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Tubulysins, New Cytostatic Peptides from Myxobacteria Acting on Microtubuli

Production, Isolation, Physico-chemical and Biological Properties†

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New cytostatic compounds, tubulysins, were isolated from the culture broth of strains of the myxobacteria *Archangium gephyra* and *Angiococcus disciformis*. The compounds are peptides partly consisting of unusual amino acids and are distantly related to the dolastatins. The tubulysins were not active against bacteria and only little against fungi, but showed high cytostatic activity against mammalian cell lines with IC_{50} values in the picomolar range. An incubation with 50 ng/ml tubulysin A led to a complete disappearance of the microtubuli network of the cells within 24 hours. The more active tubulysin D induced multipolar spindles: At 0.5 ng/ml all mitotic cells showed more than four spindle poles.

During our screening for new, biologically active metabolites from myxobacteria, we found in the culture broth of different strains of *Archangium gephyra* activities that were highly cytotoxic for L929 mouse fibroblasts. Bioassay-guided fractionation of culture extracts showed that the activities were due to a group of novel compounds which we called tubulysins. Later, we also found these compounds in certain strains of the genera *Angiococcus*, *Cystobacter*, and *Stigmatella*. In this paper we describe the production, isolation, and the physico-chemical and biological properties of the tubulysins. Fig. 1 shows the structures of the tubulysins isolated so far, the elucidation of which will be published elsewhere[1]. The tubulysins are peptides that contain unusual amino acids. The tubulysins A and B have a hydroxyl group on C-31, in contrast to the tubulysins D and E. The tubulysins are distantly related to the dolastatins, which were isolated from the marine slug, *Dolabella auricularia*[2].

Producing Organisms and Culture Conditions

The producing organism, *Archangium gephyra* strain Ar 315, was isolated in 1973 from a sample of compost from the botanical garden in Freiburg im Breisgau, Germany. *Angiococcus disciformis* strain An d48 was isolated at the GBF in 1994 from a soil sample from Crete, Greece. The strains were normally grown on a 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 M7 liquid medium[4], but glucose and vitamin B_{12} were omitted in the liquid medium for strain An d48. 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 2~5 days.

Production

Both strains were used for production on a larger scale. *Ar. gephyra* strain Ar 315 produced mainly tubulysins A and B, *An. disciformis* An d48 tubulysins D and E. Both strains were grown on the same media mentioned above for batch cultures. For example, a 500-ml culture grown on a shaker was inoculated into 10 liter medium in a 15-liter fermentor with a flat-blade turbine stirrer. After three days

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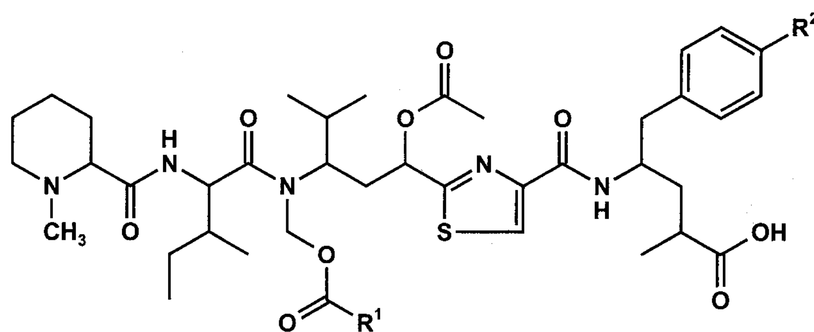
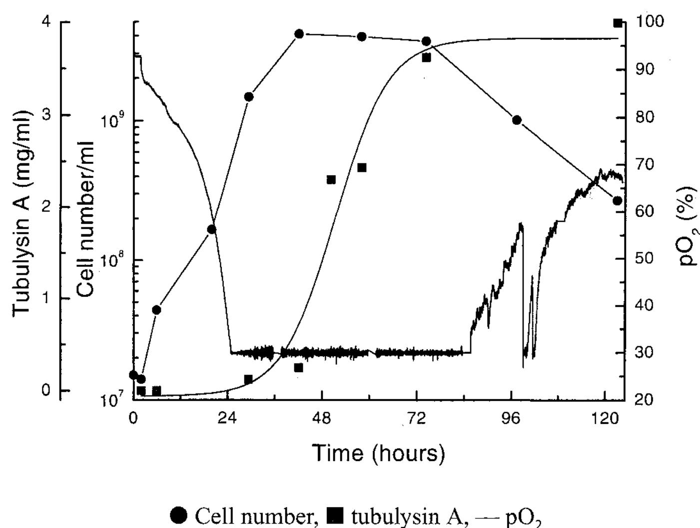
Fig. 1. The structures of tubulysins A, B, D, and E.



