MUREIDOMYCINS A~D, NOVEL PEPTIDYLNUCLEOSIDE ANTIBIOTICS WITH SPHEROPLAST FORMING ACTIVITY

I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

MASATOSHI INUKAI, FUJIO ISONO, SHUJI TAKAHASHI, RYUZO ENOKITA, YOSHIHARU SAKAIDA and TATSUO HANEISHI†

Fermentation Research Laboratories, Sankyo Co., Ltd., Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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A strain of actinomycetes identified as *Streptomyces flavidovirens* produced new antibiotics, mureidomycins (MRD's) A~D, specifically active against *Pseudomonas aeruginosa*. They were isolated from the culture filtrate by successive column chromatographies such as Amberlite XAD-2 and CG-50, Whatman DE-52 and Toyopearl HW-40. They were amphoteric white powders and soluble in methanol and water. Their molecular weights and molecular formulae in parentheses were 840 ($C_{38}H_{48}N_8O_{12}S$), 842 ($C_{38}H_{50}N_8O_{12}S$), 897 ($C_{40}H_{51}N_9O_{13}S$) and 899 ($C_{40}H_{53}N_9O_{13}S$), respectively. *m*-Tyrosine and two unknown substances were detected by amino acid analyses as their common constituents. MRD's A and C contained uracil but MRD's B and D dihydrouracil instead of uracil.

In the course of our screening program for new antibiotics with spheroplast forming activity, one strain†† of actinomycete, identified as *Streptomyces flavidovirens* SANK 60486, was found to produce new antibiotics with specific activity against *Pseudomonas aeruginosa*.

In this paper, we report the taxonomy of the producing organism, the fermentation, isolation and physico-chemical properties of mureidomycins (MRD's) A~D[1,2]. The structural elucidation and biological properties of the antibiotics will be described in the accompanying papers[3,4].

Materials and Methods

Taxonomic Studies

The producing organism, strain SANK 60486, was isolated from a soil sample collected at Miyake Island, Tokyo, Japan.

Methods adopted by WAKSMAN[5] and by the International Streptomyces Project (ISP)[6] were used for the studies of morphological and taxonomic characterizations, carbohydrate utilization and taxonomic identification. The procedure of BECKER *et al.*[7] was used for the preparation of cells and chromatographic detection of the isomers of diaminopimelic acid.

Fermentation

A loopful amount of the culture of strain SANK 60486 was inoculated into a 500-ml Erlenmeyer flask which contained 80 ml of the medium consisting of glucose 3%, pressed yeast 1%, soybean meal 3%, $CaCO_3$ 0.4%, $MgSO_4 \cdot 7H_2O$ 0.2% and Nissan Disfoam CB-442 0.01%. The inoculated flasks were incubated on a rotary shaker (220 rpm) at 22°C for 48 hours. Then a 2-ml aliquot of the culture was transferred into 2-liter flask containing 500 ml of the same medium. After 24 hours of incubation on a rotary shaker, 750 ml of this second seed culture was added to a 30-liter jar fermentor con-

† Present address: Technical Licensing Department, Sankyo Co., Ltd., Ginza, Chuo-ku, Tokyo 104, Japan.

†† FERM BP-1347 and FERM P-8636.

taining 15 liters of the medium. Fermentation was carried out at 22°C for 96 hours with an air-flow rate of 15 liters/minute and an agitation rate of 150 rpm. Antibiotic activity was determined by a paper-disc agar diffusion assay using *P. aeruginosa* SANK 70579 as the test organism. Packed cell volume was determined after centrifugation of 5 g culture broth at 3,000 rpm for 10 minutes.

