Spirofungin, a New Antifungal Antibiotic from *Streptomyces violaceusniger* Tü 4113†

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A new secondary metabolite was detected in the culture filtrate and extracts of *Streptomyces violaceusniger* Tü 4113 by HPLC-diode-array and HPLC-electrospray-mass-spectrometry screening. The compound named spirofungin has a polyketide-spiroketal structure and shows various antifungal activities, particularly against yeasts.

Our screening for new secondary metabolites is based on hyphenated HPLC techniques. HPLC-DAD analysis is used to determine the biosynthetic capacities of freshly isolated actinomycete strains which were cultivated in various complex media by means of our HPLC-UV-Vis database[2]. Unknown compounds are then analyzed by HPLC-ESI-MS to give the molecular masses of the metabolites[3]. These data permit an efficient search in commercially databases for the novelty of compounds. Screening of the culture filtrate and extracts of *Streptomyces* strain Tü 4113, which was isolated from a soil sample collected in the Otway National Park (Australia) resulted in the identification of the well known macrodiolide antibiotic elaiophylin[4,5]. In addition three different secondary metabolites were detected which could neither be identified nor classified by our spectral library database. HPLC-MS analysis indicated the novelty of these compounds. One of these compounds was isolated and its structure was determined.

In this paper we describe the taxonomy of the producing strain, fermentation and isolation, as well as characterization, structure elucidation and biological activity of the new compound, named spirofungin.

Taxonomy

The whole cell hydrolysate of strain Tü 4113 contained LL-diaminopimelic acid, which is the diagnostic diamino acid in the peptidoglycan of the genus *Streptomyces*. The aerial mycelium was well developed on all ISP media and changed in colouration from white to gray with increasing sporulation. The aerial mycelium formed entangled spirals in masses. The surface of the spores was warty, as revealed by electron microscopy. In old cultures (14 to 21 days) parts of the colonies became black and moist due to autolytic processes. According to HÜTTER[6], strain Tü 4113 was assigned to the *cinereus* colour series. The colour of the substrate mycelium varied from whitish gray, yellowish gray to greenish brown and dark grayish brown. Melanoid pigments were not produced on peptone-yeast extract-iron agar (ISP 6) and

† Art. No. 10 on biosynthetic capacities of actinomycetes. Art. No. 9: See ref. 1.

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Table 1. Cultural characteristics of strain Tü 4113.

Medium	Growth	Aerial spore mass colour	Reverse side colour	Soluble pigments
Yeast extract-malt extract agar (ISP 2)	Good	Whitish gray	Yellowish	None
Oatmeal agar (ISP 3)	Good	Whitish gray	Yellowish and greenish brown	None
Inorganic salts-starch agar (ISP 4)	Good	Brown grayish	Grayish brown	None
Glycerol-asparagine agar (ISP 5)	Flat	Whitish ~ grayish brown	Yellowish brown	None
Soil extract agar[a]	Flat	Gray ~ blackish brown	Gray	None

[a] Soil extract agar: soil extract 250 ml, yeast extract 1 g, tap water 750 ml, agar 20 g.

Table 2. Utilization of selected carbon sources of strain Tü 4113 and *Streptomyces violaceusniger* (DSM 40563^T) according to the procedure of SHIRLING & GOTTLIEB[8].

	Tü 4113	*S. violaceusniger* DSM 40563^T
L-Arabinose	+	+
Cellulose	−	−
D-Fructose	+	+
m-Inositol	+	+
D-Mannitol	+	+
Raffinose	+	+
Rhamnose	+	+
Sucrose	+	+
D-Xylose	(+)	+

Growth: +, good; (+), weak; −, none.

Table 3. Phage susceptibility.

