PC-766B, A NEW MACROLIDE ANTIBIOTIC PRODUCED BY *Nocardia brasiliensis*

II. ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE ELUCIDATION

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A new macrolide antibiotic, PC-766B, was isolated from the cells of *Nocardia brasiliensis* SC-4710 by acetone extraction, and purified by gel filtration, silica gel chromatography, HPLC and TLC. The structure of PC-766B was determined by NMR spectral analysis to be a new class of the hygrolidin family antibiotics. PC-766B had a 16-membered macrocyclic lactone ring, a 6-membered hemiketal ring and a 2-deoxy-D-rhamnose moiety. DL-α-Tocopherol, known as an antioxidant agent, significantly improved the stability of PC-766B and prevented the decomposition of PC-766B during the storage of the antibiotic.

PC-766B (**1**), a new macrolide antibiotic, was isolated from *Nocardia brasiliensis* SC-4710. In the previous paper[1], we reported the taxonomy of the producing organism, fermentation and biological activity of **1**. Here we report the isolation, physico-chemical properties and structure elucidation of **1**, which was found to be a new class of unusual 16-membered macrolides which were first described by SETO *et al.*[2]. In addition, we demonstrate the effect of antioxidant agents on the stability of **1**.

Materials and Methods

General

UV spectra were recorded on a Shimadzu UV-240 spectrometer. IR spectra were recorded on a Hitachi 270-30 infrared spectrometer. Mass spectra were obtained with a Hitachi M-80B mass spectrometer. NMR spectra were recorded using a Varian XL-200 spectrometer and a JEOL FX-90Q spectrometer. Optical rotations were measured with a Jasco DIP-181 polarimeter. Melting points were determined by using a hot-stage microscope and are uncorrected.

HPLC Analysis

Analysis of **1** was carried out by using reversed-phase HPLC as described[1].

Alkaline Hydrolysis of PC-766B (1)

A solution of **1** (101 mg) in 10 ml of 0.03 N NaOH in methanol was stirred in the dark at room temperature for 3 days. After neutralized with aqueous HCl, the reaction mixture was concentrated under reduced pressure, and put on a plate of silica gel TLC (E. Merck Silica Gel 60 F_{254}, 0.5 mm thickness). The plate was developed with benzene - ethyl acetate (1 : 1). Three bands were detected under the irradiation of UV at 254 nm. The main band with Rf 0.5 was scraped off, and eluted with benzene - ethyl acetate (1 : 1). The extract was evaporated under reduced pressure to give 18 mg of colorless crystals of an aglycone (**2**).

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The more polar band with Rf 0.1 was also scraped from the plate, and applied to a column of silica gel 60 (E. Merck). The column was eluted with chloroform - methanol - water (6 : 4 : 1). The anthrone positive fractions were concentrated under reduced pressure and recrystallized from acetone to give 11 mg of colorless amorphous powder of a sugar (**3**).

Stability Test

To a solution of **1** (10 mg) in ethyl acetate (1 ml), antioxidant agents (1 ~ 5 μg) in acetone (1 ml) were added, and the mixture was distributed in 0.2 ml volumes into 2 ml Teflon-lined screw-cap vials. After evaporation under reduced pressure, the vials were sealed and incubated in the dark at −18, 4 or 27°C. The titer of **1** was determined by reversed-phase HPLC.

Results and Discussion

Isolation

The fermentation broth (10 liters) of strain SC-4710 cultured in a 30-liter jar fermentor at 27°C for 10 days as described[1] was adjusted to pH 7.0 with 6 N HCl, and centrifuged at 20,000 rpm at 4°C. Since most of **1** produced was cell bound[1], the supernatant was discarded; the cell cake was extracted three times with 3 liters each of acetone. The acetone extract (9 liters) was concentrated under reduced pressure, and the aqueous solution was extracted with 2 liters of ethyl acetate. The extract was washed with water and concentrated to dryness under reduced pressure. This oily material was washed with *n*-hexane, dissolved in chloroform - 2-propanol (1 : 1), and put on a column of Sephadex LH-20 (Pharmacia). The column was eluted with chloroform - 2-propanol (1 : 1). Active fractions were combined, concentrated under reduced pressure, and dissolved in chloroform. The chloroform solution was applied to a column of silica gel. The column was washed with chloroform, and then eluted with chloroform - 2-propanol (95 : 5). Active fractions were combined, and concentrated to dryness under reduced pressure. About 1.2 g of curde **1** thus obtained was dissolved in 30 ml of toluene, and injected into HPLC (Waters PrepLC System 500 equipped with two PrepPAK Silica cartridges). The elution was performed with toluene - ethyl acetate (60 : 40). The eluate fractions were checked for purity by reversed-phase HPLC. Further purification was done by using TLC. The antibiotic was applied to a plate of silica gel TLC (E. Merck Silica Gel 60 F_{254}, 2 mm thickness), and developed with toluene - ethyl acetate (1 : 4). The band of Rf 0.23 was scraped off, and extracted with ethyl acetate. The extract was concentrated under reduced pressure giving a colorless powder of pure **1** (310 mg).

