A New Depsipeptide Antibiotic, Citropeptin

Yoichi HAYAKAWA, Masaya NAKAGAWA, * Yoichiro TODA*
and Haruo SETO

*Institute of Applied Microbiology, University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan*

** Pharmaceutical Laboratory, Kirin Brewery Co., Ltd.,
Miyahara, Takasaki, Gunma 370-12, Japan*

Received October 24, 1989

An actinomycete identified as *Streptomyces flavidovirens* was found to produce a new cyclic hexadepsipeptide antibiotic, designated citropeptin. Citropeptin showed antitumor activity against P388 murine leukemia.

In the course of our screening program for new antitumor antibiotics, culture K3619, identified as a strain of *Streptomyces flavidovirens*, was isolated. This organism was found to produce an antibiotic, designated citropeptin, with potent cytotoxicity. The structure of this antibiotic was elucidated by NMR spectral analysis to be a cyclic hexadepsipeptide closely related to azinothricin¹⁾ and A83586C.²⁾ This paper describes the taxonomy of the producing organism and the fermenta-

tion, isolation, physicochemical properties and biological activity of citropeptin.

Taxonomy

Culture K3619 was isolated from a soil sample collected in Brazil. Characterization of the strain was done mainly by the method described by Shirling and Gottlieb.³⁾

The cultural characteristics of strain K3619 on various media are presented in Table I. The culture grew poorly on defined media such as

Table I. CULTURAL CHARACTERISTICS OF STRAIN K3619

Medium	Vegetative mycelium	Aerial mycelium	Soluble pigments
Sucrose-nitrate agar (Waksman No. 1)	Poor, colorless	Poor, powdery, yellowish white	None
Glucose-asparagine agar (Waksman No. 2)	Poor, pale yellowish brown	Very poor, powdery, white	None
Glycerol-asparagine agar (ISP No. 5)	Poor, pale yellowish brown	None	None
Inorganic salts-starch agar (ISP No. 4)	Moderate, pale yellowish brown	Abundant, powdery, pale yellow to light olive gray	None
Tyrosine agar (ISP No. 7)	Poor, pale yellowish brown	None	None
Nutrient agar	Moderate, yellow	Very poor, powdery, white	None
Yeast extract-malt extract agar (ISP No. 2)	Moderate, pale yellowish brown	Moderate, powdery, pale yellow	None
Oatmeal agar (ISP No. 3)	Moderate, pale yellowish brown	Poor, powdery, yellowish white	None

Waksman media No. 1 and No. 2, and moderately on inorganic salts-starch agar, nutrient agar and yeast extract-malt extract agar. The aerial mass color was white or in the

Yellow color series (yellowish white to pale yellow or light olive gray). The reverse color was pale yellowish brown to yellow. Melanoid pigments or other soluble pigments were not produced.

The aerial mycelium branched monopodially. The spore chains, with 10–50 spores per chain, were very flexuous and occasionally formed imperfect or open spirals (*Retinaculiaperti*). The spores were oval to cylindrical in shape ($0.6\text{--}0.8\text{ }\mu\text{m} \times 0.8\text{--}1.0\text{ }\mu\text{m}$) with a smooth surface (Fig. 1).

The whole cell hydrolysate contained LL-diaminopimelic acid (DAP), but not meso-DAP. These were analyzed as their GITC derivatives by HPLC on a ODS column.⁴⁾

The physiological properties were as follows. Strain K3619 was able to grow at 20°C to 30°C . The culture utilized D-xylose, D-glucose, inositol, D-mannose, melibiose and trehalose, but not L-arabinose, D-fructose, sucrose, L-rhamnose, raffinose or D-ribose. The strain weakly peptonized milk, but did not either hydrolyze starch, liquefy gelatin, coagulate milk or reduce nitrate. Melanoid pigments were not produced on tyrosine agar, peptone-yeast-iron agar or tryptone-yeast-iron agar.