Phage	Tü 4113	*S. violaceusniger* DSM 40563^T
DSM 49147 (S1)	+	+
DSM 49148 (S2)	+	+
DSM 49152 (S3)	−	−
DSM 49149 (S4)	−	−
DSM 49150 (S6)	+	+
DSM 49151 (S7)	+	+
DSM 49153 (Str. III)	−	−
S5	−	−
S8	+	−
S9	+	+

+, Lysis; −, no effect.

tyrosine agar (ISP 7). No soluble pigments were found in any of the tested ISP media. The cultural characteristics of strain Tü 4113 are summarized in Table 1. The utilization of selected carbon sources in comparison with the type strain of *Streptomyces violaceusniger* (DSM 40563^T) is shown in Table 2. Based on our results, the comparison with the type strain of *S. violaceusniger* (DSM 40563^T) and the data in the literature[6,7] we propose to assign strain Tü 4113 as a strain of *S. violaceusniger* (Waksman and Curtis 1916, PRIDHAM *et al.* 1958). This affiliation was additionally confirmed by the same pattern of phage susceptibility of strain Tü 4113 and *S. violaceusniger* (DSM 40563^T) (Table 3).

Fermentation and Isolation

Batch fermentations of *S. violaceusniger* Tü 4113 were carried out in 25-liter fermenters equipped with an intensor system, using a complex medium that consisted of mannitol 2% and cottonseed 2% (pH 7.5). Spirofungin production started at about 72 hours and reached a maximum after 120 hours with a concentration of 47 mg/liter, as shown in Fig. 1.

Isolation of spirofungin was achieved by the application of Amberlite XAD-16 chromatography, ethyl acetate extraction and chromatography on Sephadex LH-20. Pure spirofungin was obtained after preparative reversed-phase HPLC using 10-micron Nucleosil-100 C-18 material with 0.5% formic acid-MeOH gradient elution.

Characterization

Culture filtrates and raw extracts from *S. violaceusniger* Tü 4113 were screened by our HPLC-DAD method (Fig. 2). Four different compounds could be detected with respect to their UV-visible spectra. The compound with retention time of 10.9 minutes was identified by the

Fig. 1. Batch fermentation of *S. violaceusniger* Tü 4113.

● Spirofungin [mg/liter]; □ mannitol [g/liter]; ○ ammonium [mM]; ◇ DNA [mg/liter].

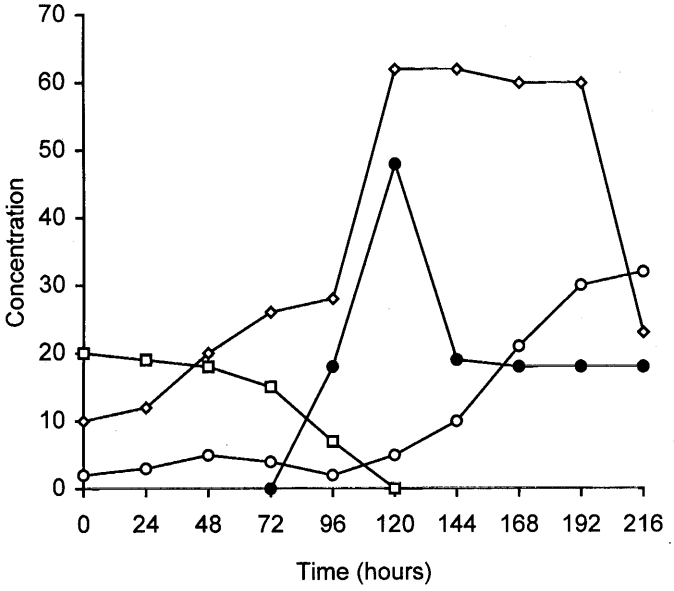
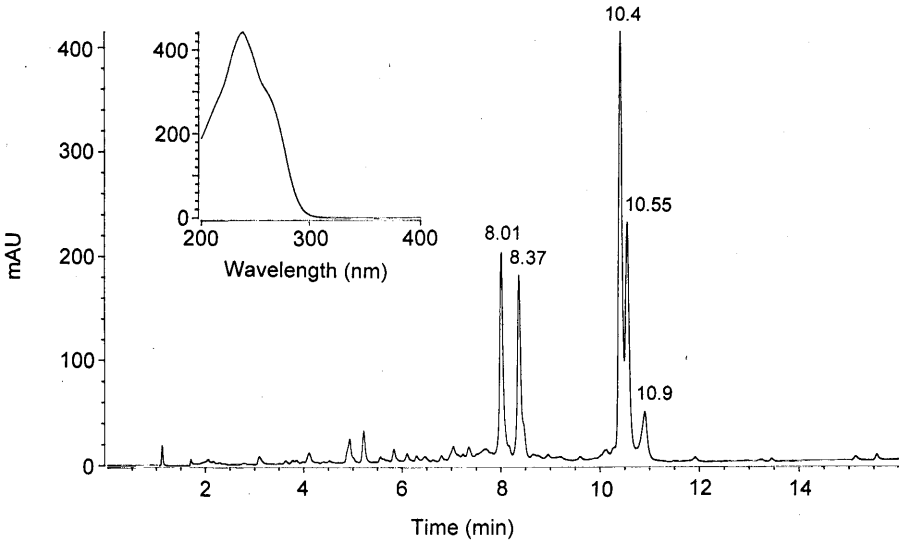


