THE MYXOVALARGINS, NEW PEPTIDE ANTIBIOTICS FROM *MYXOCOCCUS FULVUS* (MYXOBACTERALES)

I. CULTIVATION, ISOLATION, AND SOME CHEMICAL AND BIOLOGICAL PROPERTIES†

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Antibiotic activity was isolated from the culture supernatant of the myxobacterium *Myxococcus fulvus* strain Mx f65. It was active against Gram-positive bacteria (MIC 0.3~5 μg/ml), at higher concentrations also against Gram-negative ones (MIC 6~100 μg/ml), and not at all against yeasts and molds. The activity could be resolved into 4 closely related peptides, the myxovalargins. One of them, myxovalargin A, was by far the most plentiful. The compounds appear to be new antibiotics and seem to interfere with protein synthesis.

In spite of some efforts in recent years[1,11,16,17], the chemical nature of the well documented antibiotic activity of myxobacteria[8,12,18] is still largely unknown. In a screening program aimed at closing the gap, activity against Gram-positive bacteria was found in the culture supernatant of *Myxococcus fulvus* strain Mx f65*. The activity could be isolated, purified, and some of its biological and chemical properties determined (for a preliminary account see reference 9).

Production of the Antibiotic

Myxococcus fulvus strain Mx f65 (=*M. fulvus* HR3; deposited at the German Collection of Microorganisms in Göttingen under number DSM 1525) was isolated in 1969 from a soil sample collected in the Kaiserstuhl mountains (FRG).

For antibiotic production, the organism was grown in modified peptone liquid medium (1% peptone from casein, tryptically digested, Merck, Darmstadt; 0.1% $MgSO_4 \cdot 7H_2O$, 0.04% $CaCl_2 \cdot 2H_2O$, pH 7.2). Antifoam N 115 (Brenntag, Mülheim, FRG) was added at a concentration of 0.02%. Large scale fermentations were performed in type b 50 (total volume 70 liters) and type b 200 (total volume 270 liters) bioreactors from Giovanola Frères SA, Manthey, Switzerland, equipped with either a turbine plate stirrer or a circulating pump stirrer. The bioreactors were inoculated with late log-phase shake cultures (7%, v/v). The incubation temperature was 30°C. The pO_2 was initially adjusted to 95% saturation, which was achieved with a stirring rate of 400 rpm and an aeration rate of 0.1 v/v·minute. In the course of the fermentation the pO_2 decreased, reaching about 15% saturation after 48 hours. During this time the pH, which was not regulated, increased to 7.8 to 8.1, and the cell mass to 4.2 to 4.6 g per liter (wet weight, corresponding to 1.0 to 1.1 g dry weight). The cells were then separated from the broth by centrifugation. The antibiotic activity was almost all in the supernatant. With the wild strain, the yield was

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between 4 and 6 mg of antibiotic per liter. The organism was inhibited by its own antibiotic at a concentration of 6 mg/liter. After treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (40 μg/ml), mutants were obtained which were resistant to 25~50 mg of antibiotic per liter. These mutants produced up to 30 mg of antibiotic per liter in shake flasks, and up to 13 mg/liter in the bioreactor.

Synthesis of the myxovalargins seems restricted to the growth phase. The antibiotics could be demonstrated soon after inoculation, their concentration increased until the stationary phase was reached and remained then constant for several hours.

