TAN-1120, A NEW ANTHRACYCLINE WITH POTENT ANGIOSTATIC ACTIVITY

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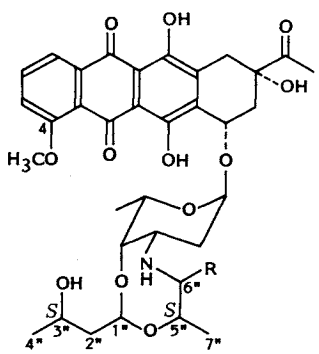
A potent angiogenesis-inhibitory compound TAN-1120 was found to be produced by a *Streptomyces* species isolated from a soil sample. The producing organism was characterized as a new subspecies of *S. triangulatus* and named *S. triangulatus* subsp. *angiostaticus* subsp. nov. due to its specific ability to produce the compound. This substance was isolated as a red powder by a combination of organic solvent extraction, silica gel column chromatography and preparative HPLC using an ODS column. Its structure was elucidated by chemical reactions and spectral analyses to be a new baumycin-group anthracycline. Reduction of TAN-1120 gave two compounds, a deoxy derivative and baumycin A1. TAN-1120 showed remarkably potent angiostatic activity in two conventional angiogenesis assay systems *in vivo*, while doxorubicin and daunomycin had far weaker activity. It strongly inhibited proliferation of vascular endothelial cells but did not prevent capillary cord formation *in vitro* by the endothelial cells on extracellular matrix-coated plates. TAN-1120 is one of the most potent angiostatic agents reported.

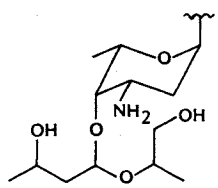
Intensive and aberrant neovascularization has been observed to be associated with various pathological conditions, such as diabetic retinopathy, trachoma, psoriasis, hemangioma, rheumatoid arthritis, atherosclerosis and solid tumors[1]. In 1971, J. FOLKMAN presented the anti-angiogenesis hypothesis that since solid tumor growth is angiogenesis-dependent, anti-angiogenesis therapy should be a potentially promising approach[2]. Since then, angiogenesis inhibitors have been sought after as a new type of antitumor agents.

During our screening program focusing on such inhibitors, we found that the *Streptomyces* strain

Fig. 1. Structures of TAN-1120 and its reduction products.



TAN-1120 (**1**) R=OH
2 R=H



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S-14519 with unique morphological properties produces a potent angiostatic substance in its culture filtrate and cell masses. The active principle, TAN-1120 (**1**), was isolated and characterized to be a new member of the baumycin-group anthracyclines[3~10] (Fig. 1). We also obtained a deoxy derivative (**2**) and baumycin A1 (**3**)[3,4] by reduction of TAN-1120.

This paper deals with the taxonomy of the producing organism, fermentation, isolation, structure elucidation and various biological activities of TAN-1120 including angiostatic activity.

Taxonomy of the Producer

The producing organism designated S-14519 was isolated from a soil sample collected at Kunisaki Peninsula in Ooita Prefecture, Japan.

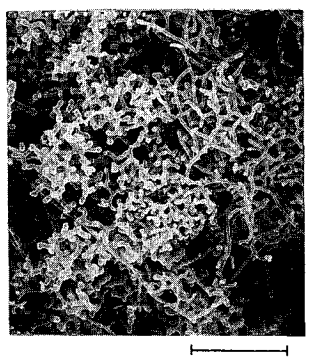
Aerial mycelia elongated with simple branching from well branched vegetative mycelia (Fig. 2). At the tips of the aerial mycelia, zigzag spore chains, each of which consisting of ten or more triangular or T-shaped spores, were observed (Fig. 3). No verticils were formed. The spores were ellipsoidal, triangular to T-shaped, $0.5 \sim 0.8 \times 0.7 \sim 1.0\,\mu m$ in size, and had a smooth surface.

Growth on various media is summarized in Table 1. Growth in the temperature range of 15 to 33°C was observed after cultivation for 7 days on yeast extract-malt extract agar. Strain S-14519 hydrolysed starch. The following characteristics were negative; liquefaction of gelatin, coagulation and peptonization of skimmed milk, and production of melanoid pigment on tyrosine agar and peptone-yeast extract-iron agar.

Sugar assimilation by strain S-14519 was examined using Pridham-Gottlieb agar. It assimilated inositol, but L-arabinose, D-xylose, D-glucose, D-fructose, sucrose, L-rhamnose, raffinose and D-mannitol. 2,6-Diaminopimelic acid in hydrolysate of the whole cells was of the LL-type.

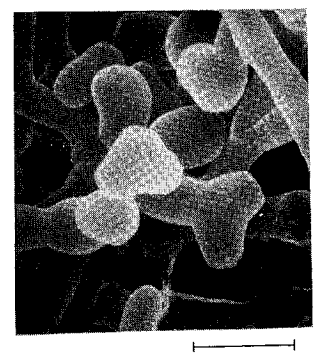
The above gross characteristics show that strain S-14519 belongs to the genus *Streptomyces*. Our extensive survey of the previously described *Streptomyces* species having triangular spores and bluish-colored aerial mycelia led to the conclusion that this strain is most closely related to *S. triangulatus* described by researchers of Meiji Seika Kaisha, Ltd., Japan[11]. Direct comparison of *S. triangulatus* IFO 13799, which they deposited at Institute for Fermentation, Osaka, with our strain showed that while minor differences were seen in the growth on glycerol-asparagine agar and tyrosine agar, they were almost identical in other respects. However, production of TAN-1120 was not observed with this IFO strain under

Fig. 2. Morphology of aerial mycelia of strain S-14519.
Bar represents 10 μm.



