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# Archazolids, New Cytotoxic Macrolactones from *Archangium gephyra* (Myxobacteria)

## Production, Isolation, Physico-chemical and Biological Properties†

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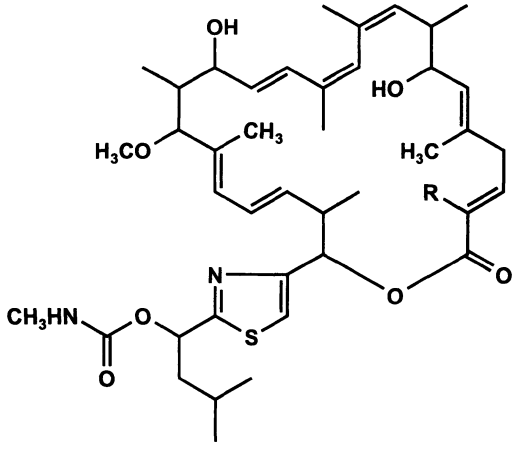
Novel cytotoxic compounds, archazolid A and B, were isolated from the culture broth of strains of the myxobacteria *Archangium gephyra* and *Cystobacter* sp. Archazolids consist of a macrocyclic lactone ring with a thiazole side chain and showed high activity against mammalian cells. The $IC_{50}$ values with different cell lines ranged from 0.1 to 1 ng/ml. An incubation of $PtK_2$ potoroo cells with archazolid A (5 ng/ml) led to the formation of vacuoles in the ER, a phenomenon that is typical for inhibitors of V-ATPases, like concanamycin and bafilomycin. We therefore assume that archazolid is an inhibitor of the V-ATPase of higher cells.

During our screening for new, biologically active metabolites from myxobacteria, we found a strain of *Archangium gephyra* that showed high cytotoxic activity in our screening assay with L929 mouse fibroblasts[1]. The adherently growing fibroblasts rounded up and detached from the surface. Bioassay-guided fractionation of culture extracts showed that the activity was due to a novel compound which we called archazolid. Up to now we found two variants, archazolid A and B, both of which occurred in strains of *Archangium*, but were also found in the related genus *Cystobacter*. Fig. 1 shows their chemical structure, the elucidation of which will be published elsewhere[2]. The archazolids consist of a macrocyclic lactone ring with a side chain that contains a thiazole ring. Archazolid B is a desmethyl derivative of archazolid A. In this paper we describe the production, isolation, and the physico-chemical and biological properties of the archazolids.

Fig. 1. The chemical structure of the archazolids.




Archazolid A; R = $CH_3$
Archazolid B; R = H

### Microorganism and Culture Conditions

The producing organism, *Archangium gephyra* strain Ar 3548, was isolated at the GBF from a soil sample collected

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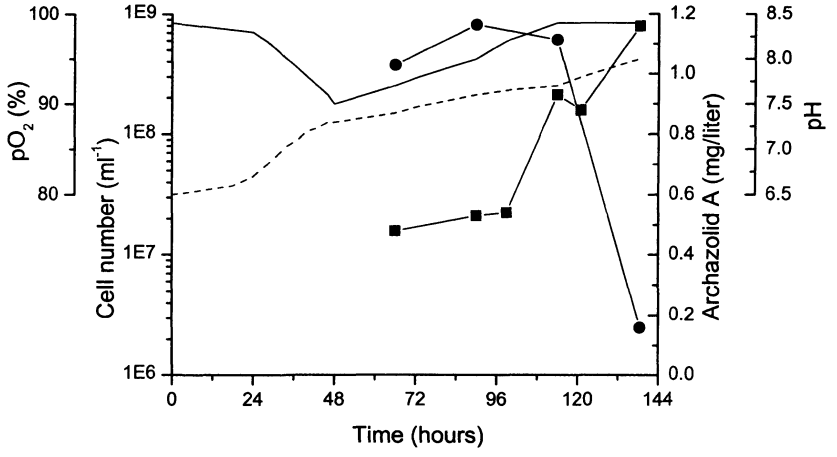
Fig. 2. Fermentation of *Archangium gephyra* Ar 3548 in a 15-liter bioreactor with 10 liters culture volume.




