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SYSTEMATIC AND APPLIED MICROBIOLOGY

The Discovery of Enfumafungin, a Novel Antifungal Compound Produced by an Endophytic *Hormonema* Species Biological Activity and Taxonomy of the Producing Organisms

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Summary

In a screening of natural products with antifungal activity derived from endophytic fungi, we detected a potent activity in a culture belonging to the form-genus *Hormonema*, isolated from leaves of *Juniperus communis*. The compound is a new triterpene glycoside, showing an antifungal activity highly potent *in vitro* against *Candida* and *Aspergillus* and with moderate efficacy in an *in vivo* mouse model of disseminated candidiasis. The agent is especially interesting since its antifungal spectrum and its effect on morphology of *Aspergillus fumigatus* is comparable to that of the glucan synthase inhibitor pneumocandin B_0, the natural precursor of the clinical candidate MK-0991 (caspofungin acetate). An additional search for other *Hormonema* isolates producing improved titers or derivatives resulted in the isolation of two more strains recovered from the same plant host showing identical activity. The producing isolates were compared with other non-producing *Hormonema* strains by DNA fingerprinting and sequencing of the rDNA internal transcribed spacers. Comparison of rDNA sequences with other fungal species suggests that the producing fungus could be an undetermined *Kabatina* species. *Kabatina* is a coelomycetous genus whose members are known to produce *Hormonema*-like states in culture.

Key words: antifungal compound – triterpene glycoside – enfumafungin – *Hormonema* – *Kabatina*.

Introduction

The increasing incidence of life-threatening fungal infections has prompted the search for new fungicidal drugs that can overcome the emerging resistance to fluconazole and the toxicity of amphotericin B, the two existing antifungal compounds now in the clinics. Our natural products screening program has focused on fermentation products from a wide ecologic and phylogenetic array of filamentous fungi, as sources of antifungal agents with therapeutic potential.

One of the most easily accessible fungal groups is that of the endophytic fungi, which inhabit internal tissues of living plants. The high number of species of endophytes considered to exist in nature makes this group an interesting target for these types of programs. The potential of endophytic fungi for the discovery of bioactive natural products has been repeatedly suggested in the literature (DREYFUSS and CHAPELA, 1994; PELÁEZ et al., 1998). In our laboratories we have discovered a considerable number of compounds from endophytic fungi showing a broad array of biological activities, from tremorgenic toxins such as paspalistrems (BILLS et al., 1992) to inhibitors of angiotensin II binding (STREVENS-MILES et al., 1996), inhibitors of caspase-1 (SALVATORE et al., 1994), inhibitors of squalene synthetase (BILLS et al., 1994) or inhibitors of ras farnesyl-protein transferase, such as the chaetomellic acids (LINGHAM et al., 1993) or the oreganic acid (SILVERMAN et al., 1997).

With respect to antifungal compounds, pramanicin has been reported to be produced by an endophytic

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Stagonospora sp. from grass (SCHWARTZ et al., 1994; ONISHI et al., unpublished results), and khafrefungin was discovered from a sterile endophyte of *Tetragastris paramensis* (BILLS et al., 1996b; MANDALA et al., 1997). Moreover, endophytic *Pezicula* and *Cryptosporiopsis* species are known to produce pneumocandin A_0 (NOBLE et al., 1991). This is a molecule similar to pneumocandin B_0, the natural precursor of the clinical candidate MK-0991, which is produced by *Glarea lozoyensis*, a fungus isolated from water (BILLS et al., 1999).

In this work, we present the discovery of a new antifungal compound, enfumafungin, isolated from extracts derived from an endophytic species of *Hormonema*. The antifungal activity *in vitro* and *in vivo* is discussed, and the producing organism is described and compared with other related fungal species by means of molecular biology techniques. The details on the purification and structural elucidation of the compound, as well as on its mode of action, have been published elsewhere (SCHWARTZ et al., 2000; ONISHI et al., 2000).

Materials and Methods

Fungal isolation

Fungal endophytes were isolated from plant materials following the method of surface sterilization by serial washing in ethanol/chlorox/ethanol described by COLLADO et al. (1996). Cultures were maintained as potato dextrose agar slants until used for fermentation for the primary screening. Strains with further interest were preserved for subsequent studies as agar plugs in 10% glycerol at –80 °C. The original culture producing enfumafungin was deposited in the American Type Culture Collection with the accession number ATCC 74360. For cultural descriptions, the strain was grown on plates of YM agar Difco), at 25 °C, with 12 hr photoperiod.

Fermentation and extraction

For the screening process, seed flasks were prepared from fresh slants as described (PELÁEZ et al., 1998). Two-ml portions of the resulting cultures were used to inoculate a solid rice-based medium in 250 ml Erlenmeyer flasks. The production medium contained (per flask) brown rice 10 g and 20 ml of a solution of yeast extract 1 g/l, sodium tartrate 0.5 g/l and KH_2PO_4 0.5 g/l. Production flasks were incubated under static conditions at 25 °C and 50% relative humidity for 28 days. Methyl-ethyl-ketone (MEK) extracts were prepared by adding 50 ml of MEK (Merck) to the flasks, disrupting the mycelium with a spoon and shaking for 1 hr. Aliquots of the organic phase (0.8 ml) were taken, dried out completely in a Savant Speed-Vac and the solid residue reconstituted in 0.5 ml of dimethylsulphoxide (DMSO).

For isolation of enfumafungin, vegetative growth of the culture ATCC 74360 was prepared by inoculating 2 ml of frozen mycelia into a 250 ml Erlenmeyer flask containing 54 ml of seed medium, prepared according to BILLS et al. (1992). Seed flasks were incubated for 3 days on a rotary shaker at 220 rpm, with a 5-cm throw, at 25 °C and 85% relative humidity in a room with constant fluorescent light. Two-ml portions of the resulting cultures were then used to inoculate the same solid rice-based medium described above, in 250 ml Erlenmeyer flasks. Production flasks were incubated under static conditions at 25 °C and 50% relative humidity for up to 21 days. At harvest, enfumafungin was extracted from the mycelial growth with 50 ml per flask of 2-propanol (isopropanol) and shaken for 1 hour. After extraction samples were entrifuged for 20 minutes at 3000 rpm. The supernatant was filtered through a 0.45 µm nylon membrane syringe filter and assayed for enfumafungin via HPLC assay.

