NOVEL ANTIMYCIN ANTIBIOTICS, URAUCHIMYCINS A AND B, PRODUCED BY MARINE ACTINOMYCETE

NOBUTAKA IMAMURA, MIYUKI NISHIJIMA,
KYOUKO ADACHI and HIROSHI SANO

Shimizu Institute, Marine Biotechnology Institute Co., Ltd.,
Shimizu 424, Japan

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Two novel antimycin antibiotics, urauchimycins A and B, were isolated from a fermentation broth of a *Streptomyces* sp. Ni-80. The strain was isolated from an unidentified sponge. Their chemical structures were determined by 2D NMR analysis. They are the first antimycin antibiotics which possess a branched side chain moiety. They exhibited inhibitory activity against morphological differentiation of *Candida albicans*.

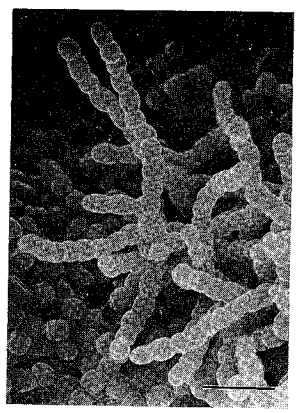
Recently, interest has intensified on marine sources as a target for new biologically active compounds, and over 3,000 natural products have been reported from marine organisms. The subjects of these studies were mainly benthos, *i.e.*, sponges, algae, coelenterates, molluscs, tunicates, echinoderms and the like[1]. It is well known that some benthos contains symbiotic microorganisms, for example, sponges include about 40% (v/v) symbiotic microbes[2]. From sponges, many unique chemicals have been obtained, but the real producer of an active compound might be a commensal microorganism.

Marine microorganisms have received much less attention than terrestrial ones[1], and because they live in quite different environment from terrestrial microbes, might be expected to produce compounds which possess unique strucure and activities. Marine bacteria isolated from benthos seem to be an especially interesting source for new antibiotics. Thus, we have started the investigations of secondary metabolites of marine bacteria commensal with some benthos.

During our screening for new biologically active substances from marine microorganisms, new antibiotics, which we have named urauchimycins A and B, were isolated from the cultured broth of *Streptomyces* sp. Ni-80. This strain was isolated from an unidentified sponge collected at Urauchi-cove, Iriomote, Japan. Their structures were determined based on spectral data.

Fig. 1. Scanning electron micrograph of spore chains of strain Ni-80.

Bar represents 2 μm.



This paper describes the taxonomy of the producing strain Ni-80 and the fermentation, isolation, physico-chemical properties and structure determination of urauchimycins A and B.

Taxonomy of the Producing Strain Ni-80

Morphology

The vegetative mycelia of strain Ni-80 showed good growth on both synthetic and complex agar

media and do not show fragmentation into coccoid or bacillary elements. The aerial mycelia grow abundantly on oatmeal agar and inorganic salts-starch agar.

The sporophores are of the *Rectiflexibiles* type and contain 10 to 30 or more spores in a chain (Fig. 1). The spores are cylindrical in shape, 0.5×0.7 μm in size and have a smooth surface (Fig. 1). No sclerotic granule, sporangium or flagellated spore was observed.

Chemical Composition

The chemical analysis of LL-2,6-diaminopimeric acid (A_2pm) in the cell wall was carried out by the method of LECHEVALIER and LECHEVALIER[3]. Strain Ni-80 showed the presence of LL-A_2pm in the cell wall.

Cultural and Physiological Characteristics

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[4] and

Table 1. Cultural characteristics of strain Ni-80.