Scanning electron microscopy was performed using a Hitachi model S-570 scanning electron microscope.

Fig. 3. Spore morphology of strain S-14519.
Bar represents 1 μm.



Scanning electron microscopy was performed using a Hitachi model S-570 scanning electron microscope.

Table 1. Growth characteristics of strain S-14519 on various media.

Medium	Growth	Reverse color	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Poor	Milky white (2ca)	Poor, powdery grayish white (2ca)	None
Glucose-asparagine agar	Moderate	Milky white (2ca)	Moderate, powdery gray (15dc)	None
Glycerine-asparagine agar	Moderate	Pale yellowish gray (2ea)	Moderate, powdery white to bluish gray (19fe)	None
Starch-inorganic salt agar	Moderate	Pale yellowish gray (2ea)	Moderate, powdery white to bluish gray (19fe)	None
Tyrosine agar	Moderate	Dark yellowish gray (31g) to dark brown (3pl)	Poor, white	Dark brown (3ng)
Nutrient agar	Moderate	Pale yellowish gray (2ea)	Poor, white	None
Yeast extract-malt extract agar	Good	Pale brown (31e)	Good, powdery bluish gray (19ih)	None
Oatmeal agar	Poor	Milky white (2ca)	Poor, powdery bluish gray (19fe)	None
Peptone-yeast extract-iron agar	Moderate	Pale yellowish gray (2ea)	Poor, white	Dark brown (3ng)

Characterization of the producer was carried out according to the methods described previously[12]. Unless otherwise stated, results after cultivation at 28°C for 14 days are described. Standard color codes in the Color Harmony Manual[13] were used to assign colors of the mature culture.

our cultivation conditions.

Based on the above results, strain S-14519 was named *S. triangulatus* subsp. *angiostaticus* and deposited under the accession number IFO 14801 at Institute for Fermentation, Osaka and under the accession number FERM BP-2199 at Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

Fermentation

Strain S-14519 was inoculated into 500 ml of the seed medium described below and cultivated at 28°C for 48 hours on a reciprocal shaker. The whole culture was transferred to 30 liters of the seed medium in a 50-liter fermentor, and cultivated at 28°C for 48 hours with aeration of 30 liters/minute and agitation of 280 rpm. The seed culture thus obtained was transferred to 100 liters of the fermentation medium described below in a 200-liter fermentor, and fermentation was carried out at 28°C for 90 hours with aeration of 100 liters/minute and agitation of 150 rpm.

The seed medium consisted of glucose 2%, soluble starch 3%, corn steep liquor 1%, raw soybean flour 1%, peptone 0.5%, NaCl 0.3% and $CaCO_3$ (precipitated) 0.5%, pH 7.0. The fermentation medium consisted of glucose 5.5%, corn gluten meal 3.5% and $CaCO_3$ (precipitated) 0.7%, pH 7.0.

Isolation

MeOH (180 liters) was added to the culture broth (90 liters) after adjustment to pH 6.6, and the mixture was stirred for 30 minutes. After filtration, the filtrate was concentrated to remove MeOH. The aqueous solution thus obtained was washed with EtOAc (2 × 20 liters at pH 5.0 and then extracted with *iso*-BuOH (2 × 7.5 liters). The *iso*-BuOH layers were combined (13 liters) and washed with 2% aq.

$NaHCO_3$ (2×3 liters) and water (2×6 liters) successively and then concentrated to give an oily residue. The residue was washed with ethyl ether (500 ml) and then extracted with $CHCl_3$ (3×500 ml). The $CHCl_3$ layers were combined and concentrated to give an oily residue (2.3 g).

The residue was chromatographed on a column of silica gel (Merck Silica gel 60, 40 g), eluting with $CHCl_3$ - MeOH - AcOH (97 : 2 : 1). The fraction containing TAN-1120 was washed with 2% aq. $NaHCO_3$ and concentrated to give a red powder (430 mg).

A 150 mg portion of the powder was divided into three portions, and each 50 mg was individually purified by preparative HPLC (column, YMC-Pack SH-343 ODS; mobile phase, 40% CH_3CN - 50 mM phosphate buffer (pH 3.0); flow rate, 10 ml/minute). Pure fractions were combined (60 ml) and concentrated to remove CH_3CN. The aqueous solution (25 ml) was extracted with $CHCl_3$ (2×15 ml) at pH 7.2. The $CHCl_3$ layers were combined and washed with water (15 ml), diluted with MeOH (100 ml) and concentrated to give TAN-1120 as a dark red powder (14.5 mg).

Characterization and Structure Elucidation

The chromatographic properties of TAN-1120 upon TLC and HPLC are shown in Table 2 along with those of daunomycin. The physico-chemical properties of TAN-1120 are summarized in Table 3. Its UV spectrum was typical for anthracyclines such as daunomycin. It gave ion peaks at m/z 694 $(M+Na)^+$ in the secondary ion mass (SI-MS) spectrum and at m/z 653 $(M^+ - H_2O)$ in the field desorption mass (FD-MS) spectrum. The molecular formula was deduced to be $C_{34}H_{41}NO_{13}$ from these mass spectra, the ^{13}C NMR spectrum and elemental analysis.