Screening of antimicrobial activities

The screening of *in vitro* antifungal activities was performed by agar-based diffusion assays, using a panel of four yeasts (*Candida albicans* MY1055, *Candida tropicalis* MY1012, *Cryptococcus neoformans* MY2062 and *Saccharomyces cerevisiae* W303) and one filamentous fungus (*Aspergillus fumigatus* MF5668). The specificity of the antifungal activity was tested using *Bacillus subtilis* MB964 as control. All these strains were from the Merck Culture Collection. Most of the microorganisms selected as target for this screening are important human clinical pathogens. *Candida albicans, C. tropicalis, Cryptococcus neoformans* and *Aspergillus fumigatus* are major causes of systematic fungal infections, particularly in immunocompromised patients, including those with acquired immunodeficiency syndrome (POLAK and HARTMAN, 1991; VARTIVARIAN et al., 1993). *Saccharomyces cerevisiae* and *B. subtilis* were used as common laboratory control species.

The inoculum for the yeast strains was obtained by seeding each culture from a cryovial, thawed in a culture flask and incubated overnight in medium containing 6.75 g/l yeast nitrogen base and 1 g/l dextrose (YNB-D) at 28 °C. The assay plates were prepared by inoculating the same cooled media YNB-D plus agar (15 g/l) with 3.3% of the inoculum adjusted to an O.D. range of 0.22–0.35 at 660 nm. The *A. fumigatus* stock conidial suspension was adjusted by quantitative colony counts at 3.5×10^9 c.f.u./ml. The conidial suspension was diluted into Yeast Nitrogen Base broth (YNB, 6.75 g/l yeast nitrogen base) to 65% transmittance at 660 nm; 10 ml of this inoculum broth was then added to 1 liter of YNB-D. The *B. subtilis* assay plates were prepared by adding 500 µl of the spore suspension (Difco) directly to 1 liter of the cooled agar medium containing 23 g/l nutrient agar and 2 g/l yeast extract. In all cases, 100 ml aliquots of the seeded agar media were poured into Nunc square plates (24 × 24 cm). MEK extracts resuspended in DMSO as described above (25 µl) were applied onto the surface of the assay plates seeded with the target microorganisms, which were incubated overnight at 28 °C. Inhibition zones around the application points were measured after 24 hours. Amphotericin B was used as internal controls of the plates.

Broth microdilution assay for minimum inhibitory concentrations (MIC) determination

MICs were determined against a panel of yeasts and filamentous fungi using a broth microdilution assay following the National Committee for Clinical Laboratory Standards (NCCLS) protocols MT27A and M38T respectively in Roswell Park Memorial Institute (RPMI) medium, modified as described by ONISHI et al. (2000).

Determination of in vivo efficacy

For the evaluation of the *in vivo* activity of the compound, a murine model of disseminated candidiasis with enhanced susceptibility to *Candida albicans* but increased sensitivity for discriminating antifungal efficacy was used (TOKA, for target organ kidney assay). Basically, immunosuppressed mice were challenged intravenously with *C. albicans* and treated with titrated dilutions of the compound administered intraperitoneally twice daily, for two days. Five mice per group were used. After 7 days the mice were sacrificed and their kidneys removed, homogenized and serial dilutions were plated. Yeast colonies were enumerated for determination of c.f.u. (colony forming units) per gram of kidneys. The details of the method have been fully described by BARITZAL et al. (1992).

HPLC assay for detection of enfumafungin

HPLC analyses of fermentation samples containing enfumafungin was performed on a system consisting of a Beckman multisolvent delivery system interfaced with a Wisp autosampler. Samples for analyses were loaded onto a Inertsil C8 column (4.6 mm × 25 cm) via the autosampler. The column temperature was maintained at 80 °C with a column heater. The flow rate was 1.0 ml per min. The isocratic solvent system was HPLC grade acetonitrile/HPLC grade water [1:1] with 0.1% TFA, and the UV of the effluent was monitored at 205 nm. Under these conditions, the retention time of enfumafungin was 9.3 min.

DNA fingerprinting

Fungal DNA was extracted as described (BILLS et al., 1999). Arbitrarily-primed PCR analyses (AP-PCR) was performed following the method described by PELÁEZ et al. (1996), using primer ITS3 (WHITE et al., 1990) for PCR amplification under low stringency conditions. Microsatellite-primed PCR was performed according to the method described by LONGATO and BONFANTE (1997), using primers $(GTG)_5$ and $(GACA)_5$. tDNA-PCR was performed following the same conditions described for the microsatellite-primed PCR, using primer T3A (WELSH and MCCLELLAND, 1991). All the primers were from Pharmacia. The amplification products were separated by electrophoresis in pre-cast gels of 12.5% polyacrylamide (GeneGel Excel 12.5/24 Kit, Pharmacia) run on a GenePhor Electrophoresis Unit (Pharmacia), and visualized by silver staining.

Amplified Ribosomal DNA Restriction Analyses (ARDRA)

The ribosomal DNA region containing the two internal transcribed spacers (ITSs) and the 5.8S rRNA gene was amplified using primers ITS1F and ITS4 (GARDES and BRUNS, 1993; WHITE et al., 1990) following standard procedures (40 cycles of 30 s at 93 °C, 30 s at 53 °C and 2 min at 72 °C). The amplification products were digested with 5–10 units of the restriction enzymes *Taq*I (Pharmacia), *Hha*I (Amersham) and *Tru*I (Fermentas) in separate reactions, following the conditions recommended by their manufacturer. The digested fragments were separated and visualized using the same system mentioned above for the DNA fingerprints.

rDNA sequencing

About 0.1 µg/ml of the double stranded amplification products containing the two ITSs and the 5.8S rRNA gene 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 ITS1F and ITS4 primers as templates. The separation of the reaction products by electrophoresis and the reading of the results were performed in an ABI 373 Automatic Sequencer (Perkin Elmer). The sequences obtained were aligned manually, and the phylogenetic analyses was performed by maximum parsimony, using the branch-and-bound algorithm of package PAUP 3.1.1 (SWOFFORD, 1993) and by neighbor joining analyses, using the options DNADIST and NEIGHBOR from PHYLIP 3.5c package (FELSENSTEIN, 1993), with *Jukes* and *Cantor* algorithm to estimate the distances between the sequences. The robustness of the branches in the trees were assessed by bootstrap analyses, resampling the data with 1000 bootstrap replicates (FELSENSTEIN, 1985).

