CADEGUOMYCIN, A NOVEL NUCLEOSIDE ANALOG ANTIBIOTIC

I. THE PRODUCING ORGANISM, PRODUCTION AND ISOLATION OF CADEGUOMYCIN

NOBUO TANAKA*, RONG TSUN WU, TAKAYOSHI OKABE, HIDE YAMASHITA, AKIRA SHIMAZU and TOSHIO NISHIMURA

Institute of Applied Microbiology, University of Tokyo, Tokyo 113, Japan

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An actinomycete strain IM7912T was found to produce cadeguomycin, a new nucleoside analog antibiotic, together with tubercidin. Morphological, cultural and physiological studies showed that the organism belongs to the species *Streptomyces hygroscopicus*. Comparisons with *S. hygroscopicus* ISP 5578 revealed that both strains possess similar characteristics except for production of yellow or brownish yellow soluble pigment in some media. Cadeguomycin was isolated from the culture filtrate, separated from tubercidin, and purified.

In the course of our screening program for new antitumor antibiotics, a soil actinomycete strain IM7912T identified as *Streptomyces hygroscopicus* was observed to produce tubercidin together with a principle which enhances [^{3}H]thymidine incorporation into DNA and [^{3}H]uridine uptake into RNA of some tumor cells in tissue culture. The latter substance was isolated and purified from the culture fluid, and found to be a new nucleoside analog antibiotic. The novel agent was designated cadeguomycin, because the chemical structure is 7-carboxy-7-deazaguanosine and the producing organism belongs to the genus *Streptomyces*. The antibiotic exhibited inhibitory effects on transplantable animal tumors, but no significant antimicrobial activity against bacteria and fungi. The taxonomy of the producing organism, fermentation and isolation of cadeguomycin are presented in the current publication. An improved purification, physicochemical properties and chemical structure will be reported in the following paper[1], and the biological activities elsewhere.

Taxonomy of the Producing Organism

The organism was isolated from a soil sample, collected at Osaka Castle, Osaka City, Japan, in May, 1979. Taxonomic studies on the culture principally followed the methods adopted by the International Streptomyces Project (ISP)[2].

Morphological Characteristics

Microscopic and electron microscopic examination showed that fairly long, straight and flexuous aerial mycelia were formed from the branched mycelia grown in yeast - malt agar and other agar media. Tight spiral spore-chains were observed in dense clusters on the mature aerial hyphae (Plates 1 and 2), and later coalesced as dark and moist masses of spores on some media. Mature spore-chains contained more than 10 spores per chain. The spore shape was short-cylindrical or elliptical ($0.5\sim0.6\times0.6\sim0.8$ μm), and the spore surface was warty or rugose[3], as seen by the electron microscope (Plate 3). Whirls and other special morphologies were not observed.

* To whom all correspondence should be addressed.

Plate 1. Aerial mycelia of strain IM7912T (yeast extract - malt extract agar, 27°C, 9-day culture; ×600).
The inserted scale is 5 μm.

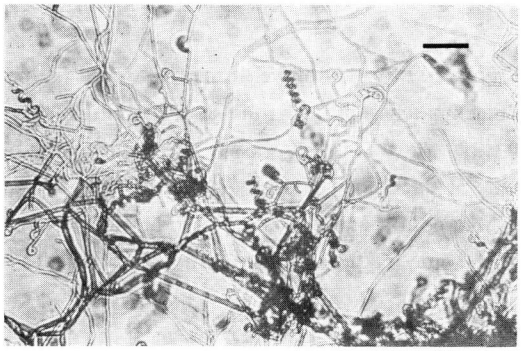


Plate 2. Electron microphotograph of tight spiral spore-chains of strain IM7912T (yeast extract - malt extract agar, 27°C, 2-week culture; A ×3,000, B ×14,000).
Inserted scales are 1 μm.

