THE MYXALAMIDS, NEW ANTIBIOTICS FROM *MYXOCOCCUS XANTHUS* (MYXOBACTERALES)

I. PRODUCTION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES, AND MECHANISM OF ACTION*

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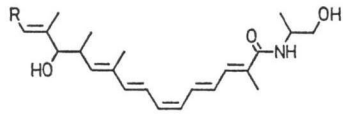
From the cell mass and culture supernatant of *Myxococcus xanthus* strain Mx X12 an antibiotic activity against yeasts, molds and some Gram-positive bacteria could be extracted. It consisted of 4 biologically active compounds which were named myxalamid A, B, C and D. The main component, myxalamid B, was shown to block in beef heart submitochondrial particles the respiratory chain at the site of complex I, *i.e.* NADH: ubiquinone oxidoreductase. The myxalamids are new antibiotics.

In the course of a screening program for antibiotics from gliding bacteria, an activity against yeasts and molds was discovered in a strain of the myxobacterium, *Myxococcus xanthus*. As it turned out, the activity consisted of a mixture of 4 chemically related new antibiotics, which were named myxalamid A to D. In this article we describe the production and the biological, biochemical and physico-chemical properties of the main component, myxalamid B (Fig. 1). The structure elucidation of the myxalamids is published elsewhere[11].

Fig. 1. The chemical structure of the myxalamids[11].



Myxalamid A: R=$CH_3CH_2(CH_3)CH$–
Myxalamid B: R=$(CH_3)_2CH$–
Myxalamid C: R=CH_3CH_2–
Myxalamid D: R=CH_3–

Organism and Culture Conditions

Myxococcus xanthus strain Mx x12 (=*M. xanthus* HR 2) was isolated in 1978 from goat dung collected near Olympia, Greece. The strain was cultivated in Peptone liquid medium (1% peptone from casein, tryptically digested (Merck, Darmstadt), 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, pH 7.2). Batch cultures of 100 ml in 250-ml Erlenmeyer flasks were incubated at 32°C on a rotary shaker at 160 cycles per minute with an amplitude of 50 mm. The organism also grew very well on certain technical substrates like bakers' yeast 3% (by fresh weight of yeast cake), Probion 1% (single cell protein from Hoechst, Frankfurt), soy flour 1%, or Zein 1% (maize gluten from Maizena, Hamburg), which give all inexpensive fermentation media. Myxalamid production was about five times higher when Mx x12 was

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grown in Probion or Zein liquid medium as compared with Peptone liquid medium. Addition of 0.3% sodium acetate resulted in a further increase of the antibiotic yield.

