A non-polyene antifungal antibiotic from *Streptomyces albidoflavus* PU 23

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In all 312 actinomycete strains were isolated from water and soil samples from different regions. All these isolates were purified and screened for their antifungal activity against pathogenic fungi. Out of these, 22% of the isolates exhibited activity against fungi. One promising strain, *Streptomyces albidoflavus* PU 23 with strong antifungal activity against pathogenic fungi was selected for further studies. Antibiotic was extracted and purified from the isolate. *Aspergillus* spp. was most sensitive to the antibiotic followed by other molds and yeasts. The antibiotic was stable at different temperatures and pH tested and there was no significant loss of the antifungal activity after treatment with various detergents and enzymes. Synergistic effect was observed when the antibiotic was used in combination with hamycin. The antibiotic was fairly stable for a period of 12 months at 4°C. The mode of action of the antibiotic seems to be by binding to the ergosterol present in the fungal cell membrane resulting in the leakage of intracellular material and eventually death of the cell. The structure of the antibiotic was determined by elemental analysis and by ultraviolet (UV), Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR) and liquid chromatography mass spectra (LCMS). The antibiotic was found to be a straight chain polyhydroxy, polyether, non-proteinic compound with a single double bond, indicating a non-polyene antifungal antibiotic.

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1. Introduction

Among the different types of drugs prevailing in the market, antifungal antibiotics are a very small but significant group of drugs and have an important role in the control of mycotic diseases. The need for new, safe and more effective antifungals is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host. The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources (Bevan *et al* 1995). This involves the screening of microorganisms and plant extracts (Shadomy 1987). The search for new, safer, broad-spectrum antifungal antibiotics with greater potency has been progressing slowly (Gupte *et al* 2002). One reason for the slow progress compared to antibacterials is that, like mammalian cells, fungi are eukaryotes and therefore agents that in-

Keywords. Antibiotic; antifungal; non-polyene; *Streptomyces albidoflavus*

Abbreviations used: BJMC, Byramjee Jeejeebhoy Medical College; DEPT, distortionless enhancement by polarization transfer; DSS, dimethyl silapentane sulfonate; FTIR, Fourier transform infrared; LCMS, liquid chromatography mass spectrum; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; MTCC, microbial type culture collection; NCCLS, National Committee for Clinical Laboratory Standards; NCIM, National Collection of Industrial Microorganisms; NMR, nuclear magnetic resonance; NRRL, Northern Regional Research Laboratory; SDA, Sabouraud dextrose agar; SDS, sodium dodecyl sulphate; TLC, thin-layer chromatography; UV, ultraviolet.

hibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity to the host as well (Georgopapadakou and Tkacz 1994). The other reason is that, until recently, the incidence of life threatening fungal infections was perceived as being too low to warrant aggressive research by the pharmaceutical companies (Georgopapadakou and Tkacz 1996). In the course of our screening programme for new antifungal antibiotics, a *Streptomyces* sp. PU 23 producing a non-polyene antifungal antibiotic was isolated from Pune University campus soil and later on identified as *Streptomyces albidoflavus* PU 23 (Augustine 2004). The taxonomy of the antibiotic producing strain and its antifungal activity in detail as well as production optimization have been described in our previous paper (Augustine *et al* 2004). In the present communication, we report the isolation, purification and characterization of the antifungal antibiotic from *S. albidoflavus* PU 23.

2. Materials and methods

2.1 *Actinomycete isolates*

Three hundred and twelve isolates of actinomycetes obtained from water and soil samples in Pune (Maharashtra), Bangalore (Karnataka) and Malapuram (Kerala) were used during the course of our investigation. All these isolates were screened for their antifungal activity against pathogenic fungi.

2.2 *Target organisms*

Aspergillus niger NCIM586, *Aspergillus flavus* NCIM1028, *Aspergillus fumigatus* MTCC2544, *Fusarium oxysporum* NCIM1072, *Candida albicans* NCIM 7102, *Cryptococcus humicolus* NRRL12944, *Cryptococcus* sp. NCIM3349, *Cryptococcus neoformans* BJMC, *Penicillium* sp. NCIM768, *Epidermophyton floccosum* MTCC613, *Trichophyton mentagrophytes* BJMC, *Trichophyton rubrum* MTCC296 and *Microsporum gypseum* MTCC283 were used as target organisms.

The following work deals with the characterization of the antibiotic from an actinomycete isolate, *S. albidoflavus* PU 23, which was selected on the basis of its strong antifungal activity against the target organisms.

2.3 *Extraction of the antibiotic from culture supernatant using different solvents*

As maximum antibiotic production was observed on the 8th day of incubation (Augustine *et al* 2004), fermentation was terminated on the 8th day and the broth was centrifuged at 10,000 rpm for 20 min to separate the mycelial biomass. Different solvents were used and tested for the extraction of the antibiotic from the culture supernatant. The solvents used were n-butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, benzene and xylene to determine the ideal solvent for extraction of the antibiotic from the culture supernatant (see table 1). The solvent was added to the supernatant in 1 : 1 proportion. Solvent-supernatant mixture was agitated for 45 min with homogenizer. The solvent was separated from broth by separating funnel. Solvent was centrifuged at 5000 rpm for 15 min to remove traces of fermentation broth. All extracts were assayed for their antifungal activity using respective solvents as control by agar well diffusion method (Augustine *et al* 2004).

2.4 *Separation of antibiotic from solvent*

The solvent, n-hexane was evaporated by subjecting the sample to rotating flash evaporator (Buchi, Switzerland) at 40°C (50 rpm) under vacuum. The dark brown gummy substance obtained was dissolved in ethanol and concentrated, following which the crude antibiotic powder was obtained. The crude antibiotic was collected and dried in vacuum oven at 40°C overnight. The residue obtained (crude antibiotic) was subjected to purification.

