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Anti-*Candida* metabolites from endophytic fungi

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Abstract

Submerged cultures of some 1500 Ascomycota and Basidiomycota isolated from their fruit-bodies or as soil-borne, coprophilous or endophytic fungi were screened for activity against *Candida albicans* and a range of other pathogenic and saprotrophic fungi. Considerably more Ascomycota (11–16%) than Basidiomycota (3.5%) produced metabolites with activity against *C. albicans*. From five species of endophytes, six bioactive compounds were isolated and identified, *viz.* cerulenin (**1**), arundifungin (**2**), sphaeropsidin A (**3**), 5-(1,3-butadiene-1-yl)-3-(propene-1-yl)-2-(5H)-furanone (**4**), ascosteroside A (formerly called ascosteroside; **5**) and a derivative of **5**, ascosteroside B (**6**). **1**, **3** and **5** were isolated from fungi belonging to different orders than previously described producers. Antifungal activities of **2** and **4**–**6** in the agar diffusion test were comparable with those of amphotericin B. Compound **6** exhibited a similar antifungal activity as **5** but its cytotoxicity towards Hep G2 cells was considerably lower. This study points to endophytic fungi related to hemibiotrophic or latent plant pathogens as an important source of bio- and chemodiversity.

Keywords: Antifungal metabolites; Ascosteroside B; Biodiversity; Cerulenin; Endophytes; Screening; Sphaeropsidin A

1. Introduction

Ever since the introduction of large-scale screenings for antifungal compounds, natural products have been playing essential roles as active principles themselves, as lead structures for the development of drugs and pesticides, and as tools for the characterisation of new and selective targets. Although numerous antifungal compounds have been discovered in the course of screening efforts as well as chemical synthesis programmes, new products applicable to human therapy are rare and urgently needed, owing to a drastic increase in the number of immunocompromised patients (AIDS sufferers, organ transplant recipients) coupled with the development of resistance among clinically relevant fungi against several of the limited number of antimycotics in current use. The situation is highlighted by the fact that amphotericin B is still in use for the treatment of systemic candidiasis and is considered to be the 'gold standard' despite its toxic side effects (Stencel, 1999).

Recent estimates have put the worldwide number of fungal species at 1.5 million (Hawksworth, 2001). Of these, only about 10% have been discovered and described as yet, and barely 1% examined for their spectrum of secondary metabolites. A major contribution to the uncharacterised fungal biodiversity is thought to be made by endophytes, i.e. fungi spending a substantial part of their life-cycle as symptomless commensals or symbionts in living plant tissues (Arnold et al., 2000). A comprehensive screening of a broad range of taxonomically diverse fungi isolated from a range of habitats, with an emphasis on endophytic strains, was therefore performed with *Candida*

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albicans as the test organism. The results are reported here, together with the identification of antifungal compounds from five selected endophytic fungi.

2. Results and discussion

2.1. Screening

Altogether 1510 fungal strains were grown in submerged culture, and the resulting mycelial and culture filtrate extracts tested against *Candida albicans*. The results of this screen are summarised in Table 1. The percentage of strains with anti-*Candida* activity was similar among various ecological groups of Ascomycota, ranging from 10.8% to 16.4%. In contrast, the proportion of Basidiomycota with anti-*Candida* activity was considerably lower (3.5%), both in comparison with the Ascomycota screened in this study and also Basidiomycota screened against other test fungi (Lorenzen and Anke, 1998).

Table 1
Antifungal activities of extracts prepared from submerged cultures of fungi of different taxonomic groups and different habitats using *Candida albicans* as test organism

Fungal group	Number of extracts	Active extracts (%)
Basidiomycota from fruit-bodies	332	3.5
Ascomycota from fruit-bodies	530	16.4
Imperfect Ascomycota from soil	803	16.4
Coprophilous fungi (mainly Ascomycota)	167	14.9
Endophytic fungi (Ascomycota)	1185	10.8

In view of the contribution of endophytes to the total fungal biodiversity and their production of bioactive metabolites (Tan and Zou, 2001; Schulz et al., 2002), five endophytic strains were selected for the isolation of active principles, *viz.* E99204, E99291, E99297, E99401 and E00175. The active compounds produced by them are shown in Fig. 1.

