HYDROXYLATION OF ANSAMITOCIN P-3

MOTOWO IZAWA, YOSHIKAZU WADA, FUMIKO KASAHARA,
MITSUKO ASAI and TOYOKAZU KISHI

Central Research Division, Takeda Chemical Industries, Ltd.,
Yodogawa-ku, Osaka 532, Japan

(Received for publication July 13, 1981)

Ansamitocin P-3 was converted into 15-hydroxyansamitocin P-3 (PHO-3), $C_{32}H_{43}ClN_2O_{10}$, and 15-*epi*-15-hydroxyansamitocin P-3 (*epi*-PHO-3), $C_{32}H_{43}ClN_2O_{10}$, by actinomycetes. PHO-3 was identical to deacetylmaytanbutacine and *epi*-PHO-3 was a new compound. The configuration at C_{15} was elucidated as *R* for PHO-3 and *S* for *epi*-PHO-3 on the basis of their physicochemical properties and X-ray analysis of *epi*-PHO-3.

Ansamitocin P-3 (P-3) is a maytansinoid antitumor antibiotic isolated from the culture broth of *Nocardia* sp. No. C-15003 (N-1)[1~4] and a mutant strain of *Nocardia* sp. No. C-14482.[5,6] 20-*O*-Demethylansamitocin P-3 (PDM-3),[7,8] a microbial conversion product of P-3, has greater antitumor activities against P 388 and L 1210 than the substrate P-3 (i.p.-i.v., s.c.-i.v.).[9]

Further studies on the microbial conversion of P-3 showed that it is converted into two 15-hydroxyl derivatives, which were designated 15-hydroxyansamitocin P-3 (PHO-3)[7] and 15-*epi*-hydroxyansamitocin P-3 (*epi*-PHO-3). PHO-3 was also isolated from a culture broth of a mutant strain of *Nocardia* sp. No. C-14482[5,6] and found to be identical to deacetylmaytanbutacine which is a partial-hydrolysis product of maytanbutacine isolated from a plant source,[10] but the stereochemistry at C_{15} had not yet been studied. This paper deals with the isolation and stereochemistry of these products on the basis of physicochemical properties and an X-ray analysis of *epi*-PHO-3.

Isolation and Structures of PHO-3 and *epi*-PHO-3

These conversion products were isolated by the general procedure for lipophilic neutral substances. The reaction mixture after conversion of P-3 was extracted with ethyl acetate and concentrated *in vacuo* to give crude substances. These substances were chromatographed on silica gel with a mixture of chloroform - methanol and crystallized with ethyl acetate, giving a mixture of crystals of the products. These mixed crystals were subjected to preparative high-performance liquid chromatography (Prep LC) on a reverse-phase column with aqueous methanol. Prep LC was performed with a recycle technique to separate PHO-3 and *epi*-PHO-3 as described in previous papers.[4,8] Each eluate was concentrated, then extracted with ethyl acetate and concentrated again, giving crystals of PHO-3 or *epi*-PHO-3. The Rf values on TLC are listed in Table 1.

Table 1. TLC Rf values of P-3, PHO-3 and *epi*-PHO-3.

	SiO_2*		RP-18** 80% aqueous MeOH
	$CHCl_3$ - MeOH (9: 1)	EtOAc saturated with H_2O	
P-3	0.52	0.42	0.42
PHO-3	0.38	0.26	0.64
epi-PHO-3	0.37	0.25	0.68

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

The physicochemical properties of PHO-3 indicate that it is identical to deacetylmaytanbutacine.[10] The mass spectrum of *epi*-PHO-3 has the same fragment peaks as those of PHO-3; *m/z*

Table 2. ^{13}C NMR spectral data of P-3, PHO-3 and *epi*-PHO-3.

