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Laschiatrion, a New Antifungal Agent from a *Favolaschia* Species (*Basidiomycetes*) Active against Human Pathogens†

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Laschiatrion (**1**), a new antifungal antibiotic, was isolated from fermentations of *Favolaschia* sp. 87129. (**1**) exhibits broad *in vitro* activity against several human pathogens while no antibacterial and cytotoxic activities could be detected. The structure was elucidated by spectroscopic techniques. As to our knowledge laschiatrion possesses a new steroid skeleton.

The genus *Favolaschia* comprises some 50 species all growing in warm-temperate to tropical zones[1]. The small and sometimes brightly orange or yellow coloured fruiting bodies develop on a variety of plant debris. Mycelial cultures are obtained from spores which readily germinate on appropriate media. A number of strongly antifungal strobilurins and other bioactive compounds have been isolated from *Favolaschia* species[2~6]. In our continuing search for new antibiotics we detected that *Favolaschia* sp. 87129 when grown in submerged culture produced, besides strobilurins A and F, oudemansin A, 9-methoxystrobilurins A and K, (+)-10α-hydroxy-4-muurolen-3-one, favolon, and a new compound named laschiatrion (**1**) active against *Candida albicans* and other human pathogens. In the following we describe the isolation, biological characterization and structure elucidation of laschiatrion.

Experimental

Fermentation and Isolation

Favolaschia sp. 87129 was maintained and grown as described previously[4]. For the production of laschiatrion 10 liters of a well grown fermentation (Biolafitte C6 fermenter) in YMG-medium (g/liter: yeast extract 4, malt extract 10, glucose 4) were used to inoculate 100 liters of 87125W182 medium (g/liter: glucose 11, malt extract 20, yeast extract 2.2, KH_2PO_4 0.5, $MgSO_4\times7H_2O$ 1; mg/liter: $FeCl_3$ 10, $ZnSO_4\times7H_2O$ 1.78, $CaCl_2$ 55) in a fermenter of Deutsche Metrohm, Filderstadt, Germany (aeration 15 liters/minute, 150 rpm, 24°C). Everyday 100 ml samples were taken. The mycelia were separated by filtration, dried for 12 hours at 80°C and weighed. The culture fluid was extracted with an equal volume of ethyl acetate, the organic phase evaporated and the residue taken up in 1 ml of MeOH. Ten μl of this solution were used for the agar plate diffusion assay with *P. variotii*. After 100~120 hours the mycelia were separated from the culture fluid, washed and lyophilised. Lyophilised mycelia (360 g) were extracted twice with 10 liters of MeOH. The resulting crude product (97 g) was distributed between water and ethyl acetate and the organic phase evaporated. The resulting extract (18 g) was subjected to a flash chromatography on silica gel 60 (column 3.5×80 cm, elution with ethyl acetate). From the resulting product (1.22 g) laschiatrion (**1**) was purified by chromatography on silica gel 60 (column 6×20 cm) and elution with cyclohexane - ethyl acetate 1 : 1 (yield 56 mg). Recrystallization from MeOH yielded 21 mg of pure crystalline laschiatrion (**1**).

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† This paper is dedicated to Professor HANS ZÄHNER on occasion of his 75th birthday.

Spectroscopy

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^{1}J_{CH}=145$ Hz and $^{n}J_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra (FAB, HRFAB) were recorded with a Jeol SX102 spectrometer, LCMS experiments were conducted with a HP 1100 system (APCI, positive mode). UV and IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer. The melting point (uncorrected) was determined with a Reichert microscope, and the optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22°C.

Laschiatrion (**1**)

Laschiatrion (**1**) was obtained as white crystals, mp >230°C. $[\alpha]_D^{22}$ +135 (c 1.2 in $CHCl_3$). UV (MeOH) λ_{max} (log ε) 232 nm (4.18). IR (KBr) 3437, 2963, 1713, 1700, 1622, 1452, 1382, 1279, 1160, 1117, 1070, 710 cm^{-1}. See Table 1 for ^{1}H and ^{13}C NMR data. HRFABMS $[M+Na]^+$ m/z 571.3029 (required for $C_{34}H_{44}O_6Na$, 571.3036).