Results and Discussion

Biological activity of enfumafungin

In our screening of natural products from endophytic fungi, we detected an antifungal activity from an extract derived from a fungus isolated from living leaves of *Juniperus communis* collected in Navalquejigo (Madrid, Spain). The activity detected was specific for yeasts and fungi (excluding *Cryptococcus*) and did not inhibit the growth of *Bacillus subtilis* (Table 1). The compound responsible for the antifungal activity was purified and identified as a new triterpene glycoside (Fig. 1), and named enfumafungin. Details on the isolation and struc-

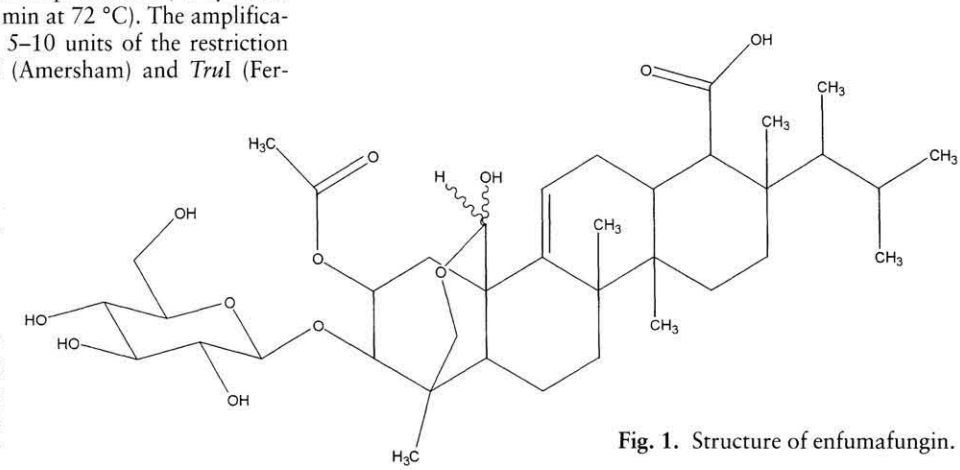


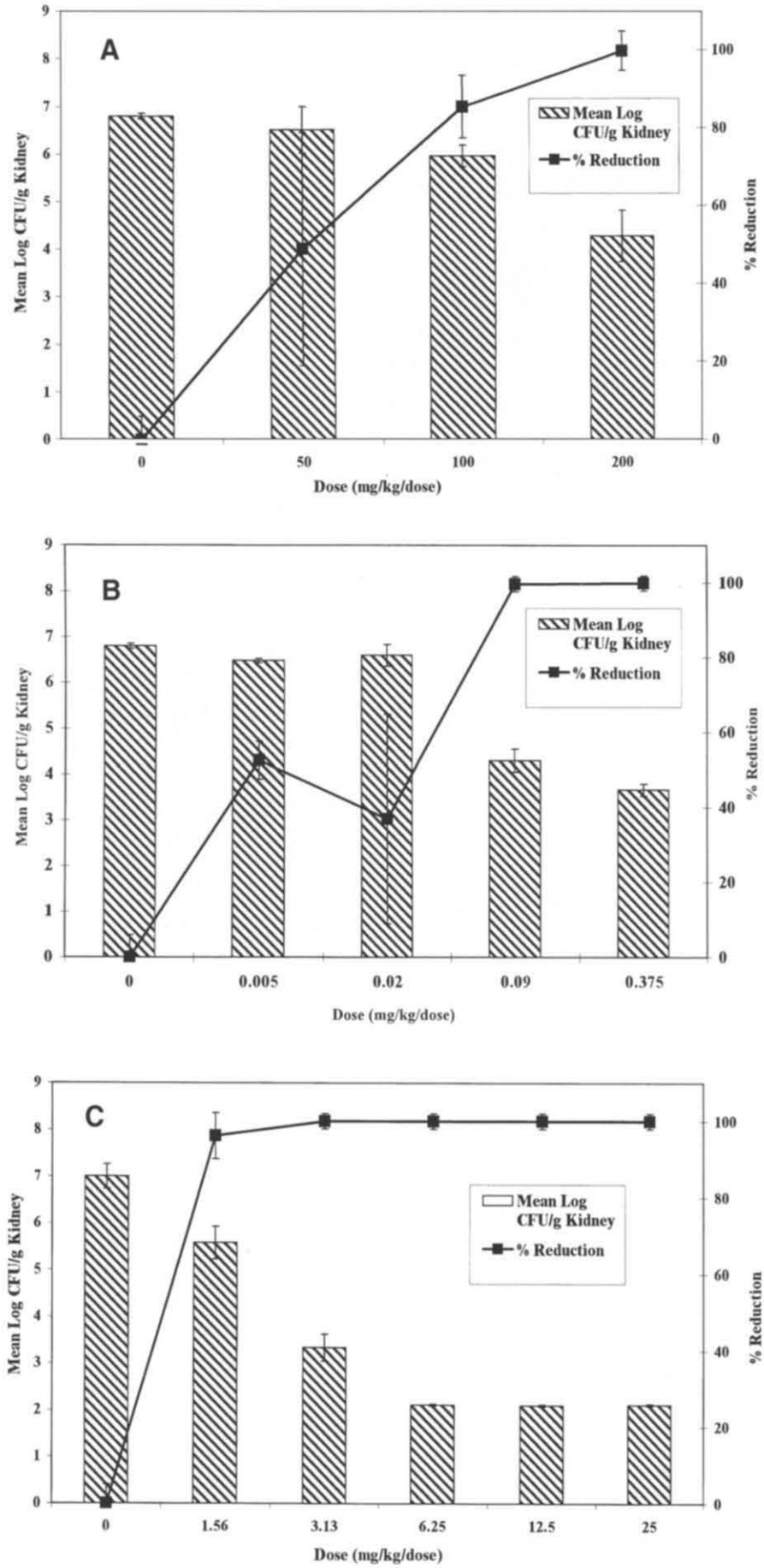
Fig. 1. Structure of enfumafungin.

Table 1. Antifungal activity of the MEK extract from isolate ATCC 74360, expressed as the diameter of the inhibition zones (in mm) in an agar diffusion assay (see Materials and Methods). The codes under each target organism are from the Merck Culture Collection.

	Bacillus subtilis MB964	*Candida albicans* MY1055	*Candida tropicalis* MY1012	*Saccharomyces cerevisiae* W303	*Aspergillus fungigatus* MF5668	*Cryptococcus neoformans* MY2062
ATCC 74360	0	19	18	26	30 h*	0
Amphotericin B†	0	21.1 ± 0.6	0	22.0 ± 1.2	23.7 ± 1.1	24.3 ± 2.3

* hazy inhibition zone

† 5 µg for all the strains except for W303 (2 µg). Data are means and standard deviations (n = 30).



ture elucidation of enfumafungin are described elsewhere (SCHWARTZ et al., 2000).

