MYCOVERSILIN, A NEW ANTIFUNGAL ANTIBIOTIC

III. MECHANISM OF ACTION ON A FILAMENTOUS FUNGUS *TRICHOPHYTON RUBRUM*

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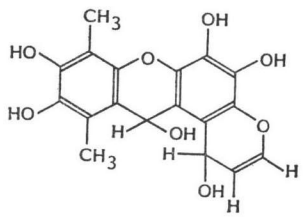
Mycoversilin is active against filamentous fungi, being specifically inhibitory to *Trichophyton rubrum*, minimum inhibitory concentration being 15 μg/ml. Mycoversilin inhibits sporulation to the extent of 28.5% even at the growth inhibitory concentration whereas inhibition of spore germination requires higher concentration. It has no effect on radial growth. Further it shows no action either on the release of UV absorbing materials or on the respiration of *T. rubrum*. However, the antibiotic inhibits *in vivo* synthesis of protein fairly strongly, DNA moderately and RNA slightly at the minimum inhibitory concentration. Cell-free protein synthesis is also strongly inhibited, the site of action being the inhibition of leucyl-tRNA formation by the antibiotic which has no action on leucine activation.

Mycoversilin (Fig. 1) is a new antifungal antibiotic isolated in this laboratory from the culture filtrate of *Aspergillus versicolor* $(N_5)_{17}$[1,2,3]. The antibiotic is specifically active against filamentous fungus *Trichophyton rubrum*, a skin pathogen. In the present paper we are presenting the results of our studies on the mechanism of action of mycoversilin on *T. rubrum*.

Fig. 1. Chemical structure of mycoversilin.



Materials and Methods

Chemicals

L-[*U*-^{14}C]Leucine with a specific activity of 240 mCi/mmol, [^{14}C]thymine 18.8 mCi/mmol, [^{14}C]-phenylalanine 432 mCi/mmol, [2-^{14}C]uracil 54 mCi/mmol and sodium [^{32}P]pyrophosphate 5 mCi/mmol were purchased from Bhaba Atomic Research Centre, Bombay, India. L-[^{14}C]Leucine, [^{14}C]thymine and [2-^{14}C]uracil were diluted with respective carrier substrates to give a 1 mM, 5 mCi/mmol solution. Adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphoenol pyruvate (PEP), polyuridylic acid (polyU), pyruvate kinase, spermine tetrahydrochloride, dithiothreitol (DTT), uracil, thymine, leucine, bovine serum albumin (BSA), 2,5-diphenyloxazole (PPO) and phenyloxazolylphenyloxazolylphenyl(POPOP) were purchased from Sigma. All other chemicals were of reagent grade.

Organisms and Growth Media

All our studies were done with the sensitive organism *T. rubrum*, obtained from the School of Tropical Medicine, Calcutta, India. The organism was maintained on Sabouraud agar slants at 28°C. Sabouraud agar medium was used for plating and harvesting of spores whereas Sabouraud broth for growth and harvesting of mycelium. For incorporation studies with intact cells of *T. rubrum*, a synthetic medium containing glucose 4%, monosodium glutamate 1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and $ZnSO_4 \cdot 7H_2O$ 0.001%, pH 5.5 was used which was subsequently named nitrate free glutamate containing Czapek-Dox medium.

Mycoversilin

Mycoversilin was used as solution in 95% ethanol. The final concentration of ethanol in the assay mixtures was kept lower than or equal to 0.7%.

Buffers Used

Buffer A, B and C contained the following ingredients:

Buffer A: 50 mM Tris-HCl, pH 8.0 containing 5 mM magnesium acetate, 10 mM KCl, 10 mM DTT and 0.5 mM spermine tetrahydrochloride.

Buffer B: 10 mM Tris-HCl, pH 7.5 containing 0.3 M sucrose, 5 mM magnesium acetate and 1 mM DTT.

Buffer C: 20 mM Tris-HCl, pH 7.5 containing 0.1 M ammonium chloride, 5 mM magnesium acetate and 1 mM DTT.

Minimum Inhibitory Concentration (MIC)

Standard serial agar plate dilution assay was used to determine the MIC. The MIC was read after 72 hours of incubation at 32°C.

