Ascosteroside, a New Antifungal Agent from *Ascotricha amphitricha*

I. Taxonomy, Fermentation and Biological Activities

JESSICA A. GORMAN[†], LI-PING CHANG[††], JUNIUS CLARK[†††], DONALD R. GUSTAVSON[††], KIN S. LAM[††], STEVEN W. MAMBER[††], DOLORES PIRNIK[††], CAROL RICCA[††], PRABHAVATHI B. FERNANDES[†] and JOSEPH O'SULLIVAN[††]

[†]Departments of Microbial Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey, U.S.A.
[††]Natural Products Research and [†††]Microbiology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut, U.S.A.

(Received for publication September 18, 1995)

Ascosteroside, a novel antifungal compound, was isolated from the culture broth of *Ascotricha amphitricha*. This compound is an alpha-linked glycoside of a lanostane type triterpenoid. It is active against yeasts such as *Candida albicans* and *Saccharomyces cerevisiae* and against filamentous fungi but shows no activity against bacteria. It is not toxic to mammalian cells at concentrations up to 150 μM. In a mouse model, the compound afforded protection comparable to that of ketoconazole.

There is a pressing need to find new antifungal agents[1] and increasing efforts are being made to develop such compounds. In the course of our natural products screening, we discovered a novel antifungal agent, ascosteroside (Fig. 1), which is produced by the ascomycetous fungus *Ascotricha amphitricha*. In this report we describe the isolation and taxonomy of the producing organism, its fermentation, and some of its biological properties. The isolation and structure determination of ascosteroside are detailed in the accompanying paper.[2]

Taxonomy

The producing microorganism, *A. amphitricha*, was isolated from a soil sample collected in Kenya. Approximately one gram of soil was suspended in 10 ml of diluent (buffered saline containing 0.01% gelatin), sonicated for 20 minutes and vortexed. This initial suspension was diluted and aliquots were plated onto six different types of nutrient agar and incubated at room temperature. After four days, colonies were subcultured onto potato-dextrose agar medium and incubated for 4 days at room temperature.

Cultures growing on potato dextrose agar show a white mycelium with dark ascocarps forming among the mycelial mass. The reverse color is a dark brown-black. Dark, globose perithecia, 180~200 μm × 200~220 μm, develop rapidly on oatmeal and cornmeal agar media. They arise from the mycelium and are ostiolate with a short discrete neck which is not readily discernible on immature ascocarps. Terminal hairs are slender, whip-like, flexuous, mostly simple with occasional dichotomous branching. The hair turns brown with age. No lateral hairs are observed. The ascogonium forms a helical coil with open spirals in several whorls. Asci are cylindrical and thin-walled measuring approximately 50 × 6 μm and contain 8 ascospores measuring approximately 6 × 11 μm. The ellipsoidal ascospores have an equatorial furrow and are uniserate (Fig. 2). The spores become dark olive brown at maturity. The asci not turn blue with Metzler's iodine stain and deliquesce after

Fig. 1. The structure of ascosteroside.

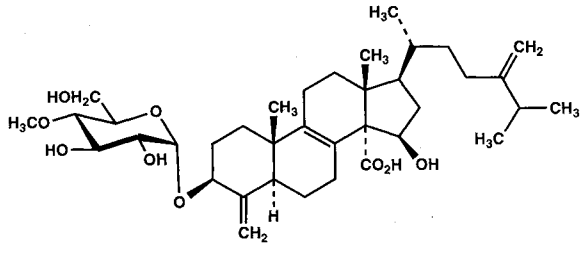
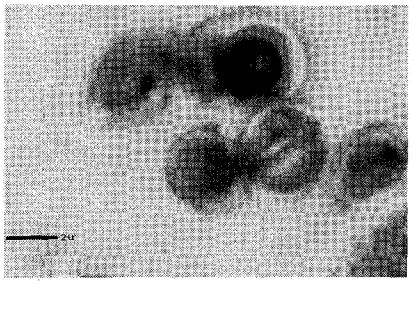


Fig. 2. Ascospore showing equatorial furrow.



maturation. The conidial, or asexual stage, is a member of the genus *Dycima* (Fig. 3). The conidia arise in clusters on pale conidiophores, appearing smooth or slightly roughened, hyaline, irregular globose to ellipsoidal, measuring 2.2 × 3.5 mm.

