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Argyrins, Immunosuppressive Cyclic Peptides from Myxobacteria

I. Production, Isolation, Physico-chemical and Biological Properties†

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A group of cyclic peptides consisting of 8 amino acid residues, named argyrins A to H, were isolated from the culture broth of strains of the myxobacterium *Archangium gephyra*. Argyrin B was found to be a potent inhibitor of T cell independent antibody formation by murine B cells and strongly inhibited the two way murine mixed lymphocyte reaction. All argyrins had slight antibiotic activity, especially against *Pseudomonas* sp., and inhibited growth of mammalian cell cultures. The growth inhibition was often incomplete and varied highly with different cell lines.

During our screening for cytostatic compounds from myxobacteria with the mouse fibroblast cell line L929, we found growth inhibiting activity in culture extracts of *Archangium gephyra*, strain Ar 8082. These activities were due to two groups of peptide compounds, the linear tubulysins[1], and the cyclic argyrins. Later, both groups of compounds were often found again in various strains of the genera *Archangium* and *Cystobacter*. In this paper we describe the production, isolation, the physico-chemical properties and the biological activities of the argyrins. Figure 1 shows the structures of argyrin A to H. The elucidation of structures and stereochemistry will be published elsewhere[2]. Argyrin A contains the building elements tryptophan, 4-methoxytryptophan, glycine, alanine, α,β-dehydroalanine, sarcosine, and 2-(1′-aminoethyl)-thiazole-4-carboxylic acid. In argyrin B, alanine is replaced by α-amino butyric acid. Argyrin C to H show alterations at three further positions.

Microorganisms and Culture Conditions

The producing organisms *Archangium gephyra* strain Ar 8082 and Ar 315 were isolated at the GBF from soil samples. They were grown on modified VY/2 agar[3] (baker's 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 40 ml or 500 ml in 200-ml or 1,000-ml Erlenmeyer flasks were incubated at 30°C on a gyratory shaker for 3~7 days at 200 rpm (40-ml cultures) and 160 rpm (500-ml cultures), respectively.

Fermentation and Quantitative Determination

Argyrin production at fermentor scale was initially performed in M7 liquid medium. Figure 2 shows a fermentation of Ar 8082 in a 15-liter draft tube bioreactor (Giovanola Fréres SA, Monthey, Switzerland) containing

† Dedicated to Prof. Dr. EKKEHARD WINTERFELDT on the occasion of his 70th birthday.

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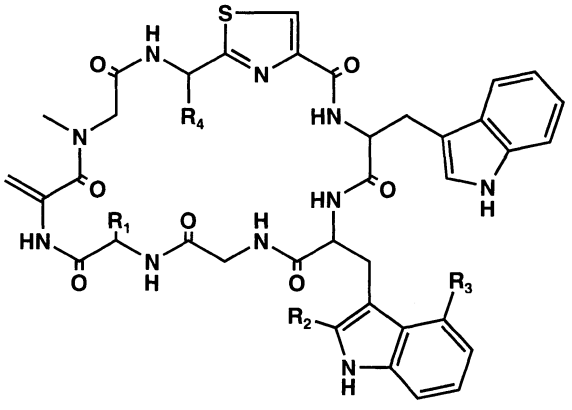
10 liters M7 medium without HEPES to which 1% (v/v) of the adsorber resin Amberlite XAD-16 (Rohm & Haas, Frankfurt, Germany) was added. The fermentor was inoculated with 1 liter shake culture, kept at 30°C and agitated at 150 rpm. In order to reduce foam formation, 0.02% silicone antifoam agent (Tegosipon, Goldschmidt AG, Essen, Germany) was added. The aeration rate was 0.1 volume of air per culture volume and minute. As the strain grew in lumps, the oxygen consumption was monitored as a growth parameter. The pO_2 value dropped fast and was kept at 30% by increasing the stirring rate. The pH was regulated between 7.2 and 7.6 by adding 5 M KOH or 5% (v/v) acetic acid. The argyrins were produced during the logarithmic and early stationary phase, the main compounds, argyrin A and B, simultaneously and in nearly equal amounts. In the given example both together reached a total yield of 10 mg/liter.

Fig. 1. The chemical structure of the argyrins.



