TAN-1323 C and D, New Concanamycin-group Antibiotics; Detection of the Angiostatic Activity with a Wide Range of Macrolide Antibiotics

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We detected potent angiostatic activity in a MeOH extract from the mycelia of microbial strain S-45628 in the chick chorioallantoic membrane (CAM) assay. The producer was taxonomically characterized as *Streptomyces purpurascens*. Active principles designated TAN-1323 A~D were isolated and determined to be 18-membered macrolide antibiotics; components C and D are new members of this group, while components A and B are identical to concanamycins C and A, respectively. When tested in the CAM assay, components B and D gave huge avascular zones at the extremely low doses of 10~100 ng/disk, although components A and C showed far weaker activity due to their preferential tissue-damaging effect on the CAM. The discovery that these 18-membered macrolide antibiotics are angiostatic substances prompted us to examine other types of macrolide antibiotics, leading to the discovery that 16-membered macrolide antibiotics such as bafilomycin C1, tylosin and leucomycin also show angiostatic activity on the CAM. Thus, angiostatic potential is widely distributed among macrolide antibiotics. The mechanism of action of these macrolide antibiotics is also discussed.

Angiogenesis, the formation of new blood vessels, is observed in various types of pathological conditions ("angiogenic deseases")[1] such as rheumatoid arthritis, diabetic retinopathy, psoriasis and solid tumors. Recent extensive research has enhanced our understanding of the process of angiogenesis and has made the anti-angiogenesis concept a promising strategy in the search for new types of agents effective against solid tumors which are dependent on angiogenesis for their growth and are refractory to presently available therapies.[1,2]

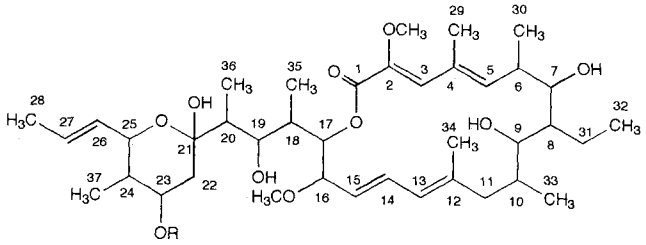
In our screening program aimed at angiogenesis inhibitors of microbial origin, a *Streptomyces* species, S-45628, was found to produce angiostatic substances mainly within the mycelia. Active principles designated TAN-1323 A~D were isolated and structually characterized as concanamycin-group antibiotics, 18-membered macrolide antibiotics.[3] Components C and D proved to be new members of this group, while components A and B are identical to concanamycin C[3] and folimycin[4] (concanamycin A),[3] respectively (Fig. 1).

This paper deals with the taxonomy of the producing organism, fermentation, isolation, structure elucidation and biological activities of TAN-1323 components. In addition, the paper describes the interesting finding that other types of macrolide antibiotics including bafilomycin C1[5], a specific inhibitor[6] of vacuolar (V-type) H^+-ATPase,[7,8] and the "classical" macrolide antibiotics such as tylosin and leucomycin also have angiostatic potential. We also discuss the mechanism of the action of these antibiotics.

Taxonomy

The producing organism, S-45628, was isolated from a soil sample collected in Miyazaki Prefecture, Japan. Taxonomical characterization was carried out accord-

Fig. 1. Structures of TAN-1323 A~D.



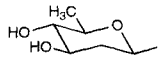
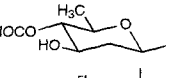
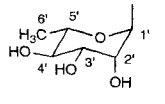
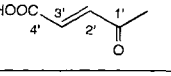
Compound	R
TAN-1323 A (**1**)	
TAN-1323 B (**2**)	
TAN-1323 C (**3**)	
TAN-1323 D (**4**)	

Table 1. Cultural characteristics of strain S-45628.