2.5 *Purification of antibiotic*

The crude antibiotic was tested for number of components present by using precoated thin-layer chromatography (TLC) plates (Polygram® Sil G/UV 254, Macherey-Nagel) using ethanol: water: chloroform (40 : 40 : 20) solvent system. Purification of the antibiotic was carried out by column chromatography using silica gel (60–120 mesh) of column chromatography grade (SRL, Mumbai). Column (35 × 10 mm) was cleaned using water and rinsed with acetone. After drying, a small piece of cotton was placed at the bottom of the column. Silica gel was then packed in the column by using ethanol: water (50 : 50) as solvent system. The crude antibiotic was loaded at the top of the column and eluted using ethanol: water (50 : 50) as solvent system. Fractions were collected at 20 min interval. TLC of each fraction was performed using precoated TLC plates and simple glass plates to detect the antibiotic. The TLC plates were exposed to iodine vapors to develop the antibiotic, if any. The fractions having same R_f value were mixed together and the solvent evaporated at 40°C in a vacuum oven. These fractions were tested for their antifungal activity by using the agar well diffusion method. The fractions showing antifungal activity were again purified by using the same above mentioned column chromatography system and purity was confirmed using TLC

plates. The brown coloured powder obtained was stored in an ampoule at 4°C.

2.6 *Determination of minimum inhibitory concentration and minimum fungicidal concentration values of the antibiotic from* S. albidoflavus *PU 23 against different target organisms*

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values (see table 2) of the antibiotic were determined by broth tube dilution procedure using two-fold dilution in Sabouraud dextrose broth (SDA) at 28°C (Cappuccino and Sherman 1999). The MFC values of the antibiotic were determined by subculturing 50 µl from tubes not visibly turbid and spot inoculating onto SDA plates (Lavermicocca *et al* 2003). MFC values were determined as the lowest concentration that prevented growth on subculture (Hammer *et al* 2002).

2.7 *Thermal stability of the antibiotic from* S. albidoflavus *PU 23*

To determine the effect of temperature on stability of the antibiotic, screw capped ampoules, each with 100 µg/ml of the antibiotic in water were kept at temperatures 30, 40, 50, 60, 70 and 80°C for 1 h in water bath. The antibiotic solutions were cooled to room temperature and volumes were brought to the original and the residual antifungal activity (see table 3) was determined against the target cultures. The antibiotic was also subjected to autoclaving temperature (121°C for 15 min).

2.8 *Effect of pH on activity and stability of the antibiotic from* S. albidoflavus *PU 23*

To determine the effect of pH on stability of the antibiotic, 100 µg of the antibiotic was mixed with 1 ml of 0·1 N phosphate buffer of varied pH (5·7–8·0) in various tubes, incubated for 1 h at 30°C and the residual antifungal activity in each tube was determined against both *A. niger* and *C. albicans* as target organisms (see table 4).

2.9 *Synergistic effect of the antibiotic from* S. albidoflavus *PU 23 with hamycin, nystatin and amphotericin B against* C. albicans

The antagonistic activity of the antibiotic from *S. albidoflavus* PU 23 in combination with hamycin was tested against *C. albicans*. Whatman paper No. 1 strips (10 × 45 mm) each containing 100 µg of the antibiotic from *S. albidoflavus* PU 23 and strips containing 100 units of hamycin per strip were prepared and placed perpendicular to each other on the agar plates already spread inoculated with *C. albicans* and incubated at 28°C for two days. The inhibition was observed in the presence and absence of hamycin (Cappuccino and Sherman 1999). Similar tests were carried out in combination with nystatin and amphotericin B.

2.10 *Effect of ergosterol on antifungal activity of the antibiotic from* S. albidoflavus *PU 23*

In order to determine the effect of the antibiotic from *S. albidoflavus* PU 23 on the ergosterol present in the fungal cell membrane, ergosterol was used as the reversal agent to test for its ability to reverse the inhibition of *C. albicans* caused by the antibiotic from *S. albidoflavus* PU 23. SDA plates with 0·5% ergosterol were prepared along with a control without ergosterol. The plates were seeded with the test organism. Wells were made with a sterile cork borer and 0·1 ml of the antibiotic (100 µg/ml) was added to the well. The plates were incubated at 28°C for 24 h and observed for the zone of inhibition.

2.11 *Effect of detergents on activity of the antibiotic from* S. albidoflavus *PU 23 against* C. albicans

Susceptibility of the antibiotic to denaturation by various detergents, viz Tween 20, Tween 80, Triton X-100, sodium dodecyl sulphate (SDS) and Cetrimide was determined by mixing the detergents with the antibiotic and incubating them at 30°C for 6 h (see table 5). Detergents were dissolved in distilled water at concentration of 0·01 g/ml. One hundred µl of the antibiotic solution (100 µg/ml) was mixed with 100 µl of detergent and incubated as mentioned above (Munimbazi and Bullerman 1998). Detergents added to distilled water were used as controls to check the effect of detergents themselves on *C. albicans*.

2.12 *Effect of enzymes on activity of the antibiotic from* S. albidoflavus *PU 23 against* A. niger *and* C. albicans

The sensitivity of the antibiotic to denaturation by enzymes proteinase K, trypsin, lipase and lysozyme was tested. All the enzymes were obtained from Sigma Chemical Co., USA and were dissolved in distilled water at concentration 1 mg/ml. One hundred µl of the antibiotic solution (100 µg/ml) was mixed with 100 µl enzyme and incubated at 30°C for 3 h. The antibiotic solution without any enzymes served as control (Munimbazi and Bullerman 1998). The residual antifungal activity of the mixture was tested against *A. niger* and *C. albicans* by the agar well diffusion method (see table 6).