Preparation of Spore Suspension of *T. rubrum*

A 21 days old slant of *T. rubrum* was washed thoroughly with distilled water. The washings were filtered through cotton to remove mycelium fragments. The spore suspension so obtained was diluted to give an absorbance of 30 Klett units at 660 nm.

Determination of Growth Curve

Effect of mycoversilin on the growth of *T. rubrum* was followed by determining the mycelial dry weight, grown under shake condition at 32°C.

Determination of Fungistatic or Fungicidal Action

Spore suspension (0.3 ml) was inoculated into 10 ml of Sabouraud broth containing desired concentrations of the antibiotic or alcohol and incubated at 32°C under shake condition. After desired intervals the growth of *T. rubrum* was filtered, washed with distilled water and resuspended in 10 ml of fresh Sabouraud broth and incubated further at 32°C for 48 hours to observe if growth had occurred.

Effect of Mycoversilin on Radial Growth, Spore Germination and Sporulation

Radial Growth: The method described by NENE[4] was followed. Discs of inoculum (6 mm) cut out of the periphery of actively growing colonies of the test fungus were used.

Spore Germination: Spore germination was studied following the method of BOSE[5]. Number of germinated spores on a microscopic slide were counted under AO phase contrast microscope after 44 hours of incubation in a moist chamber at 32°C.

Sporulation: The test fungus was allowed to grow in Sabouraud agar medium in presence of the antibiotic. After 28 days of incubation spores from discs (14 mm) of *T. rubrum* growth were harvested into 8 ml of distilled water, filtered and the number counted using NEUBAUER's counting chamber.

Effect of Mycoversilin on the Release of UV Absorbing Substances

The method used was essentially the same as described by HALDER *et al.*[6]. *T. rubrum* cells in early logarithmic phase (10 mg wet weight) were suspended in 10 ml of 0.1 M phosphate buffer, pH 7.0. The antibiotic was added and the mixture shaken at 37°C for 24 hours. The mycelia were then filtered and the absorbancy readings at 260 nm and 280 nm of the supernatants were determined.

Effect of Mycoversilin on the Respiration (Endogenous and Exogenous) of *T. rubrum*

Respiration was measured in the WARBURG apparatus following the method of HALDER *et al.*[6].

Macromolecular Synthesis in the Intact Cells of *T. rubrum*

Cells were grown to early logarithmic phase in nitrate free glutamate containing Czapek-Dox medium. Radiolabelled precursors were incorporated following the method of SINGH *et al.*[7] for 60 minutes at 30°C with 15 minutes preincubation with mycoversilin, and the radioactivity of 10% cold TCA insoluble material was determined in a liquid scintillation counter using 5 ml of toluene base scintillation counting solution. For protein and RNA synthesis, 1 ml reaction mixture contained 1 mg of cells and 0.1 μCi [^{14}C]leucine or 0.1 μCi [2-^{14}C]uracil and for DNA synthesis 2 ml reaction mixture

contained 4 mg of cells and 0.5 μCi [^{14}C]thymine.

Preparation of Enzyme for Studying Cell-free Protein Synthesis and Aminoacyl-tRNA Formation

T. rubrum cells grown to early logarithmic phase in Sabouraud broth were used to prepare cell-free extract according to SUGAWARA[8] using buffer A for homogenization. The extract was centrifuged at 10,000$\times g$ for 10 minutes, then at 20,000$\times g$ for 20 minutes and again at 20,000$\times g$ for 30 minutes discarding the pellet after each centrifugation. A part of this 20,000$\times g$ supernatant was used as the enzyme source for studying cell-free protein synthesis and the rest was again centrifuged at 105,000$\times g$ for 1.5 hours in an Ultracentrifuge (Beckman). This 105,000$\times g$ supernatant was used as the enzyme source for studying aminoacyl-tRNA formation. Protein content in the supernatant was measured by the method of LOWRY *et al.*[9] using BSA as the standard.