Yeast extract-malt extract agar[a]	G:	Moderate
	AM:	Moderate, grayish brown (10YR V8C1)
	SM:	Brownish yellow (2.5Y V7C6)
	SP:	Light brown
Oatmeal agar[a]	G:	Good
	AM:	Abundant, light brownish white (5Y V8C2)~silver gray (5Y V7C1)
	SM:	Pale yellowish brown (5Y V6C6)
	SP:	Light brown
Inorganic salts-starch agar[a]	G:	Good
	AM:	Abundant, pale whitish yellow (2.5Y V9C2)
	SM:	Bronze (5Y V4C6)
	SP:	Light brown
Glycerol-asparagine agar[a]	G:	Good
	AM:	Abundant, pale whitish yellow (2.5Y V9C2)
	SM:	Beige (2.5Y V7C4)
	SP:	Pale yellowish brown
Glucose-asparagine agar	G:	Poor,
	AM:	Poor, pale whitish yellow (2.5Y V9C2)
	SM:	Mustard (5Y V6C8)
	SP:	Pale brown
Peptone-yeast extract-iron agar[a]	G:	Moderate
	AM:	Moderate, white (2.5Y V9N)~ivory (2.5Y V8C2)
	SM:	Creamy white (2.5Y V9C3)
	SP:	Light brown
Tyrosine agar[a]	G:	Good
	AM:	Abundant, ivory (2.5Y V8C2)
	SM:	Beige (2.5Y V7C4)
	SP:	Pale brown
Sucrose-nitrate agar[a]	G:	Poor
	AM:	Poor, ivory white (5Y V9C2)
	SM:	Milky white (5Y V9C3)
	SP:	None
Nutrient agar[b]	G:	Moderate
	AM:	Moderate, pale whitish yellow (2.5Y V9C2)
	SM:	Pale whitish brown (5Y V8C4)
	SP:	None

[a] Medium recommended by ISP.
[b] Medium recommended by S. A. WAKSMAN.
G: Growth of vegetative mycelium, AM: aerial mycelium, SM: substrate mycelium, SP: soluble pigment.

Table 2. Physiological properties of strain Ni-80.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin (28°C)	+
Peptonization of milk (28°C)	+
Coagulation of milk (28°C)	+
Celluloytic activity	−
Hydrolysis of starch	+
Temperature range for growth	10~37°C

+: Active, −: not active.

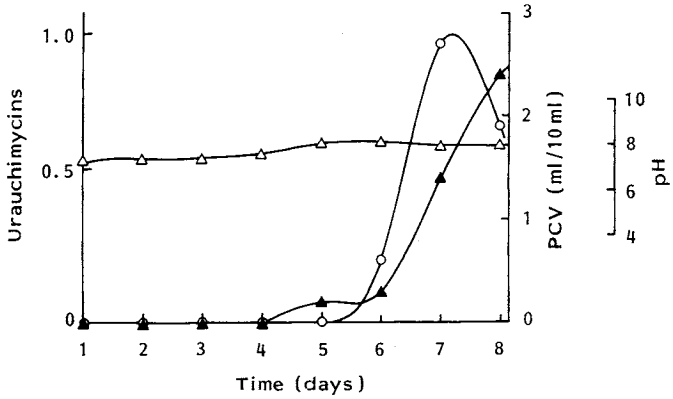
Table 3. Utilization of carbon sources by strain Ni-80.

D-Glucose	+
D-Fructose	+
L-Rhamnose	±
D-Mannitol	+
L-Arabinose	+
i-Inositol	±
Raffinose	−
D-Xylose	+
Sucrose	±

+: Utilized, ±: weakly utilized, −: not utilized.

Fig. 2. A typical time course of urauchimycins production by *Streptomyces* sp. Ni-80.

○ Urauchimycins, △ pH, ▲ PCV.



The amount of mixture of urauchimycins are demonstrated as HPLC relative peak area.

those recommended by WAKSMAN[5] were used. Cultures were observed after incubation at 28°C for three weeks. Color names and hue numbers indicated in Table 1 are adopted from the Book of JIS Color Standards (JIS Z 8721). The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% each carbon source at 28°C. The cultural and physiological characteristics and the utilization of carbon sources of strain Ni-80 are shown in Tables 1, 2 and 3, respectively.

