Gephyronic Acid, a Novel Inhibitor of Eukaryotic Protein Synthesis from *Archangium gephyra* (Myxobacteria)

Production, Isolation, Physico-chemical and Biological Properties, and Mechanism of Action†

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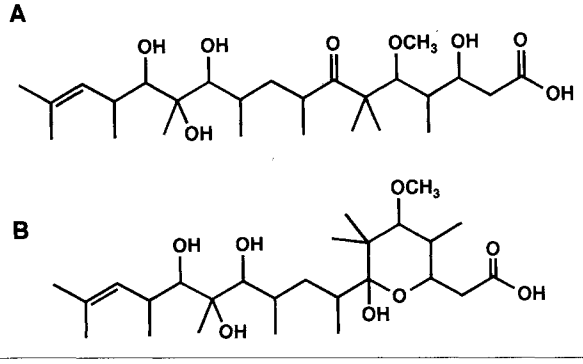
A new antibiotic compound, gephyronic acid was isolated from the culture broth of the myxobacterium, *Archangium gephyra* strain Ar 3895. Up to 3 mg/liter was produced during the logarithmic and stationary growth phase. The compound is an aliphatic acid, which tends to form a hemiacetal. Both forms inhibited growth of yeasts and molds (MIC 1~25 μg/ml) and had a cytostatic effect on mammalian cell cultures (IC_{50} 10~60 ng/ml). Gephyronic acid is a specific inhibitor of eukaryotic protein sythesis showing an IC_{50} of $1 \sim 2 \times 10^{-7}$ mol/liter in an *in vitro* translation assay.

During our screening program for antibiotics from myxobacteria, culture supernatants of different strains of *Archangium gephyra* showed antibiotic activity against yeasts and molds. The activity of strain Ar 3895 was further investigated and could be identified as a new, highly substituted aliphatic acid with a keto group. The compound showed a tendency to form an intramolecular hemiacetal (Fig. 1). It was named gephyronic acid. In this paper we describe the production, isolation, and the physico-chemical and biological properties of gephyronic acid. The structure elucidation will be published elsewhere[1].

Microorganism and Culture Conditions

The producing organism was *Archangium gephyra* strain Ar 3895 isolated in 1988 at the GBF from a soil sample collected on the island of Mallorca, Spain. It was grown in M7 liquid medium containing 0.5% Probion (single cell protein from Hoechst A.G.), 0.5% starch, 0.2% glucose, 0.1% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2 \cdot 2H_2O$, 0.1 mg/liter cyanocobalamin, 1% HEPES, pH 7.4. 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 3~7 days.

Fig. 1. The structure of gephyronic acid: keto (A) and hemiacetal form (B).



Production

Gephyronic acid production on a larger scale was performed in M7 liquid medium. For example, a 500-ml culture grown for 3 days on a gyratory shaker was inoculated into 10 liter in a 15-liter fermentor with a flat-blade turbine stirrer. After 3 days the content of the first fermentor was inoculated into a second one with 300 liter of medium to which 1% (v/v) of an absorber resin, *e.g.*, Amberlite XAD-16 (Rohm & Haas, Frankfurt) was added; also, 0.02% silicone antifoam agent (Tegosipon, Goldschmidt AG, Essen) was added in order to reduce foam formation. Both fermentors were kept at 30°C and agitated at 150 and 200 rpm, respectively. The aeration rate was 0.1 volume air per volume culture and minute. Fig. 2 shows a fermentation

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Fig. 2. Fermentation of *Archangium gephyra* Ar 3895 in a 365-liter draft tube reactor with a kaplan turbine stirrer (300 liter culture volume, 200 rpm, aeration rate 30 liter/minute).

○ Gephyronic acid, △ cell number, --- pH.