Although TAN-1120 was labile in various solvents, the ^1H and ^{13}C NMR spectra could be recorded in a mixture of $CDCl_3$ - CD_3OD (2 : 1) in which it stably exists in a carbinolamine form (Tables 4 and 5). The ^1H NMR spectrum was similar to that of barminomycin A1 (SN-07 chromophore)[8,9] except for a methoxy signal at δ_H 4.09 suggesting the presence of a methoxyl group at the C-4 position. The methine signal at δ_H 3.76 is characteristic of a carbinolamine structure which was supported by the methine signal at δ_C 94.3 in the ^{13}C NMR spectrum. These findings indicated that TAN-1120 is a 4-*O*-methyl derivative of barminomycins.

Table 2. Chromatographic properties of TAN-1120, **2**, **3** and daunomycin.

Compound	HPLC[a]	TLC[b]	
	Rt (minutes)	Rf value	
		I[c]	II[d]
TAN-1120 (**1**)	4.0	0.45	0.75
2	4.4	0.39	0.72
3	4.9	0.42	0.45
Daunomycin	2.5	0.24	0.08

[a] Column, LiChrospher 100 RP-18 (e) 5 μm 4×125 mm (Cica-Merck); solvent, 40% CH_3CN - 20 mM phosphate buffer (pH 3.0); flow rate, 1 ml/minute; detection, UV 254 nm.

[b] Merck precoated silica gel 60 F_{254} plates (0.25 mm thickness).

[c] Solvent I, $CHCl_3$ - MeOH - HCOOH - H_2O (60 : 10 : 1 : 1).

[d] Solvent II, $CHCl_3$ - MeOH - toluene (7 : 3 : 3).

To confirm the structure, TAN-1120 was reduced with sodium cyanoborohydride and, in a manner similar to that seen with barminomycins[8,9], yielded red powders of **2** and **3**. **2**: FD-MS m/z 655 (M^+); UV λ_{max}^{MeOH} (ε) 233 (36,000), 251 (27,200), 287

Table 3. Physico-chemical properties of TAN-1120.

Appearance	Dark red powder
SI-MS (m/z)	$694\ (M+Na)^+$
Molecular formula	$C_{34}H_{41}NO_{13} \cdot H_2O$
Anal Found (%)	C 58.91, H 6.08, N 2.31
Calcd (%)	C 59.21, H 6.28, N 2.03
UV λ_{max}^{MeOH} (nm) (ε)	234 (35,100), 252 (26,900), 290 (7,900), 477 (11,000), 498 (11,700), 531 (8,300), 576 (3,400)
IR KBr (cm^{-1})	1720, 1620, 1580
$[\alpha]_D^{23}$	+230° (*c* 0.01, MeOH)

Table 4. ^{1}H NMR chemical shifts of TAN-1120 and its derivatives (300 MHz).

Proton	TAN-1120 (**1**)[a]	**2**[b]	**3**[b]
1-H	8.03 br d (7.5)	8.03 dd (0.9, 7.8)	8.03 dd (0.9, 7.7)
2-H	7.83 br t (7.5)	7.78 dd (7.8, 8.5)	7.78 dd (7.7, 8.4)
3-H	7.47 br d (7.5)	7.39 dd (0.9, 8.5)	7.39 dd (0.9, 8.4)
7-H	5.25 br s	5.30 br s	5.24 br s
8-H_{eq}	2.36 br d (15.0)	2.32 br d (14.8)	2.28 br d (14.8)
8-H_{ax}	2.13 dd (4.0, 15.0)	2.09 dd (4.1, 14.8)	2.08 dd (4.1, 14.8)
10-H_{eq}	3.20 d (18.5)	3.24 dd (1.6, 18.9)	3.21 dd (1.5, 18.9)
10-H_{ax}	3.00 d (18.5)	2.97 d (18.9)	2.99 d (18.9)
13-CH_3	2.42 s	2.41 s	2.41 s
4-OCH_3	4.09 s	4.08 s	4.08 s
1′-H	5.49 br s	5.49 br d (3.4)	5.47 br d (3.6)
2′-H_2	1.75 m	1.78 dd (4.5, 13.5)	1.68 dd (4.6, 13.1)
		1.60 dt (3.4, 13.5)	1.81 dt (3.6, 13.1)
3′-H	3.15 m	2.84 m	3.01 m
4′-H	3.59 br s	3.54 br s	3.91 br s
5′-H	4.14 br q (6.4)	4.12 m	4.11 q (6.6)
6′-H_3	1.27 d (6.4)	1.28 d (6.5)	1.31 d (6.6)
1″-H	4.72 t (5.4)	4.67 t (5.2)	4.74 t (5.1)
2″-H_2	1.89 m	1.94 m	1.85 m
3″-H	4.03 m	4.12 m	4.16 m
4″-H_3	1.23 d (6.0)	1.24 d (6.3)	1.23 d (6.2)
5″-H	3.53 dq (8.1, 6.0)	3.80 m	3.79 m
6″-H_a	3.76 d (8.1)	2.77 dd (5.5, 14.9)	3.53 dd (8.7, 12.6)
6″-H_b		2.66 dd (9.9, 14.9)	3.42 dd (2.3, 12.6)
7″-H_3	1.21 d (6.0)	1.10 d (6.0)	1.06 d (6.3)
N-1, N-3			

[a] Recorded in $CDCl_3$-CD_3OD (2:1).
[b] Recorded in $CDCl_3$.

Table 5. ^{13}C-NMR chemical shifts of TAN-1120, **2** and **3** (75 MHz).