The strain exhibits the following properties. Sporophore, *Rectiflexibiles*; spores, cylindrical and smooth surface; color of vegetative mycelium, light yellow or light brown; color of aerial mycelium, beige or gray; soluble pigment, light brown; A_2pm in cell wall, LL-type. Based on the taxonomic properties described above, strain Ni-80 is considered to belong to the genus *Streptomyces*; and to be a strain of the gray series of PRIDHAM and TRESNER's system[6].

The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. Ni-80 and the accession No. is FERM P-12392.

Fermantation and Isolation

The stock culture of *Streptomyces* sp. Ni-80 was inoculated into 200 ml of a medium consisting of 1% glucose, 2% bouillon (Nissui), 0.4% $CaCO_3$, and 50% sea water in 500-ml Erlenmeyer flask. It was incubated with rotary shaking for 164 hours at 30°C. A typical time course of urauchimycins production by strain Ni-80 is shown in Fig. 2. Urauchimycins production was monitored by HPLC analysis as a mixture of A and B, which reached maximum at 164 hours.

The cultured broth (2 liters) of the strain was centrifuged to obtain about 1.8 liters of a supernatant fluid, which was extracted with EtOAc and evaporated to dryness. The resulting brown oil (107 mg) was subjected to flash silica gel chromatography and eluted with EtOAc-hexane. The active fractions were combined and then evaporated *in vacuo* to yield a yellowish amorphous material (4 mg). The amorphous material was purified on a preparative HPLC column of reversed phase silica gel (ODS) developed with the solvent (CH_3CN-H_2O-AcOH = 50:50:0.01). The active fraction was evaporated to dryness and urauchimycins A (0.3 mg) and B (1 mg) were obtained.

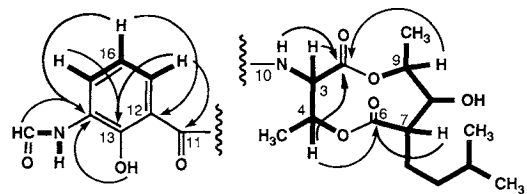
They were active inhibitors of morphological differentiation of *Candida albicans* at the concentration of 10 μg/ml.

Physico-chemical Properties and Structure Determination

Urauchimycins were obtained as colorless amorphous powder. They are soluble in EtOAc, $CHCl_3$, MeOH, and EtOH, but hardly soluble in water. They showed the similar optical rotation {A; $[\alpha]_D^{26}$ +46.7° (*c* 0.03, MeOH) and B; $[\alpha]_D^{26}$ +50.0° (*c* 0.1, MeOH)} and gave identical UV, MS, and IR data (UV λ_{max}^{EtOH} 343, 225 nm; FAB-MS *m/z* 451 $(M+1)^+$; IR ν_{max}^{KBr} 1746, 1684, 1644, 1539, 1435, 1369, 1261, 1197, 1060, 746 cm^{-1}. These facts suggested that they were isomers differing structurally from each other at some place other than the chromophore. The HRFAB-MS data of B (Found: 451.2068 $(M+1)^+$, Calcd for $C_{22}H_{31}N_2O_8$: 451.2080) suggested a molecular formula of $C_{22}H_{30}N_2O_8$. Comparison of NMR and MS data of A and B indicated they both had this molecular formula.

