ANTIBIOTICS FROM BASIDIOMYCETES. V[1]

MERULIDIAL, A NEW ANTIBIOTIC FROM THE BASIDIOMYCETE *MERULIUS TREMELLOSUS* FR.

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Merulidial, a new antibiotic, was isolated from the culture fluid of the Basidiomycete *Merulius tremellosus* FR., strain No. WQ 568. Merulidial inhibits a variety of bacteria and fungi. In cells of the ascitic form of EHRLICH carcinoma, DNA synthesis is inhibited at lower concentration as compared to RNA and protein synthesis. Merulidial shows mutagenicity when incubated with the his^--mutant TA 100 for *Salmonella typhimurium* (B.N. AMES). The molecular formula as determined by high resolution mass spectrometry is $C_{15}H_{20}O_3$.

The resupinate-reflexed growing Basidiomycete *Merulius tremellosus* FR., most frequently found on stumps and logs of deciduous trees, produces several antibiotics. One of them, merulinic acid A, isolated from the fruit bodies, has recently been described.[2] In the following we wish to describe the fermentation, isolation, physicochemical characterization, and the biological properties of a new antibiotic, which was named merulidial.

The determination of the structure will be subject of another publication.

Materials and Methods

Fermentation

Merulius tremellosus was grown on agar slants containing a yeast extract - malt extract medium (YM, yeast extract 4.0 g, malt extract 10.0 g, glucose 4.0 g per liter). From this culture three Erlenmeyer flasks with a 130-ml YM medium (pH 5.0) were inoculated and incubated on a rotary shaker (120 rpm, 22°C). These cultures were used as inoculum for a fermentor (New Brunswick FS 314) with 10 liters of the same medium. One ml of Niax polyol antifoam was added, the culture was aerated with 2 liters air per minute and stirred mechanically (120 rpm) at 22°C. During the fermentation, the antibiotic activity of the culture broth was estimated after extraction with ethyl acetate.

Isolation

The culture fluid from an 8-liter culture was separated from the mycelia and extracted three times with 2 liters of ethyl acetate. After the evaporation of the solvent, the crude extract (8.9 g) was loaded on a column (5 × 7 cm) of silica gel (Mallinckrodt) in chloroform - ethanol (99 : 1), and eluted with the same solvent. The fractions containing the antibiotic were pooled, the solvent evaporated and the residue (2 g) rechromatographed. The brown oil thus obtained was dissolved in 3 ml of chloroform, loaded on a column of silica gel (2.5 × 12 cm) and eluted with chloroform - ethanol - methanol (99 : 1 : 1).

Merulidial was obtained as a light yellow homogenous oil yielding 800 mg colorless crystals after 2 ~ 3 months at −18°C.

Thin-layer chromatography and bioautography

For thin-layer chromatography (TLC), Merck silica gel plates 60 F_{254} were used and the spots

detected under UV-light (254 nm) or by bioautography on agar plates seeded with *Bacillus subtilis*.

Test organisms and media

Clostridium pasteurianum was grown on Merck RCM medium 5411, *Corynebacterium insidiosum*, *Proteus vulgaris*, *Sarcina lutea* on YM medium, and the other bacteria on nutrient broth (Difco 8.0 g/liter). The incubation temperature was 27°C for *Arthrobacter citreus*, *Corynebacterium insidiosum*, *Micrococcus roseus*, *Sarcina lutea* and *Clostridium pasteurianum*, and 37°C for the other bacteria. *Aspergillus panamensis* and *Penicillium notatum* were grown on malt extract (20 g/liter), *Pythium debaryanum* on corn meal agar (Difco), *Saccharomyces cerevisiae* and *Rhodotorula glutinis* on yeast nitrogen base (Difco) containing 4 g glucose per liter and the other fungi on YM agar. The incubation temperature for all fungi was 27°C.

Macromolecular syntheses in cells of the ascitic form of EHRLICH carcinoma

The effect of merulidial on the macromolecular syntheses in cells of the ascitic form of EHRLICH carcinoma (ECA) was tested as described previously[3].

Test for mutagenicity

Mutagenicity was tested as described by AMES *et al.*[4] Mutants of *Salmonella typhimurium*, strain TA 100, strain TA 98, strain TA 1535, and strain TA 1537, were used for the spot test and strain TA 100 for the plate incorporation assay (3×10^8 cells/ml, without rat liver microsomes).