2.2. Cerulenin (*1*)

Strain E99401, isolated from an asymptomatic leaf of a tropical rainforest tree from French Guyana, was identified as a *Phomopsis* sp. by microscopy. Membership in the Valsaceae (Diaporthales) was confirmed by microscopy and a FASTA search with its ITS and partial 18S rDNA sequences (GenBank accession DQ872669 and DQ872670, respectively) in comparison with the data by Inderbitzin and Berbee (2001). Cerulenin, an inhibitor of fatty acid and polyketide synthases with known anti-*Candida* activity (Hoberg et al., 1986), has been reported so far only from the rice sheath rot pathogen *Sarocladium oryzae* (='*Cephalosporium caerulens*', Hypocreales; Bills et al., 2004), and from another rice pathogen, *Helicoceras oryzae* (*incertae sedis*). In *S. oryzae*, cerulenin is considered to be a phytotoxic virulence factor (Sakthivel et al., 2002), and it is also inhibitory against other fungal rice pathogens (Gnanamanickam and Mew, 1991). Since the genus *Phomopsis*

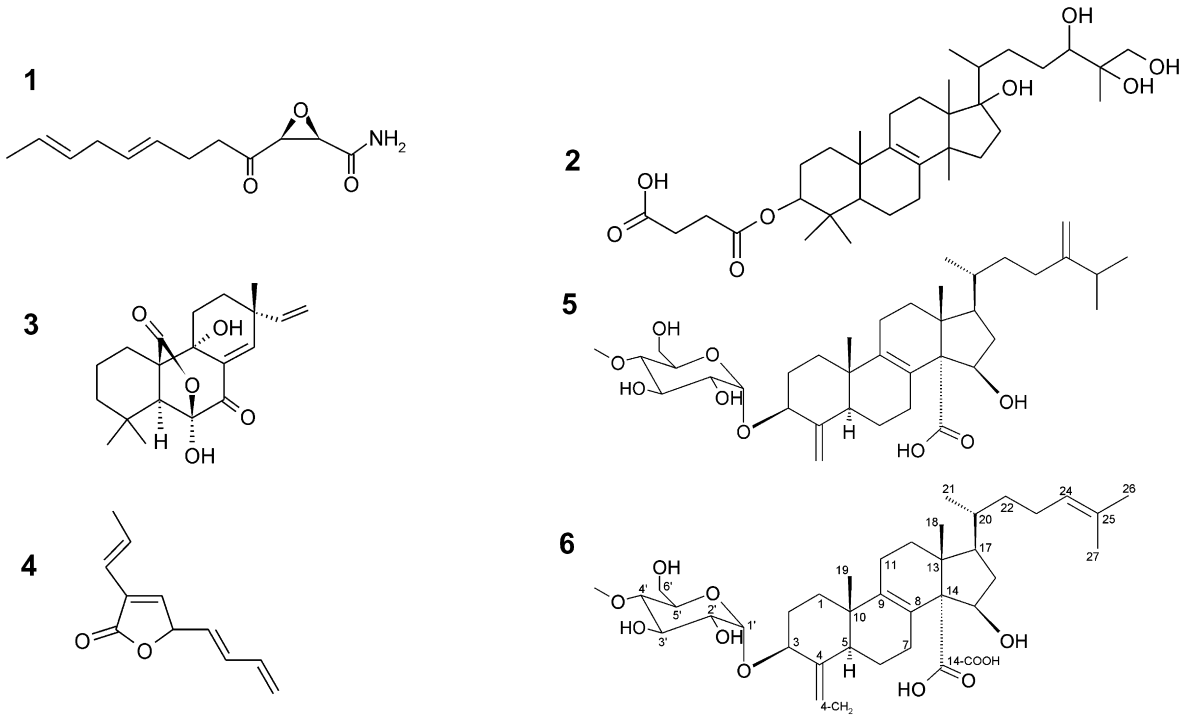

Fig. 1. Structures of the metabolites with anti-*Candida* activity isolated in the current work, cerulenin (**1**), arundifungin (**2**), sphaeropsidin A (**3**), 5-(1,3-butadien-1-yl)-3-(propen-1-yl)-2(5H)-furanone (**4**), ascosteroside A (**5**) and B (**6**).

contains important hemibiotrophic plant pathogens as well as endophytic species, it is possible that cerulenin plays a role in killing host tissue and/or in fending off competing microbes during colonisation after host death, as has been shown for other secondary metabolites in *Phomopsis* (see Weber and Anke, 2006).

