ISOLATION AND CHARACTERIZATION OF TERPENTECIN, A NEW ANTITUMOR ANTIBIOTIC

Tsuyoshi Tamamura, Tsutomu Sawa, Kunio Isshiki, Tōru Masuda, Yoshiko Homma, Hironobu Iinuma, Hiroshi Naganawa, Masa Hamada, Tomio Takeuchi and Hamao Umezawa

Institute of Microbial Chemistry
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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A new antitumor antibiotic, terpentecin was isolated from the culture broth of strain MF730-N6. Strain MF730-N6, isolated from soil, was found to belong to the genus *Kitasatosporia*. The antibiotic was extracted with chloroform, purified by column chromatography using silica gel and Diaion HP-20 successively, and finally purified by high performance reverse-phase thin layer chromatography. The molecular formula of terpentecin was determined to be $C_{20}H_{28}O_6$ (molecular weight, 364). The antibiotic inhibited the growth of Gram-positive and Gram-negative bacteria, and prolonged the survival period of mice bearing leukemia L-1210, P388 and Ehrlich ascites carcinoma.

We found a new antibiotic in a culture filtrate of strain MF730-N6 which was isolated from a soil sample collected at Nara Prefecture, Japan. Taxonomic features of this strain were identical with those of *Kitasatosporia*. As shown by the chemical structure, the antibiotic is a diterpenoid[1]. We named the antibiotic terpentecin. Terpentecin is most related to the known antibiotic clerocidin (PR-1350)[2,3] which is produced by a fungus, *Oidiodendron truncatum*. Terpentecin inhibits leukemia L-1210, P388 and Ehrlich ascites carcinoma *in vivo*. It also inhibits the growth of Gram-positive and Gram-negative bacteria. In this paper we report taxonomy of the producing strain and fermentation, purification and properties of terpentecin.

Taxonomy of Terpentecin Producing Organism

The media usually used for cultural and physiological characterizations of actinomycetes[4,5] were employed. Culture characteristics were observed after incubation at 27°C for 3 weeks. Utilization of carbon sources was examined by testing the growth and production of aerial mycelia on Pridham and Gottlieb's media containing various carbon sources (1%) at 27°C. Chemical analysis of sugars and amino acids contained in whole cells and cell walls was carried out by the method of Lechevalier and Lechevalier[6]. Analysis of GC-content was made by the method of Ulitzur[7].

Long aerial mycelia developed from the substrate mycelium. The structure was *Rectus-Flexibilis*. Mature spores were cylindrical and measured $0.4\sim0.6\times0.8\sim1.4$ μm. The spore surfaces were smooth with fine wrinkles as shown in Fig. 1. Substrate mycelia in agar media were not fragmented. Submerged spores were observed by shake culture in yeast extract - dextrose medium. Cultural characteristics in various media are given in Table 1. Aerial mycelia produced on yeast extract - malt extract agar, oatmeal agar or inorganic salts - starch agar were gray. Pale yellowish brown soluble pigment was produced in inorganic salts - starch agar. Physiological characteristics and utilization of carbon sources are shown in Tables 2 and 3.

The cell wall of the strain MF730-N6 contained LL-diaminopimelic acid and *meso*-diaminopimelic

Table 1. Cultural characteristics of strain MF730-N6.

Medium	Cultural characteristics
Yeast extract - malt extract agar	G: Pale yellowish brown A: Light brownish gray P: None
Oatmeal agar	G: Colorless A: Light brownish gray P: None
Inorganic salts - starch agar	G: Colorless ~ pale yellowish brown A: Light brownish gray P: None ~ pale yellowish brown
Glycerol - asparagine agar	G: Colorless A: Light brownish gray P: None
Sucrose - nitrate agar	G: Colorless A: Light brownish gray P: None
Nutrient agar	G: Colorless A: None P: None
Tyrosine agar	G: Colorless A: Light brownish gray P: None
Glucose - asparagine agar	G: Pale yellowish brown A: Light brownish gray ~ brownish gray P: None
Calcium - malate agar	G: Colorless A: None ~ scant, white P: None

Abbreviation: G; Growth of substrate mycelium, A; aerial mycelium, P; soluble pigment.

Fig. 1. Scanning electron micrograph of strain MF730-N6 on inorganic salts - starch agar medium (×7,800).

