NEW 5′-NUCLEOTIDASE INHIBITORS, MELANOCIDIN A AND MELANOCIDIN B

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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New 5′-nucleotidase inhibitors named melanocidins A and B were produced by *Nocardioides* sp. 1681 J. They were isolated from fermentation broth by trichloroacetic acid extraction, ethanol precipitation and CM-cellulose and DEAE-cellulose column chromatography. They inhibited 5′-nucleotidase activity from snake venom but not from rat liver membrane at 200 μg/ml. They also show antitumor activity against melanoma B16.

In the course of screening for new inhibitors, we found and reported[1] a new compound named nucleoticidin inhibiting 5′-nucleotidase activity present in snake venom and rat liver membrane. Because nucleoticidin also showed antitumor activity, 5′-nucleotidase inhibitors may form a group of compounds which are useful for the analysis of role of 5′-nucleotidase in the membrane and as antitumor substances.

Here, we will report other 5′-nucleotidase inhibitors named melanocidins A and B which also show antitumor activity.

Assay Method of 5′-Nucleotidase Inhibitors

5′-Nucleotidase and its inhibitory activities were determined by the method described previously.[1]

Taxonomic Studies

Morphology

Thestrain 1681 J grows well on various natural and synthetic media and shows the following characteristics (Table 1): On agar and liquid media, substrate mycelia grow well and branch in gray or yellowish gray in color, and 0.5 to 0.9 μm in diameter. The aerial mycelia grow on the substrate mycelia with or without branch and 0.5 to 1.9 μm in diameter. This strain is Gram-positive but not acid-fast and does not show spore and pleomorphism.

Cultural and Physiological Characteristics

The cultural and physiological characteristics of the strain 1681 J are summarized in Table 2. D-Glucose, sucrose, D-xylose, D-fructose and mannitol were used as a source of carbon, but the following compounds could not be used: L-arabinose, inositol and raffinose. The composition was analyzed on the whole-cell hydrolysate by the method of LECHEVALIER and LECHEVALIER[2] and was found to

Table 1. Cultural characteristics.

Sucrose - nitrate agar	G:	Poor
	AM:	None
	R:	Poor, colorless
	SP:	None
Glycerol - asparagine agar*	G:	Moderate, gray
	AM:	Gray
	R:	Gray
	SP:	None
Starch - inorganic salts agar*	G:	Good, gray
	AM:	Gray
	R:	Gray
	SP:	None
Tyrosine agar*	G:	Good, gray
	AM:	Gray
	R:	Gray
	SP:	None
Nutrient agar	G:	Good, gray
	AM:	Gray
	R:	Gray
	SP:	None
Yeast extract - malt extract agar*	G:	Good, gray
	AM:	Gray
	R:	Gray
	SP:	None
Oatmeal agar*	G:	Good, gray
	AM:	Gray
	R:	Gray
	SP:	None
Bennett agar	G:	Good, gray
	AM:	Gray
	R:	Gray
	SP:	None
Peptone - yeast extract - iron agar*	G:	Good, gray
	AM:	Gray
	R:	Gray
	SP:	None

* Medium recommended by International Streptomyces Project.

Abbreviation: G, growth of vegetative mycelium; AM, aerial mycelium; R, reverse; SP, soluble pigment.

Table 2. Physiological properties.

Temperature for growth	
Growth	15~37°C
Optimum temperature	33°C
Liquefaction of gelatin	+
Reaction in milk	Peptonized and coagulated
Reduction of nitrate	−
Hydrolysis of starch	+
Melanin formation	
Tyrosine agar	−
Peptone - yeast extract - iron agar	−
Hydrogen sulfide formation	−

Fig. 1. Electron micrograph of *Nocardioides* sp. 1681 J.

Bar represents 1 μm.

