Production of Antibiotics by *Collybia nivalis, Omphalotus olearius,* a *Favolaschia* and a *Pterula* Species on Natural Substrates

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Collybia nivalis, Favolaschia sp. 87129, *Pterula* sp. 82168 and *Omphalotus olearius* were cultivated on natural substrates. The antibiotic metabolites oudemansin A, strobilurins A, D, illudin S and pterulone were isolated and identified. A new antifungal metabolite, pterulone B, was described from cultures of *Pterula* sp. 82168 on wood. *Collybia nivalis* was found to be the first species of this genus to produce strobilurins and oudemansin A. As compared to rich media the cultivation on natural substrates resulted in the production of fewer metabolites. The concentrations of the antibiotics, however, were sufficient to inhibit other saprophytic fungi.

Introduction

In the search for antibiotics from basidiomycetes, Anchel, Hervey, Wilkins and their coworkers pioneered with investigations of extracts derived from fruiting bodies and mycelial cultures of approximately 2000 species (for review see Florey *et al.*, 1949). One of the most important result of their efforts was the isolation of pleuromutilin (Kavanagh *et al.*, 1951) from which the semisynthetic antibiotic tiamulin, used for the treatment of mycoplasma infections in animals, was developed (for review see Erkel, 1997). Investigations of fruiting bodies of basidiomycetes led to the isolation of toxins (Bresinsky and Besl, 1985), hallucinogens (Schultes and Hofmann, 1980), chlorinated aromatics (de Jong *et al.* 1994), pigments (Gill and Steglich 1987; Gill 1994) and sesquiterpenes (Hansson *et al.* 1995). In the last 20 years a large number of metabolites from basidiomycetes with antibacterial, antifungal, phytotoxic, nematicidal, cytotoxic, antiviral and other pharmacological activities were isolated and characterized from mycelial cultures (Erkel and Anke, 1997). The biological role of most of these „secondary" metabolites for the producer, however, remained unclear and is discussed controversially (Cavalier-Smith 1992; Vining 1992a; b; Zähner *et al.* 1983). Secondary metabolites can be defined as metabolites not essential for growth and reproduction, in the case of microorganisms this means under laboratory conditions in axenic culture. This consideration, however, does not include the survival of a given species in the natural environment. Indeed, the elaborate biosynthetic pathways, the manifold biological activities and in some cases the finding of essential functions for some metabolites, previously classified as „secondary", make it seem very unlikely that these metabolites are of no importance for their producers.

Most antibiotics have been described from soil inhabiting bacteria and fungi under axenic laboratory conditions. The microflora in natural soils is very complex and, like the metabolites produced there, very difficult to assess and quantify. Many soil organisms cannot be cultivated in axenic culture and the colonies of a given species in its natural habitat are usually so small that an identification of both the strain and its metabolites are very difficult. In this respect wood inhabiting basidiomycetes offer several advantages. The fruiting bodies can be easily recognized and collected in the field. The mycelia can be seen in the substrate (leaves and wood) and their metabolites extracted and identified, both under axenic conditions and

in nature, when the producer is exposed to competing organisms.

To address the question of possible functions of secondary metabolites the production of antibiotics by four basidiomycetes on the natural substrate was investigated and compared with the metabolites produced in rich artificial media.

