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PC-766B' and PC-766B, 16-Membered Macrolide Angiogenesis Inhibitors Produced by *Nocardia* sp. RK97-56

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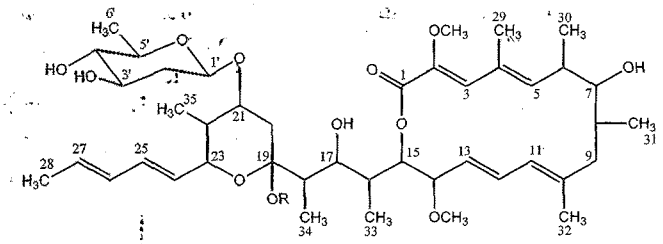
Abstract Angiogenesis is an essential event in a variety of physiological and pathological processes. Therefore, effective inhibition of this event is a promising strategy for treating angiogenesis-related diseases, including cancer. The current study investigated two unique bafilomycin-type macrolide inhibitors of angiogenesis, PC-766B' (**1**) and PC-766B (**2**). The strain RK97-56 which produced the inhibitors was identified as *Nocardia* sp. by chemotaxonomic analyses, and the purification of the inhibitors was guided by their anti-angiogenic activities. PC-766B' (**1**) and PC-766B (**2**) exhibited potent inhibitory activities towards endothelial cell migration stimulated by the vascular endothelial growth factor (VEGF).

Key words: Angiogenesis inhibitor, macrolide, *Nocardia* sp.



1: R = CH_3 (PC-766B')
2: R = H (PC-766B)

Fig. 1. Structures of PC-766B' (**1**) and PC-766B (**2**).

Angiogenesis involves the formation of new blood vessels from preexisting vessels and is necessary for tumor growth and metastasis [7, 12]. Endothelial cells, which form the inner lining of all blood vessels, play an essential role in angiogenesis. The vascular endothelial growth factor (VEGF), an inducer of endothelial cell proliferation, migration, and survival, is also considered to play a pivotal role in angiogenesis and is secreted by tumor cells and macrophages [3]. Therefore, the inhibition of cellular functions induced by VEGF in endothelial cells would appear to be a promising strategy for treating various types of cancer as well as other angiogenesis-related diseases [3, 6, 7, 12].

Previously, the current authors reported on the identification of unusual pentaketide dimers, epoxyquinols A [10] and B (H. Kakeya *et al.*, *J. Antibiot.* in press.) as well as azaspirene [1] with a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione skeleton, as novel angiogenesis inhibitors. During the continuous screening of microbial metabolites, it was found that strain RK97-56, identified as a *Nocardia* sp., produced potent angiogenesis inhibitors, PC-766B' (**1**) [15] and PC-766B (**2**) [13–15] (Fig. 1). There has been no report on the details of **1**. Accordingly, the current paper describes the taxonomy of the producing strain, and the fermentation, isolation, structural elucidation, and biological properties of **1**.

Taxonomy of Strain RK97-56

The producing organism, strain RK97-56, was isolated from a soil sample collected at Nomozaki in Nagasaki Pref., Japan. The taxonomic studies were carried out as described by the International *Streptomyces* Project (ISP) [21]. The substrate mycelia of strain RK97-56 were well-branched. The aerial mycelia consisted of long, straight, and occasionally spiral filaments (Fig. 2). No sclerotia, spores, or sporangia were observed. An analysis of the whole cell hydrolysates revealed the presence of *meso*-diaminopimelic acid.

The cultural characteristics were also observed on various media. The mature aerial mycelia were white, and the reverse side of the colony corresponded to both the

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Fig. 2. Scanning electron micrograph of spore chains of *Nocardia* sp. RK97-56 (Bar: 5 μm).

yellow and orange color series, as assigned by the *Color Harmony Manual*, 4th Ed., 1958 (Container Corporation of America, Chicago). No soluble pigment was observed. The temperature range for growth was 5–35°C with an optimum at 21–27°C. The tolerance to NaCl was less than 3.0% on Bennet's agar. The growth of the strain was observed on an ISP-9 agar supplemented with D-glucose, D-fructose, and *myo*-inositol as the carbon sources [19]. A fragment ion analysis of the menaquinone by FABMS revealed that MK-8 (H_4, ω-cycle) was the major menaquinone [2, 5], which is known to occur in the genus *Nocardia* [8].

Therefore, based on the morphological, cultural, and physiological characteristics, strain RK97-56 was determined to belong to the genus *Nocardia*. This conclusion was also supported by a nucleotide sequence analysis of the 16S rDNA (data not shown). Accordingly, the strain was named *Nocardia* sp. RK97-56.

