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Antimicrobial activity of ergokonin A from *Trichoderma longibrachiatum*

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Aims: Natural fungal products were screened for antifungal compounds. The mode of action of one of the hits found and the taxonomy of the producing organism were analysed.

Methods and Results: An extract from a *Trichoderma* species showed a more potent activity in an agar-based assay against the null mutant *fks1*::HIS strain than against the wild-type strain, suggesting that it could contain a glucan synthesis inhibitor. The active component was identified as the known compound ergokonin A. The compound exhibited activity against *Candida* and *Aspergillus* species, but was inactive against *Cryptococcus* species. It induced alterations in the hyphal morphology of *Aspergillus fumigatus*. The identification of the producing isolate was confirmed by sequencing of the rDNA internal transcribed spacers and comparison with the sequences of other *Trichoderma* species. The analysis showed that the producing fungus had a high homology with other strains classified as *Trichoderma longibrachiatum* and its teleomorph *Hypocrea schweinitzii*.

Conclusions: The antifungal activity spectrum of ergokonin A and the morphology alterations induced on *A. fumigatus* are consistent with glucan synthesis as the target for ergokonin A. The production of ergokonin A is not uncommon, but is probably restricted to *Trichoderma* species.

Significance and Impact of the Study: The discovery that ergokonin A could be an inhibitor of glucan synthesis, having a structure very different to other inhibitors, increases the likelihood that orally active agents with this fungal-specific mode of action may be developed.

INTRODUCTION

Antifungal therapies are currently limited to a small number of compounds for the treatment of a rather diverse array of pathogenic fungi, which include *Candida albicans* and other *Candida* species, *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Histoplasma capsulatum*. The standards of therapy have limitations; toxicity is an issue with treatments based on the fungicidal polyene amphotericin B and resistance is now beginning to emerge as a problem with the safer but fungistatic azoles, fluconazole and itraconazole (Carledge *et al*. 1997). Even with the most aggressive therapy available, the rate of mortality due to aspergillosis is extremely high in some patient populations, thus highlighting the need for the development of novel compounds with divergent mechanisms of action.

The synthetically modified echinocandin class (lipopeptides) of fungal cell wall biosynthesis inhibitors has constituted a recent success (Abruzzo *et al*. 1997; Zhanel *et al*. 1997) and has shown promising activity in clinics for the treatment of life-threatening infections due to *Candida* spp.

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and *A. fumigatus* (Sable *et al*. 1997; Arathoon *et al*. 1998). As a result of the development of these agents, inhibition of fungal cell wall glucan synthesis has been validated as an effective target to treat fungal infections (Kurtz and Douglas 1997). Despite these advances, they are limited in use to parenteral administration (Kurtz and Douglas 1997; Turner and Current 1997) due to their low oral absorption. In our laboratory we have continued looking for other compounds with the same mechanisms of action (inhibitors of glucan synthesis) but with higher levels of oral absorption compared with the first generation of lipopeptides, MK-0991 and LY303366 (Abruzzo *et al*. 1997; Zornes and Stratford 1997). This search resulted in the discovery of a new family of glucan synthesis inhibitors, the acidic triterpenes (Onishi *et al*. 2000). One of these compounds is ergokonin A. This antifungal agent had been previously described by Augustiniak *et al*. (1991) and Kumeda *et al*. (1994) from *Trichoderma koningii* and *T. viride*, respectively, but only a limited characterization of the compound was provided and its mode of action was not clearly identified.

In this study, we report the production of ergokonin A from extracts derived from a different *Trichoderma* species, *T. longibrachiatum*, isolated from water, and the antifungal activity of the ergokonin A against a broad panel of microorganisms as well as some details on its mode of action. The taxonomy of the producing organism was also assessed by rDNA sequence analysis.

