 liter; the pH was adjusted to 3.5 with dilute HCl before sterilization and 100-ml portions of the medium were dispensed into each flask. The flasks were then inoculated with the spores from 7-day old agar slants maintained at 25°C, and then incubated at 28°C for 9~10 days.

Determination of Cell Growth

Cell growth was measured as the dry weight of mycelium. Surface and submerged growth was

Table 1. Rf values of mycoversilin by thin-layer chromatography on Silica gel G.

Solvent system	Rf
C_6H_6 - EtOAc (1: 1)	0.00
$CHCl_3$ - EtOAc - CH_3COOH (2: 3: 0.15)	0.47
C_6H_6 - EtOAc - CH_3COOH (40: 60: 3)	0.53
C_6H_6 - MeOH - CH_3COOH (3: 2: 0.1)	0.65
$CHCl_3$ - BuOH - CH_3COOH (1: 1: 0.04)	0.89

15×2 cm, 0.25 mm thick silica gel plates (Silica gel G, E. Merck).

Detection: Iodine vapor or permanganate-BPB reagent.

Table 2. Rf values of mycoversilin on paper chromatograph.

Solvent system	Rf
C_6H_6 - CH_3COOH - H_2O (6: 7: 3)	0.17
C_6H_6 - MeOH - CH_3COOH (80: 20: 1)	0.52
BuOH - CH_3COOH - H_2O (6: 1: 2)	0.73

obtained by filtration through Whatman No. 1 filter paper and washed repeatedly with distilled water. Drying of cells was carried out at 80°C for 24 hours.

Determination of Antimicrobial Spectrum and Minimum Inhibitory Concentration (MIC)

The antimicrobial spectrum of *A. versicolor* $(N_5)_{17}$ was determined by the cross-streak technique in nutrient agar medium containing 1% peptone for bacteria, in Sabouraud medium for pathogenic fungi and in modified Czapek-Dox enriched with 0.1% peptone for other fungi.

MIC of mycoversilin against the sensitive organisms was determined by the usual agar dilution method. An alcoholic solution (1: 1) of the antibiotic was added to liquefied Sabouraud agar to give various concentrations. The resulting mixtures were poured into plates which were inoculated with the sensitive organisms by the cross streak technique; observations were recorded after 3 days of incubation.

Assay

For the assay of the antibiotic, the standard agar cup technique was used with *T. rubrum* as the test organism.

Chromatographic Studies

One-dimensional ascending chromatography was conducted as follows.

For thin-layer chromatography, glass plates (15 cm×2 cm) covered with Silica gel G (E. Merck, Germany) and activated by heating at 120°C for two hours were used. The developed plates were treated with the permanganate-BPB reagent developed by AKITA *et al.*[16] to ascertain the absence of any organic material other than the antibiotic. For paper chromatography, Whatman No. 1 paper strips (26 cm×7 cm) were used. In both cases, an alcoholic solution of the antibiotic was used and spots were detected by developing in iodine vapor. Solvent systems used are shown in Tables 1 and 2.

Results and Discussion

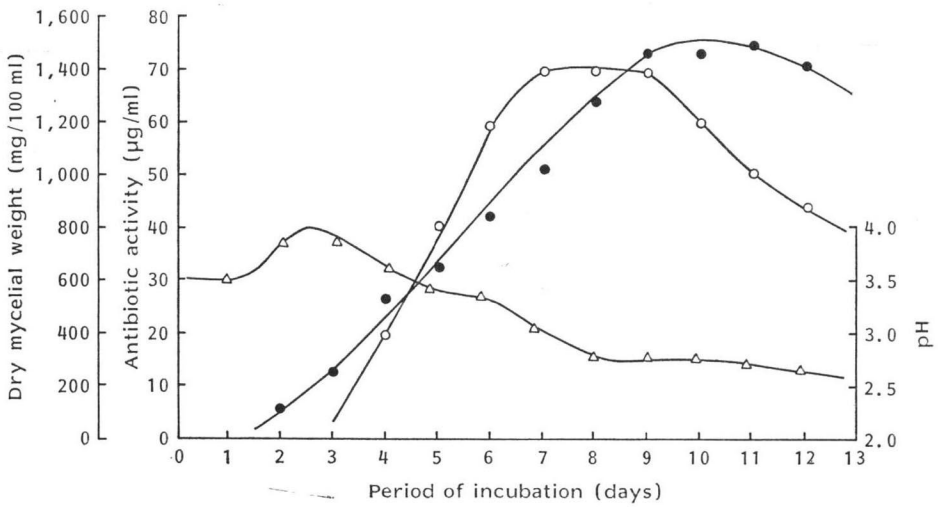
Time Course Production of Mycoversilin

The time course of mycoversilin production is shown in Fig. 1. Growth and antibiotic production were closely associated and there was no trophophase-idiophase pattern.