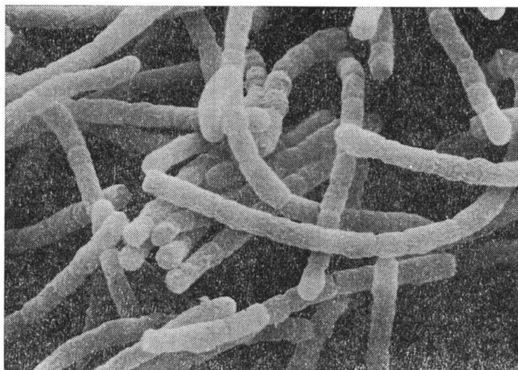


Table 2. Physiological characteristics of strain MF730-N6.

Melanin formation	Negative
Nitrate reduction	Negative
Liquefaction of gelatin	Positive
Hydrolysis of starch	Positive
Coagulation of milk	Positive
Peptonization of milk	Positive
Temperature for growth	20 ~ 37°C
Optimal temperature for growth	27 ~ 30°C

Table 3. Utilization of carbon sources by strain MF730-N6.

Carbon source	Utilization
L-Arabinose	+
D-Xylose	+
Glucose	+
Sucrose	−
D-Fructose	−
Rhamnose	−
Raffinose	−*
D-Mannitol	−

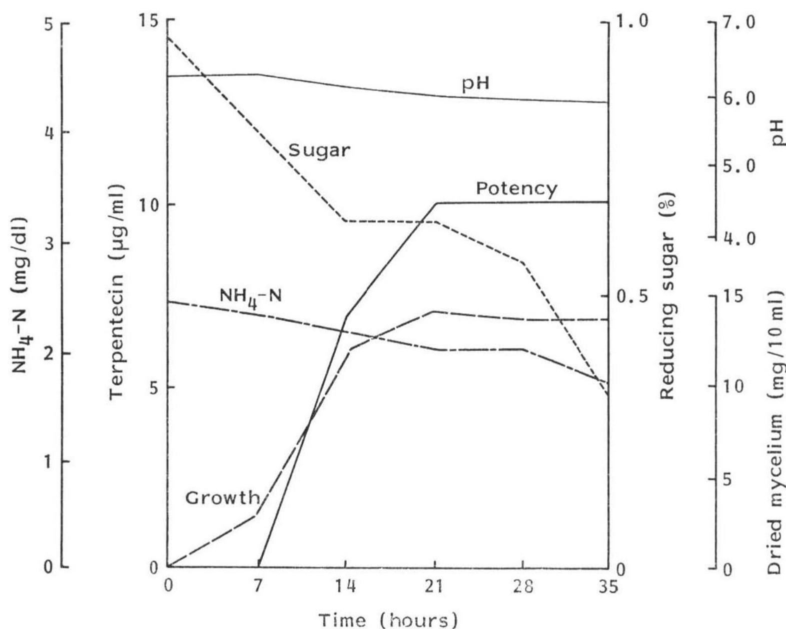
* Probably negative.

acid in almost equal molar ratio. Galactose and glycine were also contained in the cell wall. The aerial mycelia contained LL-diaminopimelic acid and the substrate mycelia contained its *meso*-form[8]. The GC-content was determined to be 70.8%. These characteristics indicated that the strain belonged to a genus of actinomycetes, *Kitasatosporia*[9]. The taxonomic comparison with *Kitasatosporia setae*[10], *K. griseola*[11] and strain MF730-N6 is in progress and will be published elsewhere.

Fermentation

The strain MF730-N6, grown on an agar medium, was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium composed of glucose 1%, yeast extract 1%. The medium was adjusted to pH 7.2 before sterilization. The organism was cultured at 27°C for 24 hours on a rotary shaker. This culture (250 ml) was inoculated into a 30-liter fermentor containing 15 liters of a medium

Fig. 2. Time course of terpentecin production.



consisting of glucose 0.04%, galactose 0.08%, maltose 0.08%, dextrin 0.16%, Bacto-Soytone (Difco Lab.) 0.08%, $(NH_4)_2SO_4$ 0.03% (pH 7.0). Fermentation was carried out for 35 hours at 27°C with agitation (150 rpm) and aeration (15 liters/minute). The antibiotic produced in the broth was determined by a cylinder plate method, using *Klebsiella pneumoniae* PCI 602 as the test organism. The time course of a typical fermentation is shown in Fig. 2.

Isolation of Terpentecin

Terpentecin in the broth filtrate (12.5 liters, pH 5.8) was extracted with an equal volume of chloroform. The active extract was concentrated to dryness under reduced pressure (950 mg). The dried material was dissolved in a small volume of chloroform, charged to a silica gel column (Wako Pure Chemical Ind., Wakogel C-200, 50 g), and eluted with chloroform - methanol (100 : 3). The active fractions were collected and evaporated to give a reddish syrup (363 mg). The syrup was dissolved in a small amount of methanol and applied on a column of non-ionic porous resin (Mitsubishi Chemical Ind. Ltd., Diaion HP-20, 10 ml). After washing with 50% aqueous methanol, terpentecin was eluted with linear gradient of methanol (50~100%) to give a crude powder (66.2 mg). The powder was spotted onto a high performance reverse-phase thin layer plate (Merck, RP-18, Art. 13724) and developed with acetonitrile - water (7 : 3). The active band was eluted with methanol to give pure terpentecin (24.1 mg).