Medium	Growth	Reverse side color	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar (Waksman 1)	Poor to moderate, grayish yellow to gray (1ea～1ca)	Gray (1ca)	Poor, white-gray (1ca)	None
Glucose-asparagine agar (Waksman 2)	Poor, pale reddish brown-grayish red-brown (3lc～4ic～5ic)	Pale yellowish brown-reddish brown (3lc～5ic～5lc)	Poor, white	None
Glycerol-asparagine agar (ISP-5)	Moderate, pale grayish yellow (2ca～2ea)	Pale grayish yellow-pale grayish brown (2ea～2ic)	Poor, pale grayish white (3cb)	None
Inorganic salts-starch agar (ISP-4)	Moderate, pale grayish brown (4ga～4ic)	Grayish yellow-dark grayish brown (3ea～4le～4nl)	Moderate, white	Pale brown (4lc)
Tyrosine agar (ISP-7)	Moderate, dark brown (3ni～3pl)	Dark grayish black-black (3pl～2po)	Poor, gray (2dc)	Pale brownish black (4pn)
Nutrient agar	Poor, pale grayish yellow (2ca～2ae)	Pale grayish yellow (2ca～2ae)	Poor, white	None
Yeast extract-malt extract agar (ISP-2)	Moderate, reddish brown (5ne～5pe)	Reddish brown-dark reddish brown (5pc～5pe)	Moderate, white	Pale reddish brown (5ia)
Oatmeal agar (ISP-3)	Moderate, pale grayish yellow-pale grayish brown (3ga～4ga)	Grayish yellow-pale grayish black (3gc～4ga)	Poor, white	None
Peptone-yeast extract-iron agar (ISP-6)	Poor, gray (3dc)	Pale grayish black (3fe)	Poor, white	Pale brownish black (4pn)

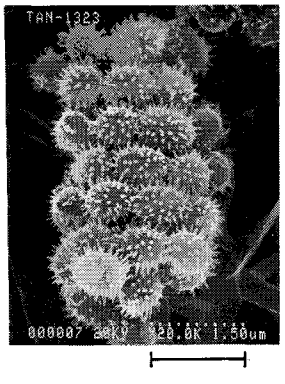
Color codes for description of colors of mature cultures were determined according to the Color Harmony Manual[10].

Fig. 2. Aerial mycelia of strain S-45628 bearing spore chains.

Bar represents 3 μm.

Fig. 3. Spores of strain S-45628.

Bar represents 1.5 μm.



ing to the method of the International Streptomyces Project (ISP).[9]

Aerial mycelia elongated from well branched basal mycelia. Spiral spore chains consisting of 10 or more spores were observed at the tips of aerial mycelia (Fig. 2). Spores were ovoid-shaped in the diameter of 0.6×0.8～0.9 μm, and the surface was spiny (Fig. 3).

Table 1 shows the growth characteristics of strain S-45628 on various agar media. Its physiological properties are summarized in Table 2. Comparison of its gross characteristics with those of the species described

Table 2. Physiological properties of strain S-45628.

Melanoid pigment production (ISP-6, 7)	+
Liquefaction of gelatin	−
Hydrolysis of starch	+
Coagulation of milk	−
Peptonization of milk	+
Temperature range for growth (ISP-2)	13～44°C (optimum, 26～35°C)
Type of diaminopimelate[a]	LL
Utilization of carbon source[b]	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
i-Inositol	+
L-Rhamnose	+
Raffinose	+
D-Mannitol	+

[a] Cell wall analysis was performed by the method of HASEGAWA *et al.*[11]

[b] Carbohydrate utilization was investigated by the method of PRIDHAM and GOTTLIEB.[12]

in BERGEY's Manual of Systematic Bacteriology[13] led us conclude that it belongs to Genus *Streptomyces* and is most related to *S. violaceus*, *S. violatus* and *S. purpurascens*. Direct comparison of the growth and physiological properties of the producer and these related species indicated that it is identical to *S. purpurascens* (data not shown). The strain was deposited at Institute for Fermentation, Osaka and National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan under the accession numbers IFO 14980 and FERM BP-2718, respectively.

Fermentation

One loopful of the spore mass of strain S-45628 grown on an ISP-2 agar slant was inoculated into a 2-liter Sakaguchi flask containing 500 ml of a seed medium consisting of glucose 2%, soluble starch 3%, corn-steep liquor 1%, soy bean flour 1%, Polypepton 0.5%, NaCl 0.3% and $CaCO_3$ (precipitated) 0.5% (pH 7.0) and cultivated for 40 hours at 28°C on a rotary shaker.