Isolation and Purification of the Antibiotic

The activity was extracted from the culture broth with butanol (0.1 v/v) using a three step counter current extractor (Westphalia, Oelpe, FRG). The butanol was removed under reduced pressure at 50°C, and the residue suspended in distilled water and stirred with an anionic exchange resin (Dowex 1X2, 50~100 mesh; 5.5 g per liter of the original culture broth) for one hour. The activity remained in the supernatant, while inactive impurities were bound to the resin. After removal of the resin, the supernatant was extracted twice with butanol (0.5 v/v). The concentrated butanol extract was chromatographed on a column of aluminum oxide (activity I; Woelm, Eschwege, FRG), first with butanol containing increasing amounts of methanol, then with methanol and increasing amounts of water. The activity eluted with methanol - water (5: 95) or with pure water and consisted of up to 70% of antibiotic substance. After this purification step between 50 and 80% of the antibiotics found in the original culture broth were recovered. Final purification was achieved by preparative HPLC on reversed phase silica gel RP-18 (10 μm, Merck, Darmstadt) with the eluant 2-propanol - acetonitrile - 3% trimethyl formiate buffer pH 6.0 (2: 2: 3). The activity was separated into four components (previously only three components were observed[9]) which were named myxovalargin A (about 90%), B, C and D in correspondence with the order of elution (Fig. 1). Analytical HPLC on silica gel RP-18 (7 μm, Merck) with the same solvent system as above was used to follow antibiotic production and purification (Fig. 2). Semiquantitative antibiotic assays were done with the paper-disc method using *Staphylococcus aureus* as the indicator organism.

Fig. 1. Preparative HPLC of the myxovalargin complex.
Detected at 254 nm.

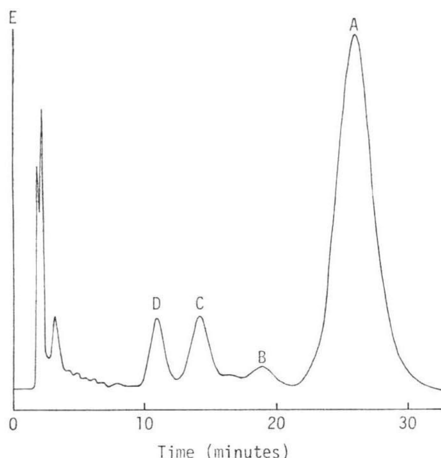
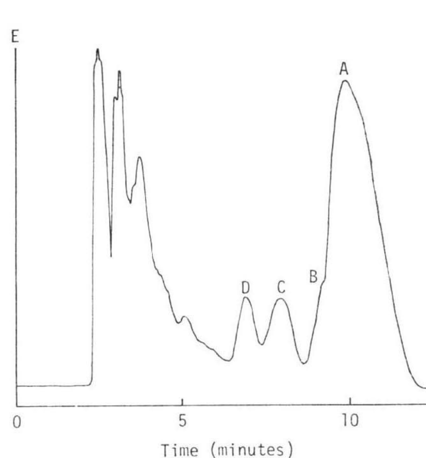


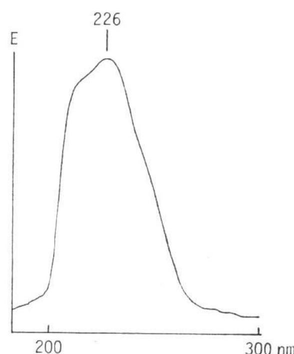
Fig. 2. Analytical HPLC of the myxovalargin complex.
Detected at 254 nm.



Physical and Chemical Properties

The myxovalargins A, B, C and D were obtained as colorless amorphous powders which were soluble in methanol, butanol, or dimethylsulfoxide. While their UV (λ_{max} 226 nm (E=1.47 for c= 10 mg/liter), methanol; Fig. 3) and IR spectra were identical, small differences were observed in the ^{1}H NMR spectra (Fig. 4). Gel permeation chromatography and ^{13}C NMR data indicated molecular weights between 1,500 and 1,700. A fast atom bombardment mass spectrum[2] of myxovalargin A showed a molecular ion at 1,676 mass units. Quantitative amino acid analysis of hydrolysates yielded Val, Ala and Arg in the ratio 5: 2: 1 (due to incomplete hydrolysis originally the ratio 3: 2: 1 was reported[9] earlier). In addition, there were several unusual amino acids like *N*-methylalanine, β-hydroxyvaline, agmatine, 3-methylbutyric acid, α,β-dehydrovaline, and α,β-dehydroleucine[9]. As the carboxyl end of the molecule is occupied by agmatine and the amino end by 3-methylbutyric acid, the myxovalargins seem to be linear peptides[9]. Their complete structure is, however, not yet known and still under investigation.