This compound showed antifungal activity against a panel of human pathogenic yeasts and filamentous fungi, as indicated in Table 2. Enfumafungin had MICs of less than 0.5 µg/ml against the *Candida* and *Aspergillus* species tested and it was inactive against *Cryptococcus*, including the decapsulated form (MY2062). It was also able to inhibit the growth of other fungal species such as *Aspergillus flavus, Fusarium oxysporum* and *Ustilago zeae* in agar diffusion assays, although MICs against these were not determined. Enfumafungin lacked antibacterial activity when tested up to 64 µg/ml. The activity of enfumafungin is comparable to that of amphotericin B for *Candida* and *Aspergillus* species. Compared with other antifungal compounds produced by fungi, tested against the same target strains, enfumafungin was a more potent fungal inhibitor than viridiofungins (ONISHI et al., 1997), sonomolides (BILLS et al., 1996a), pramanicin (SCHWARTZ et al., 1994), khafrefungin (BILLS et al., 1996b) and zaragozic acid A (BERGSTROM et al., 1991) for *Candida* spp. but not for *Cryptococcus*. On the other hand, it was slightly less potent than australifungin for *Candida* spp. and much less potent against *Cryptococcus* (MANDALA et al., 1995).

To study the *in vivo* efficacy of enfumafungin, a mouse model of disseminated candidiasis (TOKA) was used (BARTIZAL et al., 1992). Fig. 2 shows the results from the TOKA, compared with the data obtained with the antifungal compounds pneumocandin A_0 and am-

Fig. 2. *In vivo* antifungal activity (TOKA) of enfumafungin (A), amphotericin B (B) and pneumocandin A_0 (C). Results are expressed as the logarithm of the mean of yeast c.f.u. per gram of kidney and as % reduction with respect to the controls. Bars showing standard errors are provided.

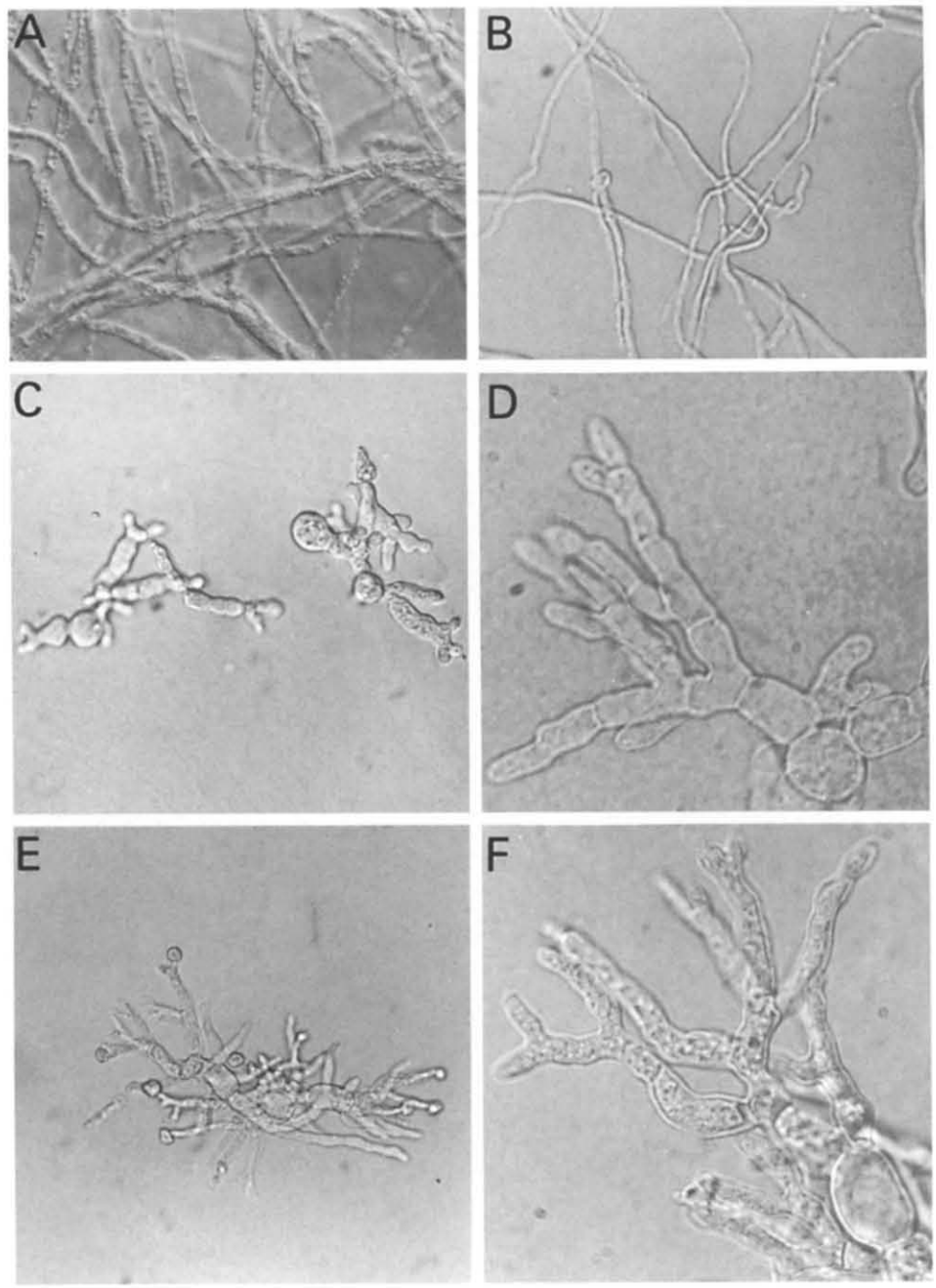


Fig. 3. Effect of enfumafungin on the growth of *Aspergillus fumigatus*. A. DMSO solvent control. B. Nystatin (25 µg). C–D. Enfumafungin (2 µg). E–F. Pneumocandin B_0 (2 µg). Microphotographs were taken at 500× (panels A–C and E) or 1,250× (D and F) with a Leitz Diaplan microscope equipped with differential interference contrast optics.

photericin B. As shown, the three compounds produced a significant decrease in the number of c.f.u. in kidneys of mice challenged with *C. albicans*, although at very different doses. Enfumafungin yielded an ED_{90} of 90 mg/Kg (ED_{99} = 162 mg/Kg). This is a modest efficacy compared to the control compounds pneumocandin A_0, which gave 0.51 mg/Kg and 1.2 mg/Kg (ED_{90} and ED_{99}, respectively) or amphotericin B, with 0.02 mg/Kg and 0.08 mg/Kg (ED_{90} and ED_{99}) in the same conditions.