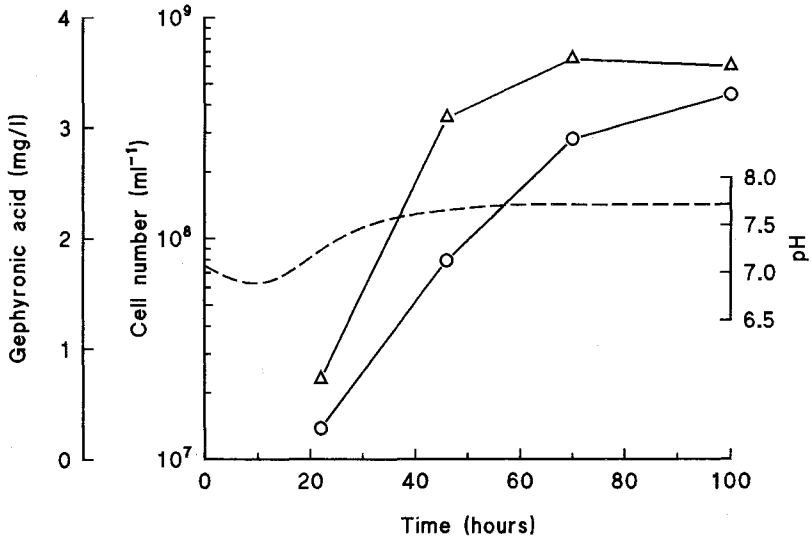
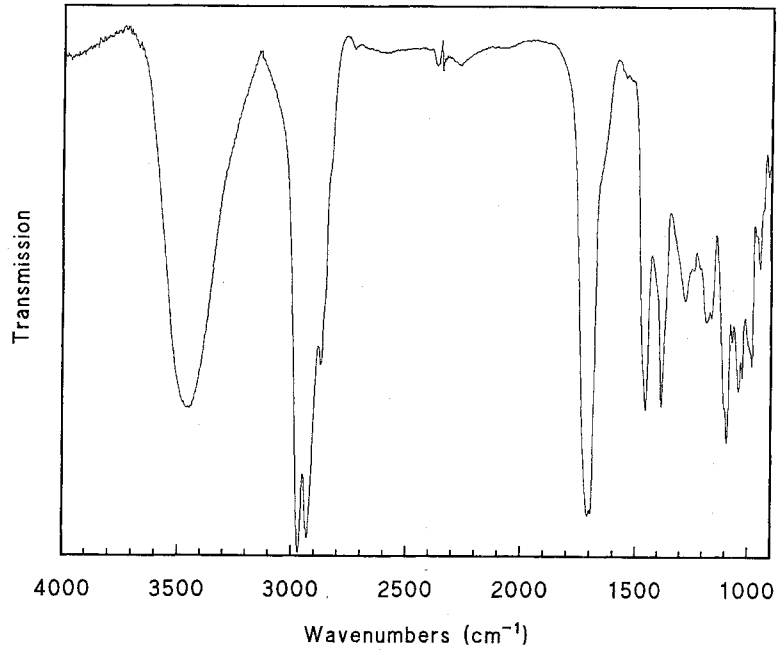


Fig. 3. IR spectrum of gephyronic acid (keto form) in KBr.



in a 365-liter draft tube bioreactor (Giovanola Frères SA, Monthey, Switzerland). The pH rose during the fermentation and was kept at 7.7 by titration with 5% H_2SO_4. Antibiotic production was followed by HPLC analysis (see below). Gephyronic acid was produced during the logarithmic and early stationary phase and reached a concentration of 3 mg/liter after 4 days. The cell density was 6×10^8/ml at that time. At harvest, the absorber resin was separated from the culture broth by passage through a process filter of 210 μm pore size.

Isolation

Gephyronic acid could be eluted from the resin with methanol and was further purified by column chromatography on Sephadex LH-20 (solvent: methanol) and silica gel RP-18 (solvent: acetonitrile - 0.05 M sodium phosphate buffer pH 4.0, 45 : 55). The antibiotic was quantitatively determined by HPLC (column 125 × 4 mm, Eurospher 100 C-18, 5 μm, Knauer, Bad Homburg; solvent: acetonitrile - 0.1% TFA in water, 1 : 1; flow rate: 1.0 ml/minute; $R_{t1} = 5.4$ minutes for the keto form, $R_{t2} = 9.5$ minutes for the hemiacetal; detection: diode-array).

