LANOMYCIN AND GLUCOLANOMYCIN, ANTIFUNGAL AGENTS PRODUCED BY *Pycnidiophora dispersa*

I. DISCOVERY, ISOLATION AND BIOLOGICAL ACTIVITY

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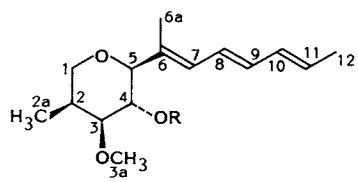
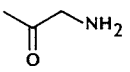
The antifungal agents lanomycin and glucolanomycin were isolated from *Pycnidiophora dispersa*. The compounds were active against species of *Candida* and dermatophytes but were inactive against *Aspergillus fumigatus* and Gram-positive and Gram-negative bacteria. The compounds inhibited the cytochrome P-450 enzyme lanosterol 14α-demethylase, and are believed, therefore, to have a mode of action similar to the azole and bis-triazole class of antifungal agents.

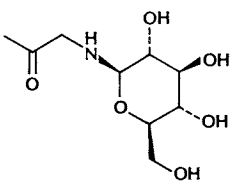
The incidence of life-threatening systemic infections caused by *Candida* species and other opportunistic fungi is growing[1]. The antifungal agents currently available are, in many instances, inadequate to deal with the problem and, consequently, there is a pressing need for novel pharmacophores which can be developed into effective drugs. The imidazoles and triazoles represent a major class of synthetic antifungal agent. These compounds act primarily by inhibiting the biosynthesis of ergosterol at the 14α-demethylation step[2]. In an effort to find novel, natural product inhibitors of this enzyme, a search was carried out in which the activity of naturally produced antifungal agents was compared on two organisms differing only in their lanosterol 14α-demethylase content[3].

In this publication we describe the identification, fermentation, isolation and partial biological characterization of lanomycin and glucolanomycin (Fig. 1), novel inhibitors of lanosterol demethylation. The taxonomy of the producing organism is also described. In separate publications the structure determination[4] and biosynthesis[5] of these compounds are described. Lanomycin is believed to correspond to M5070, a compound described in the patent literature[6] and recently, a closely related compound called restricticin, was found to be produced by *Penicillium restrictum*[7].

Fig. 1. The structures of lanomycin (**1**), glucolanomycin (**2**), and lanomycinol (**3**).



1 R = 

2 R = 

3 R=H

Isolation, Description and Taxonomy of the Producing Organism

The fungus was isolated from a soil sample collected in Culpepper, Virginia. Colonies of the fungus grew well on potato-glucose agar and

tomato juice agar. The sexual fruiting bodies (cleistothecia) were produced readily on these media but required 5 to 6 weeks for the ascospores borne within to mature. Cleistothecia were dark brown to black with an outer wall (peridium) composed of a single layer of polygonal pseudoparenchyma cells. Plates composed of radiating cells delineating lines of cleavage of the peridium were not evident. Cleistothecia occurred singly or in clusters but each had its own peridium.

Sac-like asci were produced from fertile hyphae randomly distributed within the lumen of the cleistothecium. Histological examination of thin sections of the ascus cell wall showed it to be unitunicate. Each ascus bore ascospores in multiples of eight depending on the stage of divison. The ascospores were elliptical, pigmented and partially septate with an oil globule at each end. They were not bivalve and did not possess a longtidunal germinal slit. They germinated by means of a terminal or lateral germ tube. Ascospores were $4.7 \times 2.5 \mu m$. Paraphyses, specialized filiform cells interspersed among the asci, were lacking.

The asexual cycle consisted of pycnidia which were ovoid bodies with an ostiole or pore. Conidia were borne on short conidiophores which lined the inner layer of the pycnidium. When the central cavity of the pycnidium was filled with spores, they were released through the ostiole in a slime. Conidia were elliptical, smooth, hyaline and averaged $3 \times 2 \mu m$ in size. Monoconidial isolates produced cultures which fruited both sexually and asexually, as did monoascospore cultures. This agreed with the original observation by CLUM[8] which formed the basis for the identification of *Pycnidiophora*.

