PRODUCTION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF ANGIOLAM A, A NEW ANTIBIOTIC FROM *ANGIOCOCCUS DISCIFORMIS* (MYXOBACTERALES)†

BRIGITTE KUNZE, WERNER KOHL††, GERHARD HÖFLE††
and HANS REICHENBACH

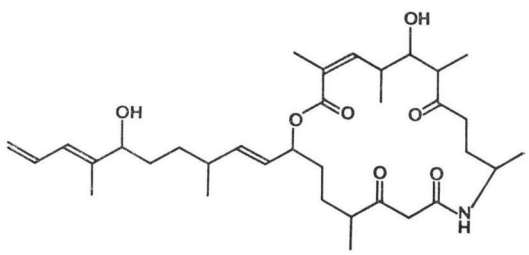
GBF, Gesellschaft für Biotechnologische Forschung,
Abt. Mikrobiologie and ††Abt. Naturstoffchemie
Mascheroder Weg 1, D-3300 Braunschweig, FRG

(Received for publication July 15, 1985)

Angiolam A, a new lactone-lactam antibiotic, was isolated from the culture broth of the myxobacterium *Angiococcus disciformis* strain An d30. It was active against a few Gram-positive bacteria and mutant strains of *Escherichia coli* with increased permeability. It appears to interfere with protein synthesis.

During our screening of gliding bacteria for new antibiotics, the myxobacterium *Angiococcus disciformis* strain An d30 was found to produce activity against fungi and bacteria. Part of the activity appeared in the culture supernatant, part within the cell mass. The activity could be extracted with ethyl acetate and acetone, respectively. Preliminary experiments showed that the activity was due to different antibiotics. One of the compounds, which was mainly found in the cell extracts, could be identified with myxothiazol, an antifungal antibiotic, which had been isolated before in our laboratory from the myxobacterium *Myxococcus fulvus* strain Mx f16[1~3]. A second activity, which was mainly in the culture supernatant inhibited the growth of a few Gram-positive bacteria and of *Escherichia coli* mutants with increased permeability. It proved to be a new antibiotic, consisting of several components, and the main component was named angiolam A (Fig. 1). A third substance with weak antibacterial activity was also new and is still under investigation. In this article we report on the production, isolation and on some physico-chemical and biological properties of angiolam A, while the structure determination is published elsewhere[4].

Fig. 1. The chemical structure of angiolam A[4].



Organism and Culture Conditions

A. disciformis strain An d30 (=AfB10 Dawid) was isolated in 1975 by Dr. W. DAWID, Bonn, from a soil sample collected in Brasilia. The organism was grown in standard peptone liquid medium (peptone from caseine, tryptically digested (Merck, Darmstadt) 1%; $MgSO_4 \cdot 7H_2O$ 0.1%; pH 6.8). Batch cultures of 100-ml or 500-ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at

† Article No. 29 on antibiotics from gliding bacteria. Article No. 28: TROWITZSCH-KIENAST, W.; V. WRAY, K. GERTH, H. REICHENBACH & G. HÖFLE: Die Biosynthese des Myxothiazols in *Myxococcus fulvus* Mx f16. Liebigs Ann. Chem., in press.

30°C on a rotary shaker at 160 rpm for 1~2 days. The strain also grew well in technical media on the basis of Probion (single cell protein prepared from *Methylomonas clara*, Hoechst Co., Frankfurt), soy flour (gift from Ciba-Geigy, Basel), or corn steep powder (from Roquette Frères, Lille, France). In Probion liquid medium the yield of angiolam A was 4~5 times higher than in peptone liquid medium. Cell suspensions in peptone liquid medium could be preserved by storing at −80°C or in liquid nitrogen.

