Studies on Cochleamycins, Novel Antitumor Antibiotics

I. Taxonomy, Production, Isolation and Biological Activities

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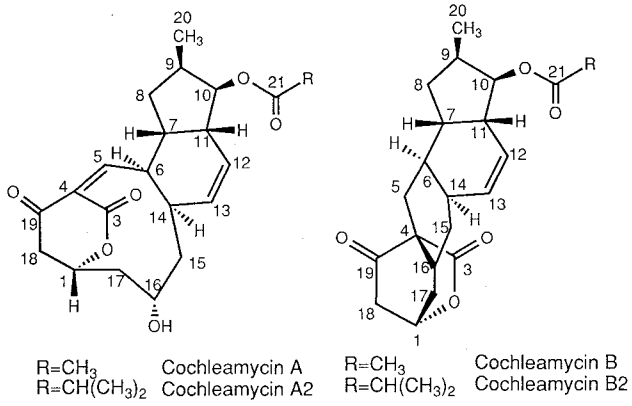
Novel antitumor antibiotics cochleamycins A, A2, B and B2 (Fig. 1) were isolated from the culture broth of *Streptomyces* sp. DT136. They were purified by column chromatography on silica gel, reversed phase HPLC and then isolated as colorless powder. Cochleamycins showed growth inhibition against tumor cells *in vitro*.

In the course of screening for new antitumor antibiotics, *Streptomyces* sp. DT136 was found to produce novel antitumor antibiotics cochleamycins A, A2, B and B2. Cochleamycins were recovered from the supernatant by EtOAc extraction and purified by chromatography.

Fig. 1. Total structures of cochleamycins.



Cochleamycins showed growth inhibition against tumor cells *in vitro*. Cochleamycins A and A2 showed antimicrobial activity against Gram-positive bacteria, while B and B2 did not.

In 1992, we report the isolation, structure determination and biological activities of cochleamycins A and B[1]. In this paper, we report the taxonomy of the producing strain, and the production, isolation and biological activities of cochleamycins. Physico-chemical properties, structure determination and biosynthetic studies will be reported in the accompanying papers[2,3].

Taxonomy of the Producing Strain

Strain DT136 was isolated from a soil sample collected at Nishimeya-cho, Aomori Prefecture, Japan. Characterization of the strain was carried out mainly by the methods described by SHIRLING and GOTTLIEB[3].

The aerial mycelium of the strain monopodially branched on the long main stem and terminated forming

Table 1. Cultural characteristics of strain DT136.

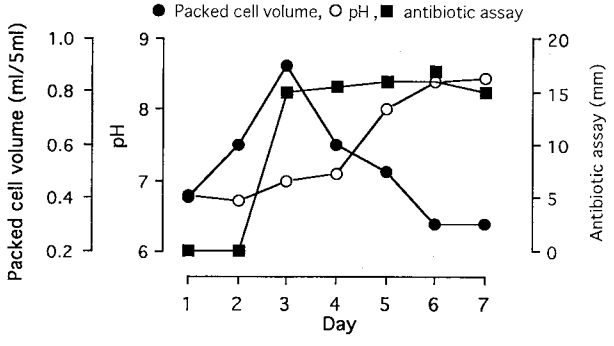
Sucrose-nitrate agar	G:	Moderate	Tyrosine agar	G:	Good
	R:	Light olive gray		R:	Pale yellow
	Am:	Poor; yellowish white		Am:	Good; light olive gray
	Sp:	None		Sp:	None
Glucose-asparagine agar	G:	Moderate	Nutrient agar	G:	Good
	R:	Light olive gray		R:	Pale yellowish brown
	Am:	Moderate; light olive gray		Am:	Good; yellowish white
	Sp:	None		Sp:	None
Glycerol-asparagine agar	G:	Good	Yeast extract-malt extract agar	G:	Good
	R:	Pale yellow		R:	Yellow
	Am:	Good; light brownish gray		Am:	Good; light olive gray
	Sp:	None		Sp:	None
Inorganic salts-starch agar	G:	Good	Oatmeal agar	G:	Good
	R:	Dull yellow		R:	Yellow
	Am:	Good; light brownish gray		Am:	Good; brownish gray
	Sp:	None		Sp:	Yellow

G: growth, R: reverse side of colony, Am: aerial mycelium, Sp: soluble pigment.

Table 2. Physiological properties of strain DT136.

Temperature for growth	15-37°C
Production of melanoid pigments:	
Tyrosine agar	Negative
Peptone-yeast extract-iron agar	Negative
Tryptone-yeast extract agar	Negative
Hydrolysis of starch	Negative
Liquefaction of gelatin	Negative
Peptonization of milk	Positive
Coaguration of milk	Negative
Utilization of carbon source:	
Utilized	L-arabinose, D-xylose, D-glucose D-fructose, sucrose, galactose D-mannose, maltose
Not utilized	inositol, L-rhamnose, raffinose D-mannitol, sorbitol

Fig. 2. The production time course of cochleamycins.



spore chains with about 30 spores per chain. The spore chains were spiral or looped. The spores were cylindrical (0.8~1.1 μm) with smooth surface. Spores were not motile, and development of whirls, sporangia (including pseudosporangia), sclerotia or other special structures were not observed. The cultural and physiological properties of strain DT136 grown on various media at 27°C are shown in Tables 1 and 2, respectively. The whole-cell hydrolysate contained the L,L isomer of diaminopimelic acid which corresponds to cell-wall type I[4]. MK-9 (H4) and MK-9 (H6) were detected as the components of menaquinones[5]. From the above characteristics, it was concluded that the strain belongs to the genus *Streptomyces*.

