STUDIES ON PENTENOMYCINS. I

PRODUCTION, ISOLATION AND PROPERTIES OF PENTENOMYCINS I AND II, NEW ANTIBIOTICS FROM *STREPTOMYCES EURYTHERMUS* MCRL 0738

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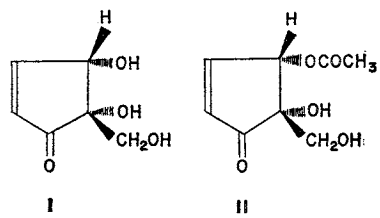
Two new antibiotics, pentenomycins I and II have been isolated from the culture broth of *Streptomyces eurythermus* MCRL 0738 as a white hygroscopic amorphous powder. Pentenomycins [I: $C_6H_8O_4 \cdot 1/2\,H_2O$ (**I**), II: $C_8H_{10}O_5 \cdot 1/2\,H_2O$ (**II**)] are moderately active against Gram-positive and Gram-negative bacteria.

During the course of our screening program for new antibiotics, two chemically-related substances which were moderately active against Gram-positive and Gram-negative bacteria were isolated from the culture broth of a streptomycetes. The antibiotics isolated were named pentenomycins I and II based on their chemical structures.

As a result of a taxonomic study, the pentenomycin-producing strain MCRL 0738 was identified as *Streptomyces eurythermus*, CORBAZ, ETTLINGER, GÄUMANN, KELLER-SCHIERLEIN, NEIPP, PRELOG, REUSSER and ZÄHNER, 1955[1], and the type strain has been deposited in the Fermentation Research Institute, Chiba, Japan and accessioned as FERM-P No. 1110.



The present paper deals with the producing strain, production, isolation and the physico-chemical and biological properties of pentenomycins I and II. Structures of pentenomycins I and II were determined as 4,5-dihydroxy-5-hydroxymethyl-(4 : S, 5 : S)-cyclopent-2-en-1-one (**I**) and 4-acetoxy-5-hydroxy-5-hydroxymethyl-(4 : S, 5 : S)-cyclopent-2-en-1-one (**II**) respectively, as will be reported in the succeeding papers[2,3].

Description of the Producing Strain

Taxonomic studies were generally carried out in accordance with the methods adopted by the International Streptomyces Project (ISP)[4] using the media prepared according to the recommendations of SHIRLING-GOTTLIEB[5] and WAKSMAN[6].

1. Morphological characteristics

On observation with a microscope, the aerial mycelia showed the presence of coils or hooks (Section *Retinaculiaperti*) on yeast extract-malt extract agar, oatmeal agar and glycerol-asparagine agar. Spiral formation was observed only on inorganic salts-starch agar instead of coils or hooks.

Not less than ten oval spores were formed in chains and the surface of the spores was smooth.

2. Cultural characteristics

Strain MCRL 0738 was aerobic and showed good growth at 20~40°C.

The cultural characteristics observed on various media for 3 weeks are shown in Table 1. Color description and the number in parentheses accord to "Color Harmony Manual"[7].

3. Utilization of carbon sources in PRIDHAM and GOTTLIEB's agar[8]

Arabinose, xylose, glucose, fructose, sucrose, inositol, rhamnose, raffinose, mannitol and starch were utilized.

4. Physiological characteristics

Starch hydrolysis (ISP medium No. 4), milk coagulation and peptonization (Difco 10 % skimmed milk), gelatin liquefaction (20 % gelatin), calcium malate solubilization, tyrosinase reaction (ISP medium No. 7 and Waksman medium No. 42), hydrogen sulfide production (Difco peptone iron agar containing 0.1 % yeast extract) and serum liquefaction (Difco LOEFFLER blood serum) were all positive, while cellulase reaction (CZAPEK's solution with a strip of filter paper as the sole carbon source) was negative.

Considering the above morphological and physiological properties, strain MCRL 0738 seems to be related to the previously reported *S. eurythermus*[1,4] which produces angolamycin.

