ANSAMITOCIN ANALOGS FROM A MUTANT STRAIN OF *NOCARDIA*

II. ISOLATION AND STRUCTURE

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Fifteen new ansamitocin analogs and deacetylmaytanbutacine were isolated from the culture broth of a mutant strain of *Nocardia* sp. No. C-14482. Their chemical structures were determined on the basis of spectroscopic and chemical evidence.

Ansamitocins are maytansinoid antitumor antibiotics isolated from the culture broth of *Nocardia* sp. No. C-15003 (N-1)[1~4]. In our continued search for novel metabolites related to ansamitocin, we isolated a mutant strain N-1231 derived from *Nocardia* sp. No. C-14482[5]. The isolation and fermentation of the mutant strain N-1231, which produced fifteen new metabolites and deacetylmaytanbutacine[6], and the anti-microbial properties of these metabolites were described in the preceding paper[7].

This paper deals with the isolation and structural elucidation of these new metabolites* by spectroscopic and chemical evidence.

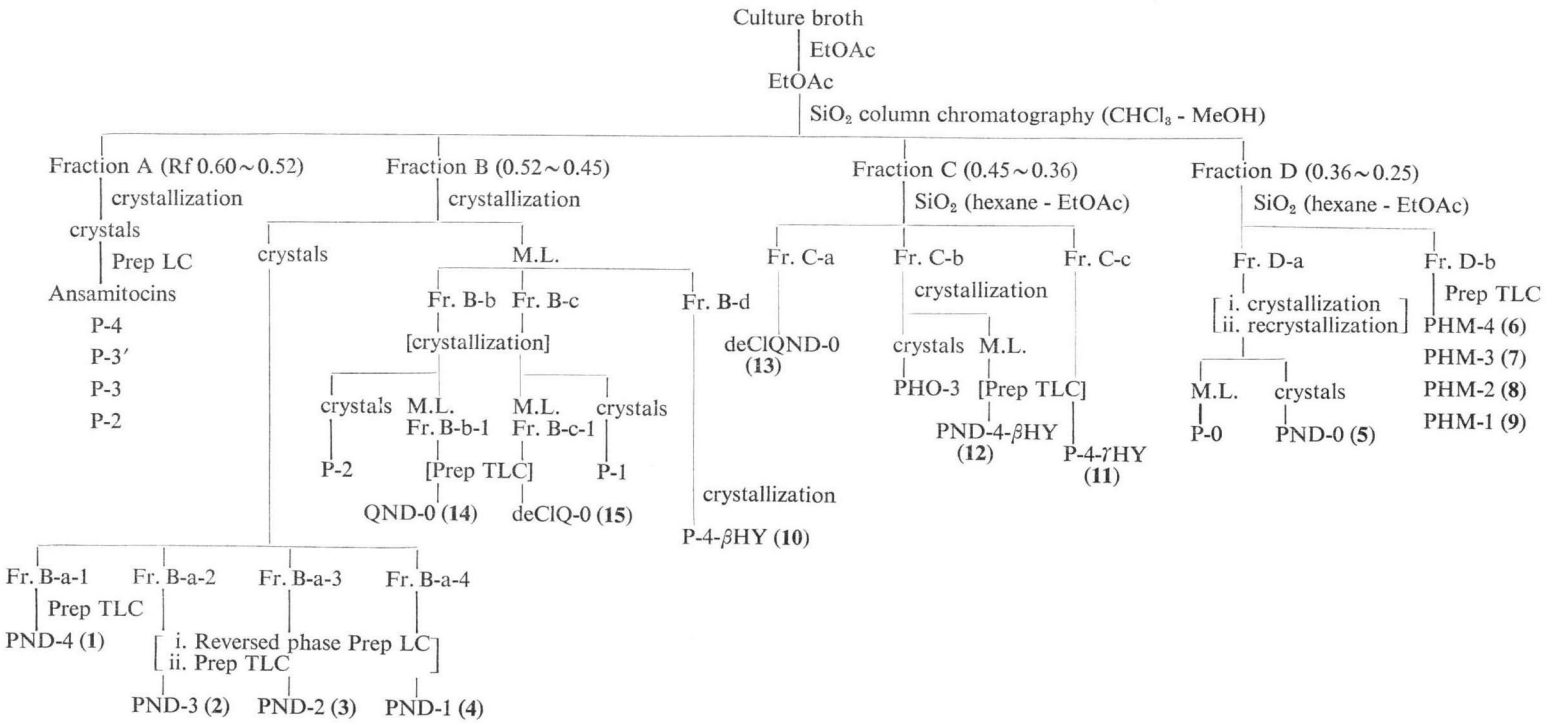
Isolation

Thin-layer chromatography (TLC) was employed to monitor the metabolites during the isolation procedure from the culture broth of the mutant strain N-1231. The metabolites on the chromatograms were detected by UV absorption or coloration with Dragendorff reagent. The metabolites were first obtained together with known ansamitocins as a mixture from the culture filtrate and mycelium. They are lipophilic and almost neutral substances. Since the metabolites are produced in very small amounts in the culture broth, they were isolated as pure substances with a combination of purification procedures; extraction, crystallization, column chromatography, preparative high performance liquid chromatography (Prep LC), and preparative thin-layer chromatography (Prep TLC), as shown in Chart 1. The TLC Rf values are presented in Table 1.