● Cell number, ■ archazolid A, —— $pO_2$, --- pH.

near the Hochobir mountain in the Karawanken, Austria. The strain was normally grown on a modified VY/2 agar[3] (bakers' yeast 0.5%, $CaCl_2 \cdot 2H_2O$ 0.1%, HEPES 1%, glucose 0.2%, vitamin $B_{12}$ 0.1 mg/liter, agar 1.8%, pH 7.2) and in M7 liquid medium[4]. Batch cultures of 100 or 500 ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at 30°C on a gyratory shaker at 160 rpm for 3~5 days.

## Production

Archazolid production on a larger scale was performed in M7 liquid medium. For example, a 500-ml culture grown on a shaker was inoculated into 10 liters M7 medium in a 15-liter fermentor with a flat-blade turbine stirrer. After three days the content of the seed fermentor was inoculated into a 350-liter bioreactor with 300 liters of medium without HEPES to which 1% (v/v) of the adsorber resin Amberlite XAD-16 (Rohm & Haas, Frankfurt) was added. The fermentors were kept at 30°C and agitated at 150 rpm. The aeration rate was 0.1 volume air per volume culture and minute. In order to reduce foam formation, 0.02% silicon antifoam agent (Tegosipon, Goldschmidt AG, Essen) was added. The pH was initially adjusted to 7.2. It rose during fermentation and was kept at pH 7.8 by adding 5% $H_2SO_4$. At harvest, the adsorber resin was separated from the culture broth by passing the culture through a process filter of 210 $\mu$m mesh size. Fig. 2 shows a fermentation of Ar 3548 in a 15-liter bioreactor with a culture volume of 10 liters (M7 medium without HEPES). Archazolid A was produced during the stationary growth phase and reached 1.2 mg/liter after 6 days.

## Isolation and Quantitative Determination

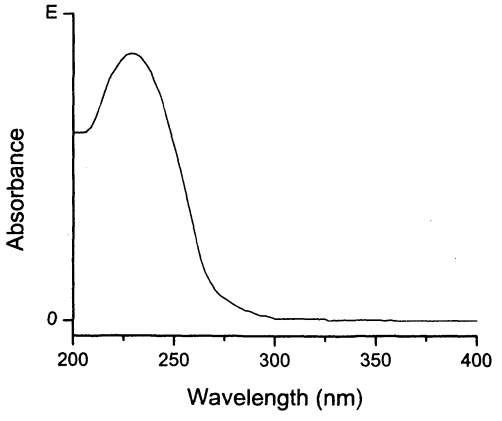
XAD-16 adsorber resin from a 300-liter fermentation was eluted with 18 liters methanol. The methanol in the eluate was evaporated *in vacuo* followed by distribution of the residue between water and ethyl acetate. Subsequently, the organic layer was concentrated *in vacuo* and the residue dissolved in methanol for separation by gel chromatography on Sephadex LH 20 (Fluka Steinheim, solvent methanol).

Biologically active fractions were separated and further purified by medium pressure RP18-chromatography (solvent: methanol/water 8:2; detection at 230 nm) yielding 240 mg archazolid A and 54 mg archazolid B. The antibiotics were analysed by HPLC (column 125×2 mm, Nucleosil 120-5 C-18, Macherey Nagel, Oensingen, Switzerland; solvent: methanol/water 82:18, flow rate: 0.3 ml/minute, detection: diode array). The retention times were Rt=11.8 minutes for archazolid A., and Rt=9.4 minutes for archazolid B.

