Int Microbiol (2001) 4: 93–102
DOI 10.1007/s101230100020

RESEARCH ARTICLE

**M. Angeles Cabello · Gonzalo Platas · Javier Collado
M. Teresa Díez · Isabel Martín · Francisca Vicente
María Meinz · Janet C. Onishi · Cameron Douglas
John Thompson · Myra B. Kurtz · Robert E. Schwartz
Gerald F. Bills · Robert A. Giacobbe · George K. Abruzzo
Amy M. Flattery · Li Kong · Fernando Peláez**

# Arundifungin, a novel antifungal compound produced by fungi: biological activity and taxonomy of the producing organisms

Received: 1 March 2001 / Accepted: 30 April 2001 / Published online: 11 September 2001


**Abstract** Echinocandins, the lipopeptide class of glucan synthase inhibitors, are an alternative to ergosterol-synthesis inhibitors to treat candidiasis and aspergillosis. Their oral absorption, however, is low and they can only be used parenterally. During a natural product screening program for novel types of glucan synthesis inhibitors with improved bioavailability, a fungal extract was found that inhibited the growth of both a wild-type *Saccharomyces cerevisiae* strain and the null mutant of the *FKS1* gene (*fks1*::HIS). The mutant strain was more sensitive to growth inhibition, suggesting that the fungal extract could contain an inhibitor of glucan synthesis. A novel acidic steroid, named arundifungin, was purified from a fungal extract obtained from a liquid culture of *Arthrinium arundinis* collected in Costa Rica. Arundifungin caused the same pattern of hallmark morphological alterations in *Aspergillus fumigatus* hyphae as echinocandins, further supporting the idea that arundifungin belongs to a new class of glucan synthesis inhibitors. Moreover, its antifungal spectrum was comparable to those of echinocandins and papulacandins, preferentially inhibiting the growth of *Candida* and *Aspergillus* strains, with very poor activity against *Cryptococcus*. Arundifungin was also detected in nine other fungal isolates which were ecologically and taxonomically unrelated, as assessed by sequencing of the *ITS1* region. Further, it was also found in two more *Arthrinium* spp from tropical and temperate regions, in five psychrotolerant conspecific isolates collected on Macquarie Island (South Pacific) and belonging to the Leotiales, and in two endophytes collected in central Spain (a sterile fungus belonging to the Leotiales and an undetermined coelomycete).

**Keywords** *Arthrinium arundinis* · Glucan synthesis · Antifungal compound · Acidic terpenoid · Psychrotolerant fungi

M.A. Cabello (✉) · G. Platas · J. Collado · M.T. Díez
I. Martín · F. Vicente · F. Peláez
Centro de Investigación Básica – Natural Products
Drug Discovery, Merck Research Laboratories,
Merck, Sharp & Dohme de España. Josefa Valcárcel 38,
28027 Madrid, Spain
E-mail: angeles_cabello@merck.com
Tel.: +34-91-3210486
Fax: +34-91-3210614

M. Meinz · J.C. Onishi · C. Douglas · J. Thompson
M.B. Kurtz · R.E. Schwartz · G.K. Abruzzo
A.M. Flattery · L. Kong
Infectious Diseases, Merck Research Laboratories,
Rahway, New Jersey, USA

R.E. Schwartz
Natural Products Chemistry – Natural Products
Drug Discovery, Merck Research Laboratories,
Rahway, New Jersey, USA

G.F. Bills · R.A. Giacobbe
Natural Products Microbiology – Natural Products
Drug Discovery, Merck Research Laboratories,
Rahway, New Jersey, USA

## Introduction

The rise in the number of immunocompromised patients has dramatically increased the incidence of human systemic fungal infections during the past two decades. Therapies used in clinic have relied mainly on the use of either amphotericin B or azole antifungal agents. Amphotericin B, an ergosterol-binding polyene, is a potent, broad-spectrum, fungicidal agent that must be used cautiously, due to dose-limiting nephrotoxicity [12]. Azoles, inhibitors of lanosterol demethylase, a late step in ergosterol biosynthesis, have improved safety [33, 35], but their widespread use has led to the appearance of strains with intrinsic or acquired resistance to these agents and sometimes cross-resistance to all azoles [32].

The therapeutic utility of echinocandins and pneumocandins, a new class of fungicidal agents that block

the formation of the fungal cell wall by inhibiting (1,3)-$\beta$-D-glucan synthase [7, 19, 40, 41, 45], is currently being evaluated. These agents are lipopeptides composed of an acyl-substituted cyclic hexapeptide. The clinical candidates caspofungin (Cancidas) and LY303366 have recently shown promising results in the clinical treatment of *Candida* infections and aspergillosis, thus providing an alternative to the ergosterol-directed agents. Since they inhibit a target unique to fungi, their therapeutic ratios are really good, but their oral absorption is low. Although considerable efforts have been made to improve oral bioavailability, they can only be used parenterally [19, 41]. A second class of glucan synthesis inhibitors, the glycolipid papulacandins [42], has antifungal activity but lacks enough in vivo efficacy for clinical use [44].

A natural product screening program searching for novel types of glucan synthesis inhibitors was conducted in our laboratory. Our screening strategy consisted of an agar-diffusion assay using a wild-type *Saccharomyces cerevisiae* strain and a null mutant of the FKS1 gene, encoding the vegetatively expressed large subunit of (1,3)-$\beta$-D-glucan synthase (*fks1*). The *fks1* strain is hypersensitive to certain types of antifungal agents, including echinocandins and papulacandins [9]; and therefore an enhanced susceptibility of this strain could reveal the presence of a potential glucan synthesis inhibitor.

## Materials and methods

### Strain isolation and characterization

Table 1 shows the substrata and geographical origins of the fungi studied. Strains were isolated from soil or leaf litter, using the particle-filtration method described by Bills and Polishook [3], in a medium that contained (per liter): 10 g malt extract, 1 g yeast extract, 20 g agar, 40 mg cyclosporin A, 5 mg streptomycin and 50 mg terramycin [6]. Isolation plates were incubated at 22 °C, except for the strains from Macquarie Island, which were incubated at 15 °C. Macquarie Island is located 1,000 km southeast of Tasmania (54° 30′ S, 158° 57′ E), close to the Antarctic Convergence. The isolates from living plant materials (F-042833, F-054289) were recovered using the surface-sterilization technique described by Collado et al. [6], on the same medium as above but without cyclosporin. Strains were transferred to potato dextrose agar (PDA, Difco) slants before screening.

Fungi were cultured at 22 °C under fluorescent light on a 12-h light/dark cycle, on plates of oatmeal agar, cornmeal agar and PDA (all prepared media from Difco) for identification. Sporulation appeared typically after 2–4 weeks under these conditions, except for the sterile isolates, which were kept in the incubator for several months without producing any sporulation. These sterile isolates were also grown on different cellulosic materials, such as sterilized leaves, filter paper and small pieces of wood, but in all cases they failed to sporulate.