MATERIALS AND METHODS

Fungal isolation

The fungal strain producing ergokonin A was isolated from water collected in the Delta del Ebro (Tarragona, Spain), following a standard dilution plating method. The medium used for isolation contained (l^{-1}): malt extract, 10 g; yeast extract, 1 g; agar, 20 g; cyclosporin A, 40 mg; streptomycin, 5 mg and terramycin, 50 mg (Collado *et al*. 1996). The strain is preserved in the Merck Culture Collection (accession no. MF6423; Rahway, NJ, USA).

Fermentation and extraction

For the screening process, seed flasks were prepared from potato dextrose agar (PDA; Difco), fresh slants as described (Peláez *et al*. 1998). Portions (2 ml) of the resulting cultures were used to inoculate a solid rice-based medium in 250-ml unbaffled Erlenmeyer flasks. The solid rice-based production medium contained (per flask): brown rice, 10 g; yeast extract, 20 mg; sodium tartrate, 10 mg; KH_2PO_4, 10 mg and water, 20 ml. Production flasks were incubated under static conditions at 25°C and 50% r.h. for 28 d. Methyl-ethyl-ketone (MEK) extracts were prepared by adding 50 ml MEK (Merck, Darmstadt, Germany) to the flasks, disrupting the mycelium with a spoon and shaking for 1 h. Aliquots of the organic phase (0·8 ml) were taken, dried out completely in a Speed-Vac (Savant, Holbrook, NY, USA) and the solid residue reconstituted in 0·5 ml 25% dimethyl sulfoside (DMSO; Merck).

For the isolation of ergokonin, production cultures were prepared as described above. At harvest, the dried MEK extract of the solid fermentation of the *T. longibrachiatum* culture was dissolved with ethyl acetate. The main activity of the ethyl acetate-insoluble portion was followed through successive steps of sephadex LH20 and high performance liquid chromatography (HPLC) preparative Zorbax RX C-8 (Agilent Technologies, Palo Alto, CA, USA); 50% acetonitrile–water, 0·1% trifluoroacetic acid (TFA; Sigma). For the ethyl acetate-soluble portion, silica gel chromatography (20% methanol, 1% acetic acid and 79% ethyl acetate) was followed by the same LH20 and HPLC steps. The structure of the main active component was assigned as ergokonin A by comparison of proton and carbon nuclear magnetic resonance (NMR) and mass spectrophotometer (MS) with literature data.

Differential susceptibility test against *Saccharomyces cerevisiae* strains W303-1a and *fks1*::HIS

The search for potential inhibitors of glucan synthase was performed by means of an agar-based differential susceptibility test against a wild-type *Saccharomyces cerevisiae* strain (W303-1a) and a null mutant of the FKS1 gene encoding the vegetatively expressed large subunit of (1,3)-β-D-glucan synthase (*fks1*::HIS). The *fks1*::HIS strain was constructed at Merck Sharp and Dohme 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). The details of the method have been fully described by Cabello *et al*. (2000).

Screening of antimicrobial activities

The screening of antifungal activities was performed using a panel of three yeast species (*C. albicans* MY1055, *C. tropicalis* MY1012 and *C. neoformans* MY2062) and one filamentous fungus (*A. fumigatus* MF5668). The specificity of the antifungal activity was tested using *Bacillus subtilis* MB964 as control.

The inoculum and assay plates for *B. subtilis* and the yeast strains were prepared as described by Peláez *et al*. (1998). The *A. fumigatus* stock conidial suspension was adjusted by quantitative colony counts at $3{\cdot}5 \times 10^9$ colony-forming units

(cfu) ml^{-1}. The conidial suspension was diluted into yeast nitrogen base broth (YNB; 6·75 g l^{-1} YNB) to 65% transmittance at 660 nm; 10 ml of this inoculum broth was then added to 1 l YNB-dextrose. In all cases, 100-ml aliquots of the seeded agar media were poured into square plates (24 × 24 cm; Nunc, Roskilde, Denmark).

