MYXOTHIAZOL, AN ANTIBIOTIC FROM *MYXOCOCCUS FULVUS* (MYXOBACTERALES)

I. CULTIVATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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Myxothiazol (AB-Mx f16-1*), a new antifungal antibiotic, is produced by the myxobacterium *Myxococcus fulvus* strain Mx f16. It is active against many filamentous fungi, and completely inhibits growth of *Mucor hiemalis* at a concentration of 2 μg/ml. The molecular formula of myxothiazol was determined to be $C_{25}H_{33}N_3O_3S_2$.

Investigators looking for new antibiotics are becoming more and more interested in the gliding bacteria, organisms that so far have not been thoroughly studied in this respect. Already a number of biologically active compounds have been isolated and more or less characterized. The chemical structure is known for myxin, the first antibiotic from a gliding bacterium to be fully characterized[8]; for ambruticin, a very interesting antifungal antibiotic[6]; and for compound G 1499-2, which is active against some Gram-positive bacteria[3]. A fungistatic activity observed with *Myxococcus xanthus* was due mainly to *iso*-branched fatty acids excreted into the medium[5]. A partial structure has been published for myxosidin, a decapeptide inhibiting a wide variety of bacteria[2]. Furthermore there are several antibiotics that are chemically still insufficiently characterized: Antibiotic TA, inhibitory for Gram-negative and Gram-positive bacteria, from *Myxococcus xanthus*[7]; an antibiotic against Gram-positive bacteria from *Myxococcus coralloides*[1]; and a compound, thought to be a polypeptide, that is active against Gram-positive and Gram-negative bacteria and was obtained from *Cytophaga*-like bacteria in freshwater[4].

In this paper we wish to report on the production, isolation, and some physico-chemical and biological properties of myxothiazol, a new antifungal antibiotic extracted from the cells of a true myxobacterium, *Myxococcus fulvus* strain Mx f16.

Production of the Antibiotic

Myxococcus fulvus strain Mx f16 (=*Mx. fulvus* HR 2; deposited at the German Collection of Microorganisms in Göttingen under number DSM 1368) was isolated in 1971 from a soil sample obtained at Borobudur, Java.

For antibiotic production, the organism was grown in peptone liquid medium (1% peptone from casein, tryptically digested, Merck, Darmstadt; 0.3% $MgSO_4 \cdot 7H_2O$; pH 7.2). Batch cultures of 100 ml in 250 ml Erlenmeyer flasks, or of 500 ml in 1 liter flasks, were incubated at 30°C on a rotary shaker at 160 cycles per minute.

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Fermentations were performed in 70-liter fermentors (Giovanola, Manthey, Switzerland) equipped with a circulating pump stirrer system ("intensor system"). Antifoam Lb 625 (Brenntag, Mülheim/Ruhr) was added to the medium in a concentration of 0.03%. To start the culture, 63 liters of medium were inoculated with 7 liters of shake culture broth in mid-log phase. The fermentor was maintained at 30°C, and stirred at 500 r.p.m. The aeration rate was 0.1 v/vm for the first 13 hours, and 0.17 v/vm for the following 17 hours. The pH was held constant at 7.5 with acetic acid. Within 8 hours after inoculation the pO_2 fell almost to zero and remained there during the rest of the fermentation. To check antibiotic production, 100-ml samples were centrifuged and the cells were extracted twice with 50 ml of acetone. The extracts were assayed by the paper-disk method against *Mucor hiemalis*.

Isolation of the Antibiotic

At harvest time, the cells were separated from the culture broth by centrifugation at 10~20°C. The red cell mass was extracted with acetone until it was colorless. The extracts were combined and concentrated under reduced pressure at 40°C. The red and highly viscous residue was dissolved in methanol. From this solution inactive non-polar material was removed by extraction with *n*-heptane. The methanol phase was then concentrated under reduced pressure, and the residue dissolved in ethyl acetate. This solution still contained considerable amounts of fatty acids which could be eliminated by extracting twice with 25% aqueous ammonia. After neutralization, the ethyl acetate solution was concentrated, and the antibiotic was isolated by chromatography on silica gel (30 g of crude material on 2.5 kg of silica gel S, Riedel de Haen, Seelze-Hannover) with acetone - *n*-heptane = 1: 4 as the eluant. The resulting material (*ca.* 1 g from 30 g of crude product) was about 95% myxothiazol. Final purification was achieved by chromatography on reversed-phase silica gel RP-18 (Merck, Darmstadt) with methanol - water = 4: 1 as the eluant.