In the descriptions, capitalized color names are from Kornerup and Wanscher [18]. Microphotographs were taken with a Leitz Diaplan microscope equipped with Nomarski interference optics. The strains studied in this work are preserved in the CIBE Culture Collection; and the original producer of arundifungin is deposited in the American Type Culture Collection under accession number ATCC 74359. For the determination of growth rates of the sterile isolates from Macquarie Island, PDA plates were inoculated from frozen agar plugs and incubated at 18 °C for 21 days. Small agar squares (0.5 $cm^2$) containing active mycelium from the margin of the colonies growing on these plates were placed in the center of new PDA plates, which were incubated at 5, 10, 16, 18, 22 and 28 °C (two plates of each isolate at each temperature), under a 12-h photoperiod. Colony diameter was established as the average of two orthogonal measurements. Measurements were taken every other day up to 28 days.

### Fungal fermentations

Seed flasks were prepared from fresh slants as described by Peláez et al. [28]. Portions (2 ml) of the resulting cultures were used to inoculate 250-ml, unbaffled Erlenmeyer flasks containing 50 ml of the following media: CYS80 (F-044878, F-055174, F-052591, F-054289), MV8 (ATCC 74359, F-046780, F-047233), AD2 (F-046835) [8] and F1 (F-042833, F-046877) [10]. Production flasks were incubated at 28 °C and 50% relative humidity, either at 220 rpm for 21 days for CYS80 and MV8, or at statically for 28 days for F1 and AD2.

**Table 1** Origin of the fungal strains producing arundifungin

| Strain code | Taxonomy | Substrate | Geography |
|---|---|---|---|
| ATCC 74359 | *Arthrinium arundinis* | Material from bird nest | El Chayote, Alajuela, Costa Rica |
| F-044,878 | *A. phaeospermum* | Leaf litter | Kimwenza forest, Mongafula area, Congo |
| F-055,174 | *A. arundinis* | Lichen in humid forest | X Region, Natl. Park Alerce Andino, Chile |
| F-042,833 | Coelomycete undetermined | Twigs of *Olea europaea* var. *europaea* | Ontígola, Madrid, Spain |
| F-054,289 | Sterile mycelium | Leaves of *Quercus ilex* | Ontígola, Madrid, Spain |
| F-052,591 | Sterile mycelium | Soil from fernbrake community | Rookery Creek above Nuggets, Macquarie Island |
| F-047,233 | Sterile mycelium | Soil from fernbrake community | Rookery Creek above Nuggets, Macquarie Island |
| F-046,780 | Sterile mycelium | Soil from tussock community | Base of Wireless Hill, Macquarie Island |
| F-046,877 | Sterile mycelium | Soil from tussock community | Base of Wireless Hill, Macquarie Island |
| F-046,835 | Sterile mycelium | Leaf litter | Handspike Corner, Macquarie Island |

Methyl-ethyl-ketone (MEK) extracts were prepared by adding 1.4 vol of MEK (Merck Farma y Química) to each culture. Then, the mycelia were disrupted, shaken for 15–60 min and centrifuged at 3,000 rpm for 15 min. Aliquots (0.8 ml) of the organic phase were taken, dried out completely in a Savant Speed-Vac and the solid residue was then reconstituted in 0.5 ml of dimethylsulfoxide (DMSO). For the preparation of methanol (MeOH) extracts, each culture was mixed with an equal volume of MeOH (Merck Farma y Química). After shaking for 15 min, the samples were centrifuged at 3,000 rpm for 15 min. Aliquots of the supernatants (2 ml) were evaporated under nitrogen atmosphere to half their volume.

For isolation of arundifungin, vegetative mycelia from the culture ATCC 74359 were prepared as described by Peláez et al. [28]. Portions (2 ml) of the resulting culture were then used to inoculate MV8 medium, in 250-ml, unbaffled Erlenmeyer flasks. Production flasks were incubated at 22 °C and 50% relative humidity, at 220 rpm for up to 21 days. Maximum titer occurred over days 14–21. At harvest, arundifungin was extracted from the mycelial growth with an equal volume of MeOH, shaken for 1 h at 25 °C and then centrifuged for 20 min at 3,000 rpm.

### Differential susceptibility test against *S. cerevisiae* strains W303-1a and *fks1*::HIS

The search for potential inhibitors of glucan synthesis was performed by means of an agar-based differential susceptibility test against a wild-type *S. cerevisiae* strain (W303-1a) and a null mutant of the FKS1 gene encoding the vegetatively expressed large subunit of (1,3)-$\beta$-D-glucan synthase (*fks1*::HIS). The *fks1*::HIS strain was constructed at Merck & Co. and is isogenic with the wild type strain W303-1a (Mat a *ade2-1 can 1-100 his3-11,15 leu2-2,112 trp1-1 ura3-1*). Both strains belong to the Merck Culture Collection (strain codes MY2141 and MY2265 for the wild-type and mutant strains, respectively; Merck & Co.).

A frozen stock of each strain was thawed in a flask containing YPAD/KCl medium (1% yeast extract, 2% peptone, 40 mg adenine/ml, 2% glucose and 4.5% potassium chloride) and incubated overnight at 28 °C. The assay plates were prepared by inoculating each strain into separate flasks containing YPAD/KCl supplemented with 1.5% Noble agar to a final optical density of 0.01 at 600 nm. Aliquots (100 ml) of the seeded agar media were poured into Nunc square plates (24×24 cm). Aliquots of the MEK or MeOH extracts (25 µl) were applied to the surface of the seeded assay plates and incubated at 28 °C. Aculeacin (2.5 µg) and pneumocandin $B_o$ (2.5 µg) were tested as positive controls, while amphotericin B (2 µg) and nystatin (25 µg) were used as negative controls in each assay plate. The plates were incubated at 28 °C and inhibition zones were scored 24 h later. Extracts producing zones of inhibition in the *fks1*::HIS strain at least 5 mm greater than those produced in the wild-type strain were considered potential inhibitors of glucan synthesis.

### Broth microdilution assay for minimal inhibitory concentrations determination

Minimum inhibitory concentrations (MIC) were determined against a panel of yeasts and filamentous fungi, using a broth microdilution assay modified as described by Onishi et al. [27]. All fungal strains were from either the Merck Culture Collection (MY, MF, MB strains) or the Clinical Culture Collection (CLY strains), Merck & Co.