Isolation and Purification of Mycoversilin

The fermented broth obtained by filtering off the mycelium after 8 days of incubation was used for isolation of the antibiotic. Preliminary assays showed that the antibiotic was extractable by solvents at acidic pH. No adjustment of pH after fermentation was necessary, since the pH of the broth, initially adjusted at 3.5, only slightly decreased to 2.8. Fermentation broth (5 liters) at pH 2.8, free from vegetative growth, was extracted with 2.5 liters of amyl acetate. The amyl acetate extract was dried over anhydrous sodium sulfate and the remaining solvent evaporated *in vacuo* at 60~70°C to give a dark brown mass (3 g) which was dissolved in 15 ml of acid-free ethyl acetate. The solution was charged on an acid alumina (E. Merck, Germany) column No. 1 (19 cm×3 cm), previously activated at 110°C

Fig. 1. Time course of mycoversilin production.
○ Antibiotic activity, ● mycelial weight, △ pH.



for 15 hours; the active material was then eluted with 250 ml ethyl acetate at the rate of 2~3 ml/minute. After evaporation of the solvent *in vacuo* (60°C) the active material was charged on a second acid alumina column (25 cm×2 cm) and eluted with ethyl acetate (150 ml) at the rate of 2 ml/minute. The eluted material, after evaporation of the solvent *in vacuo* (60°C), was rechromatographed on a silica gel (BDH, India) column (16 cm×1.5 cm) and fractionally eluted at the rate of 1 ml/minute, first with benzene and then with a mixture of solvents (benzene and ethyl acetate) whose proportions and respective volumes were (49: 1, 50 ml), (24: 1, 50 ml), (23: 2, 50 ml) and (21: 4, 350 ml). The volume of the eluent was however varied depending on the material to be charged on the column. The active material eluted with 200~350 ml of the solvent system 21: 4 gave, on standing overnight at room temperature, white crystals of the antibiotic. These were filtered and collected. The procedure for isolation of mycoversilin is schematically represented in Fig. 2.

The crude antibiotic thus obtained was recrystallized from ethyl acetate. Fine needles, mp 242±1°C (dec) were obtained.

Physico-chemical properties and structural elucidation of mycoversilin will be reported in the next paper[17].

Homogeneity of Mycoversilin

The homogeneity was determined by thin-layer chromatography and permanganate - BPB reagent and by paper bioautography. Mycoversilin appeared to be homogeneous and contained only one biologically active component as shown in Tables 1 and 2.

Antimicrobial Spectrum and MIC of Mycoversilin

A. versicolor $(N_5)_{17}$ was found active against dermatophytes and plant pathogens while inactive against yeasts, Gram-positive and Gram-negative bacteria (Table 3). The MIC of mycoversilin against the most sensitive organism, *T. rubrum*, was 15 μg/ml (Table 4).

Fig. 2. Isolation of mycoversilin.

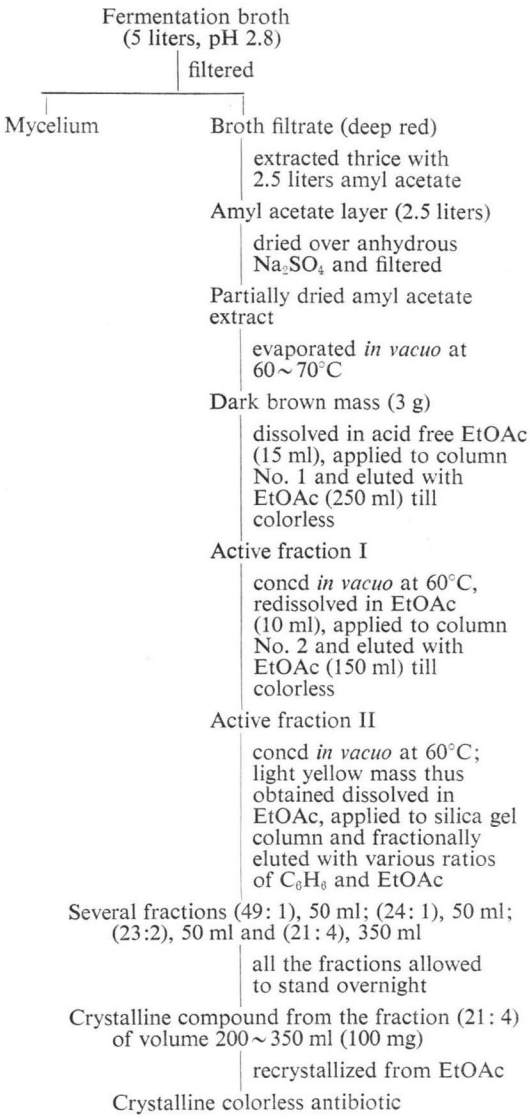


Table 3. Antimicrobial spectrum of *A. versicolor* $(N_5)_{17}$ (agar cross streak method).