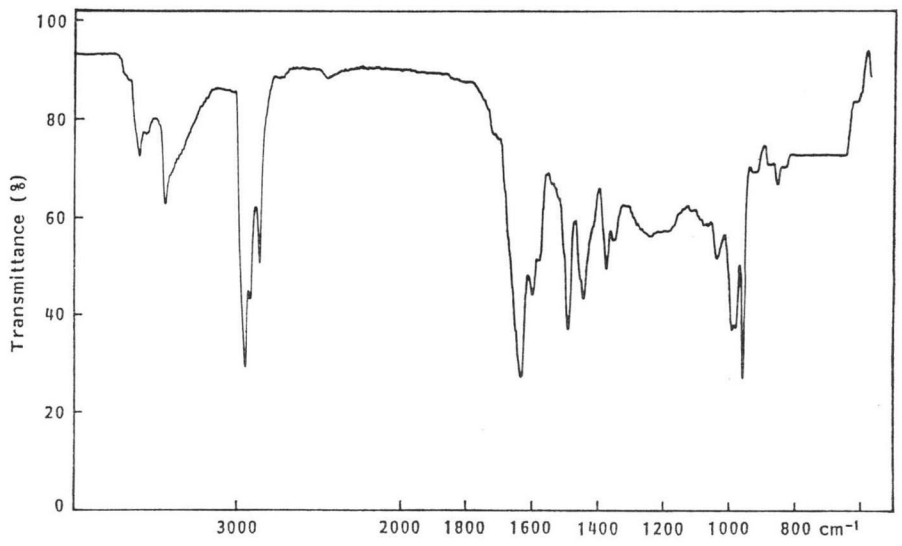
Fermentation and Extraction

Fermentations were performed in a type b 50 fermentor from Giovanola Frères (Manthey, Switzerland) containing 70 liters of modified Probion liquid medium (1% Probion L (Hoechst, Frankfurt), 0.3% sodium acetate, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, pH 7.4). Because of serious foam problems connected with this specific substrate, 0.15% of silicone antifoam agent (Merck, Darmstadt) had to be added. The fermentor was inoculated with 10 liters of a log-phase culture growing in the same medium. The fermentation was carried out at 32°C, with a stirrer speed of 400 r.p.m. and an aeration rate of 0.14 v/v/minute. The pH was kept at 7.4, during the first 12 hours by adding KOH (10%), later, when ammonia production increased, by adding acetic acid (10%). The myxalamids were produced during the growth phase and accumulated, reaching the maximal yield of 120 mg/liter at the beginning of the stationary phase. After 50 hours fermentation the cells were separated from the supernatant by centrifugation, and the antibiotics were extracted from the cell mass with acetone. The crude extract was then purified by column chromatography as described elsewhere[11]. The antibiotics were also present in the culture supernatant from which they could be extracted with ethyl acetate. As the amount was small relative to that contained in the cell mass (about 5%), the supernatant was discarded.

When 1% XAD-1180 (Röhm & Haas, Frankfurt) was added to the growing culture, the antibiotics became quantitatively adsorbed to the resin and could be eluted from it with methanol after recovery of the XAD from the culture. No myxalamid could be detected by thin-layer chromatography (TLC) in extracts of the cell mass and of the supernatant, while the carotenoids were still found exclusively in the acetone cell extract. This seems to indicate that the myxalamids are not located inside the bacteria, but are rather adsorbed to their surface.

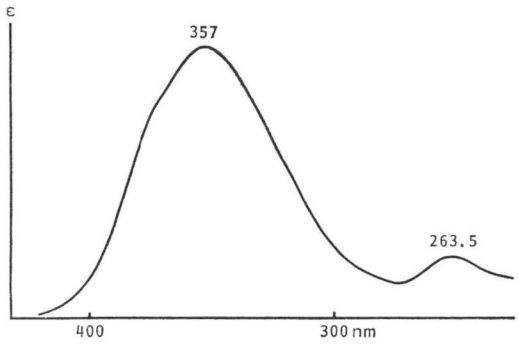
Physico-chemical Properties

Fig. 2. IR spectrum of myxalamid B dissolved in chloroform, recorded on a Perkin-Elmer 297 IR-spectrophotometer.



The isolated activity consisted of a mixture of yellow colored compounds, which could not be separated by TLC. On silica gel 60 F_{254} (precoated aluminium sheets; Merck, Darmstadt), the Rf values of the mixture were: with toluene-ethanol (9: 1) 0.27; with dichloromethane-methanol (9: 1) 0.42. The antibiotics appeared on the TLC as a pale yellow spot which turned orange-yellow upon standing in air. Under the UV lamp, there was a dark spot at 254 nm and a bright fluorescence at 366 nm. The antibiotics could also be detected by exposure to iodine vapors (deep orange) or by spraying with molybdato phosphoric acid and heating (blackish). By HPLC on reversed phase silica gel, the mixture could be separated into 4 homologues which were named myxalamid A to D. The quantitative relation of the 4 compounds was variable depending on growth media and fermentation conditions: *e.g.*, with one experiment the following proportions were found; myxalamid A: B: C: D=23: 17: 4: 1. In many cases, however, B was the main component. The infrared and the electron absorption spectra of B are shown in Figs. 2 and 3, the result of the structure elucidation[11] is given in Fig. 1.