This organism is a member of a well-known group of Ascomycetes some of which were discovered as early as 1866. There has been and continues to be considerable controversy as to their taxonomic placement, specifically at the ordinal and family levels.

When our isolate was directly compared with the type culture of *Pycnidiophora dispersa*[8] they were found to be identical. The description also matches that of the organism reported in the patent literature[6] in which the name *Westerdykella dispersa* was used. That assignment was based on their comparison with *W. dispersa* IFO strain 8431, however, the IFO strain was not the original CLUM strain[8]. These organisms all share the following characteristics;

1) the sexual fruiting body is a true cleistothecium with a discrete peridium,

2) the asci have a single layered wall,

3) the ascospores are free with up to 32 per ascus rather than disarticulated segments of eight 4-celled ascospores,

4) the asexual stage is pycnidial.

The data presented here forms the basis for the identification of SC15017 as *Pycnidiophora dispersa* sensu CLUM[8] and the designation of *Westerdykella dispersa* as a synonym. *Pycnidiophora dispersa* (CLUM) is recognized by THOMSPON and BACKUS[9] and supported by MUKERJI and SAXENA[10].

Bioassay and Measurement of Enzyme Activity

The primary assay for determining the bioactivity of lanomycin and glucolanomycin employed *Saccharomyces cerevisiae* strain SGY775, in which the gene for lanosterol demethylase was replaced by an integrated copy of the corresponding *Candida* gene[3], and *S. cerevisiae* strain SGY769, in which the native gene was replaced by the *Candida* gene carried on a multicopy vector[3]. The strain with the high copy lanosterol demethylase is significantly more resistant to econazole and other azole antifungals than *S. cerevisiae* SGY775 which is supersensitive to these compounds when compared with wild type strains.

Enzyme activity was measured by the reduced carbon monoxide versus reduced difference spectra[11]

and by monitoring the incorporation of radiolabeled acetate into solvent soluble compounds. *Candida albicans* strain SC5314 was grown on a medium consisting of Tryptone 0.5%, malt extract 0.3%, glucose 1.0% and yeast extract 0.3%. Cells were harvested by centrifugation, washed, and resuspended in water to give an absorbance of 0.3 at 600 nm. A 750 μl aliquot of this suspension was added to 300 μl of nutrient (yeast nitrogen base without amino acids 3.5% and glucose 10%), 275 μl water and 100 μl test substance and incubated at 30°C for 15 minutes. 3 μCi [*U*-^{14}C]acetic acid, sodium salt (54 mCi/mmol) were added to the mixture and incubated for 1 hour. The cells were pelleted and extracted first with 500 μl methanol then with 500 μl methanol-benzene (1:1); the extracts were pooled, brought to dryness, resuspended in chloroform-methanol (2:1) and spotted onto Whatman LK6D plates and developed in methylene chloride-acetone (50:1) which, after drying, were autoradiographed using Kodak XAR-5 film.

Fermentation

Seed cultures were prepared by transferring a loopfull of surface growth from an agar slant culture of *P. dispersa* SC15017 into 500-ml Erlenmeyer flasks containing 100 ml of sterilized medium. The medium contained toasted Nutrisoy flour 1.5%, soluble starch 1.5%, glucose 5%, $CoCl_2 \cdot 6H_2O$ 0.0005%, $CaCO_3$ 1% and distilled water. Inoculated flasks were incubated at 25°C on a rotary shaker (300 rpm; 5 cm stroke) for approximately 72 hours. A 5% transfer of this culture was then made to Erlenmeyer flasks each containing 100 ml of the following medium: glucose 2.5%, yeast extract 0.2%, N-Z amine A 0.4%, K_2HPO_4 0.1%, $NaH_2PO_4 \cdot H_2O$ 0.1%, NH_4Cl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.02% and distilled water. The pH was adjusted to 7. Flasks were incubated at 25°C on a rotary shaker at 300 rpm as before. At about 48 hours flasks were harvested by filtration through Celite.

