Lachnellins A, B, C, D, and Naphthalene-1,3,8-triol, Biologically Active Compounds from a *Lachnellula* Species (Ascomycetes)

Martin Semar[a], Heidrun Anke[a], Wolf-Rüdiger Arendholz[b], Robert Velten[c] and Wolfgang Steglich[c]

[a] LB Biotechnologie and
[b] Botanik der Universität, Paul-Ehrlich-Str. 23, D-67663 Kaiserslautern, Bundesrepublik Deutschland
[c] Institut für Organische Chemie der Universität, Karlstr. 23, D-80333 München, Bundesrepublik Deutschland

Z. Naturforsch. **51c,** 500–512 (1996); received March 1/April 29, 1996

Lachnellin A, B, C, D, Glyoxylate Cycle, Naphthalene-1,3,8-triol, Malate Synthase, Melanization, *Lachnellula* sp.

In the course of our search for new biologically active metabolites, lachnellin A (**1**), a metabolite with high cytotoxic and antimicrobial activities, the structurally related lachnellins B, C and D (**3, 4, 7**), and naphthalene-1,3,8-triol (**8**), an inhibitor of malate synthase (EC 4.1.3.2), were isolated from submerged cultures of the ascomycete *Lachnellula* sp. A 32–89. The antimicrobial, cytotoxic and phytotoxic activities of lachnellin A depended on its reactivity and could be abolished by the addition of cysteine. The enzyme inhibiting activity of (**8**) was due to reactive intermediates during melanization and was no longer observed in the presence of serum albumin. In addition, *rac*-scytalone (**9**), (+)-*trans*-3,4-dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone (**10**), 2,5-dihydroxytoluene (**11**), and (*R*)-(-)-5-methylmellein (**12**) were obtained from the same source and biologically characterized.

Introduction

The glyoxylate cycle is the essential reaction sequence for fatty seedlings for synthesis of sugars and other cellular components from the acetyl-CoA produced by β-oxidation of storage triglycerides (Zubay, 1984). The cycle plays a crucial role during growth of microorganisms on acetate, oily or fatty substrates and during the germination of uredospores of rust fungi (Kornberg, 1989; McCammon *et al.*, 1990; Frear and Chet, 1961). Since it is not found in mammals, the key enzymes of the cycle (isocitrate lyase and malate synthase) might provide ideal targets for selective herbicides and fungicides. In our *in vitro* screening for inhibitors of the malate synthase, cultures of a *Lachnellula* species, strain A 32–89, exhibited enzyme inhibiting activity. In addition, the culture broth showed strong antimicrobial activity which was not correlated with the inhibition of malate synthase.

Reprint requests to Prof. Dr. H. Anke.
Fax: 0631/205 29 99.

The enzyme inhibitor was identified as naphthalene-1,3,8-triol (**8**). The new antimicrobial compound lachnellin A (**1**) and the structurally related lachnellins B (**3**), C (**4**), and D (**7**) were isolated and their structures elucidated. Additional metabolites were identified as *rac*-scytalone (**9**), (+)-*trans*-3,4-dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone (**10**), 2,5-dihydroxytoluene (**11**) and (*R*)-(-)-5-methylmellein (**12**). In the following we describe the fermentation, isolation, physicochemical properties, and structures of these metabolites and their biological characterization.

Experimental

General

Spectral data were recorded on the following instruments: ^{1}H, and ^{13}C NMR, Bruker AC-200, AM-400 and AMX-600; EI-MS, A.E.I. MS-50; IR, Bruker FT-IR IFS 48 and Perkin-Elmer 1420; UV, Perkin-Elmer Lambda 16 and Varian Cary 17M; CD, Jobin Yvon CNRS Roussel-Jouan Dichrographe III. Optical rotations were recorded with a Perkin-Elmer 241 polarimeter. The mps were determined with a Reichert hot-plate microscope

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and are uncorrected. TLC was performed on aluminium foils coated with silica gel 60 F_{254} (Merck, Darmstadt, Germany) in toluene – acetone (7:3).

Lachnellula sp. A 32–89

Strain A 32–89 was grown and maintained on YMG agar (yeast extract 0.4%, glucose 0.4%, malt extract 1%, pH 5.5 with 2% agar). The culture is deposited in the culture collection of the Lehrbereich Biotechnology of the University of Kaiserslautern, Germany.

Biological assays

Preparation of the cell free extracts: Cell free extracts from seeds of *Ricinus communis* var. *zanzibarensis* with high malate synthase activity were prepared according to the method of Cooper and Beevers (1969). Plant material was homogenized using liquid nitrogen; cell fragments and glyoxysomes were sedimented at 270 x *g* (10 minutes) and 15000 x *g* (30 minutes), respectively.

Malate synthase assays: In order to test the inhibitory activity of the methanolic extracts an enzyme assay with the radioactive substrate ([1-^{14}C]acetyl-CoA) was used. Three µl of the concentrated (1:50) methanolic extracts were added to 100 µl of the reaction mixture (potassium phosphate buffer 100 mM, pH 7.5; sodium glyoxylate 120 µM; $MgCl_2$ 5 mM; Li_3-acetyl-CoA 100 µM [0.05 µCi]). The reaction was initiated by addition of the enzyme (500–700 ng protein) and incubated at 37 °C for 15 minutes then stopped by heating for 3 minutes at 95 °C. Malate was separated from acetyl-CoA by TLC on aluminium foils coated with cellulose F (Merck, Darmstadt, No. 5574) in diethyl ether – formic acid – H_2O (5:2:1) and quantified by an automatic TLC-linear analyzer (Berthold, Wildbad, Germany).

In addition, malate synthase activity was measured by an indirect optical assay according to the method of Hock and Beevers (1966). The reaction mixture contained in a final volume of 350 µl: potassium phosphate buffer 100 mM, pH 7.5; sodium glyoxylate 120 µM; $MgCl_2$ 5 mM; 5,5'-dithio-bis-(2-nitrobenzoic acid) 130 µM; Li_3-acetyl-CoA 100 µM; cell free extract with 1.8–2.5 µg protein. The reaction was initiated by the addition of glyoxysomes and the formation of CoA-SH was followed spectrophotometrically. Enzyme activity was recorded on a Biorad 96-well plate spectrophotometer model 2550 EIA READER at 405 nm.