Fig. 3. Electronic absorption spectrum of myxalamid B in methanol.



The myxalamids formed yellowish oils. They were well soluble in dichloromethane, acetone and ethanol, only slightly in diethyl ether, and insoluble in petroleum ether. They were sensitive to oxidation, and long term storage under air led to substantial loss of material. When exposed to light, the natural *cis*-isomers gradually changed into the more stable all-*trans* configuration[11].

Antibiotic Activity

The antibiotic spectrum of myxalamid B was determined by the agar diffusion technique using paper discs. As can be seen from Table 1, the antibiotic was active against several molds, yeasts and Gram-positive bacteria, while Gram-negative bacteria were mostly resistant.

The minimal inhibitory concentration was determined for two organisms by serial dilution assay. The bacterium, *Nocardia corallina*, was grown in a medium containing 0.5% peptone from casein (tryptically digested, Merck, Darmstadt), 0.1% meat extract (Oxoid) and 0.1% yeast extract (Difco), the yeast, *Nadsonia fulvescens*, in mycophil medium (1% Phytone peptone (BBL), 1% glucose). The test cultures were started with 5×10^6 cells/ml. Relatively high concentrations of myxalamid B, $10 \sim 20$ μg/ml, were needed for complete inhibition. All organisms were also tested against the *trans* isomer of myxalamid B. In no case was a difference in the inhibitory efficiency observed.

Toxicity

The myxalamids proved very toxic. The acute toxicity was determined in the mouse. When applied subcutaneously, the LD_{100} was 3 mg/kg (1 mg/kg LD_0); when applied *per os*, the LD_{100} was 10 mg/kg (3 mg/kg LD_0).

Mechanism of Action

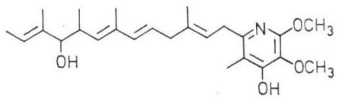
Because of a structural analogy between the myxalamids and piericidin A (Fig. 4)[15], which is an electron transport inhibitor of the eukaryotic respiratory chain[6,14], we tested the effect of myxalamid B

Table 1. The antibiotic spectrum of myxalamid B.

Test organism	Diameter of inhibition zone* (mm)	Test organism	Diameter of inhibition zone* (mm)
Paecilomyces sp.	12	*Brevibacterium ammoniagenes*	—
Mucor lusitanicus	9	*Bacillus subtilis*	8
Mucor hiemalis	—	*Bacillus megaterium*	7
Rhodotorula glutinis	18	*Bacillus polymyxa*	7
Nadsonia fulvescens	14	*Bacillus thuringiensis*	—
Nematospora coryli	—	*Staphylococcus aureus*	—
Candida albicans	—	*Rhizobium meliloti*	7
Torulopsis glabrata	—	*Aerobacter aerogenes*	9
Hansenula anomala	—	*Escherichia coli*	—
Pichia membranaefaciens	—	*Salmonella typhimurium*	—
Debaryomyces hansenii	—	*Klebsiella pneumoniae*	—
Saccharomyces cerevisiae	—	*Serratia marcescens*	—
Schizosaccharomyces pombe	—	*Proteus mirabilis*	—
Nocardia corallina	9	*Proteus morganii*	—
Nocardia flava	8	*Pseudomonas aeruginosa*	—
Corynebacterium mediolanum	8	*Pseudomonas acidovorans*	—
Mycobacterium sp.	—	*Alcaligenes eutrophus*	—
Arthrobacter rubellus	8		

* Tested by the agar diffusion method with paper discs of 6 mm diameter to which were applied 20 μg of pure antibiotic dissolved in methanol and 5 μl of dimethyl sulfoxide as a diffusion aid. The test agar for bacteria contained: peptone from casein, tryptically digested (Merck, Darmstadt) 0.5%, meat extract (Oxoid) 0.1%, yeast extract (Difco) 0.1%, agar 1.5%. The molds and yeasts were assayed on mycophil agar: Phytone peptone (BBL) 1%, glucose 1%, agar 1.6%.