These metabolites were extracted from the culture broth at neutral pH with ethyl acetate. After concentration, the extract was chromatographed on silica gel with a mixture of chloroform and methanol and afforded fractions A, B, C and D. Fraction A was treated with ethyl acetate and gave a colorless

* These metabolites were designated as follows according to their structures: N-demethylansamitocin P-4 (PND-4) (**1**), N-demethylansamitocin P-3 (PND-3) (**2**), 3-propionyl-N-demethylmaytansinol (PND-2) (**3**), 3-acetyl-N-demethylmaytansinol (PND-1) (**4**), N-demethylmaytansinol (PND-0) (**5**), 26-hydroxyansamitocin P-4 (PHM-4) (**6**), 26-hydroxyansamitocin P-3 (PHM-3) (**7**), 26-hydroxy-3-propionylmaytansinol (PHM-2) (**8**), 3-acetyl-26-hydroxymaytansinol (PHM-1) (**9**), 3-(β-hydroxyisovaleryl)maytansinol (P-4-βHY) (**10**), 3-(γ-hydroxyisovaleryl)maytansinol (P-4-γHY) (**11**), 3-(β-hydroxyisovaleryl)-N-demethylmaytansinol (PND-4-βHY) (**12**), 19-dechloro-N-demethyl-4,5-deoxymaytansinol (deClQND-0) (**13**), N-demethyl-4,5-deoxymaytansinol (QND-0) (**14**), 19-dechloro-4,5-deoxymaytansinol (deClQ-0) (**15**) and 15-hydroxyansamitocin P-3 (PHO-3), which proved to be identical to deacetylmaytanbutacine.

Chart 1. Isolation procedure for the metabolites.



crystalline mixture of ansamitocins P-4, P-3 and 3-propionylmaytansinol (P-2). The mixture was subjected to Prep LC as described in a previous paper[4] to separate pure ansamitocins P-4, P-3 and P-2.

The PND group was obtained from fraction B by repeated crystallization with ethyl acetate and ether, and the crude crystalline PND was subjected to chromatography on a silica gel column with a mixture of hexane and ethyl acetate and afforded fractions B-a-1, B-a-2, B-a-3 and B-a-4. Fraction B-a-1 was purified by Prep TLC and gave pure PND-4. Fractions B-a-2, B-a-3 and B-a-4 were subjected to reverse phase Prep LC and further purification by Prep TLC gave pure PND-3, PND-2 and PND-1. The resulting mother liquor from fraction B was subjected to silica gel column chromatography using a mixture of hexane and ethyl acetate and gave fractions B-b, B-c and B-d. P-2 in fraction B-b and maytanacine (P-1) in fraction B-c were crystallized after concentration and removed by filtration. The resulting filtrates were subjected to Prep TLC and gave pure QND-0 and deClQ-0 separately. P-4-βHY was crystallized with ethyl acetate from fraction B-d.

Table 1. TLC Rf values of the metabolites.

	SiO_2* $CHCl_3$-MeOH (9 : 1)	SiO_2* EtOAc sat. with H_2O	RP-18** 80% MeOHaq.
P-3	0.52	0.42	0.42
P-0	0.33	0.23	0.49
PND-4 (**1**)	0.51	0.55	0.55
PND-3 (**2**)	0.49	0.48	0.58
PND-2 (**3**)	0.47	0.42	0.61
PND-1 (**4**)	0.45	0.37	0.64
PND-0 (**5**)	0.30	0.25	0.61
PHM-4 (**6**)	0.31	0.19	0.40
PHM-3 (**7**)	0.30	0.16	0.42
PHM-2 (**8**)	0.29	0.13	0.44
PHM-1 (**9**)	0.27	0.09	0.47
P-4-βHY (**10**)	0.43	0.23	
P-4-γHY (**11**)	0.33	0.17	
PND-4-βHY (**12**)	0.34	0.25	
deClQND-0 (**13**)	0.40	0.34	
QND-0 (**14**)	0.47	0.38	
deClQ-0 (**15**)	0.48	0.35	
PHO-3	0.38	0.26	0.64

* Silica gel 60 F_{254} 0.25 mm (Merck).
** HPTLC RP-18 F_{254} (Merck).

Fraction C was chromatographed on a silica gel column with a mixture of hexane and ethyl acetate and separated into three fractions (C-a, C-b and C-c). The crude powder obtained from fraction C-a was treated with a small amount of ethyl acetate and methanol and gave crystalline deClQND-0. From fraction C-b, PHO-3 was crystallized with ethyl acetate and the resulting mother liquor was subjected to Prep TLC and gave pure PND-4-βHY. Fraction C-c was subjected to Prep TLC and P-4-γHY was obtained in crystalline form.

Fraction D was further separated into two fractions, D-a and D-b, by column chromatography. Fraction D-a was treated with ethyl acetate to give a crystalline mixture, consisting mainly of maytansinol (P-0) and PND-0, which was recrystallized from methanol to yield pure PND-0. Fraction D-b was purified by Prep TLC to obtain pure PHM-4, PHM-3, PHM-2 and PHM-1.

Consequently, we found that sixteen metabolites were produced together with ansamitocins and related compounds in the culture broth of the mutant strain N-1231. PND-4, PND-3, PND-2 and PND-1 were first isolated from the culture broth of *Nocardia* sp. No. C-15003 (N-1)[3].