Methyl-ethyl-ketone extracts (25 μl), obtained as described above, were applied onto the surface of the assay plates seeded with the target micro-organisms. Inhibition zones around the application points were measured after 24 h of incubation at 28°C (fungi) or 37°C (*B. subtilis*). Amphotericin B (12·5 μg ml^{-1}, 25 μl $drop^{-1}$) was used as the internal control of the plates.

Evaluation of the antimicrobial spectrum

The antifungal spectrum was determined using a panel of 14 different species of yeast and 17 species of filamentous fungi from the Merck Culture Collection. The specificity of the antifungal activity was tested using three different bacterial strains as controls (*Streptomyces* sp., *Acholeplasma laidlawii* and *B. subtilis*).

The inoculum for the yeast strains was obtained by seeding 1·0 ml of each culture from a frozen vial, thawing this at room temperature in a culture flask and incubating overnight in the appropriate medium. Most of the yeasts were grown in Sabouraud dextrose broth, except in some specific cases, as follows: *C. glabrata* MY992 and *S. cerevisiae* MY34 (yeast extract dextrose broth), *C. albicans* MY1055 (YNB plus dextrose) and *S. cerevisiae* MY410 (potato dextrose broth). The assay plates were prepared by inoculating the same cooled media used for the inoculum plus agar (15 g l^{-1}) with the yeast cultures diluted to 40% transmittance at 660 nm (except for *S. cerevisiae* MY34, which was adjusted to 65% transmittance).

For filamentous fungi, the cultures started from lyophilized tubes which were resuspended in 1–2 ml sterile H_2O, rehydrated for about 1 h and spread (0·4 ml) onto each of several PDA plates with a sterile cotton swab. Cultures were incubated for 4 d at 28°C (37°C for *Rhizomucor miehei*) or until the desired growth characteristics were achieved. Using a cotton-tipped swab, a heavy inoculum of mycelium and spores was collected and transferred to three to four fresh PDA plates. The plates were incubated at 28–37°C for another 5 d. The fungal spore suspension was diluted to 65% transmittance at 660 nm to perform the assay plates. Most of the assay plates for filamentous fungi were prepared in PDA except for *A. fumigatus* MF5668, which used YNB plus dextrose (Difco) with agar, and *A. niger* MF442, which employed yeast extract dextrose (Difco).

For bacteria, the *B. subtilis* MB964 strain was purchased as a spore suspension from Difco. To prepare the *Bacillus* plates, 0·1 ml of the *B. subtilis* spore suspension was inoculated into 100 ml molten nutrient yeast extract agar cooled to 48°C. *Acholeplasma laidlawii* MB4558 was suspended in brain heart infusion broth supplemented with 2·5% yeast extract and 20% heat-inactivated horse serum and adjusted to a final concentration of 60% transmittance at 660 nm. Assay plates were prepared by diluting the inoculum into appropriate molten agar medium to yield a final concentration of 4%. The *Streptomyces* sp. MA4798 culture was adjusted to 65% transmittance at 660 nm. The inoculum and plates of the *Streptomyces* sp. were prepared in yeast extract dextrose.

In all cases except *B. subtilis*, 4 ml of the diluted culture were added to the appropriate flask of molten agar, the flask swirled to mix the culture thoroughly and 11-ml aliquots of the seeded agar media poured into Petri dishes.

Methyl-ethyl-ketone extract samples dissolved in 25% DMSO (20 μl) were directly applied onto the surface of the assay plates seeded with the target micro-organisms, which were incubated at the appropriate temperature (28–37°C) for 20–24 h. Amphotericin B (250 μg ml^{-1} disolved in MeOH, 20 μl $drop^{-1}$) was used as the positive control.

The inhibition zones surrounding each application point were measured and qualified as clear (no qualifying letter; abrupt, distinct edges, no visible growth within the zone), hazy (H) or very hazy (V).