Fig. 4. Chemical structure of piericidin A[15].



on NADH oxidation in beef heart submitochondrial particles. The isolation of submitochondrial particles and other experimental details were as described elsewhere[19]. As can be seen in Fig. 5, myxalamid B inhibited NADH oxidation with a linear concentration dependence. At a concentration of 170 pmol/mg protein (12 pmol/ml), NADH oxidation was inhibited by 50%.

To determine the site of action within the respiratory chain, the effect of myxalamid B on the reduction of the cytochromes was investigated by means of difference spectroscopy[19]. Treatment of submitochondrial particles with the antibiotic completely inhibited reduction of cytochrome aa_3 (α band at 605 nm), cytochrome b (α band at 563 nm) and the cytochromes $c+c_1$

Fig. 5. Inhibition of NADH oxidation in beef heart submitochondrial particles by myxalamid B.

The antibiotic was dissolved in methanol. The methanol concentration in the tests did not exceed 2%. The test suspension (70 μg protein/ml) was preincubated with the antibiotic for 4 minutes before the reaction was started by addition of NADH. The rate of NADH oxidation in the control without myxalamid was 1,501 nmol/minute/mg protein. Each point in the figure gives the average of 2~4 experimental determinations.

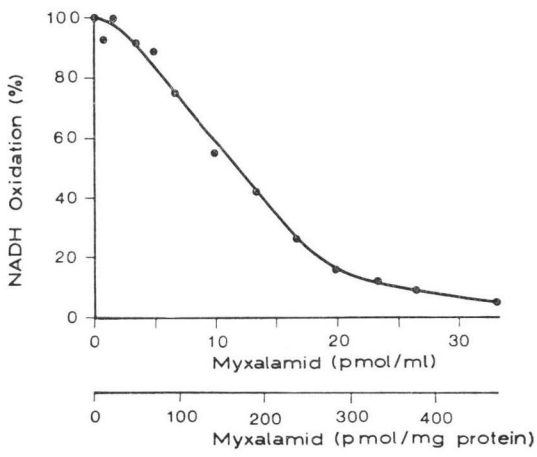


Fig. 6. The effect of myxalamid B on the reduction of cytochromes by NADH.

Air-oxidized beef heart submitochondrial particles were diluted to give a density of 2.1 mg protein/ml. Cuvettes with an optical pathway of 1 cm were filled with 1.1 ml of the suspension and placed into the reference and sample position of a double-beam spectrophotometer. After the baseline (trace 1) was recorded, myxalamid was added to the sample cuvette to a final concentration of 6.5 μM. After preincubation for two minutes, NADH (final concentration 2 mM) was added to the sample cuvette. Volume corrections were performed in the reference cuvette. After two minutes the resulting difference spectrum was recorded (trace 2). Finally a few grains of solid dithionite were added to the sample cuvette to achieve full reduction of the cytochromes (trace 3).

