Scytalols A, B, C, and D and Other Modulators of Melanin Biosynthesis from *Scytalidium* sp. 36-93

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From submerged cultures of *Scytalidium* sp. 36-93, ten metabolites were isolated due to their effects on dihydroxynaphthalene (DHN) or DOPA melanin biosynthesis. Four of the compounds, scytalols A (**1a**), B (**1b**), C (**2**), and D (**3**), are new secondary metabolites, the structures of which were determined by spectroscopy, while the other compounds are known. Scytalols A (**1a**) and D (**3**) are selective inhibitors of DHN melanin biosynthesis and exhibit no antifungal or cytotoxic activities. Nectriapyrone, 6-methoxymellein and 4-chloro-6-methoxymellein stimulated the formation of DOPA melanin in B16-F1 melanoma cells in the absence of melanin-stimulating hormone.

In plant pathogenic fungi like *Pyricularia oryzae* and *Colletotrichum* species, the formation of melanized appressoria is prerequiste for successful invasion of the host[1]. The pigments of these fungi are synthesized *via* the polyketide pathway with 1,8-dihydroxynaphthalene being the intermediate which is polymerized by laccases[2]. In *Streptomyces* species, animals or melanoma cells dark pigments are formed *via* the DOPA pathway[3]. In order to detect selective inhibitors for both pathways, a screening using *Lachnellula* sp. A32-89[4], *Streptomyces bikiniensis*[5] and B16-F1 cells[5] as test systems was conducted. Extracts of *Scytalidium* sp. 36-93 exhibited strong inhibitory activity in all three test systems. Fractionations of the extracts revealed that several compounds were responsible for the activities. Further, the extracts contained nonselective as well as selective inhibitors of DHN melanin synthesis. Activity-guided isolation led to ten metabolites, of which four are new structurally related secondary metabolites for which we propose the names scytalols A (**1a**), B (**1b**), C (**2**), and D (**3**). Six of the active metabolites are known fungal metabolites, although they have previously been reported from other species: nectriapyrone[6], mycorrhizin A[7], (3*Z*)-dechloromycorrhizin A[7], 6-methoxymellein[7], 4-chloro-6-methoxymellein[7] and linoleic acid[8]. In the present paper, the producing organism, the production and isolation of the compounds, their biological activities and structural elucidation will be described.

Materials and Methods

General

Materials used for preparative HPLC were obtained from Jasco. Preparative HPLC was performed with a Jasco MD910 diode array detector. Analytical HPLC was carried out on Hewlett-Packard HP 1090 Type II equipped with diode array detector. UV spectra were obtained with a Perkin Elmer λ 16, and IR spectra with a Bruker IFS 48. The optical rotation was measured with a Perkin Elmer 1541 polarimeter with a cell path of 10 cm. EI-MS (direct inlet, 70 eV) and FAB-MS spectra (direct inlet, positive ions) were recorded with a Jeol JMS-SX102 spectrometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX 500 spectrometer with an inverse 5 mm probe equipped with a shielded gradient coil. COSY, HMQC and HMBC experiments were performed with gradient enhancements using sine shaped gradient pulses, and for the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH} = 145$ Hz

and $^2J_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001).

Producing Organism

Mycelial cultures of *Scytalidium* sp. 36-93 were obtained from an infected fruiting body of a basidiomycete growing on wood. The strain is deposited in the herbarium of the department Biotechnology, University of Kaiserslautern. The culture is grown and kept on YMG agar containing g/liter: glucose 4, malt extract 10, yeast extract 4, agar 20.

Fermentation

Fermentations were carried out in YMG medium (see above), in a 20-liter fermenter (Braun Biostat U) at 22°C with an aeration rate of 3.2 liters/minute and agitation (120 rpm). As inoculum, a well grown shake culture (200 ml in a 500 ml-Erlenmeyer flask) in the same medium was used. Fermentations were stopped after 150 hours, when the glucose in the medium was used up and the production of active compounds, measured in agar diffusion assays with *Lachnellula* sp. A32-89 and *S. bikiniensis* had reached a plateau. During fermentations, aliquots of the culture fluid (50 ml) were extracted twice with ethyl acetate. The combined extracts were concentrated *in vacuo* (40°C). The oily residue was dissolved in 1 ml of methanol and this solution was used to determine the inhibition of melanin biosyntheses.

