ANTIBIOTICS FROM BASIDIOMYCETES

XXXI.† ALEURODISCAL: AN ANTIFUNGAL SESTERTERPENOID FROM *ALEURODISCUS MIRABILIS* (BERK. & CURT.) HÖHN.

URSULA LAUER and TIMM ANKE*

Lehrbereich Biotechnologie der Universität,
Paul-Ehrlich-Str. 23, D-6750 Kaiserslautern, FRG

WILLIAM S. SHELDRICK

Lehrbereich Anorganische Chemie der Universität,
Erwin-Schrödinger-Str. 52, D-6750 Kaiserslautern, FRG

ANGELA SCHERER and WOLFGANG STEGLICH

Institut für Organische Chemie und Biochemie der Universität,
Gerhard-Domagk-Str. 1, D-5300 Bonn 1, FRG

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Aleurodiscal, an antifungal antibiotic has been isolated from mycelial cultures of *Aleurodiscus mirabilis* (Berk. & Curt.) Höhn. It causes abnormal apical branchings of *Mucor miehei* hyphae at very low concentrations. The structure and absolute configuration of the antibiotic has been determined by a single crystal X-ray analysis and hydrolysis to D-(+)-xylose. Aleurodiscal is a hydroxysesterterpene aldehyde β-D-xyloside with a novel carbon skeleton.

The genus *Aleurodiscus* Rabenh. ex Schroet. in Cohn (Corticiaceae) is characterized by small disc-like or corticioid fruiting bodies, large amyloid spores, and conspicuous sterile elements in the hymenium. The fruiting bodies can be frequently found growing on bark or wood. Though mycelial cultures of *Aleurodiscus* species can be easily obtained, little is known about their secondary metabolism and its products. The species investigated best is *Aleurodiscus roseus* (Pers. ex Fr.) Höhn. et Litsch. from which 11 antibiotic polyacetylenes have been isolated by CAMBIE *et al.*[2].

Fruiting bodies of the species investigated here were collected from the bark of a camphor tree in Japan. In the following we wish to describe the fermentation, isolation and biological and chemical characterization of a new antifungal antibiotic from mycelial cultures derived from these specimens.

Materials and Methods

General

The MP was determined with a Büchi 510 melting point and is uncorrected. The UV spectrum was recorded on a Varian Cary 17 spectrophotometer. IR spectra were obtained using a Beckmann AccuLab 8 spectrometer. NMR spectra were recorded on a Bruker AM-400 spectrometer. MS spectra were measured with an AEI MS-50 at 70 eV. TLC was performed on aluminium foils, coated with Silica gel 60 F_{254} Merck, Darmstadt, No. 5554. The HPLC was carried out with a Waters-Millipore system. Column: 250×16 mm Nucleosil 5C18 (5 μm), Macherey, Nagel & Co., Düren. Mobile phase: CH_3CN - H_2O (21 : 1). Flow rate: 9 ml/minute. Detection: 190 nm.

† See ref 1.

Aleurodiscus mirabilis (Berk. & Curt.) Höhn., Strain 8435

Mycelial cultures were obtained from spore prints of fruiting bodies collected from the bark of *Cinnamomum camphora* (L.) Sieb. in Japan. The specimen show the characteristics of the genus[3] and species[4]. They form small orange patches of about 1 cm diameter, show acanthohyphidia and large warty amyloid ellipsoid spores (31×15 μm) which readily germinate on YMG medium. The strain is deposited in the collection of the Lehrbereich Biotechnologie, University of Kaiserslautern.

Fermentation

For maintenance and submerged cultivation *A. mirabilis* 8435 was grown in YMG medium (yeast extract 0.4%, glucose 0.4%, malt extract 1%, pH 5.5). Fermentation was carried out in a 150-liter tank (Deutsche Metrohm, Stuttgart) containing 100 liters of YMG medium with stirring (100 rpm) and aeration (10 liters air/minute) at 22°C. Antibiotic production was followed by paper disc-agar diffusion assay using *Mucor miehei* as test organism and by TLC.