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 *C. albicans* but increased sensitivity for discriminating antifungal efficacy was used (TOKA-Lite, for target organ kidney assay). Basically, immunosuppressed mice were challenged intravenously with *C. albicans* and treated with titrated dilutions of the compound administered intraperitoneally. Five mice per group were used. After 24 h the mice were sacrificed and their kidneys removed, homogenized and serial dilutions plated. Yeast colonies were enumerated for the determination of cfu per gram of kidneys. The details of the method have been fully described by Bartizal *et al.* (1992).

rDNA sequencing

About 0·1 μg ml^{-1} of the double-stranded amplification products containing the two Internal Transcribed Spacers (ITSs) and the 5·8S rRNA gene, obtained as described (Peláez *et al.* 2000), were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Boston, MA, USA), following the procedures recommended by the manufacturer. Each

strand was sequenced using primers ITS1F and ITS4 as templates. The separation of the reaction products by electrophoresis and the reading of the results was performed in an ABI 373 Automatic Sequencer (Perkin Elmer). The sequences obtained were aligned manually and the phylogenetic analysis performed by maximum parsimony analysis, using the branch-and-bound algorithm of package PAUP 3.1.1 (Swofford 1993). The robustness of the branches in the trees was assessed by bootstrap analysis (Felsenstein 1985), resampling the data with 1000 bootstrap replicates.

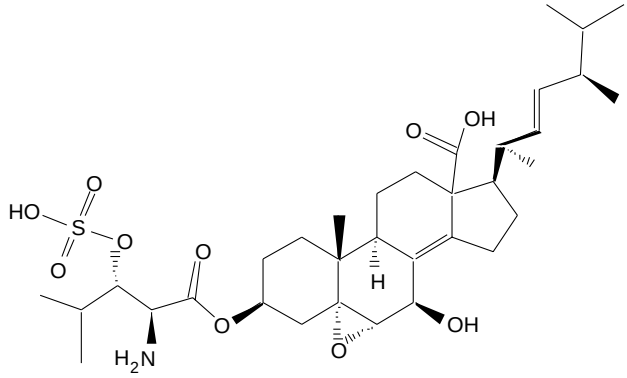

Fig. 1 Chemical structure of ergokonin A

RESULTS AND DISCUSSION

Biological activity of ergokonin A

In our search for new antifungal agents from natural fungal products we detected an antifungal activity from an extract derived from a fungus isolated from water rich in iron oxide collected in Delta del Ebro (Tarragona, Spain). The fungal activity was gathered from a screening searching for novel inhibitors of glucan synthesis and showed a more potent activity against the null mutant *fks1*::HIS strain than against the wild-type strain (Table 1). The highest sensitivity to growth inhibition shown by the mutant strain suggests that the fungal extract could contain an inhibitor of glucan synthesis. Similar behaviour was given by the known lipopeptide inhibitors of (1,3)-β-D-glucan synthase, pneumocandin Bo and aculeacin. However, antifungal agents with a different mode of action (amphotericin B and nystatin), presented practically identical activity against both *S. cerevisiae* strains (Table 1).

Table 1 Susceptibilities of the *Saccharomyces cerevisiae* strains W303-1a and *fks1*::HIS to the methyl-ethyl-ketone extract from isolate MF6423

	W303-1a (MY2141)	*fks1*::HIS (MY2265)
MF6423	16H	27H
Amphotericin B (2 μg)	22	22
Nystatin (25 μg)	32	33
Aculeacin (2 μg)	17	23
Pneumocandin Bo (2 μg)	16	24

H, Hazy inhibition zone.
Data are sizes of inhibition zones scored in the agar diffusion assay described in Materials and Methods, expressed in mm.

The active substance exhibited an antifungal activity against *Candida* and *Aspergillus* and a weak antibacterial activity against *B. subtilis*. The extract did not show activity against *C. neoformans* (Table 2). Further investigation of the active component resulted in the isolation and identification of the known antifungal agent ergokonin A (Augustiniak *et al.* 1991). As shown in Fig. 1, the structure of the ergokonin A is derived from ergosterol and characterized by an 18-carboxy group and esterified at the 3-OH group with a (2S,3S)-3-hydroxy-leucine 3-O-sulphate.