Isolation

Ten liters of culture filtrate (pH 6.5 to 7) were extracted twice with a half volume of ethyl acetate and concentrated to give 1.4 g of a brownish oil. This sample was split and 700 mg fractionated using countercurrent chromatography in heptane-ethyl acetate-methanol-pH 4.5, 0.1 M NH_4OAc buffer, 1:2:1:2, organic phase mobile, using a high-speed countercurrent chromatograph (P.C. Inc., Potomac, MD, U.S.A.) operated at 800 rpm using a 330 ml volume multilayer Teflon tube (1.6 mm i.d.)[12]. This yielded 100 mg of material sufficiently pure for spectroscopy and chemical degradation.

Following the purification of lanomycin using silica gel followed by an Ito coil step, we attempted to apply the same strategy for the purification of glucolanomycin. However, this proved not to be feasible because glucolanomycin has a half-life of less than six hours in a pH 4.5 buffered aqueous system and it was only slightly more stable in deionized water (pH 6.5). These studies served to demonstrate that glucolanomycin was converted to lanomycin in a pH dependent manner and that glucolanomycin contained the elements of lanomycin plus a more polar moiety. In subsequent workups, the oil resulting from concentration of the ethyl acetate extract was first chromatographed on a 3.0 × 25 cm bed of silica gel eluted with increasing concentrations of methanol in chloroform to give a fraction containing mostly lanomycin and another more polar band, partially purified glucolanomycin. Lanomycin was then purified using the Ito coil as described above while glucolanomycin was chromatographed using a 1.5 × 20 cm column of Sephadex LH-20 and heptane-chloroform-methanol (10:10:1) as the eluant. All of the impurities cochromatographing with glucolanomycin during the silica gel step, eluted in the first two column volumes of the Sephadex LH-20 column, while pure glucolanomycin eluted in a very broad band centered at 6 to 7 column volumes.

Using bioautography, the zone centers corresponded to TLC Rf values of 0.71 for lanomycin and 0.13 for glucolanomycin when the silica gel plates were developed with chloroform-methanol, 9:1. The compounds could also be detected by using short wave UV light, I_2 vapor and with chemical spray reagents such as Sawicki, Rydon-Smith, phosphomolybdic acid, vanillin and ninhydrin.

Lanomycin was basic by low voltage paper electrophoresis and had a UV spectrum in methanol characteristic of a triene (Fig. 2). The 270 MHz ^{1}H NMR spectrum of lanomycin in $CDCl_3$ (Fig. 3) shows resonances corresponding to olefinic, α-

Fig. 2. UV spectra of lanomycin.

Spectrum in methanol (——), in 0.1 N HCl (---) and in 0.1 N NaOH (—·—).

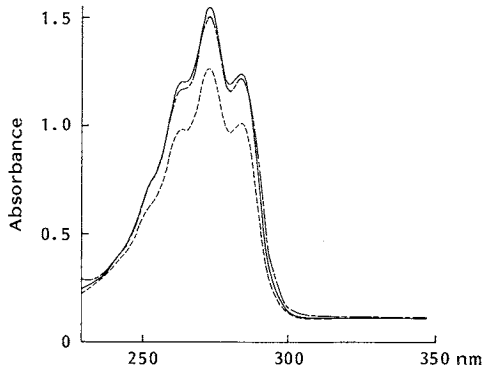


Fig. 3. ^{1}H NMR spectrum of lanomycin.