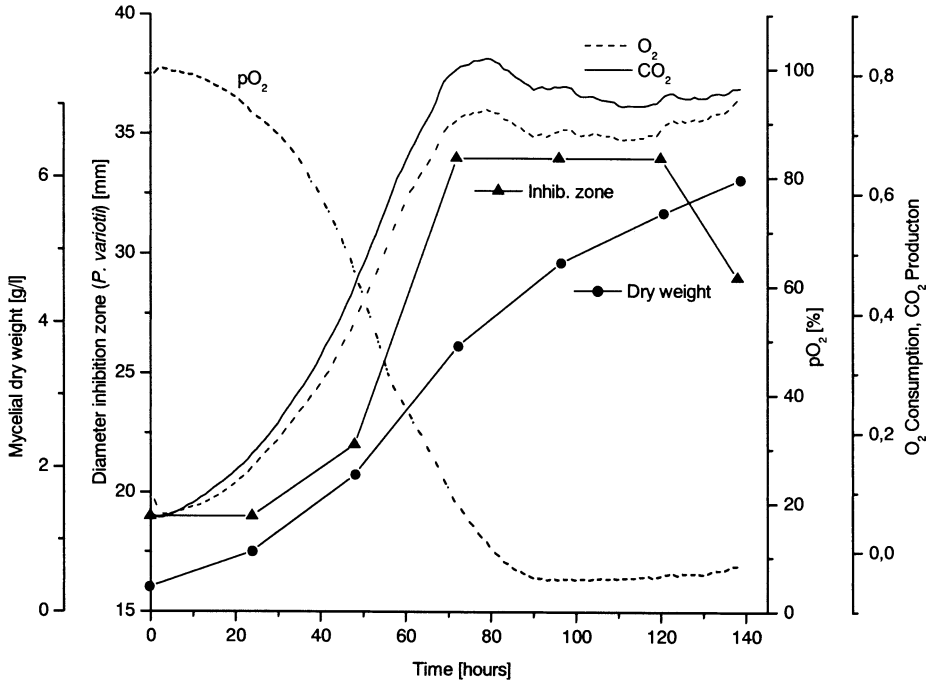
Biological Assays

The antimicrobial spectra were measured as described previously[7]. COLO-320 cells (DSMZ ACC 144, human colon adenocarcinoma) and L1210 cells (lymphocytic leukemia, mouse ATCC CCL219) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 65 μg/ml penicillin G and 100 μl/ml streptomycin sulfate in a humidified atmosphere containing 5% CO_2 at 37°C. Cytotoxicity was measured in microtiter plates with $\sim 1\times10^5$ cells/ml. Cells were incubated with or without the test compounds. After 24, 48 and 72 hours the cells were examined under the microscope and the percentage of lysed cells counted[4].

Results and Discussion

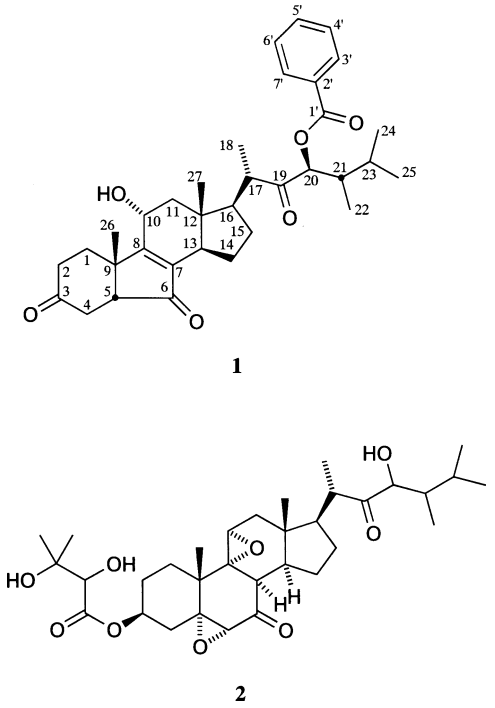
Fig. 1 shows a typical fermentation of *Favolaschia* sp. 87129. The antifungal activity of the culture broth was found to be predominantly due to strobilurins and

Fig. 1. Fermentation of *Favolaschia* 87129 in a 100-liter batch.



For experimental details see the experimental section.

Fig. 2. Structures of laschiatrion (**1**) and favolon (**2**).



1

2

oudemansins.