The antimicrobial spectrum of ergokonin A was evaluated by comparison with the polyene amphotericin B using a broad panel of yeasts and filamentous fungi, as indicated in Table 3. The antifungal spectrum of ergokonin A was very wide, inhibiting the growth of many *Candida* spp., *S. cerevisiae* and most of the filamentous fungi included in the panel (Table 3). The compound displayed no antifungal activity against *C. neoformans*, *Cr. albidus*, *C. guilliermondi*,

Table 2 Antifungal activity of the methyl-ethyl-ketone extract from isolate MF6423

	Bacillus subtilis MB964	*Candida albicans* MY1055	*Candida tropicalis* MY1012	*Aspergillus fumigatus* MF5668	*Cryptococcus neoformans* MY2062
MF6423	13	14	14H	16H	0
Amphotericin B*	0	21·1 ± 0·6	0	23·7 ± 1·1	24·3 ± 2·3

*Data are means and S.D.s ($n = 50$).
H, Hazy inhibition zone.
The antimicrobial activity was determined by the agar diffusion assay and is expressed as the diameter of the inhibition zones in mm (see Materials and Methods). The codes under each target organism are from the Merck Culture Collection.

Table 3 Antifungal and antibacterial activity of ergokonin A compared with that of amphotericin B

Target organism	Merck no.	Ergokonin A (1 mg ml^{-1})	Amphotericin B (250 μg ml^{-1})
Filamentous fungi			
Alternaria solani	MF3550	13	22H
Aspergillus flavus	MF383	24	16
Aspergillus fumigatus	MF4839	25	16
Aspergillus fumigatus	MF5668	26V	15
Aspergillus niger	MF442	34	22
Aspergillus niger	MF11	24	28
Botrytis allii	MF3587	15	23
Cephalosporium sp.	MF4641	0	10
Ceratocystis ulmi	MF4042	10H	21H
Cercospora beticola	MF4608	9	23
Fusarium oxysporum	MF4014	0	19
Penicillium sp.	MF5014	16	25
Penicillium sp.	MF5020	24	14
Penicillium sp.	MF5016	15	24
Phoma sp.	MF4332	8H	19
Rhizomucor miehei	MF4784	8V	13
Scopulariopsis communis	MF3769	8	10H
Trichoderma lignorum	MF3560	8	11
Trichoderma sp.	MF4064	8H	17
Ustilago maydis	MF1996	8H	14
Verticillium serrae	MF3794	8	10
Yeast			
Brettanomyces bruxellensis	MY315	17H	21
Candida glabrata	MY992	29	14
Candida albicans	MY1028	23	19
Candida albicans	MY1029	22	19
Candida albicans	MY1099	23H	19
Candida albicans	MY1055	19	16
Candida guilliermondi	MY1019	0	15
Candida pseudotropicalis	MY2099	19	15
Candida rugosa	MY1022	22V	18
Candida tropicalis	MY1012	22	0
Cryptococcus neoformans	MY2062	0	16
Cryptococcus albidus	MY1070	0	18
Cryptococcus laurentii	MY1077	8V	20
Kluyveromyces fragilis	MY1113	21	14
Saccharomyces cerevisiae	MY34	23	13
Saccharomyces cerevisiae	MY410	17	14
Torulopsis glabrata	MY1062	21	17
Torulospora hansenii	MY321	0	10H
Bacteria			
Streptomyces sp.	MA4798	10	9V
Acholeplasma laidlawii	MB4558	0	Not tested
Bacillus subtilis	MB964	12	0

H, Hazy inhibition zone; V, very hazy inhibition zone.
The antimicrobial activity was determined by the agar diffusion assay and expressed as the diameter of the inhibition zones in mm (see Materials and Methods).
The values are the average of duplicate samples.

Torulospora hansenii, *Fusarium oxysporum* and *R. miehi* and presented weak antibacterial activity against *B. subtilis* and *Streptomyces* sp. In general, the antifungal activity of ergokonin A was comparable with that of amphotericin B at the concentrations tested.