Argyrin A R_1= CH_3; R_2 = H; R_3 = OCH_3; R_4 = CH_3

Argyrin B R_1 = C_2H_5; R_2 = H; R_3 = OCH_3; R_4 = CH_3

Argyrin C R_1 = CH_3; R_2 = CH_3; R_3 = OCH_3; R_4 = CH_3

Argyrin D R_1 = C_2H_5; R_2 = CH_3; R_3 = OCH_3; R_4 = CH_3

Argyrin E R_1 = CH_3; R_2 = H; R_3 = H; R_4 = CH_3

Argyrin F R_1 = CH_3; R_2 = H; R_3 = OCH_3; R_4 = CH_2OH

Argyrin G R_1 = C_2H_5; R_2 = H; R_3 = OCH_3; R_4 = CH_2OH

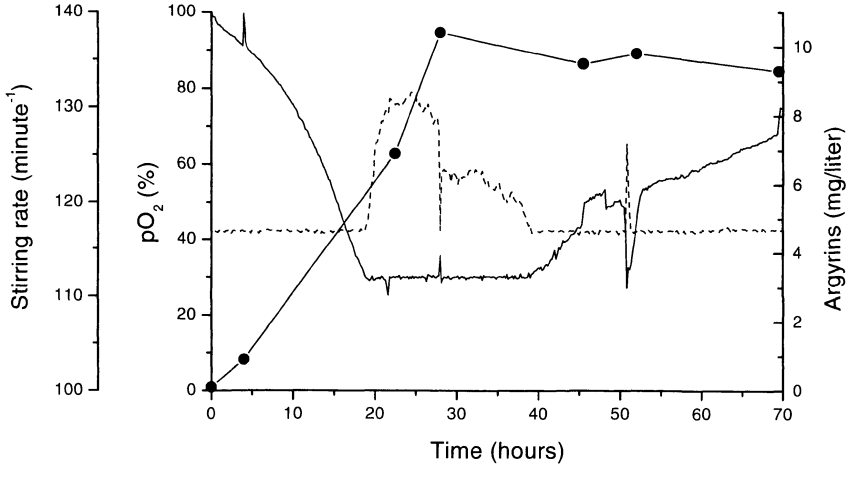
Argyrin H R_1 = CH_3; R_2 = H; R_3 = OCH_3; R_4 = H

The argyrins were quantitatively determined by HPLC (column: 125/2, Nucleosil 100-5 C18 from Macherey-Nagel, Oensingen, Switzerland; solvent: methanol - water 65 : 35 with 0.5% (v/v) acetic acid; flow rate: 0.3 ml/minute; detection: diode array at 220 nm). The retention times of argyrin A and B were 5.0 and 6.3 minutes, respectively.

Medium and Strain Improvement

To improve the fermentation yield of argyrin A and B modified media derived from the liquid medium M7 were prepared and tested with strain Ar 8082 by shake flask cultivation using 40 ml medium in 200-ml shake flasks. In these studies, an improved medium M7/14 (Table 1) was found, giving about 20% higher argyrin titers as compared to medium M7 with 1% (v/v) adsorber resin Amberlite

Fig. 2. Fermentation of *Archangium gephyra* Ar 8082 in a 15-liter bioreactor with a flat-blade turbine stirrer (10 liters culture volume, aeration rate 1 liter/minute, pH 7.2~7.6).



—— pO_2, ------ Stirring rate, ● argyrins A+B.

Table 1. Composition of medium M7/14.

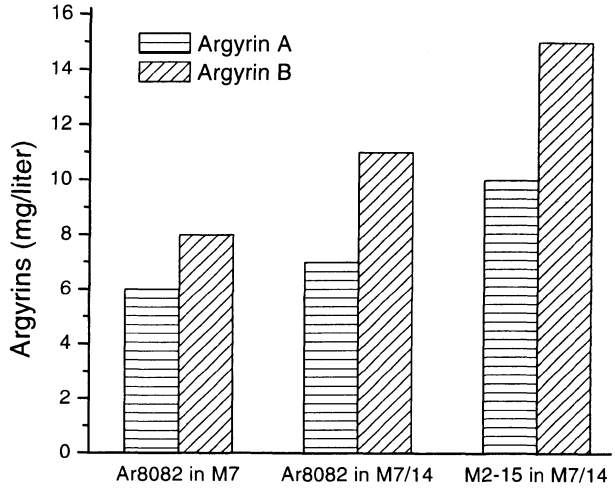
Compound	Quantity
Alburex N (Roquette, France)	5.0 g/l
$CaCl_2 \cdot 2\,H_2O$	1.0 g/l
$MgSO_4 \cdot 7\,H_2O$	1.0 g/l
Yeast extract powder w/o salt, type D (Bio Springer, France)	1.0 g/l
Potato starch Noredux A-150 (Blattmann-Cerestar, Switzerland)	5.0 g/l
HEPES	12.0 g/l
Glucose solution 50 %	4 ml/l
Vitamin B_{12} solution 0.1 %	10 ml/l