Materials and Methods

Organisms, cultivation and isolation of metabolites

Collybia nivalis (Luthi & Plomb) n.c., strain 78006: The mycelial culture was obtained from basidiospores from fruiting bodies, collected in Entringen/Germany, and maintained at 4 °C on agar slants with yeast malt glucose medium (YMG) consisting of (g/l): yeast extract (4.0), malt extract (10.0), glucose (4.0), and agar (15.0), pH 5.5. Fermentations of *C. nivalis* were carried out in 20 l of YMG medium in a Biolafitte C6 fermenter at room temperature with an aeration rate of 3.2 l/min, and agitation (120 rpm). A well grown culture of the strain (250 ml) in the same medium was used as inoculum. The fermentation was terminated after 6 days, and the mycelia were extracted with methanol/acetone 1:1 (v/v) yielding 6.5 g of crude extract. In addition, *C. nivalis* was cultivated in a Fernbach flask containing 16 g sterilized beech leaves and 100 ml water. After 14 days of cultivation, the substrate covered with mycelia (150 g weat weight) was extracted with 2 l of methanol/acetone (1:1 v/v) yielding 895 mg of crude extract. The antibiotics were further purified from the crude extracts by silica gel chromatography (yield 27 mg enriched extract) and HPLC as described for *Pterula* sp. 82168 (see below). Yields from 20 l fermentation: 22 mg strobilurin A and 4.7 mg strobilurin D; from beech leaves: 0.7 mg strobilurin D and 0.5 mg oudemansin A.

Pterula sp. 82168: The strain, its maintenance and the antibiotic production in YMG medium have been described earlier (Engler *et al.*, 1995). Here, the cultivation was carried out at 20–25 °C in a Fernbach flask containing 14 g sterilized beech leaves and 140 ml water. The leaves were inoculated with pieces of a well grown culture. After 31 days, the mycelia and the substrate were extracted twice with a total of 2 l of methanol/acetone 1:1. The crude extract (648 mg) was applied onto a silica gel column (Merck 60, 0.063–0.2 mm; 150x25 mm).

The enriched extract (68 mg) was obtained upon elution with cyclohexane/ethyl acetate (CH/EA) 8:2 and was tested in the agar diffusion assay for antimicrobial activities. Final purification was achieved by preparative HPLC on LiChrosorb Diol (Merck; 7 µm; column 250x25 mm; flow rate 5 ml/min; detection at λ= 210 nm) with a CH/*tert*-butyl methyl ether (*t*BME) gradient as described previously (Engler *et al.* 1997b). Yields: 1.2 mg pterulone, 1.9 mg strobilurin A and 2.4 mg oudemansin A. In addition, *Pterula* sp. 82168 was cultivated for 83 days on 58 g sterilized beech wood shaving with 100 ml water. 2.3 g of a crude extract were obtained from the mycelia and the substrate. The final purification by silica gel chromatography (yield 56.1 mg enriched extract) and HPLC was carried out as described above. Yields: 2 mg strobilurin A, 1.6 mg oudemansin A and 4.4 mg pterulone B.

Favolaschia sp. 87129: The mycelial culture of this strain was obtained from basidiospores of fruiting bodies collected on wood in Kolobo, Ethiopia. The production and isolation of antibiotics in YMG medium has been reported previously (Zapf *et al.*, 1995a). Here, the fungus was cultivated for 72 days on 185 g beech wood with 150 ml water. A crude extract (808 mg) was derived from the mycelia and the substrate and the final purification by silica gel chromatography (yield 50 mg enriched extract) and HPLC was performed as described for *Pterula* sp. 82168. Yield: 0.6 mg oudemansin A.

Omphalotus olearius Fayod, strain 96317: The fruiting bodies were collected growing on the base of oak trees in Belgentier/France. The mycelial culture was derived from a spore print and maintained at 4 °C on agar slants with a YMG medium. Sterilized cubes (2x2x2 cm) of oak wood (448 g) with 150 ml water were cultivated for 47 days. The mycelia and the substrate were extracted with 1 l of methanol, and the resulting crude extract (925 mg) was applied onto a column with silica gel (Merck 60, 0.063–0.2 mm; 5x7 cm) and eluted with CH/EA . The enriched extract (20.7 mg) was purified by preparative HPLC on LiChrospher RP-18 (Merck, 10 µm; column 250–10 mm; flow rate 5 ml/min; eluant: water/methanol 6:4). In addition, 55 g dried fruiting bodies were extracted with 1 l of EA, and the crude extract (1.78 g) was worked up following the purification procedure described above. Yield: 1.2 mg illudin S.

