MICROBIAL CONVERSION OF ANSAMITOCIN

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(Received for publication June 22, 1981)

Bacteria, actinomycetes, yeasts, and fungi were screened for their ability to modify the structure of ansamitocins, a group of antitumor ansamycin antibiotics. Many strains, mostly actinomycetes, were found to convert ansamitocin P-3 to one or more products. These products, compounds A, B, C, and D, were prepared using *Bacillus megaterium* IFO 12108, *Streptomyces coelicolor* IFO 3807, *Streptomyces castaneus* IFO 13670 and *Streptomyces minutiscleroticus* IFO 13361, and were identified as 20-*O*-demethylansamitocin P-3, maytansinol, 15-hydroxyansamitocin P-3 and *N*-demethylansamitocin P-3, respectively. Other maytansinoids also underwent these microbial conversions.

Ansamitocins are antitumor ansamycin antibiotics produced by *Nocardia* sp. No. C-15003 (N-1)[1~3]. Their structures are similar to those of maytansine and related maytansinoids which have been isolated from plants[4~10] (Fig. 1).

To increase their antitumor activity and decrease the toxicity of ansamitocins, we have attempted to modify their chemical structure with microorganisms. Although many antibiotics have been shown to undergo microbial conversions[11~14], little is known about the microbial conversion of ansamycins. The present paper describes the screening of microorganisms for their ability to modify ansamitocins and the preparation and identification of conversion products.

Materials and Methods

Materials

Ansamitocin P-4 (P-4), ansamitocin P-3 (P-3), maytansinol propionate (P-2), maytanacine (P-1), and maytansinol (P-0) were prepared in our laboratories[1~3]. Maytansine was obtained from Dr. N. HASHIMOTO of this research division.

Fig. 1. Structures of ansamitocins and related compounds.
P-3, ansamitocin P-3; P-3′, ansamitocin P-3′; P-4, ansamitocin P-4; P-1, maytanacine; P-2, maytansinol propionate; P-0, maytansinol.

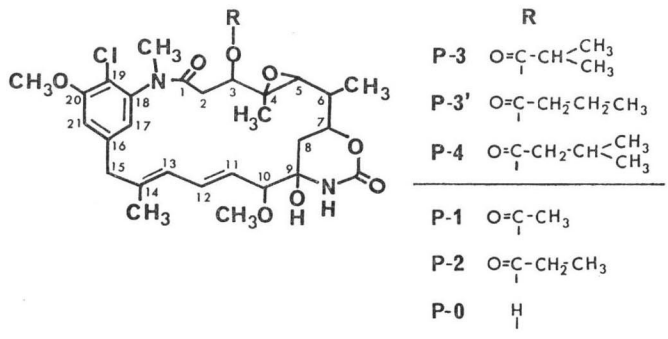


Table 1. Media for various microorganisms.

Bacteria		Actinomycetes		Yeasts		Fungi	
Dextrin	2%	Dextrin	1%	Glucose	2%	Glucose	3%
Peptone	0.5	Glucose	1	Peptone	1	Peptone	0.5
Yeast extract	0.5	Glycerol	1	Yeast extract	0.5	C.S.L.*	1
Meat extract	0.5	Peptone	0.5	pH 6.0		NaCl	0.1
pH 7.5		Yeast extract	0.5			pH 7.0	
		Meat extract	0.5				
		NaCl	0.3				
		$CaCO_3$	0.5				
		pH 7.2					

* Corn steep liquor

Microorganisms

Microorganisms were obtained from the Institute for Fermentation, Osaka, and were also isolated from soil.

Culture and Reaction Conditions

Media used for various microorganisms are shown in Table 1. For screening, each microorganism was inoculated into 5 ml of the medium in a test tube, and the tube was shaken for 1 to 3 days at 28°C. For larger scale studies, 40 ml of the medium in a 200-ml Erlenmeyer flask was inoculated, and the culture was incubated for 1 to 3 days at 28°C on a rotary shaker. A 2-ml portion was transferred to a 200-ml Erlenmeyer flask containing 40 ml of the medium, and this culture was incubated for 18 hours at 28°C on a rotary shaker. Substrates were then added at concentrations of 10 to 200 μg/ml, and the reactions were allowed to proceed under the same conditions for further 48 to 72 hours.