These characteristics serve to identify the organism as *Ascotricha amphitricha*, in accordance with the description of this organism[3,4]. The producing culture has been deposited with the American Type Culture Collection as ATCC 74237.

Fermentation

A. amphitricha was maintained as a cryopreserved culture stored at −80°C. The fungus was grown on slants for 7~10 days until uniform production of spores was obtained. The surface growth of the slant culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of medium F4 consisting of 0.5% tryptone, 0.3% malt extract, 0.3% yeast extract and 1.0% glucose, which was incubated at 28°C for 72 hours on a rotary shaker set at 250 rpm. The vegetative culture was mixed with an equal volume of cryoprotective solution consisting of 10% sucrose and 20% glycerol. Portions (4 ml) of this mixture were transferred to sterile cryogenic tubes (5-ml capacity) and were frozen in a dry ice-acetone bath. The frozen cultures were stored at −80°C until use.

A vegetative culture of *A. amphitricha* was prepared by transferring 4 ml of the cryopreserved culture to a 500-ml Erlenmeyer flask containing 100 ml of medium F4 which was incubated at 28°C and 250 rpm on a rotary shaker. After 72 hours, 2 ml aliqouts were transferred to 500-ml Erlenmeyer flasks containing 100 ml of medium F4. These production cultures were incubated at 28°C and 250 rpm on a rotary shaker for 6~7 days. For larger scale production in fermentors, 200 ml of vegetative culture was transferred to 10 liters of medium F4. These cultures were grown at 28°C, with an agitation rate of 300~500 rpm and air flow of 10 liter/minute. Polypropylene glycol P-2000 (5 ml) was used as the antifoam agent.

The production of ascosteroside in the shake flask culture (Fig. 4) or fermentor cultures (Fig. 5a and 5b) was monitored by the activity against *S. cerevisiae* strain

Fig. 3. Conidial stage of *A. amphitricha* showing the genus *Dycima*.

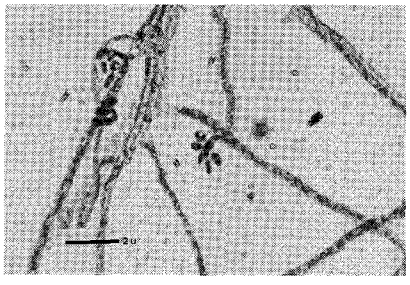


Fig. 4. Fermentation profile of *A. amphitricha* in shake flask culture.

(□), pH, (+) centrifugal solids, (△) inhibition zone size against *S. cerevisiae* SGY1242.

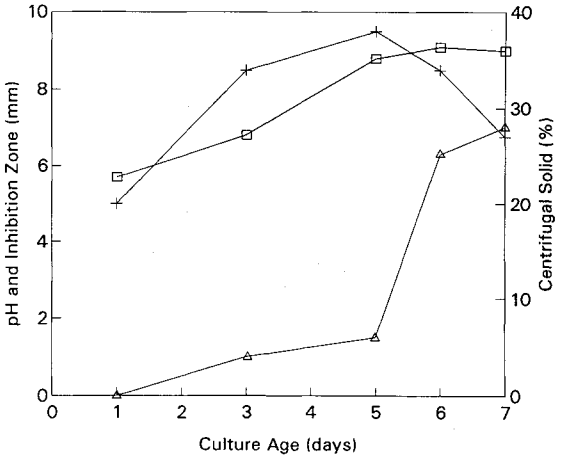
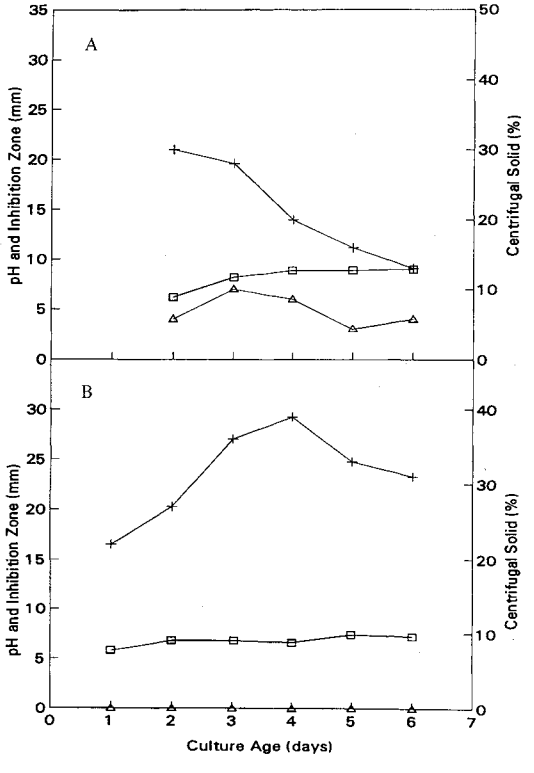