Producion and Isolation

Strain DT136 was inoculated in a 500-ml Erlenmeyer flask containing 100 ml of the fermentation medium consisting of glucose 2.5%, soy bean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4%, the pH being adjusted to 7.2 before sterilization. The fermentation was carried out at 27°C for 5 days on a rotary shaker. The time course of the production is shown in Fig. 2. Antimicrobial activity was monitored by agar diffusion assay with *B. subtilis* ATCC 6633 as the test organism. The maximum antibiotic activity was achieved at 5 days cultivation.

Fig. 3. Isolation scheme of cochleamycins.

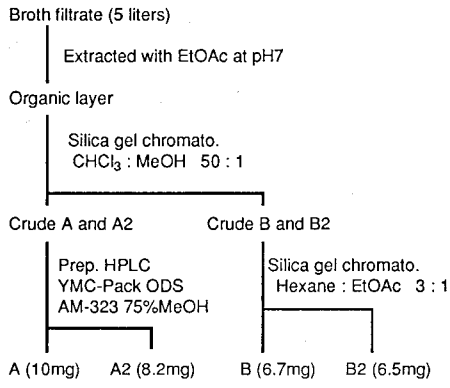


Table 3. Cytotoxicity of cochleamycins against tumor cells *in vitro*.

		IC_{50} (μg/ml)				
Cell line		P388	HL60	K562	COLO205	HT29
Cochleamycin	A	1.6	14.0	6.2	16.5	19.1
	A2	2.0	12.8	7.0	16.5	20.8
	B	2.6	4.3	6.2	16.0	9.8
	B2	4.0	6.8	6.2	18.0	12.0

Table 4. Antimicrobial activities of cochleamycins A and A2.

Organism	Medium[a]	Diameter of inhibition zone (mm)	
		Cochleamycin	
		A	A2
Staphylococcus aureus FDA 209P	I	16	18
S. aureus MS14146[b]	I	11	12
S. aureus MS14287[b]	I	15	17
Micrococcus luteus ATCC 9341	I	25	26
Bacillus subtilis ATCC 6633	I	15	15
B. subtilis PCI 219	I	15	17
Escherichia coli NIHJ	I	0	0
Pseudomonas aeruginosa NCTC 10490	I	0	0
Candida albicans Yu 1200	II	0	0
Saccharomyces cerevisiae ATCC 9763	II	0	0

[a] I: Nutrient agar (DIFCO), II: Sabouraud dextrose sgar (DIFCO).
[b] Methicillin resistant.

Plate diffusion assay: 50 μg was applied onto 8 mm filter disk. The disks were placed on plates seeded with the tested micrograms in the top of the agar.

The isolation procedures of cochleamycins A, A2, B and B2 are summarized in Fig. 3. After removal of the mycelium, the supernatant (5 liters) was extracted with EtOAc (5 liters) at pH 7. The organic layer was con-

centrated *in vacuo* and subjected to silica gel column chromatography (Wakogel C-200) using $CHCl_3$ - MeOH (50 : 1). In this chromatography, cochleamycin A group (A and A2) and B group (B and B2) were separated each other. The fractions of A group were further purified by reversed phase HPLC, using a packed column of YMC-Pack ODS AM323 (2 × 25 cm, flow rate 3 ml/minute) and 75% aqueous MeOH as the developing solvent to yield pure cochleamycin A (10.0 mg) and A2 (8.2 mg). The fractions of B group were chromatographed on a column of silica gel with Hexane - EtOAc (3 : 1) to give pure B (6.7 mg) and B2 (6.5 mg).

Biologial Activities of Cochleamycins

Cochleamycins were tested for their *in vitro* cytotoxicity. IC_{50} (μg/ml) values against tumor cells are shown in Table 3. Cochleamycin A and A2 showed antimicrobial activity against Gram-positive bacteria as shown in Table 4.

Experimental

Cytotoxicity on Tumor Cells

Tumor cells were cultured in RPMI-1640 medium supplement with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS). The cells at 5×10^4/ml were incubated with testing agents in a humidified atomosphere of 5% CO_2 in air at 37°C for 2 days. The cytotoxic activities were essentially measured according to an automated colorimetric assay based on the production of dark formazan crystals by living cells incubated with the tetrazolium salt MTT. The results are expressed as IC_{50} values which are concentrations of the drug that inhibit cell growth by 50%.

Antimicrobial Activity

The antimicrobial spectrum of cochleamycins were determined using 8 mm paper discs (Toyo Seisakusho Co., Ltd.) and Nutrient agar medium (Difco) for bacteria and Saburoud agar medium (Difco) for yeasts.

Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for yeasts.

Acknowledgments

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