2.3. Ascosterosides A (5) and B (6)

E99291, an endophyte from shoots of *Cistus salvifolius*, was assigned to the Gnomoniaceae (Diaporthales) on the basis of its ITS and 18S rDNA sequence homologies (DQ872667 and DQ872668, respectively; see Castlebury et al., 2003). Ascosteroside A (**5**) was originally isolated from *Ascotricha amphitricha* as a metabolite with anti-*Candida* activity (Gorman et al., 1996) which is now known to be due to an inhibition of β-(1,3)-glucan synthesis (Onishi et al., 2000). *Ascotricha* belongs to the Xylariaceae (Xylariales; Lee and Hanlin, 1999), and despite considerable screening efforts for inhibitors of β-(1,3)-glucan synthesis no further reports on the occurrence of ascosteroside A have appeared in the literature. E99291 is therefore a new, taxonomically unrelated producer. Since it also produced a new derivative, ascosteroside B (**6**, see below), the original compound is re-named ascosteroside A.

After dereplication studies with common databases (DNP), ^{1}H and ^{13}C NMR data (Table 2) revealed the similarity of compound **6** with ascosteroside A (**5**, Leet et al., 1996). The ^{13}C NMR spectrum of **6** showed 36 signals. Multiplicity selection by DEPT NMR analysis led to 6 methyls, 11 methylenes, 11 methins and 8 quaternary carbons. Characteristic ^{13}C chemical shift data identified one carboxyl atom at $\delta_C = 179.0$, three olefins (olefin 1, $\delta_C = 128.7$, 141.1; olefin 2, $\delta_C = 126.1$, 131.9) including one exocyclic olefin ($\delta_C = 151.6$, 104.5), and one anomeric carbon ($\delta_C = 96.9$). Additional carbon signals pointed to **6** being a steroid with a sugar unit and an aliphatic side chain. The structure of ascosteroside B was determined

Table 2
^{1}H and ^{13}C NMR data (CD_3OD) of **5** and **6**

Carbon	**5**		**6**	
	^{13}C ppm	^{1}H ppm, multiplicity *J* (Hz)	^{13}C ppm	^{1}H ppm, multiplicity *J* (Hz)
1	36.3	1.46 *m*/1.88 *m*	36.3	1.46 *m*/1.88 *m*
2	29.5	1.46 *m*/2.08 *m*	29.5	1.46 *m*/2.09 *m*
3	76.8	4.06 *m*	76.7	4.06 *m*
4	151.7	–	151.6	–
5	47.9	2.02 *m*	47.9	2.01 *m*
6	22.1	1.58 *m*/1.77 *m*	22.1	1.59 *m*/1.76 *m*
7	27.2	1.99 *m*/2.71 *m*	27.1	1.98 *m*/2.71 *m*
8	128.9	–	128.7	–
9	140.8	–	141.1	–
10	40.6	–	40.6	–
11	24.6	2.12 *m*/2.28 *m*	24.6	2.11 *m*/2.28 *m*
12	34.1	1.70 *m*/2.19 *m*	34.0	1.69 *m*/2.18 *m*
13	47.5	–	47.6	–
14	68.0	–	67.8	–
15	73.3	4.60 *d* (6.8)	73.2	4.59 *d* (6.9)
16	44.6	1.49 *m*/2.66 *m*	44.6	1.48 *m*/2.66 *m*
17	51.8	1.44 *m*	51.9	1.41 *m*
18	18.9	1.11 *s*	18.9	1.10 *s*
19	19.4	0.93 *s*	19.3	0.93 *s*
20	36.7	1.60 *m*	36.5	1.58 *m*
21	19.2	0.96 *d* (6.5)	19.2	0.94 *d* (6.6)
22	36.1	1.16 *m*/1.57 *m*	37.3	1.05 *dd* (6.6, 5.3)/1.43 *m*
23	31.9	1.92 *m*/2.10 *m*	25.6	1.89 *m*/2.01 *m*
24	157.7	–	126.1	5.09 *dd* (7.0, 7.0)
25	34.9	2.24 *hept.*	131.9	–
26	22.3	1.03 *d* (6.5)	25.9	1.66 *s*
27	22.5	1.03 *d* (6.5)	17.7	1.59 *s*
4-CH_2	104.4	4.65 *s*/5.19 *s* (br)	104.5	4.66 *s* (br)/5.190 *s* (br)
14-COOH	179.3	–	179.0	–
24-CH_2	106.9	4.65 *s*/4.72 *s*	–	–
1′	96.8	4.98 *d* (3.6)	96.9	4.98 *d* (3.7)
2′	73.7	3.41 *dd* (9.7, 3.6)	73.7	3.42 *dd* (9.8, 3.7)
3′	75.2	3.84 *dd* (9.6, 9.0)	75.2	3.85 *dd* (9.8, 9.3)
4′	81.2	3.10 *dd* (9.1, 9.0)	81.2	3.10 *dd* (9.3, 9.3)
4′-OCH_3	60.8	3.55 *s*	60.8	3.55 *s*
5′	73.0	3.61 *d* (9.1)	73.1	3.61 *m*
5′-CH_2OH	62.2	3.62 *m*/3.69 *d* (13.3)	62.3	3.62 *m*/3.68 *d* (13.1)