C-No	P-3	PHO-3	*epi*-PHO-3	C-No	P-3	PHO-3	*epi*-PHO-3
1	s 170.6	s 170.5	s 170.8	17	d 122.5	d 120.0	d 120.3
2	t 33.5	t 33.5	t 33.6	18	s 142.7*	s 142.8*	s 142.7*
3	d 77.5	d 77.4	d 77.2	19	s 119.5	s 119.8	s 120.1
4	s 61.6	s 61.4	s 61.6	20	s 157.1	s 157.2	s 156.8
5	d 67.5	d 67.3	d 67.6	21	d 114.6	d 109.7	d 113.1
6	d 39.3	d 39.3	d 39.4	22	q 12.5	q 12.6	q 12.5
7	d 75.5	d 75.4	d 75.6	23	q 14.6	q 14.7	q 14.6
8	t 37.3	t 37.2	t 37.4	24	s 154.6	s 154.4	s 156.8
9	s 81.4	s 81.2	s 81.4	25	q 56.7**	q 56.8	q 56.6**
10	d 89.5	d 89.4	d 89.6	26	q 15.7	q 10.3	q 14.8
11	d 129.0	d 130.7	d 129.6	27	q 35.8	q 35.9	q 35.8
12	d 133.7	d 133.1	d 133.4	28	q 57.0**	q 56.8	q 57.0**
13	d 125.5	d 125.7	d 120.3	29	s 177.0	s 176.8	s 177.3
14	s 140.7*	s 142.1*	s 143.1*	30	d 34.7	d 34.6	d 34.7
15	t 47.0	t 79.2	d 77.6	31, 32	q 18.3	q 18.3	q 18.3
16	s 142.2*	s 145.2*	s 145.9*		q 20.4	q 20.4	q 20.4

δ In ppm downfield from internal TMS; CD_3OD.
*, ** Tentatively assigned.

589 (M^+-a), 571 [$M^+-(a+H_2O)$], 556 [$M^+-(a+H_2O+CH_3)$], 554 [$M^+-(a+Cl)$], 536 [$M^+-(a+H_2O+Cl)$], 501 [$M^+-(a+b)$], 486 [$M^+-(a+b+CH_3)$], 483 [$M^+-(a+b+H_2O)$], 468 [$M^+-(a+b+H_2O+CH_3)$], 466 [$M^+-(a+b+Cl)$] and 448 [$M^+-(a+b+Cl+H_2O)$]. ($a=HNCO+H_2O$, $b=R_1OH$)

The ^1H NMR spectra for P-3 has signals for methylene protons of C_{15} at δ 3.22 (1H, d, $J_{gem}=15$ Hz) and 3.52 (1H, d, $J_{gem}=15$ Hz), while these signals are absent in both products but a singlet signal assignable to the C_{15}-proton appears at δ 5.37 (1H, s) for PHO-3 and at 5.11 (1H, s) for *epi*-PHO-3.

In the ^{13}C NMR spectra, a carbon assignable to C_{15} is detected at δ 79.2 for PHO-3 and 77.6 for *epi*-PHO-3 instead of 47.0 where it appeared for P-3, as shown in Table 2. ^{13}C Chemical shift assignments were based on the multiplicities in off-resonance spectra, the selective proton decoupling of these compounds, and comparison with the known chemical shift values of model compounds.

The results indicate that *epi*-PHO-3 is a stereoisomer of PHO-3 at the C_{15} position and a new compound.

Stereochemistry of PHO-3 and *epi*-PHO-3

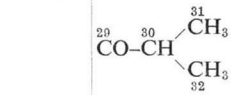
P-3 was reductively cleaved into maytansinol[1,2] as in the case of maytansine.[10,11] The structure was determined by X-ray analysis of the bromopropyl ether derivative.[12] In the ^1H NMR spectra, the chemical shifts and coupling constants of P-3, PHO-3 and *epi*-PHO-3 are almost the same as those of maytansine except for the chemical shifts of C_{13}-H. The difference is thought to result from the *N*-acetyl-*N*-methylalanine moiety at C_3 because it is near the double bond at C_{13} according to the X-ray analysis[12] and C_{13}-H is deshielded. Therefore, the stereochemistries of P-3, PHO-3 and *epi*-PHO-3 are the same as those of maytansine.

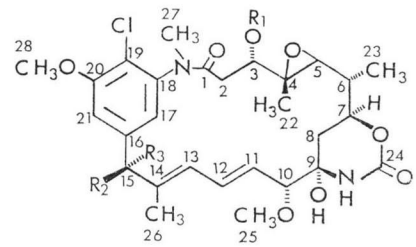
The stereochemistry of PHO-3 and *epi*-PHO-3 at C_{15} was deduced from the results of NOE experiments in acetone-d_6 and from ^{13}C NMR spectral data. In PHO-3, the C_{21}-H (δ 7.49) signal assignment was made by irradiation of C_{20}-OCH_3 (δ 4.03), resulting in 18~24% NOE, and saturation of C_{15}-H

(δ 5.37) gave enhancement of 7~10% of the integrated areas of C_{17}-H (δ 6.90) and no enhancement of C_{21}-H. On the other hand, irradiation at C_{15}-H (δ 5.11) led to enhancement of 9~12% of the integrated areas of C_{14}-CH_3 (δ 1.68) in *epi*-PHO-3. Clearly from these results, C_{15}-H should be located on the opposite side in PHO-3 and on the same side in *epi*-PHO-3 to C_{14}-CH_3 in the stereochemical relationships.