Fermentation, Isolation, and Purification

The producing strain RK97-56 was cultured in 15 liter of a medium consisting of 2% glucose, 1% soluble starch, 2.5% dried yeast, 0.3% meat extract, 0.3% cornsteep liquor, 0.05% NaCl, 0.05% $CaCO_3$, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.2) in a 30-l jar fermentor at 28°C for 4 days. The whole cultured broth (15 liter) was centrifuged to separate the supernatant and mycelium. The supernatant was adjusted to pH 3.5 with HCl and extracted with an equal volume of EtOAc. The mycelium was extracted with acetone, as most of the activity was present in this part. After removing the solvent *in vacuo*, the extract was suspended in 1 liter of water and extracted three times with an equal volume of EtOAc at pH 3.5. The combined total EtOAc extract (24 g) was applied to a silica gel column and eluted with mixtures of $CHCl_3$-MeOH (100:1, 50:1, 25:1, 10:1, 5:1, and MeOH) as a step gradient to produce six fractions. Fraction 3, eluted with a 25:1 mixture of $CHCl_3$-MeOH, was the most bioactive and was applied again to silica gel column chromatography using EtOAc-*n*-hexane (1:1) mixtures with increasing polarity. The second silica gel column chromatography produced two active fractions, A and B, containing a minor compound of **1** and a major compound of **2**, respectively. Fractions A (174 mg) and B (586 mg) were further purified by HPLC using an ODS column [Senshu Scientific Co. Ltd., Tokyo] with a gradient solvent system of MeOH-water from 85:15 to 100% MeOH to remove the impurities. Each main peak was collected and evaporated *in vacuo* to give white powders. Although fractions C (35 mg) and D (250 mg) showed high degree of purification, both fractions still contained **1** and **2**, respectively. The final purification was accomplished through preparative silica TLC (60 F_{254}, 0.5 mm, Merck) using a mixture of EtOAc-*n*-hexane (4:1) as the developing solvent to yield **1** (16 mg) and **2** (125 mg), respectively.

Physicochemical Properties of PC-766B' (1)

As yet, there has been no report on the detailed physicochemical properties and structural determination of **1**. PC-766B' (**1**), $[\alpha]_D^{25}$+2.7° (*c* 0.183, EtOH), was obtained as a colorless powder. It was soluble in MeOH, EtOAc, and DMSO, but not in *n*-hexane. PC-766B' (1) was found to have a TLC R_f value of 0.54 (silica; EtOAc-*n*-hexane, 4:1). The UV spectrum of **1** showed absorption maxima at 229 (ε 28,500), 235 (sh, ε 27,650), 250 (sh, ε 19,700), and 285 nm (ε 9,400) in MeOH, indicating the presence of an α,β,γ,δ-unsaturated ester (285 nm) and conjugated diene (250 nm). The IR spectrum exhibited two major absorption bands at 3,450 cm^{-1} and around 1,690 cm^{-1} due to a hydroxyl and carbonyl group, respectively. The molecular formula of **1** was determined to be $C_{44}H_{70}O_{12}$ based on the pseudomolecular ion peak at *m/z* 813 $(M+Na)^+$ in HR-FABMS (found *m/z* 813.4786 $[M+Na]^+$; calcd for 813.4765 $[M+Na]^+$), along with the assistance of the ^1H and ^{13}C NMR spectra.

Structural Elucidation of PC-766B' (1)

The molecular formula of **2** was determined to be $C_{43}H_{68}O_{12}$, and its structure was identified as PC-766B [13–15] based

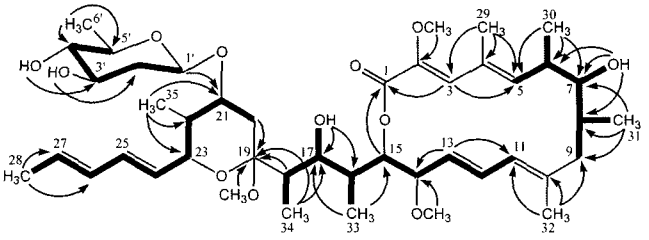

Fig. 3. Key significant correlations in PFG-DQFCOSY (bold lines) and PFG-HMBC (arrows) spectra of PC-766B' (**1**).

Table 1. ^{1}H and ^{13}C NMR data for PC-766B' (**1**) and PC-766B (**2**) in DMSO-d_6.

