LEINAMYCIN, A NEW ANTITUMOR ANTIBIOTIC FROM *STREPTOMYCES*; PRODUCING ORGANISM, FERMENTATION AND ISOLATION

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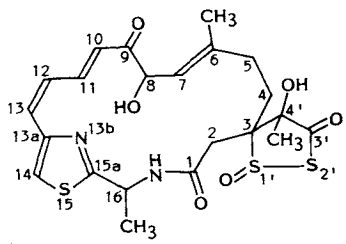
Leinamycin (DC 107) is newly discovered antitumor antibiotic with an unusual 1,3-dioxo-1,2-dithiolane structure. Five different producing strains were isolated from soils collected in Japan during 1985~1988 and were taxonomically assigned as *Streptomyces*. Fermentation studies indicate: Leinamycin was unstable in culture broth. A chemically defined medium could be designed for a preferable production. *Streptomyces* sp. S-140 grew on medium supplemented with Zn^{2+} and high porus polymer resin and accumulated 32 μg/ml of leinamycin. Improved isolation methods are described along with identification of mikamycin A co-produced with leinamycin by the strain S-140.

Leinamycin, which was formally denoted as DC 107, is a newly discovered antitumor antibiotic produced by *Streptomyces* sp.[1]. The structure of leinamycin was revealed by X-ray crystallography[2] and confirmed by spectroscopic and chemical analysis[3]. It contains an unusual 1,3-dioxo-1,2-dithiolane moiety which is connected to the 18-membered lactam ring through spiro-linkage (Fig. 1). In preliminary communication[1] we have reported its production, isolation, physico-chemical properties and potent antitumor activity on murine leukemia P388.

In the course of our continued screening program in 1987~1988 for new antitumor antibiotics, we have found that additional four actinomycetes isolated from soil samples collected in various area of Japan are producers of leinamycin which could not be detected in the screening during 1975~1984. We report here the taxonomic comparison of the producing strains, increased production through manipulation of the culture conditions and improved isolation method of leinamycin.

Fig. 1. Structure of leinamycin.



Materials and Methods

Taxonomic Studies

Growth characteristics and carbohydrate utilization were determined by the methods of the International Streptomyces Project (ISP)[4]. Color codes were assigned to the substrate and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). The spores and mycelia of the strains were observed by scanning electron microscopy (scanning microscope model S-570, Hitachi Co., Ltd.).

Analysis of diaminopimelic acid was performed on the hydrolysate of cultures grown in a medium (glucose 10 g, starch 10 g, beef extract 3 g, yeast extract 5 g, $CaCO_3$ 2 g per liter of tap water, pH 7.0) for

48 hours at 28°C.

Culture Conditions

Fermentation studies were conducted by using a high producing strain, S-140. Seed medium (glucose 10 g, soluble starch 10 g, Bacto Tryptone 5 g, yeast extract 5 g, beef extract 3 g, $CaCO_3$ 2 g per liter of deionized water, pH 7.2 prior to sterilization) was inoculated with a loopful of vegetative mycelium from ISP-4 agar slant. The seed medium was incubated at 22°C for 48 hours and was transferred into a fermentation medium. Basal components of the fermentation media were KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 20 mg, vitamine B_{12} 0.1 mg and $CaCO_3$ 5 g per liter of deionized water. The pH of medium was adjusted to 7.0 prior to sterilization. Using above basal medium, the effect of carbon, nitrogen sources in the fermentation medium was investigated in 250 ml shake-flasks at 22°C for 3 days.

Culture conditions used for 6 liters jar fermenter and 2,000 liters tank fermenter were described in figure legends. A high porus polymer resin Diaion HP-20 (5%) was added to culture at 18 hours after inoculation.

Culture growth was evaluated by centrifuging untreated fermentation broth in 10-ml conical tubes at $1,200 \times g$ for 10 minutes. The packed cell solids were reported as % of total broth volume.