### Morphological alterations in *Aspergillus fumigatus*

An agar-based assay was used to assess the morphological changes produced by arundifungin in the hyphae of the filamentous fungus *A. fumigatus* (MF5668). For the preparation of a stock spore suspension of *A. fumigatus*, a rehydrated, lyophilized culture was streaked onto Sabouraud dextrose agar (SDA, Difco) plates and incubated at 37 °C until growth was readily apparent. Slants of SDA were inoculated with colonies from the plates and incubated at 37 °C until heavy spore formation occurred. Then, 5 ml of sterile water were added to each slant and a sterile cotton swab was used to scrape the spores off the slant. The spore suspension obtained was aliquoted and kept frozen at –80 °C. The spore suspension was diluted into 6.7 g yeast nitrogen base/l (Difco) to an optical density of 0.6 at 600 nm. An aliquot (1 ml) of the diluted suspension was added to 100 ml of a medium containing 1.5% Bactoagar (Difco), 6.7 g yeast nitrogen base/l and 5 g dextrose/l. Aliquots (10 ml) of the seeded agar media were poured into Sterilin square plates (10.5×10.5 cm). Aliquots of the compounds (5 µl) dissolved in 50% DMSO were applied to the surface of the seeded assay plates and incubated overnight at 37 °C. The same volume of 50% DMSO was tested as a solvent control. Pneumocandin $B_o$ (2 µg) and nystatin (25 µg) were used, respectively, as positive and negative controls. The morphology of the treated cells within or at the edge of the inhibition zone was observed under a light microscope. Microphotographs were taken at 500× or 1,250× with a Leitz Diaplan microscope equipped with differential interference contrast optics.

### Determination of in vivo efficacy

The in vivo efficacy of the compound was evaluated in a mouse model of disseminated candidiasis with enhanced susceptibility to *C. albicans* and increased sensitivity for discriminating antifungal efficacy, following a modification of the method described by Bartizal et al. [2]. Basically, immunosuppressed mice were challenged intravenously with *C. albicans* and treated with serial dilutions of the compound administered intraperitoneally (IP), receiving three doses (t.i.d.) during 1 day. After 24 h, the mice were sacrificed and their kidneys were removed, homogenized and plated at serial dilutions. Yeast colonies were enumerated for determination of colony forming units (cfu) per gram of tissue.

### HPLC bioautograph for detection of arundifungin

HPLC bioautographs of fermentation extracts containing arundifungin were performed on a Dupont Zorbax RXC8 column (0.94×25 cm). The flow rate was 2 ml/min. The solvent system was acetonitrile/water [1:1] (0.01% trifluoroacetic acid) for the first 35 min and [9:1] for the following 10 min. The ultraviolet absorption of the eluent was monitored at 210 nm. Furthermore, mass spectrometry detection was performed on all of the active regions for identification of the $M_r$ 592 peak.

### rDNA sequencing

An aliquot (about 0.1 µg/ml) of the double stranded amplification products containing the region ITS1, obtained as described by Peláez et al. [29], using the primers ITS1F [14] and 5.8S (CGCTGCGTTCTTCATCG; R. Vylgalys, personal communication), were sequenced using the ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer), following the procedures recommended by the manufacturer. Each strand was sequenced using those same primers as templates. The electrophoretic separation of the reaction products was performed in an ABI 373 automatic sequencer (Perkin Elmer). The sequences obtained were aligned manually; and the phylogenetic analysis was performed by maximum parsimony, using the branch-and-bound algorithm of PAUP ver 3.1.1 [36]. The robustness of the branches in the trees was assessed by bootstrap analysis [11], resampling the data with 1,000 bootstrap replicates. Branch support was also assessed by calculating decay indices [4], using the SEPAL ver 1.01 [34].

## Results

### Biological activity

In the course of a natural product screening program for novel inhibitors of glucan synthesis, we detected a fungal extract that inhibited the growth of a wild-type *S. cerevisiae* strain and showed an enhanced activity against the null mutant *fks1*::HIS strain in agar diffusion assays (the diameters of the inhibition zones were 26 mm in the wild type and 31 mm in the *fks1*::HIS strain). As expected, in the same conditions, the *fks1*::HIS strain was also more sensitive than the wild-type strain to pneumocandin $B_o$ and aculeacin, the inhibition zones scored in the *fks1*::HIS strain (6–8 mm) being greater than those seen in the wild-type strain. In contrast, the inhibitory effects produced by amphotericin B and nystatin were practically identical in both *S. cerevisiae* strains. The active component from the extract was purified and identified as a new acidic terpenoid (Fig. 1), which was named arundifungin. Details on the isolation and structure elucidation of the compound are described elsewhere ([27]; Liesch JM et al., 27 January 1998, US patent 5,712,109).

Since the known glucan-synthesis inhibitors characteristically altered the morphology of filamentous fungi [20, 44], we examined the effect of arundifungin on the morphology of the filamentous fungus *A. fumigatus*. Arundifungin prevented normal polarized hyphal growth; and shortened, stunted, highly branched hyphae were observed, with bipolar or vesicular tips, swollen germ tubes and frequently balloon-like cells. Moreover, its effects on *A. fumigatus* hyphae were indistinguishable from those of the glucan-synthesis inhibitor pneumocandin $B_o$. Under the same conditions, nystatin did not cause morphological alterations in the hyphae (Fig. 2).

Arundifungin showed antifungal activity against a broad panel of human pathogenic filamentous fungi and yeast (Table 2). Its MICs ranged over 2–8 μg/ml for most of the *Candida* spp tested and were only 1 mg/ml for *A. fumigatus*, but it showed a very low activity against the *Cryptococcus* strains studied, including an unencapsulated form (MY2062). Arundifungin also inhibited the growth of other fungal species such as *A. flavus*, *Fusarium oxysporum* and *Ustilago zeae* in agar diffusion assays (results not shown), although MICs against these organisms were not measured. However, the compound lacked antibacterial activity, even when tested up to 64 μg/ml.

A mouse model of disseminated candidiasis was employed to test the in vivo efficacy of arundifungin. The effect of our terpenoid was studied in comparison with those of pneumocandin $A_o$ and amphotericin B. Effective doses giving 99% reductions ($ED_{99}$) were calculated based on the reduction on cfu/g in the kidneys of treated mice, compared to sham-treated control animals. While both amphotericin B and pneumocandin $A_o$ inhibited *Candida* growth in vivo (showing a daily $ED_{99}$ value of 0.5 mg/kg and 3.86 mg/kg; IP, t.i.d.×1 day, respectively), arundifungin was not effective in vivo when administered daily at up to 50 mg/kg (IP, t.i.d.×1 day).

### Taxonomy of the producing fungi

Arundifungin was first isolated from a tropical isolate of *Arthrinium arundinis* (= *Arthrinium* state of *Apiospora montagnei*) (Fig. 3). In culture, the original producer ATCC 74359 showed the following morphology. Colonies on yeast/malt agar (Difco) at 22 °C, with a 12-h photoperiod and attaining 24–30 mm in 14 days, were raised, velvety to lanose, with some radial sectoring, obscurely zonate, with margin even and submerged, mostly white, later pale gray (1B1) (capitals and numbers between parentheses in this Section correspond to colors in ref. [18]) to grayish brown (6E3), reverse translucent to pale brownish gray (6C2) or olivaceous gray (1D2), exudates absent. There was no growth at 37 °C. The conidiophores were extremely short, under 8 μm long, hyaline, integrated, arising as lateral or terminal branches from main hyphal axes, 1–3-celled, often with spherical to pyriform, inflated basal cells giving rise to short filamentous terminal cells that