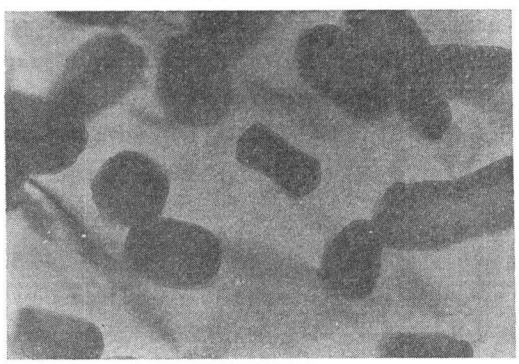


Table 3. Comparison of taxonomic characteristics of strain 1681 J and *Nocardioides albus*.

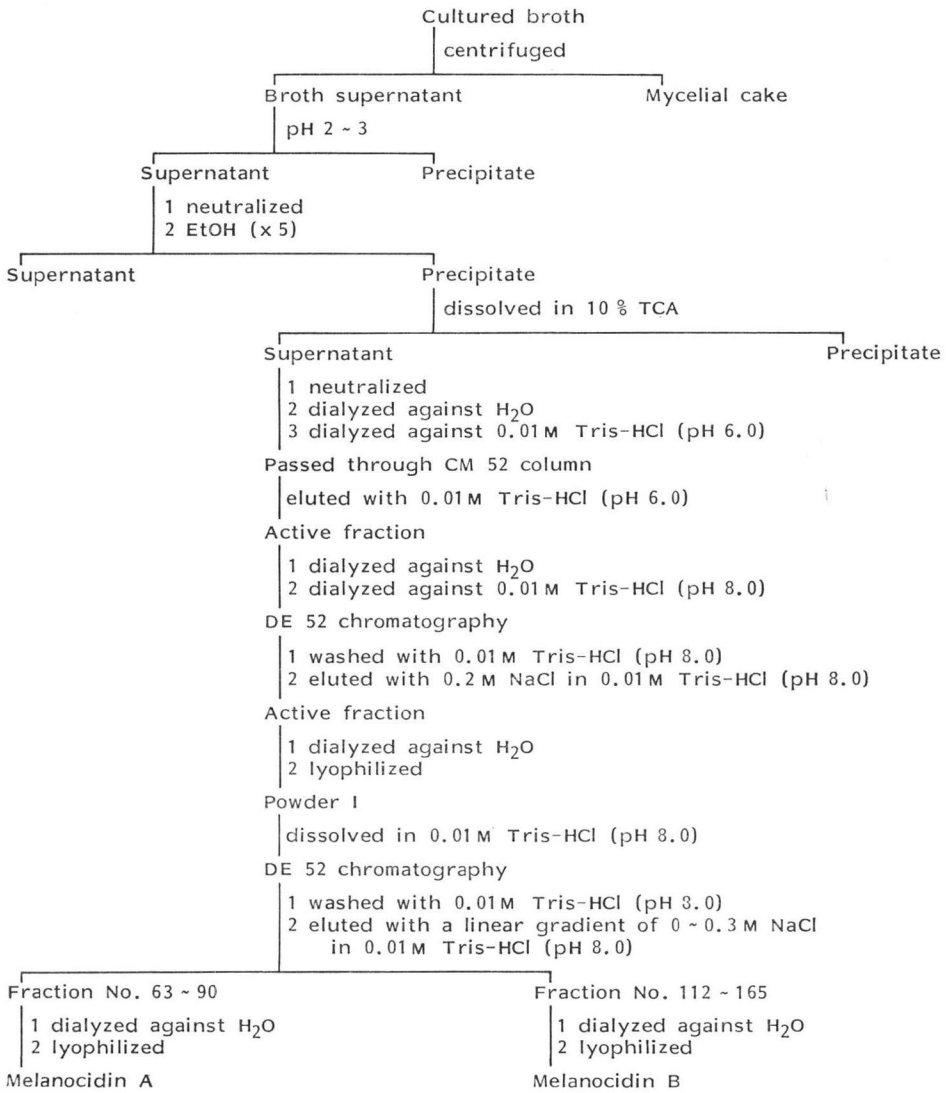
	1681 J	*N. albus*
Substrate mycelium	0.5~0.9 μm Gray	0.5~0.8 μm White
Aerial mycelium	0.5~1.0 μm Gray	0.6~1.0 μm White or pale yellow
Temperature for growth		
Growth	15~37°C	18~37°C
Optimum temperature	33°C	28°C
Hydrolysis of starch	+	+
Hydrogen sulfide formation	+	+
Melanin formation	−	−
Carbohydrate utilization		
L-Arabinose	−	+
D-Xylose	+	+
D-Glucose	+	+
D-Fructose	+	+
Sucrose	+	+
Inositol	−	−
Raffinose	−	−
D-Mannitol	+	+
Cell-wall analysis	Type I	Type I