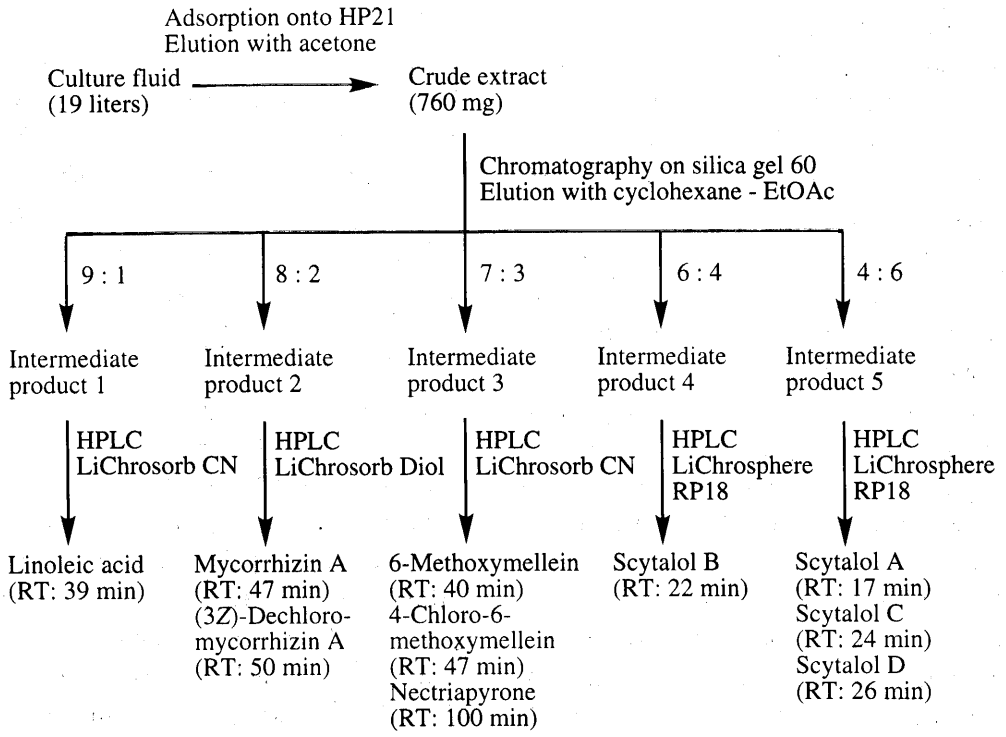
Isolation of the Compounds

The culture fluid from one fermentation (19 liters) was applied onto Misubishi HP 21 resin. The column (4 × 40 cm) was washed with 2 liters of H_2O. The bioactive compounds were eluted with 2 liters of acetone. The acetone was evaporated *in vacuo* to an aqueous residue, which was extracted twice with ethyl acetate. After evaporation of the solvent from the combined ethyl acetate extracts, 760 mg of an oily crude product was obtained. Chromatography on silica gel 60 (25~40 μm; column size 150 × 25 mm) in cyclohexane - ethyl acetate yielded intermediate products 1~5, eluting with cyclohexane - ethyl acetate 9 : 1, 8 : 2, 7 : 3, 6 : 4 and 4 : 6. From these intermediate products the pure compounds were obtained by HPLC on different phases *e.g.* LiChrosorb Diol or LiChrosorb CN (7 μm; 250 × 25 mm; flow rate 5 ml/minute) with a cyclohexane - methyl *tert*-butyl ether gradient (0~15 minutes: 30%; 15~90 minutes: 30~100%), or LiChrosphere RP 18 (7 μm; 250 × 25 mm; flow rate 5 ml/minute) with a water - acetonitrile gradient (0~120 minutes: 0~100%), for further details see Fig. 1.

Biological Assays

Inhibition of DHN melanin biosynthesis was followed

Fig. 1. Isolation scheme for the metabolites produced by *Scytalidium* sp. 36-93.



using *Lachnellula* sp. A32-89 in agar cultures[4]. DOPA melanin biosynthesis in *S. bikiniensis* and B16-F1 cells (ATCC CRL 6323) was measured according to TOMITA *et al.*[5]. Cytotoxic activities were measured as described by ZAPF *et al.*[9]. Antifungal activities were tested in the agar diffusion assay.