In the ^{13}C NMR spectra, the signals of C_{26} (C_{14}-CH_3) and C_{21} are observed higher upfield at δ 10.3 and 109.7, respectively, than P-3 because of the steric compression effect of C_{15}-OH in PHO-3. While, the signal of C_{13} appears higher upfield at δ 120.3 due to the steric compression effect of C_{15}-OH in *epi*-PHO-3. These facts indicate that the stereochemistry of C_{15} is that of Fig. 1 and the conformations at C_{15} are *R* for PHO-3 and *S* for *epi*-PHO-3.

Fig. 1. Structures of PHO-3, *epi*-PHO-3 and related compounds.

	R_1	R_2	R_3
PHO-3		OH	H
epi-PHO-3	〃	H	OH
Ansamitocin P-3	〃	H	H
Maytansinol	H	H	H
Maytansine	R_1=COCH(CH$_3$)N(CH$_3$)COCH$_3$	R_2=R_3=H	



In order to confirm the proposed stereochemistry, X-ray analysis of *epi*-PHO-3 was carried out. The structure of *epi*-PHO-3 was established to be that shown in Fig. 1.

The conformation of *epi*-PHO-3 in the crystalline state was similar to that of maytansine and also in accord with that predicted by NMR studies comparing it with maytansine. Details of X-ray study of *epi*-PHO-3 will be described in a separate paper.

PHO-3 has antiprotozoal activity against *Tetrahymena pyriformis* W[5] and *epi*-PHO-3 has equal or slightly lesser activity. Further studies on the hydroxylation mechanism by actinomycetes will be conducted.

Experimental

Melting points were determined with a Mettler PF-5 at 3°C/minute. UV Spectra were measured with a Shimadzu UV-200 double beam spectrophotometer. IR spectra were recorded with a Hitachi 285 grating spectrometer. NMR spectra were obtained using Varian XL-100-12 and Varian EM-390 instruments; chemical shifts (δ) are reported in ppm downfield from TMS. Mass spectra were determined on a JMS-OISC spectrometer equipped with a direct inlet system. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. For TLC, silica gel 60 F_{254} (E. Merck, A.G., Germany, 0.25 mm thick) and reverse phase HPTLC RP-18 F_{254} (E. Merck) were used. Preparative liquid chromatography was carried out using Prep LC/system 500 (Waters, Milford, U.S.A.).

Isolation of PHO-3 and *epi*-PHO-3

Streptomyces sclerotialus IFO 12246 was inoculated in 40 ml of a medium (pH 7.2) containing 1% dextrin, 1% glucose, 1% glycerol, 0.5% peptone, 0.5% yeast extract, 0.5% meat extract, 0.3% NaCl and 0.5% $CaCO_3$ in 200-ml Erlenmeyer flasks. The flasks were incubated for 3 days at 28°C on a rotary shaker. Culture samples of 2 ml each were transferred to 40 ml of the medium in 200-ml Erlenmeyer flasks. The flasks were incubated for 22 hours at 28°C on a rotary shaker. P-3 (900 mg) was added to the culture (18 liters) and the mixture was incubated for 48 hours at 28°C on a rotary shaker. The reaction mixture after addition of 2.2 kg of NaCl was extracted twice with EtOAc, HCl, N/10 Na_2CO_3, and