For argyrin production, 1 % (v/v) Amberlite XAD-16 adsorber resin (Rohm & Haas, Frankfurt, Germany) was added.

XAD-16.

Further increase of the argyrin titer was obtained with the mutant strain M2-15 derived through mutation by UV irridiation (1 minute at 500 μWatt per cm^2) of Ar 8082 cells streaked on VY/2 agar. Improved mutants were selected from the colonies growing on these plates by shake flask fermentation in medium M7/14 and HPLC measurement of argyrin A and B. The titer improvement of argyrin A and B obtained with the mutant M2-15 and medium M7/14 in shake flask cultures is shown in Fig. 3.

Fig. 3. Argyrin titer improvement with medium M7/14 and the mutant M2-15 derived from *Archangium gephyra* strain Ar 8082.



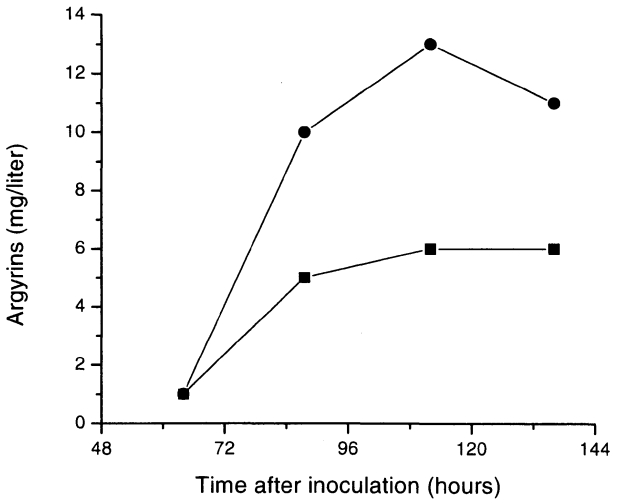
Scale-up of Production Process

For preparation of gram quantities of argyrins the fermentation and isolation processes were modified and adapted to a 600-liter scale. Seed cultures using mutant M2-15 were prepared in a 15-liter glass fermentor (Bioflow 3000, New Brunswick Scientific) with 10 liters of medium M7/14 without HEPES and XAD-16. After 3 days of cultivation at 30°C and 150 rpm, the content of the seed fermentor was transferred into a 750-liter bioreactor with 600 liters M7/14 medium without HEPES. The fermentor was kept at 30°C and 0.5 bar overpressure. The culture was aerated with 0.15 volumes of air per culture volume and minute, and agitated with a stirrer speed of 75 rpm, which was sufficient to keep the pO_2 above 50% of saturation throughout the cultivation. Excessive foam formation was avoided by controlled addition of Silicone Antifoam Emulsion B (Dow Corning, U.S.A.). The argyrins were quantitatively determined by HPLC as described above. Figure 4 shows the development of argyrin A and B titers during a 600-liter fermentation of strain Ar 8082 mutant M2-15. In this example maximum titers were reached after 5 days of cultivation with argyrin A at 6 mg/liter and argyrin B at 13 mg/liter. Numbers and yields in the

following description of the isolation process refer to this fermentation example.