Results and Discussion

Taxonomy of the Producing Organism

Strain 36-89 was isolated from the fruiting body of a basidiomycete growing on wood. The colonies are felt like and blackish with smooth hyphae. In older cultures, hyphal fragmentation yields to black arthroconidia of various shapes *e.g.* cylindrical, ellipsoidal or oblong. These characteristics fit the description of the genus *Scytalidium* Pesante[10,11]. Species of this genus are found on wood and in soil and are not described to be mycophilic or mycoparasitic. Therefore we assume that arthroconidia of a wood inhabiting strain were dispersed onto the basidiomycete growing in the same habitat.

Fermentation and Isolation of the Metabolites

During the fermentation of *Scytalidium* sp. 36-93 in YMG medium, inhibition of melanin biosynthesis in the *Lachnellula* test system was observed after 72 hours. The cultures were harvested after 150 hours, when the glucose and maltose in the medium were used up and the bioactivity in the above test had reached a plateau. At this time a red pigment was detected, which was identified as scytalone, an intermediate of the DHN melanin biosynthesis known to be produced by *Scytalidium* species and many other dematiaceous fungi[12] and which accumulates in some albino mutants of DHN forming fungi[13]. This indicated that the melanin pathway was also blocked in our cultures. In addition, cultures of strain 36-93 which did not produce melanin biosynthesis inhibitors usually became black after several days for example in corn meal medium.

When, during the isolation of the DHN melanin biosynthesis inhibitors (see Fig. 1), fractions eluting from the first silica gel column were tested in our three test systems, fraction 1 showed inhibitory activity in the DHN melanin as well as in the DOPA melanin assays. Fraction 2 exhibited antimicrobial, cytotoxic and DHN and DOPA melanin inhibitory activities. Fraction 3 however, stimulated the melanin synthesis in the absence of melanin stimulation hormone (MSH) in B16-F1 cells. Fraction 5 inhibited only the synthesis of DHN melanin. The isolation of the metabolites responsible for the different activities is shown in Fig. 1. Fraction 4 contained a metabolite with an UV spectrum similar to the spectra of the active metabolites from fraction 5 therefore, this metabolite was also purified. The yields of the compounds from 19 liters of culture filtrate were as follows: 9.7 mg of linoleic acid, 3.9 mg of mycorrhizin A, 2.1 mg of (3*Z*)-dechloromycorrhizin A, 4.2 mg of 4-chloro-6-methoxymellein, 5.5 mg of 6-methoxymellein, 14 mg of nectriapyrone, 12.1 mg of scytalol B, 46.8 mg of scytalol A, 14.8 mg of scytalol C and 32.9 mg of scytalol D. Nectriapyrone was identified by comparison of the spectral data with the literature[6]. Mycorrhizin A, dechloromycorrhizin A, 6-methoxymellein and 4-chloro-6-methoxymellein were identified by comparison with the compounds isolated from *Lachnum papyraceum* in our lab[7].

Structure Determination of Scytalols A (**1a**) and B (**1b**)

The physico-chemical properties of the four new compounds are given in Table 1, while their 1D NMR data are given in Table 2 (^1H and ^{13}C NMR data). Pertinent long-range ^1H-^{13}C correlations observed in the HMBC spectra and NOESY correlations are summarized in Figure 2. The NMR spectra of scytalols A (**1a**) and B (**1b**) are very similar, and the presence of the signals for an additional methoxy group in the NMR spectra of the latter as well as the difference in polarity between the two compounds suggested that **1b** is an *O*-methyl derivative of **1a**. This was confirmed by high resolution mass spectrometry, which showed that the elemental composition of the compounds are $C_{15}H_{18}O_6$ and $C_{16}H_{20}O_6$, respectively. The unsaturation index of 7 dictates that **1a** and **1b** possess three rings as the NMR data suggest the presence of one carbonyl group and three carbon-carbon double bonds. ^1H-^1H correlations observed in the COSY spectrum revealed the spin system from 1-H_2 to 2-H to 11-H to 10-H as well as to 12-H_2. The presence of a 1,2,3,5-tetrasubstituted benzene moiety in all four compounds is supported by the observation of the typical coupling between 6-H and 8-H (2.4 Hz). Further, two of the substituents are hydroxyl and carbonyl groups neighboring each other as suggested by the presence of a hydrogen bonded proton (observable in $CDCl_3$). The structures of **1a** and **1b** could be established after the analysis of the HMBC data (see Figure 2), from which the position of the acetal and the keto functions were determined. The relative stereochemistry of the compounds was determined by NOESY 2D NMR experiments (see Figure 2). For all four com-