Identification and structure elucidation of metabolites

The isolated metabolites were identified by their UV and IR spectra, their retention time in the analytical HPLC and their NMR data. UV spectra were recorded using a Perkin Elmer λ 16, and IR spectra with a Bruker IFS 48. For analytical HPLC, a Hewlett Packard HPLC Chemstation (HP 1090 Series II) equipped with a diode array detector and a reverse phase column (Merck; 5 µm; LiChrocart 125 × 4 mm; Lichrosher 100 RP 18) was used with a water/methanol gradient as mobile phase (0–70% in 20 min, 70–100% in 10 min; 1.5 ml/min). ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with 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. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. Mass spectra were recorded with a Jeol SX102 spectrometer, the melting point (uncorrected) was determined with a Reichert microscope, and the optical rotation measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Test for biological activities

The antimicrobial spectra were determined in the agar plate diffusion and serial dilution assays as reported before (Anke *et al.*, 1989; 1996). Tests for cytotoxicity towards L1210 cells (ATCC CCL 219) and HL60 cells (ATCC CCL 240) were carried out as described previously (Zapf *et al.*, 1995b). The inhibition of respiration of *Penicillium notatum* was measured with a Clark-type oxygen electrode as described by Weber *et al.* (1990a).

Results and Discussion

Table I shows the antimicrobial activities of the enriched extracts derived from cultivations on the natural substrates after the first silica gel chromatography. Extracts from *C. nivalis, Favolaschia* sp. 87129, and *Pterula* sp. 82168 exhibited antifungal activities, whereas the extract from *O. olearius* in-

Table I. Antimicrobial activities of the enriched extracts derived from cultures on the natural substrate. The crude extracts were applied onto a silica gel column, and the enriched extracts were obtained upon elution with EA (*O. olearius*) or CH/EA 8:2 (all other strains).

Organism	Diameter of inhibition zone [mm] (100 µg extract/disc)							
	M.m.	P.n.	P.v.	N.c.	B.b.	B.s.	M.l.	E.d.
Collybia nivalis	13	13	–	–	–	–	–	–
Favolaschia sp. 87129	9	22	27	–	–	–	–	–
Omphalotus olearius	–	–	–	13	–	–	13	–
Pterula sp. 82168[1]	32	30	30	–	–	–	–	–
Pterula sp. 82168[2]	26	20	34	–	–	–	–	–

M.m.: *Mucor miehei*; P.n.: *Penicillium notatum*; P.v.: *Paecilomyces variotii*; N.c.: *Nematospora coryli*; B.b.: *Bacillus brevis*; B.s.: *Bacillus subtilis*; M.l.: *Micrococcus luteus*; E.d.: *Enterobacter dissolvens*; –: no inhibition zone; [1]: cultivated on beech leaves; [2]: cultivated on beech wood shaving.

hibited the growth of the yeast *Nematospora coryli* and the bacterium *Micrococcus luteus*.

From the enriched extract derived from *Pterula* sp. 82168 grown on 14 g of beech leaves 1.2 mg of pterulone, 1.9 mg of strobilurin A and 2.4 mg of oudemansin A were isolated, explaining the antifungal activity of the extract (Table I). Cultivation on 58 g beech wood shaving resulted in the production of strobilurin A (2.0 mg), oudemansin A (1.6 mg), and 4.4 mg of a new compound, pterulone B. In previous studies pterulone, pterulinic acid, strobilurin A, hydroxystrobilurin A and oudemansin A, had been isolated from fermentations of the same strain in YMG medium (Engler *et al.*, 1995; 1997a; b). Pterulone B so far seems to be produced only on wood.