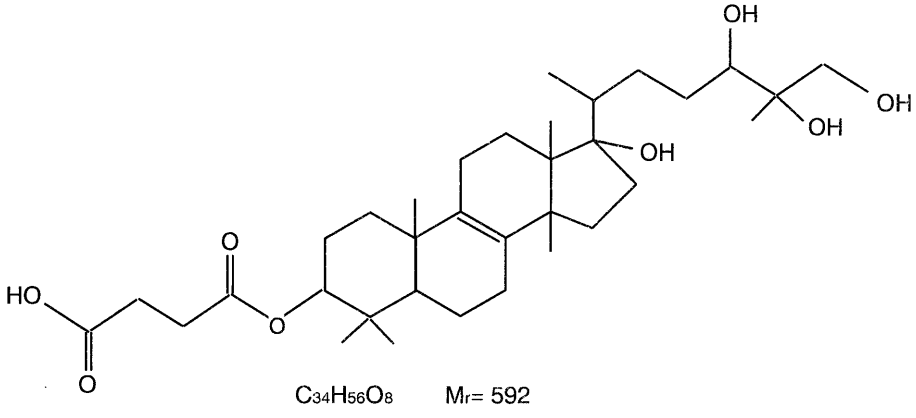


**Fig. 1** Chemical structure of arundifungin

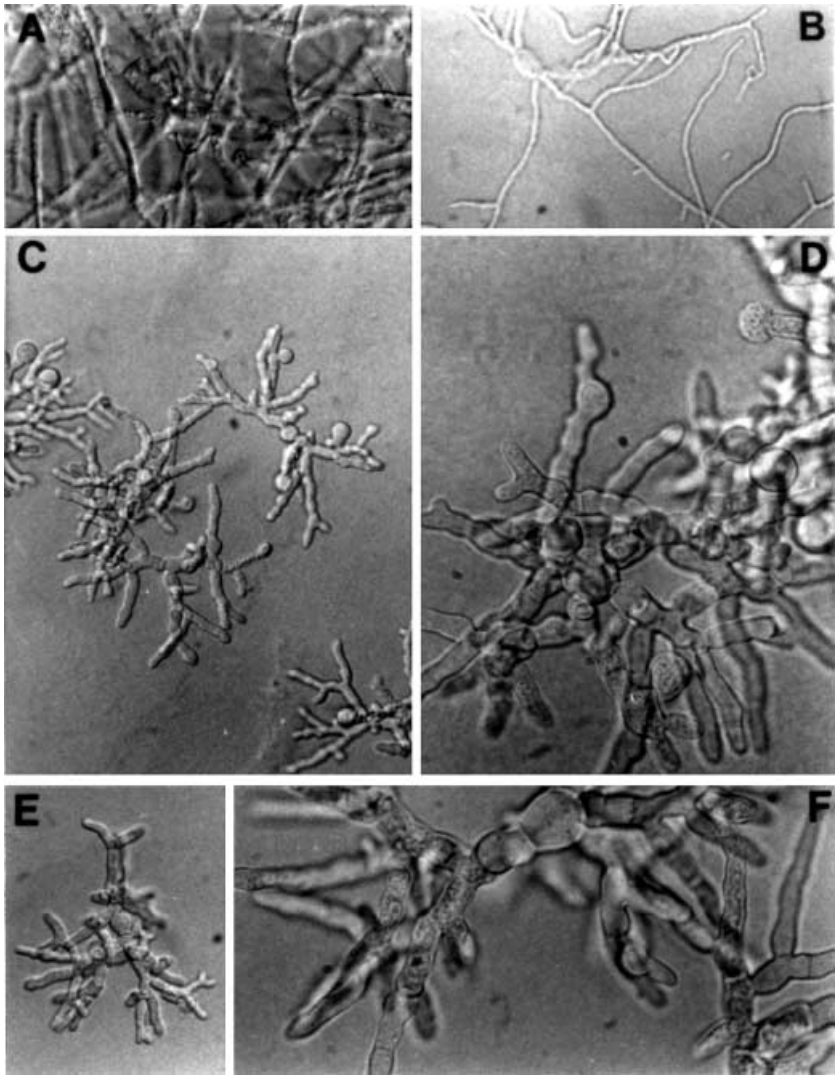


**Fig. 2A–F** Effect of arundifungin on the growth of *Aspergillus fumigatus* hyphae. **A** DMSO solvent control. **B** Nystatin (25 mg). **C–D** Arundifungin (2 mg). **E–F** Pneumocandin $B_o$ (2 mg). Photomicrographs were taken at 500× (**A–C**, **E**) or 1,250× (**D**, **F**) with a Leitz Diaplan microscope equipped with differential interference contrast optics

**Table 2** In vitro antifungal activity of arundifungin evaluated in microbroth dilution assays. All the determinations were performed after a 24-h incubation, except for *Cryptococcus neoformans*, which was incubated for 48 h. Results are expressed as the minimum inhibitory concentration (MIC)

| Strains | MIC (mg/ml) |
|---|---|
| *Candida albicans* (MY1055) | 4 |
| *C. albicans* (CLY539) | 2 |
| *C. glabrata* (MY1381) | 2 |
| *C. parapsilopsis* (MY1010) | 4 |
| *C. pseudotropicalis* (MY2099) | 8 |
| *C. tropicalis* (MY1124) | 8 |
| *C. tropicalis* (MY1012) | 16 |
| *C. krusei* (CLY549) | 4 |
| *Cryptococcus neoformans* (MY2061) | > 64 |
| *C. neoformans* (MY2062) | 32 |
| *Saccharomyces cerevisiae* (MY2140) | 8 |
| *Aspergillus fumigatus* (MF5668) | 1 |
| *Staphylococcus aureus* (MB2865) | > 64 |

tapered apically toward conidiogenous loci, solitary to densely aggregated, collapsing soon after conidium formation. Conidiogenous cells arising from inflated basal cells were holoblastic, basauxic, hyaline, thin-walled, filamentous or tapered apically. The conidia were lenticular, circular to pyriform in face view, compressed in side view, smooth, yellowish brown, often with a pale band along compressed edge, 6–8 μm in diameter. When this isolate was inoculated onto autoclaved banana leaves supported by cornmeal agar, the conidia appeared strongly aggregated into pustules that could be interpreted as sporodochia or acervuli.