From the mycelia of *Favolaschia* sp. 87129 a new compound, laschiatrion (**1**) that structurally is not related to the strobilurins, was obtained and its chemical structure (Fig. 2) was elucidated by spectroscopic techniques. Mass spectrometry with FAB ionisation gave both the ions *m/z* 531 and 549, indicating that the compound easily loses a molecule of water, and this was confirmed by LCMS measurements with APCI ionisation. However, FAB measurements with added NaAc gave a strong peak for *m/z* 571, suggesting that the molecular weight of the compounds actually is 548. High resolution measurements showed that the composition of this ion is $C_{34}H_{44}O_6Na$, and this is consistent with the number of protons and carbons suggested by 1D NMR experiments (^1H and ^{13}C NMR data are shown in Table 1). The composition of laschiatrion (**1**), $C_{34}H_{44}O_6$, reveals that the molecule has 13 unsaturations. With one benzoyl group (accounting for five unsaturations), one carbon–carbon double bond and three keto functions, laschiatrion consequently is tetracyclic. The structure of **1** was determined based on COSY and HMBC data, and pertinent HMBC correlations are shown in Fig. 3 (top). The methyl singlet (26-H_3) gives strong HMBC correlations to

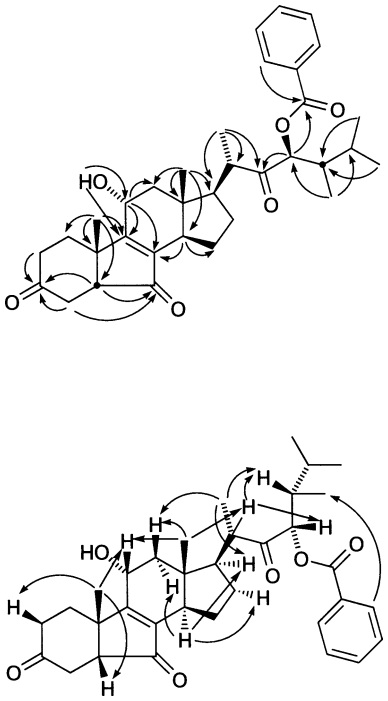
Table 1. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data for laschiatrion (**1**).

C	^1H (δ; multiplicity; *J*)	^{13}C (δ; multiplicity)
1α	1.94; m	31.0; t
1β	2.52; m	
2α	2.22; m	35.8; t
2β	1.95; m	
3	-	210.6; s
4α	2.73; dd; 4.7, 16.8	37.7; t
4β	2.57; dd; 7.3, 16.8	
5	2.34; dd; 4.7, 7.3	52.4; d
6	-	207.3; s
7	-	140.2; s
8	-	172.2; s
9	-	44.1; s
10	4.86; m	67.7; d
11α	1.62; dd; 10.1, 12.1	47.5; t
11β	2.57; m	
12	-	46.7; s
13	2.30; m	46.1; d
14α	2.27; m	22.3; t
14β	1.43; m	
15α	2.10; m	28.1; t
15β	1.41; m	
16	1.87; m	50.3; d
17	2.79; qd; 6.7, 10.4	44.5; d
18	1.17; d; 6.7	16.6; q
19	-	209.5; s
20	5.56; d; 2.0	80.5; d
21	2.00; ddq; 2.0, 7, 7	39.9; d
22	1.02; d; 6.8	12.4; q
23	1.69; dqq; 7, 7, 7	31.1; d
24	1.01; d; 6.8	20.8; q
25	1.00; d; 6.8	20.4; q
26	1.44; s	24.9; q
27	0.66; s	12.2; q
1'	-	165.7; s
2'	-	129.8; s
3'/7'	8.08; d; 8.4	129.8; d
4'/6'	7.46; dd; 8, 8	128.5; d
5'	7.59; dd.; 8, 8	133.2; d
10-OH	1.81; brs	

The spectra were recorded in $CDCl_3$, and the chemical shifts (δ) are given in ppm relative to the solvent signals (7.26 ppm for $CHCl_3$ in ^1H NMR and 77.0 for $CDCl_3$ in ^{13}C NMR). The coupling constants *J* are given in Hz. The multiplicity of the ^{13}C NMR signals were determined from the HMQC data.