Fig. 2. HPLC analysis of the culture filtrate extract from *S. violaceusniger* Tü 4113 monitored at 230 nm and UV spectrum of spirofungin (10.40 and 10.55 minutes).



HPLC-UV-Vis database as the macrodiolide antibiotic elaiophylin, showing the maximal spectral identity with a match factor of 1000 and the same retention time as the reference compound. HPLC-MS analysis revealed a molecular mass of 1025 which confirmed its identity as elaiophylin. The doubled peak with retention times of 10.40 and 10.55 minutes showed the same UV spectra with a maximum at 239 nm and identical molecular mass of 502 determined by HPLC-MS. A search in a commercially available database indicated the novelty of the compound which was confirmed by structure elucidation. The double peak of the compound, named as spirofungin, can be explained by the presence of stereoisomers.

Two further compounds with retention times of 8.01 minutes and 8.37 minutes, and having molecular masses of 1301 and 1299, respectively, are presently investigated.

Structure Elucidation

The structure elucidation of spirofungin (Fig. 3) was principally achieved using NMR spectroscopy. For this

Fig. 3. Structure of spirofungin.

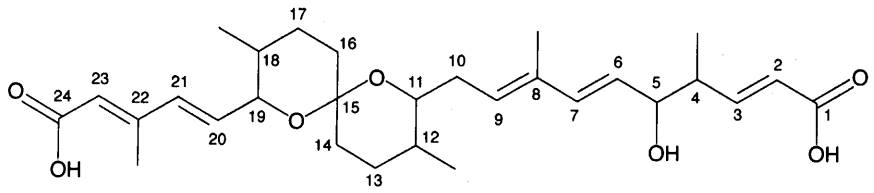


Table 4. ^{1}H and ^{13}C NMR data of spirofungin A.