HPLC Analysis of Leinamycin Production

Whole fermentation broths, which was adjusted to pH 2 with 6 N H_2SO_4 were combined with equal volume of propanol and stirred for 30 minutes. After centrifugation at 300 rpm for 10 minutes, 10 μl of supernatant was subjected to HPLC analysis. HPLC was performed using a column of Davelosil ODS-N-5 (4×25 mm, 5 μm particle size, Nomura Kagaku) at 30°C. The solvent system is 0.05 M phosphate buffer (pH 4.0) - MeOH (1 : 1), the flow rate was 1 ml/minute and UV of the effluent was monitored at 320 nm. Under those conditions the retention time of leinamycin was 16.7 minutes.

Results and Discussion

Comparison of Producing Organisms

Leinamycin has an unique structure which can be classified as a new group far from the classes of known antibiotics and moreover it is difficult to find a structural similarity with the known metabolites. Following the first discovery of leinamycin from strain S-140 in 1985, four actinomycete strains were found to produce the same antibiotic. These strains were isolated from soil samples collected at various area in Japan as noted in Table 1. Thereby we compared taxonomically the producing organisms to see whether they have similar characteristics or not.

Whole cell analysis indicated the presence of LL-diaminopimelic acid in five strains. Diagnostic sugars were absent. Based on morphology and the above analysis, all leinamycin producing strains were identified as *Streptomyces*[5~9]. The vegetative mycelium of the organisms grew abundantly on complex and synthetic agar media. Yellow diffusible pigments were formed by strain S-290 and S-419 whereas no soluble pigment was produced by other three strains. Melanoid pigment was produced abundantly by strain S-419 and moderately by S-290 on tyrosine agar. Strain S-140 and S-193 formed *Rectiflexible* or *Retinaculm-Apertum* sporophores while S-290, S-392 and S-419 formed spiral spore chains. The spore surface of strain S-419 appeared hairly whereas other strains formed spore with smooth surface (Fig. 2). Thus five strains producing leinamycin are differentiated morphologically and physiologically from each other. Especially, strain S-419 showed most clear differences from other strains.

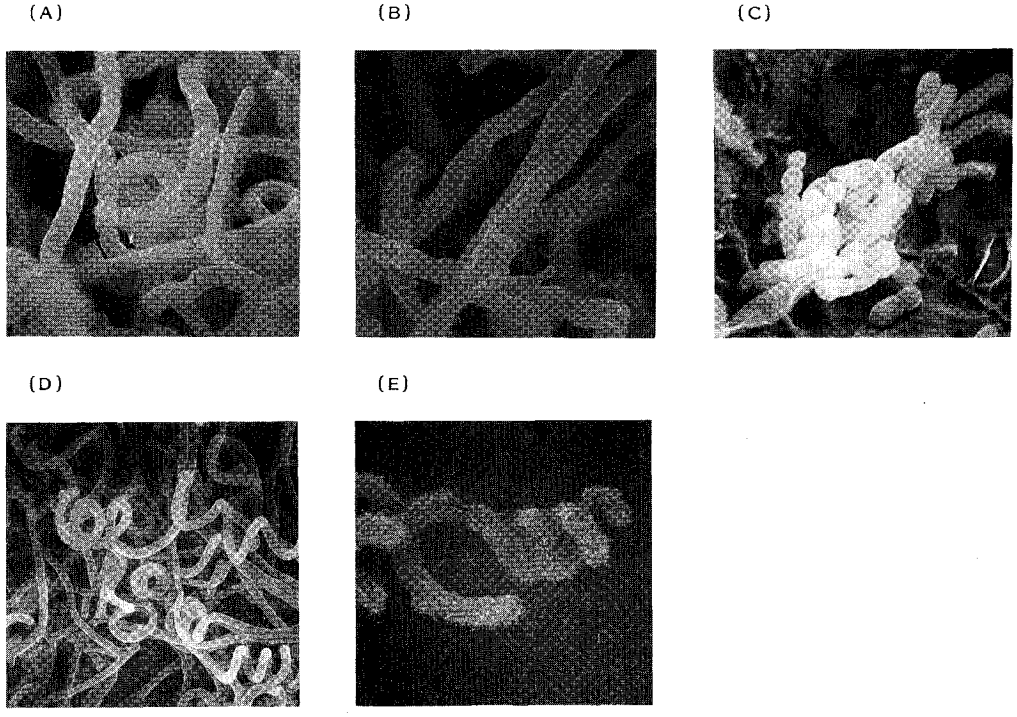
As described in following section, strain S-140 co-produced mikamycin A[10,11] while other strains did not, suggesting that biosynthesis of leinamycin and mikamycin A are not related. In preliminary experiment, we examined whether these leinamycin producing strains have some plasmid DNA, and could detect a giant plasmid in only strain S-419 (data not shown).

