QUINOLIDOMICINS A_1, A_2 AND B_1, NOVEL 60-MEMBERED MACROLIDE ANTIBIOTICS

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

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Three novel macrolide antibiotics, quinolidomicins A_1, A_2 and B_1, were isolated from the fermentation broth of *Micromonospora* sp. JY16. Quinolidomicin A_1 inhibited the growth of various tumor cells including multidrug-resistant cells. Quinolidomicin B_1 was similarly cytotoxic, while quinolidomicin A_2 was inactive against these tumor cells.

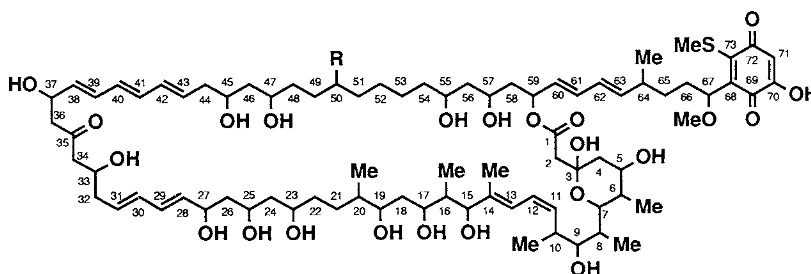
In the course of our screening for new antitumor antibiotics, an actinomycete isolated from a soil sample was found to produce three novel macrolide antibiotics designated quinolidomicins A_1[1], A_2 and B_1 (Fig. 1). This paper describes the taxonomy of the producing organism, and the fermentation, isolation, physico-chemical properties and biological activity of the quinolidomicins. The structure elucidation are described in the accompanying paper[2]. A preliminary structural study on quinolidomicin A_1 has been reported[1].

Materials and Methods

Microorganism

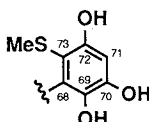
A culture designated JY16 was isolated from a soil sample collected at Yoshii-machi, Tano-gun,

Fig. 1. Structures of quinolidomicins A_1, A_2 and B_1.



Quinolidomicin A_1 R=Me
Quinolidomicin B_1 R=H

Quinolidomicin A_2 R=Me



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Gunma Prefecture, Japan. The culture has been deposited with the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Japan, under the name *Micromonospora* sp. JY16 with the accession number FERM BP-3940.

Taxonomic Studies

The characterization and identification of the culture were carried out mainly according to BERGEY's Manual[3] and the methods described by SHIRLING and GOTTLIEB[4]. For the evaluation of cultural characteristics, the strain was incubated for 21 days at 27°C. Cell wall composition was analyzed by the methods of BECKER *et al.*[5].

Spectral Analysis

Specific rotations were obtained on a Jasco DIP-140 spectropolarimeter at 589.6 nm and 24°C. Mass spectra were measured on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol-sodium chloride as matrix and polyethylene glycol as internal standard. UV and visible spectra were recorded on a Shimadzu UV-300 spectrophotometer. ^{1}H NMR spectra were obtained on a JEOL JNM-A500 spectrometer at 500 MHz. Chemical shifts are given in ppm using TMS as internal standard.

Cells and Cell Culture

K562 and KB cells were grown in EAGLE's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% Bacto-peptone. HT-29 and MKN28 cells were grown in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum. P388, P388/ADM and P388/VCR cells were grown in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum and 10 μM 2-mercaptoethanol. These cells were cultured in humidified atmosphere of 5% CO_2 in air at 37°C. K562 and KB cells were plated at 8×10^4 cells/ml and incubated for 3 days with samples, and then the viable cells were counted to determine IC_{50}s after trypan-blue staining. HT-29 and MKN28 cells were plated at 1×10^5 cells/ml and incubated for 3 days with samples. P388 cells at 3×10^4 cells/ml were incubated for 2 days with samples. The growth of these cells was measured at 570 nm with formazan formation after treatment of the cells with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C. The IC_{50}s of doxorubicin on P388, P388/ADM and P388/VCR cells were 33, 3,100 and 130 nM, respectively. The IC_{50}s of vincristine on P388, P388/ADM and P388/VCR cells were 1.3, 58 and 29 nM, respectively.