Detection and Measurement of Conversion Products

After the reactions, the cultures were extracted with ethyl acetate. The extracts were chromatographed on silica gel TLC plates (Merck 60 F-254) with chloroform - methanol (9: 1) or ethyl acetate saturated with water. Conversion products were detected by exposing the plates to UV light; the amounts were determined with a Shimadzu daul-wavelength TLC scanner CS-910.

Isolation of Conversion Products

After the reactions, the cultures were extracted with ethyl acetate. The extracts were chromatographed on silica gel columns using mainly a mixture of chloroform and methanol to give the conversion products as crystals[15~17].

Assay of Antimicrobial Activity

Antimicrobial activity of antibiotics was assayed by the paper disk method using *Hamigera avellanea* IFO 7721 as test organism[3,18].

Results

Screening of Microorganisms for Ability to Modify Ansamitocins

A total of 1,395 strains of bacteria, 730 strains of actinomycetes, 326 strains of yeasts, and 2,600 strains of fungi were screened with P-3 as substrate. Many strains of actinomycetes formed conversion products. Fig. 2 shows thin-layer chromatograms of compounds A, B, C, and D which were isolated from active cultures. Many of these strains formed more than one conversion product. Several bacteria belonging to the genus *Bacillus* were found to convert P-3 to compound A, but the substrate was inert to other bacteria. None of yeasts and fungi tested converted P-3. Representative strains of ansamitocin-converting microorganisms are shown in Table 2.

Preparation and Identification of Conversion Products

Bacillus megaterium IFO 12108, *Streptomyces coelicolor* IFO 3807, *Streptomyces castaneus* IFO 13670, and *Streptomyces minutiscleroticus* IFO 13361 were used to prepare compounds A, B, C and D, respectively, because of their high converting activities. P-3 was added to the cultures, and the reactions were carried out for 48 to 72 hours at 28°C with shaking. For the preparation of compound B, P-4 was used instead of P-3 as substrate. The conversion products were isolated from the cultures. The total yields and physico-chemical properties of the conversion products are shown in Tables 3 and 4, respectively. From the results of NMR and mass spectra, and from chemical evidence, compounds A, B, C and D were identified as 20-*O*-demethylansamitocin P-3 (PDM-3)[15], maytansinol (P-0)[1,2,7], 15-hydroxyansamitocin P-3 (PHO-3), and *N*-demethylansamitocin P-3 (PND-3)[16,17], respectively. The physico-chemical properties of PHO-3 indicate that it is identical with deacetylmaytanbutacine reported by KUPCHAN *et al.*[8]

Fig. 2. Thin-layer chromatograms of conversion products. I, $CHCl_3$-MeOH (9:1); II, EtOAc saturated with water.

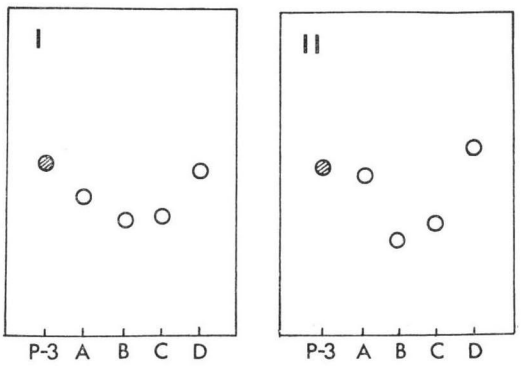


Table 2. Representative ansamitocin-converting microorganisms.

Conversion product	Microorganism	Conversion product	Microorganism
Compound A	*Bacillus megaterium* IFO 12108	Compound C	*Streptomyces castaneus* IFO 13670
	Bacillus megaterium 91277		*Streptomyces sclerotialus* IFO 12246
	Streptomyces flavotricini IFO 12770		*Streptomyces flavochromogenes* IFO 13443
	Streptomyces platensis 12901		*Streptomyces luridus* IFO 12793
	Streptomyces libani IFO 13452		*Streptomyces flaviscleroticus* IFO 13357
	Streptomyces rimosus IFO 3206		*Streptomyces olivaceiscleroticus* IFO 13484
	Streptomyces tubercidicus IFO 13090		*Chainia nigra* IFO 13362
	Streptomyces atrolaccus IFO 13667		*Streptosporangium roseum* IFO 3776
	Streptomyces toyocaensis IFO 12824		
	Actinomyces nigrescens IFO 12894		
Compound B	*Streptomyces coelicolor* IFO 3807	Compound D	*Streptomyces minutiscleroticus* IFO 13361
	Streptomyces galbus IFO 13399		*Streptomyces roseiscleroticus* IFO 13363
	Streptomyces bobili IFO 13199		*Streptomyces flaviscleroticus* IFO 13357

Table 3. Preparation of conversion products.