In vivo inhibition of the enzymes of the glyoxylate cycle in *Penicillium notatum:* An agar diffusion assay with *P. notatum* as test organism, grown on a mineral salt medium with acetate or succinate as carbon source, was developped. The composition of the medium was as follows (g/liter): $NH_4H_2PO_4$ 5 g; KH_2PO_4 2,5 g, $MgSO_4$ x 7 H_2O 1 g, $Ca(NO_3)_2$ x 4 H_2O 20 mg, $FeCl_3$ x $6H_2O$ 2 mg, 5 ml Hoagland A-Z solution [(mg/liter): H_3BO_3 611 mg, $MnCl_2$ 389 mg, $CuSO_4$ 56 mg, $ZnSO_4$ x 7 H_2O 56 mg, $Al_2(SO_4)_3$ x 18 H_2O 56 mg, $NiSO_4$ x 6 H_2O 56 mg, $Co(NO_3)_2$ x 6 H_2O 56 mg, TiO_2 56 mg, $(NH_4)_6Mo_7O_{24}$ x 4 H_20 56 mg, LiCl 28 mg, $SnCl_2$ 28 mg, KI 28 mg, KBr 28 mg] and acetate or succinate buffer (pH 5.5; 4 g free acid/liter) respectively. The pH value was adjusted to 5,5 with 5 N NaOH. The agar slants (diameter 9 cm) containing 10 ml medium with 5 x 10^5 spores/ml were evaluated after 4 days of incubation at 27 °C.

Inhibition of chitin synthase from *Coprinus cinereus* and avian myeloblastosis virus-reverse transcriptase were tested as described by Wenke *et al.* (1993) and Erkel *et al.* (1992).

The collagen stimulated bovine platelet aggregation assay was carried out as described previously (Lauer *et al.*, 1991).

Hemolytic activity was measured with bovine erythrocytes (10^5 cells/ml in phosphate buffered saline) after incubation at 37 °C for 30 minutes. Triton X-100 (2 µl/ml) was used as a positve control (100% hemolytic activity). The free hemoglobin was measured in the supernatant at 540 nm.

Tests for cytotoxicity towards L1210 cells (ATCC CCL 219), RBL-1 cells (ATCC CCL 219), HeLa S3 cells (ATCC CCL 2.2), and BHK 21 cells (ATCC CCL 10) were carried out as described previously (Erkel *et al.,* 1992). After suitable intervals the cells were examined under the microscope or after staining (monolayers) according to the methode of Mirabelli *et al.* (1985).

Macromolecular syntheses in L1210 cells were measured as described by Lorenzen *et al.* (1994).

The minimal inhibitory concentrations (MIC) were determined in the serial broth dilution assay in YMG-medium (fungi) or nutrient broth (bacteria). *Acinetobacter calcoaceticus* and *Micrococcus luteus* were incubated at 27° C other bacteria at 37° C. *Mucor miehei* and *Paecilomyces variotii*

were incubated at 37° C. Inoculum was 1 x 10^5 cells/ml (bacteria and yeasts) and 5 x 10^4 spores/ml (filamentous fungi).

Mutagenicity was tested according to the method of Ames *et al.* (1975) and Maron and Ames (1983). *Salmonella typhimurium* strain TA 98 and strain TA 100 were used for the spot test without and the plate incorporation test with and without rat liver microsomes.

Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested as described by Anke *et al.* (1989).

Fermentation

Fermentations were carried out in 5-liter Erlenmeyer flasks or in a 20-liter Biolafitte C-6 fermentor in two media. DM: malt extract 40 g/liter; MGPY (g/liter): maltose 20 g, glucose 10 g, pepton 2 g, yeast extract 1 g, KH_2PO_4 0.5 g, $MgSO_4$ x 7 H_2O 1 g, $FeCl_3$ 10 mg, $ZnSO_4$ x 7 H_2O 1.78 mg, $CaCl_2$ x $2H_2O$ (0.1 M) 5 ml. The flasks containing 2 liters of medium were inoculated with pieces of an overgrown agar slant (YMG agar), fermentors with 250 ml of a well grown shake culture (same medium as for the fermentation). Fermentors were incubated at 22 °C with aeration (6 liters air/minute) and agitation (160 rpm). Production of naphthalene-1,3,8-triol (**8**) was recorded and calculated by HPLC. Production of lachnellin A (**1**) was followed by the agar diffusion assay using *Nematospora coryli* Peglion, ATCC 10647, as a test organism.

Isolation of the metabolites

Naphthalene-1,3,8-triol (**8**), *rac*-scytalone (**9**) and 2,5-dihydroxytoluene (**11**) were obtained from cultures in MGPY-medium harvested when the concentration of **1** had reached its peak. The broth (total 5 liters) obtained by centrifugation (3000 x *g*) was extracted twice with equal volumes of EtOAc. Evaporation of the organic solvent yielded an oily crude extract (3 g) which was subjected to silica gel chromatography (Merck 60, 60–200 μm, column size: 17 x 5 cm). After elution with cyclohexane – EtOAc (1:1) an enriched product (350 mg) was obtained. Compounds **8** (25 mg) and **9** (26 mg) were separated by HPLC on LiChrosorb Diol (Merck, 7 μm, column 25 x 2.5 cm) and elution with cyclohexane – *tert*-butyl methyl ether (3:7). Elution with cyclohexane – *tert*-butyl methyl ether (4:6) yielded 5 mg of **11**. Isolation and purification of the lachnellins and cometabolites from DM-medium are shown in Fig. 2.