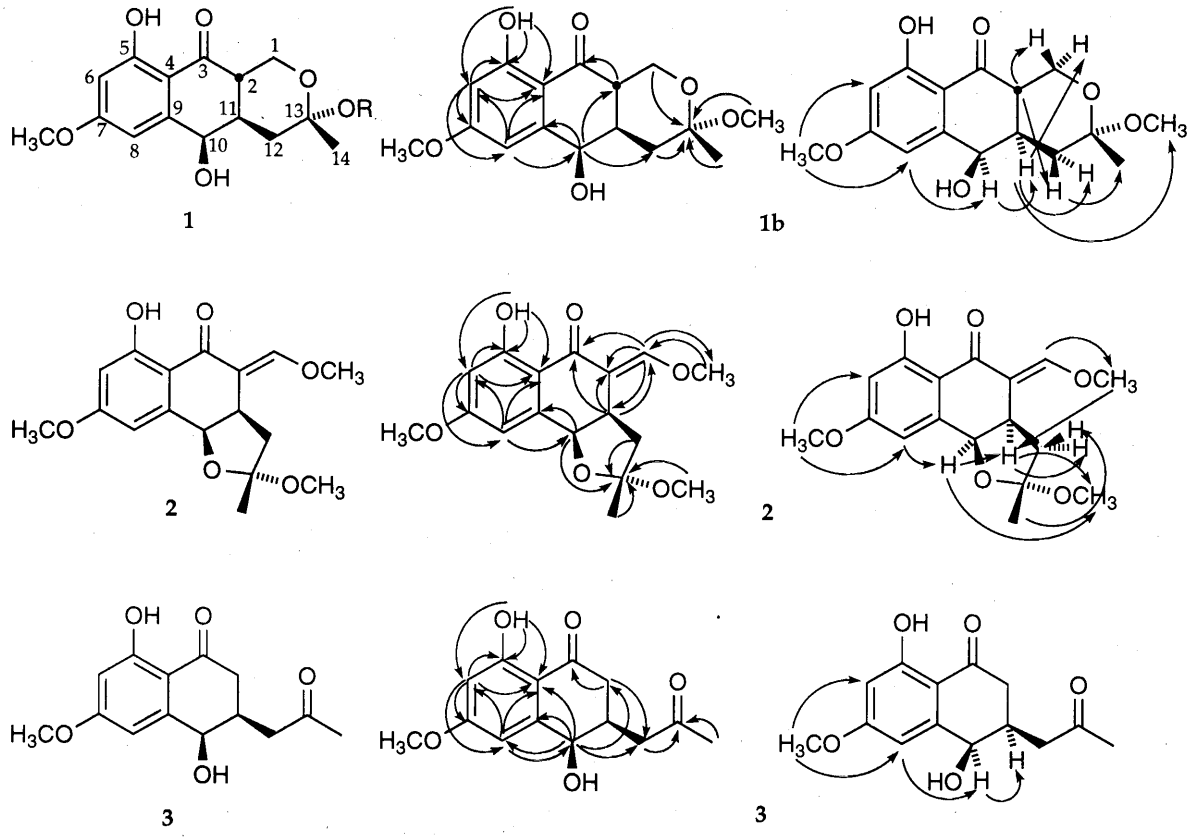
Table 1. Physico-chemical properties of scytalols A (**1a**), B (**1b**), C (**2**) and D (**3**).