Fig. 5. Fermentation profile of *A. amphitricha* in fermentor culture with an agitation rate of 500 rpm (A) and 300 rpm (B).

(□) pH, (+), centrifugal solids, (△) inhibition zone size against *S. cerevisiae* SGY1242.



SGY1242 as described below. In shake flask cultures, the maximum production of ascosteroside occurred at days 6~7 (Fig. 4). This occurred earlier in the culture grown in a fermentor with an agitation rate of 500 rpm, peaking at around days 3~4 (Fig. 5a). Ascosteroside was not detected in the fermentor culture agitated at 300 rpm (Fig. 5b), even though the growth of this culture (measured as centrifugal solids) was very similar to that of the shake flask culture. There was, however, a significant difference in pH of the fermentation in the shake flask culture and the non-producing fermentor culture. The pH of the producing tank fermentation was similar to that of the shake flask culture. However, significant cell lysis was observed after 3 days in the tank culture (500 rpm), as indicated by the drop in centrifugal solids values. The high shear force generated by the high agitation rate (500 rpm) of this tank fermentation may be responsible for the lysis of the organism. The high agitation rate appears to be required for the production of ascosteroside in the fermentor culture.

Biological Assay of Ascosteroside

S. cerevisiae strain SGY1242 was grown at 28°C overnight in minimal medium (Yeast Nitrogen Base w/o amino acids, Difco) supplemented with 2% glucose, tryptophan (10 μg/ml) and histidine (10 μg/ml). The culture was centrifuged at 3,000 rpm for 10 minutes and the medium was decanted. Cells were resuspended in 0.9% NaCl solution to an optical density of 0.6 (E600 nm) using a Coleman Junior Spectrophotometer. This working cell suspension was stored at 4°C for use as the assay inoculum. All biological assays employed agar diffusion techniques. Petri dishes or bioassay trays containing unseeded F4 agar (1.5%) medium were prepared. Overlays of soft agar (0.8% Noble agar in deionized water) containing a 3% final concentration of the working cell suspension were poured onto the surface of the assay agar and allowed to harden. Wells (7 mm diameter) were cut in the agar and 40 μl sample aliquots were added to the wells. (For some assays, 3 μl of sample were spotted directly on the surface of the agar). Plates were incubated at 28°C for 24~36 hours and zones of inhibition were measured.

Biological Activity

Table 1 shows the activity of ascosteroside in comparison with fluconazole. The compound is active against several *Candida* species and against filamentous fungi. The minimal inhibitory concentration of ascosteroside for *Candida albicans* SC5314, determined by liquid dilution assay is 0.1 μg/ml. In studies not reported here, it was seen that ascosteroside is a fungistatic agent. There is no activity against Gram negative bacteria and little or no activity against *Staphlococcus aureus* or *Bacillus cereus*. The compound is not cytotoxic to normal diploid fibroblast (SS1) cells at 100 μg/ml (150 μm).

As the structure of ascosteroside is an alpha-linked glycoside of a lanostane type triterpenoid, it was thought that the compound might interfere with sterol biosynthesis, particularly with lanosterol demethylation. Therefore, several tests to look for effects on the sterol pathway were conducted.

Table 1. The biological activity of ascosteroside.