## Physico-chemical Properties

The archazolids were soluble in methanol, acetone and ethyl acetate. TLC on silica gel 60 F-254 (Merck) with dichloromethane/methanol 9 : 1 as solvent gave Rf values of 0.63 for archazolid A and 0.64 for archazolid B. Detection was by spraying the plates with vanillin/sulphuric acid reagent and heating to 120°C, which resulted in gray blue spots. High resolution EI mass spectroscopy with a Finnigan MAT 95 gave the following molecular masses (and calculated elemental composition): 738.4239 ($C_{42}H_{62}N_2O_7S$) for archazolid A and 724.4139 ($C_{41}H_{60}N_2O_7S$) for archazolid B. The UV spectrum of archazolid A in methanol is shown in Fig. 3. $\lambda_{max}$ ($\lg \varepsilon$)= 228 nm (4.60). The IR spectrum in KBr (Fig. 4) was measured with a Nicolet 20 DXD FT-IR spectrometer.

Fig. 3. UV spectrum of archazolid A in methanol.




## Biological Activity

Archazolid A, which was tested in more detail, was inactive against bacteria and showed only low activity against fungi (Table 1). As expected from the screening data, archazolid A and B were highly effective in mammalian cell cultures (Table 2). Growth inhibition of various cell lines was determined in a microtiterplate assay. Aliquots of 120 $\mu$l of suspended cells (50,000/ml) were given to 60 $\mu$l of a serial dilution of the inhibitor. After 5 days we measured the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) or, in the case of K-562 cells, that of WST-1 (Roche, Mannheim, Germany). The $IC_{50}$ values of both archazolids ranged from 0.1 to 1 ng/ml. We compared the activity with two known inhibitors from streptomycetes, concanamycin A and bafilomycin $A_1$ (both from Sigma, Deisenhofen, Germany). Concanamycin showed nearly the same activity, bafilomycin was less active. Kinetic studies with L929 mouse fibroblasts showed that the propagation of the cells was stopped immediately after the addition of archazolid A (Fig. 5). The cells rounded up and began to detach from the substratum. Incorporation experiments with labeled metabolic precursors showed that none of the main metabolic pathways were impaired within six hours after the addition of the inhibitor (data not shown). The effect of archazolid A was reversible when the medium was replaced by fresh one without the inhibitor after one day of

Fig. 4. IR spectrum of archazolid A in KBr.

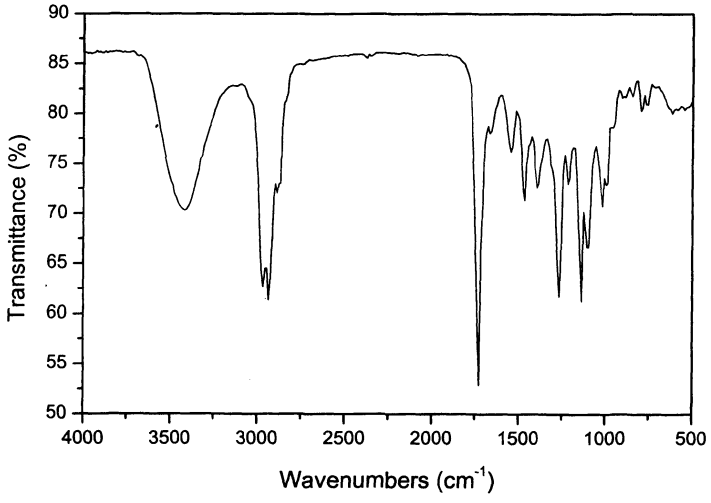


Table 1. Antimicrobial activity of archazolid A.

| | Test organisms[a] | Diameter of inhibition zone[b] (mm) |
|---|---|---|
| Gram-negative bacteria | *Escherichia coli* DSM498 | 0 |
| | *Pseudomonas aeruginosa* DSM 1117 | 0 |
| Gram-positive bacteria | *Bacillus subtilis* DSM 10 | 0 |
| | *Staphylococcus aureus* GBF 16 | 0 |
| | Micrococcus luteus GBF | 0 |
| Yeasts | *Candida albicans* GBF 129 | 0 |
| | *Hansenula anomala* DSM 70263 | 7 |
| | *Metschnikowia pulcherrima* DSM 70321 | 10 |
| | *Pichia membranaefaciens* DSM 70366 | 10 |
| | *Saccharomyces cerevisiae* GBF 36 | 9 |
| | *Torulopsis glabrata* DSM 70398 | 10 |
| Filamentous fungi | *Aspergillus niger* DSM 823 | 11 |
| | *Botrytis cinerea* DSM 877 | 8 |
| | *Mucor hiemalis* DSM 2655 | 10 |
| | *Pythium debaryanum* DSM 62946 | 20 |
| | *Trichoderma koningii* DSM 3121 | 0 |
| | *Ustilago zeae* DSM 3121 | 12 |