Carbon no.	1[a]		2[a]	
	^{13}C (mult.)	^{1}H (mult., J Hz)	^{13}C (mult.)	^{1}H (mult., J Hz)
1	164.07(s)		164.16(s)	
2	140.89(s)		140.85(s)	
3	131.72(d)	6.53(s)	131.36(d)	6.49(s)
4	130.40(s)		130.52(s)	
5	143.91(d)	5.81(d, 8.5)	143.93(d)	5.80(d, 8.4)
6	37.29(d)	2.40(br t, 7.3)	37.11(d)	2.39(br t, 7.3)
7	78.44(d)	3.16(m)	78.41(d)	3.15(m)
8	39.50(d)	1.75(m)	39.25(d)	1.70(m)
9a	41.25(t)	2.07(dd, 14.0, 11.4)	41.23(t)	2.04(dd, 13.8, 11.4)
9b		1.88(m)		1.86(m)
10	142.16(s)		142.55(s)	
11	123.95(d)	5.71(d, 11.3)	123.90(d)	5.70(d, 11.0)
12	131.54(d)	6.48(dd, 14.7, 11.3)	131.87(d)	6.50(dd, 14.9, 11.0)
13	125.59(d)	5.16(dd, 14.7, 9.6)	125.56(d)	5.13(dd, 14.9, 9.5)
14	83.49(d)	3.94(br t, 6.7)	83.00(d)	3.95(br t, 7.5)
15	75.61(d)	5.20(dd, 9.0, 2.2)	75.15(d)	5.14*
16	39.50(d)	1.75(m)	38.70(d)	1.83(dd, 10.1, 5.3)
17	68.89(d)	3.40(dd, 8.8, 7.2)	69.66(d)	3.88*
18	37.62(d)	1.90(m)	42.26(d)	1.62(dd, 14.3, 7.1)
19	103.16(s)		99.49(s)	
20a	34.55(t)	2.32(dd, 13.4, 4.6)	37.98(t)	2.21(dd, 12.3, 4.6)
20b		1.22(m)		1.04(m)
21	74.27(d)	3.55(ddd,13.4,10.8, 4.7)	74.34(d)	3.62(ddd,15.1,10.5, 4.6)
22	40.38(t)	1.13(dd, 10.8, 6.8)	41.06(t)	1.12(dd, 10.5, 6.6)
23	75.40(d)	3.50(dd, 10.8, 7.7)	74.34(d)	3.89*
24	129.77(d)	5.45(dd, 15.1, 7.7)	130.18(d)	5.43(dd, 14.9, 7.4)
25	132.40(d)	6.15(dd, 15.1, 10.6)	131.55(d)	6.08(dd, 14.9, 10.5)
26	130.85(d)	6.04(ddq,14.7,10.6, 2.2)	130.98(d)	5.97(ddq,14.6,10.5, 2.1)
27	129.64(d)	5.68(dq, 14.7, 7.0)	128.89(d)	5.61(dq, 14.9, 7.0)
28	17.88(q)	1.70(d, 6.6)	17.76(q)	1.67(d, 6.4)
29	13.49(q)	1.88(s)	13.58(q)	1.87(s)
30	18.07(q)	0.94(d, 6.8)	17.93(q)	0.94(d, 7.0)
31	22.78(q)	0.87(d, 6.8)	22.57(q)	0.86(d, 7.0)
32	18.86(q)	1.74(s)	19.08(q)	1.76(s)
33	11.15(q)	0.80(d, 7.0)	10.54(q)	0.75(d, 6.8)
34	7.41(q)	0.84(d, 7.0)	7.04(q)	0.87(d, 7.1)
35	13.29(q)	0.76(d, 6.3)	13.26(q)	0.78(d, 6.4)
1'	95.66(d)	4.49(br d, 9.7)	95.70(d)	4.50(br d, 9.7)
2'	40.24(t)	1.83(m)	40.21(t)	1.85(m)
		1.27(dd, 11.9, 10.3)		1.26(dd, 11.9, 10.1)
3'	70.43(d)	3.35*	70.40(d)	3.36*
4'	76.86(d)	2.70(m)	76.90(d)	2.70(m)
5'	71.49(d)	3.11(dd, 9.0, 6.1)	71.48(d)	3.10(dd, 9.1, 6.2)
6'	18.09(d)	1.14(d, 6.1)	18.08(d)	1.14(d, 6.2)
2-OCH_3	59.60(q)	3.57(s)	59.19(q)	3.45(s)
14-OCH_3	55.22(q)	3.14(s)	55.17(q)	3.14(s)
19-OCH_3	45.87(q)	2.92(s)		
7-OH		4.89(d, 5.3)		4.90(d, 5.3)
17-OH		4.34(d, 7.2)		4.60(d, 6.4)
19-OH				5.56(s)
3'-OH		4.83(d, 4.6)		4.80(d, 4.6)
4'-OH		4.90(d, 5.1)		4.89(d, 5.1)

[a]Chemical shifts are in ppm downfield of internal TMS in DMSO-d_6 (^{1}H, 500 MHz, ^{13}C, 125 MHz).
*Overlapped signals.