The entire seed culture thus obtained was transferred to a 50-liter fermentor containing 30 liters of the same seed medium supplemented with 0.05% Actocol (an antifoaming agent, Takeda Chem. Ind.) and cultivated for 42 hours at 28°C with aeration of 30 liters/minute and agitation of 280 rpm.

Six liters of this seed culture was inoculated into a 200-liter fermentor containing 120 liters of a fermentation medium consisting of glucose 0.5%, dextrin 5%, soy bean flour 2%, corn-gluten meal 1.5%, $CaCO_3$ (precipitated) 0.7% and Actocol 0.05% (pH 7.0). Fermentation was carried out for 66 hours at 28°C with aeration of 120 liters/minute and agitation of 150 rpm.

Isolation

The fermentation broth of *Streptomyces purpurascens* S-45628 (95 liters) was filtered, and the collected mycelia were extracted with MeOH (100 liters). The extract was concentrated to a small volume (20 liters) and then extracted twice with EtOAc (10 liters) at pH 7.0. The organic layers were combined, washed with water and then concentrated to give a crude powder (43.5 g). The powder was applied to a silica gel column (500 g) and eluted with $CHCl_3$ - MeOH (30 : 1, 20 : 1, 10 : 1 and 5 : 1) to give four fractions containing **1**, **2**, **3** and **4**, respectively.

The first fraction was chromatographed on silica gel (50 g), eluting with $CHCl_3$ - PrOH (20 : 1), to give crystals. After recrystallization from aqueous methanol, **1** was obtained as colorless thin plates (321 mg). The second fraction was chromatographed on silica gel (500 g), eluting with $CHCl_3$ - PrOH (15 : 1), to give crystals. After recrystallization from aqueous methanol, **2** was obtained as colorless thin plates (5.15 g). The third fraction was chromatographed on silica gel (50 g), eluting with $CHCl_3$ - MeOH (30 : 1 and 20 : 1), to give a white powder. After recrystallization from aqueous methanol, **3** was obtained as colorless thin plates (141 mg). Crystals obtained from the last fraction were recrystallized from aqueous methanol to give **4** as colorless thin plates (915 mg).

Characterization and Structure Elucidation

The physico-chemical properties and the behavior of **1**, **2**, **3** and **4** on TLC are summarized in Table 3. The

Table 3. Physico-chemical properties and behavior on TLC of TAN-1323 A (**1**), B (**2**), C (**3**) and D (**4**).

	1	**2**	**3**	**4**
Appearance	Colorless thin plates	Colorless thin plates	Colorless thin plates	Colorless thin plates
MP (°C)	156~157.5	169~171	167~168.5	138.5~139.5
$[\alpha]_D^{24}$	−16.8° (*c* 0.42, *i*-PrOH)	−19.4° (*c* 0.45, MeOH)	−31.9° (*c* 0.40, DMF)	−8.0° (*c* 0.58, DMF)[a]
Molecular formula	$C_{45}H_{74}O_{13}\cdot 0.5H_2O$	$C_{46}H_{75}NO_{14}\cdot 0.5H_2O$	$C_{45}H_{74}O_{14}\cdot 0.5H_2O$	$C_{43}H_{66}O_{13}\cdot 0.5H_2O$
Anal (%)	C; 64.95/64.96	C; 63.23/63.14	C; 63.76/63.73	C; 64.48/64.56
Found/Calcd	H; 9.25/9.09	H; 8.58/8.75	H; 8.98/8.91	H; 8.70/8.44
		N; 1.60/1.60		
UV λ_{max}^{MeOH} (ε)	244 (39,100), 283 (17,500)	244 (35,800), 284 (16,600)	244 (41,500), 284 (19,500)	211 (30,000), 243 (44,700), 284 (20,000)
IR ν_{max} cm^{-1} (KBr)	3430, 2980, 2940, 1700, 1625	3450, 2990, 2950, 2890, 1700, 1620	3450, 2990, 2950, 1700, 1655, 1630	3650~2500 (broad), 1730, 1700, 1650
TLC (Rf value)[b]				
Solvent I	0.54	0.46	0.35	0.08
Solvent II	0.28	0.25	0.08	0.02

[a] Measured at 23°C.