Carbon	TAN-1120 (**1**)[a]	**2**[b]	**3**[b]	Carbon	TAN-1120 (**1**)[a]	**2**[b]	**3**[b]
1	120.0	119.8	119.8	12a	135.6	135.6	135.6
2	136.2	135.6	135.7	13	212.9	211.6	212.1
3	119.0	118.4	118.4	14	24.8	24.7	24.9
4	161.3	161.1	161.1	4-OCH_3	56.8	56.7	56.7
4a	120.9	121.0	121.0	1′	101.6	101.2	101.1
5	187.2	187.0	187.1	2′	32.3	32.5	33.4
5a	111.7	111.4	111.5	3′	44.3	51.6	45.8
6	156.4	156.4	156.4	4′	80.1	79.6	82.0
6a	134.5	134.3	134.5	5′	65.8	65.7	67.9
7	70.4	69.5	69.8	6′	17.0	17.0	17.0
8	35.3	34.9	34.9	1″	107.4	107.2	106.6
9	77.0	76.9	76.8	2″	44.5	44.0	45.7
10	33.2	33.5	33.4	3″	64.7	64.6	64.2
10a	134.5	134.2	134.2	4″	23.5	23.4	23.3
11	155.6	155.8	155.9	5″	79.0	77.6	75.7
11a	111.5	111.3	111.3	6″	94.3	52.8	66.8
12	186.9	186.8	186.7	7″	21.1	22.0	18.1

[a] Recorded in $CDCl_3$-CD_3OD (2:1).
[b] Recorded in $CDCl_3$.

(8,600), 476 (11,400), 495 (11,800), 530 (8,000), 575 (2,900); IR (KBr) 1720, 1620, 1580 cm^{-1}; $[\alpha]_D^{22}$ +184° (*c* 0.08, $CHCl_3$). **3**: FD-MS *m/z* 674 $(M+H)^+$; UV λ_{max}^{MeOH} (ε) 233 (37,400), 252 (27,600), 287 (9,000), 476 (11,800), 495 (12,200), 530 (8,100), 575 (2,800); IR (KBr) 1720, 1615, 1580; $[\alpha]_D^{22}$ +144° (*c* 0.08, $CHCl_3$). **2** showed a molecular ion peak at *m/z* 655 (M^+) in FD-MS, and the carbinolamine moiety was changed to a nitrogen bearing methylene (δ_H 2.77 dd, 2.66 dd; δ_C 52.8 t). These findings indicated that **2** is a cyclic deoxy derivative. The other product (**3**) had a molecular ion peak at *m/z* 674 $(M+H)^+$ in FD-MS, and the carbinol amine moiety was changed to an oxygen bearing methylene (δ_H 3.53 dd, 3.42 dd; δ_C 66.8 t). Although we have not made a direct comparison, **3** is considered to be identical to baumycin A1[3,4] based upon the FD-MS, UV, IR, ^1H and ^{13}C NMR spectra, and specific rotation. The chromatographic properties of **2** and **3** are listed in Table 2.

Carminomycin III (identical to 4-hydroxybaumycin A1) has been reported to have the (3″*S*,5″*S*)-configuration[14]; hence, TAN-1120 should also have the same configuration as depicted in Fig. 1.

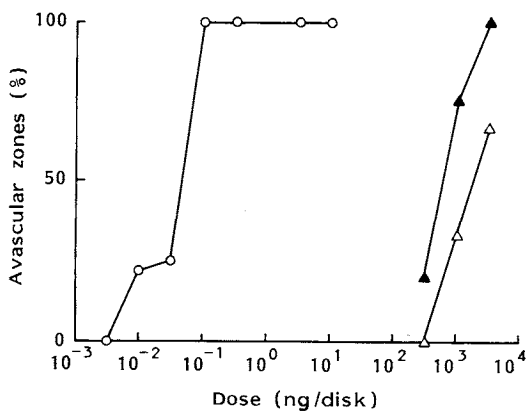
Biological Activities

Angiostatic Activity

In the CAM assay[16] which is widely used as the most conventional angiogenesis assay system *in vivo*, TAN-1120 showed remarkably potent angiostatic activity under a wide range of comparatively low doses up to 10 ng/disk without causing tissue damage (Fig. 4). While the common anthracyclines such as daunomycin and doxorubicin also showed this activity, their potency was far weaker than that of TAN-1120; the ED_{50} values of TAN-1120, daunomycin and doxorubicin were 0.046, 1750 and 560 ng/disk, respectively. In addition, the inhibitory dose ranges of daunomycin and doxorubicin were quite narrow in comparison with that of TAN-1120 (Fig. 4). One tenth μg/disk of TAN-1120 and 10 μg/disk of daunomycin or doxorubicin were toxic to the chick embryos. The angiostatic activity of

Fig. 4. Angiostatic activity of TAN-1120, daunomycin and doxorubicin in the CAM assay.

TAN-1120 (○), daunomycin (△) and doxorubicin (▲).



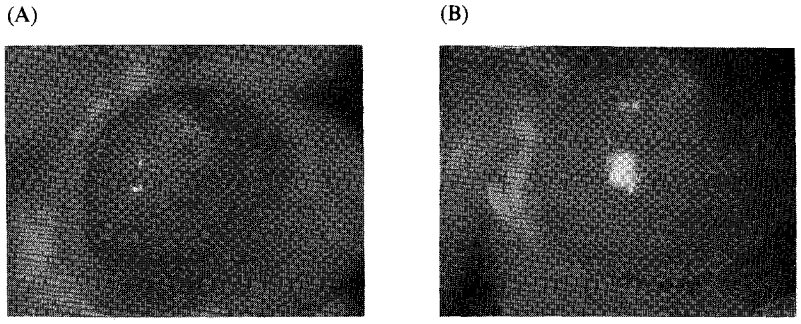
The CAM assay was carried out by the method reported by R. CRUM *et al.*[15] with some modification. Briefly, Day-3 eggs were cracked, and the embryos were placed onto hammocks of polyethylene sheets hanging in plastic cups and then incubated at 37°C under 3% CO_2 and saturated humidity for a further 6 days. On Day-9, methylcellulose (4000 Centipoise, Wako Pure Chemicals Co.) disks containing samples were placed on the CAM. After incubation for 2 days, responses induced by the samples were examined under a stereoscope (SMZ-10, Nikon). The values represent the means of three separate experiments in each of which 21 samples were tested for each dose.