Isolation of MRD's A~D

Thirty liters of the fermentation broth were filtered with the aid of Celite 545 and the cake was washed with water. Thirty liters of the filtrate thus obtained were adsorbed on a column of Amberlite XAD-2 (3 liters) and after washing with 15 liters of water and 12 liters of 15% aqueous methanol succesively, the antibiotics were eluted with 15 liters of 40% aqueous methanol. The active eluate was concentrated *in vacuo* and lyophilized to obtain 17.4 g of the crude powder. A 17-g aliquot of this crude powder was dissolved in 3 liters of distilled water and adsorbed on a column of Amberlite CG-50 (H^+ type, 800 ml). The active fractions eluted with 0.5 N NH_4OH were collected and concentrated to 1 liter under reduced pressure. The concentrate was adsorbed on a column of Whatman DE-52 (1 liter) pre-equilibrated with 0.1 M NH_4HCO_3 and eluted with 0.2 M NH_4HCO_3. The active fractions (800 ml) were collected and adsorbed on a column containing 200 ml of Diaion HP-20 resin, from which the active fraction was eluted with 500 ml of 50% aqueous acetone. The eluate was concentrated under reduced pressure and lyophilized to afford 1.6 g of the crude powder containing MRD's A~D. For separation of each component of MRD's, 1.5 g of this crude powder was dissolved in 200 ml of distilled water, adsorbed on a column of Whatman DE-52 (500 ml), pre-equilibrated with 0.05 M NH_4HCO_3, and then eluted with 0.1 M NH_4HCO_3 to give fractions each containing 20 ml of the eluate. Two fractions No. 25 to 60 and No. 80 to 130 in each were collected, desalted by Diaion HP-20 resin, concentrated and lyophilized to give 510 mg of the crude powder mainly containing MRD's B and D, and 309 mg of mainly MRD's A and C, respectively. For further separation into each component of MRD's A and C, and MRD's B and D, these crude powders, 300 mg (MRD's A and C) and 500 mg (MRD's B and D) each, were then subjected to silica gel column chromatography (100 g), respectively. The active fractions were eluted with a mixture of butanol, propanol and water (8 : 4 : 1), and separated into the fractions each containing 20 ml of the eluate. From fractions No. 13 to 36 and No. 56 to 75 of the first column and No. 37 to 55 and No. 76 to 110 of the second, partially purified single components MRD's A~D were obtained after concentration and lyophilization, respectively. Each component was then dissolved in 30% aqueous methanol, loaded on a column of 1,000 ml of Toyopearl HW-40 and developed with 30% aqueous methanol. The active eluates were collected, adsorbed on a column of 10 ml of Amberlite CG-50 (H^+) resin and eluted with 0.5 M aqueous ammonia. After concentration and lyophilization, 24, 45, 49 and 40 mg of MRD's A~D were obtained, respectively. In the course of purification, activity was monitored by HPLC, in which Aquasil SS 372-N (Senshu Kagaku Co.) was developed with a mixture of chloroform, 2-propanol, methanol and water (200 : 100 : 100 : 40) at a flow rate of 1 ml/minute. MRD's A~D were eluted at 3.92, 3.94, 6.29 and 7.24 minutes after injection, respectively.

Results and Discussion

Taxonomy of Strain SANK 60486

The vegetative hyphae of the microorganism generally branched on various agar media. The strain formed straight or flexible sporophores in monopodial branching on the aerial mycelium (Plate 1). Mature spore chains were generally long, with 10 to 50 or more spores per chain. The spores were ellipsoidal, 0.5~0.8×0.7~1.1 μm in diameter with smooth surface (Plate 2). No special morphological organs such as whirls, sclerotia, sporangia were observed on the media employed. The cultural characteristics on various agar media at 28°C for 14 days are shown in Table 1. The color of the vegetative mycelium was pale yellowish brown to yellowish gray. The strain formed an abundant amount of yellowish gray powdery aerial mycelium.

The strain grew within the temperature range of 6 to 34°C. Hydrolysis of starch and liquefaction of gelatin were positive. Other physiological properties are shown in Table 2. Strain SANK 60486 utilized D-glucose, L-arabinose, D-xylose, D-mannose, D-fructose and L-rhamnose but not inositol, sucrose and raffinose.

Detection of LL-diaminopimelic acid and glycine in the whole-cell hydrolysate of the culture indicated that this strain had Type I cell wall. Based on the taxonomic properties described above, the strain SANK 60486 is considered a member of the genus *Streptomyces*. By comparison of the description of strain SANK 60486 with those of the *Streptomyces* species described previously[8~12] and

Plate 1. Vegetative hyphae of strain SANK 60486 cultured for 14 days on water agar medium at 28°C.

