Fungal Melanin Biosynthesis Inhibitors: Introduction of a Test System Based on the Production of Dihydroxynaphthalene (DHN) Melanin in Agar Cultures

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Inhibitors of Melanin Biosynthesis, 6-Methylpurine, 3,5-Dichloro-4-methoxybenzoic Acid, 1-Methoxy-8-hydroxynaphthalene, *Stropharia squamosa*

In a screening for inhibitors of fungal melanin biosynthesis a novel test system was successfully employed. With this test system, based on the production of dihydroxynaphthalene melanin (DHN melanin) by a *Lachnellula* species in agar cultures, extracts of cultures of basidiomycetes, ascomycetes and deuteromycetes were tested. From fermentations of *Collybia dryophila*, 6-methylpurine (**1**) and 6-methyl-9-ß-D-ribofuranosylpurine (**2**) were isolated as the active principles and 3,5-dichloro-4-methoxybenzoic acid (**3**) was obtained from cultures of *Stropharia squamosa*. The corresponding alcohol (**4**) and aldehyde (**5**) previously isolated from several basidiomycetes, were also active in this test system. In a screening of fungal metabolites, 1-methoxy-8-hydroxynaphthalene and 1,8-dimethoxynaphthalene inhibited biosynthesis of DHN melanin.

Among the inhibitors identified, compounds **3** – **5** were the most selective ones. They were the only metabolites without cytotoxic activities.

Introduction

Melanins are not essential for fungal growth and development, they rather enhance the survival and competitive abilities in certain environments. In the Ascomycotina and related Deuteromycotina, the dark-brown to black melanins in cell walls are generally synthesized via the pentaketide pathway and 1,8-dihydroxynaphthalene (DHN) is the immediate precursor of the polymer (Bell and Wheeler, 1986).

Plant disease control may result from any of several mechanisms which include direct fungitoxicity, suppression of pathogenic mechanisms, or alteration of host resistance (Wolkow *et al.*, 1983). DHN melanin has been implicated as a pathogenicity factor in some fungal plant diseases and animal mycoses (Kubo and Furusawa, 1991; Dixon *et al.*, 1991). A new class of fungicides, like tricyclazole, pyroquilon, coumarin, triazoloquinoline, that inhibit melanin biosynthesis is used to prevent rice blast disease caused by *Pyricularia oryzae* (perfect sexual state, *Magnaporthe grisea*) (Kimura and Tsuge, 1993; Yamaguchi and Kubo, 1992). At non-fungitoxic concentrations these antipenetrants inhibit melanin biosynthesis in *P. oryzae*. They have no effect on the growth of the pathogen in culture or on germination of conidia and formation of appressoria on isolated *Bryophyllum pinnatum* epidermal strips, but they block melanization of the appressoria and prevent penetration of the epidermal wall (Woloshuk and Sisler, 1982). Wolkow *et al.* (1983) suggested that the unmelanized walls of treated appressoria lack the rigidity necessary to support the mechanical forces required for penetration of the plant cuticle. Experiments with inhibitors and melanin-deficient mutants have shown that fungal melanin is essential for penetration of host leaf tissues by *P. oryzae*, *Colletotrichum lagenarium*, and *C. lindemuthianum* (Kimura and Tsuge, 1993). Therefore melanin biosynthesis inhibitors meet the requirement for high biochemical specificity with activity directed at one or a few

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target sites in the pathogen and low toxicity to non target organisms.

In this report, we describe a new test system for the detection of DHN melanin biosynthesis inhibitors, its application in the screening and the isolation of inhibitors from cultures of the basidiomycetes *Collybia dryophila* and *Stropharia squamosa*. Inhibitors, which were detected by a screening of fungal metabolites are also included.