	1a	**1b**	**2**	**3**
Appearance	White crystals	White crystals	White crystals	Colourless oil
MP (°C)	165~169	135~138	169~171	
$[\alpha]_D^{22}$	+89° (*c* 0.9 in CH_3OH)	+133° (*c* 1.0 in $CHCl_3$)	−18° (*c* 1.0 in $CHCl_3$)	+9° (*c* 0.9 in $CHCl_3$)
Molecular formula	$C_{15}H_{18}O_6$	$C_{16}H_{20}O_6$	$C_{17}H_{20}O_6$	$C_{14}H_{16}O_5$
HREI-MS (*m/z*)				
Observed	294.1112 M^+	308.1261 M^+	320.1277 M^+	264.0983 M^+
Calculated	294.1103 for $C_{15}H_{18}O_6$	308.1260 for $C_{16}H_{20}O_6$	320.1260 for $C_{17}H_{20}O_6$	264.0998 for $C_{14}H_{16}O_5$
EI-MS (*m/z*)	276 (74%), 258 (29%), 234 (42%), 218 (88%), 216 (61%), 206 (75%), 204 (54%), 191 (32%), 190 (31%), 179 (81%), 151 (100%), 115 (17%)	293 (21%), 276 (72%), 258 (18%), 234 (49%), 217 (100%), 206 (47%), 180 (56%), 179 (56%), 151 (77%)	305 (15%), 289 (30%), 247 (39%), 231 (36%), 204 (28%), 149 (24%)	248 (100%), 247 (71%), 205 (54%), 204 (65%), 191 (91%), 190 (48%), 151 (25%)
UV (CH_3CN)				
λ_{max} nm (ε)	233 (20,300), 283 (29,700), 320 (14,100)	232 (18,500), 280 (25,600), 318 (12,300)	230 (19,200), 281 (26,600), 319 (12,800)	234 (15,800), 280 (18,500), 319 (8,400)
IR (KBr) cm^{-1}	3500, 3385, 2980, 1640, 1615, 1575, 1495, 1395, 1375, 1315, 1260, 1205, 1195, 1165, 1145, 1080, 1020, 875 and 845	3440, 2940, 1630, 1435, 1385, 1300, 1225, 1205, 1160, 1080, 1045, 970, 855 and 810	3025, 1935, 1690, 1630, 1590, 1505, 1230, 1195, 1155, 1030, 880 and 780	3445, 2940, 1715, 1630, 1435, 1360, 1305, 1265, 1205 and 1160

Table 2. ^1H (500 MHz) (δ; multiplicity; *J*) and ^{13}C (125 MHz) NMR data (δ; multiplicity) for scytalols A (**1a**), B (**1b**), C (**2**) and D (**3**) in $CDCl_3$ (**1b**, **2** and **3**) or $CDCl_3$:CD_3OD 9:1 (**1a**), with the $CHCl_3/CDCl_3$ signals (7.26 and 77.0 ppm) as references. The coupling constants *J* are given in Hz.

	1a	**1b**	**2**	**3**
H/C				
1α/1	3.86; dd; 11, 11/59.3; t	3.63; dd; 11.1, 11.3/59.8; t	7.61; s/160.6; d	—/—
1β	4.08; dd; 5.1, 11.3	4.23; dd; 5.1, 11.3	—	—
2α/2	—/40.9; d	—/40.8; d	—/112.8; s	2.58; dd; 4.1, 17.3/39.2; t
2β	2.90; ddd; 5, 11, 12	3.04; ddd; 5.1, 11.1, 12.2	—	2.73; dd; 9.3, 17.3
3/3	—/201.8; s	—/201.7; s	—/188.8; s	—/201.2; s
4/4	—/109.2; s	—/109.2; s	—/109.2; s	—/109.2; s
5/5	—/165.0; s	—/165.9; s	—/165.4; s	—/165.6; s
6/6	6.23; d; 2.4/100.3; d	6.35; d; 2.4/100.8; d	6.34; d; 2.4/100.2; d	6.35; d; 2.4/100.5; d
7/7	—/166.0; s	—/166.2; s	—/166.1; s	—/166.5; s
8/8	6.32; d; 2.4/108.1; d	6.38; d; 2.4/108.1; d	6.63; d; 2.4/106.6; d	6.50; d; 2.4/107.0; d
9/9	—/146.4; s	—/145.9; s	—/144.6; s	—/146.3; s
10/10	4.30; m/68.9; d	4.45; d; 2.4/69.8; d	5.06; d; 8.1/74.8; d	4.78; d; 2.6/69.5; d
11/11	2.42; m/37.0; d	2.57; dddd; 2.4, 3.8, 12.2, 13/36.8; d	3.87; m/35.0; d	2.83; m/34.9; d
12α/12	1.64; dd; 3.5, 13.2/36.6; t	1.79; dd; 3.8, 13.1/37.5; t	2.41; dd; 7.2, 12.4/44.0; t	2.82; m/44.0; t
12β	1.81; dd; 13, 13	1.96; dd; 13, 13	1.62; dd; 11.6, 12.4	2.51; d; 12.3
13/13	—/94.9; s	—/97.8; s	—/107.2; s	—/207.9; s
14/14	1.33; s/29.0; q	1.37; s/23.4; q	1.37; s/22.7; q	2.17; s/30.5; q
1-OCH_3	—/—	—/—	3.94; s/62.2; q	—/—
5-OH	—/—	12.68; s/—	13.58; s/—	12.71; s/—
7-OCH_3	3.71; s/55.3; q	3.82; s/55.6; q	3.84; s/55.5; q	3.83; s/55.6; q
13-OCH_3	—/—	3.21; s/47.9; q	3.35; s/49.0; q	—/—