Tubulysin A $R^1 = CH_2\text{-}CH(CH_3)_2$; $R^2 = OH$
Tubulysin B $R^1 = CH_2\text{-}CH_2\text{-}CH_3$; $R^2 = OH$
Tubulysin D $R^1 = CH_2\text{-}CH(CH_3)_2$; $R^2 = H$
Tubulysin E $R^1 = CH_2\text{-}CH_2\text{-}CH_3$; $R^2 = H$

Fig. 2. Fermentation of *Archangium gephyra* Ar 315 in a 6-liter bioreactor with 4.7 liter culture volume.



● Cell number, ■ tubulysin A, — pO_2

the content of the seed fermentor was inoculated into a 350-liter bioreactor with 300 liter 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 then 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 μm mesh size. Ar 315 produced up to 4 mg/liter tubulysin A, and An d48 up to 1 mg/liter tubulysin D as main compound. Fig. 2 shows a fermentation of Ar 315 in a 6-liter bioreactor (Fairmentec, Göttingen) with a culture volume of 4.7 liter (M7 medium without HEPES). The initial stirring rate was 200 rpm and was raised when the pO_2 level dropped under

Fig. 3. HPLC profile of tubulysins A, B, D and E, and corresponding UV spectra obtained by diode array detector.

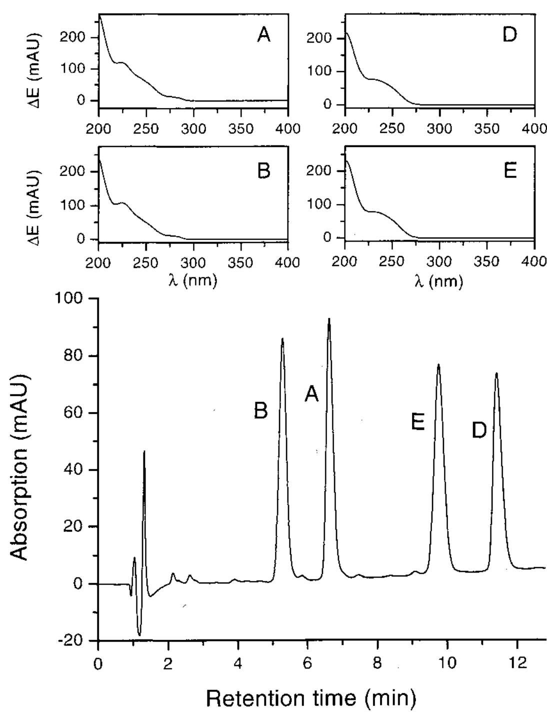
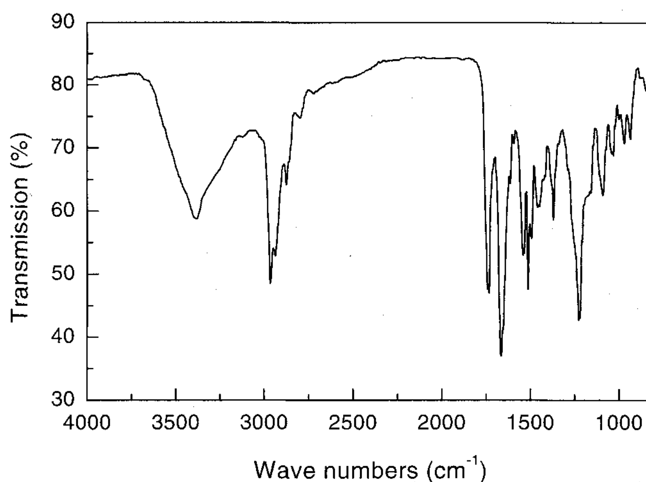


Fig. 4. IR spectrum of tubulysin A in KBr.



30%. The pH was kept at 7.4 by adding 4 M KOH or 1 M H_2SO_4. Tubulysin A was produced during the stationary growth phase and reached 4 mg/liter after 5 days.

Isolation and Quantitative Determination

The tubulysins could be eluted from the adsorber resin with methanol or acetone. The organic solvent was evaporated, and the remaining aqueous phase extracted with ethyl acetate. The organic phase was separated and evaporated again. The resulting crude extract was dissolved in acetone or dichloromethane and fractionated by column chromatography on Sephadex LH20 (solvent: methanol). Active fractions were further purified by medium pressure RP18-chromatography (solvent: methanol/ammonium acetate 0.05 M). Detection was at 285 nm.