Table 6. Angiostatic activity of TAN-1120 in the rat corneal micropocket assay.

Dose (ng/pellet)	Number of corneas with inhibition/ number of corneas tested
1	0/7
10	0/7
200	7/7
20,000	Inflammation

The rat corneal micropocket assay was performed according to the method of GIMBRONE *et al.*[16]. Briefly, an ethylene-vinyl acetate copolymer (EVA) pellet containing 250 ng of bovine basic fibroblast growth factor (bFGF) (R&D systems) was implanted into a cornea of an adult male Sprague-Dawley rat (Charles River). Another EVA pellet with or without a sample was implanted between the above pellet and the rat limbus. Ten days after implantation, the corneas were examined for capillary growth from the limbi under a stereoscope (SMZ-10, Nikon) at a magnification of 20.

Fig. 5. Prevention of capillary vessel growth by TAN-1120 in rat corneas.

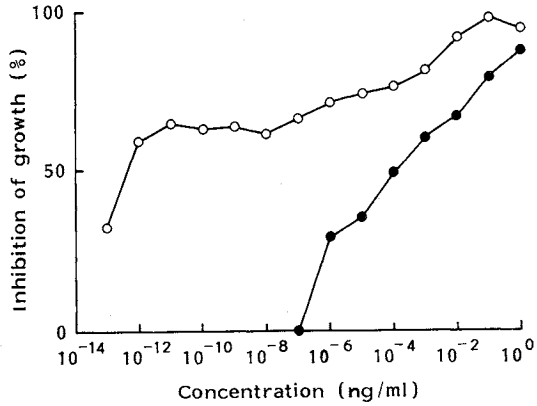


Materials and Methods, see the legend to Table 6. An EVA pellet with (B) or without (A) 200 ng of TAN-1120 was implanted between the FGF-containing pellet and the rat limbus.

the reduction products of TAN-1120, the deoxy derivative (**2**) and baumycin A1 (**3**)[4] (Fig. 1), was one tenth of that of the parent compound when evaluated in terms of ED_{50} values (data not shown).

In the rat corneal micropocket assay[16], 0.2 μg of TAN-1120 prevented capillary outgrowth induced by 250 ng of bFGF which is one of the most potent angiogenic factors known[1] (Table 6 and Fig. 5). At the dose of 20 μg/pellet, however, inflammatory responses were observed.

Fig. 6. Dose-response curve of cytotoxic activity of TAN-1120 against endothelial and fibroblastic cells.



Twenty thousand cells of endothelial cell (EC) line CRL-1395 (○)[18] or a fibroblastic cell line BALB 3T3/A31 (●) suspended in 0.5 ml of DMEM medium (Gibco Laboratories) with 10% fetal bovine serum (M.A. Bioproduct, Inc.) and 30 μg/ml of gentamicin (Sigma) were plated in each well of a 24 well-plate. After incubation at 37°C under 5% CO_2 for 24 hours, 25 μl of a sample solution was added to each well. Two days later, the cells were counted with a Coulter counter (Coulter Electronics, Inc.). The medium was supplemented with human recombinant bFGF (Takeda Chem. Ind., 25 ng/ml) for the culture of the endothelial cells.

Cytotoxic Activity Against ECs

To gain insight into the mechanism of the angiostatic action of TAN-1120, we first examined its cytotoxic activity against ECs, because proliferation of ECs is one of the most important steps in angiogenesis[1,17]. As shown in Fig. 6, TAN-1120 had a remarkably potent inhibitory effect on the growth of bovine fetal heart ECs CRL-1395[18]. Moreover, this activity was highly specific to the ECs, since the mouse fibroblast BALB 3T3/A31 cells used as a comparative cell line were much less sensitive to this compound (Fig. 6).

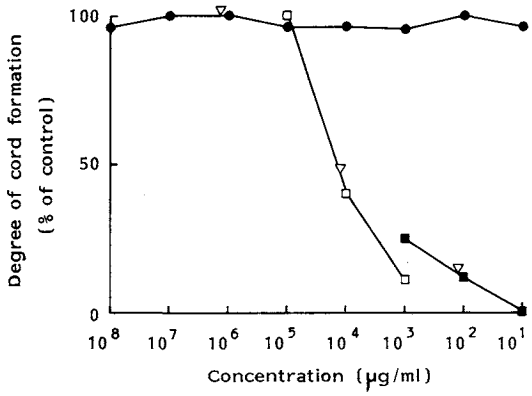
Cytotoxic activity of TAN-1120 against various human tumor cell lines was 10^3 to 10^4 times more potent than that of daunomycin and doxorubicin, and the activity of the deoxy derivative (**2**) was one tenth of that of the parent compound (data not shown).

Effect on Angiogenesis *In Vitro*

Several authors have described that in appropriate experimental conditions, ECs sprout to form

Fig. 7. Effect of TAN-1120 and microtubule inhibitors on angiogenesis *in vitro*.