Pterulone B was obtained as a yellow oil, with UV absorptions in methanol at 267 nm (log ε 3.97) and 294 nm (log ε 3.60). Its structure was determined from the ^{1}H and ^{13}C NMR data given in Table II, 2D NMR data (COSY, NOESY, HMQC and HMBC), and EIMS measurements. The presence of a chlorine in pterulone B was indicated by the typical isotope pattern in the mass spectrum, and data from high resolution EIMS measurements of the molecular ion (also the base peak) suggested that the elemental composition is $C_{17}H_{17}O_2Cl$ (observed exact mass 288.0922, $C_{17}H_{17}O_2{}^{35}Cl$ requires 288.0917). This is in agreement with the NMR spectra, in which the signals for 17 carbons and 17 protons can be seen. The ^{1}H and ^{13}C NMR chemical shifts, the ^{1}H-^{1}H
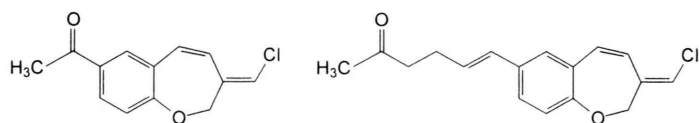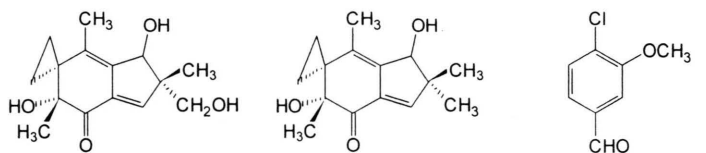
Pterulone (**1**)

Pterulone B (**2**)
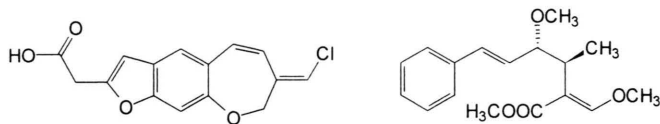
Pterulinic acid (**3**)

Oudemansin A (**4**)

R₁ = H;　　R₂ = CH₃:　Strobilurin A (**5**)
R₁ = H;　　R₂ = CH₂OH: Hydroxystrobilurin A (**6**)
R₁ = OCH₃; R₂ = CH₃:　9-Methoxystrobilurin A (**7**)

Strobilurin F (**8**)

Illudin S (**9**)

Illudin M (**10**)

4-Chloro-3-methoxy-
benzaldehyde (**11**)
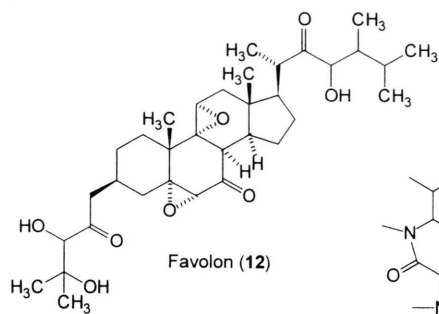
Favolon (**12**)

(+)-10a-Hydroxy-4-muurolen-3-one (**13**)

Omphalotin (**14**)

Fig. 1. Structures of the compounds **1–14**. The structures of strobilurin D (**15**) (Weber *et al.* 1990b; Nicholas *et al.* 1997) and 9-methoxystrobilurin K (**16**) (Zapf *et al.* 1995a; Nicholas *et al.* 1997) are currently under re-investigation.

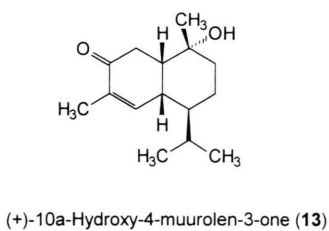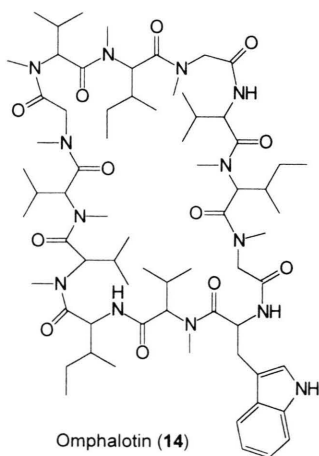Table II. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for pterulone B. The spectra were recorded in CDCl$_3$ with the solvent signals (at 7.26 and 77.0 ppm, respectively) as reference, the chemical shifts (δ) are given in ppm and the coupling constants (J) in Hz.