Isolation

After removal of the mycelia by filtration, the culture broth (90 liters) was extracted with ethyl acetate (40 liters). Evaporation of the organic phase yielded a crude extract (5.1 g) which was applied to a column of silica gel (Merck 60, 20×5 cm). Elution with CH_2Cl_2 - MeOH (9 : 1) yielded aleurodiscal which was combined with a second batch resulting from extraction of the freeze dried mycelia (370 g) with ethyl acetate (6 liters) followed by concentration. Pure aleurodiscal (1.2 g) was obtained in form of colorless crystals by repeated crystallization from CH_2Cl_2 - MeOH (9 : 1).

Aleurodiscal (**1**)

Colorless crystals: MP 249°C; $[\alpha]_D^{20}$ −86° (*c* 0.1, CH_2Cl_2 - MeOH, 9 : 1); Rf 0.57 (CH_2Cl_2 - MeOH, 9 : 1), 0.22 (toluene - acetone - AcOH, 70 : 30 : 1); UV λ_{max} (CH_2Cl_2 - MeOH, 9 : 1) nm 237; IR (KBr) cm^{-1} 3560, 2980, 2880, 1645, 1465, 1280, 1175, 1080, 980, 815; ^1H NMR (400 MHz, CD_2Cl_2 - MeOD, 9 : 1) δ 0.95~1.78 (14H, m), 0.72, 0.82 (each d, 20- and 21-CH_3), 0.82 (s, 22-CH_3), 1.18 (d, 24-CH_3), 1.45 (s, 23-CH_3), 2.05 (dd, J=16 and 9 Hz, 9-H_α), 2.13 (dd, J=13.5 and 9 Hz, 5-H_β), 2.30 (dd, J=9 and 9 Hz, 6-H), 2.77 (dd, J=16 and 7.5 Hz, 9-H_β), 3.13 (dt, J=13.5 and 9 Hz, 5-H_α), 3.24 (dd, J=11.5 and 9 Hz, 5′-H_{ax}), 3.28 (dd, J=8 and 6.5 Hz, 2′-H), 3.40 (dd, J=8 and 8 Hz, 3′-H), 3.47 (dt, J=7.5 and 9 Hz, 8-H), 3.54 (ddd, J=9, 8 and 5 Hz, 4′-H), 3.91 (dd, J=11.5 and 5 Hz, 5′-H_{eq}), 4.36 (d, J=6.5 Hz, 1′-H), 6.49 (dd, J=9 and 9 Hz, 4-H), 9.25 (s, 25-H). The assignments given have been proved by selective decoupling experiments. ^{13}C NMR (100.6 MHz, CD_2Cl_2 - MeOD, 9 : 1) δ 15.34 (q), 16.51 (q), 18.36 (q), 19.36 (q), 22.82 (q), 24.28 (t), 29.83 (t), 30.20 (d), 30.91 (t), 36.26 (d), 39.19 (t), 40.55 (t), 43.58 (s, C-15), 45.35 (t), 47.01 (d), 47.17 (d), 47.32 (d), 48.45 (d), 52.28 (d), 66.60 (t, C-5′), 71.04 (d, C-4′), 74.47 (d, C-2′), 77.75 (d, C-3′), 86.92 (d, C-8), 105.53 (d, C-1′), 131.40 (s), 131.87 (s), 150.54 (s), 152.89 (d, C-4), 197.45 (d, C-25). The multiplicities were derived from a distortionless enhancement by polarization transfer (DEPT) experiment. Electron impact mass spectra (EI-MS) (direct inlet, 180°C) *m/z* (relative intensity %) 502.3303 (3, M^+, calcd for $C_{30}H_{46}O_6$ 502.3280), 484 (2), 370 (32), 352 (100), 335 (14), 324 (15), 260 (69), 245 (16), 133 (48), 128 (30), 121 (38), 120 (41), 107 (33), 95 (34), 81 (41), 73 (78), 57 (34).

Hydrolysis of **1**

A suspension of aleurodiscal (**1**) (5 mg) in methanol (5 ml) and two drops of 16 N HCl was refluxed for 3 hours. After evaporation of the solvent, the D-(+)-xylose was separated by isocratic reversed-phase HPLC. $[\alpha]_D^{20}$ +17.6° (*c* 0.03, MeOH) (ref 5; D-(+)-xylose: $[\alpha]_D^{20}$ +19° (H_2O)).