Physico-chemical Properties

Gephyronic acid is soluble in methanol, acetone and

ethyl acetate, sparingly in chloroform and ethyl ether, and almost insoluble in hexane. In TLC on silica gel $60\,F_{254}$ with ethylacetate-methanol-water (85:15:10) as the solvent the R_f values were 0.42 (keto form) and 0.75 (hemiacetal). After spraying the plates with vanillin/sulfuric acid reagent and heating to 120°C, both forms of gephyronic acid gave purple spots. ^{13}C NMR and high resolution FAB-MS gave a molecular formula of $C_{26}H_{48}O_8$. Detailed data will be published with the structure elucidation[1]. The optical rotation of gephyronic acid (keto form) was $[\alpha]_D^{20}$ +46.5° (*c* 1.0, MeOH). The UV spectrum of gephyronic acid in methanol only shows end absorption. The IR spectrum (Fig. 3) was measured with a Nicolet 20 DXB FT-IR spectrometer.

Biological Activity

As shown in Table 1, gephyronic acid (keto form) had no effect on bacteria, but was active against all yeasts and most filamentous fungi tested. The MIC values for yeasts were generally lower than those for molds. Gephyronic acid also showed a cytostatic effect in mammalian cell cultures (Table 2). The IC_{50} values varied from 10 to 60 ng/ml. The hemiacetal was also tested against different fungi and the mouse cell line L929, but there was no significant difference between both forms of gephyronic acid concerning their biological activities in these assays.

Figures 4 and 5 show growth kinetics of the yeast *Metschnikowia pulcherrima* (DSM 70238) and the human leukemia cell line K-562 (ATCC CCL 243), respectively. The yeasts were cultivated in DSM 90 medium (3% malt extract, 0.3% peptone) at 30°C, the leukemia cells in RPMI 1640 (GIBCO) + 10% newborn calf serum at 37°C

Table 1. Antimicrobial activity of gephyronic acid (keto form).

	Test organism[a]	Diameter of inhibition zone[b] (mm)	MIC[c] (μg/ml)
Gram-negative bacteria	*Escherichia coli* DSM 498[d]	0	
	Pseudomonas aeruginosa DSM 1117	0	
Gram-positive bacteria	*Bacillus subtilis* DSM 10	0	
	Micrococcus luteus GBF[e]	0	
	Nocardia flava Zü 13086[f]	0	
	Staphylococcus aureus GBF	0	
Yeasts	*Candida albicans* CBS 1893[g]	18	2.5
	Debaryomyces hansenii DSM 70238	30	
	Hansenula anomala DSM 70263	34	0.8
	Kloeckera corticis GBF	22	
	Lipomyces lipofer GBF	24	1.6
	Metschnikowia pulcherrima DSM 70321	20	1.2
	Nadsonia fulvescens CBS 2596	12	
	Pichia membranaefaciens DSM 70366	15	
	Saccharomyces cerevisiae BT 27C-2AYGSC[h]	25	
	Schizosaccharomyces pombe Tü 501[i]	25	
	Torulopsis glabrata DSM 70398	30	0.8
Filamentous fungi	*Aspergillus niger* DSM 823	21	25
	Athelia rolfsii DSM 63030	10	
	Botrytis cinerea DSM 877	13	25
	Cercospora beticola DSM 62107	14	
	Fusarium oxysporum DSM 2018	11	
	Mucor hiemalis DSM 2655	0	
	Pythium debaryanum DSM 62946	0	
	Rhizopus oryzae DSM 905	12	
	Trichoderma koningii DSM 63060	21	25
	Ustilago zeae DSM 3121	40	2.5

[a] The organisms were grown in standard 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).
[b] Determined by the agar diffusion test using paper discs of 6 mm diameter with 20 μg gephyronic acid.
[c] Determined by a serial broth dilution assay.
[d] Deutsche Sammlung von Mikroorganismen.
[e] Collection at the GBF.
[f] Collection University of Zürich.
[g] Centraalbureau voor Schimmelcultures, Baarn.
[h] Yeast Genetic Stock Center, Berkeley.
[i] Collection University of Tübingen.