Structural Elucidation and Discussion

The UV, NMR and mass spectra and physicochemical properties of these minor metabolites are similar to those of ansamitocins. The structures of ansamitocins have been determined previously[1,2] and the assignment of the proton signals in the NMR spectra and characteristic mass fragment peaks

Table 2. Characteristic mass fragment peaks of metabolites.

	M^+	$-H_2O$	$-a$	$\overline{(a+CH_3)}$	$\overline{(a+H_2O)}$	$\overline{(a+Cl)}$	$-(a+b)$	$-(a+b+CH_3)$	$-(a+b+Cl)$
P-3			573				485	470	450
P-0			503		485	468	485	470	450
PND-4 (**1**)	634		573	558			471	456	436
PND-3 (**2**)	620		559	544			471	456	436
PND-2 (**3**)	606		545	530			471	456	436
PND-1 (**4**)	592		531	516			471	456	436
PND-0 (**5**)	550		489	474	471	454	471	456	436
PHM-4 (**6**)	664		603	588	585	568	501	486	466
PHM-3 (**7**)	650		589	574	571	554	501	486	466
PHM-2 (**8**)	636		575	560	557	540	501	486	466
PHM-1 (**9**)	622		561	546	543	526	501	486	466
P-4-βHY (**10**)	664		603	588	585	568	485	470	450
P-4-γHY (**11**)	664		603	588		568	485	470	450
PND-4-βHY (**12**)	650		589	574	571	554	471	456	436
deClQND-0 (**13**)	500		439	424	421		421	406	
QND-0 (**14**)	534	516	473		455	438	455		
deClQ-0 (**15**)	514	496	453		435		435		
PHO-3			589	574	571	554	501	486	466

a=NHCO+H_2O, b=R_1OH.

Table 3. NMR spectral data of metabolites.

	CH_3 22	CH_3 23	CH_3 26	N-CH_3 27	O-CH_3 25	O-CH_3 28	H 5	Others
P-3	0.84 s	1.28 d	1.71 s	3.18 s	3.38 s	4.00 s	2.95 d	1.27 (3H, d), 1.28 (3H, d)
P-0	0.86	1.30	1.70	3.22	3.38	4.00	2.63	
PND-4 (**1**)	1.14	1.26	1.76	—	3.39	3.94	2.88	0.95 (3H, d), 0.97 (3H, d)
PND-3 (**2**)	1.10	1.20	1.75	—	3.35	3.95	2.90	1.17 (3H, d), 1.19 (3H, d)
PND-2 (**3**)	1.12	1.26	1.76	—	3.37	3.95	2.90	
PND-1 (**4**)	1.13	1.24	1.76	—	3.38	3.95	2.82	
PND-0 (**5**)	0.98	1.27	1.67	—	3.33	3.92	2.65	
PHM-4 (**6**)	0.79	1.19	—	3.14	3.35	3.97	2.85	1.01 (3H, d), 1.03 (3H, d)
PHM-3 (**7**)	0.77	1.18	—	3.13	3.34	3.96	2.86	1.23 (3H, d), 1.26 (3H, d)
PHM-2 (**8**)	0.78	1.19	—	3.15	3.36	3.97	2.86	
PHM-1 (**9**)	0.77	1.18	—	3.13	3.34	3.96	2.80	
P-4-βHY (**10**)	0.83	1.25	1.67	3.11	3.36	3.96	2.81	1.34 (6H, s)
P-4-γHY (**11**)	0.83	1.26	1.67	3.13	3.35	3.96	2.87	1.28 (3H, d)
PND-4-βHY (**12**)	1.10	1.25	1.71	—	3.30	3.92	2.78	1.29 (6H, s)
deClQND-0 (**13**)	*1.54 br.s	1.03	1.65	—	3.24	3.71	5.28	
QND-0 (**14**)	1.43 br.s	1.10	1.78	—	3.31	3.85	5.44	
deClQ-0 (**15**)	*1.45 br.s	1.07	1.67	3.18	3.28	3.84	5.31	
PHO-3	**0.88	1.26	1.68	3.13	3.36	4.03	2.85	5.37 (1H, s, C_{15}-H)

δ in ppm downfield from internal TMS.
Measured in $CDCl_3$.
* In DMSO-d_6.
** In acetone-d_6.

were clarified in those reports. On the basis of our results, the NMR spectral data and mass fragment peaks of the metabolites were summarized as shown in Tables 2 and 3. According to their characteristics, these metabolites can be divided into the following groups containing N-demethyl (PND) (**1**, **2**, **3**, **4**, **5** and **12**), 26-hydroxy (PHM) (**6**, **7**, **8** and **9**), modified acyl (**10**, **11**, and **12**), 4,5-deoxy (Q) (**13**, **14** and **15**) and 15-hydroxy (PHO).

The structural elucidation of the metabolites are discussed below in detail for each group using the results of NMR and mass spectroscopic studies.