Fig. 3 shows the effect of enfumafungin and other antifungal agents on the growth of *Aspergillus fumigatus*. The compound produced basically the same set of morphological alterations as pneumocandin B_0, i.e., hyphae abnormally grown, shortened, stunted and highly

Table 2. *In vitro* antifungal activity of enfumafungin, evaluated in microbroth dilution assay. All the determinations were done after 24 h incubations except for *Cryptococcus neoformans*, that was incubated for 48 h.

Strain	MIC (µg/ml)	
	Enfumafungin	Amphotericin B
Candida albicans (MY1055)	0.25	0.125
Candida tropicalis (MY1012)	0.5	2.0
Cryptococcus neoformans (MY2061)	>64.0	0.125
Cryptococcus neoformans (MY2062)	>64.0	0.125
Aspergillus fumigatus (MF5668)	<0.03	0.125

branched with bipolar or vesicular tips, swollen germ tubes and frequent balloon-like cells. According to the correlation that has been established between this pattern of morphological alterations and the mode of action of the antifungal agents (KURTZ et al., 1994), these results suggest that the compound is acting on the fungal cell wall, as the pneumocandins and other inhibitors of glucan synthesis. Nystatin, which has a different mode of action, does not produce these morphological changes when compared with the control. The spectrum of activity exhibited by enfumafungin, which is active against *Candida* and *Aspergillus* while inactive against *Cryptococcus*, is also similar to the spectrum shown by the lipopeptidic inhibitors of β-(1,3)-glucan synthase (BARTIZAL et al., 1992; KURTZ et al., 1994; PFALLER et al., 1998). Furthermore, the compound was inactive against a mutagenized strain of *C. albicans* (CA2) resistant to echinocandins (ANGIOLELLA et al., 1992). These results suggest that glucan synthase is the target for enfumafungin mode of action, a hypothesis that has been further confirmed by using other more direct approaches (ONISHI et al., 2000).

Description of the producing organism

The producing organism (ATCC 74360) exhibited the following morphology in culture in YM medium.

Colonies growing slowly, attaining 20–24 mm in 21 days, raised, moist, mucoid to waxy at the center, but becoming velvety towards the margin, which are submerged, even to undulating. Some radial plications or radial sectors, which may alternate between predominantly mycelium or yeast-like, were usually observed. The colonies are translucent at the margin at first, but soon become dark olivaceous gray to nearly black, Dark Olive Gray, Olivacreous Black, Blackish Green Gray (capitalized color names from RIDGWAY, 1912). Reverse similar in color. Odors or exudates absent. No growth at 37 °C.

Microscopical observation showed the absence of conidiophores. Conidiogenous cells were holoblastic, in-

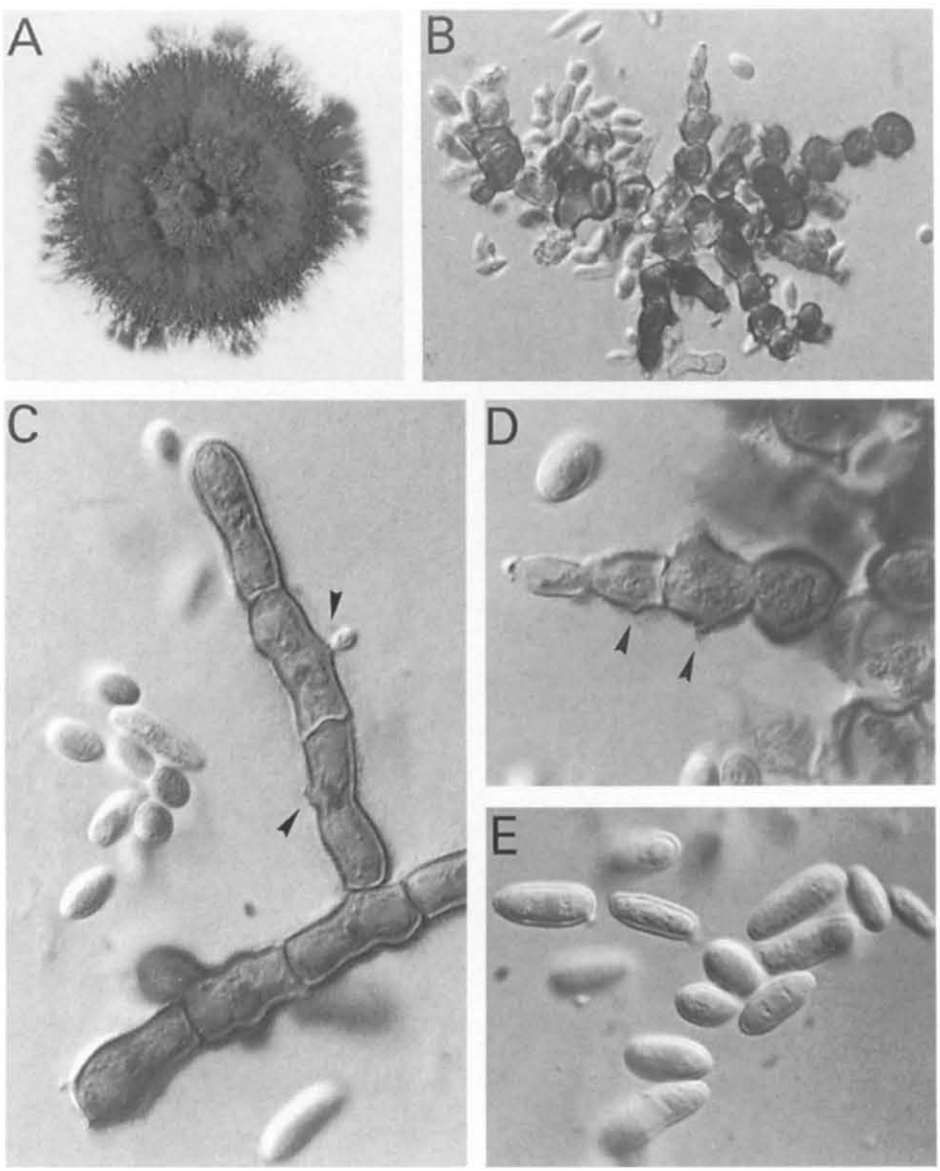


Fig. 4. *Hormonema* sp. ATCC 74360. A. Colony on potato dextrose agar, 3 week old. B. Conidia and conidiogenous cells (500×). C–D. Conidia and conidiogenous cells (1,250×). Note the collarettes, indicated by arrows. E. Conidia (1,250×). Microphotographs were taken with a Leitz Diaplan microscope equipped with differential interference contrast optics.