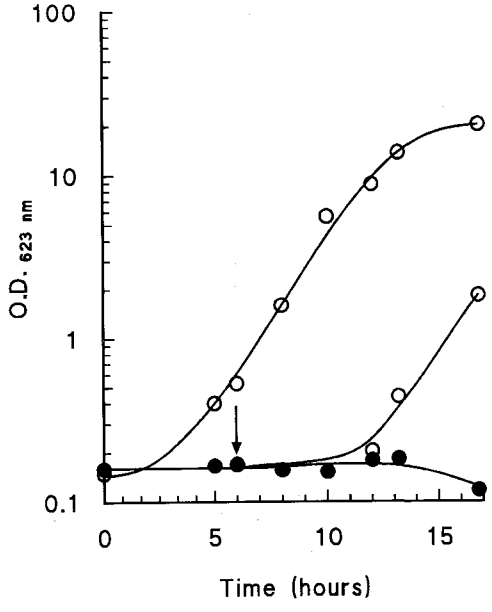
Table 2. Cytostatic effects of gephyronic acid (keto form) on mammalian cell lines.

Cell line	Origin	IC_{50} (ng/ml)
HeLa (ATCC CCL 2)[a]	Human, cervix carcinoma	10
K-562 (ATCC CCL 243)[b]	Human, myelogenous leukemia	10
BHK-21 (ATCC CCL 10)[a]	Hamster, kidney	30
CHO (DSM ACC 126)[c]	Hamster, ovary	60
L929 (ATCC CCL 1)[a]	Mouse, connective tissue	30
Vero (ATCC CCL 81)[a]	African green monkey, kidney	20

[a] DULBECCO's modified EAGLE (DME) medium (high glucose; GIBCO).
[b] RPMI 1640 medium (GIBCO).
[c] DME medium (GIBCO) plus thymidine, adenosine and 2'-desoxyadenosin (10 mg/liter); all media containing 10% newborn calf serum.

Fig. 4. Growth of the yeast *Metschnikowia pulcherrima* in the presence and absence of gephyronic acid. After 6 hours, the medium of one culture was exchanged against fresh medium without the inhibitor (indicated by an arrow).

● Gephyronic acid (10 μg/ml), ○ without gephyronic acid.

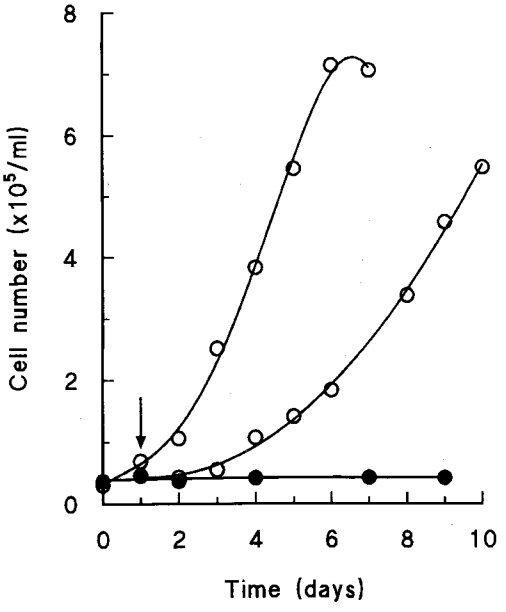


and 10% CO_2. Growth of the yeast and of the human cells stopped immediately, when gephyronic acid was added to the culture medium. But in both cases, the effect was reversible. The cells started to grow soon after the culture medium was exchanged against fresh medium without antibiotic and reached the same cell density as the control, *i.e.*, gephyronic acid had a cytostatic effect on eukaroytic cells.