●, TAN-1120; □, ansamitocin P3; ■, vincristine. The triangles (▽) represent the IC_{50} values which were obtained from the cell proliferation assay using HUVE cells.



The procedure described in ref 21 was followed with slight modification. Briefly, 2 ml of human umbilical vein endothelial (HUVE) cell suspension (1×10^5 cells/ml) in M199 medium (Gibco Laboratories) with 20% fetal bovine serum and 30 μg/ml of gentamicin was added to each well of a 24-well plate precoated with 200 μl of Engelbreth-Holm-Swarn tumor extract (12 mg protein/ml) overnight at 37°C. Immediately after plating, 25 μl of a sample solution or 40% MeOH solution as a control was added to the culture. After overnight incubation at 37°C under 3% CO_2, the wells were examined for capillary cord formation under a phase-contrast microscope (Olympus), and several fields in each well were photographed. Crossing points on the microphotographs covered with a transparent plastic plate with appropriate grids were counted to quantitate the degree of cord formation as described by TSURUOKA *et al.*[22].

Table 7. Antibacterial activity of TAN-1120.

Organism	MIC (μg/ml) at 10^6 cfu/ml
Escherichia coli NIHJ JC-2	3.13
Salmonella typhimurium IFO 12529	6.25
Citrobacter freundii IFO 12684	3.13
Klebsiella pneumoniae IFO 3317	1.56
Enterobacter cloacae IFO 12937	3.13
Serratia marcescens IFO 12648	3.13
Proteus mirabilis ATCC 21100	25
P. vulgaris IFO 3988	6.25
Morganella morganii IFO 3168	1.56
Pseudomonas aeruginosa IFO 3080	3.13
Alcaligenes faecalis IFO 13111	0.39
Acinetobacter calcoaceticus IFO 12552	3.13
Staphylococcus aureus FDA 209P	0.1
Micrococcus luteus IFO 12708	<0.1
Bacillus subtilis NIHJ PCI 219	0.1
Streptococcus faecalis IFO 3989	0.78

MICs were determined by the conventional agar dilution method with DYAB agar[25].

capillary-like tubes *in vitro*[19,20]. This "differentiation" process of the ECs is considered to be one of the key steps in angiogenesis. Therefore, we examined the effect of TAN-1120 on this process using HUVE cells in the rapid assay system reported by KUBOTA *et al.*[21]. TAN-1120 showed no inhibitory activity in a wide range of concentration even higher than the IC_{50} (0.7 pg/ml) obtained in the cell proliferation assay (Fig. 7). In contrast, the microtuble inhibitors such as vincristine[23] and ansamitocin P3[24] prevented this process at their respective IC_{50} concentrations (Fig. 7).

Antibacterial Activity

As shown in Table 7, TAN-1120 showed potent antibacterial activity against a wide range of pathogenic bacteria including *Pseudomonas aeruginosa*.

Discussion

Recently, angiogenesis inhibitors have been receiving a great deal of attention as agents to manage "angiogenic deseases" in which aberrant neovascularization contributes to the pathological state[1,26,27]. In this context, such inhibitors have been sought after, and several proteineous inhibitors, mainly from animal avascular tissues such as cartillage[28] and eye tissues[29], have been reported. In 1983, J. FOLKMAN *et al.* reported that a class of steroids which they coined "angiostatic steroids"[15] inhibits angiogenesis and causes regression of solid tumors in the presence of heparin or a heparin fragment[30]. Since then, several other low MW angiogenesis inhibitors have been described[26,27]. However, none of the angiogenesis inhibitors has reached the stage of clinical use.

During our extensive search for angiogenesis inhibitors of microbial origin, we encountered the potent angiostatic substance TAN-1120 produced by a unique *Streptomyces* species. Taxonomical characterization of the producer indicated that it is a new subspecies of *S. triangulatus*[11], and was named subsp. *angiostaticus* because of its specific ability to produce TAN-1120. Although several species of *Streptomyces* and *Actinomadura* have been reported as producers of the baumycin-group anthracyclines[3~10], the TAN-1120 producer is distinct. It is interesting that all anthracycline compounds produced by *Streptomyces* species have a methoxyl substituent at the 4-position of the anthracycline nucleus, whereas those produced by *Actinomadura* species do not have this substituent at this position[3~10]. Since CONNORS *et al.* very recently clarified using *Streptomyces* species that 4-*O*-methylation is the last step in the biosynthesis of daunomycin[31], *Actinomadura* species seem to commonly lack 4-*O*-methyltransferase.

Among the baumycin-group anthracyclines, carminomycins[6,14] D326 compounds[7], barminomycins[8] (SN-07 chromophore[9]) and SN-706[10] have a hydroxyl group at the C-4 position. Although only baumycins[3,4] have the methoxyl substituent at the C-4 position, their C-6″ group is a hydroxymethyl (A1 and A2) or a carboxylic acid (B1 and B2). Therefore, TAN-1120 is a new baumycin-group anthracycline having the methoxyl group at the C-4 position and the carbinolamine group in the sugar moiety. KIMURA *et al.* reported that the SN-07 chromophore is in equilibrium with the aldehyde, carbinolamine, imine and enamine forms[32]. Although we observed such structural alteration of TAN-1120 on HPLC, the carbinolamine form was found to be dominant and stable in MeOH solution.