Results and Discussion

Producing organism

The producing organism was isolated from the bark of *Polylepis sericea* (Rosaceae), Laguna Negra, Venezuela. The culture was obtained from ascospores of the stalked, hairy, orange coloured fruiting bodies measuring 3 mm in diameter and up to 4 mm in height. The structure of the ectalexcipulum of a thin-walled textura porrecta, the 3- to 5-celled cylindrical and obtuse, wholly granulated hairs and the filiform paraphyses strongly suggest a membership of *Lachnellula*, although the substrate is not part of a conifer, which is the typical host for most members of this genus (Dennis, 1962; Dharne, 1965; Raitviir, 1970). The poor preservation of the hymenia and the scanty material did not allow a reliable classification of the species. Formation of apothecia or conidia was not observed either on agar or in submerged cultures.

Fermentation and isolation of metabolites

During fermentations of *Lachnellula* sp. A 32–89 in MGPY medium, the disappearance of maltose and the simultaneous increase of the glucose concentration indicated the production of an α-glucosidase (Fig. 1, upper part). Inhibition of malate synthase was observed after 170 hours and the production of naphthalene-1,3,8-triol (**8**) could be measured by HPLC after 190 hours (Fig. 1, lower part). The production of lachnellin A (**1**) started after 120 hours, reached a first maximum and during production of **8**, **1** disappeared completely, even though this compound is not a direct precursor of **8**. After 260 hours, the concentration of **8** decreased, at the same time the culture filtrate turned dark green. During this stage of the fermentation, biomass formation ceased while production of **1** restarted and reached a second peak after glucose was completely used up. Whereas the production of naphthalene-1,3,8-triol (**8**) in flasks

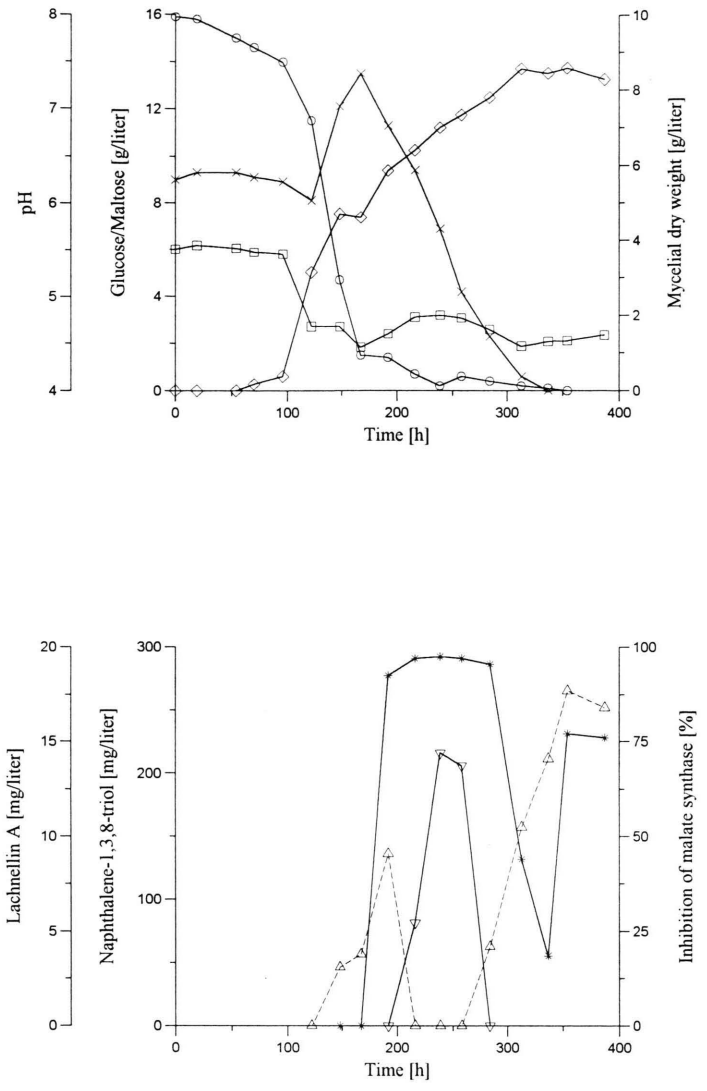


Fig. 1. Fermentation of *Lachnellula* sp. A32–89 in 2-liter MGPY-medium (5-liter Erlenmeyer flask). o–o: maltose; x–x: glucose; □–□: pH value; ◊–◊ mycelial weight; *–*: inhibition of malate synthase; ▽–▽: naphthalene-1,3,8-triol; △– – –△: lachnellin A.

was reproducible, fermentations in 20-liter scale yielded different amounts ranging from 0 mg/liter to 4 mg/liter. Yields in DM medium were considerably lower (max. 2 mg/liter). From flask cultures compounds **8** (5 mg/liter) and **9** (5 mg/liter) were isolated (see experimental section).

Whereas 2,5-dihydroxytoluene (**11**) was only detected in MPGY medium, production of lachnellins A-D (**1**, **3**, **4**, **7**) was observed in MGPY and DM medium. The metabolites were located in the broth. The isolation and purification of the lachnellins and of their cometabolites **9**, **10** and **12** from the culture broth is shown in Fig 2.

Structure elucidation

Lachnellin A (**1**), $C_9H_{12}O_3$, is an optically active oil, $[\alpha]_D^{20}$ -170° (*c* 0.45, $CHCl_3$), with UV-maxima at 236 (lg ε = 3.88) and 284 nm (4.04). The IR spectrum (KBr) contains a broad band in the OH-region at 3440 cm^{-1} and three intensive carbonyl absorptions at 1718, 1663 and 1635 cm^{-1}. From an analysis of the ^1H and ^{13}C NMR data (Tables I and II) formula **1** can be proposed for the antibiotic. It is in accord with the formation of a bis-(2,4-dinitrophenyl)hydrazone derivative and strong MS fragments at *m/z* 125 and 43 (base peak) which result from α-cleavage at the acyloin moiety. The