[a] The organisms were grown on standard agar (1.5 %) or in liquid media (bacteria: peptone 1%, meat extract 0.1%, yeast extract 0.1%, pH 7.0; fungi: malt extract 3 %, peptone 0.3%, pH 5.6). The strains were from the collection at the GBF or from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ).

[b] Determined by agar diffusion assay using paper discs of 6 mm diameter with 20 µg archazolid A.

incubation (Fig. 5). After two days the cells had begun to die and disintegrate. When the medium was exchanged at that time, only a slow culture growth was observed. After five days the cells had died, and the cultures did not recover any longer.

Investigation of the internal morphology of the cultured cells revealed that archazolid A induced formation of vacuoles in the endoplasmatic reticulum of $PtK_2$ potoroo kidney cells. This was best seen by labeling the cells with an antibody against GRP-94, a marker protein of the ER (Fig. 6). $PtK_2$ cells were seeded into four-well plates with glass coverslips (13 mm in diameter) at the bottom of the wells. Exponentially growing cells were incubated with the inhibitor for different periods of time. Cells were fixed with cold (−20°C) methanol/acetone (1 : 1) for 10 minutes, incubated with a primary monoclonal antibody against GRP-94 (1 : 1000; Affinity BioReagents, Golden, U.S.A.), and then with a secondary goat anti-rat immunoglobulin G antibody conjugated with Alexa 488 (1 µg/ml; Molecular Probes, Leiden, The Netherlands) at 37°C, each for 1 hour. Cells were rinsed with phosphate-buffered saline (GIBCO BRL, Eggenstein, Germany) between two incubations. The coverslips were mounted in ProLong Antifade (Molecular Probes), and examined with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany). In the control cells the ER, labeled by the GRP-94 antibody, was seen as a

Table 2. Growth inhibition by archazolid, bafilomycin and concanamycin of different mammalian cell lines.

| Cell line | Origin[a] | $IC_{50}$ (ng/ml) | | | |
|---|---|---|---|---|---|
| | | Archazolid A | Archazolid B | Bafilomycin $A_1$ | Concanamycin A |
| L-929 | murine connective tissue DSM ACC 2 | 0.6 | 0.8 | 2 | 0.2 |
| $PtK_2$ | *Potorous tridactylis* kidney ATCC CCL-56 | 0.5 | 1.0 | -- | -- |
| KB-3.1 | human cervix carcinoma DSM ACC 158 | 0.3 | 0.2 | 2.5 | 0.5 |
| SK-OV-3 | human ovarian adenocarcinoma ATCC HTB-77 | 0.15 | 0.4 | 0.5 | 0.1 |
| PC-3 | prostate adenocarcinoma ATCC CRL-1435 | 0.03 | 0.25 | 0.1 | 0.02 |
| U-937 | human histiocytic lymphoma DSM ACC 5 | 0.1 | 0.2 | 2.5 | 0.1 |
| K-562 | human myelogenous leukemia ATCC CCL-243 | 0.3 | 0.3 | -- | 0.4 |

