A NOVEL MACROMOLECULAR ANTIBIOTIC, SN-07

TAXONOMY OF PRODUCING ORGANISM, ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

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A novel macromolecular antibiotic SN-07 was obtained from the cultural supernatant of *Actinomadura roseoviolacea* var. *miuraensis* nov. var. The antibiotic was soluble in water, had a molecular weight of 18,000~22,000 daltons in 50 mM Tris-HCl buffer, pH 7.0, containing 2.0 M KCl as compared with authentic proteins. Its major constituents were nucleic acids. The substance had antibacterial activity against Gram-positive bacteria. It was also effective against lymphocytic leukemia P388 *in vivo*.

Among antibiotics that have antitumor activity, some are macromolecules[1]. Most of them are polypeptides such as neocarzinostatin[2], auromomycin[3], macromomycin[4], and largomycin[5]. Recently, it has been reported that a mixture of high molecular weight single and double stranded DNA extracted from *Clostridium saccharoperbutylacetonicum* cells represents an antitumor activity against Sarcoma-180[6]. We here have found a novel macromolecular antibiotic whose major constituents were nucleic acids. This report describes the producing organism, isolation and initial characterization of antibiotic SN-07.

Taxonomic Studies

Most of the studies of strain 07 were carried out according to the methods of the International Streptomyces Project[7]. Additional media recommended by WAKSMAN[8] were also used. The chemical analyses of sugars and amino acids in cell wall hydrolysates were carried out as reported by BECKER *et al.*[9] and LECHEVALIER and LECHEVALIER[10]. Strain 07 was isolated from a soil sample collected in Miura-city, Kanagawa, Japan. When strain 07 was grown on an oatmeal agar medium, aerial mycelia were well developed, long, straight to wavy and monopodially branched. The aerial mass color was pink. Substrate mycelia were orange, well developed and branched. Scanning electron microscopy revealed that the spore chains formed tightly closed, spirals with 1 to 2 turns. Pseudosporangia were also formed. The spores had a smooth surface, and they were cylindrical to ovoidal with a size of 0.4~0.6×0.6~1.0 μm. No sporangium, sclerotium and flagellum were observed (Figs. 1A and 1B).

Growth characteristics on different media are summarized in Table 1. Yeast extracts or vitamin B complex was essential for growth. The strains were grown at 32°C for 14 to 21 days. Physiological characteristics are given in Table 2. The cell wall composition indicated that strain 07 was a species of the cell wall type III and distinct from genus *Streptomyces* or *Nocardia*.

From the taxonomic studies described above, strain 07 was classified in the genus *Actinomadura*.

Fig. 1. Scanning electron micrographs of the strain 07 on oatmeal agar.

A) Spores B) Spores chains

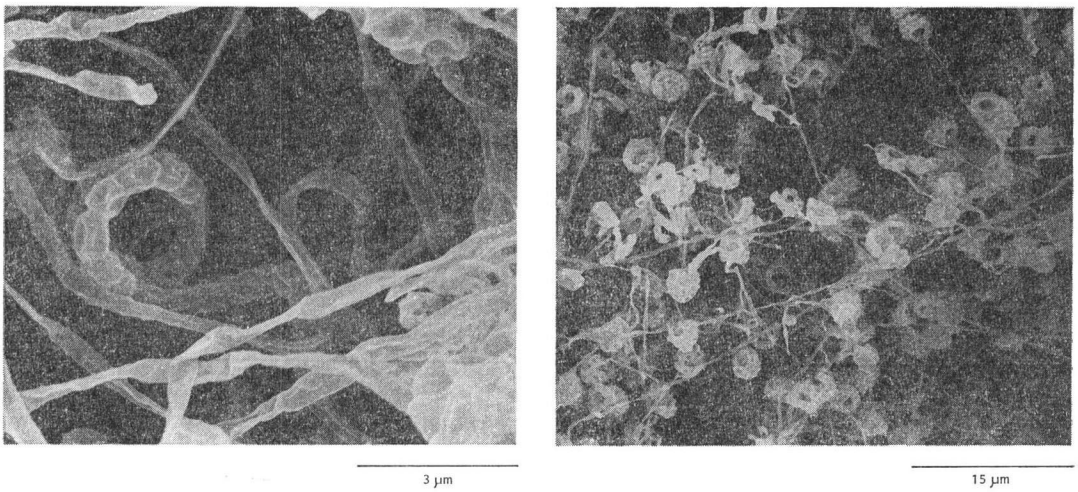


Table 1. Growth characteristics of strain 07.