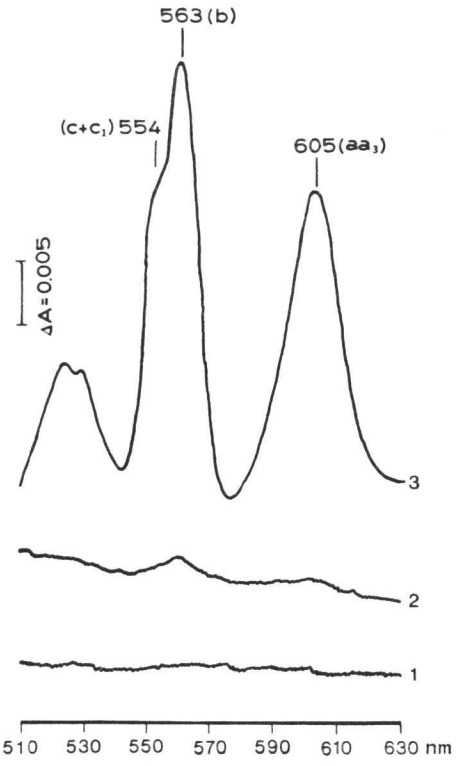
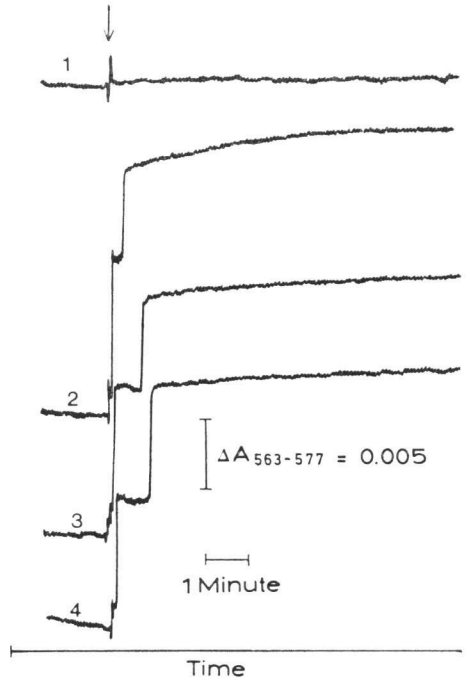


Fig. 7. The effect of myxalamid B on the kinetics of cytochrome *b* reduction.

Air-oxidized submitochondrial particles were diluted as described for Fig. 6. The time course of cytochrome *b* reduction was measured by dual wavelength spectroscopy at the wavelength pair 563 minus 577 nm. The suspension was preincubated as described in Fig. 6. The temperature was 5~10°C. At the time indicated by the arrow, either NADH or succinate were added to a final concentration of 2 mM or 5 mM, respectively. The sample cuvette contained NADH plus myxalamid B (trace 1) or plus methanol (trace 2); or succinate plus myxalamid B (trace 3) or plus methanol (trace 4).



(α band at 554 nm) by NADH (Fig. 6). This indicated that the site of action of myxalamid B is on the substrate side of cytochrome *b*.

Cytochrome *b* can be reduced by NADH *via* complex I (NADH: ubiquinone oxidoreductase), or by succinate *via* complex II (succinate: ubiquinone oxidoreductase). To decide whether myxalamid B interferes with complex I, with complex II, or with both of them, we investigated the effect of the antibiotic on the reduction kinetics of cytochrome *b* using either NADH or succinate as the substrate. As can be seen in Fig. 7, myxalamid B inhibited the reduction of cytochrome *b* only when NADH was the electron donor.

The NADH oxidation of mitochondria from commercial bakers' yeast proved insensitive to myxalamid B. The mitochondria were isolated as described before[18]. A 50% inhibition of NADH oxidation was achieved only at a concentration of 60 μM, corresponding to a dose of 260 μmol/mg protein.