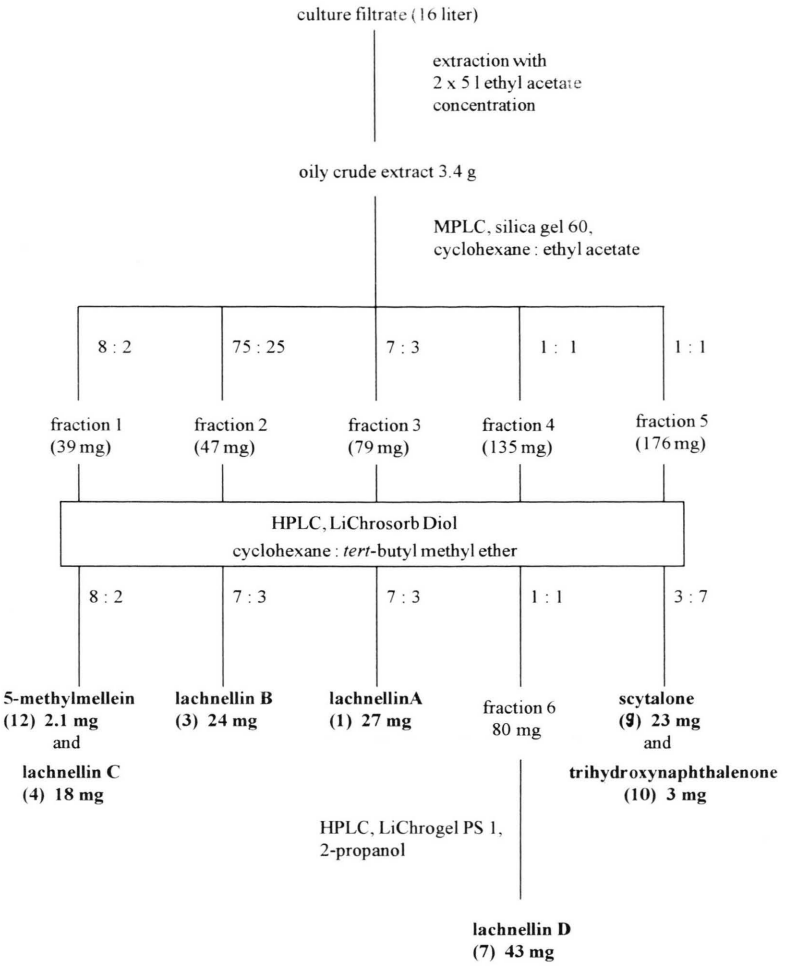


Fig. 2. Isolation and purification of the lachnellins and co-metabolites from DM-medium.

Table I. ^{1}H NMR spectral data for lachnellins A~D (**1, 3, 4, 7**) (in $CDCl_3$, 400 MHz, δ in ppm).

	1[a]	**3**	**4**	**7**[a,b]
1-H	2.01 (s)	1.43 (s)	1.43 (s)	1.50[f]/1.53[f]/2.20[g] (s)
3-H	4.96 (d)[c]	4.07 (s, br)	4.19 (d, br)[c]	4.12~4.66 (m)
3-OH	4.05 (d)[d]	[e]	2.14 (d)[d]	[e]
5-H	7.10 (dd)	6.25 (dm)	6.08 (dm)	6.07~6.45 (m)
6-H	7.00 (ddq)	5.87 (ddq)	5.89 (ddq)	5.62~5.99 (m)
7-H	6.28 (dqd)	5.74 (dq)	5.70 (ddm)	5.62~5.99 (m)
8-H	1.95 (dd)	1.76 (d, br)	1.75 (d, br)	1.77/1.81/1.82 (d)
9α-H	10.22 (s)	4.31 (dd)	4.40 (dm)	4.12~4.66 (m)
9β-H	–	4.58 (d, br)	4.44 (dm)	4.12~4.66 (m)
2-OCH_3	–	3.23 (s)	3.24 (s)	–

1: *J* (Hz): 3,3-OH = 4.5; 5,6 = 11.2; 5,7 = 1.0; 6,7 = 12.8; 6,8 = 1.2; 7,8 = 6.8
3: *J* (Hz): 5,6 = 11.0; 5,9α = 2.5; 6,7 = 15.0; 6,8 = 1.5; 7,8 = 6.5; 9α,9β = 13.5
4: *J* (Hz): 5,6 = 11.0; 6,7 = 14.7; 7,8 = 6.5; 9α,9β = 13.5; 3,3-OH = 11.7
7: *J* (Hz): 7,8 = 6.5

[a] 200 MHz; [b] solvent: $CDCl_3$; [c] singlet after addition of D_2O; [d] signal disappears after addition of D_2O; [e] not observed; [f] keto form; [g] ketal form.

Table II. ^{13}C NMR spectral data for lachnellins A~D (**1,3,4,7**) (100.6 MHz, δ in ppm).

	1[a]	**3**[a]	**4**[a]	**7**[b]	
				ketal form	keto form
C-1	25.08	15.59 (q, 127[c])	18.04 (qd, 127[c], 2)	21.57/23.91	25.46
C-2	206.48	108.99 (m)	103.28 (m)	102.70/106.99	[h]
C-3	74.60	77.95 (dm, 154[c])	78.48 (dm, 147[c])	78.01/79.25	82.08
C-4	132.74	139.64 (m)	138.80 (m)	139.74/140.77	136.34
C-5	148.91[d]	125.24[e] (dm, 154[c])	121.27[f] (dm, 152[c])	122.51/125.76[g]	127.66
C-6	144.18[d]	131.79[e] (dm, 154[c])	130.21[f] (dm, 154[c])	130.60/131.94[g]	134.14
C-7	124.12	127.38[e] (dm, 152[c])	127.13[f] (dm, 150[c])	128.59/128.87[g]	133.70
C-8	19.38	18.29 (qdd, 126[c], 6, 4)	18.29 (qdd, 126[c], 6, 5)	18.41/18.44	18.52
C-9	188.57	66.46 (tdd, 148[c], 11, 4)	66.34 (td, 148[c], 9)	66.72/67.33	57.12
H_3CO-2	–	48.39 (q, 142[c])	48.19 (q, 142[c])	–	–

[a] Solvent: $CDCl_3$; [b] solvent: CD_3OD; [c] $^1J_{C,H}$ in Hz; [d–g] assignments may be interchanged; [h] carbonyl signal not clearly observed.