1. PND-4 (**1**), PND-3 (**2**), PND-2 (**3**), PND-1 (**4**) and PND-0 (**5**)

In the mass spectra of this group, the common fragment peaks for maytansinoids *m/z* 471 [$M^+-(a+b)$], 456 [$M^+-(a+b)-CH_3$] and 436 [$M^+-(a+b)-Cl$] were observed, as shown in Table 2. These values are 14 mass units less than those of the corresponding ansamitocins. Therefore, the PND groups must be a group in which one methyl group in the skeleton of ansamitocins is replaced by hydrogen. Furthermore, the NMR spectra of ansamitocins show an N-methyl signal at about δ 3.1 ~ 3.2, whereas its signal disappears in the PND group, as shown in Table 3. Thus, the results showed that the PND group contains an –NH– group instead of the $-NCH_3$ group at C_{18} of ansamitocin. R_1, acyl groups at C_3, of PND proved to be isovaleryl in PND-4, isobutyryl in PND-3, propionyl in PND-2, acetyl in PND-1 and hydrogen in PND-0, respectively, corresponding to P-4, P-3, P-2, P-1 and P-0. In addition, the *gem*-dimethyl group of isobutyryl in PND-3 is observed at δ 1.17 (3H, d, J=7 Hz) and 1.19 (3H, d, J=7 Hz). The chemical shifts of C_4–CH_3 are δ 0.84 for P-3 and 1.10 for PND-3. The mutual stereochemical relations in PND were thought to be the same as those among ansamitocins, because P-3 was microbiologically transformed into PND-3[8]. Moreover, PND-0 treated with isobutyric anhydride in pyridine yielded 3-isobutyryl-PND-0. The mass spectrum and Rf value of the latter were identical with those of PND-3. These data show that PND-4, PND-3, PND-2, PND-1 and PND-0 are new maytansinoids and their structural differences from ansamitocins exist only in NH at C_{18} as shown in Fig. 1.

PND-4-βHY (**12**) also shows the same common mass numbers as these five compounds and is described below.

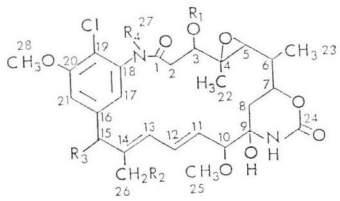
2. PHM-4 (**6**), PHM-3 (**7**), PHM-2 (**8**) and PHM-1 (**9**)

The metabolites of this group give *m/z* 501, 486 and 466 as the common mass numbers and the peaks of each M^+ and (M^+-a) are observed as listed in Table 2. Thus, these compounds have the same skeletal structure and differ only in ester residues. On the other hand, in comparing PHM-3 with the corresponding P-3, PHM-3 gives an additional 16 mass units for each fragment, suggesting that it is a compound including one oxygen atom in the skeletal moiety of P-3. In NMR spectra, P-3 shows signals of methyl protons at δ 0.83 (s, C_4–CH_3), 1.12 (d, J=7 Hz, C_6–CH_3) and 1.71 (br. s, C_{14}–CH_3), while the signal at δ 1.71 is absent for PHM-3. It is therefore evident that the CH_3 group at C_{14} in P-3 is replaced by $-CH_2OH$ in PHM-3. The ester moieties at C_3 are assumed to be isovaleryl for PHM-4, isobutyryl for PHM-3, propionyl for PHM-2 and acetyl for PHM-1. Also, the *gem*-dimethyl group of isobutyryl in PHM-3 is observed at δ 1.23 (d, J=7 Hz) and 1.26 (d, J=7 Hz). The same can be said for PHM-4, PHM-2 and PHM-1. Therefore, their probable structures are as shown in Fig. 1.

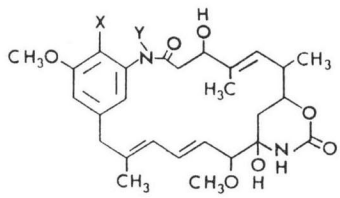
3. P-4-βHY (**10**) and P-4-γHY (**11**)

In the mass spectra of P-4-βHY and P-4-γHY, the common mass numbers, *m/z* 485, 470 and 450, are the same as those of ansamitocins, and thus both metabolites are considered to have the same

Fig. 1. Structures of ansamitocin analogs.



		R_1	R_2	R_3	R_4
1	PND-4	$COCH_2CH(CH_3)_2$	H	H	H
2	PND-3	$COCH(CH_3)_2$	H	H	H
3	PND-2	$COCH_2CH_3$	H	H	H
4	PND-1	$COCH_3$	H	H	H
5	PND-0	H	H	H	H
6	PHM-4	$COCH_2CH(CH_3)_2$	OH	H	CH_3
7	PHM-3	$COCH(CH_3)_2$	OH	H	CH_3
8	PHM-2	$COCH_2CH_3$	OH	H	CH_3
9	PHM-1	$COCH_3$	OH	H	CH_3
10	P-4-βHY	$COCH_2C(OH)(CH_3)_2$	H	H	CH_3
11	P-4-γHY	$COCH_2CH(CH_2OH)CH_3$	H	H	CH_3
12	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
13	deClQND-0	H	H
14	QND-0	Cl	H
15	deClQ-0	H	CH_3

skeleton structure as ansamitocins. In comparison with $(M^+-a)=587$ of the corresponding ansamitocin P-4, P-4-βHY and P-4-γHY show 16 additional mass units for this fragment, thus suggesting that both metabolites are compounds having one oxygen atom introduced into different parts of the side chain moiety of P-4. Furthermore, comparison of the NMR spectra shows that P-4 produces the signal of *gem*-dimethyl of the isovaleryl group at δ 1.03 (6H, d, $J=7$ Hz), whereas the signal appears as a singlet at δ 1.34 (6H, s) in P-4-βHY and only three protons as a doublet at δ 1.28 (3H, d) in P-4-γHY, indicating that P-4-βHY is a compound wherein hydrogen at the β-position of the isovaleryl group is replaced by a hydroxy group and P-4-γHY is a compound wherein one hydrogen at the γ-position of the isovaleryl group of P-4 is replaced by a hydroxy group as shown in Fig. 1.