Crystal Structure Determination

Aleurodiscal, $C_{30}H_{46}O_6$, crystallizes orthorhombic, space group $P2_12_12_1$ with a=12.996(2), b=34.701(6), c=6.233(2) Å, V=2811(2) Å^3, Z=4, D_{calc}=1.19 gcm^{-3}. Intensity data were collected on an Enraf-Nonius CAD4 diffractometer at varied scan rates in the ω-mode with graphite-monochromated CuKα radiation (λ=1.54184 Å, $2\theta_{max}$=130°; 1640 observed reflections with $F_0^2 > 2\sigma(F_0^2)$ from 2,811 measured). Three monitor reflections were measured at regular intervals; crystal decay was not observed. Empirical absorption corrections were applied to the reflection intensities (μ=6.1 cm^{-1},

crystal size $0.12\times0.13\times0.42$ mm). The structure was solved by direct methods (MULTAN 82) and refined by full matrix least-squares. Hydrogen atoms on oxygen atom 20 - oxygen atom 22 were located in a difference synthesis and included at these sites. The other hydrogen atom positions were calculated geometrically; joint isotropic temperature factors were assigned to analogous hydrogens. The remaining atoms were refined anisotropically. Final reliability indices were $R=0.058$ and $R_w=0.055$ with weights given by $w=(\sigma^2(F_0)+p^2F_0^2)^{-1}$, $p=0.014$. Atom coordinates and equivalent isotropic temperature factors for the non-hydrogen atoms are listed in Table 1. The structure is depicted in Fig. 1.

Further details of the crystal structure determination have been deposited at the Fachinformationszentrum Energie, Physik, Mathematik GmbH, D-7514 Eggenstein-Leopoldshafen 2. Any request for this material should be accompanied by a full literature citation and the reference number CSD to be added in proof.

Biological Assays

The antimicrobial spectra, the cytotoxicity against cells of the ascitic form of Ehrlich carcinoma and the effect of aleurodiscal on the radial growth of *M. miehei* were measured as described previously[6~8].

Test for Mutagenicity

Mutagenicity was tested as described by AMES *et al.*[9]. Mutants of *Salmonella typhimurium*, strain TA 98 and strain TA 100 were used for the spot test without rat liver microsomes.

Effect of Aleurodiscal on RNA, Protein, and Chitin Syntheses of *M. miehei*

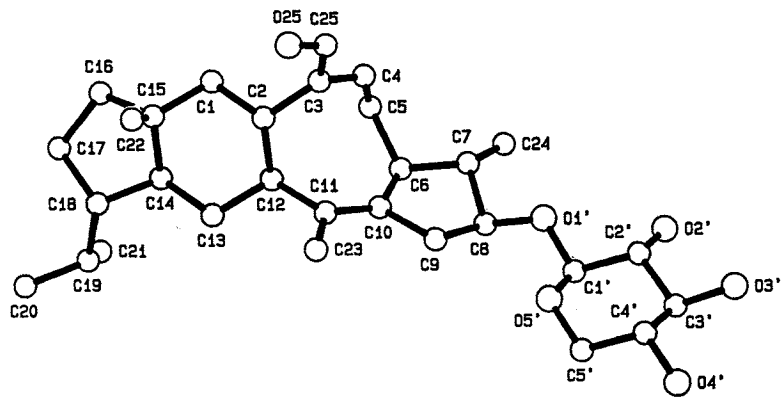
The 200-ml of a malt extract medium (1.5% malt extract, pH 5.5) were inoculated with 2×10^7 spores of *M. miehei* and incubated for 10 hours at 37°C. Then 5 ml of this culture (1.3 mg dry weight of mycelium) were preincubated with or without aleurodiscal (freshly dissolved in DMSO) for 30 minutes at 37°C on a water-bath shaker at 100 rpm. 1-ml portions of these cultures were withdrawn and incubated 90 minutes at 37°C with 0.1 μCi of [1-^{14}C]uridine, [1-^{14}C]leucine, or [1-^{14}C]-*N*-acetyl-D-glucosamine respectively. Then 1 ml of 10% TCA were added to each tube and the mycelia collected on cellulose nitrate filters. After washing with 5 ml of 5% TCA and drying, the radioactivity on the filters was determined by liquid scintillation counting.