Production

For mass production of angiolam A, fermentations were performed in Probion liquid medium (Probion PS 1.0%; $MgSO_4 \cdot 7H_2O$ 0.1%; final pH 7.0) up to a volume of 3,800 liters. In some fermentations 1% (v/v) of a neutral adsorber resin, XAD-1180 (Röhm and Haas, Darmstadt), was added to the medium. Cultivation of An d30 in the presence of adsorber resin increased the yield of the antibiotic and simplified its isolation, as has also been demonstrated in other cases[5,6]. The course of a 700-liter fermentation was as follows. A seed fermentor (type b 50, Giovanola Frères, Monthey, Switzerland) with 60 liters of Probion liquid medium + 0.01% silicone antifoam agent (Merck, Darmstadt) was inoculated with 5 liters of shake cultures grown for 36 hours in the same medium. The fermentation was carried out at 30°C, with an aeration rate of 0.05 v/v·minute and a stirring rate of 200 rpm (turbine plate stirrer). After 27 hours 35 liters of this culture were pumped into the production fermentor (type b 500, Giovanola Frères) containing 650 liters of Probion liquid medium + 0.005% silicone antifoam agent and 1% (v/v) of the adsorber resin, XAD-1180. Because of serious foam problems connected with the substrate, silicone antifoam agent had to be added from time to time up to about 0.01%. The fermentor was maintained at 30°C and agitated by a turbine plate stirrer with 300~450 rpm. The aeration rate was 0.03~0.036 v/v·minute. The pH which initially drifted slightly into the acid range was left to rise to 8.0 and was then kept constant by titration with 30% acetic acid. The pO_2 in the culture was recorded continuously with a polarographic oxygen electrode. At the beginning of the fermentation it was at about 90% saturation, it fell within the first 16 hours to about 20% and was then adjusted to 30% by increasing the aeration rate. Towards the end of the fermentation at 37 hours, the pO_2 rose to 45%. The antibiotic activity was monitored during the fermentation by the paper disc diffusion assay using *Escherichia coli* K 12 W 495 as test organism, and further was quantitatively determined by HPLC analysis (columns; 7 μm Lichrosorb RP-18, 25 cm×4 mm (Merck, Darmstadt), solvent; methanol - water, 65: 35). At the end of the fermentation the concentration of angiolam A was 5.8 mg/liter. Over 90% of the antibiotic was adsorbed to the resin.

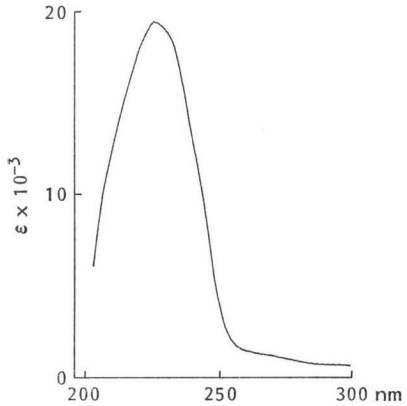
Isolation

The adsorber resin was separated from the culture by passing the content of the fermentor through a sieve of 0.25 mm pore size. The resin was then transferred into a chromatography column of 10-cm diameter and washed with 20 liters of methanol - water (40: 60). The crude antibiotic was then eluted with 40 liters of methanol - water (90: 10) and purified by different chromatography steps (Sephadex LH-20; silica gel 100) and distribution between water and heptane. Further details of the purification procedure will appear elsewhere[4].

Physico-chemical Properties

Angiolam A crystallized from methanol, yielding colorless crystals with a melting point of 178~180°C. Solubility was high in methanol, acetone, ethyl acetate and chloroform, low in ether, and

Fig. 2. Electronic absorption spectrum of angiolam A in methanol.



very low in hexane and water. In aqueous solution angiolam A was stable at neutral and slightly alkaline pH. Thin-layer chromatograms on Silica gel 60 F_{254} (Merck) showed the following Rf values; with dichloromethane - heptane - 2-propanol (45: 45: 10) as the solvent; 0.40, with dichloromethane - methanol (90: 10); 0.6. After spraying with glacial acetic acid containing 1% anisaldehyde and 2% sulfuric acid and heating at 110°C the antibiotic gave a blue-violet spot. A negative ion fast-atom bombardment mass spectrum showed a (M−H) ion at 586 mass units which together with the elemental analysis, established the molecular formula $C_{34}H_{53}NO_7$. The electronic absorption spectrum of angiolam A in methanol recorded with a Zeiss DMR 21 spectrophotometer had a maximum at 227 nm (log ε 4.29) (Fig. 2). The IR spectrum was measured in KBr

Fig. 3. IR spectrum of angiolam A in KBr.