contain LL-2,6-diaminopimelic acid, alanine, glutamic acid and glycine. Mycolic acid can not be detected. This indicates that the strain belongs to the cell-wall type I bacteria. Based on the properties described above, the strain 1681 J was similar to *Nocardioides albus*; the only differences were noted for the color of aerial mycelium, optimum temperature for growth and utilization of carbon sources, as shown in Table 3. From these results, strain 1681 J was designated as *Nocardioides* sp. 1681 J. The electron microscopic picture of the strain is shown in Fig. 1.

Fermentation

A loopful of mycelial growth of strain *Nocardioides* sp. 1681 J on an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 50 ml of a seed medium composed of dextrin 4.0%, corn steep liquor 0.5%, glucose 0.5%, Polypeptone 0.5%, yeast extract 0.5%, meat extract 0.5%, brain heart infusion 1.0% and $CaCO_3$ 0.2%. The pH of the medium was adjusted to pH 8.0 before sterilization.

Fig. 2. Purification of melanocidins A and B.



The flask was incubated on a rotary shaker at 27°C for 2 days. A 2 ml aliquot of the culture was inoculated into a 500-ml Erlenmeyer flask containing 60 ml of a production medium composed of starch 4.5%, glycerol 0.75%, corn steep liquor 0.1%, gluten meal 0.1%, soybean meal 2.5%, Casamino Acids 0.3% and $FeSO_4$ 0.002% (pH 7.2 before sterilization) and fermentation was continued at 27°C for 4 days with shaking.

Isolation of Inhibitors

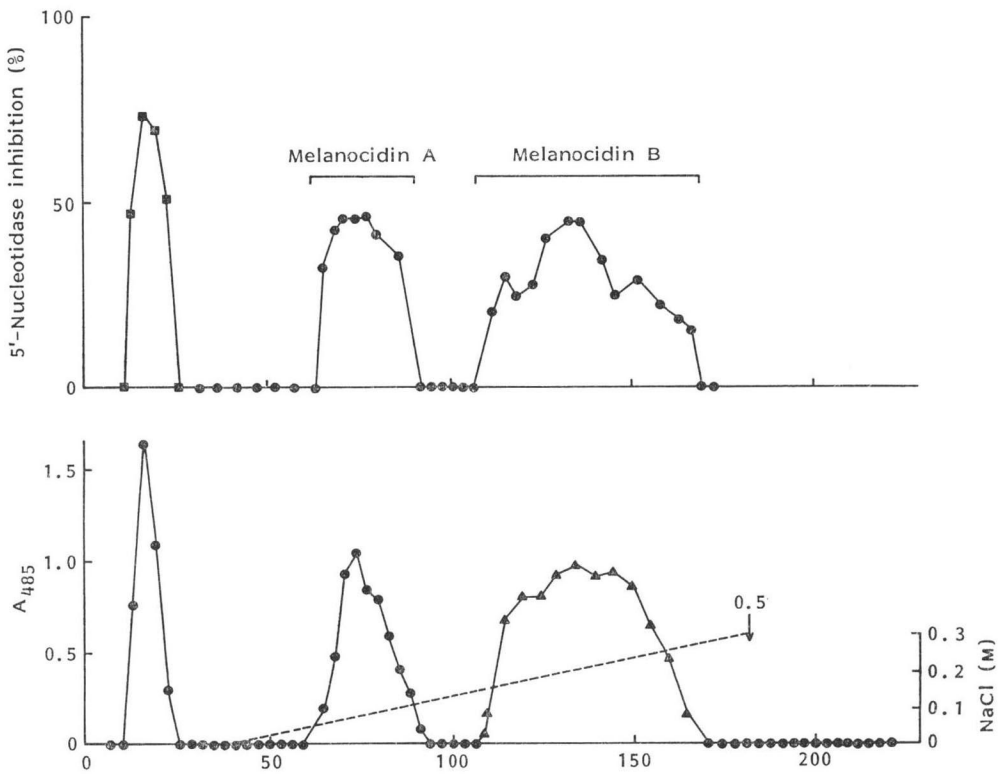
The purification procedure of melanocidins A and B is outlined in Fig. 2 and Table 4.