C-1, C-5, C-8 and C-9, and the clear COSY correlations between 1-H_2 and 2-H_2 as well as between 4-H_2 and 5-H together with the HMBC correlations from 2-H_2, 4-H_2 and 5-H to C-3 close the first ring (the A-ring) of laschiatrion (**1**). Both 10-OH and 10-H give HMBC correlations to C-8, and 10-H also correlates to C-7 and C-11, and together with the HMBC correlations observed from 27-H_3 to C-11, C-12, C-13 and C-16 as well as the HMBC correlation between 13-H and C-7 closes a second six-membered ring

Fig. 3. Pertinent HMBC (top) and NOESY (bottom) correlations observed with laschiatrion (**1**).



(the C-ring). COSY as well as HMBC correlations between 13-H and 14-H_2, between 14-H_2 and 15-H_2, and between 15-H_2 and 16-H_2 close the third ring (the D-ring), which is five-membered. HMBC correlations from 18-H_3 to C-16, C-17 and C-19 as well as COSY correlations between 16-H and 17-H, and between 17-H and 18-H_3, together with the HMBC correlation from 20-H to C-19 and the clear COSY/HMBC correlations in the remaining part of the open system places the oxidised and substituted 2-heptyl group on C-16. The 20-OH is obviously acylated, as no signal for the proton can be observed in the ^{1}H NMR spectrum and a strong HMBC correlation between 20-H and C-1′ is present, and the nature of the acyl group as a benzoyl is evident from the COSY/HMBC correlations in the aromatic ring and to the carbonyl group. This leaves nothing but the forth ring (the B-ring) to close with the two remaining loose ends, C-6 and C-7. Although no HMBC correlations from for example 10-H or 13-H to C-6 could be observed, this is the only remaining alternative.

The relative configuration shown in Fig. 2 was suggested by the NOESY correlations observed, and summarised in Fig. 3 (bottom). 26-H_3 give a NOESY correlation to 5-H indicating that the A-ring and the B-ring are *cis*-fused, and this is supported by the relatively small ^{1}H-^{1}H coupling constants observed between 4-H_2 and 5-H suggesting that 5-H is equatorial. In addition, 26-H_3 give a NOESY correlation to 10-H suggesting that the C-9 methyl group is positioned on the same side of the molecule as 10-H. Also 27-H_3 give a NOESY correlation to 10-H, as well as to 11-Hβ and (weakly) to 5-H, placing 27-H_3 on the same side. 13-H gives NOESY correlations to 11-Hα, 15-Hα and 16-H, suggesting that they all are on the other side of the molecule, and the large ^{1}H-^{1}H coupling constant between 10-H and 11-Hα, confirm their *trans* relationship and that both are axial. The C-ring and the D-ring are consequently *trans*-fused. There are strong NOESY correlations between 27-H_3 and 17-H, and between 18-H_3 and 11-Hβ as well as 16-H, showing that the configuration of C-17 is as indicated in Fig. 2. 18-H_3 also give a strong NOESY correlation to 21-H while 17-H correlates with 20-H as well as with 21-H. This shows that the predominant conformation of **1** is as depicted in lower structure of Fig. 3, with 20-H and 21-H close to 17-H and 18-H_3. The NOESY correlation between 3′/7′-H_2 and 22-H_3 indicate that the benzoyl group and the C-21 methyl group are on the same side in this predominant conformation, and that is only possible with the proposed configuration. However, to determine the relative configuration of a non-cyclic part of a molecule based only on NOESY data is difficult, and the configuration of the open-chained part of laschiatrion (**1**) shown in Figs. 2 and 3 should be regarded as a proposition.

To our knowledge laschiatrion (**1**) possesses a new steroid skeleton, although the taiwaniasterols isolated from *Taiwania cryptomerioides*[8] also have a steroid ring system with a contracted B-ring but with different side chains. Chemically it should be noted that **1** contains an α,β-unsaturated keto functionality, a Michael acceptor, that could be suspected to be responsible for its biological activity. However, when examining a molecular model of the compound it is evident that the β-carbon of this function is sterically very hindered which makes it unlikely that laschiatrion (**1**) has electrophilic properties.