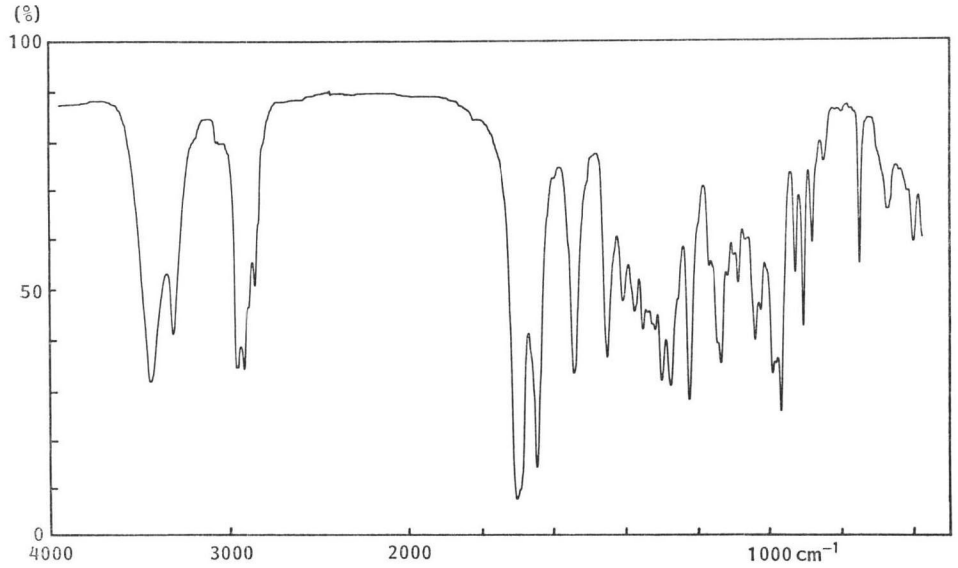
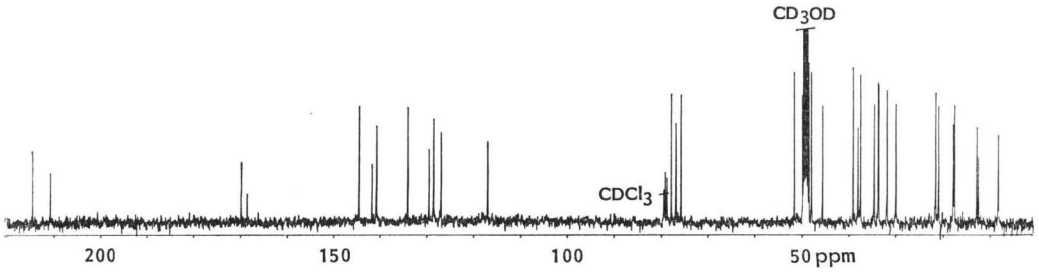


Fig. 4. ^{13}C NMR spectrum of angiolam A in $CDCl_3$.



with a Perkin Elmer 297 IR spectrometer (Fig. 3), and the ^{13}C NMR in deuteriochloroform with a NMR spectrometer WH 400 at 100.6 MHz (Bruker, Karsruhe) (Fig. 4). The result of the structure elucidation[4] is shown in Fig. 1.

Biological Properties

The antimicrobial spectrum of angiolam A was determined by the agar diffusion test using paper discs (Table 1). Among the Gram-positive bacteria tested, only some members of the Bacillaceae were sensitive. Gram-negative bacteria were in general not affected by concentrations up to 40 μg/disc, with the exception of some mutants of *E. coli* with increased permeability. Yeasts and fungi also were not inhibited. The minimal inhibitory concentration (MIC) was determined by the serial dilution assay. The sensitive Gram-positive bacteria, including anaerobic *Clostridium perfringens* were inhibited at a MIC of 0.78 μg/ml; the *E. coli* strains with increased permeability at a MIC of 2.5 μg/ml. Angiolam A had no acute toxicity for mice up to a concentration of 300 mg/kg (sc).

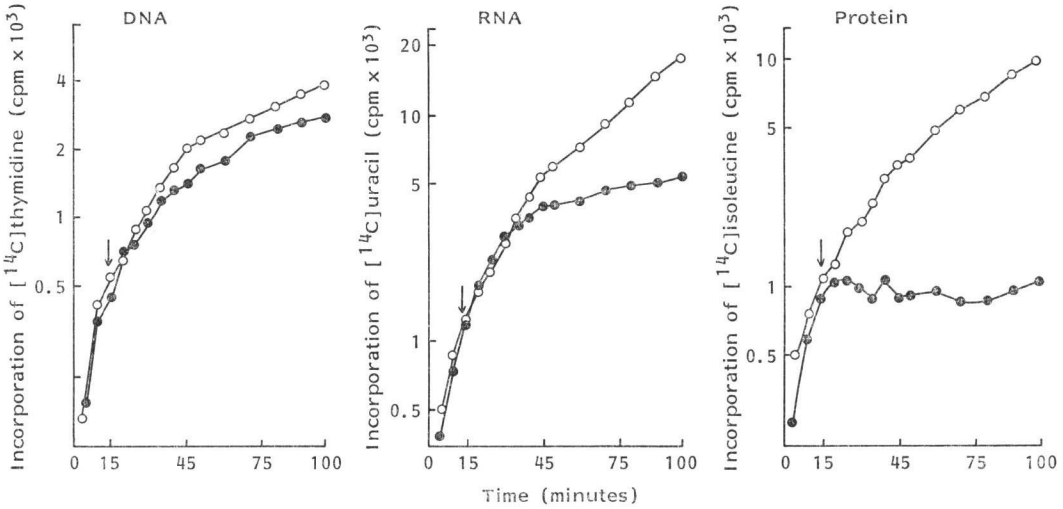
The effect of angiolam A on *Bacillus thuringiensis* was bacteriostatic. When the antibiotic was added to a log-phase culture at concentrations up to 10 μg/ml, the cells stopped to multiply, but the number of cells able to form colonies upon plating remained constant during several hours. To test the influence of angiolam A on the macromolecular syntheses, either [*U*-^{14}C]thymidine or [*U*-^{14}C]uracil, or [*U*-^{14}C]isoleucine (0.1 μCi/ml each; from Amersham-International, Braunschweig) was added to