At gephyronic acid concentrations around the IC_{50} value, the cells of the fibroblast-like mouse cell line L929 (ATCC CCL 1) growing in DME (high glucose) medium + 10% newborn calf serum became elongated

Fig. 5. Growth of K-562 human leukemia cells in the presence and absence of gephyronic acid. After 24 hours the medium of one culture was exchanged against fresh medium without the inhibitor (indicated by an arrow).

● Gephyronic acid (100 ng/ml), ○ without gephyronic acid.



and partly sickle-shaped, and formed a monolayer with the cells partly aligned in parallel. By screening for this characteristic morphological effect, we found that gephyronic acid was apparently present in 19 of the 55 *Archangium* strains tested. When positive extracts were analyzed by HPLC, the antibiotic indeed was found in each case. But so far it has not been detected in any other genus of myxobacteria.

In order to examine the mode of action of gephyronic acid, feeding experiments with radioactive precursors of the main biomacromolecules were done. Fig. 6 shows the incorporation of leucine, uridine, and thymidine by the human leukemic cell line K-562 into TCA insoluble material with and without gephyronic acid. While incorporation of leucine was drastically reduced in the presence of the antibiotic, uridine incorporation was only gradually reduced, and that of thymidine was hardly influenced. These findings suggested that protein synthesis is the primary target of gephyronic acid. Feeding experiments with yeasts supported this assumption.

Therefore the effect of gephyronic acid was examined in two *in vitro* translation systems: in a rabbit reticulocytes lysate (Fig. 7) and in wheat germ extract (both from Boehringer, Mannheim). Leucine or methionine incorporation into the translation product of TMV RNA (Boehringer, Mannheim) was measured in the presence of different concentrations of gephyronic acid and cycloheximide, a known inhibitor of eukaryotic

Fig. 6. Effect of gephyronic acid on the incorporation of radioactively labelled precursors (3.7 kBq/ml) of the main biomacromolecules by K-562 leukemia cells (1.5×10^5/ml).

(a) [*Me*-^{3}H]thymidine (3 TBq/mmol), (b) [5,6-^{3}H]uridine (1.4 TBq/mmol), (c) [*U*-^{14}C]leucine (12 GBq/mmol); ● in presence of gephyronic acid (600 ng/ml), ○ controls without gephyronic acid. All radiochemicals were purchased from Amersham Life Science.

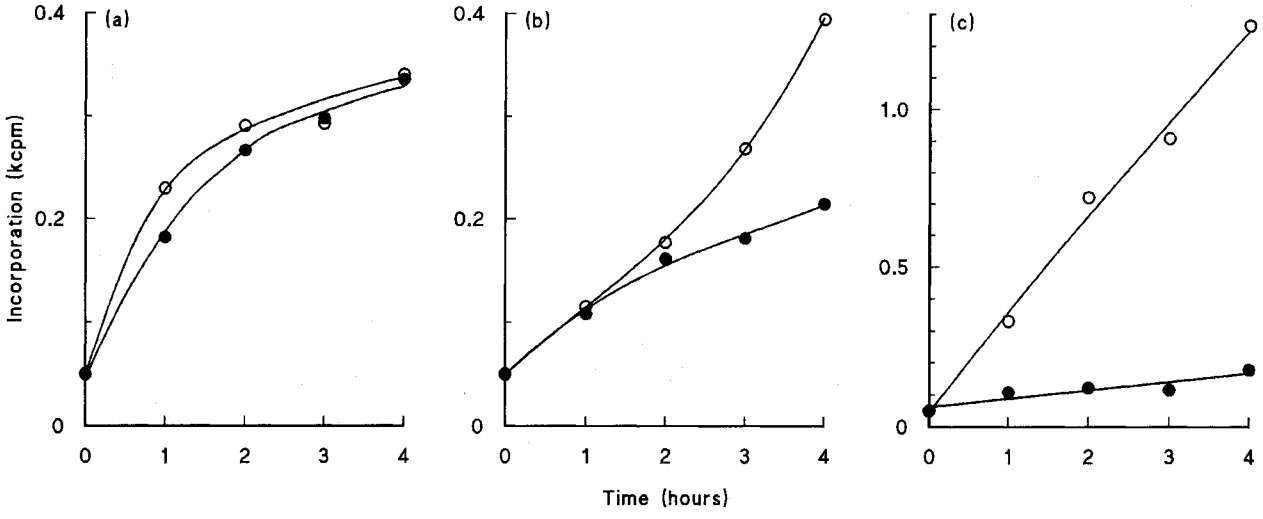
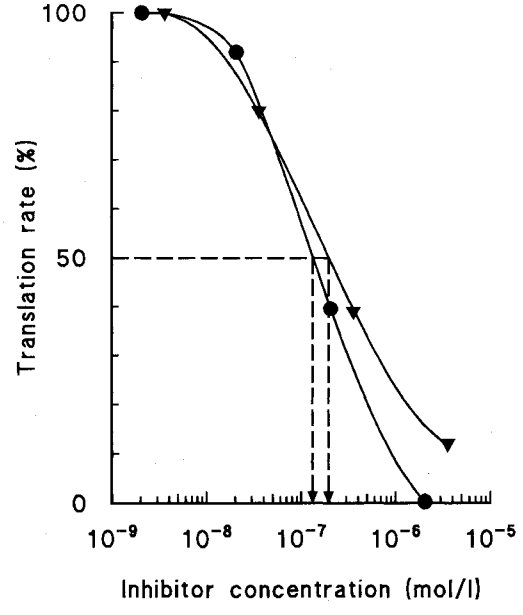