[b] Silica gel $60F_{254}$ plates (Merck, 0.25 mm) were used.
Solvent I: $CHCl_3$ - MeOH (10 : 1).
Solvent II: EtOAc - benzene (4 : 1).

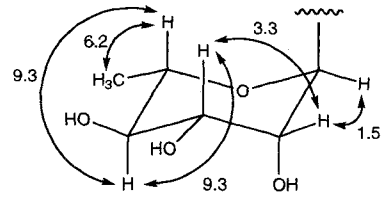
Table 4. ^{13}C and ^{1}H NMR chemical shifts for TAN-1323 C (**3**) and D (**4**) in DMSO-d_6.

Position	3		4		Position	3		4	
	^{13}C	^{1}H	^{13}C	^{1}H		^{13}C	^{1}H	^{13}C	^{1}H
1	164.0		164.2		23	72.1	3.57	74.1	4.93
2	141.6		141.6		24	41.0	1.22	40.2	1.40
3	129.0	6.26	129.2	6.27	25	74.6	3.86	74.4	3.97
4	129.9		130.0		26	130.8	5.30	130.3	5.33
5	141.0	5.64	141.1	5.63	27	127.3	5.56	127.8	5.60
6	34.6	2.58	34.7	2.58	28	17.4	1.59	17.4	1.60
7	72.1	3.64	72.1	3.64	29	13.9	1.89	13.9	1.88
8	43.7	1.27	43.8	1.27	30	17.2	0.92	17.2	0.93
9	78.2	2.97	78.2	2.97	31	22.1	1.04	22.2	1.06
10	34.7	2.42	34.7	2.42	32	11.9	0.76	11.9	0.75
11	45.5	1.85	45.6	1.85	33	21.9	0.95	22.0	0.95
12	142.1		142.2		34	16.2	1.84	16.2	1.83
13	122.4	5.68	122.5	5.68	35	9.8	0.77	9.8	0.76
14	132.9	6.64	133.0	6.64	36	6.9	0.87	6.9	0.87
15	126.6	5.09	126.6	5.09	37	13.5	0.82	13.0	0.73
16	82.0	3.90	81.9	3.90	2-OMe	58.6	3.48	58.6	3.48
17	74.3	5.12	74.4	5.11	16-OMe	55.1	3.15	55.1	3.15
18	37.9	1.90	37.9	1.92	1′	95.4	4.70	164.1	
19	69.3	3.84	69.4	3.84	2′	71.0	3.51	132.6	6.39
20	42.3	1.62	42.1	1.66	3′	70.7	3.42	134.7	6.72
21	99.3		99.3		4′	71.8	3.19	165.6	
22	36.7	1.01	38.0	1.31	5′	68.7	3.48		
		2.25		2.15	6′	17.8	1.13		

molecular formulae were determined from elemental analysis and the number of carbons observed in the ^{13}C NMR spectra. While **1**, **2** and **3** are neutral substances, **4** is acidic. UV absorption maxima of these components at 243~244 and 283~284 nm are characteristic and resemble those of bafilomycins[5] and concanamycins[3,14~16] which are 16- and 18-membered macrolide antibiotics, respectively. From these data together with the NMR spectral data, **1** and **2** were identified as concanamycins C and A[3] (folimycin[4]), respectively, while **3** and **4** were considered to be new concanamycin-group antibiotics.