Organism		Zone diameter (mm)		
		Ascosteroside	Fluconazole	Tetracycline
Saccharomyces cerevisiae	SGY1242	20	24	0
S. cerevisiae	SGY1243	14	23	0
Candida albicans	SC5314	21	35	0
C. tropicalis	SC8159	20	35	0
C. glabrata	SC9342	21	23	0
Trichophyton mentagrophytes	SC2637	20	>35	0
Aspergillus nidulans	SC12914	20	>35	0
Staphylococcus aureus	SGB42	5	0	21
Escherichia coli	SGB1267	5	0	8
E. coli	SGB1316	0	0	0

The activity of ascosteroside was determined by agar diffusion assay. Molten agar (yeast extract 1%, peptone 2%, glucose 2%, agar 1.5% for fungi; Luria Broth, tryptone 1%, yeast extract 0.5%, NaCl 0.5%, agar 1.5% for bacteria) was seeded with test organism at about 10^4 cfu/ml; wells (3 mm diameter) were bored in the agar and 5 μl (ascosteroside at 5 μg/ml, tetracycline and fluconazole at 25 μg/ml), were placed in the well. After overnight incubation at 30°C for yeast and fungi and 37°C for bacteria, zones of inhibition were measured. The values are the average of duplicate samples determined in at least 3 separate experiments. SGB, SGY and SC refer to the Bristol-Myers Squibb culture collection holdings.

First, a microbial bioassay system was used. This employs *S. cerevisiae* strain SGY775, in which the chromosomal copy of the gene for cytochrome P450 14-α-lanosterol demethylase (*ERG11*) was replaced with the *C. albicans ERG11* gene and strain SGY 769, which contains an *erg11* deletion and the *C. albicans ERG11* gene on a multi-copy vector[5,6]. Strain SGY 775 is much more sensitive to econazole and other azole antifungals than either wild type *S. cerevisiae* or strain SGY769, which is highly resistant due to the overproduction of lanosterol demethylase. However, ascosteroside gave equivalent zones against each of these strains (data not shown), suggesting it is not acting as an inhibitor of lanosterol demethylase. Second, the incorporation of ^{14}C-acetate into solvent soluble materials[7] (including lanosterol and ergosterol) was monitored in *C. albicans*. It was found that ascosteroside at levels up to fifty times the minimal inhibitory concentration, did not perturb the sterol biosynthetic pathway. Under the same conditions, econazole and other azoles at the minimal inhibitory concentration inhibited ergosterol biosynthesis with an accumulation of lanosterol. Finally, a cell free system using a microsomal preparation[7] was employed to examine the incorporation of ^{14}C-mevalonic acid into sterols. Again, ascosteroside failed to show any effect on the demethylation of lanosterol.

To examine the possibility that ascosteroside might affect membrane integrity, a test examining leakage of ^{14}C-aminoisobutyric acid from drug treated cells was employed[7]. As shown in Table 2, nystatin caused extensive membrane damage, as evidenced by the presence of radioactivity in the extracellular medium. Antifungal agents such as econazole and papulacandin or solvents such as methanol and dimethylsulfoxide did not show any significant effect on cell integrity. Ascosteroside at levels up to 20 μg/ml did not cause any significant increase in leakage of compounds from the cell under the conditions employed, thus does not appear to act by damaging the cell membrane.

Potential effects of ascosteroside on the synthesis of protein or nucleic acids were looked for by measuring the incorporation of ^{14}C-leucine and ^{14}C-uridine into

Table 2. The effect of ascosteroside on membrane integrity.

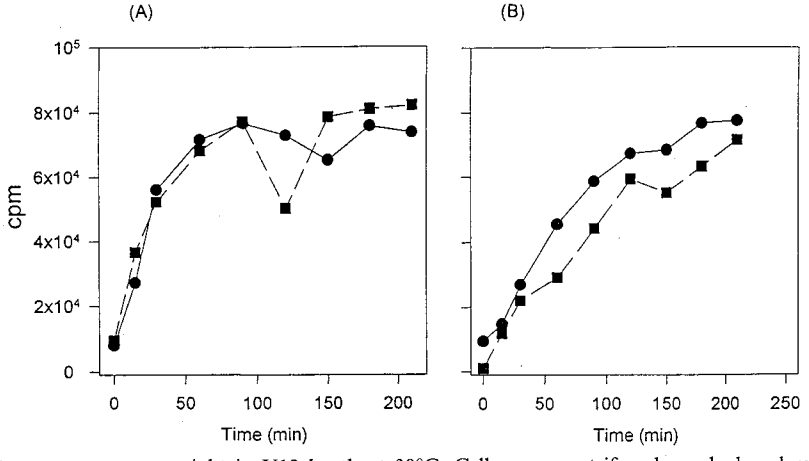
Substance	cpm/25 μl	% leakage
Water	267	0
Methanol	414	5
DMSO	335	3
Chromomycin (10 μg/ml)	206	0
Papulacandin (10 μg/ml)	295	0
Azasterol (10 μg/ml)	612	23
Econazole	300	1
Nystatin (10 μg/ml)	2,946	100
Ascosteroside (10 μg/ml)	352	3
Ascosteroside (20 μg/ml)	331	3