After 6 days the XAD-16 adsorber resin (*ca.* 6 liters) was separated from the culture broth. After washing with water the resin was extracted 3 times with 8 liters of isopropanol. The unified isopropanol extracts were evaporated under simultaneous addition of water to a final volume of 10 liters. This water phase was extracted with 3×12 liters ethyl actetate. The organic phase was evaporated until dry resulting in 92 g extract containing 2.7 g argyrin A and 5.9 g argyrin B. At this stage, extracts of several fermentations were unified for further treatment. Next step was separation on 10 kg Sephadex LH-20. The peak fractions, containing nearly 30% of each argyrins A and B, were further purified by RP-HPLC with 2 kg Daisogel SP-120-10/20-ODS-A (Daiso Co., Ltd.). Argyrin A peak fractions contained argyrin A at 45~55%. Argyrin B peak fractions were already >99% pure. Argyrin B was crystallized from *tert*-butylmethyl ether resulting in a crystalline product with a purity >99%.

Fig. 4. Titer development of argyrins A and B during fermentation at 600-liter scale.



■ Argyrin A, ● argyrin B.

Rechromatography of polar side fractions on Nucleosil C18 with the solvent system acetonitrile/water 35 : 65 in addition to argyrin A gave small amounts of argyrins E, F, G, and H.

Fermentation of the wild-type strain Ar 315 on a 300-liter scale, worked up under similar conditions as described above, yielded argyrin C (0.42 g) and D (0.22 g) instead of the structural analogs argyrin A and B.

Physico-chemical Properties

The argyrins are soluble in methanol, acetone, dichloromethane, and ethyl acetate. They could be detected by TLC (silica gel 60 F_{254}, solvent: dichloromethane-methanol 9 : 1) and HPLC. Retention times and molecular masses were determined by HPLC/ESI-MS (on Nucleosil C18, 5 μm, 125×2 mm with the solvent system

Table 2. Rf and Rt values, optical rotation (α_D), elemental composition, and calculated and found molecular masses of argyrin A to H.

Argyrin	R_f	R_t	α_D	Elemental composition	Mass calculated	(M^+)	Mass found
A	0.50	5.0	100 (c = 1.7, aceton)	$C_{40}H_{44}N_{10}O_8S$	825.3142	(M^+)	825.3159
B	0.50	5.9	104 (c = 2.9, aceton)	$C_{41}H_{46}N_{10}O_8S$	838.3221	(M^+)	838.3194
C	0.48	6.3	90 (c = 0.14, aceton)	$C_{41}H_{46}N_{10}O_8S$	838.3220	(M^+)	838.3243
D	0.51	8.2	113 (c = 0.26, DMF)	$C_{42}H_{48}N_{10}O_8S$	852.3377	(M^+)	852.3358
E	0.48	3.0	127 (c = 0.73, aceton)	$C_{39}H_{42}N_{10}O_7S$	812.3302	($M+NH_4^+$)	812.3382
F	0.39	3.4	79 (c = 0.74, aceton)	$C_{40}H_{44}N_{10}O_9S$	839.2	($M+H^+$)	841.3
G	0.39	3.8	68 (c= 0.24, aceton)	$C_{41}H_{46}N_{10}O_9S$	853.2	($M+H^+$)	855.3
H	0.50	3.9	102 (c = 2.0, aceton)	$C_{39}H_{42}N_{10}O_8S$	809.4	($M+H^+$)	811.2

methanol/water 65 : 35 containing 0.5% acetic acid, flow rate 0.3 ml/minute). High resolution mass spectra and ($EIDCI^+$), measured on a Finnigan MAT 95 mass spectrometer. The optical rotation was determined with the Perkin Elmer Polarimeter 241. The results are summarized in Table 2. The UV spectrum of argyrin A was recorded with a Shimadzu UV-2102 PC spectrophotometer (Fig. 5), the IR spectrum with a Nicolet 20 DXB FT-IR spectrometer (Fig. 6).

Biological Activity

The argyrins showed slight antibacterial and antifungal activity (Table 3). Yeasts were insensitive, and if growth inhibition of filamentous fungi took place, it was always incomplete. The most sensitive of the tested organisms were two species of the genus *Pseudomonas*.