The ^{13}C and ^{1}H NMR chemical shifts for **3** and **4** are summarized in Table 4. The signals observed with **3** were quite similar to those for concanamycins except for a sugar moiety (C-1′ to C-6′). The ^{1}H-^{1}H decoupling experiments with **3** suggested the presence of a rhamnopyranosyl moiety instead of the 2-deoxy-β-D-rhamnopyranosyl moiety in concanamycins (Fig. 4). Upon treatment of **3** with sodium hydroxide, L-rhamnose was liberated through β-elimination to yield the anhydroaglycone (**6**) which was identical to that obtained from **2** (Fig. 5). This showed that the L-rhamnopyranosyl moiety was linked to C-23 and the other moiety was the same as that in **2**. The anomeric configuration of the sugar moiety was determined to be α from the J_{CH} value

Fig. 4. ^{1}H-^{1}H coupling constants (J in Hz) of the rhamnosyl moiety of TAN-1323 C (**3**).



(167 Hz) of the anomeric carbon (C-1′) in 3.[17]

In the NMR spectrum of **4**, no sugar moiety was observed, but the presence of a fumaric acid moiety (C-1′ to C-4′) was indicated. This was also suggested by UV absorbance at 211 nm and IR absorption bands at 3650~2500 (broad) and 1730 cm^{-1}. Indeed, alkaline hydrolysis of **4** gave fumaric acid (**7**) and the anhydroaglycone (**6**) (Fig. 5). The position of the ester linkage was estimated to be C-23 from the downfield shift of H-23 (δ 4.93). Thus, the structures of the two new 18-membered macrolide antibiotics, **3** and **4**, were determined to be as shown in Fig. 1.

In the case of bafilomycin-group antibiotics (16-membered), the attachment of an α-L-rhamnosyl moiety has been reported.[18] However, **3** is the first example of a concanamycin-group antibiotic (18-membered) having this moiety. TAN-1323 D (**4**) which has a fumaric acid

Fig. 5. Alkaline degradation of TAN-1323 C (3) and D (4).

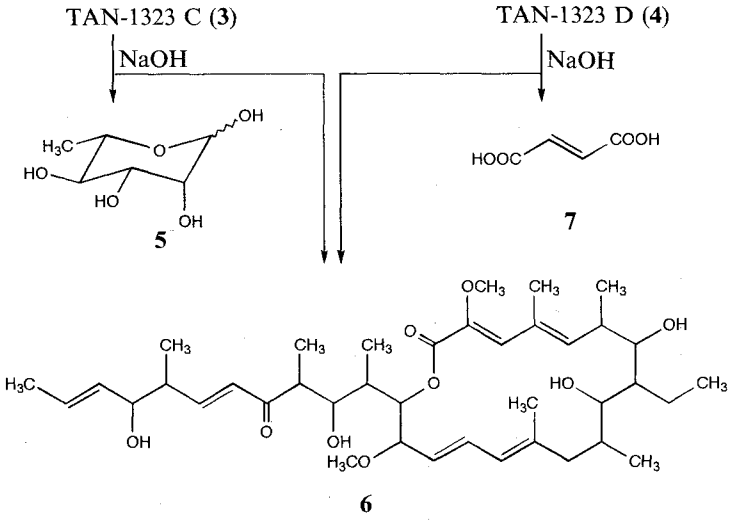
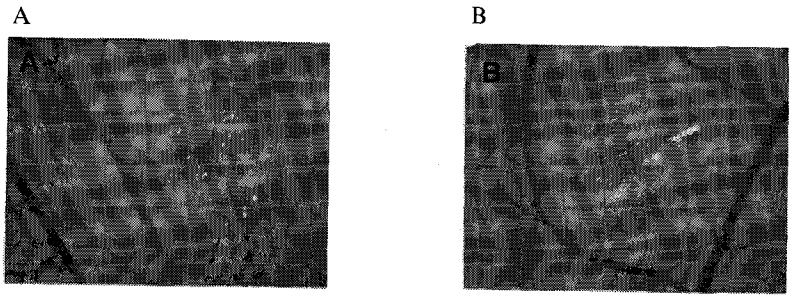


Fig. 6. Inhibition of angiogenesis by TAN-1323 B and D on the CAM.
A; TAN-1323 B, 0.1 μg/disk. B; TAN-1323 D, 0.1 μg/disk.