tegrated, intercalary, usually not differentiated from main axes of vegetative mycelium, occasionally arising from an undifferentiated lateral cell or filament, and sometimes bearing 1–2 collarettes relatively inconspicuous. Conidia were up to 14.5 µm long and up to 3.5 µm wide, aseptate, usually ellipsoidal with tapered apex and base, but quite variable, occasionally pyriform or subglobose, smooth, often with one or more budding scars, hyaline to pale olivaceous gray, often budding to produce 1 or 2 secondary conidia, accumulating in yeast-like masses along radial axes of vegetative hyphae. Mycelium consisted of contored, wide, often thick-walled, hyaline to dematiaceous, short-cylindrical to subglobose cells, often with hyphal cells longitudinally septate, with individual cells up to 18 µm in diameter.

The strain ATCC 74360 was assigned to the anamorph genus *Hormonema* based on a combination of morphological characteristics that include filamentous mycelium composed of wide, dematiaceous hyphae that do not separate into arthrospores, absence of differentiated conidiophores, conidiogenous cells usually intercalary, bearing 1–2 collarettes scarcely prominent, basipetal conidiogenesis and conidia producing abundant secondary budding (DE HOOG and HERMANIDES-NIJHOF, 1977). This isolate (Fig. 4), with its slow radial growth and large conidia, is most similar to a group of unnamed *Hormonema* species which are anamorphs of plant-inhabiting loculoascomycetes from the genera *Dothiora* and *Pringsheimia* (HERMANIDES-NIJHOF, 1977).

Search of other enfumafungin producers

The strain ATCC 74360 produced low titers of enfumafungin in the medium (30 µg/ml). Looking for wild isolates that could present a higher production, as well as natural derivatives of the compound with improved characteristics, we performed a screening of antifungal activities from other *Hormonema* strains. A total of 53 isolates were isolated from different substrates and geographical locations, covering not only endophytes from different plant species (including other *Juniperus* species) but also leaf litter and lichens (Table 3). Antifungal activ-

Table 3. *Hormonema* isolates tested for antifungal activity. Sources are sorted by types of substrate and countries, and then by number of isolates tested.

Type of substrate	Geographical location	Plant species	No. of isolates tested	No. of active isolates
Living plants	Spain	*Pinus* spp.	9	–
		Quercus ilex	4*	–
		Juniperus communis	2*	2
		Juniperus thurifera	3	–
		Alnus glutinosa	2	–
		Cystus sp.	1	–
		Erica sp.	1	–
		Eucalyptus camaldulensis	1	–
		Helichrysum stoechas	1	–
		Prumus spinosa	1	–
		Urtica dioica	1	–
	Chile	*Nothofagus pumilio*	3	–
	Taiwan	Undetermined	2	–
	Alaska	*Alnus sinuata*	1	–
	Canada	*Pinus* sp.	1	1
Lichens	Mexico		3	2
	USA		2	–
	Ecuador		1	–
	Puerto Rico		1	–
	Spain		1	–
	Sri Lanka		1	–
Leaf litter	USA		4	–
	Spain		2	–
	Chile		1	–
	India		1	–
	Namibia		1	–
Moss	Sri Lanka		1	–
Fungal fruitbody	Spain		1	–

* Samples collected in the same area as the original producer of enfumafungin.

ity was detected in five strains, but only two of them were shown to produce enfumafungin by HPLC analyses, in similar amounts to ATCC 74360 (data not shown). These two isolates were recovered from the same plant species and in the same area as the original producer. They also showed identical morphology macro- and microscopically, suggesting that they could represent the same species. However, the fact that morphological criteria for this type of fungi in culture are not unequivoval, made it desirable to use other tools to compare the three isolates.

The three producing strains were further compared by means of restriction analyses of the ITS1-5.8S-ITS2 region, a technique frequently used to assess phylogenetic relationships in fungi. As shown in Fig. 5A, the three isolates showed identical restriction profiles with all of the enzymes tested, and were very different to other non-producing *Hormonema* isolates. This indicated that the three producing strains shared a very similar DNA sequence in that region, suggesting that they were phylogenetically extremely close and that they could be conspecific. Other DNA fingerprinting techniques, which are able to discriminate at lower taxonomic levels, were used to compare these strains. The amplification patterns obtained by AP-PCR, microsatellite-primed PCR and tDNA-PCR were almost identical for the three strains (Fig. 5B), supporting that all of them would belong to the same biological species.