A B

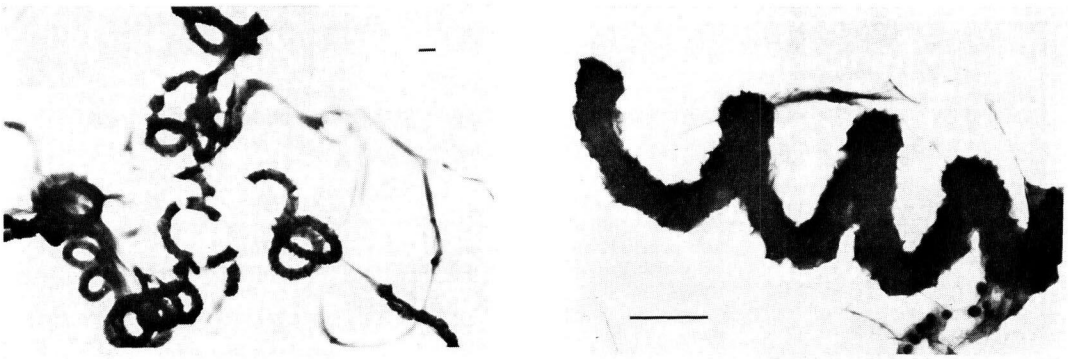
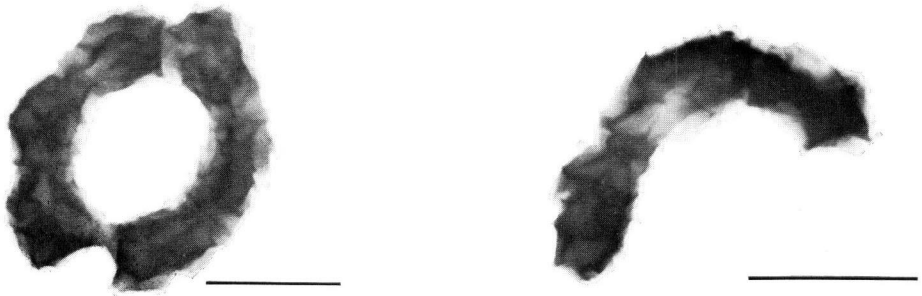


Plate 3. Electron microphotograph of spore-chains of strain IM7912T (yeast extract - malt extract agar, 27°C, 2-week culture; A ×24,000, B ×30,000).
Inserted scales are 1 μm.

A B



Cultural Properties

For experiments on cultural characteristics, all the cultures were incubated at 27°C and were observed on 3, 7, 14 and 21 days. The color recorded for mature cultures was described according to the "Color Harmony Manual"[4].

Aerial mycelium was abundantly formed on yeast-malt agar and oatmeal agar media, showing hygroscopic property or moist black patches in the mature stage of culture. The color of mature sporulated aerial mycelium was in the Gray series[5] (whitish gray to gray), but immature aerial hyphae displayed a reddish tint. The reverse side of the colony had no distinctive pigment (pale yellow to pale yellowish brown or brownish gray). Melanoid pigment was not produced, but yellow or yellowish brown diffusible pigment was formed in some media. The cultural characteristics grown on various media at

Table 1. Cultural characteristics.

Medium		Strain IM7912T	Strain ISP 5578
Sucrose - nitrate agar	G*	moderate	moderate
	AM	moderate, powdery; whitish color	moderate, powdery; ashes (5fe)
	R	pearl (2ba)	ashes (5fe)
	SP	none	none
Glucose - asparagine agar	G	moderate	moderate
	AM	moderate, powdery; beige (3ge)	moderate, powdery; silver gray (3fe)
	R	beaver (3il)	pearl (3ba) to ashes (5fe)
	SP	light yellow (1ea)	none
Glycerol - asparagine agar (ISP-5)	G	poor	poor
	AM	poor, powdery; whitish color	poor, powdery; whitish color
	R	whitish color with pearl (2ba)	pussywillow gray (5dc)
	SP	none	none
Inorganic salt - starch agar (ISP-4)	G	good	good
	AM	abundant, powdery; beige (3ge) and pearl (1ba)	abundant, velvety; ashes (5fe) and beaver (3li)
	R	brownish gray (2ig) to ivory (2db)	pussywillow gray (5dc) to ashes (5fe)
	SP	trace	none
Tyrosine agar (ISP-7)	G	poor	moderate
	AM	poor, powdery; whitish color	moderate, powdery; shadow gray (5ih)
	R	beige (3ge)	ashes (5fe)
	SP	cinnamon (3le)	none
Nutrient agar	G	poor	poor
	AM	poor, powdery; whitish color and ivory (2db)	poor, powdery; whitish color and pearl (2ba)
	R	light ivory (2ca)	pearl (2ba) and ivory (2db)
	SP	none	none
Yeast extract - malt extract agar (ISP-2)	G	good	good
	AM	abundant, velvety; ashes (5fe)	abundant, velvety; ashes (5fe)
	R	yellow (2ia) and mustard (2le)	silver gray (3fe)
	SP	gold (1 ½ lc)	none
Oatmeal agar (ISP-3)	G	good	good
	AM	abundant, velvety; beige (3ge)	abundant, powdery; beige gray (3ih) and ashes (5fe)
	R	silver gray (3fe)	shadow gray (5ih)
	SP	cream (1 ½ ca) or dusty yellow (1 ½ gc)	none