After fermentation was completed, the cultured broth was centrifuged to remove mycelial cake. The supernatant was acidified (pH 2~3) with hydrochloric acid, and the resulting precipitate was removed by centrifugation. The clear supernatant was neutralized and 5 volumes of ethanol were added. The precipitate was dissolved in 10% trichloroacetic acid, and insoluble material was removed

Table 4. Purification of melanocidins A and B.

Purification step	Total weight (mg)	Total activity ($\times 10^3$ units)	Specific activity (units/mg)	Yield (%)
Broth supernatant	4,000 ml (20 mg/ml)	216	2.7	
EtOH	15,007	120	8	56
CM 52	650 ml	71	109 (units/ml)	33
DE 52 (0.2 M NaCl)	3,034	52	17	24

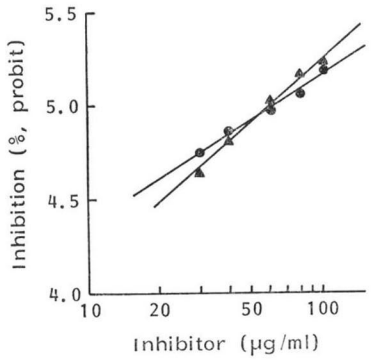
Fig. 3. DE 52 chromatography of melanocidins A and B.



by centrifugation. The supernatant was made neutral and then dialyzed against water for 3 days and then against 0.01 M Tris-HCl (pH 6.0) for 1 day. The inner solution in dialysis bags was passed through a column of CM-cellulose (CM 52, Whatman), equilibrated with 0.01 M Tris-HCl (pH 6.0), and the effluent was dialyzed against water for 3 days and then against 0.01 M Tris-HCl (pH 8.0) for 1 day. The non-dialyzable fraction was adsorbed on a column of DEAE-cellulose (DE 52, Whatman), equilibrated with 0.01 M Tris-HCl (pH 8.0). After washed with the same buffer, the column was eluted with 0.2 M sodium chloride in the same buffer, and the active fraction was dialyzed against water for 3 days and then lyophilized (powder I).

The powder I (4 g) was dissolved in 0.01 M Tris-HCl (pH 8.0), and the solution was fractionated by column chromatography on DE 52, equilibrated with the same buffer. After washed with the same buffer, the column was eluted with a linear gradient of 0.1~0.3 M NaCl in the same buffer. The eluted fractions were monitored by the 5'-nucleotidase inhibition assay and phenol-sulfate method.[3] Melanocidins A and B were separately eluted, as shown in Fig. 3. The fractions containing each component were combined, dialyzed and lyophilized. Thus, two components were obtained from powder I: 1.12 g of melanocidin A (18 units/mg), and 1.97 g of melanocidin B (17 units/mg).

Fig. 4. Effect of melanocidins A (▲) and B (●) on 5'-nucleotidase activity.



Biological Properties

Melanocidins A and B inhibited 5'-nucleotidase activity from snake venom (*Crotalus atrox*, Sigma) (Fig. 4) but not that from rat liver membrane[4] at 200 μg/ml. They also show antitumor activity against melanoma B16, but they did not reduced the growth of Sarcoma 180 solid tumor in rat (10 mg/kg). The details of the biological studies will be published elsewhere.

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