from 2D NMR (Fig. 2) using COSY, HSQC and HMBC pulse sequences. The position of the sugar at C3 was given by C3/1′-H in the HMBC spectrum. The carboxylic acid group at position 14 was determined by long-range ^{1}H–^{13}C coupling of the carboxyl carbon and 15-H. In comparison to **5** the olefin group in the side chain of **6** is in a different position, shown by signals for a CH ($\delta_C = 126.1/\delta_H = 5.09$) and a quaternary carbon ($\delta_C = 131.9$). The elucidated structure of ascosteroside B (**6**) in comparison with **5** is shown in Fig. 1.

2.4. Arundifungin (*2*)

All five closest hits from FASTA searches of the ITS and 18S rDNA sequence of E00175 (DQ872671 and DQ872672, respectively) belonged to members of the Amphisphaeriaceae in the order Xylariales (see Kirk et al., 2001; Jeewon et al., 2002, 2003). Arundifungin is related to ascosterosides (**5**, **6**) in structure and mode-of-action as a β-(1,3)-glucan synthesis inhibitor. It was originally isolated from *Arthrinium arundis* (Apiosporaceae, Xylariales) by Cabello et al. (2001) who also reported its presence in a wide range of unrelated fungi, including endophytes, from diverse habitats and geographic origins.

2.5. Sphaeropsidin A (*3*)

The 18S rDNA sequence of E99204, an endophyte from an asymptomatic leaf of *Quercus ilex*, differed from that of E99291 at only one position (nt 854, G deleted in E99291). Sphaeropsidins are a group of pimarane diterpenes known from the anamorphic fungi *Sphaeropsis sapinea* f. sp. *cupressi* and *Diplodia mutila* (Sparapano et al., 2004), two closely related phytopathogenic species belonging to the Botryosphaeriaceae (Pleosporales, Loculoascomycetes; Zhou and Stanosz, 2001). There have been suggestions that antibiotics LL-S491β and LL-S491γ from *Aspergillus chevalieri* are identical to sphaeropsidins A and B, respectively (Evidente et al., 1996, 1997). Nonetheless, E99291 is a new, taxonomically unrelated producer, belonging to the Diaporthales. Sphaeropsidins are phytotoxic and fungitoxic and have been suggested to play a role in the development of symptoms (tree cankers) and in warding off competing phytopathogenic fungi (Sparapano et al., 2004) akin to that discussed above for cerulenin.

2.6. 5-(1,3-Butadien-1-yl)-3-(propen-1-yl)-2(5H)-furanone (*4*) from E99297

Strain E99297, isolated as a symptomless endophyte from a twig of *Cistus salvifolius*, has previously been shown by Köpcke et al. (2002) to belong to the Sarcosomataceae (order Pezizales). Compound **4** was isolated on the basis of its anti-*Candida* activity and was identical to compound **3** of Köpcke et al. (2002).