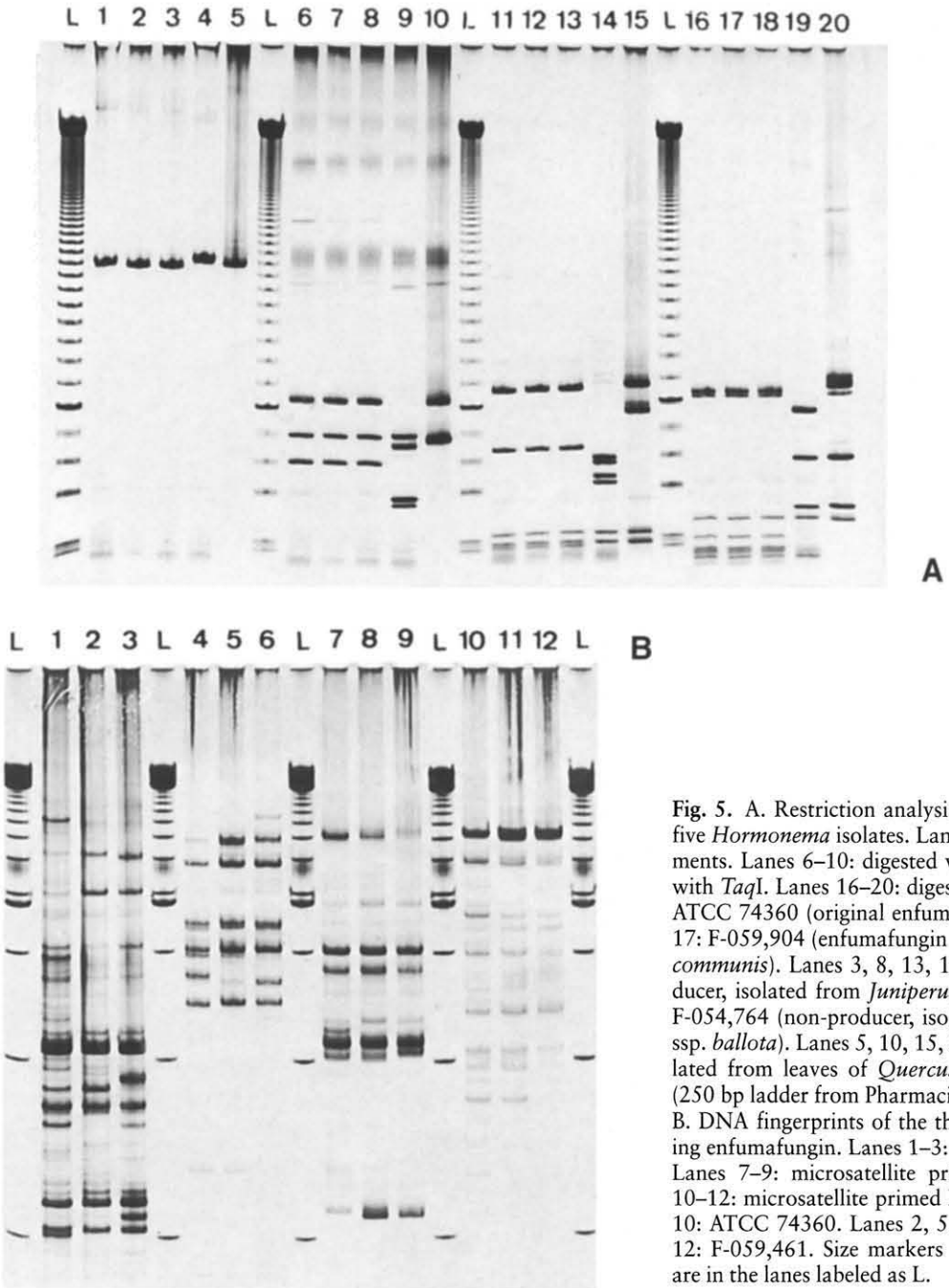


Fig. 5. A. Restriction analysis of the ITS1-5.8S-ITS2 region of five *Hormonema* isolates. Lanes 1–5: undigested amplified fragments. Lanes 6–10: digested with *Hha*I. Lanes 11–15: digested with *Taq*I. Lanes 16–20: digested with *Tru*I. Lanes 1, 6, 11, 16: ATCC 74360 (original enfumafungin producer. Lanes 2, 7, 12, 17: F-059,904 (enfumafungin producer, isolated from *Juniperus communis*). Lanes 3, 8, 13, 18: F-059,461 (enfumafungin producer, isolated from *Juniperus communis*). Lanes 4, 9, 14, 19: F-054,764 (non-producer, isolated from leaves of *Quercus ilex* ssp. *ballota*). Lanes 5, 10, 15, 20: F-054,258 (non-producer, isolated from leaves of *Quercus ilex* ssp. *ballota*). Size markers (250 bp ladder from Pharmacia) are in the lanes labeled as L.
B. DNA fingerprints of the three *Hormonema* isolates producing enfumafungin. Lanes 1–3: tDNA-PCR. Lanes 4–6: AP-PCR. Lanes 7–9: microsatellite primed PCR using $(GAC)_5$. Lanes 10–12: microsatellite primed PCR using $(GTG)_5$. Lanes 1, 4, 7, 10: ATCC 74360. Lanes 2, 5, 8, 11: F-059,904. Lanes 3, 6, 9, 12: F-059,461. Size markers (250 bp ladder from Pharmacia) are in the lanes labeled as L.

Molecular phylogenetic analysis of the producing fungus

The phylogenetic affinities of the original producing organism were assessed in more detail through complete sequencing of the ITS1-5.8S-ITS2 region. Other *Hormonema* strains not producing the compound of interest, but isolated from the same area, were also sequenced for comparison. The sequences obtained were contrasted with GenBank database using FastA application of GCG Sequence Analyses Software Package, and the most related sequences were retrieved and used for building the phylogram shown in Fig. 6. All the fungi included in the analyses are morphologically related. *Kabatina* and *Rhizosphaera* species are coelomycetes, but they produce *Hormonema*-like states in culture (HERMANIDES-NIJHOF, 1977; MARTINEZ and RAMIREZ, 1983; FUNK, 1985). *Aureobasidium pullulans* is a hyphomycete morphologically closely related with *Hormonema* (HERMANIDES-NIJHOF, 1977), and *Dothidea* species are ascomycetes which produce *Aureobasidium*-like states in culture (BARR, 1972).

It can be seen that the *Hormonema* species producing enfumafungin appears in a branch supported by a high bootstrap index, containing the sequences from *Hormonema dematioides, Dothidea insculpta* and two species of *Kabatina (K. thujae* and *K. juniperi)*. The topology of the tree was virtually identical when the sequences were compared by neighbor joining analyses (data not shown).

Although the phylogenetic analyses was not able to provide further resolution within this branch, our fungus seems to be especially close to the *Kabatina* species. Percentages of nucleotide divergence in the complete region were 6.64% and 7.75% with respect to *K. thujae* and *K. juniperi*, respectively, while they were above 8% when compared with *H. dematioides* and *D. insculpta*. The distance between the two *Kabatina* species was 1.92%. Thus, the possibility that the producing strain could be conspecific with any of them seems highly unlikely, as the range of infraspecific variability for these two species would be expectedly quite small (at least lower than 1.92%). However, the percentages of nucleotide divergence between the enfumafungin producing strain and the *Kabatina* species are close to or even within the range of infraspecific variation described for many fungal species (ARENAL et al., 2000), suggesting that they are close relatives. It seems reasonable that the producing fungus could be another *Kabatina* species, but confirm-