The cytostatic activity of the argyrins was tested with different mammalian cell lines. Sequentially diluted concentrations of the argyrins were incubated with approximately 6000 cells of the test cell line that were seeded into each well of a 96-well microtiter plate. The final culture volume was 180 μl/well. The metabolic activity of the cells was measured by an MTT assay[5] after 5 days of incubation at 37°C and 10% CO_2. The resulting inhibition curves with different cell lines showed a high variability. The curves often were very flat, and sometimes we observed a retardation of growth at low argyrin concentrations that did not further increase even with the highest applied amounts of argyrin. Therefore Table 4 gives the percentage of growth that was observed with 100 ng of the argyrins A to D. Figure 7 shows kinetic studies with L929 mouse fibroblasts. The cells were cultivated in 24-well plates with 0.75 ml/well. The inoculum was 50,000 cells/ml. Protein amount was measured as a parameter of growth[6]. The studies showed that the propagation of the cells slowed down only slightly after the addition of argyrin A. In order to get information about the structure activity relationship, we compared the IC_{50} of all derivatives with *P. aeruginosa* and *P. acidovorans* and the human KB-3.1 cell line (Table 5). Bacteria were inoculated at an OD (620 nm) of 0.01 in 96-well microtiter plates with serial dilutions of the argyrins, and incubated at 30°C. After one day the OD of each well was measured in an ELISA reader.

Fig. 5. The UV spectrum of argyrin A.

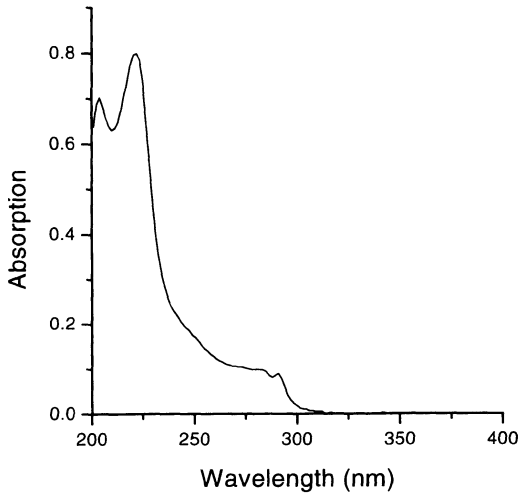


Fig. 6. IR spectrum of argyrin A in KBr.

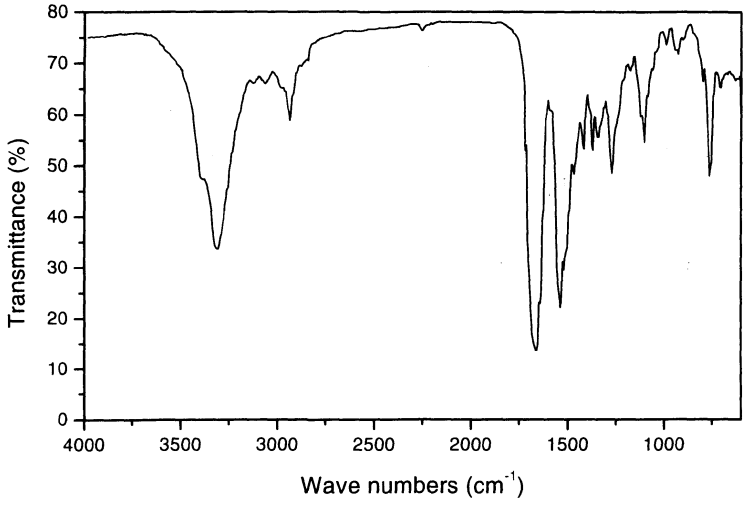


Table 3. Antimicrobial activity of the argyrins A to D.

Test organism[a]	Diameter of inhibition zone[b] (mm)			
	A	B	C	D
Bacteria:				
Bacillus subtilis DSM 10[c]	0	0	0	0
Escherichia coli DSM 498	0	0	0	0
E. coli tol C GBF[d]	9	8	0	0
Micrococcus luteus GBF	0	0	-	7
Mycobacterium phlei GBF	0	0	0	0
Pseudomonas aeruginosa DSM1117	13	15	14	13
P. acidovorans GBF	16	15	15	14
Staphylococcus aureus GBF	7	0	7	0
Yeasts:				
Candida albicans CBS 1893[e]	0	0	-	-
Hansenula anomala DSM 70263	0	0	0	0
Metschnikowia pulcherrima DSM 70321	0	0	0	0
Filamentous fungi:				
Botrytis cinerea DSM 877	12i	13i	11i	0
Mucor hiemalis DSM 2655	0	0	-	-
Pythium debaryanum DSM 62946	10i	10i	0	0

[a] The organisms were grown in standard media (bacteria: caseine peptone 0.5%, proteose peptone 0.5 %, meat extract, 0.1%, yeast extract 0.1%, pH 7.0; fungi: malt extract 3%, casein peptone 0.3%, pH 5.6). Agar in solid media was 1.5%.
[b] Determined by an agar diffusion test using paper discs of 6 mm diameter with 20μg of the compound in 20 μl methanol (i; incomplete inhibition).
[c] Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig.
[d] Collection of the GBF.
[e] Centraalbureau voor Schimmelcultures, Baarn.