(*E*)-configuration of the terminal double bond ($^3J_{H\text{-}6,H\text{-}7}$ = 12.8 Hz) is supported by a strong IR absorption at 972 cm^{-1} (KBr) and the position of the 8-methyl carbon resonance at δ 19.06 (Kalinowski *et al.*, 1984). The H-6 signal appears at δ 7.00 which indicates deshielding of this proton by the aldehyde group (Ananthasubramanian *et al.*, 1978) and defines the (*Z*)-configuration of the trisubstituted double bond.

In the presence of acids lachnellin A (**1**) equilibrates with the (4*E*,6*E*)-compound **2** ($[\alpha]_D^{20}$ -35°), which was identified as the enantiomer of the known fungal metabolite (*R*)-(+)-avellaneol ($[\alpha]_D^{25}$ +39°) (Ananthasubramanian *et al.*, 1978). Since the absolute and relative configuration of the latter is known, the (3*S*,4*Z*,6*E*)-stereochemistry of lachnellin A (**1**) is established.

Lachnellins B and C, $C_{10}H_{16}O_3$, are stereoisomers which lack major IR absorptions in the carbonyl region. The ^{13}C NMR spectra (Table II) indicate that the acetyl and formyl groups in **1** have been transformed into acetal and CH_2OR moieties, which suggests structures **3** and **4** for lachnellin B and C, respectively.

The relative configurations of these isomers can be deduced from the chemical shifts of the acetalic carbons. Whereas the C-2 signal of **3** appears at δ 108.99, that of the epimer **4** resonates at δ 103.28. This is in agreement with values of δ 109.1 and 104.7 for methyl α- (**5**) and β-D-fructofuranoside (**6**), respectively (Angyal and Bethell, 1976).

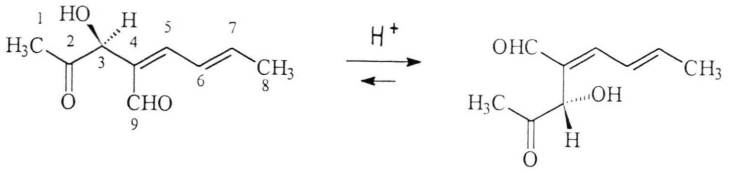
The assignment is supported by the 11.7 Hz coupling between the 3-OH and the vicinal methine proton in the ^1H NMR spectrum of lachnellin C (**4**). The formation of a hydrogen bridge between the hydroxy group and the *cis*-methoxy substituent at C-2 causes a nearly antiperiplanar arrangement of 3-H and 3-OH which is responsible for the large coupling constant (Jackman and Sternhell, 1969).

Lachnellin D, $C_9H_{14}O_3$, exhibits complex NMR spectra, in which signals of the two hemiacetals **7a** and **7c** and the open chain form **7b** can be identified. The equilibrium mixture in deuterochloroform contains approximately 20% of ketone **7b** as determined by integration of suitable ^1H NMR signals.

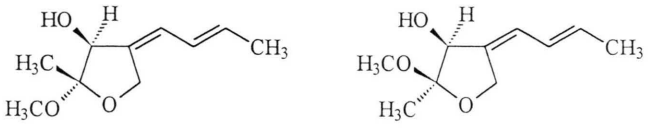
The co-occurrence of the lachnellins A-D with the non-branched pentaketide derivative scytalone (**9**) is in accord with the biosynthesis proposed for (*R*)-(+)-avellaneol in *Hypocrea avellanea* (Nair *et al.*, 1982).

The known metabolites **8, 9, 11**, **12** were identified by comparison of their physical and spectroscopic data with those reported in the literature. (+)-*trans*-3,4-Dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone (**10**) is a new compound of undetermined absolute configuration. Its properties agree well with those of the known (-)-enantiomer from *Pyricularia oryzae* (Iwasaki *et al.*, 1972).

The isolation of **10** together with naphthalene-1,3,8-triol (**8**) and scytalone (**9**) classifies *Lachnellula* sp. A 32–89 as a representative of the naphthalene-1,8-diol (DHN) melanin type (Bell and Wheeler, 1986). DHN melanin is the predominant dark pigment in ascomycetes including many phytopathogens (Yamaguchi *et al.*, 1992). Secondary metabolites from other strains of the genus *Lach-*

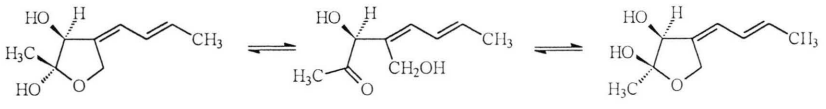


1 **2**

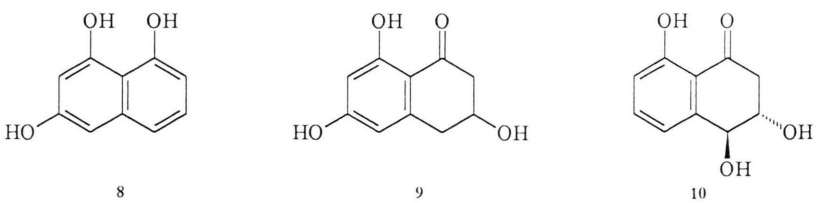


3 **4**

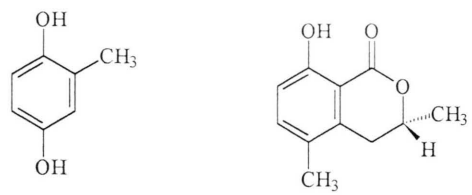
5 **6**



7a **7b** **7c**



8 **9** **10**



11 **12**

nellula are lachnelluloic acid and lachnellulone (Ayer and Villar, 1985).