The tubulysins A and B could quantitatively be determined by HPTLC (silica gel 60 F-254; solvent: dichloromethane/methanol 9 : 1). The Rf values were 0.27 and 0.25, respectively. Purified fractions could be determined by HPLC (column 125/2, nucleosil 120-5 C-18, Macherey-Nagel, Oensingen, Switzerland; solvent: water/acetonitrile + 0.1% trifluoroacetic acid, gradient from 60/40 to 40/60 in 20 minutes; flow rate: 0.3 ml/minute; detection: diode array). The retention times were as follows: tubulysin A - 6.6 minutes, B - 5.3 minutes, D - 9.8 minutes, and E - 11.4 minutes (Fig. 3).

Physico-chemical Properties

The tubulysins were soluble in methanol, acetone, and ethyl acetate. High resolution EI mass spectroscopy with a Kratos MS 9/50 or a Finnigan MAT 95 gave the following molecular masses (and calculated elemental compositions): tubulysin A - 844.4543 for $[M+H]^+$ ($C_{43}H_{65}N_5O_{10}S$), B - 830.4361 for $[M+H]^+$ ($C_{42}H_{63}N_5O_{10}S$), D - 736.3963 for $[M+H]^+$ ($C_{43}H_{65}N_5O_9S$), and E - 722 for $[M+H]^+$ (ESI-MS, $C_{42}H_{63}N_5O_9S$). The UV spectra of the four tubulysins are shown as inserts in Fig. 3. The IR spectrum of tubulysin A was recorded with a Nicolet 20 DXB FT-IR spectrometer (Fig. 4).

Biological Activity

The antimicrobial activities of the tubulysin A and B were determined by agar diffusion tests. Both tubulysins were completely inactive against bacteria and yeasts, and had only low activity against some filamentous fungi (Table 1). As expected from the screening data, all four tubulysins were highly effective in mammalian cell cultures (Table 2). Growth inhibition of various cell lines was determined in a microtiter-plate assay and compared to dolastatin-10.

Table 1. Antimicrobial activity of tubulysins A and B.

	Test organisms[a]	Diameter of inhibition zone[b] (mm)	
		A	B
Gram-negative bacteria	*Escherichia coli* DSM498	0	0
	Pseudomonas aeruginosa DSM 1117	0	0
Gram-positive bacteria	*Bacillus subtilis* DSM 10	0	0
	Staphylococcus aureus GBF 16	0	0
Yeasts	*Candida albicans* GBF 129	0	0
	Hansenula anomala DSM 70263	0	0
	Metschnikowia pulcherrima DSM 70321	0	0
	Saccharomyces cerevisiae GBF 36	0	0
Filamentous fungi	*Aspergillus niger* DSM 823	20	18
	Botrytis cinerea DSM 877	23	18
	Pythium debaryanum DSM 62946	20	-
	Trichoderma koningii DSM 3121	0	0
	Ustilago zeae DSM 3121	11	-

[a] The organisms were grown on standard agar (1.5 %) or 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 (DSM).

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

Aliquots of 120 μl of the suspended cells (50,000/ml) were given to 60 μl 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 (Boehringer Mannheim, Germany). The IC_{50} values of the tubulysins ranged from 1 ng/ml to 20 pg/ml. Tubulysin A was as active as dolastatin-10, tubulysin D had a 10 times higher activity. In general, tubulysins D and E were approximately by one order of magnitude more toxic than the corresponding hydroxylated compounds A and B. Kinetic studies showed that the propagation of all tested cell lines was stopped immediately after the addition of the tubulysins. The cells did not divide any longer, but they increased clearly in size.

Incorporation experiments with labeled metabolic precursors showed that none of the main metabolic pathways were impaired within 6 hours after the addition of the inhibitors. Investigation of the cytoskeleton of the cultured cells revealed that the tubulysins induced a disruption of the microtubuli. PtK_2 potoroo kidney 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 α-tubulin (1 : 500; Sigma Chemical Co., Deisenhofen, Germany), and then with a secondary goat anti-mouse 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

Table 2. Growth inhibition by tubulysins (A, B, D, E) and dolastatin-10 of different mammalian cell lines.

Cell line	Origin[a]	IC_{50} (ng/ml)				
		A	B	D	E	Dola-statin-10
L-929	murine connective tissue DSM ACC 2	0.2	0.4	0.03	0.1	0.1
PtK_2	*Potorous tridactylis* kidney ATCC CCL-56	0.2	0.2	0.03	0.15	1
KB-3.1	human cervix carcinoma DSM ACC 158	0.2	0.3	0.02	0.03	0.2
KB-V1	multidrug resistant KB clone DSM ACC 149	0.4	1	0.08	0.1	1.2
K-562	human myelogenous leukemia ATCC CCL-243	0.07	0.2	0.02	0.05	0.1