Pos.	^{1}H (δ, mult., J)	^{13}C (δ, mult.)
1	2.16; s	30.0; q
2	–	207.8; s
3	2.60; t; 7.2	43.2; t
4	2.47; dt; 7,7	27.1; t
5	6.10; m	128.2; d
6	6.34; d; 15.9	129.6; d
7	–	132.6; s
8	7.16; d; 8.3	126.7; d
9	6.91; d; 8.3	120.2; d
10	–	158.5; s
11	–	126.7; s
12	7.19; s	130.9; d
13	6.52; d; 11.8	131.2; d
14	6.80; d; 11.8	123.9; d
15	–	136.5; s
16	6.10; m	119.1; d
17	4.53; s	72.7; t

Table III. Antifungal activity of pterulone B in the agar diffusion assay.

Organism	Diameter of inhibition zone [mm]	
	10[a]	50[a]
Aspergillus ochraceus	–	–
Botrytis cinerea	–	–
Curvularia lunata	–	–
Fusarium fujikuroi	–	–
Fusarium oxysporum	–	–
Mucor miehei	8	12
Nadsonia fulvescens	–	–
Nematospora coryli	–	–
Paecilomyces variotii	–	–
Penicillium notatum	–	10
Phoma clematidina	–	–
Pythium irregulare	–	11
Saccharomyces cerevisiae αS288c	–	–
Saccharomyces cerevisiae is 1[b]	–	–
Ustilago nuda	–	–
Trichoderma virie	–	–
Zygorrhynchus moelleri	10d	16d

–: no inhibition zone.
[a]: μg/disc, [b]: gift of Prof. F. Lacroute, Strasbourg.
d: diffuse inhibition zone.

coupling constants and the COSY correlations established the presence of a 1,3,4-trisubstituted benzene ring, an isolated *cis* double bond, a *trans* double bond that at one end is connected to a –CH$_2$-CH$_2$-unit, an isolated olefinic proton, an isolated methyl group, an isolated -O-CH$_2$- group and a keto function. These could be out together by examining the HMBC correlations (data not shown), and the resulting structure leaves only one position for the remaining chlorine (C-16). The C-15/C-16 double bond is E, as a strong correlation in the NOESY spectrum can be observed between 16-H and 17-H$_2$.

The biological activities of pterulone B were much weaker as compared to the activities of pterulone (Engler *et al.*, 1997b). As shown in Table III, in the plate diffusion assay only *Mucor miehei*, *Penicillium notatum*, *Pythium irregulare* and *Zygorrhynchus moelleri* were inhibited at concentrations starting at 10 μg/disc or 50 μg/disc, respectively. The compound did not exhibit antibacterial activities. In the serial dilution assay, at 100 μg/ml no inhibition of growth of *Acinetobacter calcoaceticus*, *Bacillus brevis*, *Bacillus subtilis*, *Corynebacterium insidiosum*, *Escherichia coli* K12, *Micrococcus luteus*, *Mycobacterium phlei*, *Salmonella typhimurium*, *Streptomyces* sp. ATCC 23836 was observed. Unlike pterulone, which is an inhibitor

of the mitochondrial NADH:ubiquinone oxidoreductase (complex I), oxygen uptake by freshly germinated spores of *Penicillium notatum* (30 mg wet weight/ml in 1% glucose solution) was not inhibited by pterulone B at concentrations up to 100 μg/ml. Pterulone, used for comparison, totally blocked the oxygen uptake at 25 μg/ml. Thus the insertion of a C$_4$-chain between the benzene ring and the keto group appears to be responsible for the markedly altered activities of pterulone B. In contrast to pterulone, pterulone B exhibited cytotoxic activities. The growth of L1210 cells and HL60 cells was reduced to 50% at concentrations of 50 μg/ml and 25 μg/ml, respectively.

The antibiotic production of *Collybia nivalis* is reported here for the first time. *C. nivalis* was grown both in submerged culture in complex YMG and on beech leaves, the natural substrate. 22 mg of strobilurin A and 4.7 mg of strobilurin D were isolated from the mycelia grown in 20 l of YMG medium. Strobilurin D (0.7 mg) and oudemansin A (0.5 mg) were isolated from cultures on beech leaves (16 g).