The CAM assay was performed according to the method of J. FOLKMAN *et al.*[19] with some modification.[20] Briefly, 3-day old fertilized embryos were placed onto hammocks of commercial polyethylene wrap film hanging in plastic cups (6 cm in height, 7.5 cm in diameter) with two side windows and then incubated at 37°C under 3% CO_2 and saturated humidity for 6 days. A sample solution was mixed with an equal volume of 1% methylcellulose (4000 Centipoise, Wako Pure Chemicals) solution, and 10 μl of the mixture was air-dried on a polypropylene sheet for 1 hour in a hood. The disks thus prepared were applied to the 9-day old CAMs. The responses caused by the samples were examined one day later under a stereoscope (Nikon SMZ-10-1). Antiangiogenic activity was expressed as % avascular zones which was calculated by dividing the number of disks giving avascular zones by the number of disks tested.

moiety is also a new member of this group of antibiotics.

Biological Activities

Angiostatic Activity

TAN-1323 A~D were assayed in the chick chorioallantoic membrane(CAM) assay system: the most conventionally used *in vivo* angiogenesis assay system of those reported.[19] As shown in Table 5, TAN-1323 B and D showed potent angiostatic activity in this assay system. Fig. 6 shows that methylcellulose disks containing as little as 0.1 μg of TAN-1323 B or D give nice avascular zones without affecting the preexisting larger blood vessels. As for components A and C, effective dosage ranges could not be detected, since their tissue-damaging effect overshadowed the angiostatic activity.

Table 5. Angiostatic activity of TAN-1323 A~D in the CAM assay.

Component	% Avascular zones			
	ng/disk			
	0.1	1	10	100
A	0	8	0	25
B	0	0	17	100
C	8	17	8	—[a]
D	0	0	75	100

Experimental procedure, see the legend to Fig. 6.
[a] All chick embryos died due to the toxicity of the sample.

TAN-1323 D, the most potent component, was examined in the rat corneal micropocket assay system[21] in which bovine basic fibroblast growth factor (bFGF)

Table 6. Effect of TAN-1323 A~D on proliferation of HUVE cells and *in vitro* capillary tube formation by HUVE cells.

Component	Growth[a]	Degree of tube formation[b]			
		ng/ml			
	IC_{50} (ng/ml)	0.01	0.1	1	10
A	1.6	++	++	++*	++*
B	0.61	++	++	+*	+*
C	1.4	++	++	++	+*
D	0.89	++	++	++*	++*

[a] Growth of HUVE cells was measured colorimetrically by the tetrazolium salt (MTT) method.[24] Briefly, HUVE cells suspended in Medium 199/20% fetal calf serum supplemented with 10 ng/ml of human recombinant basic fibroblast growth factor (Takeda Chem. Ind.) were plated in a 96-well plate (3,000 cells/0.1 ml/well). One day later (Day 1), a sample was added. On Day 3, MTT solution was added, and the mixture was incubated for 5 hours. The reaction was then stopped by adding 10% SDS/0.01 N HCl solution. After overnight incubation, optical density at 620 nm was measured.

[b] Assay was performed as described previously.[23]

* Denotes fragmentation of capillary tubes. The degree of tube formation in the drug-free control is expressed as ++.

Table 7. Angiostatic activity of some macrolide antibiotics in the CAM assay.

Component	% Avascular zones						
	μg/disk						
	0.03	0.1	0.3	1	3	10	30
14-Membered							
Erythromycin		0		11	0	8[a]	0[a]
Oleandomycin				0	0	0	0[a]
Triacetyl-oleandomycin				0	0	0[a]	0[a]
16-Membered							
Maridomycin				0	0	0	0[a]
Leucomycin				0	38	77	46[a]
Tylosin			0	44	95	100	100[b]
Bafilomycin C1	0	33	100	—[c]			

[a] Hemorrhage. [b] Tissue damage. [c] Toxic death.
Experimental procedure, see the legend to Fig. 6.

(R & D Systems, Inc.) was used as an inducer of capillary blood vessel growth from limbi. Ethylene-vinyl acetate copolymer pellets containing 0.1 or 1 ng of this component, however, did not show inhibitory activity against capillary growth induced by the angiogenic factor. Pellets containing 10 ng of this component would not remain in place on the cornea due to the inflammatory response induced.