Results and Discussion

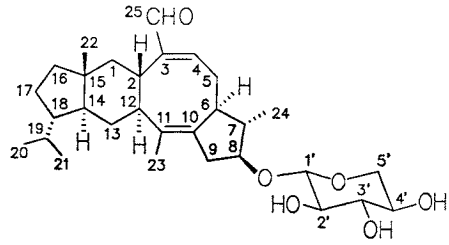
Properties and Spectroscopic Data

Aleurodiscal (**1**), mp 249°C, is only slightly soluble in water and the common organic solvents. Its high resolution (HR)EI-MS exhibits a molecular ion at *m/z* 502 which corresponds to the

Fig. 1. Molecular structure of aleurodiscal.



molecular formula $C_{30}H_{46}O_6$. Prominent ions are visible at m/z 484 (M^+-H_2O), 469 ($M^+-H_2O-CH_3$), and 352 (base peak, $M^+-C_5H_{10}O_5$). The ^{13}C NMR spectrum of **1** shows thirty carbon signals which could be attributed to an aldehyde carbonyl, four olefinic carbons, one quaternary carbon, twelve methine carbons, seven methylene carbons and five methyl carbons. The number and chemical shifts of the carbon signals and the loss of a $C_5H_{10}O_5$ fragment in the MS indicate that aleurodiscal is



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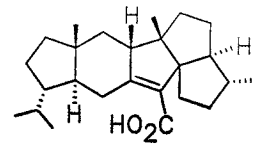

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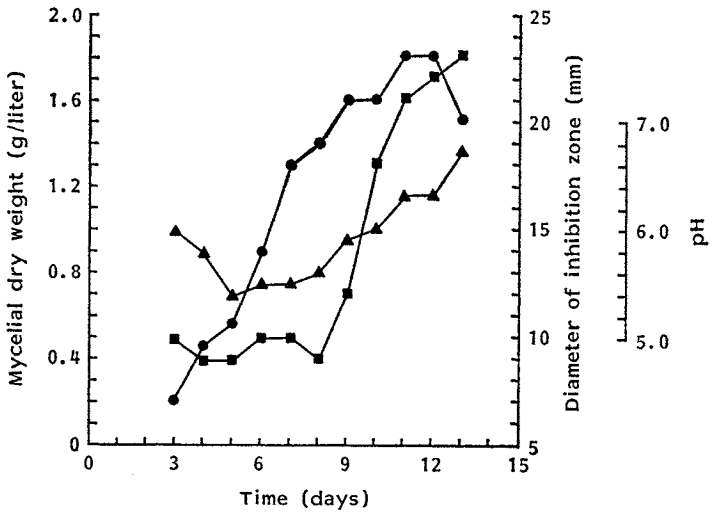
Table 1. Atom coordinates with equivalent isotropic temperature factors.

