ANSAMITOCINS, MAYTANSINOID ANTITUMOR ANTIBIOTICS

PRODUCING ORGANISM, FERMENTATION, AND ANTIMICROBIAL ACTIVITIES

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(Received for publication October 8, 1979)

Ansamitocins are new maytansinoid antitumor antibiotics produced by an actinomycete strain No. C-15003 (N-1). The organism is characterized by coremia formation on solid media, release of the rod-shaped motile spores with peritrichous flagella in liquid media, the formation of some branched mycelia at a later stage of growth in liquid culture which break up into motile elements, lysozyme resistance, the occurrence of *meso*-diaminopimelic acid in the cell wall, and guanine-cytosine content of 71 ± 1 mol%. From these results, the organism has been designated as *Nocardia* sp. No. C-15003 (N-1). In the fermentation fluids, activity against eukaryotic microorganisms was detected. Three of the purified materials, which have the activity against *Tetrahymena pyriformis* strain W and *Hamigera avellanea* IFO 7721, were new ansamycin antibiotics with antileukemic activities and were named ansamitocins P-3, P-3′ and P-4. Ansamitocins show growth inhibitory activity against several eukaryotic microorganisms but no activity against prokaryotic microorganisms. The acyl moieties at the C-3 position of ansamitocins are essential for their antifungal activities.

In screening for antitumor antibiotics, two distinct antimicrobial activities, *i.e.* active against eukaryotic microorganisms such as *T. pyriformis* W and against coliphage T5 were detected in culture fluids of an actinomycete strain No. C-15003 (N-1). The culture fluid having activity inhibiting the growth of eukaryotic microorganisms had antimitotic property and potent antileukemic activity against leukemia P-388 in mice (Ootsu, unpublished data). The active compounds were found to be a new group of maytansinoid antibiotics with antitumor, antiprotozoal and antifungal activities and were named ansamitocins P-3, P-3′ and P-4[1].

This paper deals with the preservation and properties of the producing organism, the fermentation studies and antimicrobial activities of ansamitocins.

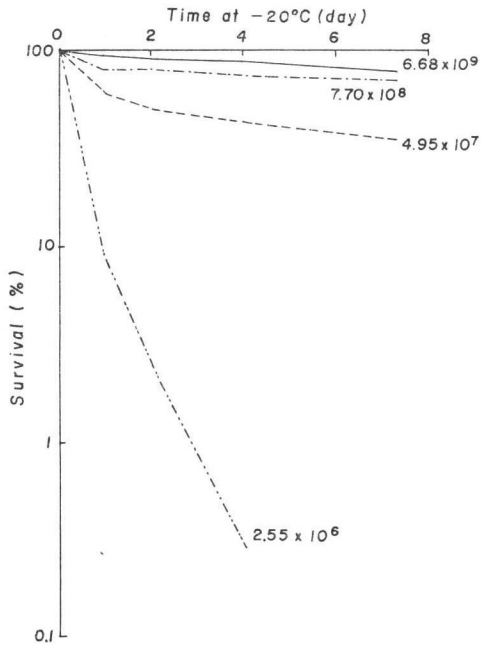
Preservation of the Producing Organism

An actinomycete strain No. C-15003 (N-1) was isolated from a natural source collected in Shiga Prefecture, Japan. The organism grew poorly in liquid media. Grown on Bennett's agar slants and preserved for 10 days at 10°C, the organism showed extremely poor growth. When cells were suspended in sterilized water or some buffer solutions and stored for 2 days at -20°C to 5°C, colony forming ability was reduced to less than one percent. In order to maintain its growing ability, cells grown on yeast-malt agar (ISP-2) for 4 days at 28°C were suspended in 40% glycerol (v/v) and stored at -20°C at which temperature the suspension did not freeze. As shown in Fig. 1, the initial cell concentration affected the survival of the organism. At an initial cell concentration of 2.55×10^6 colony

forming units (CFU)/ml, the survival ratio decreased drastically, whereas at 7.70×10^8 CFU/ml or 6.68×10^9 CFU/ml the survival decreased only slightly after 7 days. Based on these results, the organism was stored safely at $-20°C$ as a glycerol suspension at about 1×10^9 CFU/ml of the initial cell concentration. Under these conditions, an actinomycete strain No. C-15003 (N-1) has been maintained its growing ability and antibiotic productivity for more than three months.

Fig. 1. Effect of initial cell concentration on survival of *Nocardia* sp. No. C-15003 (N-1).

Cells grown on yeast extract-malt extract agar were suspended in 40% glycerol (v/v) and stored at $-20°C$. Colony forming ability was tested on yeast extract-malt extract agar plates. Numbers by lines indicate initial cell concentrations (colony forming unit/ml).