2,5-Dihydroxytoluene (**11**) is a common fungal metabolite isolated from *Scopulariopsis brumptii* (Huang *et al.*,1989), *Nectria erubescens* (Carney and Nair, 1978), *Penicillium patulum* (Scott and Yalpani, 1967) and a *Phoma* species (Sèquin-Frey and Tamm, 1971). In addition it has been reported from the beetle *Tribolium confusum* (Engelhardt *et al.*, 1965). 5-Methylmellein (**12**) is known from *Hypoxylon illitum* (Anderson *et al.*, 1983), *Valsa ceratosperma* (Okuno *et al.*, 1986), *Fusicoccum amygdali* (Ballio *et al.*, 1966) and from the wood of a *Semecarpus* species (Carpenter *et al.*, 1980).

Physico-chemical properties of the compounds

Lachnellin A (**1**)

Colourless oil; R_f 0.61; $[\alpha]_D^{20}$ -170° (*c* 0.45, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 236 (3.88), 2.84 (4.04); CD λ_{max}^{MeCN} nm (Δε) 230 (-1.76), 242 (0), 260 (+1.99), 270 (0), 289 (-9.86), 311 (0), 355 (+1.03), 450 (0); IR (KBr) cm^{-1} 3440, 1718, 1663, 1635, 1359, 1138, 1072, 972; ^1H NMR, Table I; ^{13}C NMR, Table II; EI-MS (180°C) *m/z* (%) 168.0788 (1, M^+, calcd for $C_9H_{12}O_3$ 168.0790), 150 (2), 139 (3), 125 (51), 108 (41), 80 (44), 79 (59), 43 (100).

Isomerization of lachnellin A (**1**)

Lachnellin A (**1**) (2.5 mg) in MeOH (3 ml) was treated with 1 N aqueous HCl (3 ml) at 20 °C for 30 minutes. After neutralization with 0.2 N aqueous NaOH and extraction with EtOAc, usual work-up gave (*S*)-(-)-avellaneol (**2**) (2 mg) as colourless oil; R_f 0.61; $[\alpha]_D^{20}$ -35° (*c* 0.11, $CHCl_3$), {(*R*)-(+)-avellaneol: $[\alpha]_D^{25}$ +39° (*c* 2.58, $CHCl_3$)}[10]; UV, IR, ^1H and ^{13}C NMR spectral data are in good agreement with those reported in the literature (Ananthasubramanian *et al.*, 1978).

Lachnellin B (**3**)

Colourless oil; R_f 0.61; $[\alpha]_D^{20}$ -80° (*c* 1.20, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 239 (4.48); IR (KBr) cm^{-1} 3430, 2943, 1381, 1160, 1125, 1099, 1072, 1020, 963, 852; ^1H NMR, Table I; ^{13}C NMR, Table II; EI-MS (180°C) *m/z* (%) 184.1101 (1, M^+, calcd for $C_{10}H_{16}O_3$ 184.1100), 153 (3), 135 (17), 110 (59), 109 (27), 95 (100), 81 (55), 79 (30), 75 (57), 67 (59), 43 (77).

Lachnellin C (**4**)

Colourless microcrystals: m.p. 54–56 °C; R_f 0.87; $[\alpha]_D^{20}$ +149° (*c* 0.52, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 239 (4.30); IR (KBr) cm^{-1} 3470, 3414, 1382, 1184, 1158, 1136, 1108, 1097, 1055, 1007, 973, 742; ^1H NMR, Table I; ^{13}C NMR, Table II; EI-MS (180°C) *m/z* (%) 184.1099 (1, M^+, calcd for $C_{10}H_{16}O_3$ 184.1100), 153 (2), 152 (2), 135 (15), 127 (22), 110 (62), 109 (48), 95 (100), 81 (64), 79 (47), 75 (50), 67 (55), 43 (82).

Lachnellin D (**7**)

Colourless oil; R_f 0.40; $[\alpha]_D^{20}$ +42° (*c* 0.95, MeOH); UV λ_{max}^{MeOH} nm (log ε) 239 (4.22); IR (KBr) cm^{-1} 3340, 3280, 2978, 1410, 1191, 1172, 1152, 1136, 1120, 1110, 1086, 1075, 1009, 961, 876, 818; ^1H NMR, Table I; ^{13}C NMR, Table II; EI-MS (180°C) *m/z* (%) 170.0943 (2, M^+, calcd for $C_9H_{14}O_3$ 170.0943), 152 (2), 127 (57), 110 (48), 109 (83), 95 (100), 81 (99), 79 (71), 67 (54), 43 (69).

Naphthalene-1,3,8-triol (**8**)

Colourless oil; R_f 0.45; UV λ_{max}^{MeCN} nm (log ε) 231 (4.62); IR (KBr) cm^{-1} 3390, 1640, 1599, 1478, 1458, 1383, 1278, 1233, 1186; ^1H NMR (200 MHz, CD_3OD) δ 6.46 (1H, dd, J = 7.2 and 2.0 Hz, 7-H), 7.01 (1H, dd, J = 8.2 and 2.0 Hz, 5-H), 7.10 (1H, dd, J = 8.2 and 7.2 Hz, 6-H), 2-H and 4-H signals not observed.

The structure of **1** was confirmed by conversion to the peracetyl derivative. **1** (4 mg) was treated with acetic anhydride (0.5 ml) in THF (5 ml) for 3 hours at 20 °C. Usual work-up and purification by PTLC on silica gel using toluene – acetone (7:3) afforded 1,3,8-triacetoxynaphthalene (3.7 mg) as colourless oil; R_f 0.60; EI-MS (180°C) *m/z* (%) 302.0803 (12, M^+, calcd for $C_{16}H_{14}O_6$ 302.0816), 260 (16), 219 (46), 218 (72), 177 (69), 176 (100); UV, IR and ^1H NMR spectral data are in agreement with those reported in the literature (Findlay and Kwan, 1973).

rac-Scytalone (**9**)

Colourless oil; R_f 0.25; $[\alpha]_D^{20}$ ±0° (*c* 0.20, MeOH), {(Bell *et al.*, 1976) (+)-scytalone $[\alpha]_D^{25}$ +32° (*c* 0.25, EtOH)}; EI-MS (180°C) *m/z* (%) 194.0586 (68, M^+, calcd for $C_{10}H_{10}O_4$ 194.0579), 176 (100), 150 (47); UV, IR, ^1H and ^{13}C NMR

spectral data are in good agreement with those reported in the literature (Findlay and Kwan, 1973; Sankawa *et al.*, 1972; Bell *et al.*, 1976).