Strain ATCC 74359 was readily assigned to the anamorph genus *Arthrinium*, based on its dark, lenticular conidia borne on short, filamentous conidiogenous cells and its lack of setae, stroma or ascomata. From about 20 described species of *Arthrinium*, *A. arundinis* was distinguished by its smooth, round, lenticular conidia, which were smaller than most other species of the genus, and by thin, filamentous, hyaline conidiogenous cells. Arundifungin was found in nine more fungal isolates (see Table 1), two of which were also *Arthrinium* species. One of them (F-044878), isolated from leaf litter collected in the Congo, was identified as *A. phaeospermum*, based on a combination of characteristics,

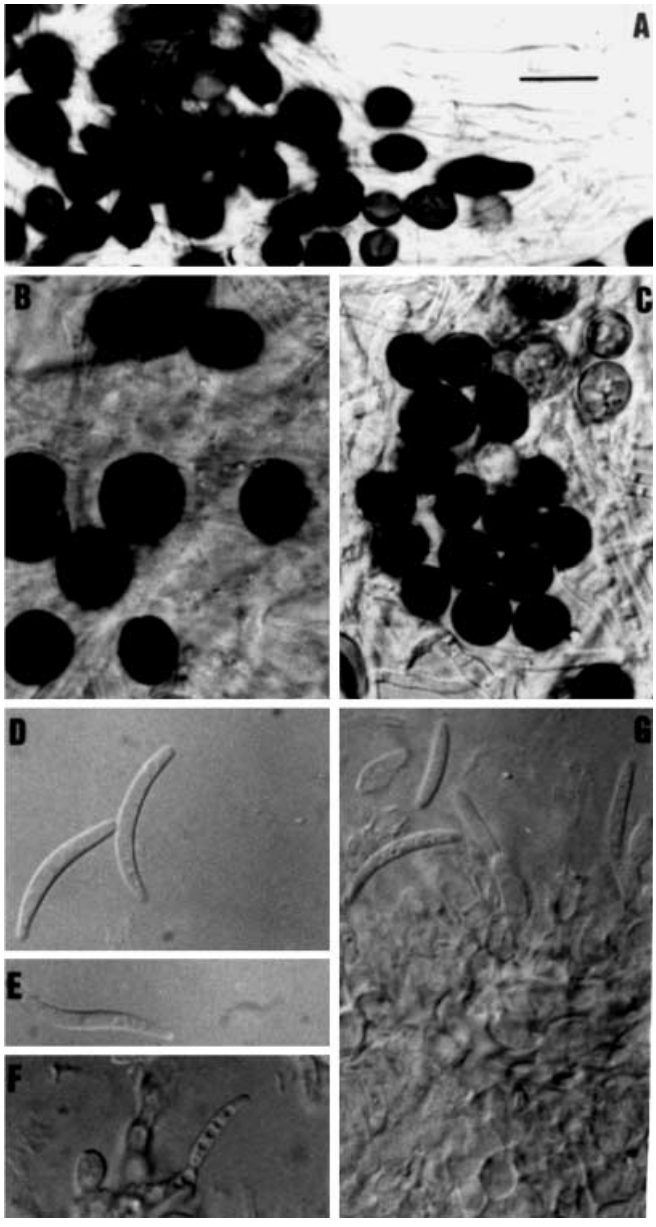


**Fig. 3A–G** Fungi producing arundifungin. **A** Conidia of *Arthrinium arundinis* F-055174. Note the presence of aberrant conidia. **B** Conidia of *A. phaeospermum* F-044878. **C** Conidia of *A. arundinis* ATCC 14359. Note the lenticular shape of the conidia and the clear equatorial rings in side view for the three strains. **D–G** Conidia and conidiogenous cells of F-042833 (*Selenophoma*-like coelomycete). All photomicrographs were taken at 1,250×; *bar* in **A** indicates 10 μm

including dark brown, 10–12 μm diameter, lenticular conidia, with a hyaline equatorial germ slit, arising from hyaline, filamentous and narrow conidiophores (1–1.5 μm thick; Fig. 3). The third *Arthrinium* isolate (F-055174), from a lichen specimen collected in Chile, was conspecific with the original producer. However, it showed some differences in culture, especially because of the proliferation of aberrant dark cells (Fig. 3).

The arundifungin producer isolated as an endophyte from twigs of *Olea europaea* (F-042833) was identified as a coelomycete, reminiscent of the genus *Selenophoma*. In culture, this strain had the following morphology. Colonies on PDA were slow-growing and reached 12 mm diameter after 21 days with a 12-h photoperiod at 22 °C. The center was dark brown (7F6), cerebriform with an irregular, grayish red (7B3) edge of folded and almost totally immersed mycelium. The colony reverse was reddish grey (7B2). Conidiomata consisting of pycnidia were produced in concentric circles around the center of the colony. Pycnidia were mostly aggregated but sometimes solitary and were colorless to fleshy when first formed, becoming dark brown with age. They were globose, unilocular, textura angularis and lacking an ostiole, dehiscence being by irregular rupture of the upper wall of the pycnidium. Spore masses were whitish or cream. Conidiophores were formed from the inner cells of the pycnidial wall and were hyaline, sparsely branched, septate, smooth and cylindrical to slightly irregular. Conidiogenous cells were hyaline enteroblastic, phialidic, integrated or discrete, indeterminate, cylindrical or ampulliform. Conidia were hyaline, aseptate (rarely with one septum), fusiform, mostly falcate but some irregularly curved, tapered to the apex, smooth and guttulate.

The rest of the producing isolates were sterile in pure culture. Strain F-052289 was an endophyte from leaves of *Quercus ilex* (holm oak) from the same area as the coelomycete described above (Ontígola, near Madrid, Spain) and it had the following morphology in pure culture. Colonies on PDA attaining 36 mm diameter after 21 days of incubation at 22 °C were partially immersed and their texture was slightly floccose, being whitish gray (1B1) at the center and becoming pale green (3E3) towards the border. The colony edge was white and regular. The reverse was grayish brown (5F3) at the center, turquoise grey (24F2) towards the edge and becoming pale orange (5A3) at the margins. Odorless; and diffusible pigments and exudates were absent. Stromata and appresoria were not observed. Some erect tufts of darkened hyphae were visible regularly throughout the surface. The mycelium was mostly hyaline, rarely brown, septate and branched dichotomously, 3.2 μm wide. Chlamydospores were absent.

The remaining five sterile isolates producing arundifungin were recovered from soil or leaf litter collected on Macquarie Island. All the strains recovered from these samples were isolated at low temperatures (15 °C) and showed optimal growth at temperatures of 16–18 °C (Fig. 4), being unable to grow at 28 °C. Thus (and consistent with their geographical origin), these isolates would be psychrotolerant fungi, according to the widely accepted criteria established by Morita [24] for bacteria. One of these strains (F-047233) could even be considered as a real psychrophile *sensu* Morita [24], since its optimum growth was clearly obtained at about 16 °C and the maximum tolerated temperature was under 22 °C. All of these strains presented similar cultural characteristics, although they differed slightly in growth rate and mycelial texture, with colonies on PDA almost covering a 90-mm Petri dish after incubation at 16 °C for 30 days.