Properties of the Producing Organism

For cultural and physiological characterization, International Streptomyces Project (ISP) media suggested by SHIRLING and GOTTLIEB[2] and several media recommended by WAKSMAN[3] were used. Cultures were incubated at 28°C for up to 14 days and color determinations were done by reference to the Color Harmony Manual[4]. Carbohydrate utilization was investigated by the method of PRIDHAM and GOTTLIEB[5]. Cell walls were analyzed by the method of ŠUPUT *et al.*[6] DNA prepared by the method of MARMUR[7] was hydrolyzed by perchloric acid and the base composition was analyzed by paper chromatography (Whatman No. 1 with methanol - conc.HCl - H_2O (7: 2: 1), ascending).

The organism is characterized by the following properties; coremia formation on both complex and synthetic agar media, fragmentation of some branched mycelia at a later stage of growth in liquid culture and motility of some fragments with peritrichous flagella, release of the rod-shaped motile spores having peritrichous flagella upon immersion of the matured aerial hyphae into liquid media, containing *meso*-diaminopimelic acid, alanine, glutamic acid and galactose as major constituents in the cell wall, growth in lysozyme broth, no growth on 5% NaCl, hydrolysis of starch, casein, tyrosine and gelatin, production of nitrate reductase and alkaline phosphatase, utilization of glucose, fructose, mannose, sucrose and mannitol, and guanine-cytosine content of 71 ± 1 mol%.

From the taxonomic point of view, the actinomycete strain No. C-15003 (N-1) could not be consistent with any species of the known genera, but delayed fragmentation of vegetative mycelium, the gross appearance of colonies, lysozyme resistancy and GC content necessitated the creation of a new species placed in Group III[8] of the genus *Nocardia* and the strain was proposed as *Nocardia* sp. No. C-15003 (N-1). Detailed studies are under way to characterize *Nocardia* sp. No. C-15003 (N-1) and to compare the organism with other resembled organisms. The results of these studies will be published elsewhere.

Fermentation and Isolation

Forty milliliters of a vegetative medium (No. 6 or 7 in Table 1) in 250 ml Erlenmeyer flasks were inoculated with 1 ml of the glycerol suspension of *Nocardia* sp. No. C-15003 (N-1), and incubated on a rotary shaker for 2 days at 28°C. In preliminary fermentation studies to find suitable media, 2 ml of the vegetative culture were transferred to 250 ml flasks containing 40 ml media (No. 1 to 5 in Table 1) and incubated for 4 days at 28°C. Antibiotics produced by the organisms were assayed with *Tetrahymena pyriformis* W, *Hamigera avellanea* IFO 7721 and coliphage T5. The activity inhibiting the growth of *T. pyriformis* was assayed with PPYG (Proteose-peptone (Difco), 2%; yeast extract (Difco), 0.1%; and glucose, 0.2%) supplemented with 1 M potassium phosphate buffer (10 ml/liter) and streptomycin sulfate (100 μg/ml). A 0.5 ml portion of a culture fluid of *Nocardia* sp. No. C-15003 (N-1) was added in 4.5 ml of the medium, then 0.1 ml of *Tetrahymena* grown in PPYG was inoculated. After incubation for 2 days at 28°C, growth inhibition was judged with the naked eyes. The activity inhibiting the growth of *H. avellanea* was assayed by the paper disk method[9] for 2 days at 28°C. Antiphage activity against coliphage T5 was assayed by a paper disk method[10].

Table 2 shows antimicrobial activities of the fermentation fluids. For the production of antibiotics active against *T. pyriformis* and *H. avellanea* medium 2 in Table 1 was the most suitable. On the other hand, the activity inhibiting the plaque formation of coliphage T5 was detected in the culture of medium 1. The major antiphage component was identified with a known antibiotic tomaymycin[11].

In order to prepare the antibiotics active against *T. pyriformis* and *H. avellanea*, 1,000 ml of a seed culture with a vegetative medium (No. 6 in Table 1) were transferred to a 200-liter fermentor containing 100 liters of the vegetative medium and cultured for 2 days at 28°C. Then a 50-liter portion of the culture was transferred to a 2,000-liter fermentor containing 1,000 liters of a fermentation

Table 1. Culture media.

Component	Medium (%, w/v)						
	1	2	3	4	5	6	7
Soluble starch	3				2	3	
Dextrin		5	3	3			
Glucose	2					2	1
Glycerol							1
Soybean flour	1					1	
Corn steep liquor	1	3				1	
Peptone	0.5	0.1	0.1			0.5	1
Meat extract			2		0.4		
Yeast extract				1			0.5
$NaNO_3$				0.2			
Na_2HPO_4					0.35		
K_2HPO_4	3.5			0.1			
KH_2PO_4	1				0.15		
KCl				0.05			
NaCl	0.3					0.3	
$MgSO_4 \cdot 7H_2O$				0.05	0.1		
$FeSO_4 \cdot 7H_2O$				0.001			
$CaCO_3$	0.5	0.5				0.5	

Table 2. Fermentation results in various media.