Experimental

Test system, test organisms and screening

Streptomyces bikiniensis DSM 40581 was obtained from DSM, Braunschweig. *Lachnellula* sp. A 32–89 was obtained from fruiting bodies and has been described previously (Semar 1993). Strain A 32–89 was grown in MGPY medium composed of (g/l): maltose 20, glucose 10, peptone 2, yeast extract 1, KH_2PO_4 0.5, $MgSO_4$ x 7 H_2O 1, $FeCl_3$ 0.01, $ZnSO_4$ 0.002, and $CaCl_2$ (0.1 M solution) 5 ml/l. For submerged cultivation, 250 ml of MGPY medium in a 500 ml-Erlenmeyer flask with one indentation were inoculated with 5 % from a well grown shake culture in the same medium and incubated on a rotary shaker (22 °C, 120 rpm) for 3 days. The mycela were centrifuged (10 min at 2000 x g), resuspended in 100 ml sterile 0.9 % sodium chloride solution and homogenized with an ultra turax (30 sec) under aseptical conditions. 100 ml of the mycelial suspension were resuspended in 500 ml modified MGPY medium (with maltose 10 g/l and glucose 20 g/l) with 2 % agar at 48 °C and 15 ml were poured into Petri dishes (90 mm). Paper discs (6 mm), bearing the compounds to be tested, were placed onto the plates which were incubated at 22 °C for 48–72 hours; the resulting white zone of inhibition of melanin formation was measured from the reverse side of the plate. Triazoloquinoline, pyroquilon and coumarin were used as standards.

For the confirmation of positive results obtained with *Lachnellula* spec. A 32–89, *Pyricularia oryzae* was used in a similar agar diffusion assay. In order to confirm the selectivity of the detected inhibitors, *Streptomyces bikiniensis* which forms DOPA melanin (Tomita *et al.*, 1990) was included in the screening.

Fermentation

Mycelial cultures of *Collybia dryophila*, TA 90314, were derived from fruiting bodies collected in Friday Harbour, USA. Fruiting bodies of *Stropharia squamosa*, TA 91045, were collected in Ethiopia. The strains are deposited in the collection of the Lehrbereich Biotechnology, University of Kaiserslautern. The cultures were maintained on agar slants with YMG agar (yeast extract 0.4 %, malt extract 1 %, glucose 0.4 %, agar 2 %; pH 5.5). For submerged cultivation 250 ml of YMG medium were inoculated with mycelium from agar plates and incubated for 14 days on a rotary shaker (22 °C, 120 rpm). These cultures were used to inoculate 20 l of the same medium in a Biolafitte fermentation apparatus. Silicone antifoam was added if necessary. Fermentations were carried out at 22 °C, 160 rpm and an aeration rate of 4 l/min.

For the determination of the biological activities, aliquots (100 ml) of the culture were taken under sterile conditions. After filtration, the culture fluid was extracted with ethyl acetate (100 ml). The extracts were dried *in vacuo* and the oily residues were redissolved in methanol (0.5 ml). Ten µl of these concentrated extracts were tested in the agar diffusion assay towards *Lachnellula* spec. A 32–89 as described above.

Isolation and identification of the active compounds

After separation of the culture broth by filtration, the fluid (16 l) was applied onto Mitsubishi DIAION HP 21 resin (500 g). The HP 21 was washed with H_2O and the active compounds were eluted with H_2O – methanol (1:1, 2 l) in the case of *Collybia dryophila* and H_2O – acetone (1:1, 2 l) for the metabolite from *Stropharia squamosa*. The eluates were concentrated to an aqueous residue.

Compounds **1** and **2** from *C. dryophila* were extracted from this residue with *n*-butanol (2 x 500 ml). The organic solvent was evaporated *in vacuo* to yield 4.1 g of an oily crude product. Compounds **1** and **2** were purified by column chromatography on silica gel (Merck 60) with ethyl acetate – methanol (1:1) as eluant and MPLC on silica gel (LiChroprep Si 60) with ethyl acetate – methanol (95:5) as eluant. From the fractions containing **1** and **2**, **2** could be crystallized from methanol yielding 2 mg

of **2** per litre of culture fluid. **1** was purified from the mother liquor by preparative thin-layer chromatography (TLC) on silica gel (Merck 60 $WF_{254}S$) developed in 2-propanol – NH_3 (25 %) – H_2O (80:15:5). Yield: 0.6 mg/l culture fluid.

Compound **3** was extracted from the aqueous extract with ethyl acetate. Purification was achieved by HPLC on reverse phase RP-18 (LiChrosorb, 7 µm; column size 250 x 25 mm; flow rate 5 ml/minute) with a water – acetonitrile gradient (0–40 minutes: 0–30 %; 40–90 minutes 30–100%) and HPLC on LiChrogel PS1 (7 µm; 250 x 25 mm; flow rate 5 ml/minute) in 2-propanol. Retention time for compound 3 was 38 minutes.