Results and Discussion

Isolation and Physico-chemical Properties

Merulidial could not be found in the fruit bodies of the fermentation strain of *Merulius tremellosus* FR. However, the fruit bodies of *Merulius tremellosus* FR. and *Phlebia radiata* FR. both contain the same antibiotic, merulinic acid A[2], suggesting a close relationship of these species. We therefore tried to detect merulidial in liquid cultures of *Phlebia radiata* FR. grown under identical conditions, but did not succeed. Merulidial, obtained as described in the experimental section, melts at 34°C and is soluble in chloroform, diethylether, ethyl acetate and methanol, less soluble in petrolether, carbon tetrachloride and water.

Table 1. Chromatographic behaviour of merulidial

Solvent system	Rf value
Benzene - acetone - acetic acid 70 : 30 : 1	0.67
Benzene - acetone - diethylamine 70 : 30 : 1	0.71
Cyclohexane - ethyl acetate - formic acid, 120 : 40 : 5	0.11

Fig. 1. IR-spectrum of merulidial (oil on a NaCl window).

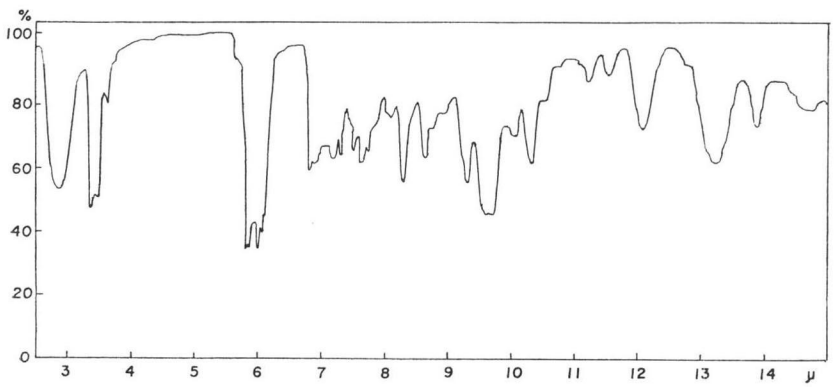


Fig. 2 Mass spectrum of merulidial.
Mass spectrum was determined on an A.E.I. MS 50 mass spectrometer (70 eV, direct insertion, 150°C).

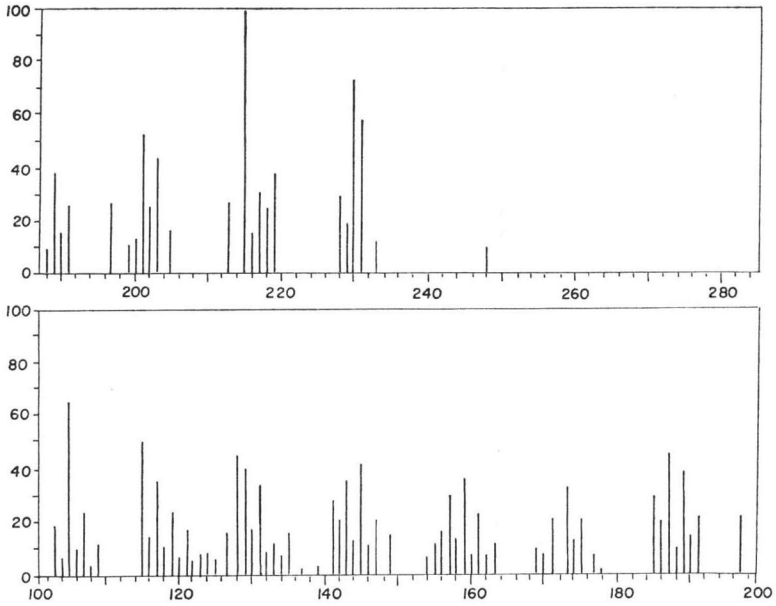


Table 1 shows the Rf values on TLC with several solvent systems. Merulidial gives a positive reaction with CARR PRICE reagent (blue), 50% H_3PO_4 (violet), aniline phthalate (blue, after heating), and anisaldehyde H_2SO_4 (purple).

The UV-spectrum in methanol shows a maximum at 270 nm ($\varepsilon = 3{,}660$). Fig. 1 shows the IR-spectrum of merulidial.

The mass-spectrum (Fig. 2) and high resolution of the molecular ions *m/e* 248.1412 yielded the formula $C_{15}H_{20}O_3$.

Biological Properties

Table 2 shows the antimicrobial spectrum of merulidial. Merulidial is active against *Micrococcus roseus, Corynebacterium insidiosum, Bacillus brevis, Bacillus subtilis, Streptomyces viridochromogenes, Sarcina lutea, Arthrobacter citreus* (Gram-positive bacteria), *Proteus vulgaris* (Gram-negative bacterium) and against a variety of filamentous fungi and yeasts.