2.7. Antifungal and cytotoxic activities of the isolated compounds

The antifungal activities of **1**–**6** were determined against a wide range of saprophytic, plant- and human pathogenic fungi, using the agar-diffusion assay. The results (Table 3) indicate comparable activity spectra and active concentrations between amphotericin B and several of the compounds isolated in the course of our work, notably **2** and **4**–**6**. Cytotoxicity data of **1**–**6** are presented in Table 4. The reduced cytotoxicity of ascosteroside B (**6**) as compared to the structurally related ascosteroside A (**5**) is noteworthy and warrants further structure–activity experiments.

2.8. Conclusions

The potential of endophytic fungi as sources of novel bioactive substances is now widely recognised, and our results pinpoint species related to hemibiotrophic or latent pathogens as particularly prolific producers. This feature was observed to transcend ordinal boundaries (e.g. Xylariales,

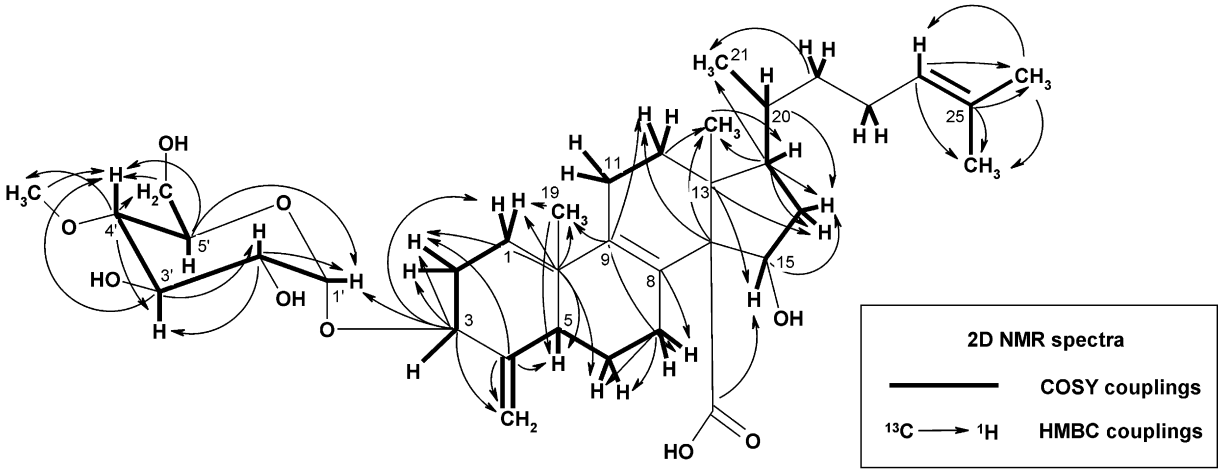

Fig. 2. 2D NMR correlations in ascosteroside B (**6**).

Table 3
Antifungal activities of compounds **1**–**6** against pathogenic and nonpathogenic yeasts and filamentous fungi in the agar diffusion assay

Test organisms	**1** 5 μg	**2** 5 μg	**3** 10/50 μg	**4** 5/20 μg	**5** 5/20 μg	**6** 5/20 μg	amphB 20 μg
Aspergillus flavus	–[a]	22	–/8	10/12	8/10	11/13	12
A. fumigatus	–	24	–/8	8/11	8/11	9/10	14
Candida albicans	16	16	–/20	–/11	17/18	14/15	14
C. glabrata	17	13	13/16	9/15	14/15	16/17	10
C. krusei	14	14	15/17	9/11	19/20	20/22	9
C. parapsilosis	28	17	13/15	–/12	17/19	14/16	10
Cryptococcus neoformans	40	n.t.[b]	n.t.	–/10	–/8	10/13	15
Fusarium solani	–	n.t.	n.t.	10/14	–/–	–/–	10
Microsporum canis	30	n.t.	n.t.	n.t.	13/15	14/16	8
Mucor miehei	42	n.t.	8/10	n.t./40	8/11	8/10	19
Nematospora coryli	40	–	13/14	n.t./25	13/15	14/15	8
Paecilomyces variotii	38	17	–/13	n.t./30	–/–	–/8	20
Rhizomucor pusillus	36	8	10/16	9/14	–/–	–/8	14
Trichophyton rubrum	–	n.t.	–/8	8/12	9/11	9/10	8

Amphotericin B (amphB) was used as a standard. Diameters of inhibition zones are given in mm.
[a] Denotes the absence of an inhibition zone around the filter paper disk (6 mm).
[b] n.t. = not tested.