Physical and Chemical Properties

Myxothiazol was a white, neutral, amorphous powder which liquefied between 50 and 55°C. It was soluble in methanol, ethanol, acetone, ethyl acetate, chloroform and dichloromethane. It was slightly soluble in benzene and water.

The antibiotic could be separated by thin-layer chromatography (Silica Gel 60 F_{254}, Merck, Darmstadt) with the following solvent systems: ethyl acetate (Rf 0.29); dichloromethane - isopropanol = 19: 1 (Rf 0.26); or toluene - dioxane - acetic acid = 30: 10: 1 (Rf 0.32). When the chromatograms were treated with iodine vapour, the antibiotic gave a brownish spot which turned dark green within 2~3 hours.

The optical rotation of myxothiazol was $[\alpha]_D^{25}$ +43.4° (*c* 6.0, methanol).

Accurate mass measurement of the molecular ion gave *m/z* 487.1982 and was in good agreement with the calculated mass 487.1980 for $C_{25}H_{33}N_3O_3S_2$. The ions of myxothiazol at *m/z* 487 (100%), 488 (31.4%), 489 (14.2%), and 490 (3.7%) (M^+, $M+1^+$, $M+2^+$, $M+3^+$) observed in the electron impact mass spectrum were in good agreement with the expected distribution of isotopes (Fig. 1).

Elementary analysis gave: C, 61.9; H, 6.8; N, 8.5; O, 9.7; S, 13.0

Calculated values for $C_{25}H_{33}N_3O_3S_2$: C, 61.6; H, 6.8; N, 8.6; O, 9.8; S, 13.1

The electronic absorption spectrum of myxothiazol (dissolved in methanol) had maxima at 234 nm (ε 46,970) and at 313 nm (ε 10,530) (Fig. 2). The infrared spectrum is shown in Fig. 3, and the 270

Fig. 1. Mass spectrum of myxothiazol (Varian MS-9, 12 eV, 200°C).

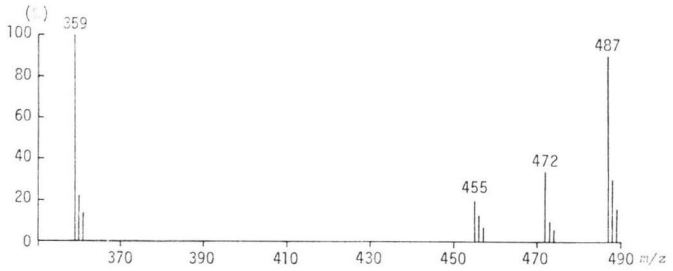


Fig. 2. Electronic absorption spectrum of myxothiazol in methanol.

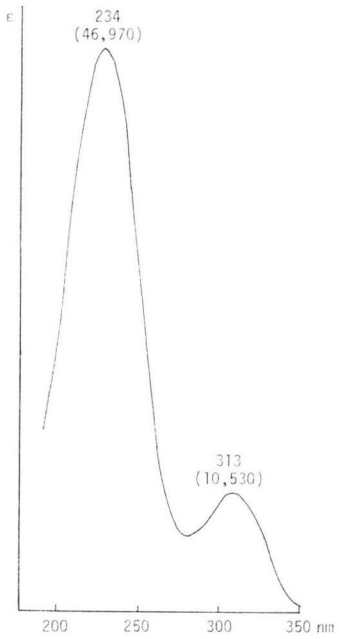


Fig. 3. IR spectrum of myxothiazol in chloroform.