Physico-chemical Properties of Terpentecin

Terpentecin was obtained as a colorless powder, which melted at 115~119°C with decomposition. The optical rotation $[\alpha]_D^{26}$ was −28.5° (*c* 0.46, $CHCl_3$). It was soluble in methanol, acetone, ethyl acetate, benzene and insoluble in water and hexane. The color reactions are as follows; positive in 2,4-dinitrophenylhydrazine, 2,3,5-triphenyltetrazolium chloride, Fehling and silver nitrate-ammonia

Fig. 3. IR spectrum of terpentecin (KBr).

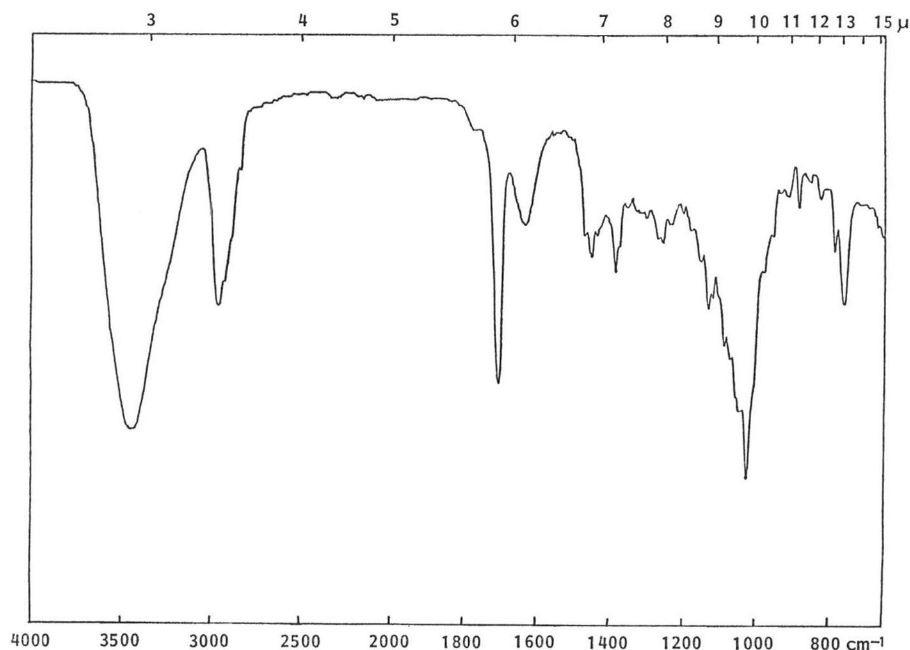
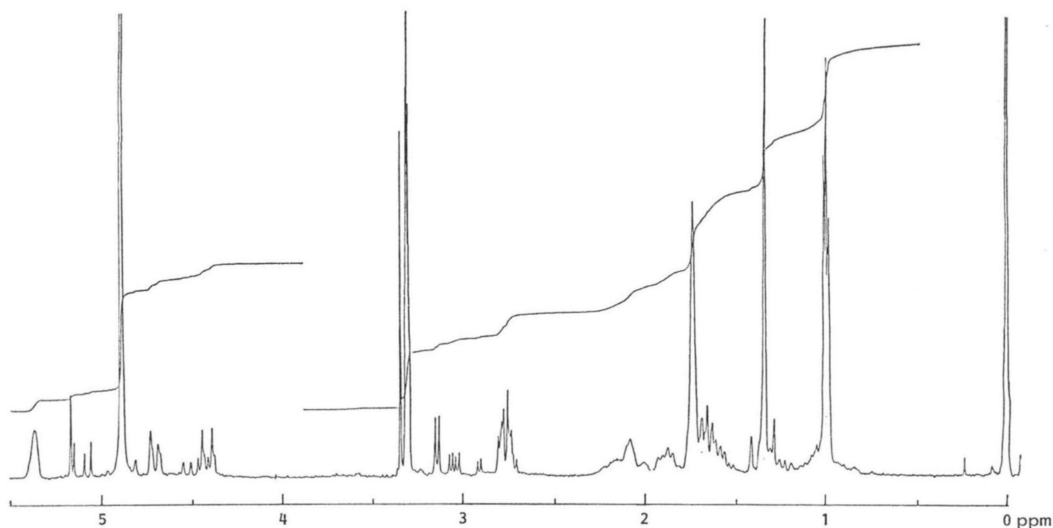