Medium	Growth and reverse color	Aerial mycelium	Soluble pigment
Sucrose - nitrate agar*	Good, light brownish orange (10R, 7/8)	Poor, pale pink (2.5R, 9/2)	None
Glucose - asparagine agar*	Good, dull brownish orange (10R, 6/10)	Poor, pale pink (2.15R, 9/2)	None
Glycerol - asparagine agar*	Good, dull orange (2.5YR, 6/8) inside, to pale yellowish (2.5YR, 7/8) outside	Poor, pale pink (2.5R, 9/2)	None
Inorganic salts - starch agar*	Good, pale yellowish brown (2.5YR, 7/8)	Poor, pale pink (2.5R, 9/2)	None
Tyrosine agar*	Good, brownish gray (2.5YR, 6/4) inside, to pale brownish gray (2.5YR, 7/4)	Poor, pale pink (2.5R, 9/2)	None
Nutrient agar*	Good, dull reddish brown (2.5R, 4/4)	Moderate, dark violet (5P, 2/1)	None
Oatmeal agar	Good, whitish orange (2.5YR, 7/6)	Abundant, gray pink (7.5R, 8/4)	None
Yeast extract - malt extract agar	Good, light brown (10R, 3/6)	Moderate, pale pink (2.5R, 9/2)	Yellowish brown (7.5YR, 6/10)
Peptone - yeast extract - iron agar	Good, black (5R, 2/N)	Moderate, dark violet (5P, 2/1)	None

Color code was assigned according to the color standard of Japan Industrial Standard Z8721.
* Containing yeast extract 0.1%.

It resembled three species of *Actinomadura roseoviolacea*. They are *A. roseoviolacea* Nonomura and Ohara[11], *A. roseoviolacea* var. *biwakoensis* nov. var.[12], and *A. roseoviolacea* var. *rubescens* nov. var.[13]. Strain 07 was different from the three strains in physiological details and sugar utilization as shown in Table 3. However, the differences were not sufficient to designate strain 07 as a new species. Therefore we named the strain 07 *Actinomadura roseoviolacea* var. *miuraensis* nov. var.

Table 2. Physiological characteristics of strain 07.

Temperature range for growth	20~45°C	Salicine	+
Optimum temperature	32~40°C	Sucrose	+
Starch hydrolysis	+	Raffinose	−
Nitrate reduction	+	L-Ramnose	−
Melanin production	−	D-Mannitol	−
Gelatin liquefaction	−	Cell wall composition	
Coagulation of skimmed milk	+	*meso*-2,6-Diaminopimeric acid	+
Peptonization of skimmed milk	+	Glucose	+
Sugar utilization		Mannose	+
D-Glucose	+	Madurose	+
D-Fructose	+	Glycine	−
D-Xylose	+	Arabinose	−
L-Arabinose	+	Xylose	−
Inositol	+	Galactose	−

Table 3. Comparison of strain 07 with related species.

	Strain 07	*A. roseoviolacea* Nonomura and Ohara[11]	*A. roseoviolacea* var. *biwakoensis* nov. var.[12]	*A. roseoviolacea* var. *rubescens* nov. var.[13]
Starch hydrolysis	+	−	+	+
Gelatin liquefaction	−	+	−	+
Sugar utilization				
Sucrose	+	−	+	+
Salicin	+	−	+	+
Mannitol	−	+	+	+
Rhamnose	−	+	+	+
Raffinose	−	−	−	+

Fermentation and Isolation

The producing organism was grown in an oatmeal starch medium at 32°C for 4 days in a 200-liter fermentor (120 liters of medium). The medium contained 40 g oatmeal, 40 g soluble starch, 7 g K_2HPO_4, 3 g $NaH_2PO_4 \cdot 2H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $FeSO_4 \cdot 7H_2O$ in 1,000 ml deionized water, and the pH was adjusted to 7.0. The fermentation broth was centrifuged and the supernatant was subjected to ammonium sulfate precipitation. The precipitate between 45 and 100% saturation was collected and dissolved in 25 liters of distilled water followed by ethanol precipitation at −20°C. The precipitate was dissolved in 50 mM sodium phosphate buffer, pH 6.5, and applied to a DEAE-Sephadex A-25 column (5.0×37.0 cm) chromatography equilibrated with the same buffer. It was then eluted with 0 to 1.0 M linear gradient of NaCl. The active fractions were collected and ammonium sulfate was added to the eluate at a final concentration of 2.0 M. Further, Phenyl-Sepharose CL-4B column chromatography was performed. The column (1.6×26.0 cm) was equilibrated with 50 mM sodium phosphate buffer, pH 6.5, containing 2.0 M ammonium sulfate and the sample was fractionated with 2.0 to 0 M ammonium sulfate linear gradient. The active fractions were collected and dialyzed against 50 mM sodium phosphate buffer, pH 6.5. The non-dialyzate was applied to a hydroxyapatite (Bio-gel HT, Bio-Rad Laboratories) column (2.6×38.0 cm) chromatography. The material was eluted with 400 mM phosphate buffer, pH 6.5. The active fractions were collected, concentrated, and applied to a Sephacryl S-200 Superfine column (3.6×138.8 cm) chromatography. The purified principle, SN-07,

Fig. 2. Procedure for isolation of SN-07.