Atom	X/a	Y/b	Z/c	U_{eq} (Å$^2\cdot10^3$)
O1′	0.9191(3)	0.0520(1)	0.5742(8)	45(3)
O2′	1.0819(3)	−0.0001(1)	0.6230(9)	53(3)
O3′	1.2061(3)	−0.0060(1)	0.2366(8)	47(3)
O4′	1.2409(4)	0.0644(1)	0.0132(8)	44(3)
O5′	1.0062(4)	0.0859(1)	0.3218(9)	52(3)
C1′	1.0161(5)	0.0613(2)	0.5024(13)	42(5)
C2′	1.0666(5)	0.0235(2)	0.4390(12)	39(4)
C3′	1.1667(5)	0.0299(2)	0.3212(12)	36(4)
C4′	1.1491(5)	0.0569(2)	0.1353(11)	37(4)
C5′	1.1013(5)	0.0937(2)	0.2182(14)	52(5)
C1	0.3670(5)	0.1453(2)	0.8018(13)	43(5)
C2	0.4627(5)	0.1378(2)	0.6597(13)	39(4)
C3	0.4804(5)	0.0955(2)	0.6260(14)	44(5)
C4	0.5381(5)	0.0720(2)	0.7492(16)	54(5)
C5	0.5998(5)	0.0824(2)	0.9396(13)	47(5)
C6	0.7095(5)	0.0957(2)	0.8744(13)	41(4)
C7	0.7787(5)	0.0621(2)	0.8132(13)	41(4)
C8	0.8658(5)	0.0811(2)	0.6948(14)	43(5)
C9	0.8139(5)	0.1116(2)	0.5548(13)	46(5)
C10	0.7170(5)	0.1230(2)	0.6829(12)	37(4)
C11	0.6534(5)	0.1508(2)	0.6203(12)	36(4)
C12	0.5569(4)	0.1610(2)	0.7432(13)	37(4)
C13	0.5310(5)	0.2043(2)	0.7390(13)	40(4)
C14	0.4333(5)	0.2114(2)	0.8748(12)	36(4)
C15	0.3395(5)	0.1880(2)	0.7997(12)	37(4)
C16	0.2611(5)	0.2017(2)	0.9676(14)	53(5)
C17	0.2832(5)	0.2452(2)	0.9975(14)	52(5)
C18	0.3906(5)	0.2523(2)	0.8967(12)	42(5)
C19	0.4605(6)	0.2812(2)	1.0135(14)	49(5)
C20	0.4165(7)	0.3220(2)	1.0008(17)	77(6)
C21	0.4833(6)	0.2705(2)	1.2487(14)	65(6)
C22	0.3000(5)	0.2000(2)	0.5741(13)	50(5)
C23	0.6714(6)	0.1723(2)	0.4137(12)	51(5)
C24	0.8125(6)	0.0362(2)	0.9969(14)	60(6)
C25	0.4284(6)	0.0774(2)	0.4491(15)	53(6)
O25	0.3735(6)	0.0950(2)	0.3211(10)	70(4)

Fig. 2. Fermentation of *Aleurodiscus mirabilis*.

● Dry weight, ■ activity, ▲ pH.



The antifungal activity was followed up by agar diffusion assay using *Mucor miehei* as test organism.

a doubly unsaturated sesterterpene aldehyde connected to a pentose moiety. Because of the high complexity of the ^{1}H NMR spectrum and the low solubility of aleurodiscal in the common NMR solvents its structure was determined by a single-crystal X-ray diffraction analysis.

X-Ray Structure Determination

The eight membered cod-ring displays a twisted boat conformation in which C5, C6, C12 and C2 are respectively displaced by the following distances from the best least-squares plane through the four sp^2-hybridised C-atoms: 1.12, 0.62, 1.21 and 0.70 Å. The following ring torsion angles were calculated (commensing with C2–C3–C4–C5): −2.3, −87.0, 43.6, 34.6, 1.6, −91.4, 46.1 and 37.4°.

Table 2. Antibacterial and antifungal activity of aleurodiscal in the serial dilution assay.

Organism	MIC (μg/ml)
Bacteria:	
Acinetobacter calcoaceticus	>100
Bacillus brevis	>100
B. subtilis	50~100
Corynebacterium insidiosum	>100
Micrococcus luteus	>100
Staphylococcus aureus	>100
Escherichia coli K-12	>100
Pseudomonas fluorescens	>100
Mycobacterium phlei	>100
Streptomyces sp.	>100
Fungi:	
Mucor miehei	0.3~1
Rhodotorula glutinis	>100
Saccharomyces cerevisiae iS 1[a]	>100

[a] Strain obtained from Prof. F. LACROUTE, Straßburg.

Inoculum 10^6 cells/ml.

For the determination of its absolute configuration, **1** was hydrolysed with methanolic HCl. The xylose formed was separated by reversed-phase HPLC and its D-configuration determined by the positive optical rotation observed. This establishes the absolute configuration of aleurodiscal as given in formula **1**.