Examination of *A. fumigatus* cells after treatment with ergokonin A revealed some relevant changes in hyphal morphology, in comparison with the control without antifungal agents (Fig. 2a). Ergokonin A (Fig. 2d) produced basically the same set of morphological alterations as the semisynthetic pneumocandin L-733560 (Fig. 2c), i.e. hyphae abnormally grown, shortened, stunted and highly branched with bipolar or vesicular tips, swollen germ tubes and frequent balloon-like cells. According to the correlation that has been established between the pattern of morphological alterations and the mode of action of the antifungal agents (Kurtz *et al.* 1994), these results suggest that ergokonin A is acting on the fungal cell wall, as do the echinocandins. Nystatin, which has a different mode of action, did not produce these morphological changes (Fig. 2b) when compared with the control. The spectrum of activity shown by ergokonin A, which is active against *Candida* and *Aspergillus* but not *Cryptococcus*, is similar to that shown by the lipopeptidic inhibitors of β-(1,3)-glucan synthesis (Bartizal *et al.* 1992; Kurtz *et al.* 1994; Pfaller *et al.* 1998). These results would be consistent with glucan synthesis as the target for ergokonin A, a hypothesis that has been further confirmed by using more direct approaches (Onishi *et al.* 2000).

The *in vivo* efficacy of ergokonin A was studied in an abbreviated 24-h version of a murine model of disseminated candidiasis with enhanced susceptibility to *C. albicans* (Bartizal *et al.* 1992). Ergokonin A did not produce any reduction in the number of cfu even when high doses were used (data not shown).

Taxonomy of the producing organism

The organism producing ergokonin A was identified as *T. longibrachiatum*, based on a combination of characteristics including sparsely branched conidiophores with a few long primary branches and short secondary branches, usually unbranched, nearly cylindrical phialides which were unconstricted at the base, frequently arising singly, and smooth-walled conidia, which were ovoid to ellipsoidal (4–5 × 2·5–3·5 μm; Rifai 1969; Bissett 1984, 1991).

Sequence analysis of the ITS regions has clarified the relationships among the species of *Trichoderma* and *Hypocrea* anamorphs ascribed to section *Longibrachiatum* (Kuhls *et al.* 1997; Samuels *et al.* 1998). Thus, it seemed desirable to assess the phylogenetic affinities of the ergokonin-producing isolate by sequencing the same region. The sequence obtained was contrasted with the sequences stored