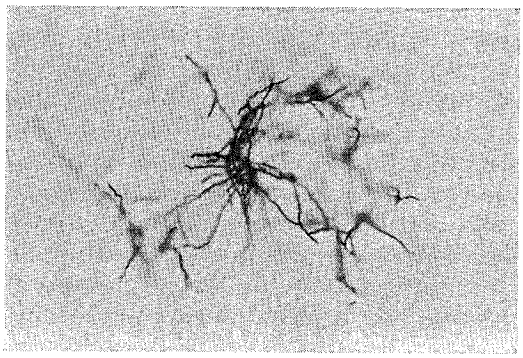

Plate 2. Spore chains of strain SANK 60486 cultured for 14 days on water agar medium at 28°C. Bar represents 5 μm.

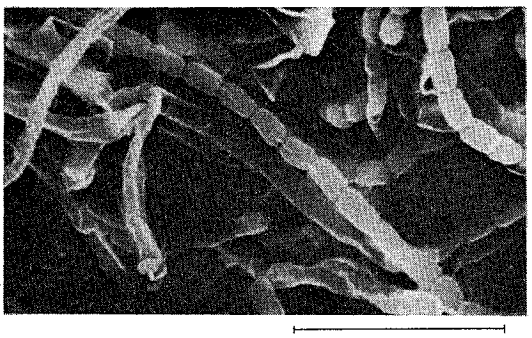

Table 1. Cultural characteristics of strain SANK 60486.

Culture medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Sucrose - nitrate agar	Limited	Good, powdery yellowish gray	Pale yellowish orange	None
Glucose - asparagine agar	Good	Good, powdery pale yellowish orange	Yellowish brown	None
Glycerol - asparagine agar (ISP 5)	Good	Plenty, powdery pale yellowish brown	Yellowish brown	None
Starch - inorganic salt agar (ISP 4)	Very good	Plenty, powdery pale yellowish orange	Pale yellowish orange	None
Tyrosine agar (ISP 7)	Very good	Plenty, powdery brownish white	Yellowish brown	None
Peptone - yeast extract - iron agar (ISP 6)	Very good	Slightly formed white	Pale yellowish brown	None
Nutrient agar (Difco)	Very good	Good, powdery white	Pale yellowish orange	None
Yeast germ - wheat agar (ISP 2)	Very good	Plenty, powdery yellowish gray	Yellowish brown	None
Oatmeal agar (ISP 3)	Good	Plenty, powdery yellowish orange	Pale yellowish brown	Pale yellowish brown
Water agar	Poor	Poor, powdery white	Pale yellowish orange	None
Potato extract - carrot extract agar	Poor	Good, powdery pale yellowish orange	Pale yellowish orange	None

Table 2. Physiological properties of strain SANK 60486.

Hydrolysis of starch	Positive
Liquefaction of gelatin	Positive
Reduction of nitrate salt	Positive
Coagulation of milk	Positive
Peptonization of milk	Positive
Temperature range of growth (medium 1)	6~34°C
Sodium chloride resistance (medium 1) 7%	Growth
10%	No growth
Decomposition of casein	Positive
Decomposition of tyrosine	Positive
Decomposition of xanthine	Negative
Productivity of melanin-like pigment	
Medium 2	Negative
Medium 3	Negative
Medium 4	Negative

Medium 1: Yeast germ - wheat agar (ISP 2), medium 2: Tryptone - yeast extract broth (ISP 1), medium 3: peptone - yeast extract - iron agar (ISP 6), medium 4: tyrosine agar (ISP 7).

by direct comparison with *S. flavidovirens* ATCC 19900 as the most related one, the strain was identified as *S. flavidovirens* and designated *S. flavidovirens* SANK 60486.

Fig. 1. Fermentation of strain SANK 60486.
○ Potency, □ pH, △ packed cell volume (PCV).

