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Zelkovamycin, a New Cyclic Peptide Antibiotic from *Streptomyces* sp. K96-0670

I. Production, Isolation and Biological Properties

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A new antibiotic termed zelkovamycin was isolated from the fermentation broth of *Streptomyces* sp. K96-0670 by solvent extraction, ODS column chromatography and preparative HPLC. Zelkovamycin showed antibacterial activity against *Xanthomonas oryzae*, *Acholeplasma laidlawii*, *Pyricularia oryzae* and *Staphylococcus aureus*.

During our screening for novel bioactive compounds of microbial origin, we have isolated a new antibiotic termed zelkovamycin (Fig. 1), that is produced by *Streptomyces* sp. K96-0670. From comparison of their physicochemical properties, the structure of zelkovamycin appeared similar to that of CP-21.635, which was previously isolated as an antibiotic from the culture broth of *Streptomyces olivaceus* sensu at Pfizer[1]. However, no definite conclusion was obtained as to whether they were identical since the structure of CP-21.635 has not been elucidated. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of zelkovamycin are described. The structure elucidation of zelkovamycin will be described in the accompanying paper[2].

Materials and Methods

General Experimental Procedures

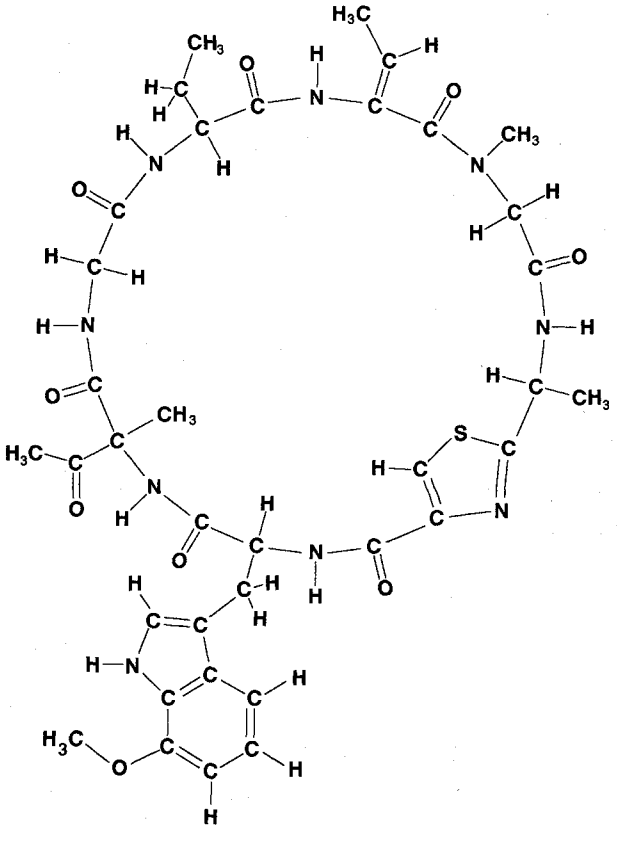
Actinomycete strain K96-0670 was isolated from a soil sample collected at Keyakidaira, Toyama, Japan, and was used for production of zelkovamycin. HPLC was carried out using a JASCO (TRI ROTAR V) system.

Taxonomic Studies

The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL).

The isomer of diaminopimelic acid (DAP) was determined by the method of TAKAHASHI *et al.*[3] Major menaquinones were extracted and purified by the method of COLLINS *et al.*[4] and analyzed by HPLC [column, Capcell Pak C18 SG (4.6 × 150 mm); solvent, methanol-2-propanol (7 : 3); detection, UV at 270 nm; flow rate, 1.0 ml/minute]. To investigate the cultural characteristics

Fig. 1. Structure of zelkovamycin.



and physiological properties, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[5] and media recommended by WAKSMAN[6] were used. Cultures were observed after incubation at 27°C for two weeks. Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago)[7] was used for color names and hue numbers. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[8] containing 1% carbon sources.

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (6 mm, ADVANTEC). Bacteria were grown on Müeller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-hour incubation at 37°C for bacteria and after a 48-hour incubation at 27°C for fungi and yeasts.