In general, baumycin-group anthracyclines having the carbinolamine moiety are more potent in both cytotoxicity and antitumor activity than the classical anthracyclines, such as daunomycin and doxorubicin, lacking this moiety[3~10]. While TAN-1120 was discovered as an angiogenesis inhibitor, its cytotoxic activity is also far more potent than that of daunomycin and doxorubicin which were used as the comparative compounds in this study. Furthermore, its angiostatic activity in the CAM assay was also remarkably strong in comparison with that of the control antibiotics (Fig. 4).

It is well established that angiogenesis *in vivo* proceeds in several distinct steps such as proliferation and migration of ECs, vascular cord formation, canalization of the cords, and maturation of the basement membrane surrounding the newly formed vessel walls[1]. Among them, vascular cord formation is a very unique differentiation process of ECs[1,20]. However, TAN-1120 did not have any inhibitory effect on this process even at concentrations higher than its growth inhibitory concentration (Fig. 7). Since the microtubule inhibitors strongly inhibited this process at their respective IC_{50} values, reassembly of cytoskeltons in the ECs seems to take place in this process[33].

In conclusion, TAN-1120 seems to be a promising angiostatic agent, since complete inhibition without any sign of tissue damage was observed under a wide range of low doses, $0.01 \sim 10$ ng/disk, in the CAM assay (Fig. 4) and since it showed highly specific inhibition of proliferation of the ECs (Fig. 6). The deoxy derivative was one tenth as active as the parent compound in cytotoxic and angiostatic activity.

TAN-1120 had also an extensive antibacterial spectrum (Table 7). It is clear that the activity against Gram-negative bacteria is dependent on the presence of the carbinolamine moiety attached to daunosamine, since barminomycin I is active but carbinomycin III is not[8].

Until now, fumagillin and its analogues[34~36], a sulfated polysaccharide-peptidoglycan complex[37], herbimycin A[38], eponemycin[39] and staurosporin[40] have been reported as angiogenesis inhibitors of microbial origin. Although the precise mechanism of the angiostatic action of TAN-1120 remains to be elucidated, this substance seems to be the most potent angiogenesis inhibitor known to date.

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References

1) FOLKMAN, J. & M. KLAGSBRUN: Angiogenic factors. Science 235: 442~447, 1987
2) FOLKMAN, J.: Tumor angiogenesis: Therapeutic implications. N. Engl. J. Med. 285: 1182~1186, 1971

3) KOMIYAMA, T.; Y. MATSUZAWA, T. OKI, T. INUI, Y. TAKAHASHI, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Baumycins, new antitumor antibiotics related to daunomycin. J. Antibiotics 30: 619~621, 1977
4) TAKAHASHI, Y.; H. NAGANAWA, T. TAKEUCHI, H. UMEZAWA, T. KOMIYAMA, T. OKI & T. INUI: The structure of baumycins A1, A2, B1, B2, C1 and C2. J. Antibiotics 30: 622~624, 1977
5) BRAZHNIKOVA, M. G.; V. B. ZBARSKY, V. I. PONOMARENKO & N. P. POTAPOVA: Physical and chemical characteristics and structure of carminomycin, a new antitumor antibiotic. J. Antibiotics 27: 254~259, 1974
6) ZBARSKY, V. B.; N. P. POTAPOVA, M. G. BRAZHNIKOVA, B. V. ROZYNOV, L. A. SIBELDINA & N. F. SEPETOV: Structure of carminomycins II and III. Antibiotiki 25: 488~492, 1980
7) MATSUZAWA, Y.; A. YOSHIMOTO, K. KOUNO & T. OKI: Baumycin analogs isolated from *Actinomadura* sp. J. Antibiotics 34: 774~776, 1981
8) UCHIDA, T.; M. IMOTO, Y. TAKAHASHI, A. ODAGAWA, T. SAWA, K. TATSUTA, H. NAGANAWA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: New potent anthracyclines, barminomycins I and II. J. Antibiotics 41: 404~408, 1988
9) KIMURA, K.: S. NAKAYAMA, N. MIYATA, Y. TAKESHITA & G. KAWANISHI: The structure of SN-07 chromophore. J. Antibiotics 41: 411~414, 1988
10) KIMURA, K.; T. KOYAMA, S. NAKAYAMA, K. TAMURA, N. MIYATA & G. KAWANISHI: A new anthracycline antibiotic SN-706. J. Antibiotics 41: 1918~1921, 1988
11) SHOMURA, T.; Y. YAJIMA, S. AMANO & T. NIIDA: A new species of *Streptomycetateceae*: *Streptomyces triangulata* nov. sp. Sci. Reports of Meiji Seika Kaisha No. 13: 72~79, 1973
12) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
13) Color Harmony Mannul. 4th Ed. Container Corporation of America, 1958
14) SPIRIDONOVA, I. A. & N. N. LOMAKINA: Spatial configuration of carbohydrate part of carminomycins II and III. Antibiotiki 27: 258~263, 1982
15) CRUM, R.; S. SZABO & J. FOLKMAN: A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230: 1375~1378, 1985
16) GIMBRONE, Jr., M. A.; R. S. COTRAN, S. B. LEAPMAN & J. FOLKMAN: Tumor growth and neovascularization: An experimental model using the rabbit cornea. J. Natl. Cancer Inst. 52: 413~427, 1974
17) D'AMORE, P. A. & M. KLAGSBRUN: Angiogenesis. Factors and mechanisms. *In* The Pathobiology of Neoplasia. *Ed.*, A. E. SIRICA, pp. 513~531, Plenum Press, New York, 1989
18) GOSPODAROWITZ, D.; J. MORAN, D. BRAUN & C. BIRDWELL; Clonal growth of bovine vascular endothelial cells: Fibroblast growth factor as a survival agent. Proc. Natl. Acad. Sci. U.S.A. 73: 4120~4124, 1976
19) FOLKMAN, J. & C. HAUDENSCHILD: Angiogenesis *in vitro*. Nature 288: 551~556, 1980
20) INGBER, D. E. & J. FOLKMAN: Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis *in vitro*: Role of extracellular matrix. J. Cell Biol. 109: 317~330, 1989
21) KUBOTA, Y.; H. K. KLEINMAN, G. R. MARTIN & T. J. LAWLEY: Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J. Cell Biol. 107: 1589~1598, 1988
22) TSURUOKA, N; M. SUGIYAMA, Y. TAWARAGI, M. TSUJIMOTO, T. NISHIHARA, T. GOTO & N. SATO: Inhibition of *in vitro* angiogenesis by lymphotoxin and interferon-γ. Biochem. Biophys. Res. Commun. 155: 429~435, 1988
23) WILSON, L; K. M. CRESWELL & D. CHIN: The mechanism of action of vinblastine. Binding of [*acetyl*-^{3}H]-vinblastine to embryonic chick brain tubulin and tubulin from sea urchin sperm tail outer doublet microtubules. Biochemistry 14: 5586~5592, 1975
24) OOTSU, K.; Y. KOZAI, M. TAKEUCHI, S. IKEYAMA, K. IGARASHI, K. TSUKAMOTO, Y. SUGINO, T. TASHIRO, S. TSUKAGOSHI & Y. SAKURAI: Effects of new antimitotic antibiotics, ansamitocins, on the growth of murine tumors *in vivo* and on the assembly of microtubules *in vitro*. Cancer Research 40: 1707~1717, 1980
25) NOZAKI, Y.; A. IMADA & M. YONEDA: SCE-963, a new potent cephalosporin with high affinity for penicillin-binding proteins 1 and 3 of *Escherichia coli*. Antimicrob. Agents Chemother. 15: 20~27, 1979
26) MAIONE, T. E. & R. J. SHARPE: Development of angiogenesis inhibitors for clinical applications. TIPS 11: 457~461, 1990
27) MOSES, M. A. & R. LANGER: Inhibitors of angiogenesis. Bio/Technology 9: 630~634, 1991
28) LANGER, R.; H. BREM, K. FALTERMAN, M. KLEIN & J. FOLKMAN: Isolation of a cartilage factor that inhibits tumor neovascularization. Science 193: 70~72, 1976
29) TAYLOR, C. M. & J. B. WEISS: Partial purification of a 5.7K glycoprotein from bovine vitreous which inhibits both angiogenesis and collagenase activity. Biochem. Biophys. Res. Commun. 133: 911~916, 1985
30) FOLKMAN, J.; R. LANGER, R. J. LINHARDT, C. HAUDENSCHILD & S. TAYLOR: Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. Science 221: 719~725, 1983
31) CONNORS, N. C.; P. L. BARTEL & W. R. STROHL: Biosynthesis of anthracyclines: carminomycin 4-*O*-