(+)-*trans*-3,4-Dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone (10)

Colourless microcrystals: m.p. 191–193 °C, [(Iwasaki *et al.*, 1972) m.p. 191–192 °C]; R_f 0.25; $[\alpha]_D^{20}$ +36° (*c* 0.18, MeOH), {(Iwasaki *et al.*, 1972) $[\alpha]_D$ -36° (MeOH)}; EI-MS (180°C) *m/z* (%) 194.0579 (41, M^+, calcd for $C_{10}H_{10}O_4$ 194.0579), 150 (57), 122 (58), 121 (100); UV, IR and ^1H NMR spectral data are in good agreement with those reported in the literature (Iwasaki *et al.*, 1972).

2,5-Dihydroxytoluene (**11**)

Colourless microcrystals: m.p. 124–125 °C, [(Goodwin and Witkop, 1957) m.p. 125–127 °C]; R_f 0.52; UV λ_{max}^{MeOH} nm (log ε) 203 (4.18), 216 (sh, 3.71), 226 (sh, 3.64), 293 (3.50); IR (KBr) cm^{-1} 3320, 1502, 1385, 1200, 819, 772, 726; ^1H NMR (200 MHz, CD_3OD) δ 2.12 (3H, s), 6.44 (1H, dm, J = 8.6 Hz), 6.54 (1H, m), 6.57 (1H, d, J = 8.6 Hz); EI-MS (180°C) *m/z* (%) 124.0526 (100, M^+, calcd for $C_7H_8O_2$ 124.0524).

(*R*)-(-)-5-Methylmellein (**12**)

Colourless microcrystals: m.p. 123–126 °C, [(Ballio *et al.*, 1966; De Alvarenga *et al.*, 1978) m.p. 126–127 °C]; R_f 0.83; $[\alpha]_D^{20}$ -136° (*c* 0.23, $CHCl_3$), [(De Alvarenga *et al.*, 1978) $[\alpha]_D^{21}$ -115° ($CHCl_3$)]; EI-MS (180°C) *m/z* (%) 192.0786 (100, M^+, calcd for $C_{11}H_{12}O_3$ 192.0786), 174 (24), 148 (26), 120 (21); UV, IR and ^1H NMR spectral data are in good agreement with those reported in the literature (Ballio *et al.*, 1966; Carpenter *et al.*, 1980; De Alvarenga *et al.*, 1978).

Biological properties

Among the metabolites isolated from cultures of *Lachnellula* sp. A 32–89, naphthalene-1,3,8-triol (**8**) was the only compound to inhibit malate synthase from *Ricinus communis in vitro*. In methanolic solutions, **8** easily underwent polymerization (melanization) and the inhibition of malate synthase was correlated to the degree of melanization which was measured spectrophotometrically at 500 nm (Table III). The disappearance of **8** could be followed by HPLC. HPLC analysis revealed no additional low molecular weight products. Melanin (polymerization products) could not be detected under these conditions. As soon as naphthalene-1,3,8-triol (**8**) was totally polymerized equalling an optical density of 9.4, the inhibition of malate synthase was drastically reduced. Higher substrate concentrations had almost no effect on the inhibition. Addition of bovine serum albumin (400 μg/ml) protected the enzyme and no inhibiton was observed. These results are in agreement with reports on noncompetitive inhibition of various enzymes by melanin and its precursors and the formation of irreversible enzyme-melanin complexes (Bell and Wheeler, 1986). One of the natural function of melanin is the protection of fungal walls against UV damage and hydrolytic attack (Huang *et al.*, 1989). It also functions as a radical sink. Thus it was not surprising, that **8** was also not active in the *in vivo* assay with *Penicillium notatum* grown on acetate or succinate supplemented mineral salt medium (Table IV). Known inhibitors of the enzymes of the glyoxylate cycle for example oxalate, glycolate (Zollner, 1989) and itaconate (McFadden and Purohit, 1977), exhibited selective effects and growth was only affected when acetate as carbon source was used, conditions under which the glyoxylate cycle is induced and essential for growth. Fungicides like nystatin, actidione or mi-

Table III. Effect of the degree of melanization of naphthalene-1,3,8-triol (**8**) (1 mg/ml MeOH) on the inhibition of malate synthase (optical test). The amount needed for 50% inhibition of the enzyme activity is given.

Degree of melanization E_{500}	Enzyme inhibition IC_{50} [μg/ml]
0.18	120.0
0.25	60.0
0.37	35.0
0.57	27.0
0.68	15.0
0.87	10.0
1.14	7.5
1.43	5.0
2.29*	2.5–5
3.70*	2.5
6.04*	1.25–2.5
7.74*	1.25–2.5
8.84*	1.25–2.5
9.04*	>20.0

*: Solution diluted (1:4).

Table IV. Inhibition of *Penicillium notatum* grown on acetate or succinate supplemented mineral salt medium by inhibitors of the glyoxylate cycle, standard inhibitors and naphthalene-1,3,8-triol (**8**).

Inhibitor (target*)	Concentration (μg/disc)	Inhibition zone acetate [mm]	succinate [mm]
Inhibitors of the glyoxylate cycle			
Oxalate (m)	2000	25	–**
Glycolate (m)	2000	24	–
Itaconate (i)	2000	16	–
Standard fungicides			
Nystatin (cm)	2	20	21
Actidion (pb)	100	20	18
Miconazol (eb)	1	18	20
Nikkomycin z (cw)	6	–	30
Strobilurin A (r)	1	60	35[d]
Naphthalene-1,3,8-triol (**8**)	400	–	–

*m = malate synthase; i = isocotrate lyase; cm = cytoplasm membrane; pb = protein biosynthesis; eb = ergosterin biosynthesis; cw = cell wall; r = respiration cain.
**: no inhibition zone; [d] = inhibition zone diffuse.

conazol were equally effective on both media. Strobilurin A, an inhibitor of respiration (Anke, 1995), was more effective on acetate medium.