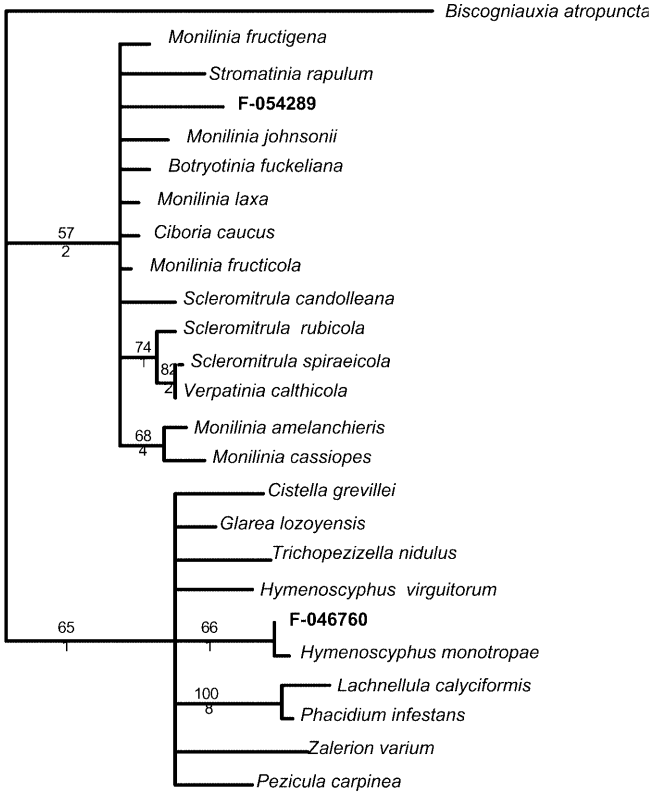


**Fig. 4** Phylogenetic tree showing the relationships between the producers of arundifungin, F-046760 and F-054289, and several fungi from the order Leotiales, based on ITS1 sequence. Bootstrap and decay indexes are *indicated above and below* each branch, respectively. The following sequences were retrieved from GenBank: *Monilinia fructigena* (AF150677), *Stromatinia rapulum* (Z73801), *M. johnsonii* (Z73783), *Botryotinia fuckeliana* (Z73764), *M. laxa* (AF150673), *Ciboria caucus* (U21829), *M. fructicola* (Z73777), *Scleromitrula candolleana* (Z80877), *S. rubicola* (Z80891), *S. spiraeicola* (Z80882), *Verpatinia calthicola* (U21826), *M. amelanchieris* (Z73769), *M. cassiopes* (Z73776), *Cistella grevillei* (U57089), *Glarea lozoyensis* (AF169304), *Trichopezizella nidulus* (U57813), *Hymenoscyphus virguitorum* (Z81434), *H. monotropae* (AF169309), *Lachnellula calyciformis* (U59145), *Phacidium infestans* (U92305), *Zalerion varium* (AF169303), *Pezicula carpinea* (AF169306); and *Biscogniauxia atopunctata* (AJ390411) was selected as the outgroup, to root the tree

Colonies had a regular border, mostly flat but folded in some cases; and the mycelium around the center was mostly aerial, floccose, its color varying from white to pale yellow (4A3), orange white (5A2) or brown (6E5), becoming immersed and hyaline towards the colony margin. Odorless; and exudates were absent. The reverse varied from brown (6E5) or light brown (6D4) over the center to white or orange white (5A2) towards the edge.

In order to investigate their phylogenetic affinities, all the sterile isolates producing arundifungin and the *Selenophoma*-like coelomycete were subjected to sequencing of the ITS1 region [13]. The five isolates recovered from the Macquarie Island had identical sequences in the ITS1 region, suggesting that they are conspecific. All the sequences obtained were compared with the GenBank database. The closest sequences found for the sterile isolates from the Macquarie Island were within the Leotiales. The ITS1 sequence showed a high similarity with respect to that of *Hymenoscyphus monotropae* (93.5%). The sequences from these species and from other related fungi were retrieved and used to build the dendrogram shown in Fig. 4. The sequence of one representative of these sterile isolates (F-046760) appears in a branch together with *H. monotropae*, strongly suggesting that this fungus belongs to the Leotiales; and it could probably represent a *Hymenoscyphus* species close to *H. monotropae*. The sequence of the sterile endophyte from *Q. ilex* (F-054289) showed also the best matches with species within the Leotiales, although in this case with species of the Sclerotiniaceae. Thus, it showed 83% similarity with *Stromatinia rapulum* and about 80% with *Scleromitrula spiraeicola* and *S. calthicola* and, when a phylogenetic tree was built using these sequences (Fig. 4), this fungus clustered within the Sclerotiniaceae. Finally, the sequence from the coelomycete F-042833 showed some similarity with *Phaeoacremonium chlamydosporium* and some *Exophiala* strains. However, the matches were weak (lower than 75% similarity) and no attempt at phylogenetic reconstruction has been made.

## Discussion

Cell walls are essential elements to maintain the shape and viability of yeasts and filamentous fungi. The fungal cell wall is an attractive target for drug therapy, since the synthesis of glucan and chitin are processes absent from mammals [16, 39]. Indirect evidences presented in this paper suggest that the novel molecule arundifungin is a new type of glucan-synthesis inhibitor. It has been reported that the specific activity of (1,3)-$\beta$-D-glucan synthase in *Saccharomyces cerevisiae fks1* mutants is 5- to 8-fold lower than that seen in the wild-type strain and that the residual activity of the mutants was 3-fold more sensitive to echinocandins [9]. Accordingly, the fact that the *fks1*::HIS strain was more sensitive than the wild-type strain to the growth inhibition caused by arundifungin provided the first line of evidence suggesting that arundifungin could be an inhibitor of glucan synthesis. Cell wall synthesis inhibitors are known to produce hallmark alterations in fungal growth that can be detected microscopically, either in liquid- or in agar-based cultures on the stage of a light microscope [20, 44]. According to the correlation established between these morphological changes and the mode of action of the antifungal agents [20], our results suggest that arundifungin, like echinocandins, inhibits the synthesis of glucan and thus blocks the fungal cell wall formation.

The antifungal spectrum of arundifungin is comparable with those of the known glucan synthesis inhibitors, MK-0991 (Cancidas, caspofungin acetate), the semisynthetic echinocandin L-733560 and papulacandins, although arundifungin was less potent. Like

lipopeptides, arundifungin preferentially affected several pathogenic *Candida* and *Aspergillus* strains, while *Cryptococcus* and *Staphylococcus aureus* were not inhibited. A common feature of all glucan synthesis inhibitors discovered so far is their very poor activity against *Cryptococcus* strains. It has been postulated that *C. neoformans* may have 1,6-$\beta$-glucan or other non-1, 3-$\beta$-D-glucans in its cell wall, thus explaining its relative lack of sensitivity to the known glucan synthesis inhibitors [16, 39]. Recently, genetic and biochemical evidence has suggested that the unique FKS1 gene of this organism is essential [38]. Differences between the biochemical properties of the *Cryptococcus* enzyme and those of *C. albicans* and *Saccharomyces cerevisiae* may account for the lack of anti-*Cryptococcus* activity.

Ascosteroside [14, 21], enfumafungin [29] and ergokonin A [43], three other terpenoid compounds, were detected in our screening of natural products. Further biochemical and genetic evidence has been reported recently [27] showing that arundifungin, ascosteroside, enfumafungin and ergokonin A constitute a new class of glucan synthesis inhibitors. These terpenoids are the first non-lipopeptide or papulacandin-type glucan synthesis inhibitors described for 20 years. With the discovery of this third class of glucan synthesis inhibitors, opportunities to develop orally active agents with improved pharmacokinetic profiles and enhanced in vivo efficacy in animal models may arise. Although arundifungin lacked in vivo efficacy, ascosteroside and enfumafungin have detectable anti-*Candida* activity in animal models [14, 29]. Further studies on the oral absorption of the acidic terpenoids and trials to increase their in vivo antifungal activity will aid assessment of the therapeutic potential of these agents.