2.13 *Elemental analysis and melting point of the compound*

The elemental analysis was carried out in the micro analytical laboratory of the National Chemical Laboratory, Pune. The analysis was done using CHNS analyzer (Vario-El, Germany). Melting point was determined on electrically heated oil bath (Thomas Hoover, USA).

2.14 *Ultraviolet and Fourier transform infrared spectra*

Ultraviolet (UV) spectrum were recorded on Shimadzu UV-170 spectrophotometer. One milligram of sample was dissolved in 10 ml water and the spectra were recorded at 200–400 nm range. The infrared spectra were recorded on Shimadzu IR-470 model. The spectra were scanned in the 400 to 4000 cm^{-1} range. The spectra were obtained using potassium bromide pellet technique. Potassium bromide (AR grade) was dried under vacuum at 100°C for 48 h and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra were plotted as intensity versus wavenumber.

2.15 *Nuclear magnetic resonance and liquid chromatography mass spectra*

The pure antibiotic sample was subjected to ^{1}HNMR, ^{13}CNMR and DEPT spectra (500 MHz, Brucker Biospin, Switzerland) The antibiotic sample was dissolved (3 mg for ^{1}HNMR and 10 mg for ^{13}CNMR) in 3 ml of D_2O and analysed by nuclear magnetic resonance (NMR) with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard. The liquid chromatography mass spectrum (LCMS) was obtained from HP CHEM instrument (GC-LC/MS).

2.16 *Determination of shelf life of the antibiotic from* S. albidoflavus *PU 23*

The effect of storage time on antifungal activity of the antibiotic (100 µg/ml) was determined by storing the antibiotic at 4°C in different ampoules for 1 h, 24 h, 1 week, 1 month, 2 months, 3 months, 6 months and 12 months. After the specified storage period, 100 µl from each tube was added in wells prepared in SDA plates already seeded with the target organisms i.e. *A. niger* and *C. albicans* separately. The wells were prepared by using a sterile cork borer of 10 mm diameter. The results were recorded after incubation at 28°C for four days (see table 8).

3. Results

3.1 *Actinomycete isolates*

Out of total 312 actinomycetes 22% of the isolates exhibited activity against fungi. One promising isolate, *S. albidoflavus* PU 23 with strong antifungal activity

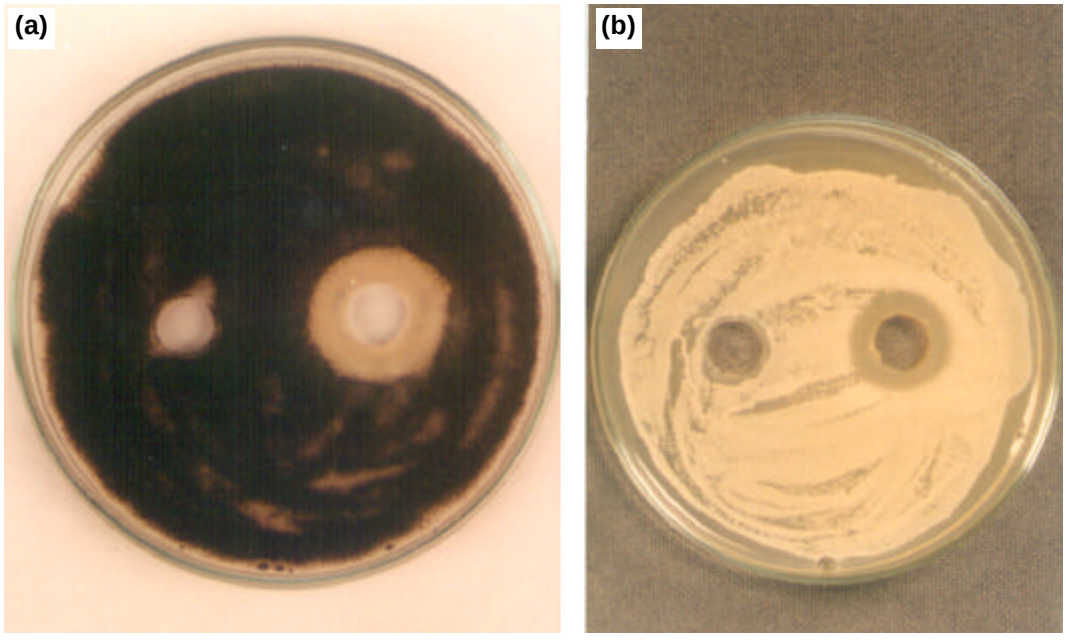

Figure 1. Antifungal activity exhibited by n-hexane extract of supernatant from *S. albidoflavus* PU 23 against (**a**) *A. niger* and (**b**) *C. albicans* (100 µl of the n-hexane extract was added in the wells. The well without the zone of inhibition is the control i.e. n-hexane).

against pathogenic fungi was selected for further studies (figure 1).

3.2 *Extraction of the antibiotic*

Different solvents were used and tested for the extraction of the antibiotic. In case of *S. albidoflavus* PU 23, maximum antibiotic yield was observed in residue, which was extracted by using n-hexane (table 1).

3.3 *Purification of the antibiotic*

The fractions collected by column chromatography technique were checked for their antifungal activity. The active fraction had a R_f value 0·78. The pure powder thus obtained was stored at 4°C.

3.4 *MIC and MFC values of the antibiotic from* S. albidoflavus *PU 23 against different target organisms*

Aspergillus spp. were most sensitive to the antibiotic produced by *S. albidoflavus* PU 23 followed by other molds and yeasts as seen by the MIC and MFC values (table 2).