Vegetative medium	Fermentation medium	Antimicrobial activity					
		*T. pyriformis**		*H. avellanea***		Coliphage T5**	
		4 days	6 days	4 days	6 days	4 days	6 days
6	1	−	−	0	0	0	21
	2	++	++	18	20	0	0
	3	−	−	0	0	0	0
	4	+	+	13	14	0	0
	5	+	+	14	15	0	0
7	1	−	−	0	0	0	16
	2	++	++	19	19	0	11
	3	−	−	10	13	0	0
	4	+	+	15	16	0	0
	5	+	+	13	15	0	0

The activity inhibiting the growth of *T. pyriformis* W was determined by a broth dilution method. Activity against *H. avellanea* IFO 7721 and coliphage T5 were measured by paper disk methods.

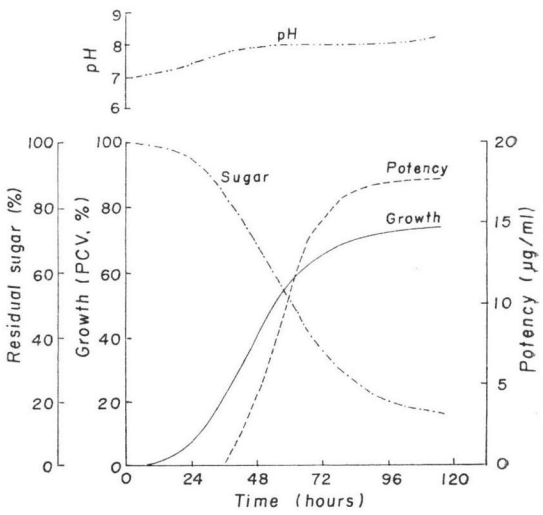
* ++, Complete inhibition; +, partial inhibition; −, no inhibition.

** Inhibition zone diameter.

medium No. 2 in Table 1 and cultured for 4 days at 28°C. Fig. 2 shows a typical time course of the fermentation. The antibiotic activity appeared in the culture fluid at about 24 hours and reached a maximum at about 96 hours. The active substances were extracted with ethyl acetate from the culture filtrate, and were chromatographed on a silica gel column using a mixture of $CHCl_3$-methanol and aqueous ethyl acetate. Five crystalline products were obtained and named P-1, P-2, P-3, P-3′ and P-4, of which P-3 and P-4 were the most abundant. P-1 and P-2 were identified with maytanacine and maytansinol propionate, respectively, which have been obtained from a plant source[12]. P-3, P-3′ and P-4 were new ansamycin antibiotics with potent antileukemic activity and named ansamitocins P-3, P-3′ and P-4. Details of isolation, physicochemical properties and chemical structures of ansamitocins have been described in the report by ASAI *et al.*[13] Fig. 3 shows the chemical structure of ansamitocins and related compounds produced by *Nocardia* sp. No. C-15003 (N-1).

Fig. 2. Time course of the production of ansamitocins.

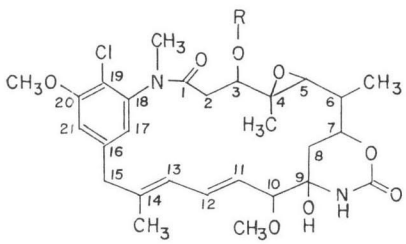
Potency was determined by a paper disk method using *Hamigera avellanea* IFO 7721 as the test organism. Growth was measured as the packed cell volume (PCV) by centrifugation at 3,000×*g* for 10 minutes.



Biological Activities

Antibacterial and antifungal activities of the purified materials were assayed by the agar dilution

Fig. 3. Structures of ansamitocins and related compounds.



R

P-3 $O=C-CH<^{CH_3}_{CH_3}$

P-3' $O=C-CH_2CH_2CH_3$

P-4 $O=C-CH_2-CH<^{CH_3}_{CH_3}$

P-1 $O=C-CH_3$

P-2 $O=C-CH_2CH_3$

P-0 H

Fig. 4. Inhibition of growth of *Tetrahymena* by ansamitocins.

Tetrahymena pyriformis W was cultured in PPYG with varying concentrations of ansamitocin P-3 or P-4. After incubation at 28°C for 2 days, 1 ml of the culture was fixed with glutaraldehyde and diluted to 10 ml with 0.9% NaCl. Cell density in the diluent was determined with a Celloscope electric particle counter model 302 (AB Lars Ljungberg, Sweden).

●, Ansamitocin P-3; ○, ansamitocin P-4.