Fig. 3. Electronic absorption spectrum of myxovalargin A in methanol.



Biological Properties

The antibiotic spectrum of the myxovalargins is shown in Table 1. The antibiotics were active particularly against Gram-positive bacteria (MIC 0.3~5 μg/ml). At high concentrations they were also active against Gram-negative ones

Fig. 4. ^{1}H NMR spectrum of myxovalargin A in CD_3OD (Brüker WH 400 MHz).

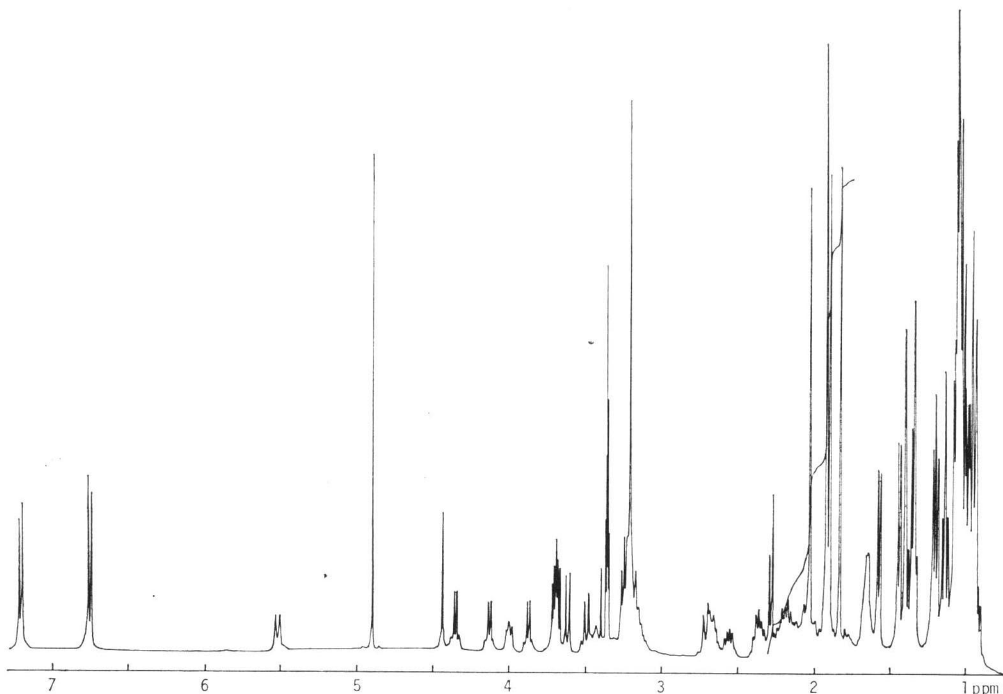


Table 1. The antibiotic spectrum of myxovalargin[1)].

Test organism	Diameter of inhibition zone[2)] Concentration of the antibiotic 1 μg/disc	2 μg/disc	MIC in liquid[3)] (μg/ml)
Mycobacterium phlei	17	18	
Corynebacterium mediolanum	8	11	5
Bacillus megaterium	11	14	
Bacillus subtilis			0.6
Staphylococcus aureus	14	17	0.6
Brevibacterium ammoniagenes	17	21	
Arthrobacter simplex	12	19	
Arthrobacter rubellus	16	20	
Micrococcus lysodeikticus	19	22	
Micrococcus luteus			0.3
Nocardia flava	15	20	
Nocardia corallina	15	19	
Rhizobium 1–595	19	22	
Rhizobium meliloti	11	15	
Myxococcus fulvus Mx f65			6
Myxococcus fulvus Mx f65–M9			25～50
Salmonella typhimurium	8	10	6
Escherichia coli	7	8	6
Klebsiella sp.	11	13	
Serratia marcescens			25
Pseudomonas fluorescens	7	8	
Pseudomonas aeruginosa			12
Pseudomonas aerobacter			25
Proteus morganii	0	7	50
Proteus mirabilis			100
Mucor hiemalis	0	0	
Schizosaccharomyces pombe	0	0	>100
Candida albicans	0	0	>100

1) The experiments were done with the antibiotic mixture containing 90% myxovalargin A.