Fermentation broth
↓ Centrifuge
Supernatant
↓ Ammonium sulfate fractionation (45~100%)
↓ EtOH precipitation at −20°C
↓ DEAE-Sephadex A-25 column chromatography
↓ Phenyl-Sepharose CL-4B column chromatography
↓ Hydroxyapatite column chromatography
↓ Sephacryl S-200 Superfine column chromatography
↓ Lyophilization
Purified material

Table 4. Physico-chemical properties of SN-07.

Appearance	Pinkish red powder
MP	235~245°C (dec)
$[\alpha]_D^{20}$	+76° (*c* 0.5, H_2O)
Molecular weight	18,000~22,000 (in 50 mM Tris-HCl buffer, pH 7.0, containing 2.0 M KCl)
	50,000~60,000 (in 50 mM Tris-HCl buffer, pH 7.0)
UV absorption spectra	
H_2O	258, 505
0.1 N NaOH	258, 563
Solubility	
Soluble	H_2O, 0.1 N NaOH
Insoluble	0.1 N HCl, ethanol, methanol, acetone, 2-propanol, ether
Constituents*	DNA, 64%
	RNA, 29%
	Neutral sugars, 13%
	Amino acids, 4%
	Amino sugars, not detected
*Anal.**; Found	C 36.97, H 6.79, N 12.27

* Optical density at 505 nm of SN-07 (Lot No. 9) was 0.80 at 1 mg/ml solution.

was dialyzed and lyophilized. Ninety milligrams of material was obtained from 120 liters of fermentation broth. The outline of the isolation is summarized in Fig. 2.

Physico-chemical Properties

The purified SN-07 was a pinkish red powder and its physico-chemical properties are summarized in Table 4. The absorption spectra are shown in Fig. 3. Since SN-07 had the absorption at 505 nm and 260 nm (Fig. 3), both wavelength was measured in checking the purity. Fig. 4 illustrates the high-performance liquid chromatography (HPLC) on TSK-GEL DEAE-5PW (Toyo Soda Manufacturing Co.). The absorption in both wavelength was observed at the same retention time. No extra peaks were observed in either wavelength. The molecular weight of SN-07 was estimated by gel filtration. A Sephadex G-100 column (1.5 × 110.5 cm) was equilibrated and eluted either with 50 mM Tris-HCl buffer, pH 7.0, or with 50 mM Tris-HCl buffer, pH 7.0, containing 2.0 M KCl, at a flow rate of 10 ml/hour. Molecular weight markers used were bovine serum albumin, ovalbumin, chymotrypsinogen A and lysozyme. SN-07 of which optical density at 505 nm was 0.80 at 1 mg/ml solution contained 64% DNA measured by the diphenylamine method[14], 29% RNA by the orcinol method[14], 13%

Fig. 3. Absorption spectra of SN-07.
SN-07 was dissolved in distilled water (—) or in 0.1 N sodium hydroxide (---) at a concentration of 100 μg/ml in each case.

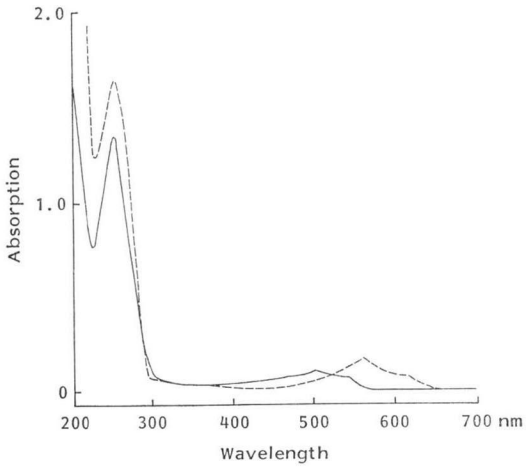
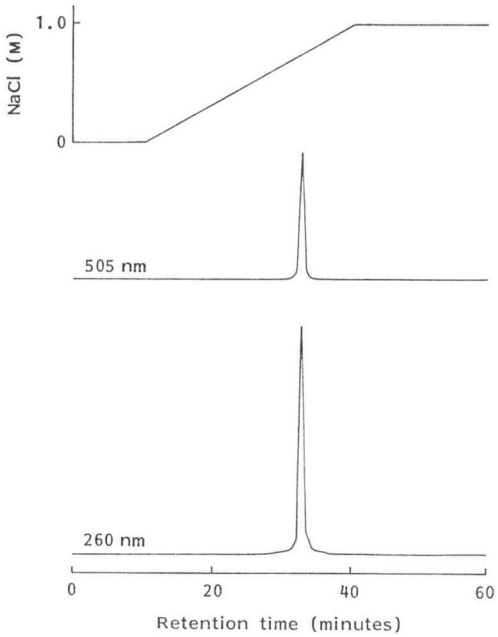