Table 1. Comparison of leinamycin producing strains.

	Strain				
	S-140	S-193	S-290	S-392	S-419
Spore chain	RF, RA	RF	SP	SP	SP
Spore surface	sm	sm	sm	sm	ha
Aerial mass color	Gy, W, Y	Gy, W	Gy, W, Y	Gy, W	Gy
Melanoid pigment					
Tyrosine agar	−	−	+	−	+
Peptone-yeast extract-iron agar	−	−	−	−	+/−
Soluble pigment	−	−	Y	−	Y
Liquefaction of gelatine	−	−	−	−	−
Peptonization of milk	−	+	+	+	+
Decomposition of cellulose	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+
Carbon utilization					
L-Arabinose	+	+	+	+	+
D-Xylose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	−	+	+	+
D-Mannitol	+	−	+	+	−
Sucrose	−	+	−	+	+
m-Inositol	+	−	+	−	−
Raffinose	+	+	−	+	−
L-Rhamnose	+	−	+	+	+
Collected place	Miyagi	Okinawa	Toyama	Kanagawa	Tokyo

Gy: Gray, W: white, Y: yellow, SP: *Spira*, RF: *Rectiflexible*, RA: *Retinaculm-Apertum*, sm: smooth, ha: hairly.

Fig. 2. Scanning electron micrographs of strain S-140 (A), S-193 (B), S-290 (C), S-392 (D) and S-419 (E).



Fermentation

The original media and culture condition used for the leinamycin production failed to support titers greater than 1 μg/ml[1]. Thus we developed production media for increased production of leinamycin using strain S-140 and the fermentation titers were increased to about 30 times through the following manipulation of the ingredients of culture media and addition of high porus polymer resin Diaion HP-20.

One early conclusion from experiments for optimization of leinamycin production was that the addition of a trace element dry mixture[12] to culture media stimulated the titer markedly and the effective component of the mixture was $ZnSO_4$ (Table 2). Presence of $ZnSO_4$ at $10 \sim 1{,}000$ μg/ml in media resulted in the similar increase of leinamycin production. Supplement of vitamin B_{12} increased the titer significantly. The maximum titer depend heavily upon the nature of the carbon and nitrogen sources. Soluble starch was the best carbon source and soybean meal as well as corn steep liquor were good nitrogen sources for production of leinamycin (Table 3). Strain S-140 could grow on chemically defined media containing ammonium sulfate as a sole nitrogen source and produced an almost comparable amount of leinamycin to that of the best complexed media with soybean meal or corn steep liquor. From these result, LF1 basal medium was desinged as follows: Soluble starch 40 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $ZnSO_4 \cdot H_2O$ 20 mg, vitamin B_{12} 0.1 mg and $CaCO_3$ 5 g in 1 liter of water.

Table 4 shows a typical time course of leinamycin production in LF1 with 1% soybean meal (LF1-S medium) and LF1 with 0.5% ammonium sulfate (LF1-A medium) in 6 liters jar fermenter. Leinamycin production started at 24 hours and increased reaching a maximum at about 48 hours and then decreased rapidly while cell growth continued gradually until 72 hours. The peak titers of leinamycin in LF1-S medium and LF1-A medium were 3.2 and 2.5 μg/ml, respectively. The chemically defined medium LF1-A will be useful to examine the biosynthetic pathway of the unusual structure of leinamycin.

Leinamycin was found to be unstable in culture broth; 50% degradation of leinamycin in culture filtrate were observed after 20 hours at 30°C, pH 7,

Table 2. Effect of trace metals and vitamin on production of leinamycin.