The IC_{50} data show a clear drop in activity of the argyrins E to H, and give a hint that the 4-methoxy group of the tryptophan and the methyl group besides the thiazole ring are important for a good activity.

Further tests showed a high immunosuppressive activity. Argyrin B was found to be a potent inhibitor of T-cell independent antibody formation by murine B cells (Table 6). Spleen cells from C57BL/6 nu/nu and C57BL/6 mice (BRL, Fullingsdorf, Switzerland) were cultured in duplicate in flat bottomed microtiter plates. The cells were stimulated by TNP-LPS (trinitrophenyl-lipopolysaccharide; 2×10^5 cells per well) or by DAGG-Ficoll (2,4-dinitrophenyl-β-alanyl-glycyl-glycyl-Ficoll; 4×10^5 cells per well)[7~10]. Serial dilutions of the drug in fetal calf serum supplemented (10%) culture medium (RPMI 1640) were added to a final volume of 0.2 ml in the presence of optimal concentration of TNP-LPS (0.2 μg/ml) or DAGG-Ficoll (0.2 ng/ml). TNP-LPS stimulated spleen cells were cultured at 37°C in a humidified CO_2 (5.5%) incubator for 4 days, and DAGG-Ficoll stimulated spleen cells were cultured in a gaseous environment of 83% N_2, 7% O_2 and 10% CO_2 for five days. On day four or day five, depending on the antigen, supernatants from each of the two replicate cultures were pooled and diluted 4-fold using bovine serum albumin (2%) or human serum albumin (5%) in phosphate buffered saline and then assayed for the presence of IgM antibodies against dinitrophenyl-ovalbumin by ELISA. Interestingly, argyrin B inhibited the T-cell independent

Table 4. Growth inhibition of mammalian cell cultures by the argyrins A to D.

Cell line[a]	Growth in the presence of argyrins (100ng/ml) compared to control cells [%][b]			
	A	B	C	D
L929 (ATCC CCL1), mouse, connective tissue	54	66	53	58
KB-3.1 (DSM ACC 158), cercix carcinoma	43	65	36	42
KB-V1 (DSM ACC 149), multidrug-resistant KB line	83	82	93	108
PC-3 (ATCC CRL-1435), prostate adenocarcinoma	60	65	60	61
SK-OV-3 (ATCC HTB-77), ovarian adenocarcinoma	78	100	85	86
K-562 (ATCC CCL 243), chronic myelogenous leukemia	81	82	77	78
U-937 (DSM ACC 5), histiocytic lymphoma	78	107	91	93

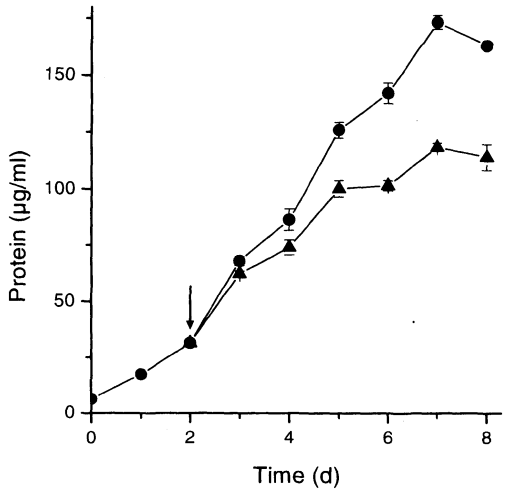
[a] All cell lines with the exception of L929 were of human origin.
[b] Growth inhibition was measured by an MTT assay. Data are means of 2 ~ 6 independent experiments.

antibody production against TNP-LPS more potently than in response to DAGG-Ficoll. Moreover, argyrin B strongly inhibited the two way murine mixed lymphocyte reaction (MLR), an *in vitro* model for alloantigen-mediated T cell activation and proliferation[11]. Argyrin B also potently inhibited IgG production of human B cells stimulated either with soluble CD40L/IL-4 (CD40L 3 mg/ml; IL-4 100 U/ml; both Novartis) or by *S. aureus* Cowan I (SAC; dilution 1 : 10,000; Sigma)[12,13]. These results indicate that argyrin B has potent immunosuppressive activity.