C. albicans was grown overnight in F4 medium. The harvested cells were resuspended in 50 mM phosphate buffer, pH 7 containing 2% glucose at OD600 nm of 1.5.

^{14}C-aminoisobutyric acid was added to the cells and incubated for 30 minutes at 37°C. The cells were washed and resuspended in the buffered glucose and test samples were added. Cells were harvested by centrifugation after being allowed to incubate at 37°C for one hour. 25 μl aliquots of the supernatant were counted for radioactivity[7].

Fig. 6. Macromolecular biosynthesis: the incorporation of leucine into protein (A) and the incorporation of uridine into nucleic acids (B).

Ascosteroside (■) and control (●).



S. cerevisiae was grown overnight in Y13 broth at 30°C. Cells were centrifuged, washed and resuspended in defined medium (Y14) to an OD 600 nm of 0.5. Ascosteroside was added to 5 μg/ml and samples were removed at intervals, added to cold trichloroacetic acid and filtered. Filters were washed, dried and counted.

Table 3. The effect of ascosteroside in a eucaryotic cell-free translation system.

Sample	Concentration	% Inhibition
Control	1 μg/ml	0
Tetracycline	1 μg/ml	0
Cycloheximide	1 μg/ml	84.4
Ascosteroside	1 μg/ml	0
	20 μg/ml	0
	5 μg/ml	0

The cell-free translation system contained in 100S ribosomal samples from yeast, polyU, s-RNA, phenylalanine and an energy generating system.[8]

trichloroacetic acid precipitable material. As shown in Fig. 6, there was no significant inhibition of the incorporation of either precursor in the presence of ascosteroside. In addition, in a cell-free translation assay[8] to measure *in vitro* protein synthesis, ascosteroside also failed to give any inhibition of the incorporation of [^{3}H] phenylalanine into protein, while cycloheximide showed inhibition at 1 μg/ml (Table 3).

In Vivo Activity

The *in vivo* activity of ascosteroside was determined using female Swiss-Webster mice (Taconic Farms, Germantown, NY) weighing between 18~20 grams. *C. albicans* strain SC5314 was grown overnight in Sabouraud dextrose broth at 25°C with shaking. The yeast cells were washed 3 times with physiological saline and the number of cells counted in a hemocytometer. Mice were infected by the intravenous injection of 10^5 yeast cells contained in 0.2 ml of saline. Therapy was begun at 1 hour post-infection and given once a day for 5 consecutive days. Surviving animals were sacrificed at 28 days and the efficacy of the test compound represented as mean day of death (MDD) for the animals that died.

Ascosteroside was dissolved in DMSO and diluted with water (Water for Injection, Henry Shine, Inc.) so that the animals received a 5% DMSO-water solution. Animals were treated intraperitoneally (IP) with 0.5 ml of the DMSO-water solution at a dose of 30 mg/kg/day of ascosteroside. Ketoconazole (Janssen) was suspended in 0.5% carboxymethylcellulose and administered orally at 7.5 mg/kg/day.

It was found that ascosteroside was effective in increasing survival time in *Candida* infected mice when administered at 30 mg/kg/day as indicated by an MDD of 16.3 days compared to an MDD of 10.2 for the infection control group. Ketoconazole administered at 7.5 mg/kg/day was also effective with an MDD of 13.3.

Conclusion

The novel antifungal agent ascosteroside was identified in the fermentation broth of *A. amphitricha*. The compound was specific for fungi, having little or no activity against bacteria and it was not cytotoxic to normal diploid fibroblast (SS1) cells. Studies to date help in ruling out possible modes of action, but the biochemical target of ascosteroside remains to be determined. In mice infected with *Candida*, ascosteroside increased survival time comparable to that found with ketoconazole.