Preparation of pH 5 Enzyme

The pH 5 enzyme from filamentous *T. rubrum* was prepared following the method of HOAGLAND *et al.*[10] as further modified by STAEHELIN and FALVEY[11]. *T. rubrum* early log phase cells in Sabouraud broth were used to prepare cell-free extract according to SUGAWARA[8] using buffer B for homogenization. The post-mitochondrial supernatant was prepared by centrifugation of the extract at 20,000$\times g$ for 30 minutes which was then further centrifuged in an Ultracentrifuge (Beckman) at 105,000$\times g$ for 1.5 hours. The supernatant (post-microsomal) was then diluted with two volumes of cold distilled water containing 1 mM DTT and the pH was adjusted to 5.1$\sim$5.2 by dropwise addition of 1 M acetic acid with constant stirring. The precipitate which formed was immediately centrifuged at 20,000$\times g$ for 10 minutes and then dissolved in buffer C. The pH was adjusted to 7.5 by addition of small amounts of 1 N KOH. The final volume was approximately 1 ml per 6 ml of post-microsomal supernatant. Traces of insoluble material were removed by centrifugation. Protein content in the pH 5 enzyme fraction was determined by the method of LOWRY *et al.*[9]

Protein Synthesis in Cell-free System

The 20,000$\times g$ supernatant was used as the enzyme source for studying polyU directed cell-free protein synthesis. Incorporation studies were carried out according to the method of SINGH *et al.*[7]. After incubation at 37°C for 30 minutes, one volume of ice cold 10% TCA was added to terminate the reaction. Tubes were placed in a boiling water bath for 15 minutes. After cooling the insoluble protein fraction was filtered, washed and dried according to SIEKEVITZ's[12] method. Radioactivity on the dried filter papers was counted in a liquid scintillation counter using toluene base scintillation counting solution.

Amino Acid Activation by Amino Acid Dependent Pyrophosphate Exchange Reaction

The pH 5 enzyme was used as the enzyme source for studying leucine dependent activation reaction following the method of ROTH and AMES[13]. The reaction mixture contained in a volume of 500 μl: 100 mM Tris-HCl pH 7.6, 10 mM KF, 5 mM magnesium chloride, 2 mM DTT, 2 mM ATP, 2 mM leucine, 2 mM sodium pyrophosphate, ^{32}P-labelled sodium pyrophosphate 15 μCi, mycoversilin solution or alcohol and pH 5 enzyme (1 mg protein). After incubation at 37°C for 30 minutes, the reaction was stopped by adding 0.5 ml of ice-cold 10% TCA containing Norit A (30 mg/ml) and sodium pyrophosphate (0.1 mmol). After standing for 15 minutes at 0°C, the mixture was collected by Millipore filtration and the radioactivity was counted in a liquid scintillation counter.

Method of Following Aminoacyl-tRNA Formation in Presence of Mycoversilin

The 105,000$\times g$ supernatant was used as the enzyme source for studying [^{14}C]leucyl-tRNA formation by the process as developed by FANGMAN and NEIDHARDT[14]. The reaction mixture contained in a volume of 500 μl: 50 mM Tris-HCl pH 7.5, 3.5 mM magnesium acetate, 7.0 mM KCl, 2 mM DTT, 0.15 mM GTP, 2 mM ATP, 5 mM PEP, 25 μg pyruvate kinase, [^{14}C]leucine 1 μCi, mycoversilin solution or alcohol and 105,000$\times g$ supernatant fraction (1 mg protein). After incubating for 20 minutes at 30°C, the reaction was terminated by addition of 3.5 ml cold 6% TCA. The acid insoluble precipitate was filtered through glass filter (Whatman GF/C) in the cold (4°C) and washed with 5% TCA containing 0.5% of DL-[^{12}C]leucine. The radioactivity on glass filter was counted in a liquid scintillation counter.

Results

Effect of Mycoversilin on the Growth of *T. rubrum*

The minimum inhibitory concentration (MIC) of mycoversilin against *T. rubrum* was found to be 15 μg/ml.

Fig. 2 shows that at subinhibitory concentrations the antibiotic had no effect on the growth, while at MIC level it affected the lag period only slightly but maximum growth supportable was very strongly affected. At 20 μg/ml, the specific effect was particularly on the lag period which was almost 4 days and the maximum growth supportable was also very adversely affected, being inhibited to 59% of the control.