Table 4
Cytotoxic activities of compounds **1**–**6** towards human cell lines

Cell line	**1** LD_{50}/LD_{90}	**2** LD_{50}/LD_{90}	**3** LD_{50}/LD_{90}	**4** LD_{50}/LD_{90}	**5** LD_{50}/LD_{90}	**6** LD_{50}/LD_{90}
Hep G2	5/10	>5/n.t.[a]	2.5/5	10/25	n.t./25	100/>100
Colo 320	<2.5/5	>5/n.t.	2.5/10	25/50	>100/n.t.	>100/n.t.
MCF-7	2.5/5	>5/n.t.	2.5/5	50/>100	50/100	100/>100
HeLa S3	5/10	>5/n.t.	1/2.5	50/>100	>100/n.t.	>100/n.t.

LD_{50} and LD_{90} values are concentrations (μg ml^{-1}) at which cell proliferation was reduced by 50% and 90%, respectively.
[a] n.t.: not tested.

Hypocreales, Diaporthales, Pleosporales). Our study has also shown that metabolites of particular research interest such as **1**, **3** and **5**, previously known from only one or a few species, are produced by other, taxonomically unrelated fungi. In evolutionary terms, the conversion of a pathogen to an endophyte may require as little as the loss of a single pathogenicity-relevant gene (Redman et al., 1999) or colonisation of a plant species which does not permit the expression of disease symptoms (Freeman et al., 2001). Such endophytes may retain their ability to produce potent metabolites involved in habitat defence.

3. Experimental

3.1. Isolation and identification of fungi

Cultures of Basidiomycota and Ascomycota from fruitbodies and from the dung of herbivorous animals were obtained by germinating spores. Anamorphic fungi were isolated from soil samples by dilution-plating, and endophytic fungi from surface-sterilized living plant material as described by Köpcke et al. (2002). The isolation medium was 2% malt-extract agar (MEA) augmented with 200 mg l^{-1} each of penicillin G and streptomycin sulphate for both groups of fungi. All strains were cultivated on YMG agar (10 g malt extract, 10 g glucose, 4 g yeast extract l^{-1}), and are being maintained in the culture collection of the IBWF.

Methods and reagents for DNA extraction and PCR amplification of the ITS region (primers ITS4 and ITS5) and the 5′ end of the 18S ribosomal RNA gene (primers NS1 and SR4) were as described previously (Köpcke et al., 2002; Schwarz et al., 2004). PCR products were purified with the JetQuick spin kit (Genomed, Löhne, Germany) and sequenced externally by MWG Biotech (Martinsried, Germany). Searches in GenBank were performed with the FASTA function of the GCG Wisconsin package.

3.2. Screening and fermentation

For initial screenings, fungi were inoculated into 1000 ml conical flasks with four indentations, containing 500 ml YMG medium adjusted to pH 5.5 before autoclaving. These flasks were incubated at 22 °C with shaking (120 rpm). After consumption of the carbon source, the cultures were harvested by filtration. The culture fluid was extracted with EtOAc or by adsorption onto HP21 diaion resin (Mitsubishi, Düsseldorf) and elution with MeOH and acetone, whereas wet mycelium was extracted by shaking in MeOH for 2 h. After solvent evaporation, the crude extracts were dissolved in MeOH to give a 100-fold concentration relative to the original culture volume.

Antifungal activity was determined with the agar diffusion assay, applying 20 µl crude extract per filter paper disk (see Section 3.5).

Fermentations were carried out at 22 °C in 5-l Erlenmeyer flasks containing 2 l YMG medium (strain E99401) or in a stirred (120 rpm) and aerated (3.3 l sterile air min^{-1}) fermentor (Biolafitte C6) containing 20 l YMG medium (other strains). A well-grown culture (200 ml YMG medium in a 500 ml Erlenmeyer flask) incubated on a rotary shaker at 22 °C was used as inoculum for fermentors. Cultures were harvested when no further increase in bioactivity could be observed. Mycelium and culture fluid were separated by filtration and extracted separately with MeOH and EtOAc, respectively. The organic phases were concentrated in vacuo. The compounds were purified from these crude extracts by bioactivity-guided fractionation.