4. PND-4-βHY (12)

The mass spectrum of PND-4-βHY has the same *m/z* 471, 456 and 436 fragments as those of PND-4. However, PND-4 shows $(M^+-a)=573$, whereas PND-4-βHY shows $(M^+-a)=589$, its 16 additional mass units suggesting that the relationship of the ester moiety between PND-4 and PND-4-βHY is the same as that between P-4 and P-4-βHY. In the NMR spectra, PND-4-βHY is very similar to PND-4, but PND-4 shows the *gem*-dimethyl signal of the isovaleryl group at δ 0.95 (3H, d, $J=7$ Hz) and 0.97 (3H, d, $J=7$ Hz), whereas PND-4-βHY shows the signals as a singlet at δ 1.29 (6H, s), thus suggesting that PND-4-βHY is a compound including one oxygen atom introduced into the β-position of the isovaleryl group of PND-4 as shown in Fig. 1.

5. deClQND-0 (13), QND-0 (14) and deClQ-0 (15)

In the mass spectra of these metabolites, fragment peaks of (M^+-H_2O) are observed along with the characteristic fragment peaks (M^+-a) and $[M^+-(a+b)]$, as shown in Table 2. Thus, the residue at C_3 is assumed to be hydroxy in these compounds. Comparing QND-0 and PND-0 shows that the difference is 16 mass units, suggesting that QND-0 is a compound where one oxygen atom is removed from skeleton moiety of PND-0. Comparison of the NMR spectra shows that PND-0 has the signal of the C_4–CH_3 group at δ 0.98 (s), whereas QND-0 shows it downfield at δ 1.43 (br. s). Also, PND-0 shows the signal of C_5–methine at δ 2.65 (d), while it disappears in QND-0 and newly appears at δ 5.44 (br. d) as olefinic proton coupled with methyl protons at δ 1.43, indicating that QND-0 is a compound wherein the epoxy ring at C_4 and C_5 is replaced by double bond. In comparing of QND-0 with deClQND-0, deClQND-0 shows 34 mass units less than QND-0, and consequently, deClQND-0 seems to be a compound wherein chlorine atom at C_{19} of the corresponding QND-0 is replaced by hydrogen.

On the other hand, in comparing deClQ-0 with deClQND-0, deClQ-0 has 14 mass units more than deClQND-0, and therefore deClQ-0 seems to be a compound where one hydrogen in the skeleton moiety of deClQND-0 is replaced by methyl group. When we compare the NMR spectra of deCl Q-0 and deClQND-0, deClQND-0 shows methyl signals at δ 3.24 (C_{10}–OCH_3) and 3.71 (C_{20}–OCH_3), whereas the corresponding methyl signals at δ 3.28 and 3.84 and new methyl signal at δ 3.18 appear in deClQ-0, indicating that deClQ-0 is a compound wherein the NH group at C_{18} is replaced with an NCH_3 group in deClQND-0. The signals of C_4–methyl and C_5–olefinic proton in deClQ-0 appear δ 1.45 and 5.31, respectively.

All of this evidence shows that QND-0, deClQND-0 and deClQ-0 are new maytansinoids, as shown in Fig. 1.

6. PHO-3

The physicochemical properties of PHO-3 indicates that it is identical to deacetylmaytanbutacine[6]. When treated with acetic anhydride in pyridine, PHO-3 yielded a monoacetate, m.p. 227~229°C, with the same physicochemical properties as maytanbutacine[6]. PHO-3 was also obtained by microbiological transformation of P-3[8].

The elucidation of the chemical structures of these metabolites attracted our interest in the biosynthesis of ansamitocins, especially in the methylation at C_{18}–N, chlorination at C_{19} and epoxydation at C_4–C_5. The most reasonable precursor for ansamitocins is presumed to be deClQND-0. Also these structures show noteworthy similarities to those of macbecins I and II[9,10], benzoquinoid ansamycins, which are also produced in a culture broth of *Nocardia* sp. No. C-14919.

The structure-activity relationships of these metabolites were discussed in another paper[7].

Experimental

Melting points were determined with a Metler FP-5 at 3°C/minute. UV spectra were measured with a Shimadzu UV-200 double beam spectrophotometer. NMR spectra were obtained using a Varian XL-100-12 and a Varian EM-390 instrument; chemical shifts (δ) are reported in ppm downfield from TMS. Mass spectra were determined on a JEOL JMS-OISC spectrometer equipped with a direct inlet system. For TLC, silica gel 60 F_{254} (E. Merck, A. G., Germany, 0.25 mm thick) and reversed phase RP-18 F_{254} (E. Merck) were used. Preparative separation was carried out using a PrepLC/system 500 (Waters, Milford, U.S.A.) and a column packing of silica gel (E. Merck).