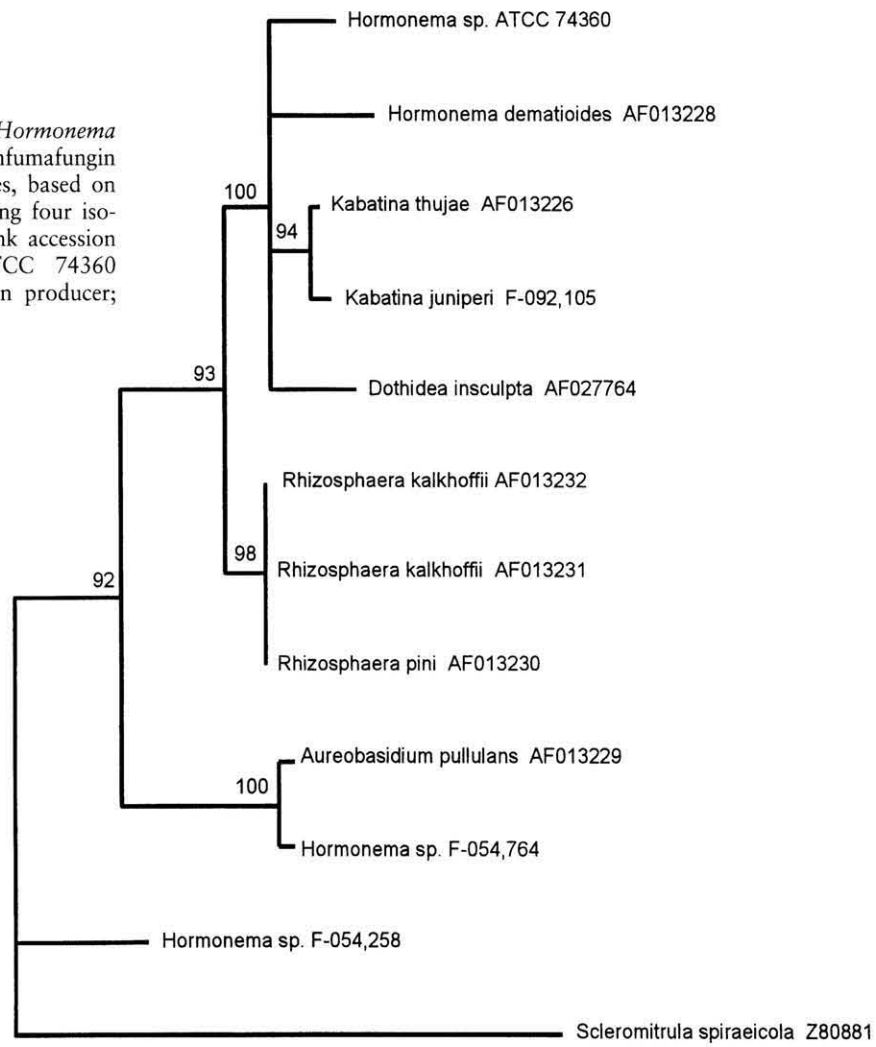


Fig. 6. Phylogenetic analysis of different *Hormonema* strains isolated in this study (including the enfumafungin producing isolate) and other related species, based on the ITS1-5.8S-ITS2 sequences. The following four isolates were sequenced in this work (GenBank accession numbers are between parenthesis): ATCC 74360 (AF182375) *Hormonema* sp. enfumafungin producer; F-054,258 (AF182378) and F-054,764 (AF182377), *Hormonema* spp. not producers (isolated from *Quercus ilex* ssp. *ballota*, Navalquejigo, Madrid, Spain); F-092,105 (AF182376), *Kabatina juniperi* (unknown substrate, Germany). The following sequences were retrieved from GenBank: AF013228, *Hormonema dematioides* (from *Pinus sylvestris*, Greenville, Michigan, USA); AF013226, *Kabatina thujae* (from *Thuja occidentalis*, Germany, CBS 238.66); AF027764, *Dothidea insculpta* (from *Clematix vitalba*, France, CBS 189.58); AF013229, *Aureobasidium pullulans* (from *Pinus sylvestris* Greenville, Michigan, USA); AF013230, *Rhizosphaera pini* (from *Abies fraseri*, Gran Rapids, Minnesota, USA); AF013231, *Rhizosphaera kalkhoffii* (from *Picea mariana*, Lake City, Michigan, USA); AF013232, *Rhizosphaera kalkhoffii* (from *Pucea pungens*, Michigan, USA). *Scleromitrula spiraeicola* (Z80881) was chosen as outgroup to root the tree. Bootstrap indexes are indicated for each branch (for values greater than 50%).

ing this hypothesis would require identifying the coelomycete on the plant, isolating the culture and establishing their co-specificity by molecular methods. The other two *Hormonema* isolates recovered in this work that do not produce enfumafungin are comparatively more distant, with about 20% nucleotide divergence in the same region with respect to the producing strain.

Occurrence of enfumafungin and other fungal natural products

In our study, enfumafungin was restricted to a single *Hormonema* species, not being produced by other closely related fungi. This is suggested not only by the results from our search for enfumafungin or related compounds in other *Hormonema* strains (Table 3), but also by the overall results of our screening program. We tested about 6,000 fungal endophytic strains from tropical and temperate regions, and only one isolate produced the compound. In addition, 9,000 fungal strains from different ecological groups (coprophilous, aquatic, leaf litter, soil and others), as well as almost 8,000 actinomycetes isolates, were tested in the same assay during the same period, and none of them was able to produce the compound.

Secondary metabolites may be produced at different taxonomic levels. For instance, the squalene synthase inhibitors known as zaragozic acids (squalestatins) are synthesized by many different species of ascomycetes and their anamorphs, phylogenetically very distant, from Dothideales to Leotiales, Diaporthales and Onygenales (BILLS et al., 1994; HOSOYA et al., 1997). This is also the case of many other compounds, some of them economically relevant, such as mevinolin, cephalosporins or cyclosporins, produced by several taxonomically unrelated species (TURNER and ALDRIDGE, 1983; DREYFUSS and CHAOELA, 1994). Other compounds are only produced by species phylogenetically closer, such as the strobilurins, isolated from species of the basidiomycetous genera *Strobilurus, Cyphellopsis* and *Mycena* (TURNER and ALDRIDGE, 1983). On the opposite extreme of this spectrum, many metabolites have been recorded only from a single species or strain (TURNER and ALDRIDGE, 1983; DREYFUSS and CHAPELA, 1994). In our own screening program, we have characterized bioactive metabolites that have appeared only once through thousands of isolates screened. A good example would be the flutimide, an inhibitor of influenza virus cap-dependent transcription (TOMASSINI et al., 1996), produced by *Delitschia confertaspora*, a new and unusual ascomycete species (PELÁEZ et al., 1994). Enfumafungin would join this group of metabolites restricted to a single species. Interestingly, *Hormonema* strains isolated from holm-oak *(Quercus ilex)*, growing in the same location, lacked antifungal activity (Table 3) and they were phylogenetically quite distant from the producing strain (Fig. 6). This suggests that the species producing the compound is a specific endophyte of *Juniperus communis*. This finding supports the interest of this fungal group for screening programs of bioactive natural products, based on the rationale that host-specific endophytes may provide additional chemical diversity to those programs.

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