The effect of zelkovamycin on the growth of several microoganisms was determined using 96-well microplates[9] and the following media: peptone 0.5% and meat extract 0.5% (pH 7.0) for *Xanthomonas oryzae* and *Staphylococcus aureus*; PPLO broth (Difco) 3.0%, phenol red (5 mg/ml) 0.2%, glucose 0.1%, penicillin 1% and horse serum 15% for *Acholeplasma laidlawii*; and glucose 1.0% and yeast extract 0.5% (pH 6.0) for *Pyricularia oryzae*. Microorganisms were inoculated in each well at about $2 \times 10^4 \sim 1 \times 10^5$ cells/ml and incubated in the presence of the drug ($0 \sim 150\,\mu$g/ml). The growth was measured as OD_{600} (OD_{405} for *A. laidlawii*) after a 24-hour incubation at 27°C for *X. oryzae*, after a 24-hour incubation at 37°C for *A. laidlawii* and *S. aureus*, or after a 48-hour incubation at 27°C for *P. oryzae*.

Results

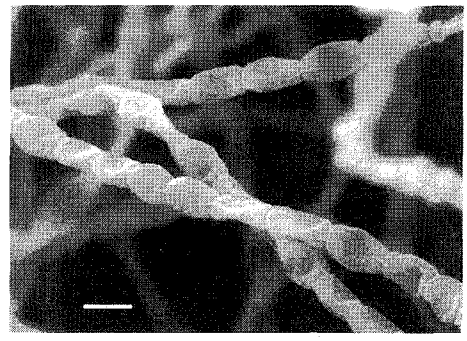
Taxonomy of the Producing Strain K96-0670

Morphological Properties

The vegetative mycelia of strain K96-0670 grew abundantly on both synthetic and complex media and did not show fragmentation into coccoid or bacillary elements. The aerial mycelia grew abundantly on oat meal agar and inorganic salts-starch agar. The spore chains were *Rectiflexibiles* type and each had more than 20 spores per chain. The spores were cylindrical in shape, $1.1 \times 0.7\,\mu$m in size, and had a smooth surface (Fig. 2). Whirls, sclerotic granules, sporangia and flagellated spores were not observed.

Fig. 2. Scanning electron micrograph of spore chains of strain K96-0670 grown on inorganic salts-starch agar for 14 days.

Bar represents 1.0 μm.



Chemical Composition

The DAP isomer in whole cells of the strain was determined to be the LL-type. Major menaquinones were MK-9(H_6).

Cultural Characteristics and Physiological Properties

The cultural characteristics and the physiological properties are shown in Tables 1 and 2. The vegetative mycelia showed beige to brown color on various media. The aerial mass color showed white to gray. Soluble pigment was produced on tryptone-yeast extract broth. The utilization of carbon sources is shown in Table 3.

Based on the taxonomic properties described above, strain K96-0670 is considered to belong to the genus *Streptomyces*[10].

Fermentation

A slant culture of strain K96-0670 grown on Seino agar (starch 1.0%, N-Z amine 0.3%, yeast extract 0.1%, meat extract 0.1%, $CaCO_3$ 0.3%, agar 1.0%, pH 7.0) was used to inoculate a 50-ml test tube containing 10 ml of the seed medium (glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, pH 7.0). The tube was shaken on a reciprocal shaker for 3 days at 27°C. One ml of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the production medium (soluble starch 4.0%, defatted soybean meal 2.0%, $FeSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%, KCl 0.03%, 0.1 N sodium thiosulfate 32 μg/liter, pH 6.5). The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 3. The production of zelkovamycin was measured by HPLC under the following conditions: column,