Table 5. Antibacterial activity of SN-07*.

Microorganism	MIC (μg/ml)**
Salmonella typhimurium LT2	>100
S. typhimurium TA1535	0.39
Escherichia coli BE1186	0.39
Micrococcus luteus ATCC 9341	6.25
Staphylococcus aureus ATCC 6538P	25
S. aureus (SM^r, KM^r, PCs^r, TC^r, SA^r, CM^r)	25
Bacillus subtilis (rec$^+$, SM^r)	12.5
B. subtilis (rec$^-$, SM^r)	3.13

* Optical density at 505 nm of SN-07 (Lot No. 9) was 0.80 at 1 mg/ml solution.
** Minimum inhibitory concentration (MIC) was determined by broth dilution method.

Fig. 4. HPLC chromatogram of SN-07.
Thirty μg of purified material was loaded on a TSK-GEL DEAE-5PW column (7.5×75 mm) equilibrated with 20 mM Tris-HCl, pH 7.0. SN-07 was eluted with 0 to 1.0 M NaCl linear gradient at a flow rate of 1 ml/minute. Absorbance at 505 nm and 260 nm was measured at the same time by using two detectors. The sensitivity of the detector when 505 nm was measured was 8 times higher than in the case of 260 nm.



neutral sugars by the phenol-sulfuric acid method[15], and 4% amino acids by a Hitachi 835 amino acid analyzer. No amino sugars were detected under our analytical conditions.

Biological Activities

The antibacterial activities of SN-07 are summarized in Table 5. When *Escherichia coli* BE 1186[16] that has mutations in *uvrA*, *ruv*, and *tolC* genes and *Salmonella typhimurium* TA1535[17] that has mutation in *rfa* and lacks *uvrB* gene were adapted to the test, they showed high susceptibility to the antibiotic. The minimum inhibitory concentrations (MIC) were 0.39 μg/ml for each organism. SN-07 had no activity against the Gram-negative bacteria tested, but inhibited the growth of Gram-positive bacteria. The repair mutant of *Bacillus subtilis* was more sensitive than the parent strain. SN-07 possesses antitumor activity against lymphocytic leukemia P388. The evaluation was performed according to the NCI protocol[18]. The antitumor activity designated by increased life span (ILS) was 51.9% at a dose of 80 μg/kg mice. The biological activities of SN-07 dissolved in water were stable at 27°C for at least 3 weeks.

Discussion

In the search for macromolecular antibiotics, a new substance, SN-07, of which major constituents were nucleic acids, was found in the broth filtrate of genus *Actinomadura*. SN-07 showed a cytotoxicity against cultured HeLa cells. It was effective at a concentration of 0.5 μg/ml in a preliminary examination. The cytotoxicity of commercially purchased DNA (from salmon sperm, Wako Pure Chemical Industries Ltd.) on HeLa cells was not observed up to a concentration of 100 μg/ml. HAYASHIDA and WATANABE reported that a mixture of single and double stranded DNA possessed antitumor activity against Sarcoma-180[6].

The absorption spectra of SN-07 might suggest that it contained a chromophore of which absorption was at 505 nm in distilled water (Fig. 3). The absorption at 505 nm and 260 nm was observed at the same retention time (Fig. 4), and it demonstrated that the chromophore binds to the rest of the molecule. Though the chromophore-like substance has not been separated from SN-07 yet, it may play important roles in expressing its specific biological activities as in the case of neocarzinostatin[19], largomycin FII[20], auromomycin, and macromomycin[21].

Since the homogeneity of SN-07 was demonstrated by gel filtration and HPLC, it was concluded that the substance was a new macromolecular antibiotic whose major constituents were nucleic acids.

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