Physico-chemical Properties

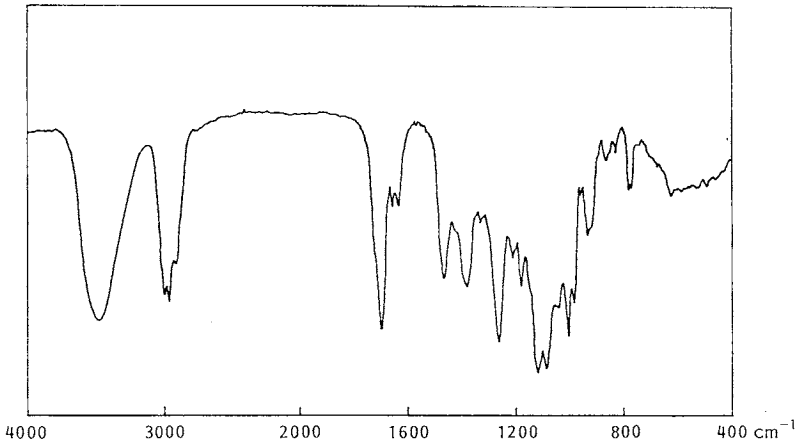
The physico-chemical properties of **1** are summarized in Table 1. The antibiotic gave a single peak on reversed-phase HPLC column and a single band on silica gel TLC. The molecular formula of **1** was determined to be $C_{43}H_{68}O_{12}$ on the basis of mass and NMR spectra and elemental analysis.

Table 1. Physico-chemical properties of PC-766B (**1**).

Appearance	Colorless powder
MP (°C)	182 ~ 184
$[\alpha]_D^{20}$ (*c* 0.5, EtOH)	+26.0°
Molecular formula	$C_{43}H_{68}O_{12}$
FD-MS (*m/z*)	799 $(M+Na)^+$, 815 $(M+K)^+$
Elemental analysis	
Calcd for $C_{43}H_{68}O_{12}\cdot 3H_2O$	C 62.14, H 8.97
Found	C 61.29, H 8.81
UV $\lambda_{max}^{2\text{-PrOH}}$ nm (ε)	228 (36,700), 235 (sh 35,200), 247 (sh 28,900), 285 (13,700)
IR ν_{max} (KBr) cm^{-1}	3448, 2936, 1690, 1456, 1368, 1250, 1166, 1102, 1072, 990
Reversed-phase HPLC[a]	
Retention time (minutes)	16.0
Silica gel TLC Rf	
Benzene - EtOAc (1 : 4)	0.23
$CHCl_3$ - MeOH (15 : 1)	0.14

[a] Column, Waters μBondapak C_{18} (3.9 × 250 mm); mobile phase, MeOH - water (80 : 20); flow rate, 1 ml/minute; detection, UV at 254 nm.

Fig. 1. IR spectrum of PC-766B (1) (KBr).



The UV spectrum of **1** measured in 2-propanol showed absorption maxima at 228, 235 (sh), 247 (sh) and 285 nm. The antiobiotic was soluble in chloroform, benzene, toluene, ethyl acetate, acetone and lower alcohols but insoluble in *n*-hexane and water. It gave positive color reaction to anisaldehyde-sulfuric acid, but negative to ninhydrin, ferric chloride, and Erhlich and Dragendorff reagents.