Aleurodiscal constitutes a new type of sesterterpenoid[10]. It is biogenetically closely related to retigeranic acid A (**2**)[11~13] a pentacyclic sesterterpenoid from lichens which possesses the same relative and absolute stereochemistry.

Table 3. Antifungal activity of aleurodiscal in the agar diffusion assay.

Organism	Diameter of inhibition zone (mm)			
	2 μg/disc	10 μg/disc	50 μg/disc	100 μg/disc
Absidia glauca (+)	11	12		
A. glauca (−)	—	10		
Alternaria porri	8	11		
Ascochyta pisi	15	21		
Aspergillus ochraceus	20	24		
Botrytis cinerea	—	—	—	—
Cladosporium cladosporioides	—	—	—	10
Curvularia lunata	—	—	—	—
Epicoccum purpurascens	—	—	—	—
Eurotium cristatum	—	—	—	20
Fusarium fujikuroi	—	—	—	—
F. oxysporum	10	13		
Mucor miehei	14	17		
Nematospora coryli	—	—	—	—
Neurospora crassa	—	—	—	—
Paecilomyces varioti	—	12		
Penicillium islandicum	—	14		
P. notatum	—	—	—	—
Phoma clematidina	—	—	—	—
Phytophthora infestans	—	—	—	—
Pythium debaryanum	—	—	—	—
Ustilago nuda	—	—	—	—
Venturia cerasi	—	—	8	
Verticillium sp. 153-83	10			
Zygorrhynchus moelleri	13	16		
Rhodotorula glutinis	—	—	—	10
Saccharomyces cerevisiae iS 1[a]	—	—	—	—

[a] Strain obtained from Prof. F. LACROUTE, Straßburg.
—: No inhibition zone.

During fermentation (Fig. 2) the production of aleurodiscal by *A. mirabilis* 8435 starts 9 days after inoculation. At this time the free glucose in the medium has been used up almost completely. The highest antibiotic content is reached after 13 days. When grown under comparable conditions the European species *Aleurodiscus disciformis* (DC. ex Fr.) Pat. did not produce detectable amounts of antibiotics. The antimicrobial spectra of aleurodiscal are shown in Tables 2 and 3. In the serial dilution assay *M. miehei* is inhibited at very low concentrations, whereas yeasts and with the exception of *Bacillus subtilis* bacteria are not affected up to concentrations of 100 μg/ml. In the plate diffusion assay (Table 3) most of the fungi tested are strongly

Fig. 3. Effect of aleurodiscal on the radial growth of *Mucor miehei*.

● Control, ■ 1 μg/ml, ▲ 10 μg/ml.

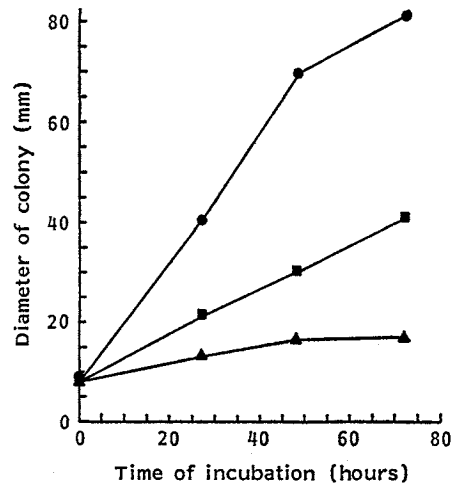
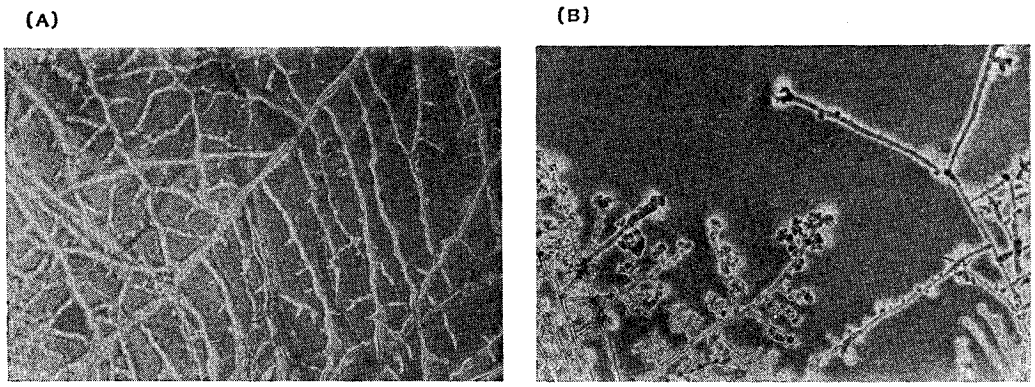
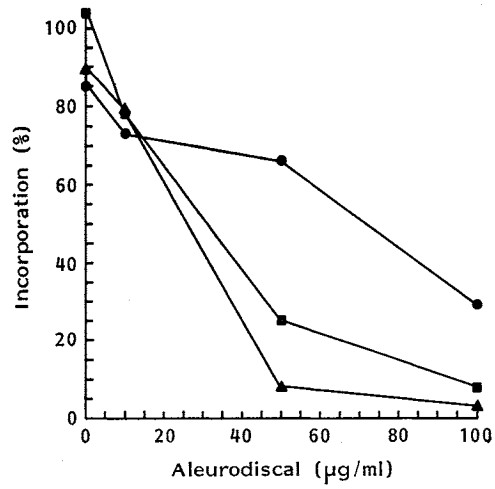