3.5 *Thermal stability of the antibiotic*

The antibiotic was stable at different temperatures, however the antibiotic lost its antifungal activity completely after autoclaving at 121°C for 15 min (table 3).

3.6 *Effect of pH on activity and stability of the antibiotic from* S. albidoflavus *PU 23*

The antibiotic was quite stable within this pH range as tested against both *A. niger* and *C. albicans*. After incubation of the antibiotic at pH in the range of 5·7–8·0, maximum residual activity was observed at pH range 6·8–8, whereas the activity decreased at pH 6·3 and below (table 4).

3.7 *Synergistic activity of the antibiotic from* S. albidoflavus *PU 23 in presence of hamycin, nystatin and amphotericin B against* C. albicans

The strip containing hamycin (100 unit/strip) and another one containing the antibiotic from *S. albidoflavus* PU 23 (100 μg/strip) were kept at right angles on the agar plate, seeded with *C. albicans*. The inhibition zone was 16 mm along the length of the strip containing the antibiotic

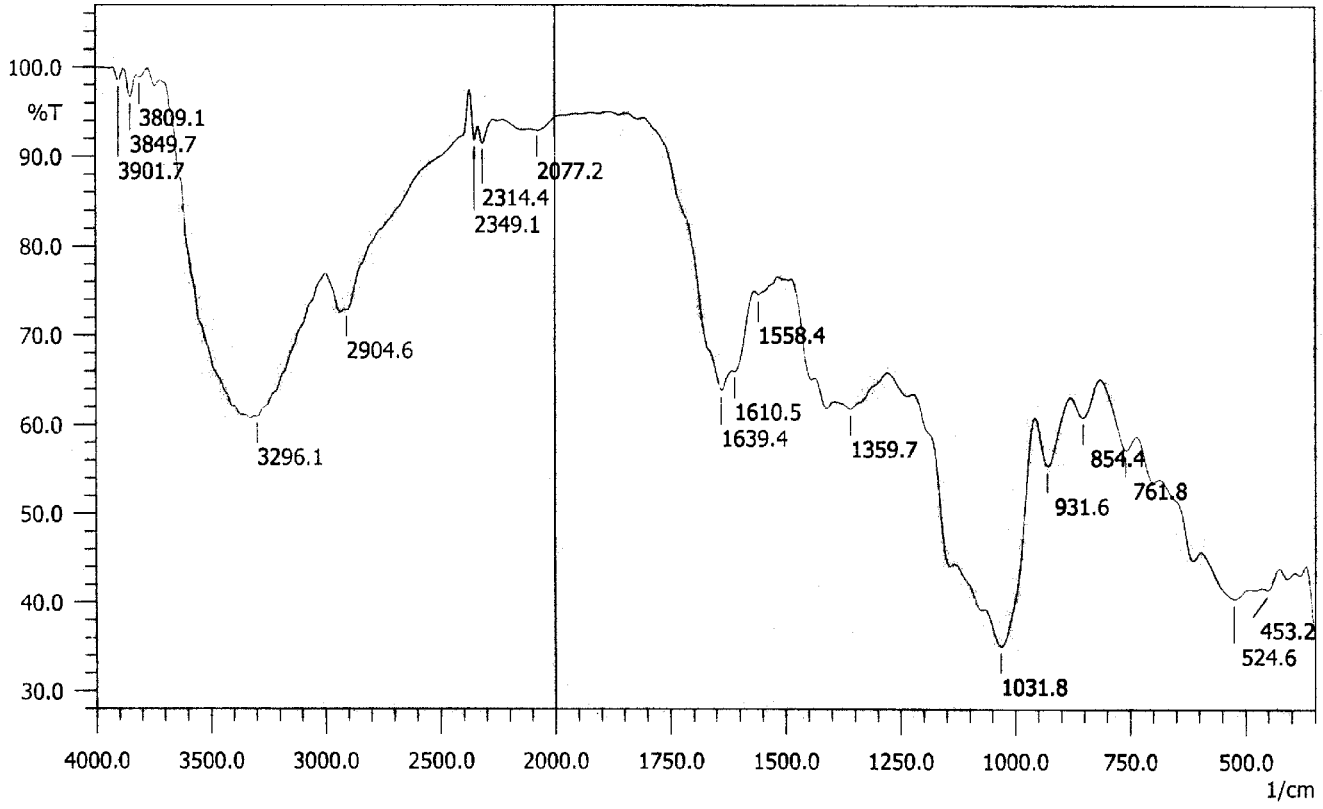

Figure 2. FTIR spectrum of the antibiotic from *S. albidoflavus* PU 23.