Test organism	Activity
Bacillus subtilis (B_3)	−
Escherichia coli	−
Micrococcus luteus	−
Serratia marcescens	−
Trichophyton rubrum	+
T. tonsurans	+
T. mentagrophytes	+
T. interdigitale	+
Microsporum gypseum	+
Epidermophyton floccosum	+
Candida albicans	−
Saccharomyces cerevisiae	−
Macrophomina phaseoli	+
Helminthosporium oryzae	−
Colletotrichum gloeosporioides	+
Penicillium chrysogenum ITCCF 1495	−
P. italicum ITCCF 1507	±
Aspergillus niger	−

+ Activity, − no activity.

Table 4. MIC of mycoversilin (agar dilution method).

Test organism	MIC (μg/ml)
Trichophyton rubrum	15
T. tonsurans	20
T. mentagrophytes	40
T. interdigitale	45
Microsporum gypseum	30
Epidermophyton floccosum	30
Colletotrichum gloeosporioides	70
Macrophomina phaseoli	250

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References

1) SHIBATA, S.; S. NATORI & S. UDAGAWA: List of Fungal Products. Univ. of Tokyo Press, Tokyo, 1964
2) BIRKINSHAW, J. H.; J. C. ROBERTS & P. ROFFEY: Studies in mycological chemistry. XIX. Product B (averantin) [1,3,6,8-tetrahydroxy-2-(1-hydroxyhexyl)anthraquinone], a pigment from *Aspergillus versicolor* (Vuillemin) Tiraboschi. J. Chem. Soc. (C) 1966: 855~857, 1966
3) ROBERTS, J. C. & P. ROFFEY: Studies in mycological chemistry. XVII. Averythrin, an anthraquinonoid pigment from *Aspergillus versicolor* (Vuillemin) Tiraboschi. J. Chem. Soc. 1965: 3666~3672, 1965
4) HAMASAKI, T.; Y. HATSUDA, N. TERASHIMA & M. RENBUTSU: The structure of two new metabolites of *Aspergillus versicolor*. Agric. Biol. Chem. 29: 166~167, 1965

5) CIEGLER, A.; S. KADIS & S. J. AJL: Microbial Toxins. Vol. VI. Fungal Toxins. pp. 152~154, Academic Press, New York and London, 1971
6) COLE, M. & G. N. ROLINSON: New antibiotic from *Aspergillus versicolor* fermentation. Ger. Offen. 1,803,037, Aug. 14, 1969
7) COLE, M. & G. N. ROLINSON: Microbial metabolites with insecticidal properties. Appl. Microbiol. 24: 660~662, 1972
8) DHAR, A. K. & S. K. BOSE: A new antifungal antibiotic from *Aspergillus versicolor*. J. Antibiotics, Ser. A, 21: 156~157, 1968
9) DHAR, A. K. & S. K. BOSE: Studies on versicolin, a new antifungal antibiotic from *Aspergillus versicolor*. II. Isolation and purification. Appl. Microbiol. 16: 749~752, 1968
10) DHAR, A. K. & S. K. BOSE: Effect of trace elements upon the fermention of versicolin. Appl. Microbiol. 16: 1429~1430, 1968
11) DHAR, A. K. & S. K. BOSE: Studies on versicolin, a new antifungal antibiotic from *Aspergillus versicolor*. I. Structure of versicolin. Tetrahedron Lett. 1969: 4871~4874, 1969
12) DHAR, A. K. & S. K. BOSE: Mutagens for regeneration of an antibiotic-producing strain of *Aspergillus versicolor*. Appl. Microbiol. 16: 340~342, 1968
13) KOLE, H. K. & S. K. BOSE: A note on the development of a technique for regeneration of a degenerated culture. J. Appl. Bacteriol. 48: 433~436, 1980
14) KOLE, H. K.; A. K. SAMANTA, S. K. GOSWAMI & S. K. BOSE: Attempt at gene reversion of a desired phenotype as a rescue against strain degeneration. J. Appl. Bacteriol. 53: 163~167, 1982
15) SAMANTA, A. K.; H. K. KOLE, S. K. GOSWAMI & S. K. BOSE: Mycoversilin, a new antifungal antibiotic from a mutant derivative of *Aspergillus versicolor*. Ind. J. Exptl. Biol. 21: 577~578, 1983
16) AKITA, E. & T. IKEKAWA: A new colour reaction with $KMnO_4$/bromophenol blue on thin layer chromatography. J. Chromatogr. 12: 250~251, 1963
17) SAMANTA, A. K.; S. K. BOSE & S. B. MAHATO: Mycoversilin, a new antifungal antibiotic. II. Structure elucidation. J. Antibiotics 37: 733~737, 1984