The results of the screening program suggest that arundifungin is a relatively common metabolite, produced by fungi which are taxonomically and ecologically unrelated. Thus, it has been found in several *Arthrinium* isolates, both from tropical and temperate regions. *Arthrinium* species are anamorphs of the Lasiosphaeriaceae (Sordariales) [15]. Likewise, it was found in several conspecific isolates obtained from soil and leaf litter from a cold environment (Macquarie Island), that were shown to belong to a species of the Leotiales, close to *H. monotropae* and very far apart from the Lasiosphaeriaceae. A sterile endophyte from *Q. ilex* also seems to be akin to the Leotiales, although not close to the same species but within the Sclerotiniacea. Finally, another endophytic fungus from *O. europaea*, collected in the same area as the *Quercus* endophyte, was a coelomycete whose phylogenetic affiliation was not solved by sequence analysis of the ITS region (other more conserved regions could provide a more accurate idea about that). In any case, this latter fungus was completely unrelated to the other fungi sequenced.

This widespread occurrence of arundifungin contrasts with what has been found in the related fungal metabolite, enfumafungin [27, 29]. This compound has so far been found in three isolates belonging to a *Hormonema* species, collected in the same location. *Arthrinium* species are prolific producers of biologically active secondary metabolites. For example, the papulacandins, antifungal agents with the same mode of action as arundifungin and pneumocandins but a totally different structure (glycolipids), were initially isolated from *Papularia sphaerosperma* (= *Arthrinium sphaerospermum*) [41]. The metabolite CAF-603, a carotene sesquiterpene which is a potent modulator of the Maxi-K channels and has also antifungal properties, was isolated from the same species [26]. The antifungal agent apiosporamide has been reported from *Apiospora montagnei* (teleomorph of *Arthrinium arundinis*) [1], which also produces several cyclic peptides that are nanomolar inhibitors of the 20S proteasome [17]. Other examples are terpestacin (produced by a strain of *Arthrinium* sp.), a bicyclic sesterterpene that inhibits the formation of syncitia by HIV-infected T4 cells, thus being a potential anti-HIV agent [25].

Psychrophilic and psychrotolerant fungi are typically isolated from substrates collected in alpine regions, Antarctica and other cold regions [22, 30, 31]. Although these fungi have been studied as sources of enzymes [5, 37], apparently they have not been reported to produce secondary metabolites. There are even reports showing the loss of secondary metabolite production by strains of a given species recovered from cold areas, compared to the isolates from the same species recovered from temperate or tropical environments. For instance, Möller et al. [23] found that Antarctic isolates of *Chaunopycnis alba* produced fewer metabolites than temperate or tropical strains from the same species, based on HPLC profiles and data from biological assays. Losses might be due to the adaptation to extreme conditions during evolution, which would result in a loss of ability to adapt and to produce secondary metabolites [23]. Our study provides evidence indicating that psychrophilic and psychrotolerant fungi may present and express the same secondary metabolic pathways as other phylogenetically very distant mesophilic fungi. This should encourage the exploration of these habitats as sources of potentially useful novel metabolites.

**Acknowledgement** The contribution of the CIBE technician staff who assisted in the screening process is gratefully acknowledged.

## References

1. Alfatafta AA, Gloe, JB, Scott JA, Malloch D (1994) Apiosporamide, a new antifungal agent from the coprophilous fungus *Apiospora montagnei*. J Nat Prod 57:1696–1702
2. Bartizal K, Abruzzo G, Trainer C, Krupa D, Noll-Stadt K, Schmatz D, Schwartz R, Hammond M, Balkovec J, Vanmiddlesworth F (1992) In vitro antifungal activities and in vivo efficacies of 1,3-$\beta$-D-glucan-synthesis inhibitors L-679,329, L-646,991, tetrahydroechinocandin B, and L-687,781, a papulacandin. Antimicrob Agents Chemother 36:1648–1657
3. Bills GF, Polishook JD (1994) Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. Mycologia 86:187–198