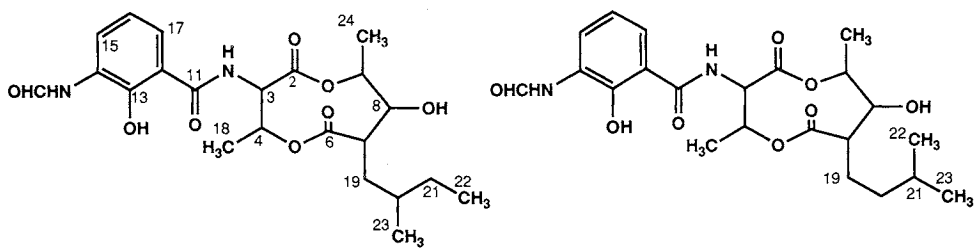
The ^1H-^1H COSY spectrum of B indicated the partial structures as shown by the bold lines in Fig. 3. In the HMBC spectrum of B, the two carbonyl carbons C-2 (170.1 ppm) and C-6 (173.9 ppm) showed multiple bond couplings with three protons (H-3 (5.32 ppm), H-4 (5.69 ppm), and H-9 (4.87 ppm)) and two protons (H-4 and H-7 (2.32 ppm)), respectively. This led to the fomulation of the 9-members dilactone moiety characteristic of the antimycin antibiotics. The trisubstituted benzene chromophore was confirmed by the HMBC data as shown by arrows in Fig. 3. Finally, the NOESY spectrum of B showed a cross peak between the NH proton signal (H-10) at 7.07 ppm and aromatic proton signal (H-17) at 7.26 ppm, and this allowed the connection to be made between the dilactone and substituted benzene moieties. Thus, the structure of B was determined as shown in Fig. 4. The determination

Fig. 3. Summarized results of COSY (bold lines) and HMBC (arrows) spectra of urauchimycin B.



⟶ Long range coupling.

Fig. 4. Structures of urauchimycins A and B.



A B

Table 4. ^{1}H and ^{13}C chemical shifts of urauchimycins A and B. (Varian UNITY 500, in $CDCl_3$, δ ppm)

Position	Urauchimycin A		Urauchimycin B		Position	Urauchimycin A		Urauchimycin B	
	Proton	Carbon	Proton	Carbon		Proton	Carbon	Proton	Carbon
2		170.1		170.1	14-NH	7.90		7.88	
3	5.24	53.8	5.32	53.7	14-NHCHO	8.49	158.9	8.49	158.9
4	5.69	70.7	5.69	70.8	15	8.55	124.8	8.55	124.8
6		173.8		173.9	16	6.92	119.0	6.92	119.0
7	2.49	50.0	2.32	52.3	17	7.24	120.1	7.26	120.1
8	2.59	77.1	3.60	77.1	18	1.30	15.0	1.31	15.0
8-OH	1.93		1.93		19	1.51,	35.8	1.68,	26.8
9	4.88	76.3	4.87	76.3		1.84		1.78	
10	7.09		7.07		20	1.32	32.4	1.15	36.2
11		169.4		169.4	21	1.27	30.5	1.54	28.0
12		112.6		112.6	22	0.89	11.3	0.88	22.6
13		150.6		150.6	23	0.86	18.4	0.89	22.2
13-OH	12.63		12.40		24	1.45	18.5	1.46	18.4
14		127.4		127.4					

of structure of A shown in Fig. 4 was also carried out by the ^{1}H-^{1}H COSY data and comparison with the data of B. The total assignments of ^{1}H and ^{13}C NMR signals of urauchimycins are listed in Table 4, and these results agree with those reported for antimycin A_1[7].

Discussion

Many antimycin group antibiotics produced by terrestrial *Streptomyces* sp. have been reported, and they possess C_4 or C_6 straight side chains. Urauchimycins are the first antimycin type antibiotics reported in which the side chain contains an odd number of carbons. Moreover they are the first reported antimycins in which this side chain is branched.

The effects of salinity on production of urauchimycins were examined. The growth rate of the strain in diluted sea water medium was faster than that in sea water medium, but the final packed cell volumes were equivalent. The production of urauchimycins were almost the same under the conditions examined ($0 \sim 100\%$ sea water). The strain may have originated from a terrestirial one and modified its secondary metabolism in a marine environment.

Urauchimycins are targets for biosynthetic studies, because the origin of methyl group of the branched side chain is uncertain, and because the substituted benzene chromophore is structurally a m-C_7N unit. This type of structure can be derived from one of several pathways[8]. Their biosynthesis is currently under investigation.

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