Table 1. Cultural characteristics of strain K96-0670.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract-malt extract agar[a]	G: Good, clove brown (3ni) R: Dark brown (4nl) AM: Abundant, powder rose, pink tint (6ec, 7ba) SP: Brick red (61/2ne)	Tyrosine agar[a]	G: Good, mustard brown (2ni) R: Covert brown (2nl) AM: Abundant, natural (3dc) SP: None
Oatmeal agar[a]	G: Good, bisque (3ec) R: Light tan (3gc) AM: Abundant, white ~ light gray (a ~ c) SP: Coral (61c)	Sucrose-nitrate agar[b]	G: Moderate, brick red (6ng) R: Dark redwood (61g) AM: Moderate, white (a) SP: None
Inorganic salts-starch agar[a]	G: Good, yellow maple (3ng) R: Adobe brown, beaver (31g, 3li) AM: Abundant, white ~ light gray (a ~ c) SP: None	Glucose-nitrate agar[b]	G: Poor, adobe brown (31g) R: Adobe brown (3lg) AM: Poor, pussywillow gray (5dc) SP: None
Glycerol-asparagine agar[a]	G: Good, chestnut brown (3ni) R: Deep brown (3pl) AM: Abundant, pussywilow gray (5dc) SP: None	Glycerol-calcium malate agar[b]	G: Moderate, adobe brown (31g) R: Yellow maple (3ng) AM: Moderate, white (a) SP: None
Glucose-asparagine agar	G: Good, bisque (4ec) R: Bisque (4ec) AM: Moderate, white (a) SP: None	Glucose-peptone agar[b]	G: Moderate, camel (3ie) R: Camel (3ie) AM: Moderate, white (a) SP: None
Peptone-yeast extract-iron agar[a]	G: Moderate, light mustard tan (2ie) R: Light mustard tan (2ie) AM: None SP: None	Nutrient agar[b]	G: Moderate, bisque (3ec) R: Light wheat (2ea) AM: Moderate, white (a) SP: None

[a] Medium recommended by ISP.
[b] Medium recommended by S. A. WAKSMAN.
Abbreviations: G, growth of vegetative mycelium; R, reverse side color; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of strain K96-0670.

Melanin formation	
Tyrosine agar	−
Peptone-yeast extract-iron agar	−
Tryptone-yeast extract broth	+
Reduction of nitrate	−
Liquefaction of gelatin (21 ~ 23°C)	+
Hydrolysis of starch	+
Coagulation of milk (27°C)	+
Peptonization of milk (27°C)	+
Decomposition of cellulose	−
Temperature range for growth	10 ~ 36°C

+: Positive, −: negative.

Table 3. Utilization of carbon sources by strain K96-0670.

Utilized:	D-Glucose, L-Arabinose, D-Xylose, D-Fructose
Not utilized:	D-Mannitol, *i*-Inositol, Raffinose, Melibiose, Sucrose, L-Rhamnose

PEGASIL ODS (4.6 × 50 mm); solvent, 55% aq CH_3CN; detection, UV at 220 nm; flow rate, 1.0 ml/minute. Under these conditions, zelkovamycin was eluted as a peak with a retention time of 8.0 minutes. The production of zelkovamycin was observed at day 1 after inoculation, and reached a maximum at day 4.

Isolation

The 6-day old whole broth (3 liters) was centrifuged at 3000 rpm for 15 minutes. The supernatant was extracted with ethyl acetate (2.3 liters). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a brown oil (318 mg). The mycelium was extracted with 500 ml of acetone. After centrifugation, the extracts were concentrated to remove acetone, and the remaining aqueous solution was extracted with ethyl

Fig. 3. Time course of zelkovamycin production in a 500-ml Erlenmeyer flask.

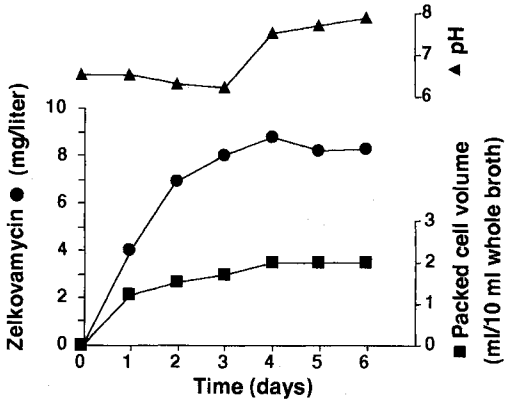
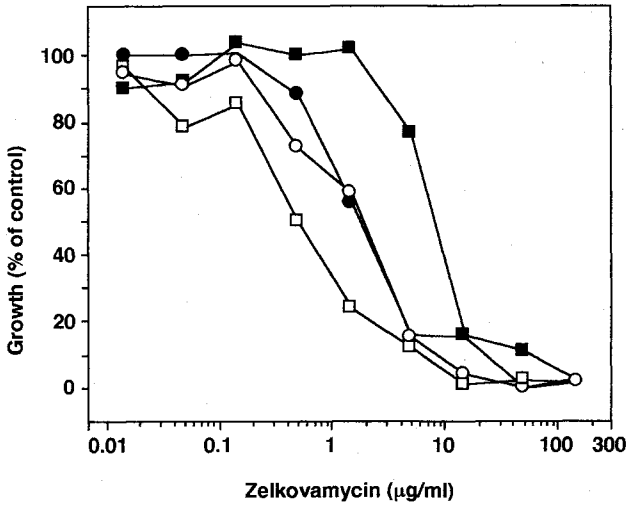


Fig. 4. Inhibition of microorganism growth by zelkovamycin.