The UV spectra were recorded with a Perkin Elmer Lambda 16, the IR spectra with a Bruker IFS 48. The NMR spectra were recorded in $CDCl_3$ – CD_3OD (95:5) with a Bruker ARX500 spectrometer, and the solvent signals at 7.26 and 77.0 ppm, respectively, were used as reference. The mass spectra (70 eV EI ionisation) with a Jeol SX102 spectrometer. The analytical HPLC system consisted of a Merck LiChrospher RP 18 column (5 µm, 125 mm x 4 mm) eluted with 1.5 ml/min of a H_2O – methanol gradient. The injection volume was 10 µl, and the compounds were detected using Diode Array Detection (Hewlett Packard 1090 Series II).

Biological assays

The inhibition of seed germination and the antimicrobial activities were tested as described previously (Anke *et al.*, 1989). The cytotoxicity against L1210 (ATCC CCL 163), HL-60 (ATCC CCL 240), RBL-1 (ATCC CRL 1378), and BHK 21 cells (ATCC CCL 10) were measured as described by Erkel *et al.* (1991).

Results and Discussion

Selection of test organisms and screening

The ascomycete *Lachnellula* spec. A 32–89 produces DHN melanin in agar and liquid cultures. This was proven by the identification of two precursors of the DHN melanin pathway, scytalone and 1,3,8-trihydroxynaphthalene. In addition two metabolites closely related to the melanin pathway, flaviolin, and 3,4,8-trihydroxytetralone, were also isolated from cultures of strain A 32–89 (Semar, 1993). The melanin production in agar cultures occurred within a short period of time, ca. 48–72 hours. In submerged cultures, the colour of the broth became brownish-black after 5–6 days of incubation.

The effects of known melanin biosynthesis inhibitors like triazoloquinoline, coumarin and pyroquilon on the production of melanin by *Lachnellula* spec. A 32–89 in agar cultures are shown in Table I (part A).

In a screening of 61 natural products, mainly fungal metabolites, 5 compounds were active: lachnumon A (Stadler *et al.*, 1993a, b), lachnumon B_2 (Stadler *et al.*, 1995a, b), illudin M (McMorris *et al.*, 1989), 1-methoxy-8-hydroxy-naphthalene, and 1,8-dimethoxynaphthalene (Allenport and Bu'Lock, 1960; Anke *et al.*, 1995). Their effects on the synthesis of melanin are also shown in Table 1 (Part B). With the exception of the naphthalene derivatives, all compounds inhibited mycelial growth when tested in submerged cultures with *Lachnellula* spec. A32–89 and thus do not fulfil the criteria for selective inhibitors of melanin bio-

Table I. Inhibition of melanin biosynthesis in agar cultures of *Lachnellula* spec. A 32–89 by reference compounds and fungal metabolites.

Compound	(µg/paper disc)	Inhibition zone [mm]
A		
Triazoloquinoline	10	10
	20	18
	50	21
Coumarin	100	12*
	150	22
	200	29
Pyroquilon	100	–**
	200	9
	300	11
B		
Illudin M	20	17
Lachnumon A	20	24
	50	29
Lachnumon B	20	25
	50	28
1-Methoxy-8-hydroxy-naphthalene	50	13
	100	15
1,8-Dimethoxy-naphthalene	50	10

* Diffuse light brown zone; ** no inhibition zone.

synthesis inhibitors. The inhibitory activity of the mono- and di-methyl ether of 1,8-DHN might be due to the structural similarities of these compounds to 1,8-DHN, the immediate precursor of the black pigment. It remains to be elucidated which enzyme in the pathway is inhibited. The most likely candidate is the wall-bound laccase catalysing the polymerization reaction. Methylation of the immediate precursor of melanin yielding an inhibitor of the polymerization might reveal means by which pigment synthesis can be regulated in fungi.

In the screening of 586 fungal cultures, extracts of *Stropharia squamosa* and several *Collybia* species (basidiomycetes) were found to inhibit the synthesis of melanin in *Lachnellula* spec. A 32–98 but not or less in *Streptomyces bikiniensis*. The extracts from *S. squamosa* and *C. dryophila* were the most active ones and therefore these fungi were chosen for the production of the inhibitors.