I. Separation of Ansamitocins and Minor Metabolites

The filtrate (900 liters) of culture broth of the mutant strain N-1231 of *Nocardia* sp. No. C-14482 was adjusted to pH 6.5 and extracted with one third volume of EtOAc. The EtOAc extract was washed with N/10 hydrochloric acid, M/5 aqueous sodium carbonate, then water and concentrated *in vacuo* to obtain about 500 ml of concentrate. Five liters of petroleum ether added to the concentrate, giving a precipitate which was washed with one liter of ether. Two liters of chloroform were added to the resulting precipitate followed by stirring and the insoluble materials were removed by filtration. The filtrate was concentrated to dryness and one liter of hot EtOAc was added to the resulting residue which was then left standing at room temperature until crystals (about 50 g) containing ansamitocins were removed by filtration. The filtrate was concentrated to dryness giving crude material of minor metabolites (238 g). The crystal was subjected to Prep LC using PrepLC/system 500 as described in a previous paper[4] to obtain ansamitocins and propionyl maytansinol.

The crude material (238 g) obtained above was chromatographed on silica gel column (1 kg, E. Merck, 0.063~0.2 mm) successively with chloroform (6 liters), chloroform - MeOH (50: 1) (4 liters), (30: 1) (4 liters), (9: 1) (3 liters), (4: 1) (3 liters) and (1: 1) (3 liters). Each fraction of effluent (1 liter) was examined by TLC using the solvent system of chloroform - MeOH (9: 1). Fraction A (Nos. 4~13) which was detected as absorption spots of 2537 Å, having Rf values of 0.52~0.60, was concentrated to dryness. EtOAc (80 ml) was added to the residue, which after being left standing at room temperature, gave a mixture of P-4, P-3′, P-3 and P-2 as crystals (23 g). Fraction B (Nos. 14~17) of Rf 0.45~0.52, fraction C (Nos. 18~21) of Rf 0.36~0.45 and fraction D (Nos. 22~25) of Rf 0.25~0.36 were respectively concentrated.

II. Isolation of PND-4 (1), PND-3 (2), PND-2 (3) and PND-1 (4)

To the residue obtained from fraction B, 200 ml of EtOAc was added and the insoluble materials were removed by filtration. The filtrate was concentrated *in vacuo* to 40 ml and left to stand to crystallize the co-produced P-3, P-2 and P-1, which were obtained by filtration (14 g). This filtrate was concentrated *in vacuo*, 15 ml of EtOAc and 10 ml of ether were added to dissolve the residue, then this solution was left to stand to obtain a second crop of crystals containing PND (**1**, **2**, **3** and **4**, 6.2 g). These crystals (6 g) were chromatographed on silica gel (200 g) with hexane - EtOAc (1: 1) (800 ml), (1: 3) (2400 ml), (1: 4) (800 ml) and EtOAc (800 ml). Each fraction of effluent (150 ml) was examined by TLC using the solvent system of EtOAc saturated with water. Fraction B-a-1 having an Rf value of about 0.55 was concentrated to obtain 58 mg of residue containing PND-4. Also, fraction B-a-2 containing PND-3 and having an Rf value of 0.48, fraction B-a-3 containing PND-2 and having an Rf value of 0.42 and fraction B-a-4 containing PND-1 and having an Rf value of 0.37 were concentrated to dryness and residues of 760 mg, 4100 mg and 86 mg, respectively, were obtained.

Fraction B-a-1 was chromatographed on Prep TLC using EtOAc saturated with water as the developing solvent. The band of Rf 0.55 was extracted with the mixture of EtOAc and water. The EtOAc layer was separated, washed with water and concentrated *in vacuo*. Upon addition of petroleum ether to the concentrate, 23 mg of PND-4 was obtained as a white powder: $C_{32}H_{43}ClN_2O_9$=635.17; $[\alpha]_D^{22}$ −56.6° (*c* 0.415, EtOH); UV (MeOH) 232 nm (ε sh. 31500), 239 (31000), 252 (sh. 27600), 279 (37600), 288 (3690).

Fraction B-a-2 (750 mg) was chromatographed on Prep LC using PrepLC/system 500 equipped with a reversed phase gel column (Waters, U.S.A., Prep PAK-500/C 18; 5.7 cm×30 cm). The solvent, 70% aqueous MeOH, was passed through the column at a flow rate of 50 ml/minutes and the eluate

between 20 and 30 minutes after initiation of elution was fractionated. MeOH was distilled from the fraction *in vacuo* and the residue was extracted with 150 ml of EtOAc. The EtOAc layer was concentrated to dryness *in vacuo* and 88 mg of a white powder was obtained. The powder was chromatographed on Prep TLC using EtOAc saturated with water as the developing solvent. The bands of Rf 0.55 (PND-4) and 0.48 (PND-3) gave the white powder of PND-4 (18 mg) and the crystals of PND-3 (43 mg). PND-3: $C_{31}H_{41}ClN_2O_9$=621.14; m.p. 226~228°C (decomp.); $[\alpha]_D^{22}$ −57.1° (*c* 0.14, EtOH); UV (MeOH) 232 nm (ε 32500), 239 (33000), 252 (sh. 28400), 279 (3880), 288 (3790).

Fraction B-a-3 (4 g) was subjected to Prep LC in the same manner described above and the fraction eluted between 20 and 30 minutes after initiation of elution was collected to obtain 730 mg of a powder. This was also subjected repeatedly to Prep LC until 64 mg of a white powder was obtained. This powder was subjected to Prep TLC as described above and 17 mg of PND-3 of Rf 0.48 and 48 mg of a white powder of PND-2 of Rf 0.42 were obtained.