	δ (^{13}C)	δ (^{1}H)	Observed C–H long range correlations
1	171.42	—	H-2, H-3
2	121.24	5.85	H-3, H-4
3	153.00	7.09	H-2, H-4, Me-4, H-5
4	42.50	2.58	H-2, H-3, Me-4, H-5, H-6
4-Me	14.32	1.08	H-3, H-4, H-5
5	76.25	4.13	H-2, H-3, H-4, Me-4, H-6, H-7
6	125.38	5.48	H-4, H-5, H-7
7	137.84	6.19	H-5, Me-8, H-9
8	133.42	—	H-6, H-7, Me-8, H-9, H-10
8-Me	12.74	1.70	H-7, H-9
9	130.40	5.49	H-7, Me-8, H-10, H-11
10	33.11	2.33	H-9, H-11
11	74.86	3.40	H-9, H-10, Me-12
12	34.94	1.33	H-10, Me-12, H-14a, H-14b
12-Me	17.60	0.76	H-11, H-12, H-13
13	27.81	1.47	H-11, Me-12, H-14a, H-14b
14	36.07	a: 1.67 b: 1.43	H-12, H-13
15	95.72	—	H-11, H-13, H-14a, H-14b, H-16a, H-16b, H-17a, H-19
16	36.29	a: 1.77 b: 1.55	H-14a, H-17b
17	23.52	a: 1.75 b: 1.46	H-16a, H-16b, Me-18
18	33.11	1.91	H-16a, H-16b, H-17a, Me-18, H-19, H-20
18-Me	17.31	0.74	H-17a, H-18, H-19
19	78.46	4.16	H-17a, H-17b, H-18, Me-18, H-20, H-21
20	135.42	6.53	H-18, H-19
21	136.70	6.27	H-19, Me-22, H-23
22	154.50	—	H-20, H-21, Me-22, H-23
22-Me	14.54	2.21	H-21, H-23
23	118.55	5.81	H-20, H-21, Me-22
24	171.77	—	Me-22, H-23

purpose, the two closely eluting HPLC peaks showing identical UV spectra and molecular masses were not separated. As a consequence, all NMR spectra exhibited two signal sets, which are further referred to as spirofungin A and B. The relative intensity of the two signal sets suggests a ratio A : B of about 4 : 1.

HR-FAB-MS spectra (m/z [M + Li^+] exp.: 509.31567, calc.: 509.32227) in combination with ^{13}C NMR and DEPT data revealed $C_{29}H_{42}O_7$ as the molecular formula for spirofungin. Combination of data extracted from ^{1}H NMR, ^{13}C NMR, DEPT and HSQC spectra indicated that spirofungin consists of five methyl groups, five methylene groups, three ordinary methine groups, three oxygenated methine groups, eight olefinic methine groups, two olefinic quarternary carbons, two carbonyl carbons, and one additional quarternary carbon atom.

Connectivities between CH_n-fragments were established by the use of TOCSY and HMQC-TOCSY experiments, both carried out using short and long spinlock times to differentiate between vicinal and long range connectivities. HMQC-TOCSY experiments are advantageous in the assignment procedure of complex spectra because of their enhanced signal dispersion due to the introduction of ^{13}C as the indirect dimension. Data

Table 5. ^{1}H and ^{13}C NMR data for spirofungin B.

	δ (^{13}C)	δ (^{1}H)	$\Delta\delta$ (^{13}C)
1	171.27	—	+0.15
2	121.32	5.86	−0.08
3	152.84	7.09	+0.16
4	42.76	2.60	−0.26
4-Me	14.49	1.09	−0.17
5	75.90	4.17	+0.35
6	125.65	5.51	−0.27
7	137.33	6.29	+0.51
8	133.74	—	−0.32
8-Me	12.80	1.72	−0.06
9	130.88	5.66	−0.48
10	32.49	a: 2.48 b: 2.32	+0.62
11	78.45	3.16	−3.59
12	34.08	1.48	+0.86
12-Me	17.60	0.87	+0.00
13	28.44	a: 1.75 b: 1.28	−0.63
14	35.79	a: 1.69 b: 1.62	+0.28
15	97.68	—	−1.96
16	23.14	a: 1.95 b: 1.39	**13.15**
17	25.53	a: 1.89 b: 1.40	−2.01
18	31.50	1.78	+1.61
18-Me	11.61	0.89	**+5.70**
19	72.36	4.79	**+6.10**
20	137.43	6.07	−2.01
21	132.72	6.29	+3.98
22	154.62	—	−0.12
22-Me	13.98	2.25	+0.56
23	118.09	5.77	+0.46
24	171.98	—	−0.21

gained from a HMBC experiment confirmed the assignment of the CH_n-fragments and allowed us to determine the positions of the quarternary carbon atoms within the molecule. Table 4 summarizes ^{1}H and ^{13}C chemical shifts of spirofungin A as well as the C–H long range connectivities observed in the HMBC spectrum for spirofungin A. ^{1}H and ^{13}C chemical shifts of spirofungin B are given in Table 5†††.