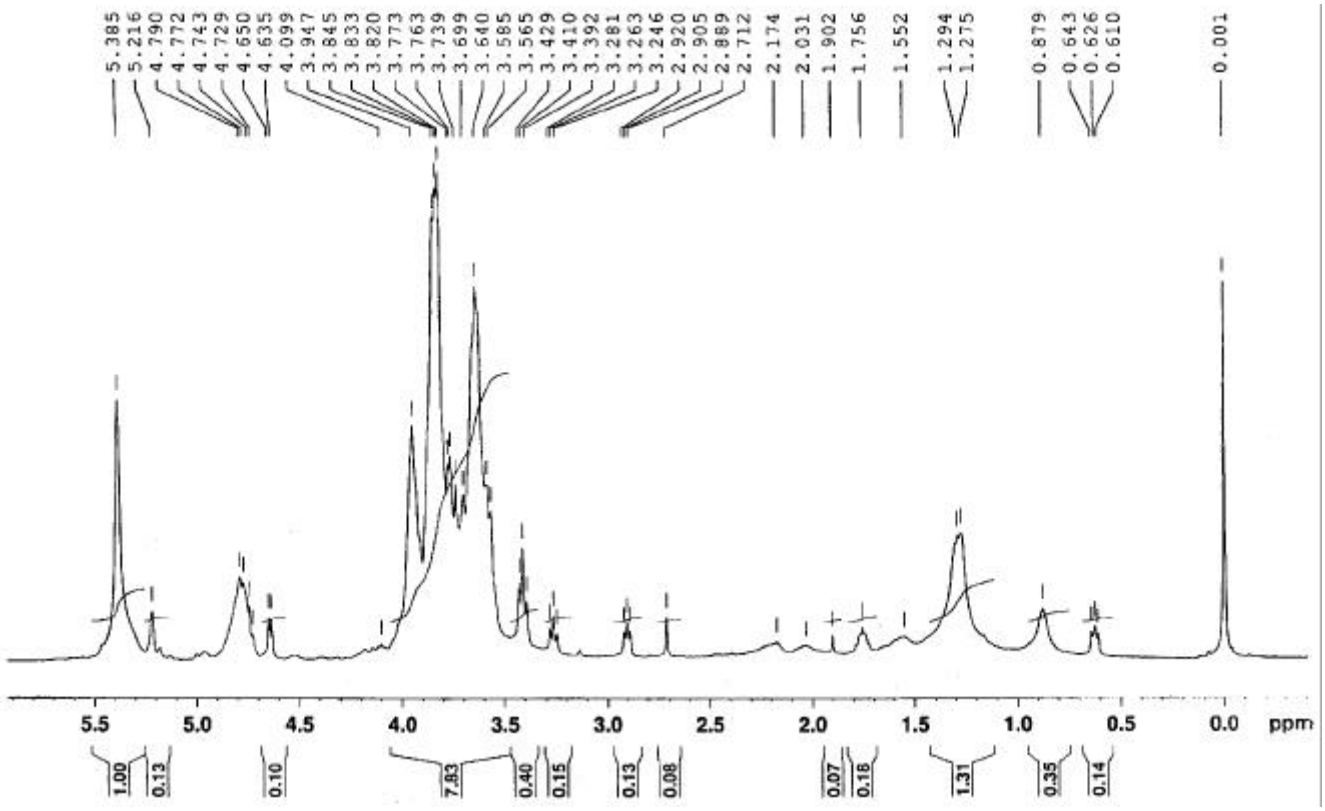

Figure 3. ^{1}H NMR spectrum of the antibiotic from *S. albidoflavus* PU 23. (The spectrum shows more than the expected peaks as it includes the peaks of the internal standard, DSS, and the solvent used.)

(100 μg/strip) and was 24 mm along the length of hamycin strip (100 unit/strip); however, at the intersection, widths of inhibition zone increased along both the strips, thus indicating the synergistic effect of the antibiotic from *S. albidoflavus* PU 23 with hamycin. The antifungal effect of each drug is enhanced when the two drugs are used in combination. No synergistic effect was observed when nystatin or amphotericin B was used.

3.8 *Effect of ergosterol on antifungal activity of the antibiotic from* S. albidoflavus *PU 23*

The control plate without ergosterol showed an inhibition zone diameter of 16 mm, whereas the plate containing the reversal agent, ergosterol, showed a reduced inhibition zone diameter i.e. 7 mm. Thus the antibiotic from *S. albidoflavus* PU 23 probably binds to the ergosterol present in the fungal cell membrane resulting in the leakage of intracellular material and eventually death of the cell.

3.9 *Effect of detergents on activity of the antibiotic from* S. albidoflavus *PU 23 against* C. albicans

The antibiotic alone showed 16 mm inhibition zone diameter against *C. albicans*, while inhibition zone diameter obtained with mixture of antibiotic and detergents ranged between 15 to 17 mm. This clearly indicated that there was no significant loss of antifungal activity of the antibiotic after treatment with detergents (table 5).

3.10 *Effect of enzymes on activity of the antibiotic from* S. albidoflavus *PU 23 against* A. niger *and* C. albicans

The antibiotic alone showed 22 mm and 16 mm inhibition zone diameter against *A. niger* and *C. albicans* respectively, while inhibition zone diameter obtained with mixture of antibiotic and enzymes ranged between 21 to 22 mm in case of *A. niger* and 16 to 17 mm in case of *C. albicans* (table 6). This clearly indicated that there was no significant loss of antifungal activity of the antibiotic after treatment with the enzymes.

3.11 *Elemental analysis and melting point of the compound*

The elemental analysis showed the presence of carbon 37·09% and hydrogen 6·42%. The antibiotic is a brown coloured, amorphous powder having a melting point of 218–220°C.