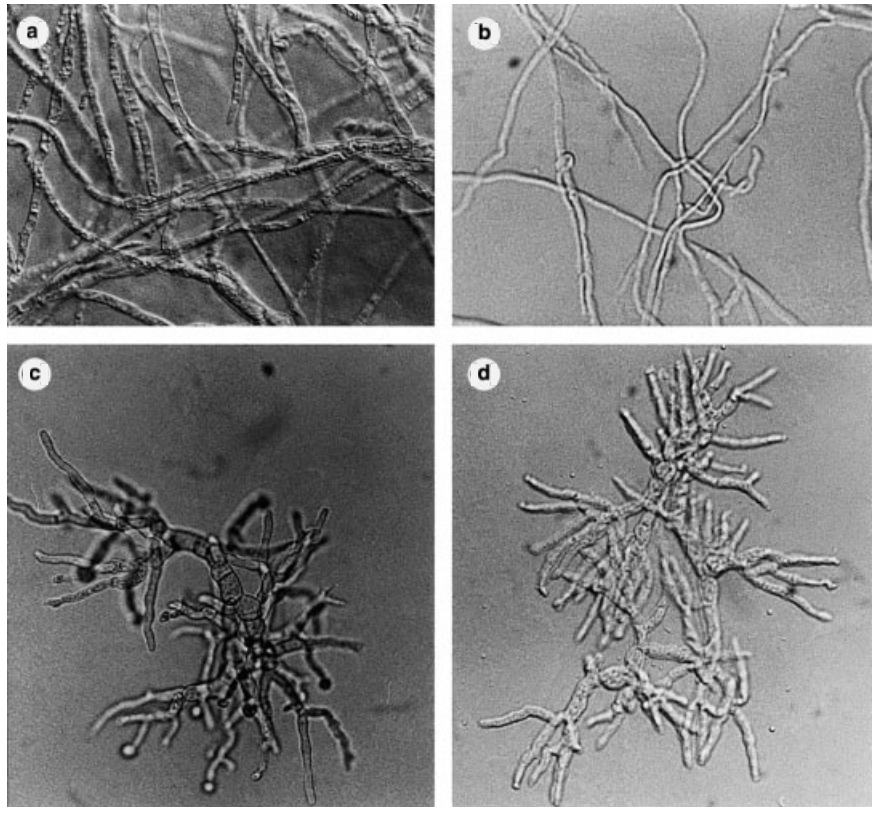

Fig. 2 Effect of ergokonin A and other antifungal agents on the morphology of *Aspergillus fumigatus*. (a) DMSO solvent control; (b) nystatin (25 μg); (c) ergokonin A (2 μg) and (d) semi-synthetic pneumocandin L-733560 (2 μg). Microphotographs were taken at 500× with a Diaplan (Leitz) microscope equipped with differential interference contrast optics

in the GenBank (Bethesda, MD, USA) database, by using the FastA application of the GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, WI, USA), and the most related sequences were retrieved and used for building the phylogram shown in Fig. 3. As expected, the best matches were found with the ITS sequences from other members of section *Longibrachiatum*, including some *Hypocrea* anamorphs.

As shown in Fig. 3, the sequences sharing a higher homology with the producing strain (0·4–1·5% of nucleotide divergence) were classified as *T. longibrachiatum* or as its teleomorph *Hypocrea schweinitzii*. The percentages of nucleotide divergence in this range are widely accepted to be within the limits of the infraspecific variation for many fungal species (Arenal *et al*. 2000).

During our screening programme, several hundred examples of *Trichoderma* spp. were assayed to seek for new antifungal agents, but only one of these isolates screened produced ergokonin A. In contrast, other antifungal metabolites were recovered from a variety of fungal strains during the same period (Cabello *et al*. 2000). This would suggest that ergokonin A is a metabolite not very often found in nature. However, as mentioned above, ergokonin A had been previously described as being produced by a strain identified as *T. koningii* (Augustiniak *et al*. 1991). As shown in Fig. 3, a strain of the same species is relatively distant from our producing isolate (more than 15% of nucleotide divergence). Likewise, Kumeda *et al*. (1994) have also reported ergokonin A from *T. viride*. These results, provided that the identifications are correct, suggest that the production of ergokonin A is not uncommon, but probably restricted to *Trichoderma* species. The related compound ergokonin C was isolated from *Tolypocladium inflatum* (Gräfe *et al*. 1991). This fungal species shares some morphological traits with the genus *Trichoderma* (Bissett 1983) and is phylogenetically not very distant, being the anamorph of *Cordyceps subsessilis* (Clavicipitaceae, Hypocreales).

Other antifungal compounds structurally similar to ergokonin A and with the same mechanism of action (Onishi *et al*. 2000) include ascosteroside, produced by *Ascotricha amphitricha* and *Mycoleptodiscus atromaculans* (Gorman *et al*. 1996; Leet *et al*. 1996; Clapp *et al*. 1997); enfumafungin, produced by a *Hormonema* species (Schwartz *et al*. 2000; Peláez *et al*. 2000) and arundifungin, produced by *Arthrinium arundinis* and other fungi (Cabello *et al*. 2000). These compounds are also acidic triterpenes, modified at the 3-OH with glycosides (in enfumafungin and ascosteroside) or a succinate (in arundifungin) instead of the sulpho-derivative of an amino acid in ergokonin A.