Table 1. Antimicrobial spectrum of angiolam A*.

Test organism	Diameter of inhibition zone (mm)	Test organism	Diameter of inhibition zone (mm)
Bacillus brevis DSM 30	0	*Pseudomonas aeruginosa* DSM 1117	0
B. cereus DSM 31	20	*Enterobacter aerogenes*	0
B. cereus DSM 626	20	*Escherichia coli* K 12 DSM 498	0
B. megaterium DSM 32	0	*E. coli* K 12 W 495**	17
B. mycoides DSM 2048	25	*E. coli* DSM 423	0
B. polymyxa DSM 36	0	*E. coli* DSM 789	0
B. subtilis DSM 10	0	*E. coli* tol C**	17
B. thuringiensis DSM 2046	20	*E. coli* NF 1128	0
Clostridium pasteurianum DSM 525	22	*Proteus mirabilis* VI	0
C. perfringens DSM 756	0	*P. mirabilis* L VI***	>40
Micrococcus luteus GBF 38	0	*Klebsiella pneumoniae*	0
Staphylococcus aureus GBF 16	0	*Salmonella typhimurium*	0
Enterococcus faecalis	0	*Serratia marcescens*	0
Leuconostoc mesenteroides DSM	0	*Xanthomonas campestris*	0
Arthrobacter aurescens DSM 20116	0	*Candida albicans*	0
Brevibacterium ammoniagenes DSM 20306	0	*Debaryomyces hansenii* DSM 70238	0
B. linens DSM 20425	0	*Nadsonia fulvescens* Tü 561	0
Corynebacterium fascians DSM 20131	0	*Rhodotorula glutinis* DSM 70398	0
C. glutamicum DSM 20300	0	*Saccharomyces cerevisiae* GBF 36	0
Mycobacterium phlei	0	*Polyporus* sp. GBF 224	0
Nocardia corallina	0	*Mucor hiemalis* Tü 189	0
Streptomyces fulvissimus DSM 40593	0	*Polystictus* sp. GBF 223	0
S. griseus DSM 40695	0	*Paecilomyces varioti* GBF 159	0
Azotobacter vinelandii DSM 2289	0	*Rhizoctonia solani* CBS 177.4	0

* Angiolam A was dissolved in methanol and applied at 40 μg/disc.
** Mutant strain with increased permeability.
*** Stable protoplast-L-form of *Proteus mirabilis* VI.

Fig. 5. Effect of angiolam A on DNA, RNA and protein synthesis in *Bacillus thuringiensis*.
●: At the time indicated by the arrow the antibiotic (3 μg/ml) was added to the culture.
○: Control culture without antibiotic.



parallel cultures of *B. thuringiensis* cells, growing logarithmically in nutrient broth. After an incubation period of 15 minutes the antibiotic (3 μg/ml, dissolved in methanol) or corresponding amounts of methanol were added. In certain time intervals samples of 0.5 ml were poured into 2 ml of ice-cold trichloroacetic acid (TCA). After 120 minutes the precipitates were collected on Whatman GF/B glass microfiber filters presoaked with 5% TCA and washed three times with 5 ml of 5% TCA. The radioactivity of the dry filters was determined in a Packard Tri-Carb liquid scintillation spectrometer. Fig. 5 shows that the incorporation of labeled isoleucine stopped completely 5 minutes after addition of the antibiotic, while DNA and RNA synthesis continued for a considerable time. Similar results were obtained with *E. coli* tol C as test organism.

