ANSAMITOCIN ANALOGS FROM A MUTANT STRAIN OF *NOCARDIA*

I. ISOLATION OF THE MUTANT, FERMENTATION AND ANTIMICROBIAL PROPERTIES

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A mutant having a high ability to produce ansamitocins was derived from a dnacin-producing strain, *Nocardia* sp. No. C-14482 (N-1001), by treatment with ethidium bromide. Mutant N-1231 produced ansamitocins P-3 and P-4 as major components, but was deficient in its ability to produce dnacins. Strain N-1231 also produced fifteen novel ansamitocin analogs as minor components. These analogs showed no activity against prokaryotic microorganisms. The results of determining the activity inhibiting cilia regeneration of deciliated *Tetrahymena pyriformis* suggest that hydroxylation of C_{15}, C_{26} and the acyl moiety at C_3 of ansamitocins may cause marked reduction of their antitubulinic activities whereas demethylation of $-NCH_3$ at C_{18} slightly affected their activities.

Ansamitocins are maytansinoid antitumor antibiotics isolated from the culture fluid of *Nocardia* sp. No. C-15003 (N-1).[1~3] In our search for new ansamitocin-related compounds, components inhibiting cilia regeneration in deciliated *Tetrahymena*[4,5] were detected in the culture fluid of a dnacin-producing organism *Nocardia* sp. No. C-14482 (N-1001),[6] but only in small amounts. In an attempt to obtain active materials, a high-producing mutant was derived from the parent strain, N-1001, by treatment with ethidium bromide. Mutant strain N-1231 produced large amounts of ansamitocins in its culture fluid, but the strain had no ability to produce detectable amounts of dnacins. The results of isolation and characterization of the active metabolites revealed that strain N-1231 had the ability to produce ansamitocins P-3 and P-4 as major components and 19 ansamitocin analogs as minor components.

This paper deals with the isolation of mutant N-1231, fermentation studies, and the antimicrobial properties of the isolated metabolites.

Materials and Methods

Isolation of a High Producing Mutant

The cell suspension of the parent strain, N-1001, prepared as described previously[3,6] was plated on yeast extract-malt extract agar (YMA) plates and incubated for 4 days at 32°C. One hundred of the resultant colonies were picked at random from YMA plates, and were then cultivated with 40 ml of a liquid medium (medium 3 in Table 1) in 200 ml flasks. After cultivation for 5 days at 28°C with shaking, a strain having a high ability to produce ansamitocins was selected by assaying the inhibitory activity against cilia regeneration of deciliated *Tetrahymena*[4,5] and growth of *Hamigera avellanea* IFO 7721.[3] The selected strain was cultivated further in a liquid medium (TYG) containing 1% glucose, 1% tryptone, 0.6% yeast extract, pH 7.0. After cultivation for 24 hours at 28°C with shaking, a 2 ml aliquot of the culture was transferred to 40 ml TYG supplemented with 5 μg/ml of ethidium bromide (Aldrich Chemicals Co. Inc.) in a 20 ml flask and cultivated for 24 hours at 28°C with shaking. The culture was filtered through filter paper (Toyo, No. 2). The filtrate was diluted with 0.02 M potas-

sium phosphate buffer, pH 7.0 and plated on YMA plates. One hundred colonies picked at random from YMA plates were cultivated with 40 ml of a liquid medium (medium 4 in Table 1) in 200 ml flasks. After cultivation for 3 days at 28°C with shaking, the mutant strain deficient in its ability to produce dnacins was selected by assaying the inhibitory activity against *Proteus mirabilis* ATCC 21100.[6]