3.3. General methods for purification and analysis

Silica gel 60 (63–200 µm; Merck, Darmstadt) in cyclohexane was used for low-pressure chromatography of crude extracts. Elution was achieved in cyclohexane with increasing concentrations of EtOAc. Sephadex LH-20 (Pharmacia LKB, Uppsala) packed in MeOH was used for gel filtration chromatography. Preparative HPLC was performed with a Jasco modular HPLC system (Gross-Umstadt, Germany) consisting of two PU-1586 high-pressure pumps and the multi-wavelength detector UV-1570M. The system was fitted with LiChrosorb RP-18 or Diol columns (250 × 25 mm, 7 µm particle size; Merck).

NMR spectra were recorded in MeOH-d_4 at room temp. using a Varian VNMRS 600 NMR spectrometer fitted with a 3 mm inverse triple resonance probehead and a 5 mm dual broadband probehead (both equipped with gradient selection coils). Solvent signals ($\delta_H = 3.30$ and $\delta_C = 49.0$) were used as internal reference. HR-MS spectra were recorded with a Bruker Apex IV system equipped with an ESI source, and UV spectra with a Varian Cary 100 Bio.

3.4. Isolation of ascosteroside B (6)

Culture filtrate and mycelial crude extracts of strain E99291 were processed separately. Crude product from 16 l culture filtrate (185 mg) was further separated by chromatography on silica gel. Elution with cyclohexane/EtOAc (45:55) resulted in 108 mg of an intermediate fraction. Final purification was accomplished by HPLC with the RP18 column in a linear H_2O/acetonitrile gradient from 80% to 100% acetonitrile in 60 min. Two active fractions yielded 6.4 mg compound **5** (elution at 32–33 min) and 16.8 mg compound **6** (elution at 35–36 min). Mycelial crude extract (2.2 g) was processed by chromatography on silica gel; elution with cyclohexane/EtOAc (40:60) yielded 416 mg intermediate product which was separated by HPLC as above, affording 24 mg compound **5** and 62 mg compound **6**. Compound **5** was found to identical to ascosteroside previously isolated from the ascomycete *Ascotricha amphitricha* (Leet et al., 1996) while compound **6** was new.

Compound **6** appeared as a colourless amorphous solid. Its molecular formula was established as $C_{36}H_{56}O_9$ based on 1D NMR and HR-MS spectra. Melting point, 137 °C. HR-MS (ESI pos.) found 655.38161 $[M+Na]^+$, calculated 655.38165. IR (KBr) 3436, 2922, 2874, 1693, 1652, 1458, 1381, 1202, 1148, 1069, 1033, 977, 908, 785, 726, 685 and 622 cm^{-1}. UV (MeOH) λ_{max} (ε) 202 (13257), 230 (sh, 4498), 280 (77) nm.

Following purification of substances **1**–**4** (not described in detail), these were identified by reference to the relevant literature, *viz.* Arison and Omura (1974) for **1**, Cabello et al. (2001) for **2**, Evidente et al. (1996) for **3** and Köpcke et al. (2002) for **4**.

3.5. Biological assays

Disk diffusion assays were carried out with YMG agar containing 10^6 cells or spores ml^{-1}. Extracts or pure compounds dissolved in MeOH were added to filter paper disks (6 mm diam.) which were placed on the agar surface after solvent evaporation. The diameter of the inhibition zones was measured after 24 h incubation.

Cytotoxic activity was assayed as described previously (Zapf et al., 1995) with slight modifications. HeLa S3 (ATCC CCL 2.2) and Hep G2 (DSMZ ACC 180) cells were grown in D-MEM (Gibco) supplemented with 10% foetal calf serum (Gibco), 65 µg penicillin G ml^{-1} and 100 µg streptomycin sulphate ml^{-1}. These assays contained 10^5 cells ml^{-1} medium. Test substance concentrations at which cell proliferation was reduced by 50% or 90% are given as IC_{50} and IC_{90} values, respectively.

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