Fig. 7. Effect of gephyronic acid on the *in vitro* translation of TMV RNA in rabbit reticulocytes lysate in comparison with cycloheximide. [^{3}H]Leucine (5.4 TBq/mmol; Amersham) incorporation was measured according to instructions from Boehringer Mannheim.

● Gephyronic acid, ▼ cycloheximide.



protein synthesis. The experiments proved that gephyronic acid inhibited the eukaryotic translation reaction. The IC_{50} values for gephyronic acid derived from the inhibition curves were 1.4×10^{-7} M with the reticulocytes lysate (leucine incorporation) and 1.7×10^{-7} M with the wheat germ extract (methionine incorporation). Nearly identical values were found for cycloheximide.

Discussion

Gephyronic acid is the first antibiotic published from an *Archangium* species. Our screening results suggest that it is widely distributed in this genus, but seems to be restricted to it. Thus, it could be used as a taxonomic marker. Gephyronic acid also is the first antbiotic reported from myxobacteria that specifically inhibits eukaryotic protein synthesis. Inhibitors of the prokaryotic translation system have been reported from different genera of myxobacteria, *viz.*, angiolam[2] from *Angiococcus disciformis*, althiomycin[3] from *Cystobacter fuscus* and myxovalargin[4] and althiomycin[3] from *Myxococcus* strains.

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References

1) STEINMETZ, H.; SASSE, H. REICHENBACH & G. HÖFLE: Gephyronsäure, ein neues Antibiotikum aus *Archangium gephyra*. Liebigs Ann. Chem., in preparation
2) KUNZE, B.; W. KOHL, G. HÖFLE & H. REICHENBACH: Production, isolation, physico-chemical and biological properties of angiolam A, a new antibiotic from *Angiococcus disciformis* (Myxobacterales). J. Antibiotics 38: 1649～1654, 1985
3) KUNZE, B.; H. REICHENBACH, H. AUGUSTINIAK & G. HÖFLE: Isolation and identification of althiomycin from *Cystobacter fuscus* (Myxobacterales). J. Antibiotics 35: 635～636, 1982
4) IRSCHIK, H.; K. GERTH, T. KEMMER, H. STEINMETZ & H. REICHENBACH: The myxovalargins, new peptide antibiotics from *Myxococcus fulvus* (Myxobacterales). I. Cultivation, isolation, and some chemical and biological properties. J. Antibiotics 36: 6～12, 1983