Structure Elucidation

The UV absorption maxima (228, 285 nm) of **1** indicated the presence of an acyclic conjugated diene and an $\alpha,\beta,\gamma,\delta$-unsaturated ester functionality. The IR spectrum of **1** (Fig. 1) was very similar to that of concanamycin C[3], which is an 18-membered macrolide antibiotic isolated from *Streptomyces diastatochromogenes*, suggesting that there is a structural resemblance between the two compounds. As shown in Table 2, ^{13}C NMR spectrum of **1** indicated 43 carbons; 9 C-methyl carbons, 3 methylene carbons, 5 methine carbons, 2 methoxy carbons, 9 oxymethine carbons, an acetal and a ketal carbons (δ_C 96.7 and 99.7), 12 olefinic carbons and an ester carbon. Therefore, the number of carbon atoms of **1** was two less than that of concanamycin C ($C_{45}H_{74}O_{13}$).

Table 2. ^{13}C NMR assignments of PC-766B (1) (50.3 MHz, $CDCl_3$)[a].

Assignments	δ (ppm)	Assignments	δ (ppm)
C-1	167.1	C-16-CH_3	9.6
C-2	141.3	C-17	70.3
C-2-OCH_3	59.7	C-18	41.6
C-3	132.7	C-18-CH_3	7.1
C-4	143.3	C-19	99.7
C-4-CH_3	14.0	C-20	40.0
C-5	142.5	C-21	76.1
C-6	36.6	C-22	41.5
C-6-CH_3	17.2	C-22-CH_3	13.4
C-7	81.1	C-23	75.2
C-8	40.3	C-24	130.3
C-8-CH_3	21.7	C-25	132.5
C-9	41.3	C-26	131.1
C-10	133.2	C-27	129.2
C-10-CH_3	20.2	C-28	18.0
C-11	125.2	C-1′	96.7
C-12	133.1	C-2′	39.6
C-13	127.1	C-3′	72.0
C-14	81.9	C-4′	77.5
C-14-OCH_3	55.6	C-5′	71.4
C-15	76.4	C-5′-CH_3	17.6
C-16	37.3		

[a] Chemical shifts are given in ppm downfield of internal TMS.

Treatment of **1** with 0.03 N NaOH in methanol under conditions similar to those used for concanamycin C[4] gave two products; an aglycone (**2**) and a sugar (**3**). The ^1H NMR spectrum of **2** indicated 11 olefinic protons, a methylene proton, 10 methine protons, 8 methyl groups, 2 methoxy groups and 3 hydroxyl protons. The assignments of these proton signals were carried out by ^1H-^1H COSY and spin decoupling experiments. The results are summarized in Table 3. **2** had the same 16-membered macrolide ring as

Table 3. ^{1}H NMR assignments of PC-766B (**1**) and its aglycone (**2**) (200 MHz, $CDCl_3$)[a].

Assignment	1	2	Assignment	1	2
C-2-OCH_3	3.48 s	3.60 s	18-H	1.70 m	2.89 dq (4.0, 6.9)
3-H	6.56 s	6.57 s	C-18-CH_3	1.02 d (7.0)	1.14 d (6.9)
C-4-CH_3	1.93 br s	1.91 br s	20-H_a	2.28 m	6.21 dd (0.9, 15.7)
5-H	5.73 dm (10.0)	5.69 br d (10.2)	20-H_b	1.15 m	6.21 dd (0.9, 15.7)
6-H	2.48 ddm (2.0, 7.0, 10.0)	2.50 m	21-H	3.72 m	6.79 dd (7.8, 15.7)
			22-H	1.28 m	2.42 m
C-6-CH_3	1.03 d (7.0)	1.00 d (7.0)	C-22-CH_3	0.87 d (6.3)	0.87 d (6.9)
7-H	3.25 m	3.25 m	23-H	3.99 dd (7.6, 10.5)	3.93 t (6.9)
8-H	1.90 m	2.10 m	24-H	5.37 dd (7.6, 14.9)	5.45 dd (6.9, 14.6)
C-8-CH_3	0.90 d (7.0)	0.87 d (6.8)	25-H	6.06 dd (10.3, 14.9)	6.13 dd (10.5, 14.6)
9-H_a	2.15 m	2.10 m	26-H	5.87 ddq (1.5, 10.3, 14.7)	6.00 ddq (1.2, 10.5, 14.3)
9-H_b	1.95 m	2.10 m			
C-10-CH_3	1.91 br s	1.84 br s	27-H	5.56 dq (6.5, 14.7)	5.70 dq (7.0, 14.3)
11-H	5.78 d (10.5)	5.74 d (10.9)	28-H	1.67 br d (1.5, 6.5)	1.69 br d (1.2, 7.0)
12-H	6.48 dd (10.5, 14.6)	6.41 dd (10.9, 15.2)	1′-H	4.55 br d (9.5)	
13-H	5.12 dd (9.3, 14.6)	5.11 dd (8.9, 15.2)	2′-H_{ax}	1.56 m	
14-H	3.88 dd (9.0, 9.3)	3.74 t (8.9)	2′-H_{eq}	2.05 m	
C-14-OCH_3	3.22 s	3.15 s	3′-H	3.55 m	
15-H	4.94 dd (1.0, 9.0)	4.99 dd (1.4, 8.9)	4′-H	3.02 t (8.9)	
16-H	2.08 m	2.05 m	5′-H	3.18 m	
C-16-CH_3	0.80 d (6.8)	0.87 d (6.8)	5′-CH_3	1.26 d (6.1)	
17-H	4.04 m	3.70 m			