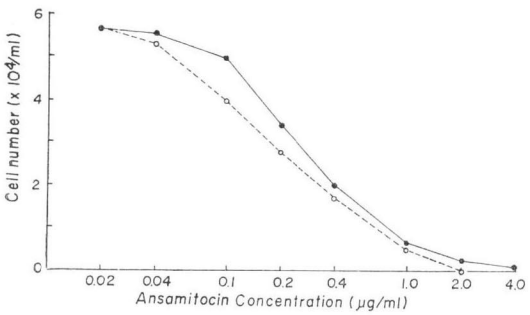


Table 3. Antimicrobial activity of ansamitocins.

Organism	MIC (μg/ml)	
	P-3	P-4
Escherichia coli K 12	>100	>100
Escherichia coli NIHJ JC-2	>100	>100
Proteus vulgaris IFO 3045	>100	>100
Proteus mirabilis IFO 3849	>100	>100
Pseudomonas aeruginosa IFO 3080	>100	>100
Salmonella typhimurium IFO 12529	>100	>100
Enterobacter cloacae IFO 12009	>100	>100
Serratia marcescens IFO 3046	>100	>100
Bacillus subtilis PCI 219	>100	>100
Bacillus cereus IFO 3514	>100	>100
Staphylococcus aureus FDA 209P	>100	>100
Mycobacterium avium IFO 3143	>100	>100
Mycobacterium smegmatis ATCC 607	>100	>100
Aspergillus niger IFO 4066	>100	>100
Penicillium chrysogenum IFO 4626	>100	>100
Hamigera avellanea IFO 7721	20	20
Tricophyton mentagrophytes IFO 7522	20	20
Candida albicans IFO 0583	>100	>100
Cryptococcus neoformans IFO 0410	5	5
Saccharomyces cerevisiae IFO 0209	>100	>100
Tetrahymena pyriformis W	2	2

The activity against bacteria and fungi were determined by the agar dilution method. The activity against *T. pyriformis* W was determined by a broth dilution method. Details were described in the text.

Table 4. Antifungal activity of ansamitocins and related compounds.

Compound	Inhibition zone (mm)
Maytansinol	0
Maytanacine	18
Maytansinol propionate	28
Ansamitocin P-3	34
Ansamitocin P-3′	34.5
Ansamitocin P-4	36

The activity was measured by a paper disk method with *Hamigera avellanea* IFO 7721 as the test organism.

method. Trypticase soy agar (BBL) was used as the assay medium for common bacteria. In the assay for acid-fast bacteria and fungi, the medium was supplemented with 3% glycerol and 1% glucose, respectively. The activity inhibiting the growth of *T. pyriformis* was assayed by broth dilution method with PPYG. Bacteria were grown for 18 hours at 37°C whereas fungi and protozoan were grown for 2 days at 28°C.

As shown in Table 3, ansamitocins P-3 and P-4 show antifungal and antiprotozoal activities but no antibacterial activity. Ansamitocins did not show any activity inhibiting the plaque formation of coliphages Qβ, MS2, f2, f1, ϕX174, T2, T4, T5 and T7 (unpublished data). As shown in Fig. 4, the antiprotozoal activity of ansamitocin P-4 against *T. pyriformis* was slightly greater than P-3. In order to compare the antifungal activities of ansamitocins and related compounds, growth inhibitory activity against *H. avellanea* was assayed by paper disk method with 100 μg/ml of the compounds (Table 4). Maytansinol (P-0) was obtained as described previously[1]. Ansamitocin P-4 showed the strongest activity and maytansinol showed no activity. Therefore, the inhibitory activities depended on their acyl moieties at the C-3 position. Similar results have been obtained from the studies on the cilia regeneration of *Tetrahymena*[14].

Discussion

Extensive efforts to find clinically useful antitumor agents have been made in many laboratories. Recently, maytansine and related maytansinoids with interesting antitumor activities were observed[12,15,16]. These compounds were isolated from tropical plants of the genus *Maytenus*, and have structural similarity of benzenic ansamycins produced by microorganisms[17]. However, the content of maytansinoids in the plants is very low, and the possibility that a microorganism might be involved in their production has been considered[9]. Our attempts to detect antibiotic producers among rarely occurring actinomycetes resulted in the isolation of *Nocardia* sp. No. C-15003 (N-1), the first microorganism to produce maytansinoid antibiotics. It is attractive to hypothesize that microorganisms such as the *Nocardia* may be involved in the production of maytansine and other maytansinoids in plants. Since ansamitocins are microbial products, *Nocardia* sp. No. C-15003 (N-1) is a useful tool for investigating the biosynthesis of maytansinoids.

Acknowledgments

We thank Drs. E. OHMURA and M. ISONO for their continued interest and encouragement, and Dr. M. ASAI and Dr. K. OOTSU for their fruitful discussion. We also thank Mr. Y. NAKANO for his technical assistance.

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