* Abbreviation: G, growth; AM, aerial mycelium; R, reverse color of substrate mycelium; SP, soluble pigment.
Incubation at 27°C for 2 weeks.
Color and number in parenthesis followed the color standard in Reference 4.

27°C for 2 weeks are summarized in Table 1.

Physiological Studies

The organism grew in a range of 20~37°C on yeast - malt agar medium: temperature optimum 25~35°C.

The utilization of carbon compounds was investigated by the method of PRIDHAM and GOTTLIEB[6]. The following carbohydrates supported growth as a sole carbon source in PRIDHAM-GOTTLIEB basal agar medium: D-glucose, L-arabinose, D-fructose, D-xylose, D-mannitol, sucrose, rhamnose, raffinose and inositol (Table 2). However, the requirement of carbon compounds for the growth could not be absolutely determined, because the organism grew to a considerable extent without carbohydrate. Gelatin was liquefied in glucose-peptone-gelatin medium, with liquefaction starting about a week after inoculation.

Table 2. Utilization of carbon sources.

Carbohydrate	Growth	
	IM7912T	ISP 5578
D-Glucose	+	+
L-Arabinose	+	±
D-Fructose	+	+
D-Xylose	+	+
D-Mannitol	+	+
Sucrose	+	±
Rhamnose	+	+
Raffinose	+	±
Inositol	+	±

The basal medium used was ISP medium 9.

Skim milk was hardly coagulated, but was peptonized in *ca.* 2-week culture. Starch was hydrolyzed in inorganic salt-starch medium. The hydrolysis began to appear approximately 5 days after inoculation (Table 3).

The cell wall composition was analyzed by the procedure of BECKER *et al.*[7], showing LL-diaminopimelic acid. It was classified as Type I, indicating that strain IM7912T belongs to the genus *Streptomyces*.

Comparison with *Streptomyces hygroscopicus* ISP 5578

The morphological, cultural and physiological characteristics of the organism, described above, revealed that strain IM7912T is similar to *Streptomyces hygroscopicus* according to KÜSTER's classification[8]. Therefore, comparative studies with *S. hygroscopicus* ISP 5578 were carried out by simultaneous culture experiments with both organisms. Strain ISP 5578 was kindly given by Dr. M. HAMADA, Institute of Microbial Chemistry, Tokyo.

Both strains IM7912T and ISP 5578 possessed the following common characteristics:

(1) The aerial mycelium formed tight spiral spore-chains.
(2) The spore surface was warty or rugose.
(3) Growth characteristics on various media were similar (Table 1).
(4) The color of mature sporulated aerial mycelium was in the Gray series[5] (whitish gray to gray). However, the gray color of ISP 5578 was darker than that of IM7912T (Table 1).

Table 3. Physiological characteristics.

	Medium	IM7912T	ISP 5578
Starch hydrolysis	inorganic salt - starch agar	+	+
Gelatin liquefaction	glucose - peptone - gelatin	+	+
Milk coagulation	skim milk	−	−
Milk peptonization	skim milk	+	+
Melanoid pigment production	tryptone - yeast extract broth	−	−
	tyrosine agar	−	−
	peptone - yeast extract iron agar	−	−

(5) The mature culture showed hygroscopic property or moist black patches on yeast-malt agar, oatmeal agar and other agar media (Table 1).