Fermentation and identification of the inhibitors from Collybia dryophila

During fermentation of *C. dryophila*, inhibitors of melanin biosynthesis were detected in the extracts of the culture fluid after 115 h. The fermentation was terminated after 12 days, when the glucose and maltose in the culture broth were used up and growth ceased. Although the biological activity had not reached its maximum the termination of the fermentation was necessary because of the production of slime substances which hampered the extraction of the active compounds.

Two bioactive metabolites (**1** and **2**) were isolated as described in the experimental section. Structural elucidation revealed the identity of **1** with 6-methylpurine and **2** with 6-methyl-9-ß-D-ribofuranosylpurine first described as natural products from cultures of *C. maculata*. For both compounds strong cytotoxic and antiviral activities have been reported (Leonhardt *et al.*, 1987).

Fermentation and identification of the inhibitor from Stropharia squamosa

A typical fermentation diagram of *S. squamosa* is depicted in Fig. 1. The production of 3,5-dichloro-4-methoxybenzoic acid started after 8 days, small amounts <1 mg/l could be detected as early as on the forth day of the fermentation. The peak of 12 mg/l was reached after 12 days. Following the isolation scheme given in the experimental section, 191 mg of compound **3** were obtained.

The structure of 3,5-dichloro-4-methoxybenzoic acid was determined by NMR spectroscopy and mass spectrometry. The molecular ion in the EI mass spectrum showed the typical isotope pattern of a molecule containing two chlorines, and high resolution measurements suggested the molecular composition to be $C_8H_6O_3Cl_2$ and that the molecule contains 5 unsaturations. Only two signals were visible in the ^1H NMR spectrum, a singlet at 7.91 ppm integrating for 2 protons and a singlet at 3.89 ppm integrating for 3 protons, indicating that

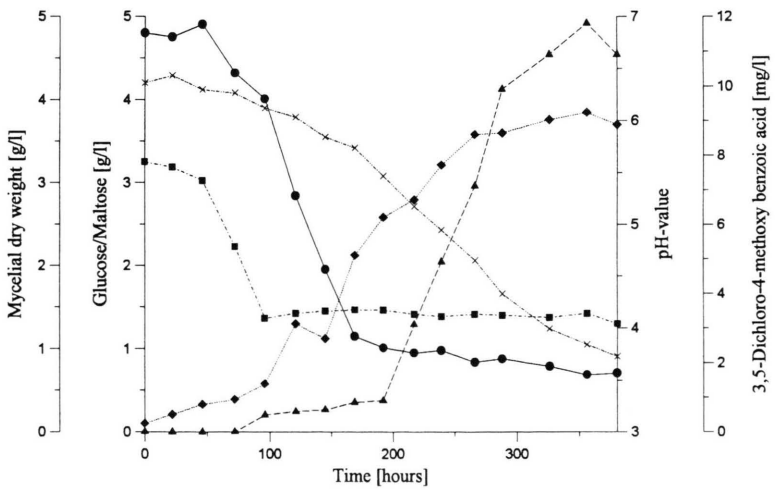

Fig. 1. Fermentation of *Stropharia squamosa* TA 91045 in 20 l scale and production of 3,5-dichloro-4-methoxybenzoic acid. ◆–◆ Mycelial dry weight; ●–● glucose; x-x maltose; ■–■ pH; ▲–▲ 3,5-dichloro-4-methoxybenzoic acid.

Table II. Inhibition of DHN-melanin synthesis in agar cultures of *Lachnuella* spec. A 32–89 and of DOPA-melanin synthesis in *Streptomycenes bikiniensis* by compounds **1, 2, 3, 4,** and **5.**

Compound	Diameter of inhibition zone [mm]				
	Lachnellula spec.			*S. bikiniensis*	
(µg/disk)	5	10	50	10	50
1	11*/20	12*/25	13*/30	8*/18	12*/23
2	11*/20	12*/24	13*/28	17	24
3	18	19	25	–	12
4	16	18	24	–	12
5	12	16	22	–	8

* Inhibition zone of growth.

the compound is a symmetrical benzoic acid derivative containing one methoxy and two chloro substituents. The strong correlations observed in the HMBC spectrum between the aromatic protons and the carbon to which they are attached (130.4 ppm) as well as to the carbon to which the methoxy group is attached (155.8 ppm) determine that the aromatic protons are meta to each other and to the methoxy group, and thereby the structure of the compound.