Nature of Antifungal Action, Fungistatic or Fungicidal

The antibiotic was fungistatic at the concentrations of 15, 30 and 60 μg/ml. It's fungicidal activity was observed only at 120 μg/ml (data not shown).

Effect of Mycoversilin on Radial Growth, Spore Germination and Sporulation of *T. rubrum*

It appears from Fig. 3 that mycoversilin had no inhibitory effect on radial growth even at a concentration as high as 300 μg/ml. However, it had some inhibitory effect at exceptionally high concentrations viz. 480 μg/ml or 600 μg/ml.

The effect of mycoversilin on spore germination was not very marked, inhibition being 16.6% at the MIC level. However, elevated concentrations of the antibiotic increased the inhibitory effect, which was as high as 91% at an unusually high concentration viz. 180 μg/ml (Fig. 3).

Fig. 3 also shows that mycoversilin inhibited sporulation strongly, inhibition being 28.5% at MIC. At 30 μg/ml it inhibited sporulation by 73.7%. With a further increase in concentrations, the degree of inhibition (%) also increased but slowly.

Fig. 2. Effect of mycoversilin on the growth of *T. rubrum*.

Growth was determined from dry weight of mycelia.

○ Control, □ mycoversilin 5 μg/ml, ▲ 10 μg/ml, ■ 15 μg/ml, ● 20 μg/ml.

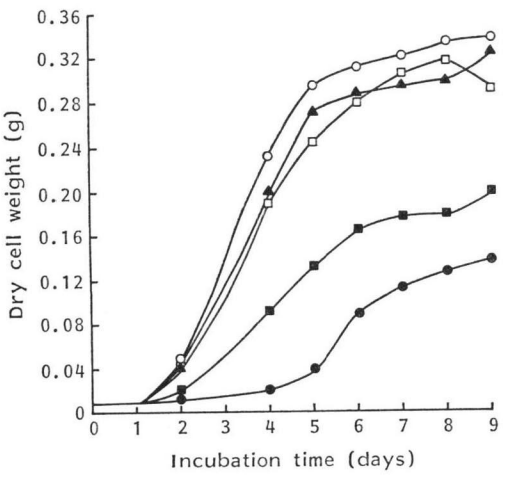


Fig. 3. Effect of mycoversilin on the different stages in the life cycle of *T. rubrum*.

● Sporulation, ○ spore germination, △ radial growth.

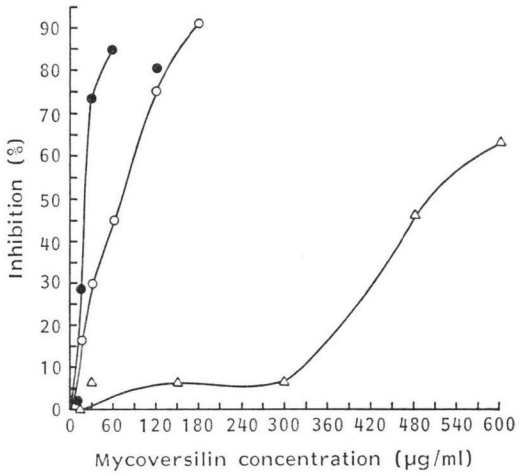


Table 1. Effect of mycoversilin on incorporation of L-[*U*-^{14}C]leucine, [2-^{14}C]uracil and [2-^{14}C]thymine into the protein, RNA and DNA of *T. rubrum*.

Mycoversilin (μg/ml)	L-[*U*-^{14}C]Leucine incorporation into protein		[2-^{14}C]Uracil incorporation into RNA		[2-^{14}C]Thymine incorporation into DNA	
	cpm/mg cell	Inhibition (%)	cpm/mg cell	Inhibition (%)	cpm/mg cell	Inhibition (%)
0	8,476	—	6,516	—	4,690	—
15	4,309	49.1	5,671	13.1	3,835	18.3
30	3,513	58.5	5,048	22.5	2,524	46.1
60	2,803	67.0	4,854	25.5	2,178	53.6

Incubation 1 hour at 30°C. The mean of triplicate assay of each experiment are presented.

Effect of Mycoversilin on the Release of UV Absorbing Substances

Mycoversilin at any concentrations tested (ranging from 15~150 μg/ml) did not cause release of 260 nm and 280 nm absorbing materials from mycelia of *T. rubrum* (data not shown).