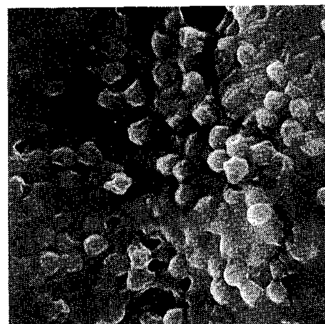
Results

Taxonomy

The substrate mycelium of strain JY16 was long and monopodially branched, but no aerial mycelium was observed on various media. Spores were borne singly on the substrate mycelium and were spherical (0.8 μm) with a slightly warty surface (Fig. 2). The culture showed no special morphology such as sporangia, whirls or sclerotia.

Fig. 2. Scanning electron micrograph of spores of strain JY16.

Bar represents 3.8 μm.



The hydrolysate of the purified cell walls contained *meso*-diaminopimelic acid, glycine and a trace of 3-hydroxydiaminopimelic acid. The whole-cell sugar hydrolysate contained arabinose and xylose, indicating cell-wall type II D.

The cultural and physiological properties of strain JY16 are shown in Tables 1 and 2, respectively. These properties can be summarized as follows:

Table 1. Cultural properties of strain JY16.

Medium	Growth	Reverse color	Soluble pigments
Sucrose-nitrate agar	Good	Yellowish white	None
Glucose-asparagine agar	Moderate	Yellowish white	None
Glycerol-asparagine agar	Moderate	Dark yellowish brown	None
Inorganic salts-starch agar	Good	Orange	None
Tyrosine agar (ISP No. 7)	Moderate	Dark yellowish brown	None
Nutrient agar	Good	Dark yellowish brown	None
Yeast extract-malt extract agar	Moderate	Black	None
Oatmeal agar	Good	Light yellow ~ orange ~ dark yellowish brown	None
CZAPEK's-sucrose agar	Good	Dull orange ~ yellowish orange	Grayish brown

no aerial mycelium was observed; the color of the culture was orange ~ yellowish white ~ dark yellowish brown ~ black; melanoid pigments were not produced in either tyrosine agar, peptone-yeast-iron agar or tryptone-yeast broth; neither inositol, D-mannitol nor L-rhamnose was utilized.

The results of these morphological and chemotaxonomic studies indicate that strain JY16 belongs to the genus *Micromonospora*. Among the species of *Micromonospora* described in BERGEY's Manual, strain JY16 appeared to be the most closely related to *Micromonospora halophytica* subsp. *nigra*, although we could not identify it because of differences in the utilization of glycerol and D-ribose.

Table 2. Physiological properties of strain JY16.

Growth temperature	15~45°C
Melanoid pigment formation in:	
Tyrosine agar (ISP No. 7)	−
Peptone-yeast-iron agar (ISP No. 6)	−
Tryptone-yeast broth (ISP No. 1)	−
Starch hydrolysis	+
Gelatin liquefaction	−
Milk coagulation	+
Milk peptonization	+
Nitrate reduction	+
Carbon utilization	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
myo-Inositol	−
L-Rhamnose	−
Raffinose	+
D-Mannitol	−
D-Galactose	+
D-Sorbitol	±
D-Mannose	+
Maltose	+
D-Ribose	+
Glycerol	+

+: Positive, −: negative, ±: doubtful.

Fermentation

The seed medium consisted of soluble starch 1.0%, molasses 1.0%, meat extract 1.0% and Polypepton 1.0% (pH 7.2 before sterilization). Seed tubes containing 15 ml of the medium were inoculated with a stock culture of *Micromonospora* sp. JY16 maintained on a BENNET's agar slant and were incubated on a reciprocal shaker at 27°C for 2 days. The seed culture at 2% was transferred to 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of glycerol 2%, molasses 1%, meat extract 1%, Polypepton 1% and calcium carbonate 0.4% (pH 7.2 before sterilization). The flasks were incubated on a rotary shaker at 27°C for 3 days. A 600-ml portion of the culture was inoculated into 50-liter jar fermentor containing 30 liters of a production medium having the same composition as the flask fermentation medium. The fermentation was carried out at 27°C for 2 days under agitation of 300 rpm and aeration of 30 liters/minute.