Other Biological Activity Related to Angiogenesis

Since it has been established that endothelial cells (ECs) play a pivotal role in the process of new blood vessel formation,[1,2,22] we examined the effect of TAN-1323 components on EC proliferation and *in vitro* capillary tube formation by ECs[23] using human umbilical vein endothelial (HUVE) cells. Each component strongly inhibited HUVE cell growth (Table 6), but the inhibition does not appear to be specific for ECs, because growth of fibroblastic cells, Balb/3T3-A31, was also inhibited with the same potency (data not shown).

All the components induced fragmentation of capillary tubes formed by the ECs (Table 6). The effect, however, seems to be due to cytotoxic activity, because it was observed at concentrations greater than the respective IC_{50} values for EC growth (Table 6).

Angiostatic Activity of Other Macrolide Antibiotics

The discovery that TAN-1323 compounds, 18-membered macrolide antibiotics, show angiostatic activity in the CAM assay system prompted us to examine other macrolide antibiotics having various types of ring structures. Table 7 shows that the 16-membered macrolide antibiotics other than maridomycin were also angiostatic, although far weaker than TAN-1323 B and D, while the 14-membered macrolide antibiotics were totally inactive; as expected, bafilomycin C1 which is

structurally related to the concanamycins showed angiostatic activity, but it was surprising that the classical macrolide antibiotics such as tylosin and leucomycin were active in the CAM assay. Since they did not inhibit proliferation of ECs (IC_{50} values of tylosin and leucomycin in the EC proliferation assay were 40 and $>100\,\mu g/ml$, respectively), it is likely that their angiostatic activity is elicited by a mechanism different from that of TAN-1323 B and D.

Discussion

Eleven 18-membered (concanamycin-group) macrolide antibiotics have previously been discovered from microbial sources, based on antifungal[4] and antiviral[14] activities, cytotoxic activity against mammalian cells[3,15,16] and inhibition of biosynthesis of cholesterol ester in macrophages.[25] We can now add two new members to this group of antibiotics, TAN-1323 C and D, which were found in our screening program based on angiostatic activity. Besides the activities described above, concanamycin-group antibiotics have been reported to show various biological activities such as inhibition of the acidification of endosomes and lysosomes in macrophages[26] and blockade of cell-surface expression of virus-envelope glycoprotein.[27]

In this study, we showed novel biological activity, angiostatic activity, with TAN-1323 B and D and with bafilomycin C1 which is a 16-membered macrolide antibiotic structurally related to the concanamycins.[5] We did not, however, detect angiostatic activity with TAN-1323 A or C at the doses tested (Table 5). There must be difference in the active dosage range, "a pharmacologically active window", with concanamycin group antibiotics, because they were toxic to chick embryos at higher doses. In the rat corneal micropocket assay, we could not detect angiostatic activity with TAN-1323 D, the most active compound in the CAM assay. This may be due to the difference in the neovascularization mechanism between the cornea and the CAM: the former was forcibly induced by bFGF, while the latter is part of the physiological process of chick development.

In 1988, E. J. BOWMAN *et al.* reported that bafilomycins are highly specific inhibitors of the V-type H^+-ATPase from microorganisms, animal and plant cells.[6] As expected from the structural similarity to concanamycins, many research groups have reported similar types of biological activities with bafilomycins[28~33]. Very recently, S. ORÖSE showed that concanamycins are more potent and specific inhibitors than bafilomycins and clarified structural motifs of bafilomycins and concanamycins important for the inhibitory activity against the V-type H^+-ATPase using various types of derivatives.[34] It is highly likely that these pleiotrophic effects of concanamycins and bafilomycins result from the inhibition of the V-type H^+-ATPase which plays an important role in maintaining the acidity of cellular vacuolar compartments.[7,8]