Fermentation Studies

Forty milliliters of a vegetative medium containing 2% glucose, 3% soluble starch, 1% soybean flour, 1% corn-steep liquor, 0.5% meat extract, 0.3% NaCl and 0.5% $CaCO_3$ in a 200 ml flask were inoculated with 2 ml of the glycerol cell suspension[3,6] of the producing organism, and cultivated on a rotary shaker for 2 days at 28°C. Two milliliter aliquots of the vegetative culture were transferred to 200 ml flasks containing 40 ml of fermentation media (Table 1) and cultivated for 3~5 days at 28°C. The culture was centrifuged at 3,000×*g* for 10 minutes at room temperature, and the amount of antibiotics in the supernatant was measured by paper disk methods[3,6] with ansamitocin P-3 and dnacin B_1 as the standard. Some of the fermentations were subjected to thin-layer chromatography. In large scale fermentations, 1 liter of the vegetative culture was transferred to a 200-liter fermentor containing 100 liters of the vegetative medium and cultured for 2 days at 28°C. A 50-liter aliquot of this culture was transferred to a 2,000-liter fermentor containing 1,000 liters of fermentation medium 5 and cultured for 5 days at 28°C. Growth was measured by its DNA content described previously.[6]

Table 1. Fermentation media.

Component	%, w/v						
	1	2	3	4	5	6	7
Glycerol	3						
Sucrose					5	5	
Dextrin		5	5	5			
Soluble starch							2
Corn-steep liquor		3	3	3	3	3	
Peptone		0.1	0.1				
Yeast extract	1						0.4
$(NH_4)_2SO_4$				0.1	0.1		
$NaNO_3$	0.2					0.2	
K_2HPO_4	0.1						
KH_2PO_4							0.15
Na_2HPO_4							0.35
KCl	0.05						
$MgSO_4$	0.05						0.01
$FeSO_4$	0.001						
$CaCl_2$			1	1	1	1	
$CaCO_3$		0.5	0.5	0.5	0.5	0.5	

Thin-Layer Chromatography

Five ml of the supernatant of the culture was extracted with an equal volume of ethyl acetate. The extract was then washed with 2.5 ml of water and evaporated to dryness. The dried materials was dissolved in 50 μl of methanol, and 5 μl of this solution was subjected to thin-layer chromatography by using silica gel plates (Merck, F254) with water saturated ethyl acetate as the solvent. Densitometric analysis of the chromatogram was carried out with a Shimadzu dual wave-length TLC scanner CS-910 at 255 nm.

Assay for Antimicrobial Activities

The antibacterial activity of the ansamitocin analogs was determined by the agar dilution method.[3] Inhibitory activity against the growth of *Tetrahymena pyriformis* W was assayed by the broth dilution method.[4] Activity against cilia regeneration of deciliated *Tetrahymena* was measured by the method described previously.[4,5]

Results

Fermentation Studies

Fermentation studies were carried out in seven media (Table 1). The antibiotic yields are shown in Table 2. As compared with the parent strain N-1001, strain N-1231 produced large amounts of ansamitocins but no detectable dnacins in various media. A typical time course of the fermentation is shown in Fig. 1. After 6 days cultivation, total ansamitocins in the supernatant of the culture of N-1231 reached a maximum at about 90 μg/ml, which was more than 90 times the maximum titer of

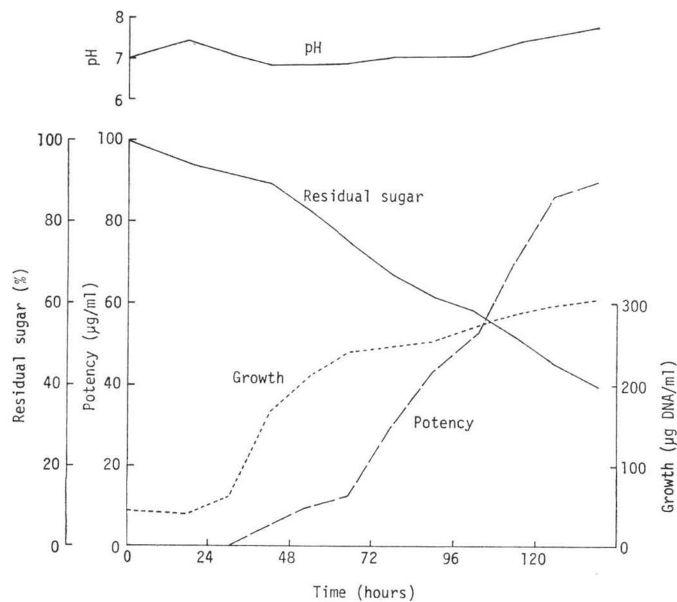
Table 2. Fermentation results.