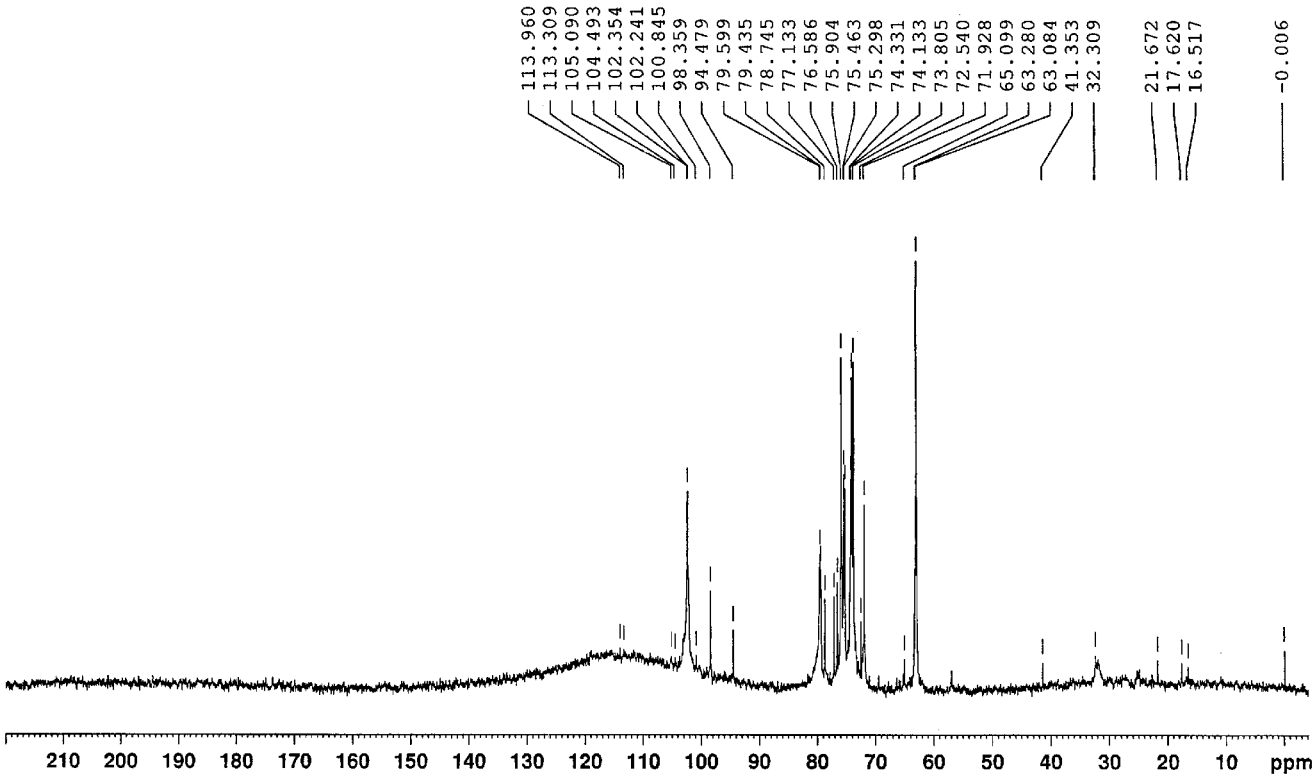

Figure 4. ^{13}C NMR spectrum of the antibiotic from *S. albidoflavus* PU 23. (The spectrum shows more than the expected peaks as it includes the peaks of the internal standard, DSS, and the solvent used.)

3.12 *Structural analysis of the antibiotic*

The UV spectral data exhibited strong absorption at l_{max} 211, 216 and 218 nm, suggesting a carbon-carbon double bond. The FTIR spectrum exhibited absorption bands at 3296 and 1031·8 cm^{-1}, which indicates hydroxyl groups, and at 1639 cm^{-1} indicating a double bond (figure 2). ^1H NMR (500 MHz) spectrum of the antibiotic in D_2O has peaks in the region 2·7 to 5·4 *d* and major peaks between 3·3 and 4·4 *d*, which probably indicates –CHOH protons (figure 3). The ^{13}C NMR (500 MHz) and DEPT (500 MHz) spectra showed only one $–CH_2$ group at 63 ppm and two olefinic carbons at 102·241 and 102·354 ppm (figures 4 and 5). The LC-Mass spectrum showed a molecular ion at m/z 528 indicating that the molecular weight is probably 528 (table 7).

With the above spectral data, the compound probably has a molecular formula of $C_{16}H_{32}O_{19}$ having one $–CH_2OH–$ (hydroxymethyl) group, one olefinic double bond and the rest being –CHOH– (hydroxymethylenes), which are linked to each other by ether linkages. Thus the antibiotic probably is a straight chain polyhydroxy polyether compound with a single double bond, indicating a non-polyene (lacking conjugated double bonds) antifungal antibiotic.

3.13 *Determination of shelf life of the antibiotic from* S. albidoflavus *PU 23*

The antibiotic was fairly stable for a period of 12 months at 4°C (table 8).

4. Discussion

Actinomycetes have been recognized as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man. They have provided about two-thirds (more than 4000) of the naturally occurring antibiotics discovered, including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, *b*-lactams, macrolides, tetracyclines etc. (Gupte *et al* 2002). Fungi are eukaryotic and have machinery for protein and nucleic acid synthesis similar to that of higher animals. It is, therefore, very difficult to find out compounds that selectively inhibit fungal metabolism without toxicity to humans (Glazer and Nikaido 1995; Dasgupta 1998). Fungal infections have been gaining prime importance because of the morbidity of hospitalized patients (Beck-