As with other biological effects exerted by concanamycins and bafilomycins, the angiostatic activity may also result from the inhibition of the V-type H^+-ATPase. It is well established that the V-type H^+-ATPase in the vacuolar compartments is involved in many fundamental steps of cellular metabolism such as receptor-mediated endocytosis of macromolecules, intracellular transport of proteins through the trans-Golgi network, protein degradation in lysosomes and coupled transport of small molecules such as epinephiline and neurotransmitters in secretory organelles.[7,8,35] Furthermore, the V-type H^+-ATPase has also been shown to reside on the plasma membrane of some specialized cells such as distal kidney tubule cells, MDBK cells, osteoclasts and macrophages and to play an essential role in regulating the cytoplasmic pH of these cells.[7,8,35] Since the function of the enzyme is so pleiotrophic and the process of angiogenesis is highly complex, it is at present unclear which functional role of this enzyme is responsible for the embryonic blood-vessel formation on the CAM. However, the angiostatic activity seems to be limited to inhibitors of this type of H^+-ATPase, since we did not detect any activity with oligomycin A or vanadate, which are specific inhibitors of the F_1, F_0-ATPase in the mitochondria and E_1, E_2-ATPase in the plasma membrane, respectively[7,8] (data not shown).

In this study, we also found that classical macrolide antibiotics such as tylosin and leucomycin show angiostatic activity in the CAM assay, though not as potent as TAN-1323 B and D and bafilomycin C1. Structurally, however, tylosin and leucomycin are quite different from concanamycins and bafilomycins, and the former antibiotics lack the hemiketal ring structure which was shown to be important for inhibition of the V-type H^+-ATPase.[34] Furthermore, while TAN-1323 compounds and bafilomycin C1 potently inhibited the proliferation of HUVE cells, tylosin and leucomycin did not (Table 6 and the text). The mechanism of the angiostatic action of the classical macrolide antibiotics may, therefore, be completely different from that of the V-type H^+-ATPase inhibitors, but it remains to be elucidated.

Experimental

General

Melting points were determined using a Yamato MP-21 melting point apparatus and are uncorrected. UV spectra were taken on a Hitachi 320 spectrophotometer. Optical rotations were determined with a JASCO DIP-181 digital polarimeter. IR spectra were measured with a Hitachi 285 grating IR spectrophotometer using KBr pellets. NMR spectra were recorded on a Bruker AC-300 instrument (^1H, 300 MHz; ^{13}C, 75 MHz) in

DMSO-d_6 at 24°C. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane. Desalination was carried out with a Micro Acilyzer G1 (Asahi Chem. Ind., Japan) using an AC-110-10 cartridge.

Alkaline Hydrolysis of **3**

A 0.1 M solution of NaOH in MeOH (3.0 ml, 0.30 mmol) was added to a hot solution of **3** (100 mg, 0.12 mmol) in MeOH (7.0 ml), and the reaction mixture was stirred for 3.5 minutes at 50°C. The mixture was neutralized with 1 M HCl (0.3 ml) and then concentrated. The residue obtained was suspended in H_2O and extracted with EtOAc. The aqueous layer was desalted and freeze-dried to give L-rhamnose as a white powder (12 mg). The EtOAc layer was concentrated, and the residue obtained was chromatographed on a silica gel column (7.0 g) eluting with $CHCl_3$-MeOH (50:1 and 20:1). The pure fraction containing the anhydroaglycone (**6**) was concentrated to give a white powder. A portion of the starting material (37 mg) was recovered.

Alkaline Hydrolysis of **4**

A suspension of **4** (100 mg) in 0.03 M NaOH/MeOH (5.0 ml) was stirred for 20 minutes at room temperature, and then water (50 ml) was added. The mixture was extracted with EtOAc at pH 7.1. The EtOAc layer containing **6** was concentrated, and the residue obtained was chromatographed on a silica gel column (7.0 g) eluting with $CHCl_3$-MeOH (50:1). The pure fraction was concentrated to give **6** (68 mg) as a white powder. The aqueous layer containing **7** was extracted with EtOAc at pH 2.5, and the EtOAc layer thus obtained was dried over anhydrous Na_2SO_4 and then concentrated to give crystals. Recrystallization from water afforded **7** (8.5 mg) as colorless crystals.

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