water, then concentrated *in vacuo* to afford about 30 ml of concentrate. Addition of 300 ml of petroleum ether to the concentrate produced a precipitate (2.34 g). This precipitate was chromatographed on silica gel (E. Merck, 100 g, 0.063~0.2 mm) successively with $CHCl_3$ (200 ml) and $CHCl_3$ - MeOH (50: 1) (300 ml) and (20: 1) (300 ml). Each fraction of effluent (10 ml) was examined by TLC using the solvent system of $CHCl_3$ - MeOH (9: 1). The fraction which was detected as absorption spots of 2537 Å, having Rf values of 0.37~0.38, was concentrated to dryness (640 mg). A small amount of warm EtOAc was added to the residue and this mixture, after standing at room temperature, gave crystals containing PHO-3 and *epi*-PHO-3 (470 mg). These crystals were subjected to Prep LC using a Prep LC/system 500 equipped with a reverse-phase column (Waters, Prep PAK-500/C-18; 5.7×30 cm). The solvent, 55% aqueous MeOH, was passed through the column at a flow rate of 50 ml/minute and the eluate emerging during the period of 20 to 25 minutes from the start of elution was recovered (Fraction A). The eluate from 25 to 35 minutes was recycled and the eluate from 35 to 47 minutes was recovered (Fraction B). The eluates from 55 to 73 minutes (Fraction C) and from 77 to 93 minutes (Fraction D) were also recovered. Fractions A and C, of Rf 0.68 on reverse-phase TLC using 80% aqueous MeOH as solvent, and also Fractions B and D (Rf 0.64) were concentrated to remove MeOH. Each concentrate was extracted with EtOAc and the EtOAc layer, concentrated to a small volume and left standing at room temperature, gave crystals of *epi*-PHO-3 (52 mg, from Fractions A and C) and PHO-3 (134 mg, from Fractions B and D).

PHO-3: $C_{32}H_{43}ClN_2O_{10}$=651.17; m.p. 227~229°C (EtOAc), 234~236°C (CH_2Cl_2 - ether); $[\alpha]_D^{23}$ −95.9° (*c* 0.515, EtOH); UV (MeOH) 233 nm (ε 26600), 252 (23100), 281 (4520), 289 (4520); ^{1}H NMR (acetone-d_6+D_2O) δ 0.88 (3H, s, C_4-CH_3), 1.19 (3H, d, J=7 Hz, C_{31}- or C_{32}-H_3), 1.21 (3H, d, J=7, C_{31}- or C_{32}-H_3), 1.26 (3H, d, J=7, C_6-CH_3), 1.68 (3H, s, C_{14}-CH_3), 1.4~1.8 (3H, C_6-H, C_8-H_2), 2.1~2.4 (1H, C_{30}-H), 2.15 (1H, dd, J=15, 3, C_2-H), 2.62 (1H, dd, J=15, 11, C_2-H′), 2.85 (1H, d, J=9, C_5-H), 3.13 (3H, s, N-CH_3), 3.36 (3H, s, C_{10}-CH_3), 3.63 (1H, d, J=9, C_{10}-H), 4.03 (3H, s, C_{20}-CH_3), 4.26 (1H, m, C_7-H), 4.70 (1H, dd, J=11, 3, C_3-H), 5.37 (1H, s, C_{15}-H), 5.62 (1H, dd, J=15, 9, C_{11}-H), 6.38 (1H, d, J=11, C_{13}-H), 6.68 (1H, dd, J=15, 11, C_{12}-H), 6.90 (1H, d, J=1.5, C_{17}-H), 7.49 (1H, d, J=1.5, C_{21}-H).

epi-PHO-3: $C_{32}H_{43}ClN_2O_{10}$=651.17; m.p. 220~222°C (EtOAc), 211~214°C (CH_2Cl_2 - ether); $[\alpha]_D^{23}$ −123.2° (*c* 0.47, EtOH); UV (MeOH) 233 nm (ε 28100), 253 (25400), 281 (5220), 289 (5220); ^{1}H NMR (acetone-d_6+D_2O) δ 0.88 (3H, s, C_4-CH_3), 1.19 (3H, d, J=7 Hz, C_{31}- or C_{32}-H_3), 1.21 (3H, d, J=7, C_{31}- or C_{32}-H_3), 1.26 (3H, d, J=7, C_6-CH_3), 1.68 (3H, s, C_{14}-CH_3), 1.4~1.8 (3H, C_6-H, C_8-H_2), 2.1~2.4 (1H, C_{30}-H), 2.16 (1H, dd, J=15, 3, C_2-H), 2.62 (1H, dd, J=15, 3, C_2-H′), 2.83 (1H, d, J=9, C_5-H), 3.12 (3H, s, N-CH_3), 3.34 (3H, s, C_{10}-CH_3), 3.62 (1H, d, C_{10}-H), 4.01 (3H, s, C_{20}-CH_3), 4.21 (1H, m, C_7-H), 4.70 (1H, dd, J=11, 3, C_3-H), 5.11 (1H, s, C_{15}-H), 5.58 (1H, dd, J=15, 9, C_{11}-H), 6.63 (1H, d, J=11, C_{13}-H), 6.76 (1H, dd, J=15, 11, C_{12}-H), 7.20 (1H, d, J=1.5, C_{17}-H), 7.27 (1H, d, J=1.5, C_{21}-H).