Discussion

Angiococcus disciformis strain An d30 is the second myxobacterium after *Stigmatella aurantiaca* strain Sg a15[7] which we found to produce several structurally unrelated antibiotics. The antifungal compound, myxothiazol[1~3], which was first isolated from *Myxococcus fulvus* strain Mx f16, has a bisthiazol system and was produced by *A. disciformis* up to 6 mg/liter. Angiolam A is a new macrocyclic lactone-lactam antibiotic and was produced by *A. disciformis* up to about 6 mg/liter. In some fermentations minor amounts of angiolam A derivatives also were detected. While the antibiotics from *Stigmatella aurantiaca* strain Sg a15 all interfere with the respiration[5,8], the antibiotics from An d30 show quite different biological properties. Myxothiazol is mainly an antifungal antibiotic and selectively inhibits the cytochrome b-c_1 segment of the respiratory chain[3]. Angiolam A is active against only a few bacteria. The insensitivity of most bacteria is probably due to an impermeability of their cell wall for angiolam A. This is suggested a) by the sensitivity of *E. coli* strains with increased permeability, and b) by the sensitivity of a stable protoplast-L-form of *Proteus mirabilis* VI which as an intact organism was totally resistant. Angiolam A appears to interfere with the prokaryotic protein synthesis. It shows no acute toxicity for mice. It might be possible to synthesize derivatives of angiolam A, with a broader antimicrobial spectrum.

Acknowledgments

Strain An d30 (=AfB10 Dawid) was obtained from Dr. W. DAWID, Bonn. The fermentations for antibiotic production were performed by H.-G. RENG and his collaborators in the fermentation Service of the GBF. The mass and NMR spectra were run by Drs. L. GROTJAHN and V. WRAY. The toxicity data were supplied by colleagues at the Ciba-Geigy A.G. in Basel. *P. mirabilis* strain VI and its stable protoplast-L-form was obtained from Prof. Dr. H. H. MARTIN, Darmstadt. Mrs. O. EBELMANN and Mrs. B. WITTE provided excellent technical assistance. We wish to thank all these persons for their collaboration.

References

1) GERTH, K.; H. IRSCHIK, H. REICHENBACH & W. TROWITZSCH: Myxothiazol, an antibiotic from *Myxococcus fulvus* (Myxobacterales). I. Cultivation, isolation, physico-chemical and biological properties. J. Antibiotics 33: 1474~1479, 1980
2) TROWITZSCH, W.; G. REIFENSTAHL, V. WRAY & K. GERTH: Myxothiazol, an antibiotic from *Myxococcus fulvus* (Myxobacterales). II. Structure elucidation. J. Antibiotics 33: 1480~1490, 1980
3) THIERBACH, G. & H. REICHENBACH: Myxothiazol, a new inhibitor of the cytochrome b-c_1 segment of the respiratory chain. Biochim. Biophys. Acta 638: 282~289, 1981
4) KOHL, W.; B. WITTE, B. KUNZE, V. WRAY, D. SCHOMBURG, H. REICHENBACH & G. HÖFLE: Angiolam A — ein neues Antibiotikum aus *Angiococcus disciformis* (Myxobacterales). Liebigs Ann. Chem. 1985: 2088~2097, 1985.
5) GERTH, K.; R. JANSEN, G. REIFENSTAHL, G. HÖFLE, H. IRSCHIK, B. KUNZE, H. REICHENBACH & G. THIERBACH: The myxalamids, new antibiotics from *Myxococcus xanthus* (Myxobacterales). I. Production, physico-chemical and biological properties, and mechanism of action. J. Antibiotics 36: 1150~1156, 1983
6) STEINMETZ, H.; N. BEDORF, W. TROWITZSCH, R. KRÜTZFELDT, K. GERTH, H. REICHENBACH & G. HÖFLE: Large scale isolation of myxovirescins. 3rd European Congress on Biotechnology. Vol. I, pp. 643~647, 1984
7) KUNZE, B.; T. KEMMER, G. HÖFLE & H. REICHENBACH: Stigmatellin, a new antibiotic from *Stigmatella aurantiaca* (Myxobacterales). I. Production, physico-chemical and biological properties. J. Antibiotics 37: 454~461, 1984
8) THIERBACH, G.; B. KUNZE, H. REICHENBACH & G. HÖFLE: The mode of action of stigmatellin, a new inhibitor of the cytochrome b-c_1 segment of the respiratory chain. Biochim. Biophys. Acta 765: 227~235, 1984