[a] Chemical shifts are given in ppm downfield of internal TMS. Coupling constants (J=Hz) are in parentheses.

bafilomycins[5]). Two olefinic methine signals at 3-H (δ_H 6.57) and 5-H (δ_H 5.69) and three olefinic methine signals at 11-H (δ_H 5.74), 12-H (δ_H 6.41) and 13-H (δ_H 5.11) were assigned to an $\alpha,\beta,\gamma,\delta$-unsaturated ester and a conjugated diene of the 16-membered macrolide ring, respectively. Four other olefinic methine signals at 24-H (δ_H 5.45), 25-H (δ_H 6.13), 26-H (δ_H 6.00) and 27-H (δ_H 5.70) were assigned to an isolated conjugated diene attached to the 6-membered hemiketal ring of **1**. The configurations of these double bonds were deduced as all *E* from the large coupling constants between 12-H and 13-H (J=15.2 Hz), 24-H and 25-H (J=14.6 Hz), and 26-H and 27-H (J=14.3 Hz).

Another alkaline degradation product **3** was identified as α,β-anomeric mixture of 2-deoxy-D-rhamnose[6]) on the basis of ^{1}H and ^{13}C NMR spectral analyses and its optical rotations: ^{1}H NMR (D_2O) δ 5.30 and 4.90 (1′-H); 3.94 and 3.42 (5′-H); 3.87 and 3.66 (3′-H); 3.09 and 3.04 (4′-H); 2.25 and 2.13 (2′-H_{eq}); 1.69 and 1.49 (2′-H_{ax}); 1.27 and 1.25 (5′-CH_3): ^{13}C NMR (D_2O) δ 94.0 and 92.0; 77.7 and 77.1; 72.7 and 71.1; 68.8 and 68.5; 40.6 and 38.4; 17.8: $[\alpha]_D^{20}$ +56.3° (*c* 0.73, acetone). This sugar moiety was identical to that of concanamycin C. The chemical shifts of 21-H (δ_H 3.72) and C-21 (δ_C 76.1) in **1** indicated the connectivity of the sugar in **1** as an *O*-glycoside linkage between C-21 and C-1′. The stereo configuration of the sugar moiety in **1** was determined to be β-anomer from its proton resonances which were almost identical to those of the β-anomer of **3**. Based on these results, the structure of PC-766B was determined as **1** (Fig. 2). Therefore, PC-766B, which was first reported in 1987[7]), is a new class of the hygrolidin family. Recently, a related compound HS-6 has been isolated from *Nocardia otitidiscaviarum* by MIKAMI *et al.*[8]).

Chemical Stability of PC-766B and Stabilizing Effect of Antioxidant Agents

PC-766B (**1**) proved to be unstable in the atmosphere (Fig. 3). Even at low temperatures, a gradual decomposition was observed. To prevent the decomposition during the storagte of **1**, various additives were tested for their stabilizing effects. Some antioxidant agents were found to prevent the decomposition of **1**

at concentrations of 0.01 ~ 0.05% (Fig. 4). DL-α-Tocopherol (vitamin E) was found to be the most effective. In the presence of 0.02% DL-α-tocopherol, **1** could be stored with no decomposition for 30 days at 4°C, and for 12 months at −18°C (data not shown). The fact that **1** was remarkably stabilized by DL-α-tocopherol suggests that **1** is sensitive to the air oxidation. DEEG *et al.*[9] have reported in a study on chemical modifications of bafilomycins that they were unable to isolate some derivatives of bafilomycins owing to their instability. Our finding should help in the preparation of derivatives of this type and may contribute to the study of their structure-activity relationships.