Organism	Medium	Ansamitocins (μg/ml)		Dnacins (μg/ml)	
		3 days	5 days	3 days	5 days
N-1001	1	<1	<1	0.8	<0.5
	2	<1	<1	0.5	<0.5
	3	<1	1	1.0	<0.5
	4	<1	1	1.2	<0.5
	5	<1	<1	1.0	<0.5
	6	<1	<1	<0.5	<0.5
	7	<1	<1	<0.5	<0.5
N-1231	1	<1	<1	<0.5	<0.5
	2	11	33	<0.5	<0.5
	3	22	71	<0.5	<0.5
	4	19	59	<0.5	<0.5
	5	26	91	<0.5	<0.5
	6	<1	<1	<0.5	<0.5
	7	6	28	<0.5	<0.5

The amounts of ansamitocins and dnacins in the supernatants of the cultures were measured by paper disk methods with ansamitocin P-3[3] and dnacin B_1[6] as the standard, respectively.

Fig. 1. Time course of the fermentation with a 2,000-liter fermentor.

The strain N-1231 and the medium 5 in Table 1 were used for the fermentation. Potency was measured by a paper disk method[3] with ansamitocin P-3 as the standard.

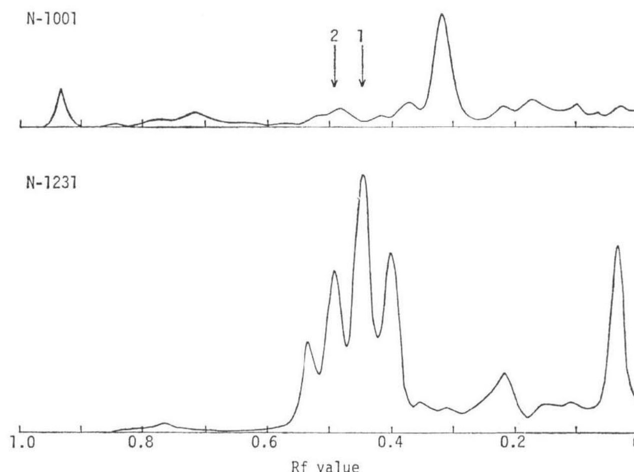


N-1001 under these conditions.

Densitometry of the chromatogram scanned at 255 nm revealed that strain N-1231 produced several UV absorbing materials in its culture fluid (Fig. 2). The results of purification and structural elucidation showed that the major components of the products were identical with ansamitocins P-3

Fig. 2. Densitometry of the thin-layer chromatogram.

The culture filtrate of *Nocardia* sp. No. C-14482 (N-1001) or the mutant strain N-1231 was extracted with ethyl acetate and subjected to silica gel thin-layer chromatography with water saturated ethyl acetate as the solvent. The developed plate was scanned at 255 nm with a TLC scanner. Arrows 1 and 2 indicate the position of ansamitocins P-3 and P-4, respectively.



and P-4. Furthermore, strain N-1231 also produced ansamitocin P-3′ and 19 analogs as minor components (Fig. 3). Among them, P-0, P-1, P-2 and PHO-3 were identical with maytansinol, maytanacine, propionyl maytansinol and deacetyl maytanbutacine, respectively.[2,7,8] Novel analogs, PND-1, -2, -3, and -4 were also obtained from a culture fluid of *Nocardia* sp. No. C-15003 (N-1). Details of the isolation and chemical characterization of these analogs will be described in a separate paper.[9]