Accordingly, the microbiological characteristics of strain MCRL 0738 were directly compared with those of *S. eurythermus* strain ISP-5014 kindly provided by the Institute for Fermentation, Osaka, and it was found that strain MCRL 0738 differs from strain ISP-5014 only in the following cultural characteristics: on sucrose-nitrate agar strain ISP-5014 form adobe brown (5pl) vagetative growth, chestnut brown (4ni) reverse growth and produces a adobe brown (5pl) soluble pigment. In spite of these minor differences, strains MCRL 0738 and ISP-5014 showed numerous similarities in other properties, including morphology, physiology and carbon utilization pattern. Thus, the pentenomycin-producing strain (MCRL 0738) was identified as *Streptomyces eurythermus*.

Production of the Antibiotics

The antibiotic-producing strain grows well in shaken culture or aerated submerged culture at 27°C. The fermentation medium for seed and production was as follows: 1.5 % soybean meal,

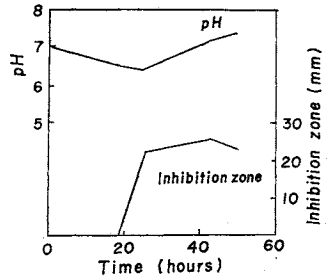
Table 1. Cultural characteristics of strain MCRL 0738

Culture medium	Vegetative growth	Mycelium reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar (WAKSMAN medium No. 1 at 27°C)	Colorless transparent	Colorless	Powdery, silver gray (3fe)	None
Glucose-asparagine agar (WAKSMAN No. 2 medium at 27°C)	Light amber (3ic)	Bamboo (2fb)	Cottony, silver gray (3fe)	Dark luggage tan (4pg)
Glycerol-asparagine agar (ISP medium No. 5, at 27°C)	Chestnut brown (4ni)	Chestnut brown (4ni)	Covert gray (2fe)	Amber (3nc)
Glycerol calcium-malate agar (WAKSMAN medium No. 7, at 27°C)	Ivory (2db)	Ivory (2db)	None	None
Inorganic salt-starch agar (ISP medium No. 4, at 27°C)	Chestnut brown (4ni)	Golden brown (3pg)	Beige (3ge)	Cinnamon (31e)
Tyrosine agar (ISP medium No. 7, at 27°C)	Ebony brown (8pn)	Ebony brown (8pn)	Powdery, white to natural (2dc)	Chocolate brown (4pn)
Yeast extract-malt extract agar (ISP medium No. 2, at 27°C)	Oak brown (4pi)	Dark spice brown (4pl)	Covery gray (2fe)	Cinnamon (31e)
Oatmeal agar (ISP medium No. 3, at 27°C)	Covert brown (3pl)	Golden brown	Covert gray (2fe)	Adobe brown (31g)*
Nutrient agar (WAKSMAN medium No. 14, at 37°C)	Gold flate (21e)	Light wheat (2ea)	Powdery, natural (2dc)	Cinnamon (31e)

* This pigment is not pH indicator.

1.0 % glucose, 1.0 % starch, 1.0 % glycerine, 0.3 % NaCl and 0.1 % $CaCO_3$ (pH 7.0). Five hundred ml shake flasks containing 100 ml of the media were inoculated from an agar slant and harvested to prepare a vegetative inoculum on a reciprocating shaker (180 strockes/min., 7 cm amplitude) at 27°C for 48 hours. Then, the vegetative inoculum (500 ml) was transferred to 30-liter jar fermentor containing 15 liters of the above medium and fermentation was carried out aerobically (aeration: 8 liters/min., pressure: 0.5 kg/cm²) under stirring (400 r.p.m.) at 26~28°C. Antibiotic production was assayed by a cup plate method using *Staphylococcus aureus* Terashima as a test organism. Maximum production was usually obtained after 42 hours of fermentation. A typical time course of the fermentation is shown in Fig. 1.