The presence of one hydroxy group and two carboxy groups in spirofungin was independently proven by methylation of the carboxy groups with diazomethane followed by trimethylsilylation of the hydroxy group, and subsequent EI-MS analysis.

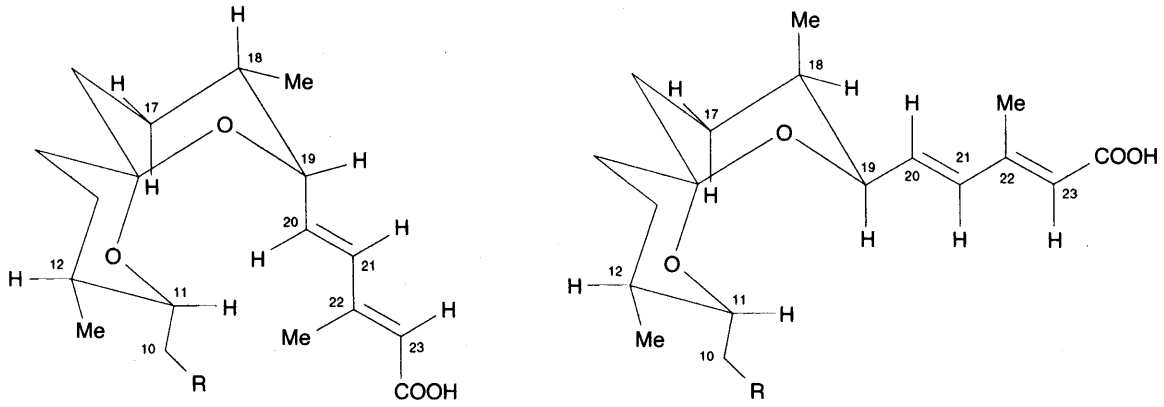
The stereochemistry of the disubstituted double bonds in both components could easily be determined as *E* configuration by the vicinal coupling constants of the concerned protons. Coupling constants were extracted from a P.E. COSY spectrum as well as from the resolution-enhanced 1D ^{1}H NMR spectrum (spirofungin A: H-2 and H-3: $^{3}J_{HH}=15.8$ Hz, H-6 and H-7: $^{3}J_{HH}=15.6$ Hz, H-20 and H-21: $^{3}J_{HH}=15.8$ Hz; spirofungin B: H-2 and H-3: $^{3}J_{HH}=15.8$ Hz, H-6 and H-7: $^{3}J_{HH}=15.8$ Hz, H-20 and H-21: $^{3}J_{HH}=15.9$ Hz). The configuration of the trisubstituted double bonds could be deduced by comparison of the intensity of cross-peaks in a Jump Symmetrized (JS)-ROESY spectrum. The JS-ROESY experiment[9] overcomes artifacts and drawbacks of the conventional ROESY technique, and is favorable for quantitative investigation of spatial proximities between protons in smaller molecules, for which the NOE effect is intrinsically weak. The observation of intense cross-peaks between H-7 and H-9, and between H-21 and H-23, respectively, lead to the conclusion that the trisubstituted double bonds in spirofungin A and B possess *E* configuration.