2) A methanolic solution of the antibiotic was applied to paper discs of 6 mm diameter. The test bacteria were seeded into the following medium: peptone from casein, tryptically digested (Merck) 0.5%, Proteose peptone (Difco) 0.5%, meat extract (Oxoid) 0.1%, agar 1.5%, pH 7.0. *Myxococcus fulvus* was seeded in a medium containing Casitone (Difco) 0.3%, yeast extract 0.1%, $CaCl_2 \cdot 2H_2O$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.15%, agar 1.5%. *Mucor* and the yeasts were suspended in Mycophil agar (Phytone peptone BBL 1%, glucose 1%, agar 1.6%).

3) With *Myxococcus fulvus*, initial cell density was 10^6 cells/ml. Incubation time was 2 days at 30°C. The other test organisms were started with 10^5 cells/ml and incubated for 18 hours.

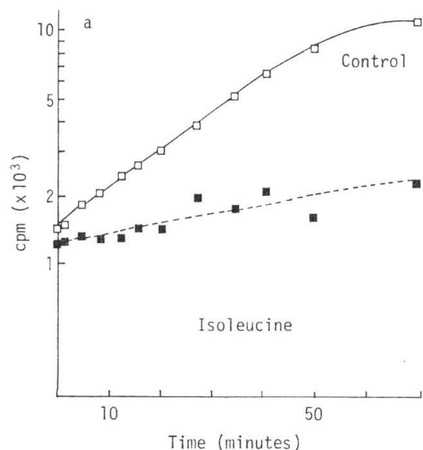
(MIC 6～100 μg/ml). Yeasts and molds were insensitive. The cell density had an unusually strong influence on the MIC values: With 10^7 cells/ml the values were 2 to 5 times higher than those given above and obtained with 10^5 cells/ml. Results of therapeutical experiments are given in Table 2. The antibiotics were relatively toxic: The LD_{50} for mice (s.c.) was 10 mg/kg, the LD_{100} 30 mg/kg.

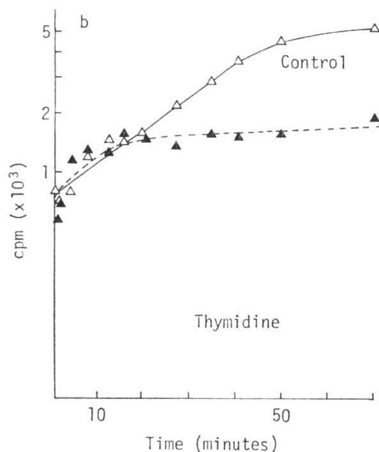
To investigate the effect of the myxovalargins on DNA, RNA and protein synthesis, *Staphylococcus aureus* was grown in shake flasks in a medium containing Casitone (Difco) 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%

Fig. 5. The effect of myxovalargin A on protein (a), DNA (b) and RNA (c) synthesis in *Staphylococcus aureus* measured as incorporation of ^{14}C-labeled isoleucine, thymidine and uracil into TCA insoluble material.

The antibiotic was added at time 0.

Solid signs: with 3 μg myxovalargin per ml; open signs: control without the antibiotic.





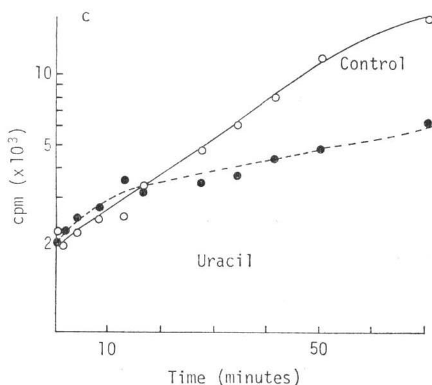
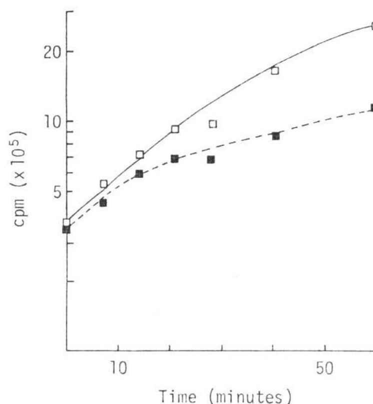


Table 2. Therapeutical effect of myxovalargin in the mouse.