Discussion

The argyrins are the fourth group of natural compounds obtained from strains of the myxobacterial genus *Archangium*. While gephyronic acid[4] seems to be restricted to that genus, the melithiazols[14] were first found in *Melittangium*. The argyrins also occur in the genus *Cystobacter*, which is closely related to *Archangium*. The argyrins are very similar to antibiotics A21459 A and B,

Fig. 7. Influence of argyrin A (500 ng/ml) on the growth of L929 mouse fibroblasts measured as increase of protein. Argyrin was added after 2 days as indicated by the arrow.



● Control, ▲ in the presence of argyrin A.

Table 5. Structure activity relationship of argyrins A to H.

Argyrins	IC_{50} (μg/ml)		
	P. aeruginosa	*P. acidovorans*	KB-3.1
A	0.12	0.07	0.1
B	0.08	0.05	0.3
C	0.1	0.05	0.1
D	0.14	0.07	0.3
E	1.4	1.5	10
F	5	5	1.5
G	4	4	>20
H	0.8	0.5	>20

Table 6. Inhibiting effect of argyrin B on the *in vitro* activity of B and T lymphocytes; IC_{50} (μM).

Compound	murine			human	
	TNP-LPS[a]	DAGG-Ficoll[a]	MLR[b]	SAC[a]	CD40L/IL-4[a]
Argyrin B	0.02	0.2	0.05	0.005	0.003
Cyclosporin A	> 1	0.005	0.006	-	0.3

[a] Immunoglobulin production was measured after stimulation by the given agents.

[b] Growth was measured according to a standard mixed-lymphocyte reaction (MLR) procedure.[10]

which were isolated from *Actinoplanes* sp. and shown to be inhibitors of the bacterial protein biosynthesis[15]. Like the A21459 complex, the argyrins also showed an unsual spectrum of antibacterial activity. They clearly inhibited *Pseudomonas* sp., which was surprising, because such activities were seldom found during our screening program. The other peculiarity was the incomplete inhibition of mammalian cell growth. Thus, we often observed a slight inhibition that gave flat inhibition curves, which did not reach a growth reduction of 50%.

Argyrin B was found to be a very active immunosuppressant. Several autoimmune diseases are mediated by autoantibody production from B cells. In addition, T-cell independent antibody-mediated rejection is a major obstacle to solid organ xenotransplantation, because production of xenogeneic antibodies is a key event during both, the hyperacute and the acute rejection phase of xenotransplantation[16,17]. Moreover, chronic allograft rejection (graft vessel disease, late graft rejection)[18~20] is another syndrome associated with the presence of graft-reactive antibodies, which leads to organ loss due to obstruction of arterial vessels of the graft. Thus, drugs which selectively inhibit antibody formation by B cells can be effective in certain autoimmune diseases, have potential to make xenotransplantation possible, and may also alter the progression of chronic rejection. Argyrin B inhibited the T-cell independent B-cell responses of murine B cells and blocked alloantigen-induced proliferation of murine T

cells at submicromolar concentrations. Antibody production of SAC- or CD40L-stimulated human B cells was even more powerfully inhibited, with IC_{50} values in the low nanomolar range. Therefore, argyrin might bear potential for the treatment of antibody mediated autoimmune diseases and for xenotransplantation. Moreover, argyrin blocked the alloantigen-induced murine mixed lymphocyte reaction suggesting a possible use in allo-transplantation as well.

In view of the potent immunosuppressive activity, argyrin B might preferentially inhibit cell differentiation rather than cell proliferation or alternatively might exert selective inhibition of proliferation of only certain cell types. This latter possibility is in the line with the observed difference in effect on the growth of different human cell lines. Therefore, argyrins could become tools to specifically inhibit growth of certain cell types. This possibility has to be further investigated including studies in animal models.

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