The production of ascosteroside in fermentation broths was seen to vary between shake flask culture where an optimum was reached at days 6 to 7 and fermentor culture, where the optimum production was seen at days 3 to 4. In the fermentor, agitation rates of 500 rpm were necessary for production and ascosteroside was not detected at an agitation rate of 300 rpm. Ascosteroside appears to be a static agent. There are a number of recent reports of antifungal compounds with structures similar to ascosteroside; Ergokonin C produced by *Tolypocladium inflatum*[9], PF1032 produced by *Neosartorya*[10], UCA1064-B produced by *Wallemia*[11] and squalamine produced by the dogfish shark *Squalus acanthias*[12]. A synthetic cholesterol derivative, 7-aminocholesterol, has recently been described with antifungal and anti Gram-positive activity[13].

References

1) PFALLER, M. A.: Epidemiology and control of fungal infections. Clinical Infectious Diseases 19 (Suppl. 1): 5~13, 1994
2) LEET, J. E.; S. HUANG, S. E. KLOHR & K. D. MCBRIEN: Ascosteroside, a new antifungal agent from *Ascotricha amphitricha*. II. Isolation and structure elucidation. J. Antibiotics 49: 553~559, 1996
3) CHIVERS, A. H.: A monograph of the genera *Chaetomium* and *Ascotricha*. Memoirs of the Torrey Botanical Club Vol. 143: 3, 1915
4) HAWKSWORTH, D. L.: A revision of the genus *Ascotricha*. Ber. Mycological Papers #126, pp. 1~33, 1971
5) O'SULLIVAN, J.; D. W. PHILLIPSON, D. R. KIRSCH, S. M. FISCHER, M. H. LAI & W. H. TREJO: Lanomycin and glucolanomycin, antifungal agents produced by *Pycnidiophora* dispersa. I. Discovery, isolation and biological activity. J. Antibiotics 45: 306~312, 1992
6) KIRSCH, D. R.; M. H. LAI & J. O'SULLIVAN: Isolation of the gene for cytochrome P450L1A1 (lanosterol 14α-demethylase) from *Candida albicans*. Gene 68: 229~237, 1988
7) GEORGOPAPADAKOU, N. H.; B. A. DIX, S. A. SMITH, J. FREUNDENBERGER & P. T. FUNKE: Effects of antifungal agents in lipid biosynthesis and membrane integrity in *Candida albicans*. Antimicrobial Agents Chemo. 31: 46~51, 1987
8) EUSTICE, D. C.; L. P. WAKEM, J. M. WILHELM & F. SHERMAN: Altered 40 S ribosomal subunits in omnipotent suppressors of yeast. J. Mol. Biol. 188: 207~214, 1986

9) SCHLEGEL, B.; U. GRAEFE, W. IHN, E. J. BORMANN, W. R. RUDAT & K. H. HEIDENBLUTH: A novel antifungal antibiotic manufactured by cultures of *Tolypocladium* or *Sesquicillopsis*. Ger. Offen. DE4. 115: 490, 1992
10) GOM, S.; H. WATABE, Y. MITO, C. MORIYAMA, T. YUGAMI & M. KOYAMA: New antibiotic substance PF1032, with antifungal property, prepared by culturing bacteria of genus *Neosartorya*, and having low toxicity. Japanese Patent J04046188, 1992
11) TAMAOKI, T.; I. TAKAHASHI, K. ANDO, M. YOSHIDA & T. IWAZAKI: Compound UCA1064-B. Eur. Patent Appl. 92104079.6, 1992
12) WEHRLI, S. L.; K. S. MOORE, H. RODER, S. DURELL & M. ZASLOFF: Structure of the novel steroidal antibiotic squalamine determined by two-dimensional NMR spectroscopy. Steroids 58: 370～378, 1993
13) ELKIHEL, L.; I. SOUSTRÈ, F. KARST & Y. LETOURNEUX: Amino-acid aminoethylcholesterol derivatives with fungicidal activity. FEMS Microbiology Letters 120: 163～168, 1994