(6) Melanoid pigment was not produced (Tables 1 and 3).

(7) Both strains displayed similar characteristics in hydrolysis of milk, gelatin and starch (Table 3).

On the other hand, strain IM7912T was differentiated from *S. hygroscopicus* ISP 5578 in that the former produced a yellow or yellowish brown diffusible pigment on some media (Table 1).

The comparative investigation showed that strain IM7912T belongs to the species *Streptomyces hygroscopicus*.

Fermentation

Vegetative inoculum of cadeguomycin-producing culture was obtained by transferring spores of *S. hygroscopicus* IM7912T to a 500-ml Erlenmeyer flask containing 100 ml of an inoculum medium of 2% oatmeal and 0.1% yeast extract, pH 7.1. The inoculated medium was incubated at 27°C for 40 hours on a rotary shaker operating at 180 rpm. For jar fermentation, 300 ml of the inoculum growth was added to 15 liters of the same production medium used in shake flask fermentation in a 40-liter jar fermentor, which was stirred at an impeller speed of 400 rpm and aerated at 15 liters per minute. The fermentation was terminated after 114 hours of incubation at 27°C.

Isolation of Cadeguomycin

A flow diagram for isolation of cadeguomycin is illustrated in Fig. 1. The culture broth of *S. hygroscopicus* IM7912T was found to contain both tubercidin and cadeguomycin.

The antibiotics in the culture filtrate (*ca.* 12 liters) were adsorbed on an Amberlite XAD-8 column, and eluted with 18 liters of methanol, which was then concentrated to approximately

Fig. 1. Isolation and purification of cadeguomycin.

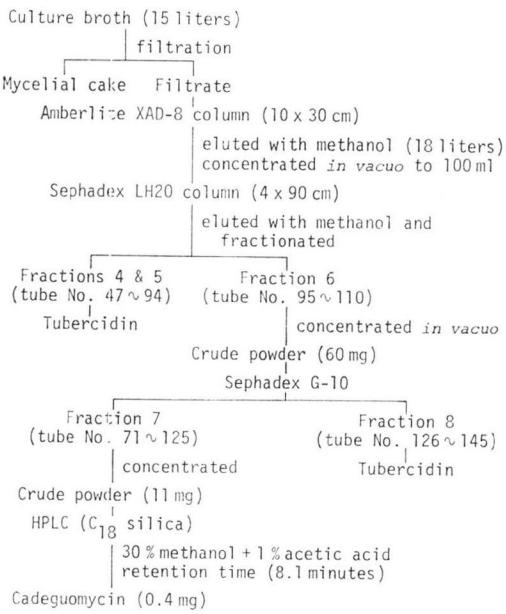
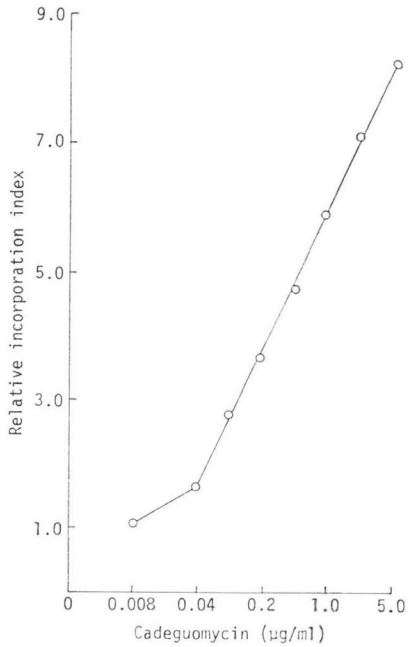


Fig. 2. Uptake of [^{3}H]thymidine into DNA of K562 human leukemic cells.

The ratio of [^{3}H]thymidine incorporation in the presence and absence of cadeguomycin was taken as the stimulation index or relative incorporation index.