Effect of Mycoversilin on Respiration (Endogenous and Exogenous)

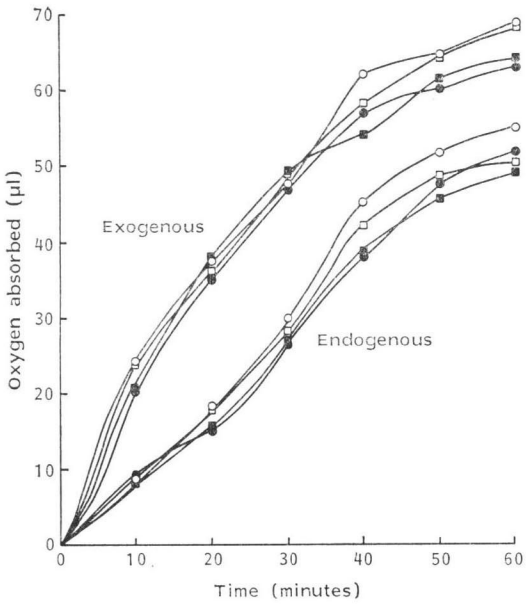
Fig. 4 shows that mycoversilin did not have any effect on respiration both endogenous and exogenous either at MIC or at higher concentrations (viz. 30 μg/ml or 60 μg/ml).

Effect of Mycoversilin on Macromolecular Synthesis in the Intact Cells of *T. rubrum*

Fig. 4. Effect of mycoversilin on respiration (endogenous and exogenous) of *T. rubrum*.

The reaction mixture in 15-ml WARBURG flask contained 30 mg wet weight of 4 days old mycelium (corresponding to 4.5 mg dry weight), mycoversilin solution or alcohol 0.1 ml, 0.1 M phosphate buffer (pH 7.0) 2.8 ml and water or 0.6 M glucose 0.1 ml. Central cup contained 0.20 ml 20% KOH solution. Temp 37°C. Gas phase air. Incubation period 1 hour.

○ Control, □ mycoversilin 15 μg/ml, ● 30 μg/ml, ■ 60 μg/ml.



It appears from the results (Table 1) that approximately 50% inhibition of protein and DNA synthesis were observed at antibiotic concentrations 15 μg/ml (MIC) and 30 μg/ml respectively.

Table 2. Effect of mycoversilin on protein synthesis in cell-free system.

System	Mycoversilin (μg/ml)	Phenylalanine incorporation (cpm/mg protein)	Inhibition or decrease (%)
Complete	0	1,971	—
Complete	15	804	59.2
Complete	30	764	61.2
Complete	60	625	68.3
−PolyU	0	265	86.55
−ATP, −PEP, −pyruvate kinase	0	187	90.5
+ATP, −PEP, −pyruvate kinase	0	405	79.4

Reaction mixture of 500 μl contained: 50 mM Tris-HCl pH 7.5, 3.5 mM magnesium acetate, 7.5 mM KCl, 0.5 mM spermine tetrahydrochloride, 0.05 mM DTT, 0.15 mM GTP, 0.5 mM ATP, 5 mM PEP, [^{14}C]phenylalanine 0.1 μCi, 100 μg polyU, 25 μg pyruvate kinase, mycoversilin solution or alcohol (desired concentrations) and enzyme extract (1 mg protein).

The mean of triplicate assay are presented.

Table 3. Effect of mycoversilin on leucine activation.

Mycoversilin (μg/ml)	cpm/mg protein	Inhibition (%)
0	27,470	—
10	21,508	21.7
15	21,976	20.0*
25	20,117	26.7
50	20,601	25.0
100	15,216	44.6

Incubation period: 30 minutes at 37°C.
The mean of triplicate assay are presented.
* This data represents the result of a single experiment, the other two being omitted because of their aberrant values.

Table 4. Effect of mycoversilin on leucyl-tRNA formation by leucyl-tRNA synthetase.

Mycoversilin (μg/ml)	cpm/mg protein	Inhibition (%)
0	1,237	—
10	900	27.2
15	679	45.1
25	529	57.2
50	400	67.7
100	322	73.1

Incubation period: 20 minutes at 30°C.
The mean of triplicate assay are presented.