Fig. 2. Structures and numbering of the scytalols (left), and pertinent correlations observed in the HMBC (middle) and NOESY (right) spectra of scytalols B (**1b**), C (**2**) and D (**3**).



The corresponding correlations were observed with scytalol A (**1a**). **1a**: R=H; **1b**: R=CH_3.

pounds a strong correlation between 10-H and 11-H was observed, indicating that the two are located at the same side of the ring. For scytalols A (**1a**) and B (**1b**), 11-H also gives NOESY correlations to 1-Hα and 12-Hα, while 2-H correlates with 1-Hβ and 12-Hβ. As $J_{2\sim11}$ is large (12 Hz), it is therefore reasonable to conclude that 2-H and 11-H are trans and diaxial in **1a** and **1b**. According to the ^{1}H-^{1}H coupling constants, 1-Hα and 12-Hβ should also be axial, and NOESY correlations between 13-OCH_3 and 1-Hα as well as 11-H shows that this methoxy group is directed as indicated in Figure 1. The methyl group (C-14) should therefore be on the same side as 2-H and equatorial, which is in agreement with the observed NOESY correlations between 14-H_3 and 12-Hα as well as 12-Hβ in both scytalol A (**1a**) and scytalol B (**1b**). In addition, the small coupling constant between 10-H and 11-H shows that 10-H is equatorial.

Structure Determination of Scytalol C (2) and D (3)

According to high resolution mass spectrometry, the elemental composition of scytalol C (**2**) is $C_{17}H_{20}O_6$. According to the ^{1}H and ^{13}C NMR data, it contains one additional double bond compared to scytalols A and B, and three methoxy groups. The chemical shifts for the tetrasubstituted benzene moiety are similar to those of scytalols A and B, although C-3 is shifted upfield and C-10 and 10-H downfield. The HMBC correlation (see Figure 2) between one of the methoxy protons and a protonated olefinic carbon resonating at 160.6 ppm in the ^{13}C NMR spectrum suggested the presence of a enol methyl ether, and this functionality turned out to be a good starting point for the unravelling of the structure. This methoxylated double bond is conjugated to the keto function, according to the correlations shown in Figure 2 while the C-10 oxygen is part of a tetrahydrofuran ring, which explains the differences in the NMR data (*vide supra*). The NOESY correlations observed between 13-OCH_3 and 10-H, 11-H as well as 12-Hα place the four on the same side of the five-membered ring, while 14-H_3 correlate to 12-Hβ. A correlation between 1-OCH_3 and 11-H, and the lack of a correlation between 1-H and

11-H, suggests that the C-1/C-2 double bond is *E*, as indicated in Figure 1. The ^{13}C NMR data of the left part (C-3 through C-10) of scytalol D (**3**) is almost identical to those of scytalols A (**1a**) and B (**1b**). However, the elemental composition of scytalol D (**3**) is $C_{14}H_{16}O_5$, suggesting that it has lost an oxygenated carbon compared to scytalol A (**1a**). Its unsaturation index is 7, and with three carbon-carbon double bonds and two keto functions this suggests the presence of two rings of which one is the benzene ring. COSY and HMBC correlations (see Figure 2) show that the missing carbon is C-1, presumably lost by decarboxylation during the biosynthesis. The similar situation as in **1a** and **1b**, with a strong NOESY correlation and a small ^1H-^1H coupling constant between 10-H and 11-H, is observed with scytalol D (**3**), and the relative stereochemistry as well as conformation should be the same.