The above morphological and chemical characteristics assign this strain to the genus *Streptomyces*. Comparative study of the characteristics of similar strains in the literature indicates that strain K3619 is most similar to *Streptomyces flavidovirens*. Strain K3619 and *S. flavidovirens* agree well on their spore chain morphology, aerial mass color, reverse color and pigment production, but are different in their utilization of L-arabinose. The reports for *S. flavidovirens* vary on the utilization of several carbon sources,⁵⁾ and it was very difficult to identify the carbon utilization of strain K3619 because of its poor growth on Pridham-Gottlieb medium. Therefore, strain K3619 was identified as *Streptomyces flavidovirens*.^{5,6)}

Fermentation and isolation

Strain K3619 was cultured at 27°C for 3

Fig. 1. Electron Micrographs of Spore Chains of Strain K3619 on Inorganic Salts-Starch Agar, 14 Days (Top: $\times 1,500$, Bottom: $\times 10,000$).

days on a rotary shaker in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of glucose, 0.4%; malt extract, 1%; and yeast extract 0.4% (pH 7.2). The above seed culture (300 ml) was transferred into a 50-l jar fermentor containing 25 l of a medium consisting of glucose, 2.5%; soybean meal, 1.5%; dry yeast, 0.2%; and calcium carbonate 0.4% (pH 7.0). The fermentation was carried out at 27°C for 5 days with aeration (10 l/min) and agitation (200 rpm).

The whole broth (50 l) was filtered through Celite and the mycelial cake was extracted with 25 l of acetone. After evaporation *in vacuo*, the extract was partitioned between 10 l each of ethyl acetate and water. The organic layer was concentrated to dryness, and then chromatographed on a silica gel column. Development of the column (5 × 60 cm) with chloroform-ethanol (50:1) gave an active fraction, which was evaporated to dryness and put on a second silica gel column (4 × 50 cm). The crude active material eluted with chloroform-ethyl acetate

(2:1) was concentrated *in vacuo* and then chromatographed on a Sephadex LH-20 column (2.5 × 50 cm) with methanol. The active eluate was evaporated to dryness and crystallized from ethanol to yield 270 mg of colorless needles of citropeptin.

Physicochemical properties

mp 109–111°C. *Anal.* Found: C, 58.11; H, 7.88; N, 10.77; O, 23.00%. Calcd. for $C_{50}H_{82}N_8O_{15}$: C, 58.01; H, 7.98; N, 10.82; O, 23.18%. FD-MS m/z 1035 ($M+H$), 1057 ($M+Na$). IR ($CHCl_3$) cm^{-1} 3400, 2950, 1730, 1665, 1640, 1495, 1460, 1390, 1315, 1280, 1120, 1005. UV λ_{max} (MeOH) nm (ϵ) 230 (sh 14,000). $[\alpha]_D^{25} + 113^\circ$ (c 0.5, $CHCl_3$). The 500 MHz 1H NMR spectrum as shown in Fig. 2 was obtained in $CDCl_3$ solution at 27°C on a JEOL GX-500 spectrometer.

The structure of citropeptin as shown in Fig. 3, except for the stereochemistry, was elucidated by NMR spectral analysis including a variety of two-dimensional techniques. The

Fig. 2. 1H NMR Spectrum of Citropeptin (500 MHz, $CDCl_3$).

Fig. 3. Structures of Citropeptin, Azinothricin and A83586C.

structural study will be reported elsewhere.

Biological activity

Citropeptin was very cytotoxic with IC_{50} s of 0.02 μ g/ml and 0.1 μ g/ml against murine P388 leukemia cells and B16 melanoma cells, respectively, which were incubated at 5×10^4 cells/ml with various concentrations of citrop-

peptin in RPMI1640 medium supplemented with 10% fetal calf serum for 2 days.

CDF₁ mice were inoculated intraperitoneally with 10^6 P388 leukemia cells and treated intraperitoneally with citropeptin on days 1 and 5. Citropeptin prolonged the survival periods of mice bearing P388 (T/C 123%) at doses of 2 mg/kg/day, but caused toxic

death at 4 mg/kg/day.

References

- 1) H. Maehr, C. Liu, N. J. Palleroni, J. Smallheer, L. Todaro, T. H. Williams and J. F. Blount, *J. Antibiot.*, **39**, 17 (1986).
 - 2) T. A. Smitka, J. B. Deeter, A. H. Hunt, F. P. Mertz, R. M. Ellis, L. B. Boeck and R. C. Yao, *J. Antibiot.*, **41**, 726 (1988).
 - 3) E. B. Shirling and D. Gottlieb, *Int. J. Syst. Bacteriol.*, **16**, 313 (1966).
 - 4) T. Yamaguchi, *J. Bacteriol.*, **89**, 444 (1965).
 - 5) E. B. Shirling and D. Gottlieb, *Int. J. Syst. Bacteriol.*, **19**, 391 (1969).
 - 6) R. Locci, "Bergey's Manual of Systematic Bacteriology," Vol. 4, ed. by S. T. Williams, Williams & Wilkins Co., Baltimore, M.D., 1989.
-