Discussion

After myxothiazol[2], the myxovalargins[10] and the myxovirescins[3], the myxalamids are the fourth new antibiotic isolated by us from myxobacteria. They were produced by *Myxococcus xanthus* Mx x12 with yields up to 120 mg/liter, which is relatively high for antibiotics from myxobacteria. After their discovery in Mx x12, the myxalamids were also found in *Stigmatella aurantiaca* Sg a15[12], in *M. xanthus* strains Mx x17 (isolated in 1978 from soil collected near Minneapolis, Minnesota, USA) and Mx x41 (isolated in 1980 from goat dung collected near Madras, India); in *M. virescens* strains Mx v18 (strain Mv BA3 isolated in 1977 by Dr. W. DAWID, Bonn, from soil collected on Long Island, Bahamas), Mx v27 (isolated in 1979 from deer dung collected near Wolfsburg, FRG), Mx v32 (strain Mv S35 isolated in 1976 by Dr. W. DAWID, Bonn, from soil collected in the Siebengebirge mountains near Bonn, FRG) and Mx v36 (strain Ne BA3 isolated in 1977 by Dr. W. DAWID, Bonn, from soil collected on Long Island, Bahamas); and in *M. stipitatus* strains Mx s3 (strain Ms B21 isolated in 1975 by Dr. W. DAWID, Bonn, from soil obtained from Brazil) and Mx s9 (isolated in 1982 from soil collected near Iguaçu, Brazil). The myxalamids seem thus fairly common among myxobacteria and are produced by members of different species and genera.

Myxalamid B is a new inhibitor of the electron transport in the respiratory chain. Like piericidin A[6], which is structurally related[15], myxalamid B specifically blocked the electron flow at complex I (NADH: ubiquinone oxidoreductase EC 1.6.5.3) of beef heart submitochondrial particles. The high toxicity of myxalamid B for mammalian systems is thus understandable; it is in the same range or even higher than that of the piericidins[16,17].

In contrast to the respiration inhibitor myxothiazol[2], which acts at the cytochrome bc_1 segment[19], myxalamid B had a relatively narrow antibiotic spectrum. Only some molds, few yeasts, and a number of bacteria, mainly Gram-positive ones, were inhibited. Piericidin A and B show a comparable toxicity for fungi, but are essentially inactive against bacteria[16].

Because of the structural relationship between piericidin A (Fig. 4) and myxalamid B (Fig. 1), it is interesting to compare the efficiencies of the two inhibitors. The dose required for a 50% inhibition of NADH oxidation by beef heart electron transport particles is 23~60 pmol of piericidin A per mg protein[9]. Myxalamid B was thus 3~7 times less effective than piericidin A. Since a FMN containing flavoprotein is a characteristic constituent of complex I[8], it may be more meaningful to use this component as the reference. The FMN content of beef heart mitochondria and submitochondrial particles is 100~150 pmol per mg protein[7]. Accordingly the dose required for a 50% inhibition of NADH oxidation was 1 to 1.7 mol myxalamid B per mol FMN.

Our data are not sufficient to determine the stoichiometry of the inhibitor-enzyme interaction. For piericidin A it is known that it binds very tightly, readily displacing *e.g.* rotenone which binds at the same site in complex I[9], and that there is a considerable amount of unspecific binding on beef heart[5,9] as well as on *Candida utilis*[1] submitochondrial particles. Working with [^{14}C]piericidin A and reducing the unspecific binding by treatment with bovine serum albumin, it could be shown that 2 mol of piericidin A bind at the specific site per mol of NADH dehydrogenase[5].

The resistance of the majority of yeasts and of isolated mitochondria of *Saccharomyces cerevisiae* to myxalamid B has its parallel in piericidin A, too. Saccharomyces yeasts and mitochondria isolated from them are insensitive to this inhibitor[18]. *Candida* (*Torulopsis*) *utilis* produces under different growth conditions two types of NADH dehydrogenases of which only one responds to piericidin A[4].

The myxalamids were produced as several homologues, which were all biologically active. In addition, all-*trans* isomers could arise during the isolation procedure; they, too, were active. It remains to be seen whether there are differences in the efficiency of the individual compounds in different test systems as is reported for piericidin A and B[16].

Acknowledgments

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tion Service of the GBF. Occurrence of myxalamids in certain strains and the toxicity data were determined by colleagues at Ciba-Geigy, Basel. Dr. U. SCHAIRER (GBF) allowed us to use his double-beam spectrophotometer. Mrs. O. EBELMANN and Mrs. E. HEINZE provided excellent technical assistance. We wish to thank all these persons for their collaboration.

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