Two of the new metabolites, scytalols B (**1b**) and C (**2**), are methyl acetals, and could be suspected to be formed chemically during extraction and work-up. However, as they are obtained also during carefully controlled extraction and work-up conditions when no methanol is present, they are believed to be true natural products. In addition, both methyl acetals were obtained as single isomers, which would not be expected it they were formed chemically. This is in agreement with previous reports about the isolation of acetals of heptaketide naphthoquinones[14,15] as true metabolites. The scytalols are heptaketides related to the fusarubins. However, while most heptaketides with this skeleton reported are naphthoquinones, with keto functions on both C-3 and C-10 (*e.g.* 5-deoxyfusarubin[16]), and with an additional hydroxyl group on C-8 as well as a C-2/C-11 double bond (*e.g.* the antibiotics fusarubin[17] and herbarin[18], or derivatives thereof), the compounds reported here are comparably less oxidized.

Biological Activities

The activities of the isolated metabolites are summarized in Tables 3 and 4. As can be extracted from

Table 3. Inhibition of growth and pigment biosynthesis in agar cultures of *Lachnellula* sp. A32-89 (DHN melanin) and *Streptomyces bikiniensis* (DOPA melanin) by metabolites from *Scytalidium* sp. 36-93.

Compound	Inhibition zone (melanin biosynthesis/growth) [mm]			
	10	50	100	200 μg
A: *Lachnellula* sp. A32-89				
Mycorrhizin A	12/—[a]	14/12	19/14	n.t.[b]
(3*Z*)-Dechloromycorrhizin A	—/—	14/10	18/13	n.t.
Scytalol A	—/—	—/—	12/—	15/—
Scytalol D	—/—	—/—	13/—	15/—
Linoleic acid	—/—	10/—	15/—	n.t.
B: *Streptomyces bikiniensis*				
Mycorrhizin A	10/8	13/10	16/13	n.t.
(3*Z*)-Dechloromycorrhizin A	9/8	12/10	15/12	n.t.
Linoleic acid	10/—	13/—	17/—	n.t.

[a] —: No inhibition.
[b] n.t.: Not tested.

Table 4. Stimulation of DOPA melanin biosynthesis in B16-F1 cells by nectriapyrone, 6-methoxymellein, 4-chloro-6-methoxymellein and rolipram in comparison to the control stimulated with 10^{-6} M α-MSH.

Compound	Melanin biosynthesis (% of the control)				
	2	5	10	50	100 (μg/ml)
Rolipram	33.6	70.9	99.5	100	100
Nectriapyrone	14.2	16.6	21.1	40.4	95.8
6-Methoxymellein	13.9	16.4	23.0	45.6	90.2
4-Chloro-6-methoxymellein	14.4	15.9	16.8	37.8	85.4

Table 3, besides the already reported antimicrobial, cytotoxic and nematicidal activities[19], the mycorrhizins inhibited the formation of pigments. This inhibition occurred at lower concentrations as compared to inhibition of growth in *S. bikiniensis* and *Lachnellula* sp. A32-89. Linoleic acid inhibited DHN and DOPA melanin biosyntheses. Only scytalols A and D selectively affected DHN melanin synthesis. Scytalols B (**1b**) and C (**2**) were not active. The fact that scytalol A (**1a**) is active and B (**1b**) is not, indicate that the open (C-13 keto) form of scytalol A (**1a**) is responsible for the activity. That would also explain why scytalols A (**1a**) and D (**3**), which only differ in the C-2 hydroxymethyl group present in the C-13 keto form of **1a**, possess similar bioactivities. In B16 F-1 cells, MSH-stimulated melanin synthesis was inhibited by 50% at 50 μg/ml of linoleic acid, the other compounds had no effect, except the mycorrhizins which were highly cytotoxic (data not shown). In cells without MSH added, melanin synthesis was stimulated by nectriapyrone and the melleins as shown in Table 4. The three compounds were equally active, but much less than rolipram[20].

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