This is the first report on the natural occurrence of 3,5-dichloro-4-methoxybenzoic acid. The corresponding alcohol (**4**) is known from *Stropharia* and *Hypholoma* species (Pfefferle *et al.*, 1990; Hautzel and Anke, 1990). *Pholiota destruens* was found to produce the alcohol (**4**) and the aldehyde (**5**) (Becker *et al.*, 1994). Among synthetic inhibitors of DHN melanin synthesis, chlorinated aromatic compounds like pentachlorobenzyl alcohol and fthalide are found (Inoue *et al.*, 1984). The structural similarity of the compounds detected in the screening with compounds used in rice blast control proves the validity of our test system.

Biological properties

6-Methylpurine (**1**), 6-methyl-9-ß-D-ribofuranosylpurine (**2**), 3,5-dichloro-4-methoxybenzoic acid (**3**), the corresponding alcohol (**4**) and aldehyde (**5**) inhibited the formation of melanin by *Lachnellula* spec. A 32–89 and *Pyricularia oryzae* (results not shown). The biosynthesis of DOPA melanin in *Streptomyces bikiniensis* was slightly affected by compounds **3**, **4**, and **5**, and moderately by compounds **1** and **2**, as shown in Table II. The latter two compounds also inhibited growth of both test organisms. The antifungal activities in the serial dilution assay are given in Table III. Both compounds from *C. dryophila* exhibited weak antifungal activities. The concentrations needed for inhibition of mycelial growth are at least 10 fold higher compared to those needed for inhibition of

Table III. Antifungal activities of compounds **1, 2, 3, 4,** and **5** in the serial dilution assay. The minimal inhibitory concentrations (MIC) were evaluated after 24 h incubation.

Testorganism	MIC [µg/ml]				
	1	**2**	**3**	**4**	**5**
Nadsonia fulvescens	>100	>100	>100	>100	>100
Nematospora coryli	100	50	100	>100	50
Saccharomyces cerevisiae is1	>100	>100	>100	>100	>100
S. cerevisiae S 288	>100	>100	>100	>100	>100
Fusarium oxysporum	50	>100	>100	>100	>100
Paecilomyces variotii	50	>100	>100	>100	100
Penicillium notatum	100	>100	>100	>100	100
Mucor miehei	50	100	>100	>100	100
Rhodotorula glutinis	>100	>100	>100	>100	>100

Table IV. Cytotoxicity of compounds **1, 2 ,3, 4,** and **5** towards L1210 cells.

Compound	IC_{90} [μg/ml]
1	1
2	0.2
3	>50
4	>50
5	>50

Tavle V. Effect of **1** and **2** in the seed germination assay. The results from four assays (each with 6 seeds) are given as a percentage of the growth of control seeds (= 100%).

Compound	Growth [%]					
	Setaria italica		*Lepdium sativum*		*Oryza sativa*	
	10	50	10	50	10	50 (μg/disk)
1	0	0	0	0	20	0
2	0	0	0	0	25	0
3	62	23	55	14	81	73
4	75	12	71	22	80	62
5	76	24	80	32	81	73

melanin synthesis. In all cases the mycelia started to grow after removal of **1** and **2** e.*g.* both compounds are fungistatic and not fungitoxic. 3,5-Dichloro-4-methoxybenzoic acid (**3**) had no activity towards filamentous fungi up to 100 μg/ml and therefore, was the most selective inhibitor of DHN melanin biosynthesis. 3,5-Dichloro-4-methoxybenzyl alcohol (**4**) was equally active and selective as **3** whereas the aldehyde (**5**) was less active in the melanin assay and slightly active towards the yeast *Nematospora coryli*. The enzymatic step inhibited in the biosynthetic pathway of melanin remains to be elucidated. In the serial dilution assay, bacteria were not sensitive to 100 μg/ml of all five compounds with the exception of *S. bikiniensis*, which was sensitive to 50 μg/ml of compound **1**.

Cytotoxic and phytotoxic activities, as shown in Tables IV and V, were pronounced in the case of compounds **1** and **2**, whereas **3**, **4**, and **5** were not toxic to L1210 cells and only weakly toxic to plants. The cytotoxic activities of compounds **1** and **2** were as high as reported by Leonhard *et al.* (1987). The same authors have described compounds **1** and **2** as inhibitors of adenosine deaminase.

The results obtained so far clearly indicate that the new test system is suitable for the detection of inhibitors of fungal DHN melanin synthesis and can be successfully applied in the search for biologically active compounds in microbial cultures.

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