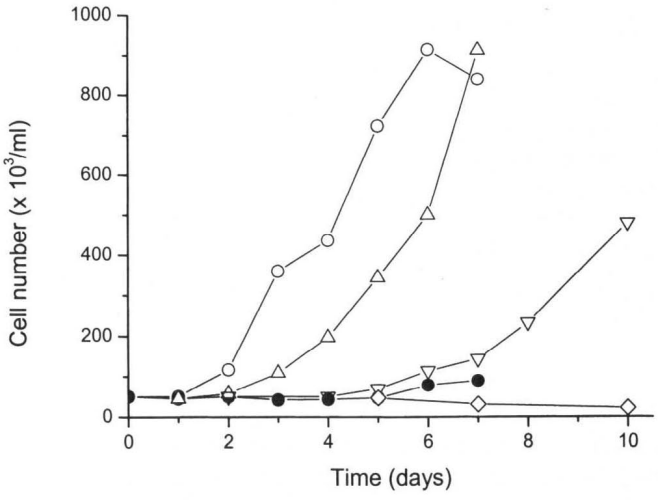
[a] The cell lines were from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) or American Type Culture Collection (ATCC), and cultivated in the media recommended by the supplier plus 10 % newborn calf serum or, in the case of $PtK_2$ cells, fetal calf serum at 37°C and 10 % $CO_2$ in a moist atmosphere.

fine network that extends throughout the entire cytosol. When the cells were incubated with archazolid A (5 ng/ml) for one day, we observed big holes in the ER network, and flakes of GRP-94 within these holes. We observed the same typical morphological alterations, the rounding up of L929 cells and the formation of vacuoles in the ER of $PtK_2$ cells, when the cells were incubated with concanamycin A and bafilomycin $A_1$. These compounds are known inhibitors of vacuolar type ATPases (V-ATPases)[5]. However, the vacuolation in the ER of $PtK_2$ cells were not observed with oligomycin A (Sigma) or thapsigargin (Sigma), which are selective inhibitors of the F-ATPase in the mitochondria[6] and the $Ca^{2+}$-ATPase of the sarcoendoplasmatic reticulum[7], respectively.

## Discussion

After gephyronic acid[8], tubulysin[9], and argyrin[10], the archazolids are the fourth group of compounds that was detected in strains of *Archangium gephyra*. A survey for producing myxobacteria showed that the archazolids occur more rarely than the other compounds. They were only found in *Archangium* and the related genus *Cystobacter*. The archazolids are new natural products that show no relation to known compounds. They are highly effective against mammalian cells. Since archazolid A induces the same morphological changes in the cells as concanamycin A and bafilomycin $A_1$, two known inhibitors of the V-ATPase[5], we assume that the archazolids act by inhibiting the V-ATPase activity of the cells.

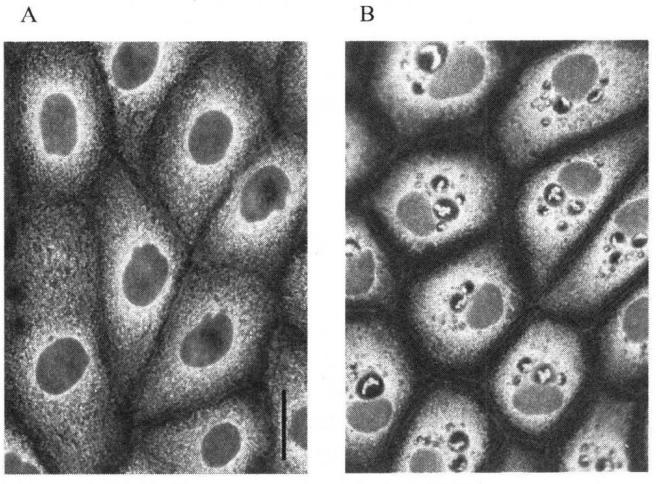
Fig. 5. Influence of archazolid A (20 ng/ml) on the growth of K-562 leukemia cells.




The culture medium was replaced by fresh one without the inhibitor after 1 day (△), after 2 days (▽) and after 5 days (◇) of incubation, in order to check the reversibility of the archazolid effect.

● Archazolid A (20 ng/ml), ○ controls without archazolid.

Fig. 6. Effect of archazolid A (5 ng/ml) on the ER structure of $PtK_2$ cells which is visualized by labeling the marker protein GRP94.




(A) Control cells. (B) Cells incubated with archazolid A (5 ng/ml) for one day.

Scale bar, 20 $\mu$m.

Acknowledgments

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