methyltransferase, the terminal enzymic step in the formation of daunomycin. J. Gen. Microbiol. 136: 1895～1898, 1990

32) KIMURA, K.; S. NAKAYAMA, N. MIYATA & G. KAWANISHI: Structural alteration of SN-07 chromophore. J. Antibiotics 42: 127～131, 1989

33) INGBER, D. E. & J. FOLKMAN: How does extracellular matrix control capillary morphogenesis? Cell 58: 803～805, 1989

34) INGBER, D.; T. FUJITA, S. KISHIMOTO, K. SUDO, T. KANAMARU, H. BREM & J. FOLKMAN: Synthetic analogues of fumagilin that inhibit angiogenesis and suppress tumor growth. Nature 348: 555～557, 1990

35) KUSAKA, M.; K. SUDO, T. FUJITA, S. MARUI, F. ITOH, D. INGBER & J. FOLKMAN: Potent anti-angiogenic action of AGM-1470: comparison to the fumagillin parent. Biochem. Biophys. Res. Commun. 174: 1070～1076, 1991

36) OTSUKA, T.; T. SHIBATA, Y. TSURUMI, S. TAKASE, M. OKUHARA, H. TERANO, M. KOHSAKA & H. IMANAKA: A new angiogenesis inhibitor, FR-111142. J. Antibiotics 45: 348～354, 1992

37) INOUE, K.; H. KORENAGA, N. G. TANAKA N. SAKAMOTO & S. KADOYA: The sulfated polysaccharide-peptidoglycan complex potently inhibits embryonic angiogenesis and tumor growth in the presence of cortisone acetate. Carbohydr. Res. 181: 135～142, 1988

38) YAMASHITA, T.; M. SAKAI, Y. KAWAI, M. AONO & K. TAKAHASHI: A new activity of herbimycin A: Inhibition of angiogenesis. J. Antibiotics 42: 1015～1017, 1989

39) OIKAWA, T.; M. HASEGAWA, M. SHIMAMURA, H. ASHINO, S. MUROTA & I. MORITA: Eponemycin, a novel antibiotic, is a highly powerful angiogenesis inhibitor. Biochem. Biophys. Res. Commun. 181: 1070～1076, 1991

40) OIKAWA, T.; M. SHIMAMURA, H. ASHINO, O. NAKAMURA, T. KANAYASU, I. MORITA & S. MUROTA: Inhibition of angiogenesis by staurosporine, a potent protein kinase inhibitor. J. Antibiotics 45: 1155～1160, 1992