X. oryzae (□), *P. oryzae* (○), *S. aureus* (■), and *A. laidlawii* (●) were grown in medium containing zelkovamycin, and growth was plotted as percent of control in the absence of inhibitor.



acetate (500 ml). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a brown oil (173 mg). Both extracts were combined and subjected to an ODS column (Senshu SS 1020T, 25 g). The materials were eluted stepwise with 30%, 45%, 60%, 80% and 100% CH_3CN (50 ml each), and each 5 ml of the elution was successively collected. The 28th to 32nd fractions contained zelkovamycin and were concentrated and extracted with ethyl acetate (300 ml). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a yellow powder (62.7 mg). In the 38th and 39th fractions glucopiericidin A[11] was obtained. Zelkovamycin was finally purified by preparative HPLC [column, YMC pack D-ODS-5 (20 × 250 mm); solvent, 55% aq CH_3CN; detection, UV at 220 nm; flow rate, 6.0 ml/minute]. The peak with a retention time of 13.8 minutes was collected and concentrated to give an aqueous solution that was extracted with ethyl acetate to yield pure zelkovamycin (15.1 mg) as a white powder.

Biological Properties

Antimicrobial Activities

Zelkovamycin showed antimicrobial activity against *X. oryzae* (diameter of inhibition zone: 26 mm), *A. laidlawii* PG8 (19 mm), *S. aureus* FDA 209P (16 mm) and *P. oryzae* (trace inhibition) at a concentration of 1000 μg/ml (10 μg/disk). No antimicrobial activity was observed against other microorganisms: *Micrococcus luteus* PCI 1001, *Bacillus subtilis* PCI 219, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Escherichia coli* NIHJJC-2 IFO 12734, *Pseudomonas aeruginosa* P-3, *Bacteroides fragilis* ATCC 23745, *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus niger* ATCC 6275 and *Mucor racemosus* IFO 4581.

The inhibitory effect on the growth of the four microorganisms was confirmed in a liquid culture using 96-well microplates. The growth of *X. oryzae*, *A. laidlawii*, *P. oryzae* and *S. aureus* was inhibited by zelkovamycin in a dose-dependent fashion (Fig. 4) with IC_{50} values of 0.5, 1.8, 1.9 and 8.0 μg/ml, respectively. The results are comparable to those by the paper disk method.

Discussion

Zelkovamycin and CP 21.635[1] showed very similar physicochemical properties, and were both produced by *Streptomyces* strains. Their antimicrobial activity was compared: CP 21.635 was reported to be active against normal and drug-resistant *S. aureus* and to be inactive against other 12 bacteria tested, while zelkovamycin showed antimicrobial activity against *X. oryzae*, *A. laidlawii*, *S. aureus*, and *P. oryzae*, suggesting a rather unusual spectrum of antimicrobial activity. Anyway, direct comparison would be necessary for identification of the two compounds.

As described in the following paper[2], zelkovamycin is a cyclic peptide containing a thiazole and a modified tryptophan residue. Thiopeptide antibiotics[12~15] having some thiazoles in the cyclic skeleton, such as thiostrepton and nosiheptide, were reported to share a common mode

of action, that is, inhibition of protein synthesis in Gram-positive bacteria[13]. Dolastatin 3, isolated from *Dolabella auricularia*, is also a cyclic peptide containing three thiazoles and showed potent cytotoxic activity against P388 cells[15]. However, the mode of action has not been reported. Antibiotics A21459 A and B, structually related to zelkovamycin in that they are cyclic peptides containing a thiazole and a modified tryptophan residue, were also reported to inhibit bacterial protein synthesis[12]. Therefore, it would be interesting to test whether zelkovamycin inhibits bacterial protein synthesis.

Acknowledgment

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