The data presented so far strongly suggest spirofungin A and B to be stereoisomers. The presence of seven chiral centers in the molecule lead to the theoretical possibility of 64 diastereomers. Significant differences in the ^{13}C chemical shifts between spirofungin A and B (Table 4) indicate differences in local structure. Relative configurations within the spiroketal ring system could be derived from known relations for coupling constants and spatial proximity of protons as well as ^{13}C chemical shifts in six-membered rings. In spirofungin A, the vicinal coupling constant between H-11 and H-12 ($^{3}J_{HH}=11.1$ Hz) reflects the diaxial position of the two protons, whereas the vicinal coupling constant between H-18 and H-19 ($^{3}J_{HH}=5.3$ Hz) indicates an axial-equatorial or diequatorial position. The ^{13}C chemical shifts of the methyl groups Me-12 and Me-18 are typical for an equatorial position in the ring from which it follows that H-18 is axial and H-19 equatorial. This assignment was also supported by observed ROEs between H-20 and H-9, H-10, H-11, and H-17 axial (H-17a), and between H-21 and H-11, which show that the side chain at C-19 exists in an axial position.

The ^{13}C chemical shifts of C-16 and Me-18 in spirofungin B are significantly lower compared to spirofungin A. This can be explained by the well-known γ-effect[10], caused by the axial position of Me-18. ROEs as described above for spirofungin A were not observed, instead the presence of ROEs between H-19 and H-9, H-10b, H-11, and H-17 axial (H-17a), and between H-20 and H-18

††† C–H long range correlations observed in the HMBC spectrum for spirofungin B are essentially the same as for spirofungin A and are therefore not shown.

Fig. 4. Relative stereochemistry of spirofungin A (left) and B (right).



R=CH=C(CH_3)–CH=CH–CH(OH)–CH(CH_3)–CH=CH–COOH

suggested the side chain at C-19 to be in an equatorial position, in agreement with the observed coupling constant between H-18 and H-19 ($^3J_{HH}$=4.2 Hz). The position of Me-12 and of the side chain at C-11 were found to be the same as in spirofungin A (Me-12: $\delta(^{13}C)$=17.60 ppm, equatorial position; coupling constant for H-11 and H-12: $^3J_{HH}$=9.5 Hz, diaxial position). The relative configuration of C-11, C-12, C-18 and C-19 in spirofungin A and B is depicted in Fig. 4.

Biological Properties

The antibiotic activity of spirofungin was determined by the agar plate diffusion assay. The compound was not active against various Gram-positive and Gram-negative bacteria at a concentration of 1 mg/ml, but showed a high activity against yeasts and a moderate activity against filamentous fungi, such as *Botrytis cinerea* and *Mucor miehei*. The minimal inhibition concentration was 15 μg/ml in case of *Candida albicans* ATCC 10231 and *Rhodotorula rubra* Tü 136.

Discussion

The combination of HPLC-DAD and HPLC-MS proved to be highly efficient in the screening for new secondary metabolites in the case of strain *S. violaceusniger* Tü 4113. One of the compounds produced by this strain was identified in the culture filtrate extract as elaiophylin, and three further compounds were predicted to be novel. The production of one of them could be successfully transferred from shaking cultures to the fermenter scale. The compound, named spirofungin, was isolated and its structure was determined as a new polyketide-spiroketal type antibiotic, related to reveromycins, antibiotics produced by a *Streptomyces* strain[11~14]. Reveromycins were detected in an antitumor screening as inhibitors of mitogenic activity induced by epidermal growth factor (EGF). Spirofungin differs in the side chain in position 18 of the spiroketal ring system from reveromycins A, C and D.

The antibiotic activity of spirofungin is restricted to an antifungal action, particularly against yeasts, such as the human pathogen *Candida albicans*. The MIC value of spirofungin is in a range similar to that described for reveromycin A. The mode of antifungal action may be the same as in case of reveromycins which is located in the inhibition of protein synthesis in eukaryotic cells[13].

Experimental

Microorganisms

Strain Tü 4113 was isolated from a soil sample taken in the Otway National Park, Australia, using HV-medium[15] with addition of nikkomycin Z and nalidixic acid, and was identified according to HÜTTER[6] and SHIRLING & GOTTLIEB[7,8] as a strain of *S. violaceusniger*. It is deposited in the culture collection of our institute.