Compounds	Leinamycin[a] (μg/ml)
None	0.6
Trace element dry mix	2.3
$FeSO_4$ (30 mg/ml)	<0.1
$MnSO_4$ (15 mg/ml)	<0.1
$ZnSO_4$ (20 mg/ml)	2.8
$(NH_4)_6MoO_7O_{24}$ (4 mg/ml)	<0.1
$CuSO_4$ (1 mg/ml)	0.2
$CoSO_4$ (0.5 mg/ml)	<0.1
Vitamin B_{12} (0.1 mg/ml)	0.9

Basal fermentation medium (g/liter): Soluble starch 50, soybean meal 10, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCO_3$ 5.

[a] Leinamycin production at 44 hours was included.

Table 3. Effect of carbon and nitrogen sources on production of leinamycin.

C or N sources	Leinamycin at 48 hours (μg/ml)
(a) With soybean meal (1%) as N source	
Soluble starch	2.3
Sucrose	<0.1
Glucose	1.3
Maltose	<0.1
Lactose	<0.1
Fructose	0.1
(b) With soluble starch (5%) as C source	
Soybean meal	1.3
Corn steep liquor	1.3
Dry yeast	0.2
Pharmamedia	<0.1
Polypepton	<0.1
Yeast extract	<0.1
Soy casein	<0.1
Meat extract	<0.1
NH_4Cl	0.1
$(NH_4)_2SO_4$	1.2
NH_4NO_3	0.2
KNO_3	<0.1
$NaNO_3$	0.1
$NaNO_2$	<0.1

Basal fermentation medium (g/liter): KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCO_3$ 0.5, $ZnSO_4 \cdot 7H_2O$ 0.02, vitamin B_{12} 0.0001.

Table 4. Time course of leinamycin production in LF1-S and LF1-A media without Diaion HP-20 resin.

Time (hours)	LF1-S		LF1-A	
	Growth (PCV)	Leinamycin (μg/ml)	Growth (PCV)	Leinamycin (μg/ml)
0	5	<0.1	2	<0.1
18	16	0.8	5	<0.1
24	19	1.3	8	1.1
42	28	2.2	16	1.6
48	32	3.2	18	2.5
66	36	0.2	20	0.3
73	38	0.1	21	0.2

Fermentation medium (3 liters) in 6 liters jar fermenter were inoculated with 0.15 liter of seed culture and incubated at 28°C with agitation at an impeller speed of 400 rpm and aeration of 3 liters/minute.
PCV: Packed cell volume.

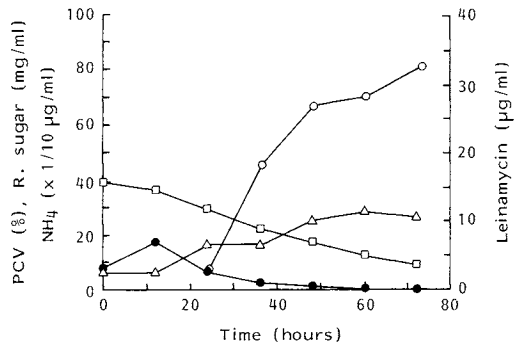
Table 5. Effect of Diaion HP-20 on production of leinamycin.

Diaion HP-20 added (%)	Leinamycin (μg/ml)	pH	PCV (%)
0	7.29	6.9	31
1.25	21.15	6.8	25
2.5	25.81	6.9	28
3.75	30.25	6.8	26
5	32.29	6.9	29
6.25	32.14	6.8	27
7.5	33.40	7.0	31

PCV: Packed cell volume.

Fig. 3. Time course of leinamycin fermentation in a 2,000-liter tank fermenter.

○ Leinamycin, △ packed cell volume (PCV), □R. (residual) sugar, ● NH_4.