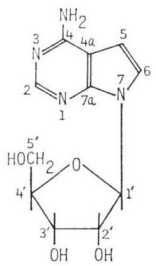
100 ml. The concentrate was applied to a column of Sephadex LH20, and eluted with methanol. The eluate was divided into 6 fractions, and each fraction was passed through a Sephadex G-10 column, from which cadeguomycin came out faster than tubercidin. Cadeguomycin was detected in the 6th fraction (tube No. 95~110, 12 g solution per tube) of the LH20 fractionation and in the 7th fraction (tube No. 71~125, 7 g solution per tube) of the G-10 chromatography. The latter fraction was subjected to reverse phase HPLC (5 μm C_{18}/Nucleosil) with 30% methanol plus 1% acetic acid, yielding fine needle crystals of cadeguomycin (*ca.* 400 μg) after removal of the solvent.

Bioassay

Cadeguomycin displayed no antimicrobial activity. The antibiotic was assayed by enhancement of [^{3}H]thymidine incorporation into DNA of K562 human leukemic cells. Since tubercidin inhibited the uptake of [^{3}H]thymidine, the activity of cadeguomycin could be detected only after both antibiotics were separated in the isolation procedure of culture broth.

A microplate method was employed for the assay. The K562 cells were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum; and was distributed into microwells (Nunc, Denmark) in the presence or absence of the fractionation samples. Each microwell contained 10^4 cells/0.2 ml. The mixtures were incubated at 37°C for 4 hours in a 5% CO_2 and 95% air atmosphere, and then [^{3}H]thymidine (0.2 μCi/well) was incorporated for 2 hours. The cells were collected and washed in phosphate-buffered saline and then in 5% cold TCA in a multiple automated sample harvester (Abe Kagaku). The radioactivity was determined in a liquid scintillation counter (Beckman); and the ratio of [^{3}H]thymidine incorporation in the presence and absence of cadeguomycin was taken as the stimulation index.

Table 4. 400 MHz ^{1}H NMR of tubercidin in CD_3OD.



Proton	Chemical shift* (ppm)	Multiplicity**		Coupling constant (Hz)
2-H	8.05	1H	s	
5-H	6.59	1H	d	$J_{5,6}=3.6$
6-H	7.30	1H	d	$J_{6,5}=3.6$
1'-H	5.97	1H	d	$J_{1',2'}=6.5$
2'-H	4.65	1H	dd	$J_{2',1'}=6.5$, $J_{2',3'}=5.4$
3'-H	4.27	1H	dd	$J_{3',2'}=5.4$, $J_{3',4'}=2.7$
4'-H	4.11	1H	ddd	$J_{4',3'}=2.7$, $J_{4',5'}=2.7$, 2.9
5'-H_2	3.73	1H	dd	$J_{5',4'}=2.7$, $J_{5',5'}=12.2$
	3.84	1H	dd	$J_{5',4'}=2.9$, $J_{5',5'}=12.2$

* Chemical shifts in ppm relative to internal TMS.

** s=singlet, d=doublet, dd=double doublet, ddd=double double doublet.

The potency of cadeguomycin was expressed by the stimulation index. A typical dose-response curve is illustrated in Fig. 2. A linear relationship was observed between the stimulation index and logarithm of antibiotic concentration in a drug concentration of 0.04~5.0 μg/ml.

Purification and Identification of Tubercidin

Fraction 4 in Sephadex LH20 chromatography (Fig. 1) was applied to a Sephadex G-10 column, from which tubercidin was eluted with 0.02 N HCl after washing the column with distilled water. A crude powder (61.9 mg) was obtained from the eluate after neutralization with Amberlite IRA-45 and removal of water, and was then subjected to HPLC C_{18} column with H_2O - methanol - acetic acid (70: 30: 1), yielding 21.5 mg of colorless needle crystals, which was identified as tubercidin by UV and IR spectra, and by ^1H NMR.

The UV spectrum showed maxima at 227 nm and 270 nm in 0.01 N HCl, and a peak at 270 nm in H_2O and in 0.01 N NaOH. The IR spectrum showed ν_{max}^{KBr} 3320, 1645, 1605, 1570, 1430, 1320, 1265, 1145, 1050, 1025 and 1000 cm^{-1}. The ^1H NMR signals are presented in Table 4.

Acknowledgements

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