The standard strains for testing the biological activity spectrum were obtained from the stock collection of our laboratory and ATCC.

Fermentation

S. violaceusniger Tü 4113 was cultivated in a 25-liter fermenter (b-20, intensor system; Giovanola) using a production medium consisting of mannitol 2% and cottonseed 2% in tap water (pH 7.5, adjusted with 1 N

NaOH). The fermenter was inoculated with 5 vol-% of shaking cultures grown for 48 hours in 500 ml-Erlenmeyer flasks with one baffle on a rotary shaker at 120 rpm and 27°C in the same medium. For production of spirofungin, the fermentation was carried out at 27°C for 120 hours with an aeration rate of 0.5 v/v/m and an agitation rate of 1100 rpm.

Isolation

Hyflo Super-cel (3%) was added to the fermentation broth which was separated by multiple sheet filtration into culture filtrate and mycelium cake. The mycelium was discarded. The culture filtrate was passed through an Amberlite XAD-16 column (10% resin volume with respect to culture filtrate volume). Impurities were washed out with H_2O-MeOH (40+60) and spirofungin was desorbed with MeOH. The eluate was concentrated *in vacuo* and extracted three times with ethyl acetate. After concentration of the organic layer to dryness, the residue was dissolved in a small amount of MeOH and purified on a Sephadex LH 20 column using MeOH as eluent. The spirofungin containing fractions were combined and concentrated to dryness and had a purity of 66%.

Pure spirofungin was obtained by preparative reversed-phase HPLC using a stainless steel column (250 × 16 mm) filled with 10-μm Nucleosil-100 C-18, and linear gradient elution with 0.5% HCOOH-MeOH, starting from 70% MeOH to 90% MeOH within 15 minutes at a flow rate of 20 ml/minute. The preparative HPLC system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald, Sinsheim), gradient unit (Sepacon GCU-311), and Valco preparative injection valve (6UW; VICI) with a 5-ml sample loop. The UV absorbance of the eluate was monitored simultaneously at 260 and 300 nm by a Gilson spectrophotometer Mod. 116 equipped with a preparative cell.

HPLC-DAD-Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a built-in diode array detector, HP 79994B Pascal-workstation (200 MB hard disk) and HP 79988A software rev. 5.3 (Hewlett-Packard, Waldbronn). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360 and 435 nm without reference wavelength; the spectrum range was from 200 to 600 nm with a 2 nm step and a sampling interval of 640 msec.

For analysis of the culture filtrate, a sample of the fermentation broth was centrifuged (10 minutes, 13,000 × *g*). For analysis of extracts, 5 ml culture filtrate were extracted with the same volume of ethyl acetate; the organic layer was concentrated to dryness and dissolved in 10 vol-% MeOH.

10 μl of the samples were injected onto an HPLC column (125 × 4.6 mm), fitted with a guard column (20 × 4.6 mm) which was packed with 5-μm Nucleosil-100 C-18 (Grom, Herrenberg). The samples were separated by linear gradient elution using solvent 0.1% phosphoric acid as solvent A and acetonitrile as solvent B. The gradient was from 0% to 100% solvent B in 15 minutes with a 1-minute hold at 100% B and a 5-minute post-time at initial conditions, at a flow-rate of 2 ml/minute.

HPLC-MS-Analysis

The system consisted of an ABI 140A HPLC gradient pump (Applied Biosystems, Weiterstadt), and an API III Taga 6000 E mass spectrometer equipped with a nebulizer-assisted electrospray source (Sciex, Thornhill, Canada). The HPLC column eluate was split by a T and introduced into the ion source at a constant flow-rate of 40 μl/minute.