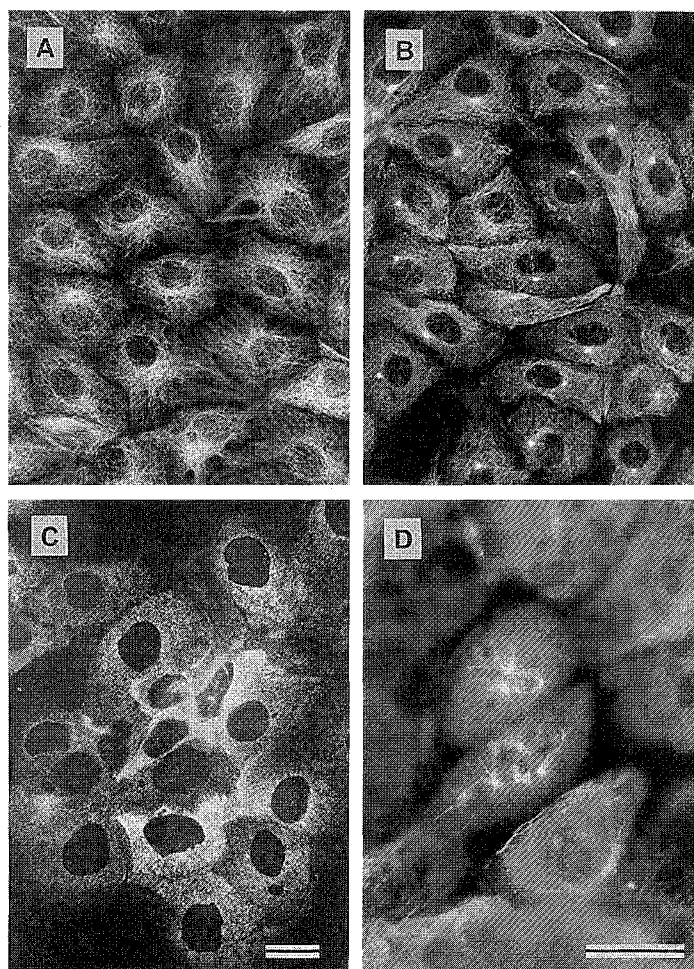
[a] The cell lines were from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) or American Type Culture Collection (ATCC), and cultivated in the media recommended by the supplier plus 10 % newborn calf serum or, in the case of PtK_2 cells, fetal calf serum at 37°C and 10 % CO_2 in a moist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

microscope (Carl Zeiss, Jena, Germany). At a concentration of 50 ng tubulysin A /ml, the microtubuli network was severely impaired after a short incubation time (Fig. 5). The first alteration of the morphology of the tubulin cytoskeleton could be observed after 2 hours. The network appeared to be thinner, and the centrosomes became visible. With longer incubation time the microtubuli continued to disappear. After 8 hours there were only a few short filaments left, while the centrosomes were still visible. After 24 hours the microtubuli had completely gone, and also most of the centrosomes. The immunostained cells showed only a faint diffuse fluorescence. The more active tubulysin D induced the formation of abnormal spindle apparatuses in dividing cells. At a concentration of 0.5 ng/ml all mitotic cells showed more than four spindle poles after an incubation time of two days. The highest number counted were 10 spindle poles per cell. The microtubuli network of interphase cells looked normal at this low concentration.

Discussion

After epothilon[5)], chondramide[6,7)], and rhizopodin[8,9)], the tubulysins are the fourth group of compounds from myxobacteria that act on the cytoskeleton of higher cells. While chondramide and rhizopodin act on actin fibers, epothilon and tubulysin interact with the microtubuli network. Epothilons increase filament formation, tubulysins induce their disruption. A survey for tubulysin producing organisms showed that tubulysins are widespread within the myxobacteria, but restricted to the suborder Cystobacterineae. Tubulysins are new antimitotic drugs, which have structural similarities with the dolastatins found in minute amounts in the marine slug, *Dolabella auricularia*. Published growth inhibition data, *e.g.*, of leukemia cell lines[10)], agree well with our data. They show that dolastatin-10 and tubulysin A are equally active. The dolastatins were taken as lead compounds to develop the potential anticancer drug LU103793[11)], which is produced by chemical synthesis. Tubulysins are new promising

Fig. 5. Influence of tubulysin A (50 ng/ml) and D (0.5 ng/ml) on the microtubuli cytoskeleton of PtK_2 potoroo cells.



The cells were fixed and immunostained for tubulin after different periods of time.

(A) Microtubuli of control cells. (B) Cells incubated with tubulysin A for 4 hours show a less pronounced microtubuli network, the centrosomes become visible. (C) After 24 hours with tubulysin A only a diffuse tubulin fluorescence is left. (D) Tubulysin D induces abnormal, multipolar spindle apparatuses in dividing cells. Scale bars, 20 μm (in C for A~C).

compounds that could be used to develop antitumor drugs.

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