The antibiotic slightly depressed incorporation of [^{14}C]uracil into the TCA-insoluble fraction only at high concentrations.

Effect of Mycoversilin on Protein Synthesis in Cell-free System

Mycoversilin was found to inhibit polyU directed [^{14}C]phenylalanine incorporation into protein very strongly. At growth inhibitory concentration (15 μg/ml) the antibiotic showed 59.2% inhibition of protein synthesis. However at the antibiotic concentrations 30 μg/ml and 60 μg/ml the inhibition was observed to the extent of 61.2% and 68.3% respectively (Table 2).

The data in Table 2 also reveals that omission of polyU, ATP or an energy generating system almost entirely eliminated [^{14}C]phenylalanine incorporation into protein.

Effect of Mycoversilin on Amino Acid Activation

It appears from the results of Table 3 that mycoversilin did not significantly affect leucine dependent ATP-$^{32}PP_i$ exchange at MIC. However, a significant inhibitory effect was observed only at a higher concentration, viz. 100 μg/ml.

Effect of Mycoversilin on Leucyl-tRNA Formation by Leucyl-tRNA Synthetase

It is observed from Table 4 that the synthesis of leucyl-tRNA formation by leucyl-tRNA synthetase was inhibited to the extent of 27.2% and 45.1% at subinhibitory (10 μg/ml) and minimum inhibitory (15 μg/ml) concentrations, respectively. On further increase in concentrations of the antibiotic, the degree of inhibition progressively increased, being 73.1% at 100 μg/ml.

Discussion

Mycoversilin showed strong growth inhibition of filamentous fungus *T. rubrum*. The nature of antifungal action, fungistatic or fungicidal depend on the concentration of the antibiotic.

At low concentrations mycoversilin inhibited sporulation specifically but spore germination slightly whereas radial growth remained unaltered in the life cycle of *T. rubrum*. It has been reported that aabomycin A exhibited inhibitory effects on each of the stages of the growth cycle of *Piricularia oryzae*, spore germination being the most inhibited one in the growth cycle[15]. Griseofulvin reduced the mycelial growth and spore germination of *Cohliobolus miyabeanus*[16]. Chloramphenicol (0.2 μg/ml) inhibited spore germination of *Anabaena doliolum* when added during the first 24 hours[17]. The action of mycoversilin is therefore found to be different from these antibiotics with regards to its specific action on sporulation.

Mycoversilin did not alter membrane permeability like polyene macrolides[18] or mycobacillin[6,19] and had no effect on endogenous or exogenous respiration of *T. rubrum*.

Mycoversilin was found to produce a preferential inhibition of protein synthesis to DNA synthesis, but it did not significantly affect RNA synthesis in the intact *T. rubrum* cells. In this respect mycoversilin resembles very strongly the antifungal antibiotics, cycloheximide[20,21], anisomycin[22] and sparsomycin[23,24] in regard to the action on *in vivo* macromolecular synthesis.

The *in vivo* inhibitory action of mycoversilin on protein synthesis was further confirmed by its action *in vitro* in inhibiting polyU directed cell-free protein synthesis. In localising the exact site of action on *in vitro* protein synthesis it has been observed that the antibiotic inhibited not the aminoacyl-AMP formation but the aminoacyl-tRNA formation. In this respect it was similar to some but dissimilar to other antibiotics. The antibiotics nalidixic acid, oxolinic acid and novobiocin[25] inhibited both pyrophosphate exchange reaction and aminoacylation of specific tRNA in yeast, while sodium pseudomonate[26] inhibited only the pyrophosphate exchange reaction and had no action on isoleucyl-tRNA formation in *Escherichia coli* B. On the other hand the antibiotic furanomycin[27], kasugamycin[28], borrelidin[29,30] and granaticin[31] were shown to inhibit aminoacylation of specific tRNA in bacteria. Thus, there appears to be no report except the present one to our knowledge revealing the inhibition of aminoacylation of a specific tRNA by an antifungal agent. However, the action of mycoversilin, which specifically inhibits sporulation, remains yet to be explained.

Acknowledgments

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