The antimicrobial spectra of compounds **8**, **1** and **11** are given in Table V. Compounds **3, 4, 7, 9** and **10** were not active at 100 μg/ml. Lachnellin A (**1**) and naphthalene-1,3,8-triol (**8**) had very weak antibacterial and antifungal activities, 2,5-dihydroxytoluene (**11**) was slightly more active towards bacteria and *Nematospora coryli*, but was inactive towards filamentous fungi. Compounds **1** and **11** were cytotoxic towards all four cell lines at 1–10 μg/ml, **3** and **4** had no effects on the cells at concentrations up to 100 μg/ml (Table VI). Compounds **7, 8, 9,** and **10** were moderately cytotoxic. Biosynthesis of all macromolecules in L1210 cells was totally inhibited at 5 μg/ml of **1** (Fig. 3). Compounds **9** and **10** had no effect on macromolecular syntheses at 100 μg/ml. Compound **7** inhibited the incorporation of leucine, uridine and thymidine

Table V. Antibacterial and antifungal activity of naphthalene-1,3,8-triol (**8**), lachnellin A (**1**), and 2,5-dihydroxytoluene (**11**) in the serial dilution assay.

Orgamism	MIC (μg/ml) Compound **8**	**1**	**11**
Acinetobacter calcoaceticus	100	50	50
Proteus vulgaris DSM 300119	100	50	25
Salmonella typhimurium Ta 98	100	50	25
Bacillus subtilis ATCC 6633	>100	>100	25
Bacillus brevis ATCC 9999	100	100	25
Staphylococcus aureus DSM 346	100	100	10
Micrococcus luteus ATCC 381	100	100	25
Penicillium notatum	>100	100	>100
Paecilomyces variotii ETH 114646	>100	100	>100
Mucor niehei TÜ 284	>100	50	>100
Candida albicans TÜ 164	>100	>100	>100
Rhodotorula glutinis ATCC 26086	>100	10	>100
Nematospora coryli ATCC 10647	25	5	5

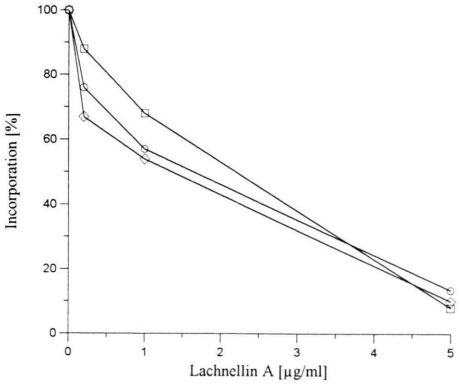


Fig. 3. Effect of lachnellin A (**1**) on the incorporation of [^{14}C]-thymidine, [^{14}C]-uridine and [^{14}C]-leucine in L1210 cells. Controls without antibiotic (100%): Thymidine, 14000 cpm; uridine, 31000 cpm; leucine, 29500 cpm; ◊–◊ leucine; o–o uridine; □–□ thymidine.

Table VI. Cytotoxic activity of compounds **1, 3, 4, 7, 8, 9,** and **11** towards L1210 cells, RBL 1 cells, BHK cells, and HeLa S3 cells.

Compound	IC_{50} [μg/ml] L1210	RBL 1	BHK 21	HeLa S3
Lachnellin A (**1**)	1–5	5–10	5–10	1–5
Lachnellin B (**3**)	>100	>100	>100	>100
Lachnellin C (**4**)	>100	>100	>100	>100
Lachnellin D (**7**)	25–50	25–50	>100	50
Naphthalene-1,3,8-triol (**8**)	25–50	25	50	10–25
rac-Scytalone (**9**)	100	>100	>100	>100
(+)-*trans*-3,4-Dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone (**10**)	25	25–50	>100	50
2,5-Dihydroxytoluene (**11**)	1–5	1–5	5	5–10

Table VII. Effect of compounds **1, 3, 4, 7, 8, 10** and **11** on germination of seeds.

Compound	IC_{50} [μg/disc] *Lepidium sativum*	*Setaria italica*
Lachnellin A (**1**)	25	25
Lachnellin B (**3**)	–	–
Lachnellin C (**4**)	–	–
Lachnellin D (**7**)	–	–
Naphthalene-1,3,8-triol (**8**)	–	–
(+)-*trans*-3,4-Dihydro-3,4,8-trihydroxy-1(2*H*)-naphthalenone (**11**)	50	100
2,5-Dihydroxytoluene (**11**)	–	25

– = less than 30% inhibition at 100 μg/disc.

rather weakly, IC_{50} values were between 50 and 100 μg/ml. Thymidine incorporation was reduced to 50% by 10 μg/ml of **11**, uridine and leucine incorporation by 50 μg/ml. Lachnellin A, the melanin precursors **9** and **10** and compound **11** were weakly phytotoxic (Table VII). Phytotoxicity of **9**, **10** and **11** has been reported before (Bell and Wheeler, 1986; Huang *et al.*, 1989) .

Among the compounds isolated from *Lachnellula* sp. A 32–89, **1** and **11** were the most active ones. The activities however seemed not to be specific. This prompted us to investigate the influence of SH-groups on the biological activities. Upon preincubation with equimolar amounts of cysteine, **1** completely lost its activity towards *Nematospora coryli*. On TLC plates, the spot was no longer detectable. Compound **11** did not react with cysteine and its activity remained unchanged. Biological activities of **11** are comparable to those of other hydroquinones (O'Brien, 1991).

In several test systems the compounds showed weak or no activity. Platelet aggregation was only inhibited by compound **1** at concentrations of 7 μg/ml or higher. None of the tested compounds (**1, 3, 4, 7–11**) was nematicidal, mutagenic or hemolytic or inhibited chitin synthase. AMV-reverse transcriptase was inhibited by (**8**) and (**1**) (100 μg/ml, 30% and 65% inhibition respectively).

Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft within the "Graduiertenkolleg Biotechnologie" and the BASF AG.

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