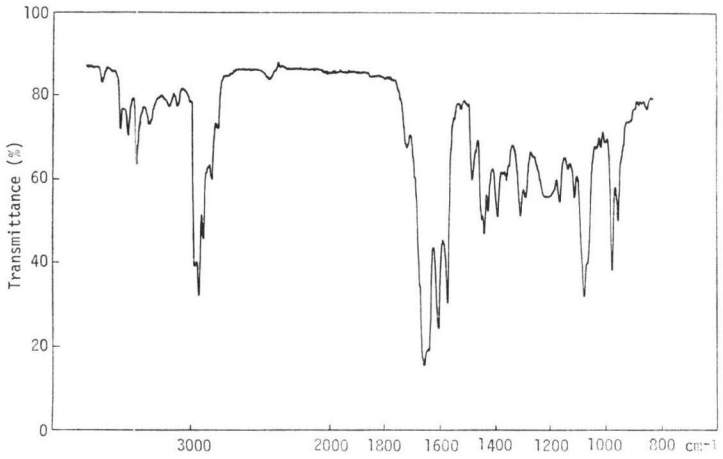
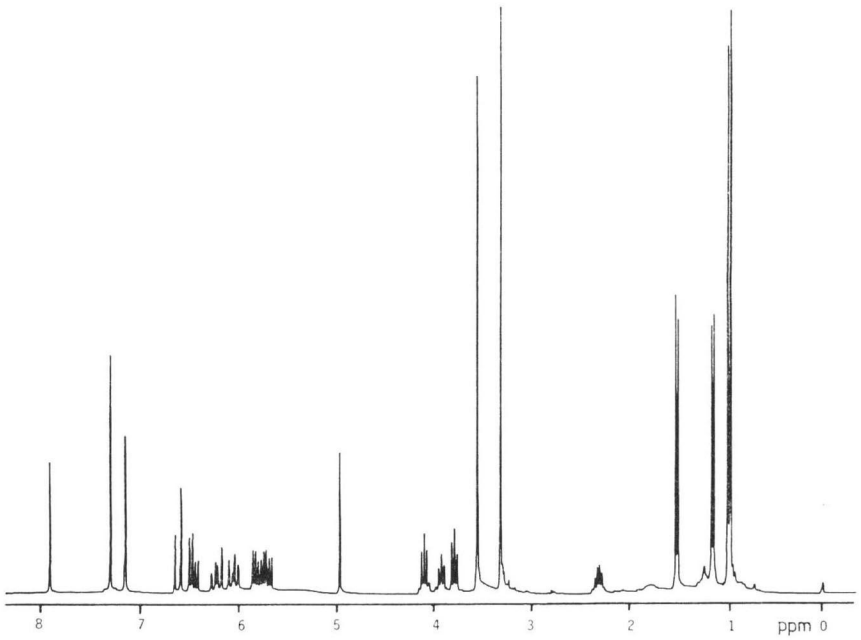


Fig. 4. ^1H NMR spectrum of myxothiazol in $CDCl_3$ (Bruker, 270 MHz).



MHz ^{1}H-NMR spectrum in Fig. 4.

Biological Properties

Myxothiazol was active against numerous fungi and a few Gram-positive bacteria. There was no inhibition of yeasts and Gram-negative bacteria under our test conditions (Table 1).

Minimal inhibitory concentrations were determined by the serial dilution assay. In the case of *Mucor hiemalis*, sporangiospores taken from plate cultures were suspended in peptone medium. Aliquots of the suspension were pipetted into reagent tubes containing varying amounts of the antibiotic. After incubation on a rotary shaker over night at 30°C, mycelia had formed in all cultures where the concentration of myxothiazol was not sufficient to suppress germination of the sporangiospores.

Table 1. The antibiotic spectrum of myxothiazol.

	Test organism*	Inhibition in plate culture**	MIC in liquid culture (μg myxothiazol/ml)
Fungi	*Botrytis cinerea* GBF 157	+	
	Microsporum gypseum CBS 424.66	+	
	Penicillium digitatum CBS 319.48	+	
	Mucor hiemalis Tü	+	1~2
	Geotrichum candidum CBS 187.38	+	
	Polyporus spec. GBF 224	+	
	Polystictus spec. GBF 223	+	
	Phycomyces blakesleeanus GBF 219	+	
	Trichophyton mentagrophytes GBF 238	+	0.02
	Piricularia oryzae GBF 158	+	0.8
	Pythium debaryanum GBF 161	+	0.02
	Rhizoctonia solani CBS 177.44	−	>50
	Alternaria brassicola CBS 106.41	−	
	Gliocladium roseum GBF 239	−	
Yeasts	*Candida albicans* CBS 1893	−	>50
	Schizosaccharomyces pombe Tü 501	−	
Bacteria	*Bacillus subtilis* ATCC 6051	(+)	
	Staphylococcus aureus GBF 16	(+)	30~50
	Escherichia coli GBF 164	−	>100
	Serratia marcescens GBF 61	−	>100
	Proteus morganii GBF 166	−	>100
	Pseudomonas aeruginosa GBF 167	−	>100
	Xanthomonas oryzae GBF 162	−	>50
	Xanthomonas vesicatoria GBF 240	−	>50

* Origin of the strains: ATCC=American Type Culture Collection in Washington, D.C., USA; CBS=Centraalbureau voor Schimmelcultures in Baarn, Netherlands; GBF=from our own culture collection; Tü=from the Institut für Biologie II of the university of Tübingen, FRG.