Microorganism	Substrate (mg)	Concentration (μg/ml)	Conversion (%)	Product (mg)	Yield (%)
B. megaterium IFO 12108	P-3 (2200)	200	100	Compound A (1500)	68
S. coelicolor IFO 3807	P-4 (36)	10	86	Compound B (20)	56
S. castaneus IFO 13670	P-3 (1000)	100	100	Compound C (670)	67
S. minutiscleroticus IFO 13361	P-3 (1000)	200	55	Compound D (360)	36

Table 4. Physico-chemical properties of conversion products.

	Compound A (PDM-3)	Compound B (P-0)	Compound C (PHO-3)	Compound D (PND-3)
Formula	$C_{31}H_{41}ClN_2O_9$	$C_{28}H_{37}ClN_2O_8$	$C_{32}H_{43}ClN_2O_{10}$	$C_{31}H_{41}ClN_2O_9$
Melting point	200~202°C	198~200°C	227~229°C	226~228°C
Optical rotation	$[\alpha]_D^{22}-120°$	$[\alpha]_D^{22}-198°$	$[\alpha]_D^{23}-95.9°$	$[\alpha]_D^{22}-57.1°$
	(*c* 0.52, MeOH)	(*c* 0.22, $CHCl_3$)	(*c* 0.515, EtOH)	(*c* 0.14, EtOH)
Mass *m/z*	559, 471, 456, 436	503, 485, 468	589, 571, 501	620, 559, 471, 456, 436
NMR*	3.19 (3H, s)	3.22 (3H, s)	3.13 (3H, s)	3.35 (3H, s)
	3.38 (3H, s)	3.38 (3H, s)	3.36 (3H, s)	3.95 (3H, s)
		4.00 (3H, s)	4.03 (3H, s)	
			5.37 (1H, s)	

* δ in ppm downfield from internal TMS, measured in $CHCl_3$-*d* except for compound C where acetone-d_6 was used. Only diagnostic signals are reported.

Conversions of Various Maytansinoids

Conversions of various maytansinoids were examined with the following strains: *B. megaterium* IFO 12108, *Streptomyces flavotricini* IFO 12770, and *Streptomyces platensis* IFO 12901 for 20-*O*-demethylation; *S. coelicolor* IFO 3807 and *Streptomyces galbus* IFO 13399 for deacylation; *S. castaneus* IFO 13670 for 15-hydroxylation; and *S. minutiscleroticus* IFO 13361 for *N*-demethylation. The rates of conversion at a concentration of 100 μg/ml are shown in Table 5.

(1) 20-*O*-Demethylation: P-4, P-3, P-2, P-1 and P-0 were converted to the corresponding 20-*O*-demethyl derivatives by all three strains tested. Maytansine was 20-*O*-demethylated by *S. platensis* and *S. flavotricini* but was a poor substrate for *B. megaterium*.

(2) Deacylation: *S. coelicolor* deacylated P-4 and P-3 better than P-2 and P-1, while the reverse was true for *S. galbus*. Maytansine was not deacylated by either strain.

(3) 15-Hydroxylation: P-4, P-3, P-2, P-1 and P-0 were converted to the corresponding 15-hydroxylated derivatives by *S. castaneus*.

(4) *N*-Demethylation : P-4, P-3, P-2, P-1 and P-0 were *N*-demethylated by *S. minutiscleroticus*.

Antimicrobial Activity of Conversion Products

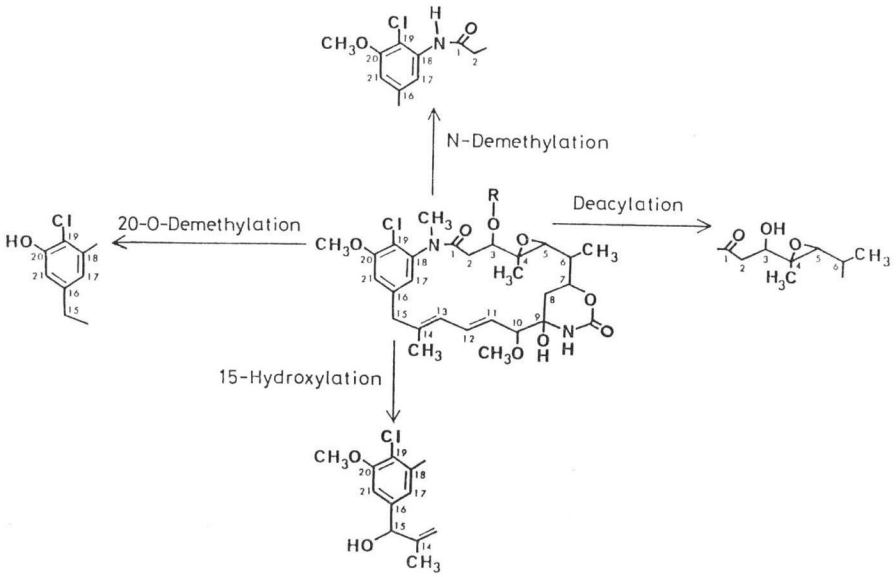
Since ansamitocins show antimicrobial activity against *Hamigera avellanea* IFO 7721[3], the activity