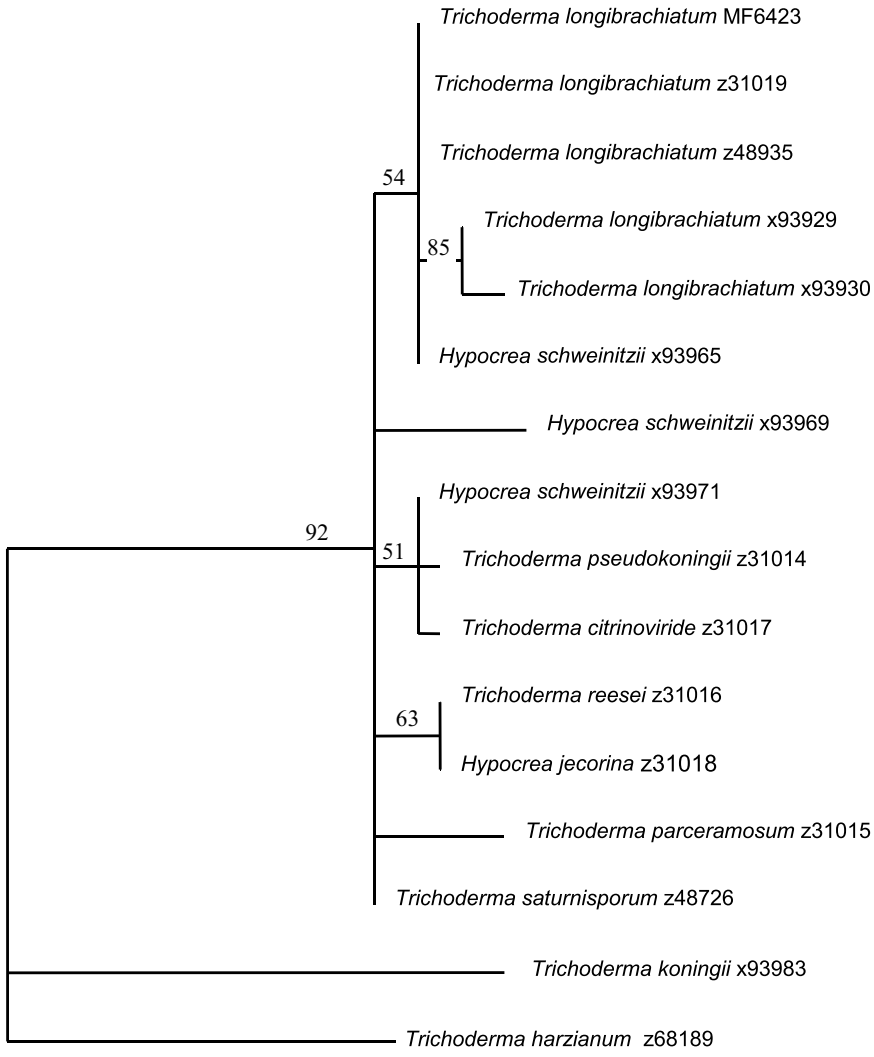

Fig. 3 Dendrogram showing the relationships between different *Trichoderma* strains (including the ergokonin A-producing isolate MF6423) and the teleomorph genus *Hypocrea*, based on the two ITSs and the 5·8S sequences. The accession numbers of the sequences follow the strain name: *T. longibrachiatum* MF6423 (producer of ergokonin A); *T. longibrachiatum* z31019; *T. longibrachiatum* z48935; *T. longibrachiatum* x93929; *T. longibrachiatum* x93930; *H. schweinitzii* x93965; *H. schweinitzii* x93969; *H. schweinitzii* x93971; *T. pseudokoningii* z31014; *T. citrinoviride* z31017; *T. reesei* z31016; *H. jecorina* z311018; *T. parceramosum* z31015; *T. saturnisporum* z48726; *T. koningii* x93983 and *T. harzianum* z68189

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