Antimicrobial Properties of Ansamitocin Analogs

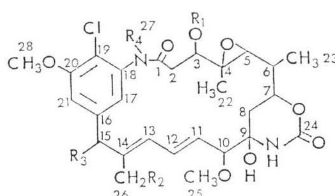
No analog showed inhibitory activity against prokaryotic microorganisms even at the concentrations up to 100 μg/ml. The antiprotozoal activity of these analogs was examined using *Tetrahymena pyriformis* W as the test organism. As shown in Table 3, PND-4, N-demethyl ansamitocin P-4, exhibited the highest activity among them, and the activity of the N-demethyl analogs depended on their acyl moieties at C_3. Although ansamitocin P-4 completely suppressed the growth of *T. pyriformis* at 2 μg/ml,[4] 16~32 μg/ml of its hydroxy analogs of the acyl moiety at C_3 (P-4-βHY and P-4-γHY, Fig. 3) were needed for complete inhibition of growth. A similar relation was also observed between PND-4 and PND-4-βHY. As compared with ansamitocin P-3, the activities of C_{26} and C_{15} hydroxy analogs (PHMs and PHO-3, Fig. 3) were markedly less. $C_{4,5}$ deoxy analogs (deClQ-0, QND-0 and deClQND-0, Fig. 3) showed slight activity against the protozoan, probably because of their deacyl-

Table 3. Antiprotozoal activity of ansamitocin analogs against *Tetrahymena*.

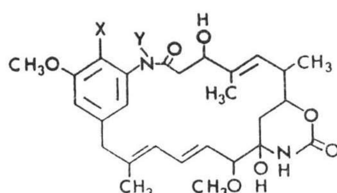
Component	MIC (μg/ml)	Component	MIC (μg/ml)
PND-0	32~64	P-4-βHY	16
PND-1	8	P-4-γHY	16~32
PND-2	4	PND-4-βHY	32
PND-3	2	deClQ-0	≧64
PND-4	1~2	QND-0	32~64
PHM-1	64	deClQND-0	64
PHM-2	64	PHO-3	32
PHM-3	32~64	Ansamitocin P-3	2~4
PHM-4	32		

Inhibitory activity against *Tetrahymena pyriformis* W was measured by the broth dilution method[4] with ansamitocin P-3 as the control.

Fig. 3. Chemical structures of ansamitocin analogs isolated from the culture fluid of the strain N-1231.



	R_1	R_2	R_3	R_4
PND-4	$COCH_2CH(CH_3)_2$	H	H	H
PND-3	$COCH(CH_3)_2$	H	H	H
PND-2	$COCH_2CH_3$	H	H	H
PND-1	$COCH_3$	H	H	H
PND-0	H	H	H	H
PHM-4	$COCH_2CH(CH_3)_2$	OH	H	CH_3
PHM-3	$COCH(CH_3)_2$	OH	H	CH_3
PHM-2	$COCH_2CH_3$	OH	H	CH_3
PHM-1	$COCH_3$	OH	H	CH_3
P-4-βHY	$COCH_2C(OH)(CH_3)_2$	H	H	CH_3
P-4-γHY	$COCH_2CH(CH_2OH)CH_3$	H	H	CH_3
PND-4-βHY	$COCH_2C(OH)(CH_3)_2$	H	H	H
PHO-3	$COCH(CH_3)_2$	H	OH	CH_3
Ansamitocin P-4	$COCH_2CH(CH_3)_2$	H	H	CH_3
Ansamitocin P-3′	$COCH_2CH_2CH_3$	H	H	CH_3
Ansamitocin P-3	$COCH(CH_3)_2$	H	H	CH_3
Propionyl maytansinol (P-2)	$COCH_2CH_3$	H	H	CH_3
Maytanacine (P-1)	$COCH_3$	H	H	CH_3
Maytansinol (P-0)	H	H	H	CH_3