Fig. 2. Structures of PC-766B (**1**) and its alkaline degradation products, an aglycone (**2**) and a sugar (**3**).

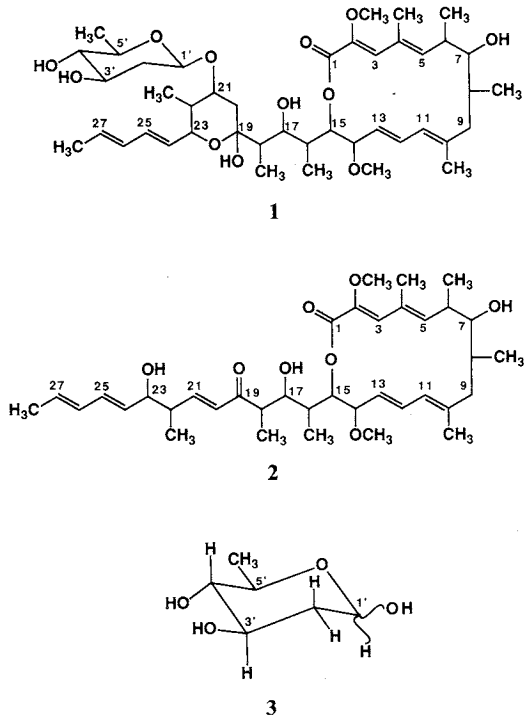


Fig. 3. Stability of PC-766B (**1**) incubated at −18°C (■), 4°C (●) and 27°C (▲).

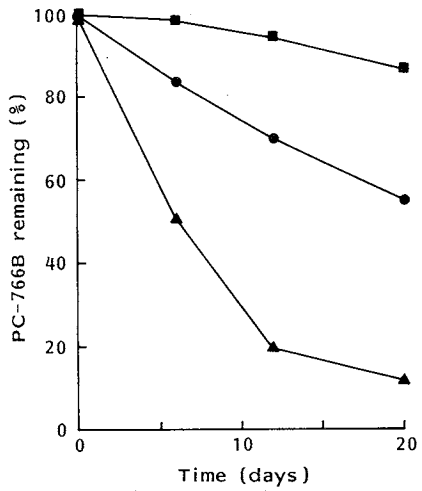
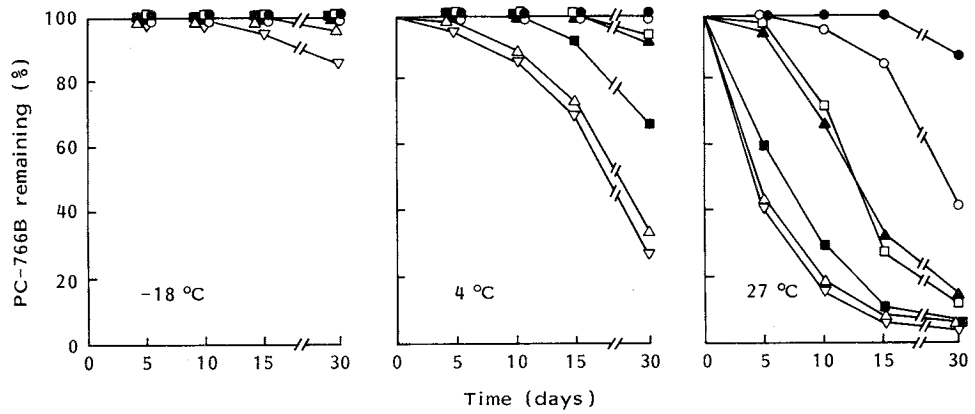


Fig. 4. Stabilization of PC-766B (**1**) by antioxidant agents.

●, DL-α-Tocopherol (0.02%); ○, butylated hydroxyanisole (0.02%); □, nordihydroguaiaretic acid (0.01%); ▲, butylated hydroxytoluene (0.02%); ■, *n*-propyl gallate (0.01%); △, sodium L-ascorbate (0.05%); ▽, no addition.



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