Fermentation medium LF2 medium (1,000 liters) in 2,000-liter tank fermenter were inoculated with 200 liters of seed culture and incubated for 72 hours at 22°C with agitation at an impeller speed of 100 rpm and aeration of 400 liters/minute. Diaion HP-20 (5%) was added to culture medium at 18 hours after inoculation.

even without stirring and aeration. Thereby we examined the addition of high porus polymer resin Diaion HP-20 in the production medium to adsorb leinamycin and found that accumulation of leinamycin was significantly increased. Diaion HP-20 resin were added at 18 hours after inoculation and fermentation was continued further for 54 hours. As shown in Table 5, leinamycin production increased to over 30 μg/ml in the presence of 5% of the resin. The cell growth was not inhibited by added resin. Leinamycin present in culture supernatant, mycelia and Diaion HP-20 resin were 0.5, 4.0 and 95% of the total amount indicating that absorption of leinamycin by the resin in the culture could prevent the degradation and resulted in the high titer after 72 hours of fermentation. Further experiments in the presence of Diaion HP-20 resin revealed: Corn steep liquor was a good nitrogen source because of low production of co-produced mikamycin A and other undesired metabolites. L-Tryptophan (1 mg/ml) and L-methionine (0.1 mg/ml) stimulated leinamycin production. Based on the above results LF2 medium was selected for large scale fermentation. LF2 medium: Soluble starch 40 g, corn steep liquor 25 g, methionine 0.1 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot H_2O$ 0.5 g, $ZnSO_4 \cdot H_2O$ 10 mg, vitamin B_{12} 0.1 mg, $CaCO_3$ 5 g in 1 liter of water, pH 7; Diaion HP-20 resin (5%) was added at 18 hours after inoculation. Fig. 3 shows the time course of leinamycin production in 2,000 liters tank fermenter under the optimum conditions according to the above data. Cell growth continued until 60 hours. Leinamycin production started at about 24 hours and 32 μg/ml of leinamycin was accumulated during 72 hours of fermentation.

Isolation of Leinamycin and Co-produced Antibiotics

Leinamycin was isolated from the culture broth of 2,000 liters fermenter by the following improved procedure. The fermentation beer (1,000-liter) was adjusted to pH 2 with HCl and passed through a large vibrating screen (Kawasaki-juko) in order to isolate resin from mycelia. The recovered Diaion HP-20 was suspended in methanol (430 liters) and stirred for 1 hour at 5°C to elute leinamycin. Then Diaion HP-20 was removed by filtration and the filtrate containing leinamycin was concentrated. This concentrate was applied to a column of Diaion HP-20 SS (28 liters). The column was washed with 75% MeOH and leinamycin was eluted with 90% MeOH. Fractions eluted with 100% MeOH contained an co-produced antibiotic, which was identified as mikamycin A[10] by spectroscopic analysis. From the structural dissimilarity between leinamycin and mikamycin A, it is unlikely that mikamycin A is biosynthetically related to leinamycin. The 90% MeOH eluate of above Diaion HP-20 column procedure were concentrated and was applied to a stainless steel column (200 × 600 mm) containing 10 kg of 30 ~ 50 μm C_{18}-reverse phase silica gel (SOKEN ODS) packed with 0.05 M phosphate buffer - MeOH (1 : 1). The column was developed at 10 kg/cm^2 pressure and flow rate 1.2 liters/minute with the same solvent. The eluate were monitored by a UV recorder and each fraction was analyzed by HPLC. The activity eluted at retention time 1.4 hours contained minor metabolite related to leinamycin. This fraction was applied to Diaion HP-20, eluted with 100% MeOH and then concentrated. The subsequent purification by EtOAc extraction afford 50 mg of pure compound, named leinamycin M. Detailed structure analysis and biological activities of leinamycin M are currently being investigated and will be published elsewhere. The fractions containing leinamycin, eluted at retention time 1.7 hours were concentrated. This concentrate was desalted using a column with 2 liters of Diaion HP-20 resin. After 70% MeOH wash to remove phosphate, leinamycin was eluted with 100% MeOH. The eluate was concentrated about 500 mg/ml and 1 liter of CH_2Cl_2 - $CHCl_3$ (2 : 10) was added. After crystallization at 5°C for 2 days, the resulting white-needled crystals were collected and dried *in vacuo* at 30°C to afford 10 g of leinamycin.

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