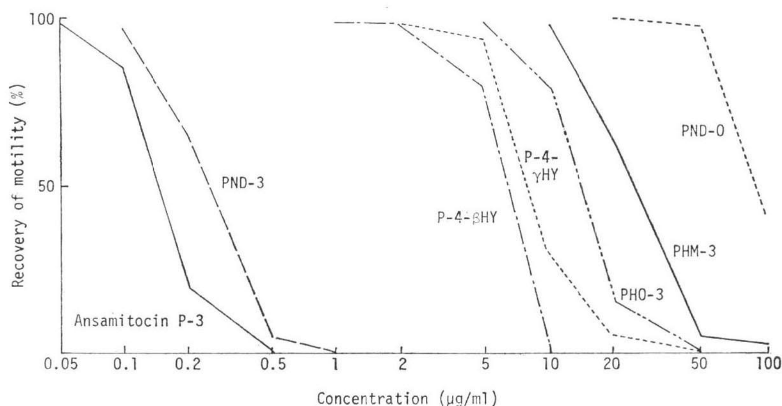


	X	Y
deClQND-0	H	H
QND-0	Cl	H
deClQ-0	H	CH_3

ated structure at C_3. In order to clarify antitubulinic properties of these analogs, the inhibitory activity of representative compounds against cilia regeneration of deciliated *T. pyriformis* W[4,5] was examined. The activity of PND-3 was slightly weaker than that of ansamitocin P-3 (Fig. 4), and its deacyl analog PND-0 did not show complete inhibition even at 100 μg/ml. A similar relation has already been observed between ansamitocin P-3 and maytansinol.[4] The hydroxy analogs of C_{26} (PHM-3), C_{15} (PHO-3) and acyl moiety at C_3 (P-4-βHY and P-4-γHY) showed marked reduction of their activities. These results suggest that hydroxylation of C_{26}, C_{15} and the acyl moiety at C_3 may cause reduction of the specific properties of ansamitocins to microtubule systems in eukaryotic cells. Since one of the deoxy analogs, deClQND-0, had the ability to inhibit cilia regeneration at 50 μg/ml and its acetylated derivative at C_3 showed complete inhibition at 8 μg/ml (data not shown), an epoxide at $C_{4,5}$ may not be essential for the antitubulinic properties of ansamitocins.

Fig. 4. Effect of ansamitocin analogs on cilia regeneration in deciliated *Tetrahymena*.

The cilia regeneration system with *T. pyriformis* W was established by the method described previously.[4,5] The regeneration of cilia was determined as the recovery of motility of the treated cells by scoring of motile cells after 90-minute incubation with a phase contrast microscope.



Discussion

A dnacin-producing strain *Nocardia* sp. No. C-14482 (N-1001) has several unique characteristics from the taxonomic point of view[6] and these characteristics closely resemble those of an ansamitocin-producing strain *Nocardia* sp. No. C-15003 (N-1).[3] On the basis of this similarity, a search for ansamitocins and their analogs in the culture fluids of the dnacin-producing *Nocardia* was made. As shown in this communication, a mutant strain N-1231 derived from the parent strain *Nocardia* sp. No. C-14482 (N-1001) by treatment with ethidium bromide has the ability to produce large amounts of ansamitocins but no detectable dnacins. Since ethidium bromide is well-known to cause a high frequency of loss of extrachromosomal DNA,[10] there is a possibility that the ability to produce dnacins in the strain N-1001 might be controlled by such DNA. However, the possibility that ethidium bromide might act as a mutagen to cause frame-shift mutation[11~13] in the organism cannot be ruled out. Cultural conditions for the production of dnacins are different from those of the ansamitocins. Metabolic correlation between dnacins and ansamitocins are of interest in investigating secondary metabolism in the *Nocardia*.

Mutant N-1231 has the ability to produce not only large amounts of ansamitocins P-3 and P-4 but small amounts of 15 novel ansamitocin analogs. The isolated analogs have antiprotozoal activities against a ciliate protozoan *T. pyriformis* W, and some of them inhibit its cilia regeneration. These properties lead us to the hypothesis that hydroxylation of maytansinoids might reduce their antitubulin activities resulting in decreasing their antitumor activities. In spite of the low activity of deClQND-0 to inhibit cilia regeneration of deciliated *T. pyriformis*, its C_3 acetylated derivative showed significant activity. This suggests that an epoxide at $C_{4,5}$ might not be essential for the activity of maytansinoids against microtubule systems in eukaryotic cells. To clarify the structure-activity relationship of maytansinoids more precisely, it is necessary to examine the biological properties of additional analogs. Mutants such as N-1231 may contribute to the supply of these analogs.

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