Table 5. Conversions of various maytansinoids.

Substrate	20-*O*-Demethylation			Deacylation		15-Hydroxylation	*N*-Demethylation
	1*	2	3	4	5	6	7
P-4	100%	52%	6.0%	23%	0%	63%	20%
P-3	98	94	23	14	2.3	100	40
P-2	94	97	68	7.0	92	95	5.8
P-1	97	98	11	8.5	98	30	5.8
P-0	18	64	1.5	—	—	64	17
Maytansine	0.5	34	64	0	0	—	0

* Microorganisms: 1, *B. megaterium* IFO 12108; 2, *S. flavotricini* IFO 12770; 3, *S. platensis* IFO 12901; 4, *S. coelicolor* IFO 3807; 5, *S. galbus* IFO 13399; 6, *S. castaneus* IFO 13670; 7, *S. minutiscleroticus* IFO 13361.

Fig. 3. Microbial conversions of ansamitocins.



of the conversion products was assayed by the paper disk method. The diameters of inhibition zones obtained with P-3, PDM-3 and PND-3 at a concentration of 100 μg/ml were 33, 14 and 27.5 mm, respectively. Neither P-0 nor PHO-3 showed inhibition at 100 μg/ml.

Discussion

The results of the present study show that ansamitocins undergo 20-*O*-demethylation, deacylation, 15-hydroxylation, and *N*-demethylation by microorganisms (Fig. 3). Most ansamitocin-converting strains were actinomycetes. This group of microorganisms has been shown to modify numerous antibiotics, and 16-membered macrolide-converting strains were also more widespread in actinomycetes than in bacteria, yeasts, and fungi[19~21]. Thus, actinomycetes appear to have a greater ability to convert antibiotics than do other microorganisms. Since, in general, the ability of actinomycetes to produce antibiotics is greater than that of other microorganisms, the two abilities might be related.

Maytansinol (P-0), maytanbutacine, colubrinol acetate, and colubrinol have been isolated from plants[7,8,10]. The plants, or microorganisms that are parasitic on the plants, might be able to deacylate and 15-hydroxylate maytansinoids synthesized in the plants.

The conversions of ansamitocins occurred with microbial cell suspensions but not with culture filtrates. Thus, the enzymes responsible are located in the cells. Except for deacylation, no conversions have yet been observed with the cell-free extracts. 20-*O*-Demethylation, 15-hydroxylation, and *N*-demethylation are believed to be catalyzed by enzymes such as cytochrome P-450[22].

Whereas *B. megaterium* 20-*O*-demethylated ansamitocins but showed little activity with maytansine, *S. platensis* and *S. flavotricini* 20-*O*-demethylated both compounds. Other strains of actinomycetes also 20-*O*-demethylated both substrates (data not shown). These results indicate that the 20-*O*-demethylating enzyme of *B. megaterium* differs from that of actinomycetes in substrate specificity. Since *S. coelicolor* deacylated P-4 and P-3 better than P-2 and P-1, while *S. galbus* deacylated P-2 and P-1 better than P-4 and P-3, the esterases of these two actinomycetes that catalyze the deacylation differ in acyl group specificity.

The antitumor activity of PDM-3 against leukemia P-388 was higher than that of P-3[23]. Furthermore, PDM-3, P-0, PHO-3 and PND-3 are available as raw materials for synthesis of new antibiotics.

Acknowledgements

We are grateful to Drs. E. OHMURA, M. YONEDA and E. HIGASHIDE for their encouragement and to Drs. T. HASEGAWA and K. HATANO for their useful advice. We also thank Mr. T. MIYAZAKI for his technical assistance.

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