Fig. 4. Hyphal branching of *Mucor miehei*.
(A) Control, (B) 1 μg/ml aleurodiscal.



inhibited by concentrations of 2~10 μg/disc. The effect of aleurodiscal on the radial growth of *M. miehei* is shown in Fig. 3. In YMG medium growth of this zygomycete is inhibited 50% at 1 μg/ml while almost no growth is observed at 10 μg/ml. At both concentrations the apical hyphae of *M. miehei* are abnormally branched as compared to the controls without antibiotic (Fig. 4). Similar effects have been reported for the scopamycines which are produced by *Streptomyces* sp. ETH 28832 using *Botrytis cinerea* as test organism[14].

Fig. 5. Effect of aleurodiscal on the incorporation of [^{14}C]uridine (●), [^{14}C]leucine (■), and [^{14}C]-*N*-acetyl-D-glucosamine (▲) in *Mucor miehei*.



Controls without antibiotic (100%): [^{14}C]Uridine 6.056 cpm, [^{14}C]leucine 91.473 cpm, [^{14}C]-*N*-acetyl-D-glucosamine 98.296 cpm.

The effect of aleurodiscal on the incorporation of [^{14}C]-*N*-acetyl-D-glucosamine, [^{14}C]leucine, and [^{14}C]uridine into TCA-precipitable material (chitin and chitosan, protein, and RNA) in mycelia of *M. miehei* is shown in Fig. 5. The incorporation of [^{14}C]-*N*-acetyl-D-glucosamine into chitin and chitosan was almost completely inhibited by concentrations of 50 μg/ml. Protein and RNA syntheses were affected to a lesser extent. However, the effects on macromolecular syntheses were low at the concentrations needed for the induction of morphological alterations (1~10 μg/ml).

The cytotoxic activity of aleurodiscal was tested with Balb/3T3 clone A31 (mouse embryo cells, ATCC CCL 163), Ehrlich ascites carcinoma cells (H. PROBST, University of Tübingen), SV-T2 (SV40 transformed Balb/3T3 mouse embryo cells, ATCC CCL 163.1), and M-MSV-Balb/3T3 (Moloney murine sarcoma virus transformed Balb/3T3 mouse embryo cells, ATCC CCL 163.2) as described previously[8]. At 40 μg/ml of aleurodiscal lysis of 50% of Balb 3T3 cells and reduced proliferation of ECA cells but no inhibition of growth of SV-T2 and M-MSV-Balb/3T3 cells was observed after 48 hours of

incubation.

In the test for mutagenicity according to AMES *et al.*[9] no induction of revertants of *S. typhimurium* TA 98 and TA 100 could be observed with 100 μg of aleurodiscal/disc (spot test without addition of rat liver microsomes).

Acknowledgments

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