Fig. 1. A typical time course of antibiotic production*

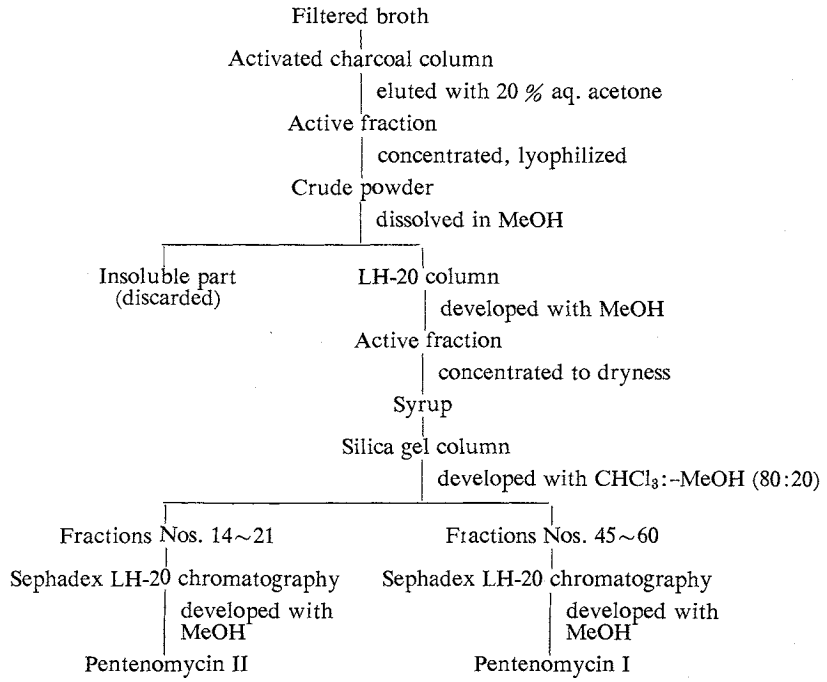


* Antibiotic production was assayed by a cup plate method using *Staph. aureus* Terashima.

Isolation of Pentenomycins

Pentenomycins were recovered from the filtered broth by the procedure outlined in Chart 1. After 42 hours of cultivation, the harvested broth was filtered with the aid of 3 % Celite-545 and the mycelial cake discarded. The antibiotics in the filtrate (30 liters) were adsorbed on an activated charcoal column (activated charcoal for chromatography: Wako Junyaku, 1.5 kg; column 20 cm × 30 cm) at a flow rate of 100 ml/min.

Chart 1. Isolation and purification of pentenomycins



The column was washed with deionized water and eluted with 20 % aqueous acetone at a flow rate of 50 ml/min. The biologically-active eluate (5.2 liters) was concentrated *in vacuo* under 40°C and the concentrate was further lyophilized to yield a crude powder (120 g). The crude powder was

dissolved in methanol (300 ml) and the methanol solution was concentrated to 60 ml. The concentrate was poured onto a Sephadex LH-20 column (4.5 cm×180 cm) and the column was developed with methanol at a flow rate of 60 ml/hour at 20°C. The biologically-active fractions were concentrated to dryness to give a colorless syrup (11 g). The syrup (10 g) thus obtained was chromatographed on silica gel column (Mallinckrodt silicic acid AR, 100 Mesh, 4 cm×62 cm), using a chloroform-methanol mixture (85 : 15) as a developing solvent. Each eluting fraction (20 ml) was assayed by the paper disc method against *S. aureus* Terashima and by spot test with silver nitrate reagent. The biologically-active and silver nitrate positive fractions were then monitered by thin-layer chromotography using silica gel (Kiesel gel G, Merck) with a solvent system of chloroform-methanol (80 : 20). Pentenomycin I (Rf 0.35) was found in fractions Nos. 45~60, while pentenomycin II (Rf 0.82) was eluted in the forerunning fractions Nos. 14~21.