4. Bremer K (1994) Branch support and tree stability. Cladistics 10:295–304
5. Cairns AJ, Howarth CJ, Pollock CJ (1995) Characterization of acid invertase from the snow mould *Monographella nivalis*: a mesophilic enzyme from a psychrophilic fungus. New Phytol 130:391–400
6. Collado J, Platas G, Pelez F (1996) Fungal endophytes of twigs, leaves and bark of *Quercus ilex* from Central Spain. Nova Hedwigia Z Kryptogamenk 63:347–360
7. Current WL, Tang J, Boylan C, Watson P, Zechkner D, Turner W, Rodríguez M, Dixon C, Ma D, Radding JA (1995) Glucan biosynthesis as a target for antifungals: the echinocandin class of antifungal agents. In: Dixon GK, Copping LG, Hollomon DW (eds) The discovery and mode of action of antifungal drugs. BIOS, Oxford, pp 143–160
8. Dombrowski A, Jenkins R, Raghoobar S, Bills GF, Polishook JD, Pelez F, Burgess B, Zhao A, Huang L, Zhang Y, Goetz M (1999) Production of a family of kinase-inhibiting lactones from fungal fermentations. J Antibiotics 52:1077–1085
9. Douglas CM, Foor F, Marrinan JA, Morin N, Nielsen, JB, Dahl AM, Mazur P, Baginsky W, Li W, El-Sherbeini M, Clemas JA, Mandala SM, Frommer BR, Kurtz MB (1994) The *Saccharomyces cerevisiae* FKS1 (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-$\beta$-D-glucan synthase. Proc Natl Acad Sci USA 91:12907–12911
10. Dufresne C, Wilson KE, Zink DL, Smith JL, Bergstrom JD, Kurtz MB, Rew DJ, Nallin M, Jenkins RG, Bartizal KF, Trainor C, Bills GF, Meinz MS, Huang L, Onishi JC, Milligan JA, Mojena M, Peláez F (1992) The isolation and structure elucidation of zaragozic acid C, a novel potent squalene synthase inhibitor. Tetrahedron 48:10221–10226
11. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
12. Gallis HA, Drew RH, Pickard WW (1990) Amphotericin B: 30 years of clinical experience. Rev Infect Dis 12:308–329
13. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118
14. Gorman JA, Chang LP, Clark J, Gustavson DR, Lam KS, Mamber SW, Pirnik D, Ricca C, Fernandes PB, O'Sullivan J (1996) Ascosteroside, a new antifungal agent from *Ascotricha amphitricha*. I. Taxonomy, fermentation and biological activities. J Antibiot (Tokyo) 49:547–552
15. Hawksworth DL, Kirk PM, Sutton BC, Pegler D (1995) *Arthrinium*. In: Ainsworth & Bisby's Dictionary of the fungi, 8th edn. CAB International, Wallingford, p 27
16. Hector RF (1993) Compounds active against cell walls of medically important fungi. Clin Microbiol Rev 6:1–21
17. Koguchi Y, Kohno J, Nishio M, Takahashi K, Okuda T, Ohnuki T, Komatsubara S (2000) TMC-95A, B, C, and D, novel proteasome inhibitors produced by *Apiospora montagnei* Sacc. TC 1093. Taxonomy, production, isolation and biological activities. J Antibiot (Tokyo) 53:105–109
18. Kornerup A, Wanscher JH (1989) Methuen handbook of colour, 3rd edn. Methuen, London
19. Kurtz MB, Douglas C (1997) Lipopeptide inhibitors of fungal glucan synthase. J Med Vet Mycol 35:79–86
20. Kurtz MB, Heath IB, Marrinan J, Dreikorn S, Onishi J, Douglas C (1994) Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-$\beta$-D-glucan synthase. Antimicrob Agents Chemother 38:1480–1489
21. Leet JE, Huang S, Klohr SE, McBrien KD (1996) Ascosteroside, a new antifungal agent from *Ascotrica amphitricha*. II. Isolation and structure elucidation. J Antibiot (Tokyo) 49:553–559
22. Möller C, Dreyfuss MM (1996) Microfungi from Antarctic lichens, mosses and vascular plants. Mycologia 88:922–933
23. Möller C, Weber G, Dreyfuss MM (1996) Intraspecific diversity in the fungal species *Chaunopycnis alba*: implications for microbial screening programs. J Ind Microbiol 17:359–372
24. Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 29:144–167
25. Oka M, Iimura S, Tenmy, O, Sawada Y, Sugawara M, Ohkusa N, Yamamoto H, Kawano K, Hu SL, Fukagawa Y, Oki T (1993) Terpestacin, a new syncitium formation inhibitor from *Arthrinium* sp. J Antibiot (Tokyo) 46:367–373
26. Ondeyka JG, Ball RG, Garcìa ML, Dombrowski AW, Sabnis G, Kaczorowski GJ, Zink DL, Bills GF, Goetz MA (1995) A carotene sesquiterpene as a potent modulator of the Maxi-K channel from *Arthrinium phaeospermum*. Bioorg Med Chem Lett 5:733–734
27. Onishi J, Meinz M, Thompson J, Curotto J, Dreikorn S, Rosenbach M, Douglas C, Abruzzo G, Flattery A, Kong L, Cabello A, Vicente F, Peláez F, Dìez MT, Martìn I, Bills G, Giacobbe R, Dombrowski A, Schwartz R, Morris S, Harris G, Tsipouras A, Wilson K, Kurtz MB (2000) Discovery of novel antifungal (1,3)-$\beta$-D-glucan synthase inhibitors. Antimicrob Agents Chemother 44:368–377
28. Peláez F, Collado J, Arenal F, Basilio A, Cabello MA, Díez MT, García JB, Gonzalez del Val A, Gonzalez V, Gorrochategui J, Hernández P, Martín I, Platas G, Vicente, F (1998) Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycol Res 102:755–761
29. Peláez F, Cabello A, Platas G, Díez MT, Gonzalez del Val A, Basilio A, Martín I, Vicente F, Bills GF, Giacobbe RA, Schwartz RE, Onishi JC, Meinz MS, Abruzzo GK, Flattery AM, Kong L, Kurtz MB (2000) The discovery of enfumafungin, a novel antifungal compound produced by an endophytic *Hormonema* species. Biological activity and taxonomy of the producing organisms. Syst Appl Microbiol 23:333–343
30. Petrini O, Petrini LE, Dreyfuss MM (1992) Psychrophilic deuteromycetes from alpine habitats. Mycol Helv 5:9–20
31. Petrovic U, Gunde CN, Zalar P (2000) Xerotolerant mycobiota from high altitude Anapurna soil, Nepal. FEMS Microbiol Lett 182:339–342
32. Rex JH, Rinaldi MG, Pfaller MA (1995) Resistance of *Candida* species to fluconazole. Antimicrob Agents Chemother 39:1–8
33. Rodríguez LJ, Rex JH, Anaissie EJ (1996) Update on invasive candidiasis. Adv Pharmacol 137:349–400
34. Salisbury BA (1999) Strongest evidence: maximum apparent phylogenetic signal as a new cladistic optimality criterion. Cladistics 15:137–149
35. Sheehan DJ, Hitchcock CA, Sibley CM (1999) Current and emerging azole antifungal agents. Clin Microbiol Rev 12:40–79
36. Spiegel FW, Stephenson SL (2000) Protostelids of Macquarie Island. Mycologia 92:849–852
37. Swofford DL (1993) PAUP: phylogenetic analysis using parsimony, version 3.1.1. Laboratory of Molecular Systematics, Smithsonian Institution, Washington, D.C.
38. Takasawa T, Sagisaka K, Yagi K, Uchiyama K, Aoki A, Takaoka K, Yamamoto K (1997) Polygalacturonase isolated from the culture of the psychrophilic fungus *Sclerotinia borealis*. Can J Microbiol 43:417–424
39. Thompson JR, Douglas CM, Li W, Jue CK, Pramanik B, Yuan X, Rude TH, Toffaletti DL, Perfect JR, Kurtz M (1999) A glucan synthase FKS1 homolog in *Cryptococcus neoformans* is single copy and encodes an essential function. J Bacteriol 181:444–453
40. Tkacz JS (1992) Glucan biosynthesis in fungi and its inhibition. In: Sutcliffe JA, Georgopapdakou NH (eds) Emerging targets in antibacterial and antifungal chemotherapy. Chapman & Hall, New York, pp 495–523
41. Turner WW, Current W (1997) Echinocandin antifungal agents. In: Strohl WR (ed) Biotechnology of antibiotics. Marcel-Dekker, New York, pp 315–334
42. Traxler P, Gruner J, Auden JAL (1977) Papulacandins, a new family of antibiotics with antifungal activity. I. Fermentation, isolation, chemical and biological characterization of papulacandins A, B, C, D and E. J Antibiot (Tokyo) 30:289–296
43. Vicente MF, Cabello A, Platas G, Basilio A, Díez MT, Bills GF, Dreikorn S, Giacobbe RA, Onishi JC, Meinz M, Kurtz MB, Rosenbach M, Thompson J, Abruzzo G, Flattery A, Kong L, Tsipouras A, Wilson KE, Peláez F. (2001) Antimi-

crobial activity of ergokonin A from *Trichoderma longibrachiatum*. J Appl Microbiol 91:1–9
44. Yeung CM, Klein LL, Lartey PA (1996) Preparation and antifungal activity of fusacandin analogs: C-6′ sidechain esters. Bioorg Med Chem Lett 6:819–822
45. Zhu WY, Gooday GW (1992) Effects of nikkomycin and echinocandin on differentiated and undifferentiated mycelia of *Botrytis cinerea* and *Mucor rouxii*. Mycol Res 96:371–377