From the enriched extract derived from the cultivation of *Favolaschia* sp. 87129 on 185 g of beech wood, 0.6 mg oudemansin A was obtained. The concentration of this antifungal metabolite in the mycelia and substrate was high enough to inhibit a substrate competitor. When *Favolaschia* sp.

87129 was co-cultivated with *Penicillium notatum* on beech wood the spreading of the mycelia of the fast-growing deuteromycete was suppressed in the peripheral area around the *Favolaschia*'s mycelia whereas the other parts of the wood were totally covered. In addition to oudemansin A, the following compounds were recently isolated from fermentations of *Favolaschia* sp. 87129 in YMG medium: strobilurins A and F, 9-methoxystrobilurins A and K, the antifungal favolon, and (+)-10α-hydroxy-4-muurolen-3-one (Zapf *et al.*, 1995a; 1996; Anke *et al.*, 1995).

The cultivation of *Omphalotus olearius* on oak wood led to the isolation of illudin S, first described by McMorris and Anchel (1965). 1.2 mg of this sesquiterpene was also isolated from 55 g of dried fruiting bodies. Besides illudin S, several strains of *Omphalotus olearius*, including the strain used in this study, were found to produce illudin M (McMorris and Anchel, 1965) and omphalotin, a nematicidal cyclic peptide (Mayer *et al.*, 1997) in complex media.

Antibiotic production on the natural substrates was observed for all four basidiomycetes. Strobilurins, oudemansin A, and pterulones were produced on the natural substrates as well as in complex media by *Pterula* sp. 82168, *Collybia nivalis*, and *Favolaschia* sp. 87129. *Omphalotus olearius* produced the antimicrobial illudin S. The physiological function of the secondary metabolites for the producers themselves may vary. The cytotoxic illudin S, for example, may deter predatory animals, and the (*E*)-β-methoxyacrylates may secure the habitat of the producer by inhibiting the growth of competing fungi. As shown for *Favo-laschia* sp. 87129, the concentrations of oudemansin A both in the mycelia of the producing basidiomycete and in the surrounding substrate were sufficient to suppress the growth of the competitor *Penicillium notatum*. A prerequisite for an assumed function of antifungal metabolites in securing a substrate is the resistance of the producing fungus against its own antibiotic. This has been shown for the strobilurin producers *Strobilurus tenacellus* and *Mycena galopoda* where resistance is conferred by amino acid replacements in two regions of cytochrome b known to contribute to the formation of center Q_p, the molecular target of this class of antifungals (Kraiczy *et al.*, 1996). The present results clearly indicate that secondary metabolites are produced by basidiomycetes in the natural substrate, although this provides only a limited amount of available carbon and nitrogen sources, trace elements and other nutrients. It is interesting that media with rich supplies of glucose and amino acids yield a wider spectrum of metabolites with different biological activities, in some cases (favolon and omphalotin) originating from different biosynthetic pathways (Table IV). It might be speculated that this would allow a flexibel response to likely competitors on different substrates.

Acknowledgements

Table IV. Comparison of the antibiotics isolated from the natural substrates with the products isolated or previously described from complex media. The structures of the compounds are shown in Fig. 1.

Organism	Leaves or wood	Antibiotics produced in Fruiting bodies	YMG-medium	
Collybia nivalis	**4, 15**	n.a.	**5, 11, 15**	
Favolaschia sp. 87129	**4**	n.a.	**4, 5, 7, 8, 12, 13, 16**	(Zapf *et al.*, 1995a; 1996; Anke *et al.*, 1995)
Omphalotus olearius	**9**	9	**9, 10, 14**	(McMorris & Anchel, 1965; Mayer *et al.*, 1997, Sterner *et al.*, 1997)
Pterula sp. 82168[1]	**1, 4, 5**	n.a.	**1, 3, 4, 5, 6,**	(Engler *et al.*, 1995; 1997a; b)
Pterula sp. 82168[2]	**2, 4, 5**	n.a.		

n.a.: not available in sufficient amounts. [1]: cultivated on beech leaves; [2]: cultivated on beech wood shaving.

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