PND-2: $C_{30}H_{39}ClN_2O_9$=607.12; $[\alpha]_D^{22}$ −56.3° (*c* 0.14, EtOH); UV (MeOH) 232 nm (ε 31000), 239 (32000), 252 (sh. 28200), 279 (3800), 288 (3760).

Fraction B-a-4 (86 mg) was subjected to Prep LC and Prep TLC as described above and 12 mg of a white powdery PND-1 of Rf 0.37 was obtained after Prep TLC.

PND-1: $C_{29}H_{37}ClN_2O_9$=593.09; $[\alpha]_D^{22}$ −55.8° (*c* 0.12, EtOH); UV (MeOH) 232 nm (ε 31500), 239 (32000), 252 (sh. 28600), 279 (3780), 288 (3700).

III. Isolation of P-4-βHY (**10**), QND-0 (**14**) and deClQ-0 (**15**)

A powder (6.8 g) from the second mother liquor of fraction B was chromatographed on silica gel column (110 g) with hexane - EtOAc (1: 4) (600 ml) and EtOAc (500 ml) saturated with water. Each fraction of effluent (20 ml) was examined by TLC using the solvent system of EtOAc saturated with water. Fraction B-b (Nos. 25~27) of Rf near 0.38, fraction B-c (Nos. 29~40) of Rf near 0.35 and fraction B-d (Nos. 46~53) of Rf near 0.25 were respectively concentrated, then crystallized giving P-2 and P-1, which were removed by filtration. Both filtrates from fractions B-b and B-c were concentrated *in vacuo* to obtain, respectively, 62 mg of a powder of B-b-1 containing QND-0 and 58 mg of powder of B-c-1 containing deClQ-0. Powder B-b-1 was subjected to Prep TLC using a mixture of chloroform - MeOH (9: 1) as developing solvent. The band of Rf 0.47 was extracted with a mixture of EtOAc and water and the EtOAc layer was separated, washed with water and concentrated to give 38 mg of a powder of QND-0: $C_{27}H_{35}ClN_2O_7$=535.02; UV (EtOH) 231 nm (ε 30300), 240 (30000), 251 (25800), 279 (3600), 288 (3560).

Powder B-c-1 was subjected to Prep TLC using the same system described above and yielded 17 mg of a white powder of deClQ-0 of Rf 0.48.

deClQ-0: $C_{28}H_{38}N_2O_7$=514.60; UV (EtOH) 231 nm (ε 27200), 243 (24800), 252 (24800), 277 (3600), 285 (3520).

A crystal obtained from fraction B-d, described above, was recrystallized with EtOAc and gave P-4-βHY (1.2 g) as colorless needles: $C_{33}H_{45}ClN_2O_{10}$=665.19; mp 201~203°C (decomp.); UV (MeOH) 231 nm (ε 30100), 240 (sh. 28400), 251 (27500), 280 (5650), 288 (5630).

IV. Isolation of P-4-γHY (**11**), PND-4-βHY (**12**), deClQND-0 (**13**) and PHO-3

To fraction C was added 100 ml of chloroform and insoluble materials were removed by filtration. The filtrate, after concentration, was chromatographed on silica gel column (50 g) with hexane - EtOAc (1: 4) (500 ml), EtOAc (500 ml) and EtOAc (500 ml) saturated with water. Each fraction of effluent (20 ml) was examined by TLC using the solvent system of EtOAc saturated with water. The fractions (Nos. 35~42) of Rf near 0.33 were concentrated to about 10 ml. To the concentrate was added 50 ml of ether and the resulting precipitate, when washed with a small amount of EtOAc and MeOH, afforded 45 mg of deClQND-0: $C_{27}H_{36}N_2O_7$=500.60; UV (EtOH) 218 nm (ε 39800), 243 (35400), 251 (sh, 30500), 280 (2800), 288 (2500).

The fractions (Nos. 45~53) of Rf 0.25~0.30 were concentrated then allowed to crystallize. The crystals were recrystallized from the mixture of EtOAc and MeOH to give PHO-3 (81 mg): $C_{32}H_{43}ClN_2O_{10}$=651.15; m.p. 227~229°C (decomp.); $[\alpha]_D^{23}$ −95.9° (*c* 0.515, EtOH); Mass (*m/z*) 589, 571, 554, 536, 501, 486, 483, 468, 466; UV (MeOH) 233 nm (ε 26600), 252 (23100), 281 (4520), 289 (4520).

The filtrate of the first mother liquor was chromatographed on Prep TLC using a mixture of chloroform and MeOH (9: 1) as developing solvent. The band of Rf 0.34 was extracted with a mixture of EtOAc and water and afforded a white powder of PND-4-βHY (18 mg): $C_{32}H_{43}ClN_2O_{10}$=651.17; UV (MeOH) 232 nm (ε 31500), 239 (32000), 252 (sh. 28500), 279 (3800), 288 (3760).

The fractions (Nos. 69~73) near Rf 0.17 were concentrated and the resulting residue was chromatographed on Prep TLC using a mixture of chloroform and MeOH (9: 1) to obtain 32 mg of P-4-γHY from the band of Rf 0.33: $C_{33}H_{45}ClN_2O_{10}$=665.19; m.p. 205~207°C (decomp.); UV (MeOH) 232 nm (ε 30000), 240 (sh. 28200), 252 (27300), 280 (5630), 288 (5610).