Isolation

The fermentation broth (60 liters) was centrifuged to give a mycerial cake, which was extracted with 30 liters of methanol. The extract was concentrated to dryness and then washed with 2 liters each of ethyl acetate and water. The residue was dried and applied to a silica gel column (600 ml), which was washed with 2.4 liters of chloroform-methanol (20:1) and then eluted with chloroform-methanol (10:1). After evaporation, the active eluate was chromatographed on a Sephadex LH-20 column (1 liter). The active eluate with chloroform-methanol (1:1) was subjected to HPLC using a normal-phase column (Senshu Pak. AQUASIL SS-762N, Senshu Scientific Co.) with chloroform-methanol (10:1). The main fraction appearing as the second peak of three fractions was evaporated to dryness to yield an orange powder of quinolidomicin A_1 (150 mg). The first fraction of the HPLC was further purified by HPLC using the same conditions and then concentrated to dryness to give an orange powder of quinolidomicin A_2 (10 mg). The third fraction of the HPLC was applied to a reverse-phase HPLC column (YMC-Pack D-ODS-7, Yamamura Chemical Laboratories Co.), which was eluted with 75% methanol. The active eluate was evaporated to dryness to yield an orange powder of quinolidomicin B_1 (2 mg).

Physico-chemical Properties

The physico-chemical properties of the quinolidomicins are summarized in Table 3. The ^{1}H NMR

Table 3. Physico-chemical properties of quinolidomicins A_1, A_2 and B_1.

	A_1	A_2	B_1
Appearance	Orange powder	Orange powder	Orange powder
MP	142~147°C	142~147°C	134~139°C
$[\alpha]_D^{24}$ $CHCl_3$-CH_3OH (1:1)	+50°	+7°	+50°
HRFAB-MS (m/z) calcd:	1,551.8771 $(M+Na)^+$, 1,551.8778	1,553.8909 $(M+Na)^+$, 1,553.8935	1,537.8699 $(M+Na)^+$, 1,537.8622
Formula	$C_{83}H_{132}O_{23}S$	$C_{83}H_{134}O_{23}S$	$C_{82}H_{130}O_{23}S$
UV λ_{max} (ε) nm in MeOH	232 (93,500), 257 (42,500), 268 (49,600), 279 (37,900), 365 (6,600)	232 (96,600), 257 (46,000), 268 (53,100), 279 (38,700), 301 (6,200), 314 (6,300)	232 (99,500), 257 (42,400), 268 (48,500), 279 (38,300), 360 (11,200)
IR (KBr) ν_{max} cm^{-1}	3400, 1720, 1710, 1630	3400, 1720, 1710, 1630	3400, 1720, 1710, 1630

Fig. 3. ^{1}H NMR spectrum of quinolidomicin A_1 in CD_3OD.

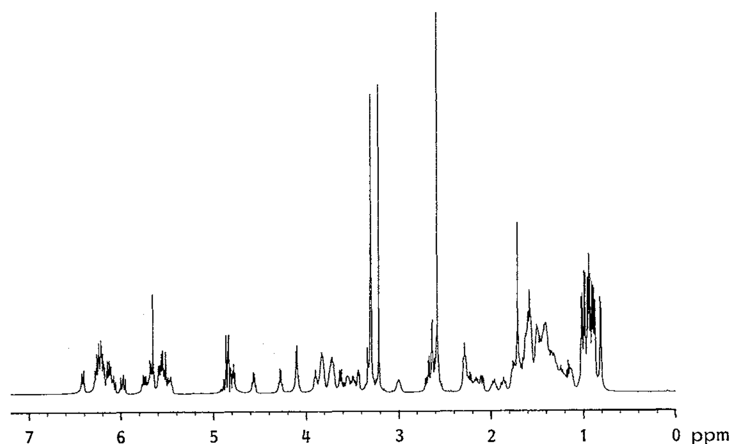


Fig. 4. ^1H NMR spectrum of quinolidomicin A_2 in CD_3OD.