Laschiatrion (**1**) exhibits broad *in vitro* antifungal activity against *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus flavus*, *Fusarium verticillioides*, *Trichphyton mentagrophytes* and *Microsporum gypseum* at concentrations of 10~50 μg/ml. Favolon (**2**), a triterpenoid with a different ring system and substitution pattern exhibits higher activities at concentrations starting from 1 μg/ml (Table 2). In the agar plate diffusion assay no antibacterial activity of laschiatrion (**1**) was detected against *Enterobacter dissolvens*, *Micrococcus luteus*, *Bacillus brevis* and *Bacillus subtilis* at 50 μg/disk. When tested as described in the experimental section, laschiatrion (**1**) exhibited no

Table 2. Antifungal activity of laschiatrion (**1**) and favolon (**2**) in the agar diffusion assay.

	Laschiatrion (**1**)		Favolon (**2**)	
	μg/disk			
	Diameter inhibition zone (mm)			
	10	50	0.1	1
Mucor miehei[a]	-	16	24	30
Paecilomyces variotii[a]	-	16	20	30
Penicillium notatum[a]	-	15i	12	23
Nematospora coryli[a]	-	-	-	-
			μg/disk	
			10	50
Candida albicans[b]	-	11	+	10
Cryptococcus neoformans[b]	-	11	-	10
Aspergillus fumigatus[b]	-	20	25	31
Aspergillus flavus[b]	10	22	22	30
Fusarium verticillioides[b]	-	10	-	19
Trichophyton mentagrophytes[b]	-	33	28	28

Diameter paper disk: 6 mm
-: no inhibition zone
i: inhibition zone incomplete
[a] YMG-medium containing (g/l): malt extract, 10; glucose, 4; yeast extract, 4; agar 20. *Penicillium notatum* and *Nematospora coryli* were grown at 27°C, *Mucor miehei* and *Paecilomyces variotii* at 37°C.

[b] Sabouraud-Agar, 37°C

After applying the paper discs (diameter 6 mm) containing the compounds, the agar plates (diameter 9 cm, containing 20 ml of medium) were incubated for 24 to 48 hours.

cytotoxic activities against Colo 320 (human) and L1210 (mouse) cells at 50 μg/ml.

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References

1) SINGER, R.: A monograph of *Favolaschia*. Beihefte zur Nova Hedwigia 50: 1～108, 1974
2) ZAPF, S.; A. WERLE, T. ANKE, D. KLOSTERMEYER, B. STEFFAN & W. STEGLICH: 9-Methoxystrobilurine-Bindeglieder zwischen Strobilurinen und Oudemansinen. Angew. Chemie 107: 255～257, 1995
3) ANKE, T.; A. WERLE, S. ZAPF, R. VELTEN & W. STEGLICH: Favolon, a new antifungal triterpenoid from a *Favolaschia* species. J. Antibiotics 48: 725～726, 1995
4) ZAPF, S.; A. WUNDER, T. ANKE, D. KLOSTERMEYER, W. STEGLICH, R. SHAN, O. STERNER & W. SCHEUER: (+)-10-a-Hydroxy-4-muurolen-3-one, a new inhibitor of leukotriene biosynthesis from a *Favolaschia* species. Comparison with other sesquiterpenes. Z. Naturforsch. 51c: 487～492, 1996
5) WOOD, K. A.; D. A. KAU, S. K. WRIGLEY, R. BENEYTO, D. V. RENNO, A. M. AINSWORTH, J. PENN, D. HILL, J. KILLACKY & P. DEPLEDGE: Novel β-methoxyacrylates of the 9-methoxystrobilurin and oudemansin classes

produced by the basidiomycete *Favolaschia pustulosa*. J. Nat. Prod. 59: 646～649, 1996

6) NICHOLAS, G. M.; J. W. BLUNT, A. L. J. COLE & M. H. G. MUNRO: Investigation of the New Zealand basidiomycete *Favolaschia calocera*: Revision of the structures of 9-methoxystrobilurins K and L, strobilurin D, and hydroxystrobilurin D. Tetrahedron Lett. 38: 7465～7468, 1997

7) ANKE H.; O. BERGENDORF & O. STERNER: Assays of the biological activities of guaiane sesquiterpenes isolated from fruit bodies of edible *Lactarius* species. Food Chem. Toxicol. 27: 393～397, 1989

8) LIN, W.-H.; J.-M. FANG & Y.-S. CHENG: Diterpenoids and steroids from *Taiwania cryptomerioides*. Phytochem. 48: 1391～1397, 1998