The pooled eluates of each antibiotic were concentrated *in vacuo* to yield pentenomycins I and II respectively as a colorless syrup (I, 5.3 g; II, 2.1 g). I was further purified by chromatography on Sephadex LH-20 column (3.5 cm×120 cm) using methanol as a developing solvent. The biologically-active eluates were combined and the combined eluate was concentrated *in vacuo* to give pure pentenomycin I (3.2 g) as an amorphous white powder.

Purified pentenomycin II (1.1 g) was obtained by a method similar to that described for pentenomycin I.

Physico-chemical Properties of the Pentenomycins

Pentenomycins I and II, obtained by the above process, are white amorphous powders which did not give sharp melting points because of their strong hygroscopic nature. However, the homogeniety of each antibiotic was demonstrated by thin-layer chromatography. The antibiotics were visualized as dark brown spots after spraying with a silver nitrate solution followed by 5 % sodium hydroxide solution. Rf values are shown in Table 2. Some of physico-chemical properties of pentenomycins I and II are summarized in Table 2.

As shown in Fig. 2, the IR spectrum of pentenomycin I shows absorption bands at 3400 cm^{-1},

Fig. 2. Infrared spectra of pentenomycins (nujol)

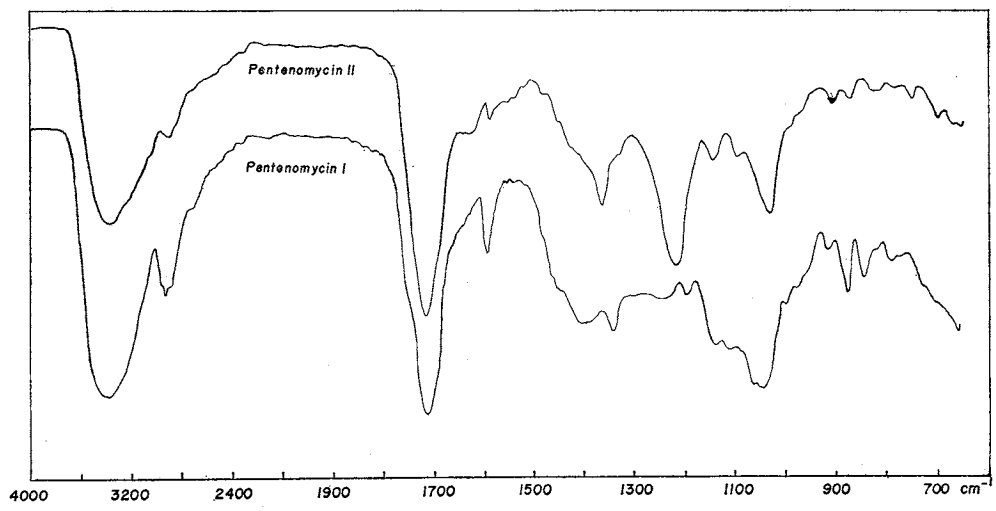


Table 2. Physicochemical properties of pentenomycins

	Pentinomycin I	Pentenomycin II
Molecular formula	$C_6H_8O_4 \cdot 1/2\ H_2O$	$C_8H_{10}O_5 \cdot 1/2\ H_2O$
Elementary analysis	Anal. calcd: C 47.07, H 5.92 Found: C 47.99, H 5.98	Anal. calcd: C 49.22, H 5.68 Found: C 49.71, H 5.69
Optical rotation	$[\alpha]_D^{21}$ −32° (*c* 0.3, EtOH)	$[\alpha]_D^{28}$ −55° (*c* 1.45, MeOH)
Ultraviolet absorption	$\lambda_{max}^{H_2O}$ 216 nm ($E_{1cm}^{1\%}$ 260)	$\lambda_{max}^{H_2O}$ 216 nm ($E_{1cm}^{1\%}$ 271)
Rf* Benzene-EtOH (7 : 3)	0.35	0.51
Chloroform-MeOH (9 : 1)	0.10	0.18
Color reaction		
FEHLING	positive	positive
TOLLENS	positive	positive
Ninhydrin	negative	negative
Ferric chloride	negative	negative

* TLC on Silica gel G (Merck)

1709 cm^{-1} and 1950 cm^{-1}, indicating the presence of hydroxyl, carbonyl and vinyl groups.