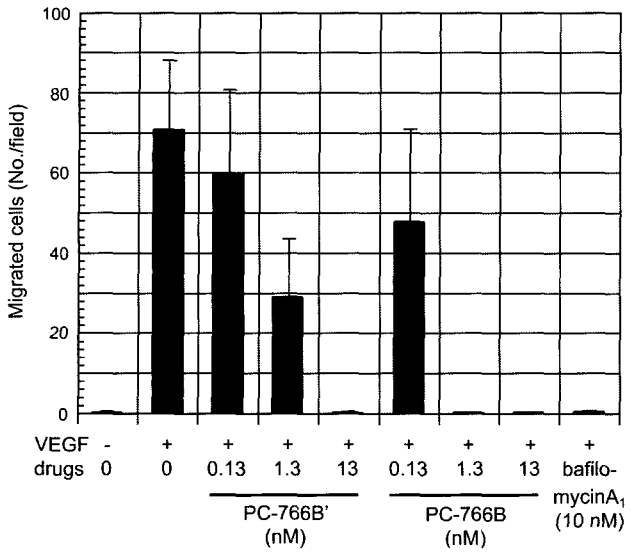

Fig. 4. Inhibition of VEGF-induced cell migration by PC-766B' (**1**) and PC-766B (**2**) in HUVECs.

Human umbilical vein endothelial cells (HUVECs) (1×10^5) suspended in a HuMedia-EG2 medium (KURABO, Osaka) with various concentrations of the test compounds were added to the upper compartment of a CHEMOTAXICELL chamber (KURABO, Osaka) and then incubated with a HuMedia-EG2 medium containing 12.5 ng/ml of VEGF in the lower compartment for 18 h at 37°C in a 5% CO_2 atmosphere. The filter was fixed with MeOH and stained with hematoxylin. The cells on the upper surface of the filter were carefully removed by wiping with cotton swabs. The cells that migrated through the filter to the areas of the lower surface were counted manually under a microscope at a magnification of ×100. Values are means±SD for triplicate samples.

on the spectroscopic data. The ^1H and ^{13}C NMR spectra of **1** were almost identical with those of **2**, but with the presence of proton and carbon signals corresponding to the methyl group at hemiketalic 19-OH, thus suggesting that **1** is a methylated form of **2** at this position. This conclusion was further confirmed by a detailed analysis of the NMR spectroscopic data, including PFG-DQFCOSY, PFG-HMQC, and PFG-HMBC, as shown in Fig. 3 and Table 1.

Anti-Angiogenic Activities of PC-766B' (1) and PC-766B (2)

The *in vitro* anti-angiogenic activities of **1** and **2** were investigated using a CHEMOTAXICELL chamber with 8.0-μm-pore-polycarbonate filter inserts. As shown in Fig. 4, the angiogenic factor, VEGF, significantly enhanced the migration in human umbilical vein endothelial cells (HUVECs). PC-766B' (**1**) and PC-766B (**2**) efficiently inhibited the migration of HUVECs stimulated by VEGF in a dose-dependent manner. The ED_{100} values of **1** and **2** were 13 nM and 1.3 nM, respectively. Ten nM bafilomycin A_1, a known vacuolar-type ATPase (V-ATPase) inhibitor [4, 16, 17] with the same 16-membered macrolide ring but with different side chain, also inhibited the VEGF-induced endothelial migration.

Discussion

The results described above showed that **1** was a 19-*O*-methyl derivative of **2** and related to 16-membered macrolides, such as bafilomycins [17, 22, 23], hygrolidins [20], and leucanicidins [9]. The fact that **2** can be converted into **1** in MeOH solution suggests the possibility that **1** may be formed by a specific positional methanolysis during the isolation procedure. However, the substitution of a diene residue at the C-23 position in **1** and **2** has not been reported in bafilomycins, concanamycins (18-membered) [11], and related macrolides bearing the same hemiketal ring. Recently, micromonospolide A, a gastrulation inhibitor of starfish embryos, was isolated from *Micromonospora* sp. [18]. This compound has the same substitution of a diene residue at the C-23 position as in **1** and **2.** PC-766B (**2**) inhibited VEGF-induced endothelial cell migration at a 10 times lower concentration than PC-766B' (**1**), as shown in Fig. 4, suggesting that the hydroxyl group at C-19 plays a key role in the anti-angiogenic activity. Although PC-766B (**2**) exhibits antitumor activity and antimicrobial activities against Gram-positive bacteria, certain fungi, and yeasts [13–15], no biological activity has yet been identified for **1**. Consequently, it should be noted that the bafilomycin-type macrolides **1** and **2** both affect the complex process of angiogenesis in endothelial cells and have potent antiangiogenic activity.

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