** The tests were carried out by the paper-disk method. Paper disks of 6-mm diameter containing 10 μg of myxothiazol were put on agar plates seeded with the respective test organisms. Fungi and yeasts were assayed on Mycophil agar (Phytone peptone, BBL, 1 %; glucose 1 %; agar 1.6 %). Bacteria were suspended in peptone agar (peptone from casein, tryptically digested, Merck, 1 %; $MgSO_4 \cdot 7H_2O$ 0.2 %; agar 1.6 %; pH 7.2).

Relatively high concentrations of the antibiotic were required to inhibit growth of *Staphylococcus aureus*. Inhibition became recognizable at 10 μg/ml and reached its maximum at 50 μg/ml, but even then the growth yield was reduced by only 35% as compared with control cultures.

The antibiotic effect of myxothiazol on *M. hiemalis* is fungistatic, for the number of viable cells remained constant over a long time when sporangiospores were incubated in the presence of 10 μg of myxothiazol/ml.

By studying the incorporation of radioactive precursors in germinating sporangiospores of *M. hiemalis*, it was demonstrated that the synthesis of RNA and of protein was stopped almost immediately upon the addition of 1.5 μg/ml of myxothiazol to the culture.

The compound proved highly toxic for chicken embryo fibroblasts. Acute toxicity for the mouse (LD_{50}) was 2 mg/kg. There was no selective toxicity towards transplantable EHRLICH ascites carcinoma in mouse: a slight growth inhibition of the tumor cells was observed only when the dosage approached the toxic level to mice (4×1 mg/kg, i.p.).

To find out which metabolites might be involved in the biosynthesis of myxothiazol, a number of radioactive amino acids and organic acids were fed to cultures of *Mx. fulvus* Mx f16. The potential precursors were added to batch cultures in mid-log phase. After 12-hours incubation, the cells were harvested and extracted with acetone. Each extract was chromatographed on a silica-gel thin-layer plate with ethyl acetate as the solvent. The myxothiazol fraction was scraped off, eluted with acetone, and rechromatographed twice on HPTLC silica-gel plates (Merck, Darmstadt) with dichloromethane - isopropanol=19: 1, and then with hexane - acetone=3: 1 as the solvents. The dried thin-layer plates were scanned with a thin-layer scanner (Berthold-Frieseke Vertriebs-GmbH für Meßtechnik, Karlsruhe). Results are shown in Table 2. Radioactivity was incorporated into myxothiazol from cysteine, threonine, leucine and isoleucine.

Table 2. Incorporation of radioactivity into myxothiazol by *Myxococcus fulvus* Mx f16.

Labelled compound tested*	Radioactivity added to 100 ml of culture (μCi)	Incorporation**
L-(^{35}S) Cysteine hydrochloride	380	+
L-(U-^{14}C) Isoleucine	50	+
L-(U-^{14}C) Leucine	50	+
L-(U-^{14}C) Phenylalanine	10	−
L-(U-^{14}C) Proline	25	−
L-(U-^{14}C) Threonine	50	+
DL-(benzene ring U-^{14}C) Tryptophan	4	−
L-(U-^{14}C) Tyrosine	40	−
(U-^{14}C) Acetic acid, sodium salt	20	−
DL-(2-^{14}C) Mevalonic acid	40	−

* All compounds from Amersham Buchler, Braunschweig.

** As judged by identity of Rf's for radioactive spot and myxothiazol reference in two solvent systems. Experimental details are given in the text.

Discussion

The data presented in this article strongly suggest that myxothiazol is not identical with any of the antibiotics obtained so far from gliding bacteria, nor with any other known antibiotic. The analysis of its chemical features (see the following paper) proved, that this is indeed the case.

The biological activity of myxothiazol was essentially restricted to eukaryotes. It was very toxic in all animal systems tested (chicken cells, ascites cells, mice), and it seems thus unlikely that the new compound could be useful for medical or veterinary purposes. Its toxicity for fungi was selective, perhaps due to differences in permeability. Toxicity for prokaryotes was restricted to a few Gram-

positive organisms. As in this case the required concentration of the antibiotic was high and the inhibition never complete, it may be surmised that the biochemical mechanism of inhibition is different in bacteria from that in eukaryotes. Investigations on the mechanism of action of myxothiazol, which are under way at present in our laboratory, should answer this question.

Acknowledgements

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