Test bacterium	ED_{50}
Staphylococcus aureus	2.3 mg/kg, s.c.
Streptococcus pyogenes Aronson	2×0.4 mg/kg, s.c.
Escherichia coli	2×10 mg/kg, s.c.

and $CaCl_2 \cdot 2H_2O$ 0.05%, pH 7.0. Either [U-^{14}C]-thymidine, or [U-^{14}C]uracil, or [U-^{14}C]isoleucine was added to parallel cultures (0.1 μCi/ml each; from Amersham-International, Braunschweig). After 30 minutes each culture was divided into two, and myxovalargin (3 μg/ml) was added to one of them. Samples of 0.5 ml were taken every 4 minutes and poured into 1 ml of ice cold trichloroacetic acid (TCA). After 30 minutes the samples were passed through Whatman GF/B glass microfiber filters presoaked with 5% TCA. The filters were washed three times with 5 ml of 5% TCA and once with 5 ml of 95% ethanol. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. As can be seen from Fig. 5, incorporation of thymidine and uracil broke off about 15 minutes after addition of the antibiotic, while incorporation of Ile stopped immediately. In a similar experiment the effect of the inhibitor on cell wall

Fig. 6. The effect of myxovalargin A on the incorporation of labeled *N*-acetylglucosamine into TCA insoluble material by *Staphylococcus aureus*: ■—■ with 3 μg myxovalargin per ml; □—□ control without the antibiotic.



synthesis was studied by adding [U-^{14}C]-*N*-acetylglucosamine (0.04 μCi/ml; from Amersham International). Samples of 8 ml were taken, the cell harvested, the murein was isolated according to PARK and HANCOCK[14] and its radioactivity determined. Murein synthesis levelled off more than 20 minutes after addition of the antibiotic (Fig. 6).

Discussion

After the polyether antibiotic ambruticin[16], the two thiazol containing antibiotics, myxothiazol[5,18] and althiomycin[10], the nitrophenylpyrrole antibiotic pyrrolnitrin[7], and the myxovirescins[6,19] which are macrocyclic lactam-lactone antibiotics, the myxovalargins are the first true peptide antibiotics from myxobacteria. For the decapeptides, myxosidine A and B, were obtained from so-called myxobacter 495[4], which is a strain of *Lysobacter enzymogenes*[3] and thus not related to the myxobacteria[15]. Obviously myxobacteria produce antibiotics of quite different chemical structure. Since their discovery in *Myxococcus fulvus* Mx f65, the myxovalargins have also been isolated from the culture broth of two *M. xanthus* strains (*viz.* Mx x4 isolated in 1966 from sheep dung collected in St. Paul. Minnesota; and Mx x48 isolated in 1980 by H. J. VOCKERODT, GBF, from soil collected in the oasis of Gabès, Tunesia) and of an *Archangium* strain (Ar D8=Ag BA2 isolated in 1977 by Dr. W. DAWID, Bonn, from soil collected on the Bahamas).

Although the complete structure of the myxovalargins is not yet known, their amino acid composition and the end groups, agmatin and 3-methylbutyric acid[9], strongly suggest that they are new antibiotics. The myxosidins differ from the myxovalargins in their amino acid composition and in that they are cyclic peptides[4].

The myxovalargins are mainly active against Gram-positive bacteria, although at higher concentration many Gram-negatives, including pseudomonads, are also inhibited. The different response is probably due to differences in permeability. The producing organism was relatively sensitive to its own antibiotic, but resistant mutants could be obtained. Apparently the myxovalargins are synthesized only during the growth phase. Their mode of action seems directed against protein synthesis. It remains to be seen whether their relatively high toxicity for warm blooded animals is based on the same mechanism.

Acknowledgements

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