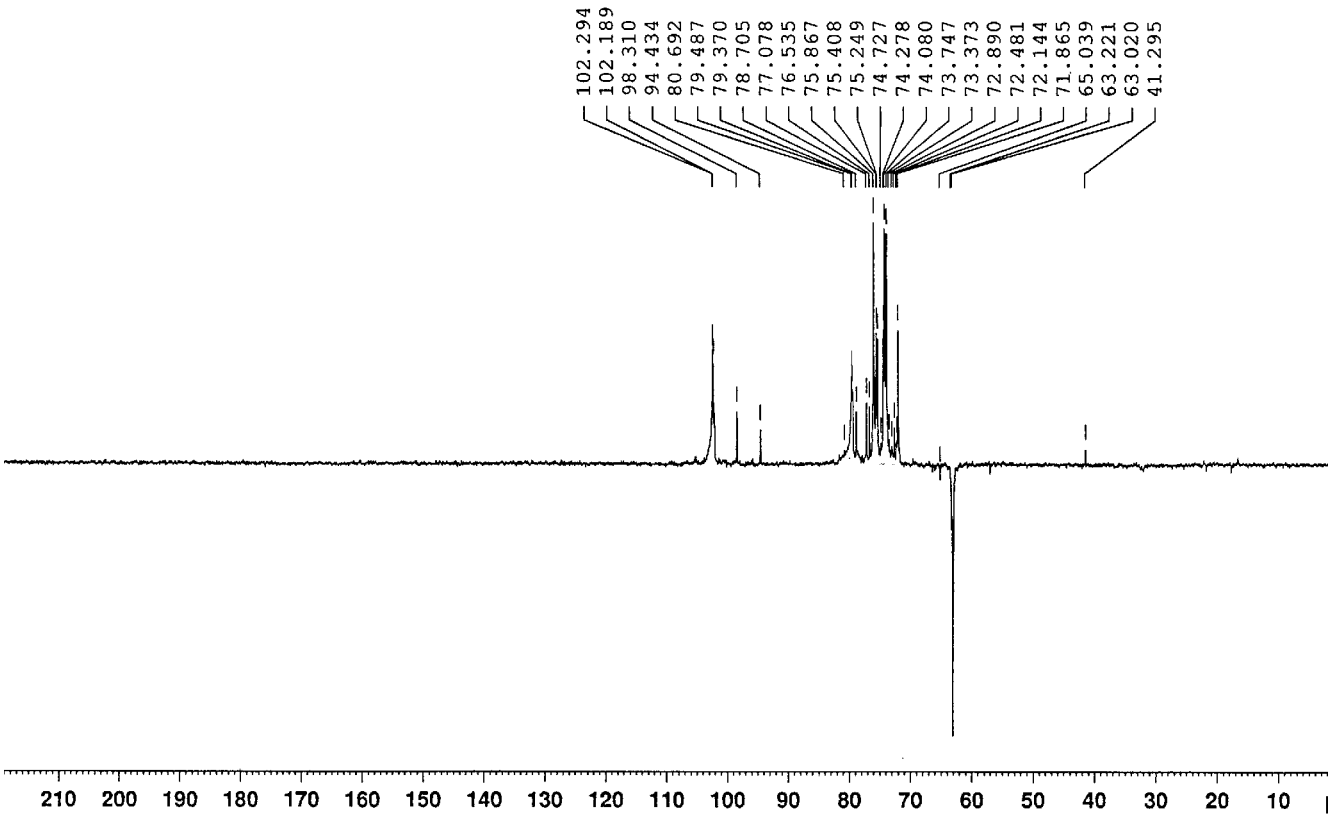

Figure 5. DEPT spectrum of the antibiotic from *S. albidoflavus* PU 23. (Internal standard-DSS.)

Table 1. Different solvents used for extraction of antibiotic from *S. albidoflavus* PU 23.

Solvents used for extraction of antibiotic	Yield of the antibiotic (mg/l)
n-hexane	1·66
Petroleum ether	0·55
Ethyl acetate	–
n-butanol	–
Chloroform	–
Benzene	–
Xylene	–

The solvents chloroform, benzene and xylene were themselves inhibitory to the target organisms and hence considered poor solvents for extraction.

Sague and Jarvis 1993; Gupte *et al* 2002). Although synthetic drugs contribute to a major proportion of the antifungals used, natural antifungals have their own place in the antimycotic market (Cragg *et al* 1997). The classical method, still used today, was followed in the present course of research programme, which includes the whole cell bioassay technique, e.g. using susceptible and resistant species of *Aspergillus* and *Candida*. MIC and MFC are determined and the best antibiotic producer is selected. Subsequently, the antifungal profile of the compound is determined, using a wide range of pathogens. Finally the structure of the compound is elucidated along with the target of the action (Barry 1980; Shadomy *et al* 1985).

The antibiotic from *S. albidoflavus* PU 23 was extracted from the supernatant using n-hexane solvent and could not be extracted using ethyl acetate. However, most of the antifungal antibiotics are extracted using ethyl acetate (Franco and Countinho 1991). The pure compound is soluble in water but the standard antifungal antibiotics, like hamycin, are insoluble in water. The peaks of the UV spectrum of the compound differs from the peaks of the standard polyene antifungal antibiotics like nystatin, amphotericin B and hamycin, thus indicating it to be a non-polyene antifungal antibiotic.

The purified antibiotic was active against a number of test organisms like *A. niger*, *A. fumigatus*, *F. oxysporum*, *C. albicans* and *Cryptoccocus* species. The Streptomycetes, producers of more than half of the 10,000 documented bioactive compounds, have offered over 50 years of interest to industry and academics (Anderson and Wellington 2001). However, the antifungal property of *S. albidoflavus* has not been much studied and reported.

The antibiotic produced by *S. albidoflavus* PU 23 was very heat stable and active over a wide range of pH. The

Table 2. MIC and MFC values of the antibiotic from *S. albidoflavus* PU 23 against different target organisms*.

Target organism	MIC (μg/ml)	MFC (μg/ml)
Aspergillus niger	20	40
Aspergillus fumigatus	20	40
Aspergillus flavus	20	40
Penicillium sp.	40	80
Fusarium oxysporum	40	80
Candida albicans	40	80
Cryptococcus sp.	40	80
Cryptococcus humicolus	40	80
Cryptococcus neoformans	40	80

*Activity was performed by using the agar well diffusion technique. Each data point represents average of three replicates. The antibiotic was not active against bacteria and dermatophytes.

Table 3. Thermal stability* of the antibiotic from *S. albidoflavus* PU 23.