Fig. 4. ^{1}H NMR spectrum of terpentecin in d_4-methanol (250 MHz).



and negative in ferric chloride. Rf value of thin-layer chromatography of terpentecin was 0.5 (Merck silica gel plate F_{254} Art. 5715, solvent; $CHCl_3$ - MeOH, 10 : 1). Field desorption mass spectrometry of terpentecin gave a peak at m/z 365 $(M+H)^+$ and the secondary ion mass spectrometry also gave a peak at m/z 365 $(M+H)^+$. UV absorption showed maxima in methanol at 203 nm ($E^{1\%}_{1cm}$ 60) and 280 nm ($E^{1\%}_{1cm}$ 3). In the IR spectrum shown in Fig. 3, characteristic absorptions attributable to hydroxyl and carbonyl groups were observed at 3400 and 1700 cm^{-1}, respectively. The ^{1}H NMR spectrum (in d_4-methanol, 250 MHz) is shown in Fig. 4. The spectrum was complicated by the tautomeric

Table 4. Antitumor activity of terpentecin against mouse leukemia L-1210, P388 and Ehrlich ascites carcinoma (EAC).

Dose (μg/mouse/day)	T/C (%)*		
	L-1210	P388	EAC
125	161	—	77
62.5	161	>175	188
31.25	170	155	>354
15.6	200	146	>369
7.8	160	139	215

* Prolongation rate (T/C, %)=mean survival period of treated/mean survival period of controls.

Table 5. Antimicrobial activity of terpentecin.

Test organism	Medium	MIC (μg/ml)
Staphylococcus aureus FDA 209P	1	6.25
S. aureus Smith	1	<0.05
Micrococcus flavus FDA 16	1	0.39
Bacillus subtilis PCI 219	1	<0.05
B. subtilis NRRL B-558	1	0.1
B. cereus ATCC 10702	1	0.1
Corynebacterium bovis 1810	1	<0.05
Escherichia coli NIHJ	1	25
E. coli K-12	1	3.12
Shigella dysenteriae JS 11910	1	<0.05
S. sonnei JS 11746	1	12.5
Salmonella enteritidis 1891	1	0.1
Proteus vulgaris OX19	1	12.5
Serratia marcescens	1	50
Pseudomonas aeruginosa A3	1	25
Klebsiella pneumoniae PCI 602	1	0.39
Mycobacterium smegmatis ATCC 607	1	0.39
Aeromonas salmonicida ATCC 14174	1*	0.05
Vibrio anguillarum NCBM 6	1*	0.05
Candida albicans 3147	2	100
Aspergillus niger	2	>100

Medium 1: Nutrient agar.
2: Nutrient agar + glucose 1%.
37°C, 17 hours.
* 27°C, 17 hours.

nature of this substance[1]. The molecular formula $C_{20}H_{28}O_6$ which was determined by mass spectrometry is shown in the next paper[1].

Biological Properties of Terpentecin

The antitumor activity of terpentecin was examined in mice. Mouse leukemia L-1210, P388 and Ehrlich ascites carcinoma were inplanted intraperitoneally in mice; the inoculum size was 1×10^5, 1×10^5 and 2×10^6 cells per mouse, respectively. After twenty-four hours, varied doses of terpentecin were administered to mice intraperitoneally ten times at one day intervals. The results are shown in Table 4. Terpentecin exhibited a therapeutic effect on mouse leukemia L-1210, P388 and Ehrlich ascites carcinoma. The concentrations of terpentecin inhibiting the growth of microorganisms were tested by a serial two-fold agar dilution method. Results are shown in Table 5. Terpentecin inhibits the growth of both Gram-positive and Gram-negative bacteria. It shows strong inhibition against *Staphylococcus aureus* Smith, *Bacillus subtilis* PCI 219, *Corynebacterium bovis* 1810, *Shigella dysenteriae* JS 11910, *Aeromonas salmonicida* ATCC 14174, *Vibrio anguillarum* NCBM 6. LD_{50} (ip) of terpentecin in mice was 100 mg/kg.

Discussion

As will be reported in the next paper[1], terpentecin is a diterpenoid compound closely related to LL-S491β and -γ[12], aphidicolin[13] and clerocidin (PR-1350)[2,3]. Although these antibiotics are structurally related, only terpentecin is produced by actinomycetes. The others are produced by fungi. The antimicrobial spectrum and antitumor activities suggest that terpentecin has stronger antibiotic activities than the other antibiotics.

Acknowledgments

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