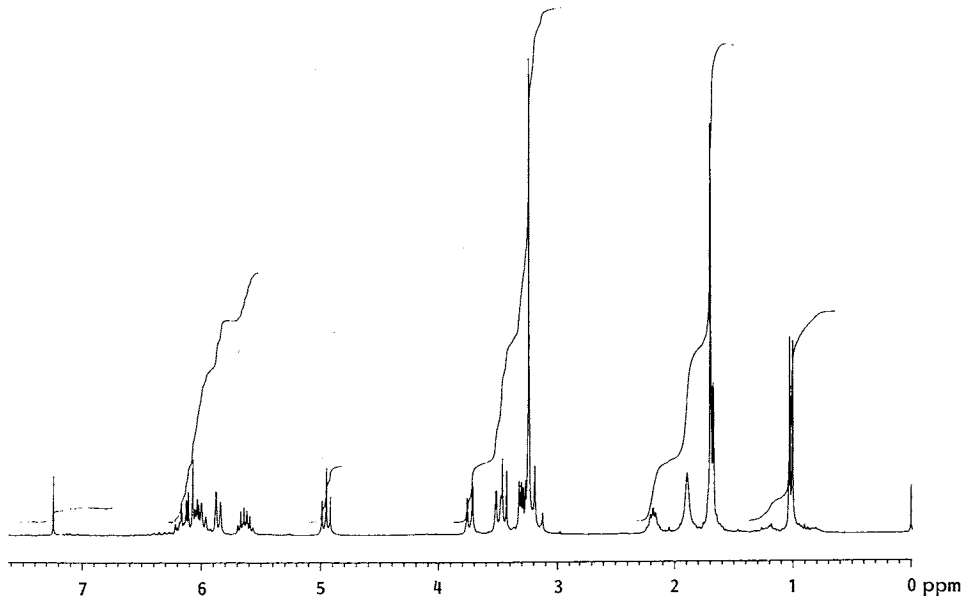


Table 1. Antibacterial and antifungal activity of lanomycin.

Organism	SC No.	MIC (μg/ml)[a]	Organism	SC No.	MIC (μg/ml)[a]
Staphylococcus aureus	1276	>100	*Candida albicans*	11422	100
S. aureus	2399	>100	*C. tropicalis*	2963	25
Micrococcus luteus	2495	>100	*C. guillermondii*	2996	1.6
Enterococcus faecalis	9011	>100	*Trichophyton mentagrophytes*	2637	0.4
Escherichia coli	8294	>100	*T. rubrum*	9199	1.6
Klebsiella aerogenes	10440	>100	*Epidermophyton floccosum*	9185	0.4
Proteus mirabilis	3855	>100	*Microsporum canis*	9237	0.4
Pseudomonas aeruginosa	8329	>100	*Aspergillus fumigatus*	2100	100
Candida albicans	5314	>100			

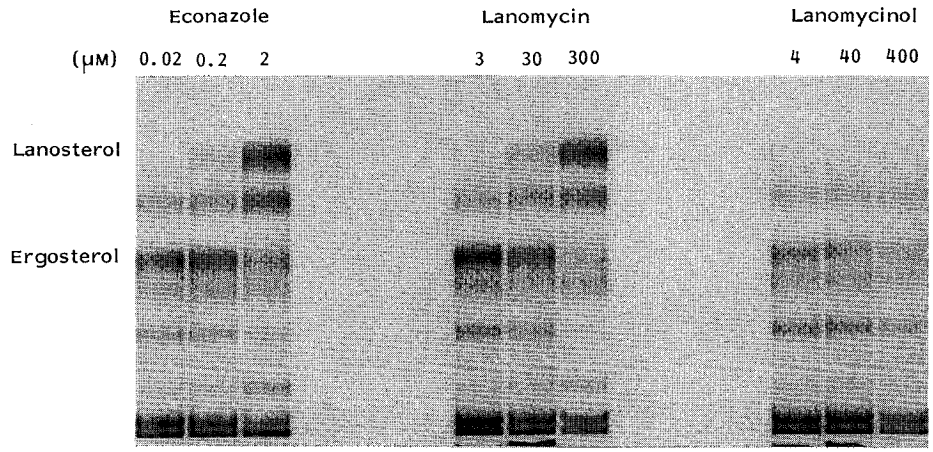
[a] Minimal inhibitory concentrations were determined by agar dilution assay.

Table 2. Anticandidal activity of lanomycin.