The NMR spectrum of pentenomycin I in deuterium oxide at 60 MHz is illustrated in Fig. 3. The spectrum suggested the presence of methylene protons (δ=3.7, 2H, singlet) and vinyl protons (δ=7.8 and 6.4 each 1 H, multiplets). The IR spectrum of pentenomycin II was illustrated in Fig. 2. The NMR spectrum (Fig. 4) of pentenomycin II indicated an acetoxy methyl signal at δ=2.17 in addition to the singnals shown by I. Pentenomycin I was soluble in water, methanol, ethanol, acetic acid, pyridine and dioxane, slightly soluble in chloroform and practically insoluble in ethyl acetate, benzene, ether, petroleum ether, ligroin and *n*-hexane. Pentenomycin II showed similar solubilities as pentenomycin I, except the former was much more soluble in chloroform and ethyl acetate. Judging from their physico-chemical properties, the antibiotics could be differentiated from other known antibiotics and afterward this conclusion was supported by the structural studies which will be reported in succeeding papers[2,3] Based on their cyclopentenone structure, the names pentenomycins I and II were proposed to these new antibiotics.

Biological Properties of Pentenomycins

The antimicrobial activities of pentenomycins I and II are summarized in Table 3. Both antibiotics are moderately active against Gram-positive and Gram-negative bacteria.

Mice survived more than 24 days without any symptom after intravenous administration of 400 mg/kg of I and 150 mg/kg of II, respectively. Administration of pentenomycin I (50 mg/kg, i.p., mice) showed protective effect against *S. aureus* Smith.