4 μl of the samples were injected onto an HPLC column (100 × 2 mm) packed with 5-μm Nucleosil-120 C-18 (Grom). Samples were separated by linear gradient elution. Solvent A was 0.05% trifluoroacetic acid and solvent B was acetonitrile. The gradient was from 25% to 60% solvent B in 15 minutes at a flow-rate of 350 μl/minute.

HR-FAB-MS

HR-FAB mass spectra were recorded on a Finnigan MAT 711A instrument (Bremen, Germany) in the positive ion mode using a mixture of nitrobenzylic alcohol as matrix. Lithium chloride was added for better ionization. The temperature of the ion source was 35°C.

Derivatization and EI-MS

A small amount of spirofungin was dissolved in MeOH. Into that solution diazomethane dissolved in ether was distilled at 0°C. After completion of the reaction, reagents and solvents were removed with N_2 at room temperature. The product was treated with 20 μl bis(trimethyl-silyl)trifluoracetamide for 15 minutes at 60°C. Again, reagents and solvents were removed with N_2 at room temperature. EI mass spectra were recorded on a Finnigan TSQ 70 instrument using an electron energy of 70 eV.

NMR Spectroscopy

One and two-dimensional NMR spectra were recorded at 300 K on a Bruker AMX2-600 spectrometer (Karlsruhe, Germany) operating at a proton frequency of 600.13 MHz using an inverse triple resonance probe equipped with z-gradients. 8.21 mg of spirofungin were dissolved in 650 μl $CDCl_3$. The spectra were referenced to the signal of residual $CHCl_3$ ($\delta(^1H) = 7.25$ ppm, $\delta(^{13}C) = 75.0$ ppm).

The data set consisted of homo- and heteronuclear NMR spectra. Homonuclear NMR experiments comprised 1D ^1H, clean-TOCSY (total correlation spectroscopy), P.E. COSY (primitive exclusive correlation spectroscopy) and JS-ROESY (jump symmetrized-rotating frame Overhauser effect spectroscopy) experiments. Two TOCSY experiments were acquired, with a spinlock duration of 15.5 ms and 65.5 ms, respectively. The JS-ROESY experiment was carried out using a spinlock of 5 kHz field strength and 190 ms duration.

Heteronuclear NMR experiments comprised 1D ^{13}C, DEPT135, HSQC (heteronuclear single quantum coherence), HMQC-TOCSY (heteronuclear multiple quantum coherence-total correlation spectroscopy) and HMBC (heteronuclear multiple bond correlation) experiments. HMQC-TOCSY experiments included a BIRD filter for suppression of signals from protons bound to ^{12}C. The TOCSY part of the experiments was analogous to that described above. The sweep width in the ^{13}C dimension was set to 220 ppm for the 1D ^{13}C NMR spectrum and to 180 ppm for the DEPT135, HSQC, HMQC-TOCSY and HMBC spectra.

In all NMR spectra the sweep width in the proton dimension was set to 5435 Hz. Two-dimensional NMR spectra were acquired as 512 t_1-increments with a data size of 2048 or 4096 complex points. Pulsed field gradients were applied for coherence selection in JS-ROESY, HSQC, and HMBC experiments. Data processing consisted of apodization with a $\pi/2$ shifted squared sine-bell function, two-fold zero-filling in both dimensions and baseline correction using a fifth order polynome.

Biological Assays

An agar-plate diffusion assay was used to determine the antibacterial spectrum of spirofungin. The test solutions were applied to filter discs (6 mm diameter) and the test plates were incubated for 24 hours at 27°C and 37°C, respectively.

A broth dilution method was used to determine the minimal inhibition concentrations of spirofungin. The antibiotic was dissolved in MeOH, giving a MeOH concentration in the cultures of not more than 5%. The yeasts were grown in a medium consisting of malt extract 1%, yeast extract 0.4% and glucose 0.4% in tap water (pH 5.5). 10^6 Cells/ml were used as inoculum of the complex media and growth inhibition was evaluated after incubation for 24 and 48 hours at 37°C on a rotary shaker.

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