Organism	SC No.	MIC (μg/ml)		Organism	SC No.	MIC (μg/ml)	
		Lanomycin	Econazole			Lanomycin	Econazole
Candida albicans	5314	>100	3.1	*Candida tropicalis*	10597	100	6.3
C. albicans	9177	100	3.1	*C. krusei*	2967	6.3	0.8
C. albicans	11422	100	1.6	*C. krusei*	2968	12.5	3.1
C. albicans	10580	>100	3.1	*C. krusei*	2969	25	3.1
C. albicans	10102	>100	3.1	*C. parakrusei*	2621	12.5	1.6
C. albicans	9721	100	3.1	*C. parakrusei*	2966	25	1.6
C. albicans	12734	>100	3.1	*C. pseudotropicalis*	11241	50	1.6
C. albicans	14021	>100	3.1	*C. guillermondii*	2210	50	6.3
C. albicans	10584	50	3.1	*C. guillermondii*	2996	1.6	0.2
C. albicans	10585	100	3.1	*C. stellatoidea*	2211	50	6.3
C. tropicalis	8159	>100	6.3	*C. glabrata*	9342	0.8	<0.05
C. tropicalis	2963	25	0.4	*C. glabrata*	11267	25	6.3
C. tropicalis	9861	100	6.3				

MICs were determined by the agar dilution method using a medium composed of Tryptone (0.5%), malt extract (0.3%), glucose (1.0%) and yeast extract (0.3%); 10^4 cfu of the test organism was applied to the agar.

Fig. 4. The incorporation of ^{14}C-acetate into lanosterol, ergosterol and other membrane components of *Candida albicans*.



monooxygenated methines and a methylene, one aliphatic methine as well as one "O", one "C" and two vinyl methyl protons.

Biological Activity

Lanomycin was not active against Gram-positive or Gram-negative bacteria but showed activity against some *Candida* species and dermatophytes (Table 1). In a fuller examination of anticandidal activity (Table 2) it was found not to be active against the *C. albicans* species tested, but showed good activity against certain strains of *C. crusei*, *C. parakrusei*, *C. guillermondii* and *C. glabrata* (Table 2). Lanomycin was active against dermatophytes but inactive against *Aspergillus fumigatus* (Table 1).

When lanomycin was administered intraperitoneally to random bred Swiss Webster female mice an LD_{50} of 385 mg/kg was calculated.

Lanomycin prevents the binding of carbon monoxide to lanosterol demethylase (cytochrome P-450)

with an I_{50} of 3.2×10^{-5} M. In contrast, lanomycinol which is also found in the culture filtrates or which can be generated by mild treatment of lanomycin with base, does not inhibit carbon monoxide binding. This compares with observed values of 4.5×10^{-7} M, 7×10^{-6} M and 1.6×10^{-5} M for econazole, itraconazole and fluconazole, respectively.

In an assay to determine the effect of lanomycin and econazole on the ergosterol biosynthetic pathway, it was found (Fig. 4) that at concentrations corresponding to the I_{50} value in the binding assay, there was an accumulation of lanosterol and a corresponding decrease in ergosterol in both cases. Lanomycinol did not inhibit the incorporation of ^{14}C-acetate into ergosterol (Fig. 4) nor was it active by disc bioassay against various *Candida* species or other fungi. Glucolanomycin was identical to lanomycin in its activity and it is likely that the glucose moiety is readily hydrolyzed to give lanomycin.

Discussion

In the course of screening for antifungal agents with a mode of action involving inhibition of lanosterol demethylase, we isolated the compounds lanomycin, glucolanomycin and lanomycinol. Lanomycin was active against *Candida* species and dermatophytes but had no activity against bacteria. The bioactivity profile against *Candida* species was analogous to that for econazole (Table 2) in that those species which were most sensitive to econazole were also sensitive to lanomycin, despite the difference in the levels of activity. Lanomycin was shown to bind to lanosterol demethylase in a spectrophotometric assay and, in a separate experiment (Fig. 4), the inhibition resulting from this binding leads to lanosterol accumulation. The failure of the lanomycinol (desglycyllanomycin) to bind to the enzyme or to inhibit the biosynthetic pathway is consistent with the interaction of the glycyl nitrogen of lanomycin with the heme moiety of the enzyme. It is believed that the accumulation of lanosterol has an adverse effect on membrane integrity which, in turn, leads to a cascade of events that cause a cessation of growth[2]. The data presented here suggest that lanomycin inhibits sterol biosynthesis and thereby prevents cell growth. To our knowledge this is the first report of a natural product having antifungal activity by virtue of its inhibition of lanosterol 14α-demethylase.

Acknowledgments

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