Fig. 3 shows the effect of merulidial on the growth of *Pythium debaryanum*. An inoculum (diameter 8 mm) of *Pythium debaryanum* grown on corn meal agar was placed in the center of Petri dishes containing 0 (control), 10 and 100 μg merulidial per ml.

Fig. 4 shows the effect of merulidial on the RNA, DNA and protein syntheses in ECA-cells. At a concentration of 1 μg merulidial per ml incorporation of thymidine into the TCA precipitable fraction of cells is reduced to 22% of the control whereas incorporation of uridine or leucine is much less affected.

Merulidial does not cause haemolysis in a test with human erythrocytes, performed as described earlier.[2]

In the spot test for mutagenicity, *Salmonella typhimurium* TA 100 proved to be the only strain

Table 2. Antimicrobial spectrum of merulidial

	Test organism	MIC (μg/ml)
Serial dilution test	*Aerobacter aerogenes*	>100
	Arthrobacter citreus	10~100
	Bacillus brevis	73
	Bacillus subtilis	10~100
	Corynebacterium insidiosum	10~100
	Escherichia coli	>100
	Micrococcus roseus	1~10
	Mycobacterium phlei	>100
	Proteus vulgaris	10
	Pseudomonas fluorescens	>100
	Sarcina lutea	10~100
	Staphylococcus aureus	>100
	Streptomyces viridochromogenes	10
Diameter inhibition zone (mm) with 100 μg per paper disc (6 mm)	*Clostridium pasteurianum*	—
	Aspergillus panamensis	20
	Botrytis cinerea	—
	Fusarium cubense	11
	Neurospora crassa	19
	Paecilomyces varioti	10
	Penicillium notatum	12
	Rhizoctonia solani	20
	Ascoidea rubescens	25
	Candida albicans	11
	Dipodascus albidus	—
	Endomyces magnusii	16 i
	Endomycopsis fibuliger	14 i
	Eremothecium ashbyi	38
	Hansenula anomala	8 i
	Nadsonia fulvescens	—
	Nematospora coryli	21
	Pichia farinosa	—
	Rhodotorula glutinis	9
	Saccharomyces cerevisiae	20
	Schizosaccharomyces pombe	8

i=incomplete inhibition.

Fig. 3. Effect of merulidial on the growth of *Pythium debaryanum*.
(1) Control without antibiotic, (2) Plate containing 10 μg/ml, (3) Plate containing 100 μg/ml.

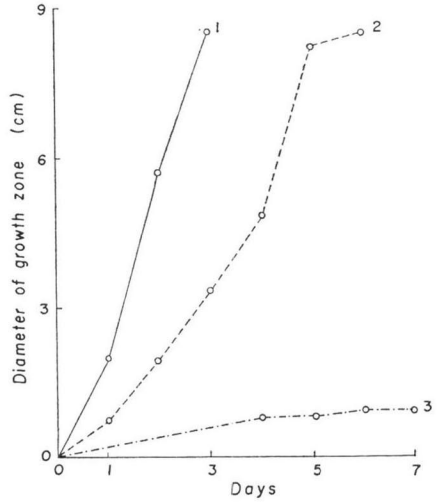
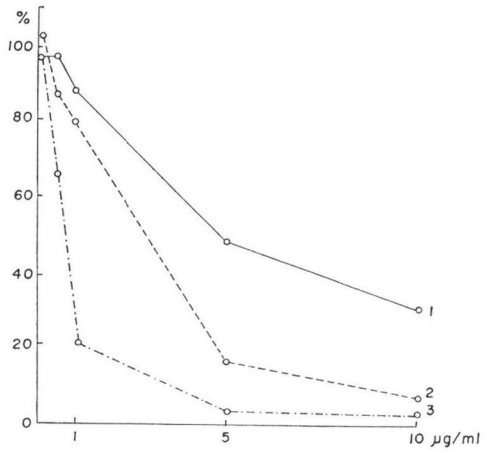


Fig. 4. Effect of merulidial on RNA, DNA and proteins syntheses of ECA-cells in % of the control without antibiotic.
(1) Protein synthesis, (2) RNA synthesis, (3) DNA synthesis.



yielding revertants after addition of merulidial. In the plate incorporation assay, 216 revertants were found when 10 μg merulidial per ml was added to the plate (revertants on a plate containing no merulidial substracted).

These findings and the preferential inhibition of DNA synthesis in ECA-cells suggest that merulidial may interact with the DNA template or DNA replication.

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