Acknowledgements

We are grateful to Drs. E. OHMURA, M. YONEDA and M. NISHIKAWA for encouragement throughout this work. We also indebted to Drs. M. KIDA, T. HASEGAWA, E. MIZUTA, K. KAMIYA, M. TAKAMOTO, K. NAKAHAMA and S. TANIDA.

References

1) HIGASHIDE, E.; M. ASAI, K. OOTSU, S. TANIDA, Y. KOZAI, T. HASEGAWA, T. KISHI, Y. SUGINO & M. YONEDA: Ansamitocin, a group of novel maytansinoid antibiotics with antitumor properties from *Nocardia*. Nature 270: 721~722, 1977

2) ASAI, M.; E. MIZUTA, M. IZAWA, K. HAIBARA & T. KISHI: Isolation, chemical characterization and structure of ansamitocin, a new antitumor ansamycin antibiotic. Tetrahedron 35: 1079~1085, 1978

3) TANIDA, S.; T. HASEGAWA, K. HATANO, E. HIGASHIDE & M. YONEDA: Ansamitocins, maytansinoid antitumor antibiotics. Producing organism, fermentation and antimicrobial activities. J. Antibiotics 33: 192~198, 1980

4) IZAWA, M.; K. HAIBARA & M. ASAI: Analytical studies and preparative separation of ansamitocins and related antibiotics using high-performance liquid chromatography. Chem. Pharm. Bull. 28: 789~794, 1980
5) TANIDA, S.; M. IZAWA & T. HASEGAWA: Ansamitocin analogs from a mutant strain of *Nocardia*. I. Isolation of the mutant, fermentation and antimicrobial properties. J. Antibiotics 34: 489~495, 1981
6) IZAWA, M.; S. TANIDA & M. ASAI: Ansamitocin analogs from a mutant strain of *Nocardia*. II. Isolation and structure. J. Antibiotics 34: 496~506, 1981
7) NAKAHAMA, K.; M. IZAWA, M. ASAI, M. KIDA & T. KISHI: Microbial conversion of ansamitocin. J. Antibiotics 34: 1581~1586, 1981
8) IZAWA, M.; K. NAKAHAMA, F. KASAHARA, M. ASAI & T. KISHI: Demethylation of ansamitocins and related compounds. J. Antibiotics 34: 1587~1590, 1981
9) KOZAI, Y. & K. OOTSU: Antitumor activities of C_{20}-demethyl derivatives of ansamitocins. Proceeding of the Japan Cancer Association; The 39th Annual Meeting, Tokyo, November, 1980
10) KUPCHAN, S. M.; Y. KOMODA, A. R. BRANFMAN, A. T. SNEDEN, W. A. COURT, G. J. THOMAS, H. P. J. HINTZ, R. M. SMITH, A. KARIM, G. A. HOWIE, A. K. VERMA, Y. NAGAO, R. G. DAILEY, Jr., V. A. ZIMMERLY & W. C. SUMNER, Jr.: The maytansinoids. Isolation, structural elucidation, and chemical interrelation of novel ansamacrolides. J. Org. Chem. 42: 2349~2357, 1977
11) KUPCHAN, S. M.; Y. KOMODA, W. A. COURT, G. J. THOMAS, R. M. SMITH, A. KARIM, C. J. GILMORE, R. C. HALTIWANGER & R. F. BRYAN: Maytansine, a novel antileukemic ansa macrolide from *Maytenus ovatus*. J. Am. Chem. Soc. 94: 1354~1356, 1972
12) BRYAN, R. F.; C. J. GILMORE & R. C. HALTIWANGER: Crystals and molecular structure and absolute configuration of maytansine (3-bromopropyl) ether. J. C. S., Perkin II 1973: 897~902, 1973