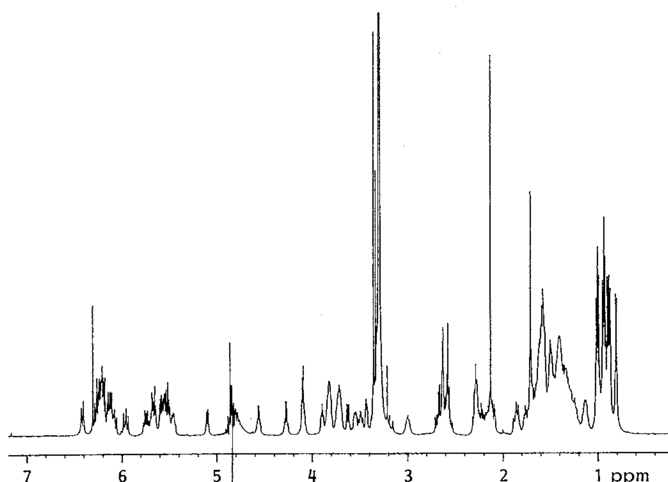
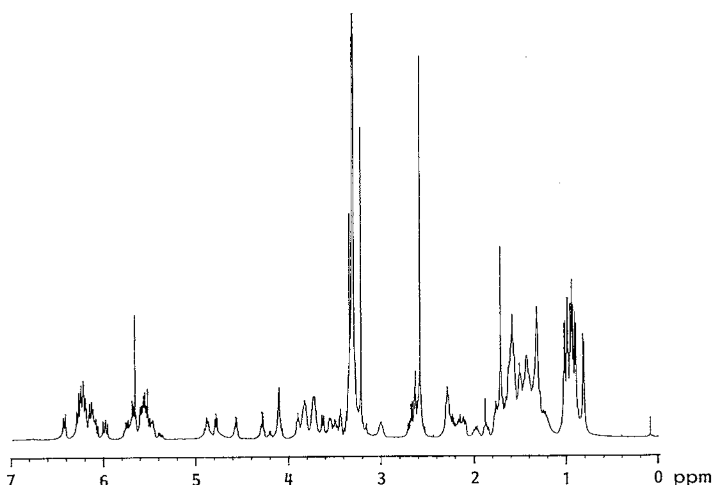


Fig. 5. ^1H NMR spectrum of quinolidomicin B_1 in CD_3OD.



spectra of quinolidomicins A_1, A_2 and B_1 are shown in Figs. 3, 4 and 5, respectively. The molecular formula of quinolidomicin A_1 was determined to be $C_{83}H_{132}O_{23}S$ by high-resolution FAB-MS, ^{13}C NMR and elemental analysis (calcd: C 65.16, H 8.70, O 24.05, S 2.10; found: C 64.21, H 8.82, O 24.98, S 1.99). The high-resolution FAB-MS also established the molecular formulae of quinolidomicins A_2 and B_1 as $C_{83}H_{134}O_{23}S$ and $C_{82}H_{130}O_{23}S$, respectively.

The UV spectrum of each quinolidomicin revealed three maximum absorption peaks at 257, 268 and 279 nm, indicating the presence of a triene moiety[6]. Both of quinolidomicins A_1 and B_1 exhibited IR absorption peaks due to hydroxyls (3400 cm^{-1}), two ester and/or ketone carbonyls (1720 and 1710 cm^{-1}) and quinone carbonyls (1630 cm^{-1}), and quinolidomicin A_2 showed a similar IR spectrum except for lower intensity at 1630 cm^{-1}.

Quinolidomicin A_2 was rapidly converted to quinolidomicin A_1 in air.

Table 4. Cytotoxic activity of quinolidomicins A_1, A_2 and B_1.

Tumor	Origin	IC_{50} (nM)		
		A_1	A_2	B_1
K562	Human leukemia	25	>500	68
KB	Human oral cancer	110	>500	145
P388	Mouse leukemia	27		
P388/ADM	Mouse leukemia	30		
P388/VCR	Mouse leukemia	47		
HT-29	Human colon cancer	170		
MKN28	Human gastric cancer	327		

Biological Activity

The cytotoxic activity of the quinolidomicins is summarized in Table 4. At low concentrations, quinolidomicin A_1 inhibited the growth of various tumor cells including two multidrug-resistant cell lines, P388/ADM (doxorubicin-resistant) and P388/VCR (vincristine-resistant). Quinolidomicin B_1 also showed cytotoxicity at approximately the same concentrations, while quinolidomicin A_2, which yielded quinolidomicin A_1 by air oxidation, was active against neither K562 nor KB cells at less than 500 nM, indicating that the benzoquinone chromophore is necessary for the cytotoxic activity of quinolidomicins.

Despite its potent cytotoxicity, intraperitoneal administration of quinolidomicin A_1 did not prolong the survival period of mice bearing P388 leukemia at the dosage level of 2~8 mg/kg/day on days 1 to 9, and showed toxicity at more than these doses.

Acknowledgment

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