Fig. 3. NMR spectrum (60 MHz) of pentenomycin I in D_2O solution

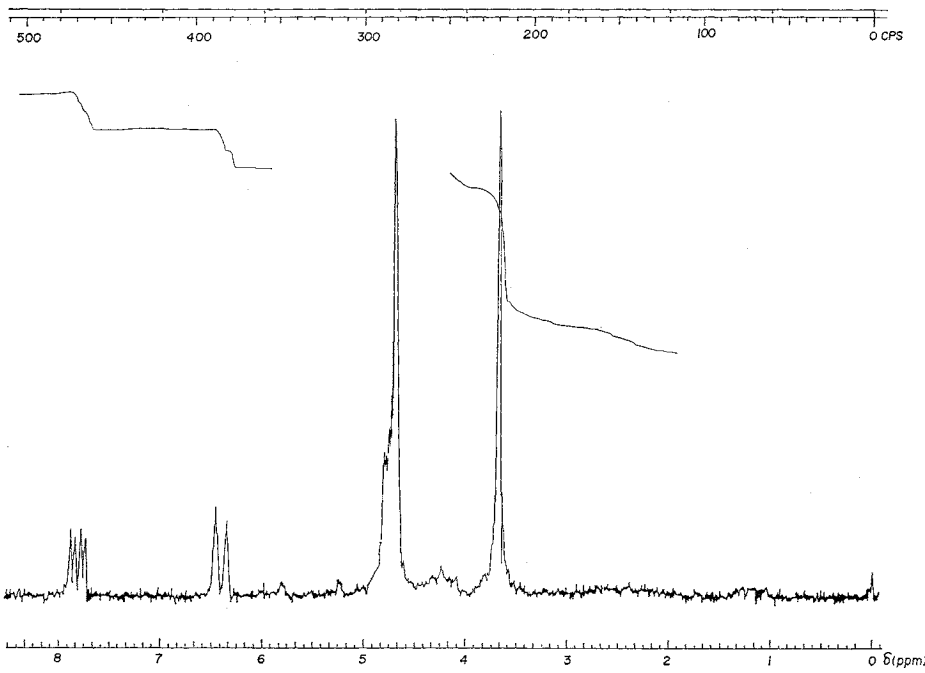


Fig. 4. NMR spectrum (60 MHz) of pentenomycin II in D_2O solution

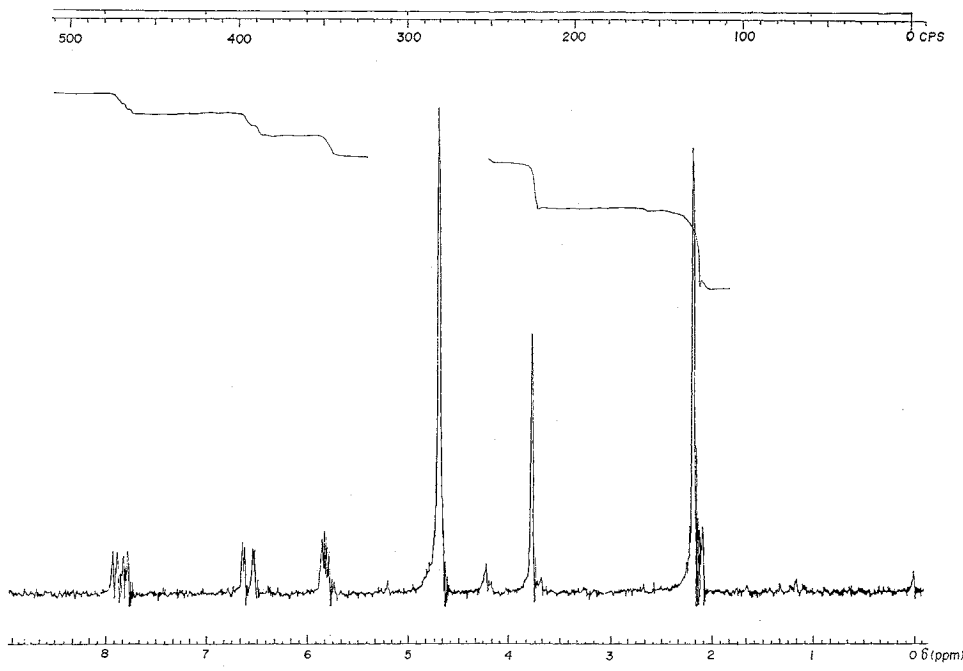


Table 3. Antimicrobial spectrum of pentenomycins I and II (serial dilution method)

Test organisms	Medium	M.I.C. (mcg/ml)	
		I	II
Staphylococcus aureus FDA 209P	I	125	250
Staphylococcus aureus TERASHIMA	I	125	125
Staphylococcus aureus SMITH	I	250	250
Streptococcus hemolyticus 9-76	II	125	125
Diplococcus pneumoniae	II	125	125
Corynebacterium diphtheriae PARK-WILLIAMS No. 8	II	31.2	62.5
Bordetella pertussis TOHAMA	II	31.2	31.2
Neisseria meningitidis 69480	II	62.5	62.5
Neisseria gonorrhoeae YOSHIOKA	II	15.6	31.2
Bacillus subtilis PCI 219	I	>500	250
Escherichia coli NIHJ JC-2	I	500	250
Shigella dysenteriae	I	250	125
Shigella flexneri 2a	I	>500	62.5
Salmonella typhosa T-58	I	500	250
Proteus vulgaris	I	62.5	62.5
Klebsiella pneumoniae	I	>500	500
Pseudomonas aeruginosa 1095	I	500	250
Mycobacterium tuberculosis $H_{37}Rv$	III	31.2	62.5

Medium: I. Heart infusion agar (Difco).
II. Heart infusion agar (Difco) supplemented with 10 % horse serum.
III. KIRCHNER's liquid medium containing 10 % horse serum.

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