V. Isolation of PND-0 (**5**), PHM-4 (**6**), PHM-3 (**7**), PHM-2 (**8**) and PHM-1 (**9**)

To 5.2 g of fraction D was added 40 ml of chloroform and the insolubles were removed by filtration. The filtrate was chromatographed on silica gel column (100 g) with EtOAc saturated with water (2 liters) and a mixture of EtOAc saturated with water - MeOH (10: 1). Each fraction of effluent (20 ml) was examined by TLC. Fraction D-a (Nos. 87~103) of about Rf 0.23~0.25 was concentrated then allowed to crystallize and 320 mg of crystals containing maytansinol and PND-0 was obtained. The crystals were recrystallized from MeOH and gave pure PND-0 (240 mg): $C_{27}H_{35}ClN_2O_8$=551.05; m.p. 189~191°C (decomp.); UV (MeOH) 231 nm (ε 32500), 239 (32500), 250 (sh. 28400), 278 (4060), 287 (3980).

Fraction D-b (Nos. 118~147) of Rf 0.09~0.19 was concentrated to dryness, then a small amount of EtOAc, MeOH and hexane was added to it and 87 mg of a powder containing PHM-4, PHM-3, PHM-2 and PHM-1 was obtained. The powder was subjected to Prep TLC using EtOAc saturated with water as the developing solvent. The bands of Rf 0.19 (PHM-4), 0.16 (PHM-3), 0.13 (PHM-2) and 0.09 (PHM-1) were extracted with the mixture of chloroform and water. Each chloroform layer was washed with water and concentrated to obtain the powders of PHM-4 (14 mg), PHM-3 (38 mg), PHM-2 (7 mg) and PHM-1 (4 mg), respectively. The powder of PHM-3 was crystallized from EtOH and gave 31 mg of crystals.

PHM-4 : $C_{33}H_{45}ClN_2O_{10}$=665.19; $[\alpha]_D^{24}$ −149° (*c* 0.23, EtOH); UV (MeOH) 232 nm (ε 25400), 249 (23600), 280 (4160) and 288 (4220).

PHM-3 : $C_{32}H_{43}ClN_2O_{10}$=651.17; m.p. 192~194°C (decomp.); $[\alpha]_D^{24}$ −148° (*c* 0.5, EtOH); UV (MeOH) 232 nm (ε 25600), 249 (23700), 280 (4190), 288 (4250).

PHM-2 : $C_{31}H_{41}ClN_2O_{10}$=637.14; UV (MeOH) 232 nm (ε 25400), 249 (23600), 280 (4200), 288 (4240).

PHM-1 : $C_{30}H_{39}ClN_2O_{10}$=623.12; UV (MeOH) 232 nm (ε 25800), 249 (23900), 280 (4210), 288 (4250).

VI. PND-0 Monoisobutyrate (synthesis of PND-3)

In 0.5 ml of pyridine was dissolved 30 mg of PND-0 and to the solution was added 0.3 ml of isobutyric anhydride. The mixture was stirred at 22°C for 8 hours. The reaction mixture was subjected to purification by Prep TLC and 8.5 mg of PND-3 was obtained: MS (*m/z*) 620, 605, 559, 471, 456, 436.

VII. PHM-3 Monoacetate (synthesis)

In 0.2 ml of pyridine was dissolved PHM-3 (13 mg) and after addition of 0.1 ml of acetic anhydride, the solution was allowed to stand at room temperature overnight. The reaction mixture to stand at room temperature overnight. The reaction mixture was subjected to Prep TLC and yielded monoacetate of PHM-3 (9 mg): $C_{34}H_{45}ClN_2O_{11}$=693.19; m.p. 240~242°C (decomp.); MS (*m/z*) 692, 631, 571, 556, 536, 483, 468, 448; NMR ($CDCl_3$, δ) 2.07 (3H, s).

VIII. PHO-3 Monoacetate (synthesis of maytanbutacine)

PHO-3 (40 mg) in pyridine (0.5 ml) was acetylated with acetic anhydride (0.25 ml) for 18 hours at room temperature and when the mixture was poured into ice water, it gave a crude acetate. This acetate was crystallized with EtOAc and gave pure crystals (32 mg) of PHO-3 monoacetate: $C_{34}H_{45}ClN_2O_{11}$=693.19; m.p. 253~255°C (decomp.); MS (*m/z*) 692, 631, 571, 556, 536, 483, 468, 448; NMR ($CDCl_3$, δ) 0.79 (3H, s), 1.20 (3H, d), 1.28 (3H, d), 1.28 (3H, d), 1.67 (3H, s), 2.23 (3H, s), 3.16 (3H, s),

3.35 (3H, s), 4.02 (3H, s), 6.29 (1H, s). The PHO-3 monoacetate was identified as maytanbutacine from its physicochemical properties.

IX. deClQND-0 Monoacetate (synthesis of deClQND-1)

In 10 ml of dry CH_2Cl_2 was suspended deClQND-0 (23 mg) and after addition of 0.2 ml of acetic anhydride and 20 mg of 4-N-dimethylaminopyridine, the suspension was stirred at room temperature for 3 hours. The reaction mixture was purified by Prep TLC and gave monoacetate of deClQND-0 (16 mg): $C_{29}H_{38}N_2O_8$=542.62; MS (*m/z*) 542, 481, 421, 406; NMR ($CDCl_3$, δ) 2.08 (3H, s), 3.32 (3H, s), 3.77 (3H, s).

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