	Inhibition zone diameter (mm) against	
Temperature (°C)	*A. niger*	*C. albicans*
30	22	16
40	22	16
50	22	16
60	22	16
70	22	16
80	22	16

*The antibiotic lost its activity completely after autoclaving at 121°C for 15 min. Each data point represents average of three replicates.

Table 4. Effect of pH on activity of the antibiotic from *S. albidoflavus* PU 23.

	Inhibition zone diameter (mm) against	
pH	*A. niger*	*C. albicans*
5·7	19	14
6·3	20	15
6·8	22	16
7·3	22	16
7·8	22	16
8	22	16

Each data point represents average of three replicates.

antibiotic was kept at various temperatures (30 to 80°C) for 1 h and was found to be stable at different temperatures, however the antibiotic lost its antifungal activity completely after autoclaving at 121°C for 15 min. Similar observations on the antifungal metabolites produced by *Bacillus* species stable at various temperatures and active under both acidic pH and basic pH have been reported

Table 5. Effect of detergents on activity of the antibiotic from *S. albidoflavus* PU 23 against *C. albicans*.

	Inhibition zone diameter (mm)	
Detergents	Without antibiotic	With antibiotic
Tween 20	0·0	16
Tween 80	0·0	16
Triton X 100	0·0	16
SDS	0·0	15
Cetrimide	8	17
Antibiotic*	–	16

*Antibiotic concentration 100 μg/ml. Each data point is the mean of three experiments.

Table 6. Effect of enzymes on activity of the antibiotic from *S. albidoflavus* PU 23 against *A. niger* and *C. albicans*.

	Inhibition zone diameter (mm)	
Test	*A. niger*	*C. albicans*
Proteinase K + antibiotic	22	16
Trypsin + antibiotic	21	16
Lipase + antibiotic	22	17
Lysozyme + antibiotic	22	16
Antibiotic*	22	16

*Antibiotic alone at concentration 100 μg/ml. Each data point is the mean of three experiments.

(Phae *et al* 1990; Lebbadi *et al* 1994; Motta and Brandelli 2002; Singh and Garg 2003).

From our studies we observed that RPMI 1640 medium buffered with MOPS as proposed by the National Committee for Clinical Laboratory Standards (NCCLS) does not adequately support the growth of the target cultures and the incubation time required is too long. Similar problems were earlier reported (Petrou and Shanson 2000). Hence MIC was determined by broth tube dilution procedure using two-fold dilutions of antibiotic in SDB (Collins *et al* 1995). It was important to establish whether the antibiotic was fungistatic as well as fungicidal, as an indication of the potential usefulness of the antibiotic in the antifungal treatment. Most isolates showed a difference of concentration between inhibitory and cidal values, indicating that although the antibiotic has fungicidal activity, at particular concentration it is fungistatic only. The MFC values were determined as per Hammer *et al* (2002). There was no significant loss of antifungal activity of the antibiotic after treatment with various detergents and enzymes (table 5).

According to our investigations, the non-polyene antibiotic from *S. albidoflavus* PU 23 probably binds to the ergosterol present in the fungal cell membrane resulting in the leakage of intracellular material and eventually

Table 7. Physico-chemical properties of the antibiotic from *S. albidoflavus* PU 23.

Properties	Results
Colour	Brown
Nature	Amorphous
Melting point	218–220°C
R_f value	0·78
Elemental analysis (%)	Carbon 37·09, Hydrogen 6·42
UV l_{max} (nm)	211, 216, 218
IR (KBr) cm^{-1}	1031·8, 1639, 3296
LC-MS (m/z)	528
Molecular formula	$C_{16}H_{32}O_{19}$

Table 8. Shelf life of antibiotic from *S. albidoflavus* PU 23 at 4°C.

	Inhibition zone diameter (mm) against	
Incubation time	*A. niger*	*C. albicans*
1 h	22	16
24 h	22	16
1 week	22	16
1 month	21	16
2 months	20	15
3 months	20	15
6 months	20	15
12 months	20	15

Each data point represents average of three replicates.

death of the cell. This is similar to the mode of action of polyene antibiotics like nystatin and amphotericin B, which interact with ergosterol, disrupting the fungal membranes (Igraham and Igraham 2000; Prescott *et al* 2002). Thus the non-polyene antifungal has similar mode of action to polyene antibiotics. We also observed that the antifungal effect of the antibiotic from *S. albidoflavus* PU 23 and hamycin is enhanced when the two drugs are used in combination against *C. albicans*.

^{1}H NMR spectrum of the antibiotic in D_2O has peaks in the region 2·7, 3·3, 4·4 and 5·4 *d*, which indicates the presence of –CHOH protons. The ^{13}C NMR and DEPT spectra shows the presence of a $–CH_2$ group at 63 ppm and two olefinic carbons at 102·241 and 102·354 ppm. From the spectral data, the compound has a molecular formula $C_{16}H_{32}O_{19}$ with one hydroxymethyl ($–CH_2OH$) group, one olefinic double bond and hydroxymethylenes (–CHOH–) linked to each other by ether linkages. The data also indicate that the antibiotic is a non-polyene, antifungal antibiotic. The NMR spectra shows more than the expected peaks as it includes the peaks of the internal standard (DSS) and the solvent used.

The need for new, safe and more effective antifungals is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host. On this background, the biotechnological potential of *S. albidoflavus* PU 23 in terms of production of antibiotic inhibiting pathogenic fungi, both yeasts and molds is noteworthy. Various studies done and results obtained in the present investigation indicated that *S. albidoflavus* PU 23 produced a non-proteinic, stable antifungal antibiotic, which is non-polyene in nature and seems to be novel, as it does not fit into any of the known classes of antifungal antibiotics. We also further propose that the actinomycetes, even today, are a source for new antifungal antibiotics.

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