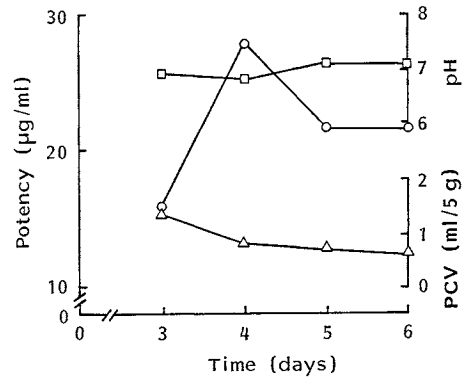

Table 3. Physico-chemical properties of MRD's A~D.

	A	B	C	D
Appearance	Amphoteric white powder	Amphoteric white powder	Amphoteric white powder	Amphoteric white powder
Molecular formula	$C_{38}H_{48}N_8O_{12}S$	$C_{38}H_{50}N_8O_{12}S$	$C_{40}H_{51}N_9O_{13}S$	$C_{40}H_{53}N_9O_{13}S$
High-MS Calcd (QM^+)	841.318855	843.334505	898.340300	900.35595
Found (QM^+)	841.31798	843.33289	898.33687	900.35617
Anal Calcd:	C 49.99,	C 49.88,	C 49.53,	C 49.43,
(As tetrahydrate)	H 6.18,	H 6.39,	H 6.13,	H 6.33,
	N 12.27,	N 12.25,	N 13.00,	N 12.97,
	S 3.51	S 3.50	S 3.31	S 3.30
Found:	C 49.73,	C 50.67,	C 49.44,	C 48.79,
	H 5.65,	H 6.36,	H 5.50,	H 5.86,
	N 12.08,	N 12.62,	N 12.53,	N 12.42,
	S 3.40	S 3.13	S 3.09	S 3.26
$[\alpha]_D^{23}$ in 70% aq MeOH	+40.9° (*c* 0.69)	−7° (*c* 0.3)	+16.7° (*c* 0.57)	−30° (*c* 0.52)
UV λ_{max} nm ($E_{1cm}^{1\%}$)				
pH 7.0	260 (348)	255 (194)	258 (292)	255 (191)
pH 2.0	258 (358)	255 (186)	259 (312)	255 (184)
pH 9.0	240 (499),	245 (325),	240 (444),	245 (346),
	265 (sh, 330),	295 (sh, 85)	265 (sh, 276),	295 (sh, 90)
	295 (sh, 78)		295 (sh, 72)	
Solubility				
Soluble:	Water, MeOH	Water, MeOH	Water, MeOH	Water, MeOH
Insoluble:	EtOAc, $CHCl_3$, benzene	EtOAc, $CHCl_3$, benzene	EtOAc, $CHCl_3$, benzene	EtOAc, $CHCl_3$, benzene
Color reaction	Ninhydrin, H_2SO_4, iodine, ferric chloride, Baeyer reaction	Ninhydrin, H_2SO_4, iodine, ferric chloride, Baeyer reaction	Ninhydrin, H_2SO_4, iodine, ferric chloride, Baeyer reaction	Ninhydrin, H_2SO_4, iodine, ferric chloride, Baeyer reaction

Fermentation of Strain SANK 60486

The time course of fermentation of MRD's in a 30-liter jar fermentor is shown in Fig. 1. Production of the antibiotics reached to the maximum level (60 μg/ml estimated as MRD A) at 96 hours after inoculation. Among four components, MRD's A and C were produced as the main components of the complex.

Physico-chemical Properties of MRD's A~D

The physico-chemical properties of MRD's A~D are summarized in Table 3. They are amphoteric white powders, and soluble in water and methanol. Their molecular weights and molecular formulae (in parenthesis) were determined to be 840 ($C_{38}H_{48}N_8O_{12}S$), 842 ($C_{38}H_{50}N_8O_{12}S$), 897 ($C_{40}H_{51}N_9O_{13}S$) and 899 ($C_{40}H_{53}N_9O_{13}S$) by mass spectrometry and elemental analysis, respectively. MRD's A and C contained a uracil moiety, and MRD's B and D contained dihydrouracil instead of uracil. *meta*-Tyrosine and two unknown substances were detected as common constituents in all MRD's and glycine was only in MRD's C and D. The differences of their molecular weights were exactly accounted for by these differences of the constituents.

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