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Anicemycin, a New Inhibitor of Anchorage-independent Growth of Tumor Cells from *Streptomyces* sp. TP-A0648

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Abstract The anchorage-independence of cells is closely related to their tumorigenicity. In the screening of inhibitors of anchorage-independent growth of tumor cells, anicemycin was isolated from the fermentation broth of an actinomycete strain TP-A0648. The producing strain was isolated from a leaf of *Aucuba japonica* collected in Toyama, Japan and identified as *Streptomyces* sp. based on the taxonomic data. The structure of anicemycin was elucidated as a new analog of spicamycin by NMR and MS analysis. Anicemycin inhibited the anchorage-independent growth of the human ovary cancer SKOV-3 cells with an IC_{50} of 0.015 μM about three times more potently than their anchorage-dependent growth.

Keywords anicemycin, anchorage-independent growth, cytotoxic, spicamycin, *Streptomyces*

Introduction

Most of the previously developed antitumor agents act mainly on the nucleic acid biosynthesis, DNA or microtubules and therefore these agents show cytotoxicity to normal tissues with significant adverse effects. Recent researches on the mechanism of cancer have been disclosing that a number of oncogenes are involved in tumorigenesis and many of these gene products are functioning as signal transduction molecules. The agents that inhibit the signal transduction of oncogenes are expected to become selective and safe anticancer drugs [1].

The normal cells derived from epithelial and endothelial cells can proliferate only when they attach to the firm substrate. When these cells detach from the substrate and lose contact with the matrix, they die in an apoptotic process termed 'anoikis'. On the other hand, cancer cells usually have developed resistance to anoikis and show growth ability without the firm substrate attachment, namely the anchorage-independence. Cell viability under anchorage-dependent conditions is regulated by integrin signal transduction through its interaction with the extracellular matrix. Various oncogenes expressed in transformed and cancer cells cause constitutive activation of the integrin signaling pathway which results in anoikis resistance [2, 3].

In our screening program of new antitumor compounds from microbial secondary metabolites, anicemycin (Fig. 1) was isolated as an inhibitor of anchorage-independent growth from *Streptomyces* sp. TP-A0648. In this paper, we describe the fermentation, isolation, structure and biological activity of anicemycin.

Materials and Methods

General Experimental Procedures

NMR experiments were performed on a JEOL JNM-LA400 NMR spectrometer. The MS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectra were

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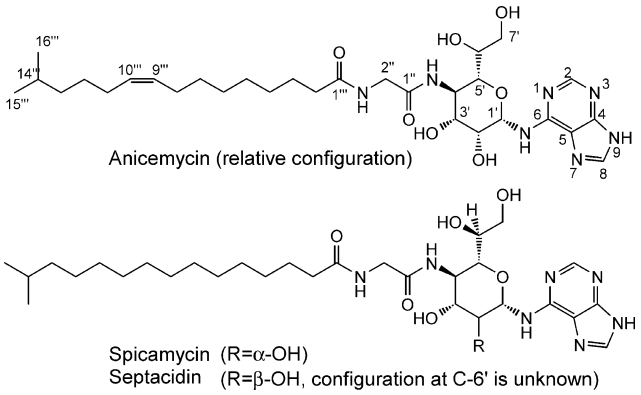

Fig. 1 Structures of anicemycin and related compounds.

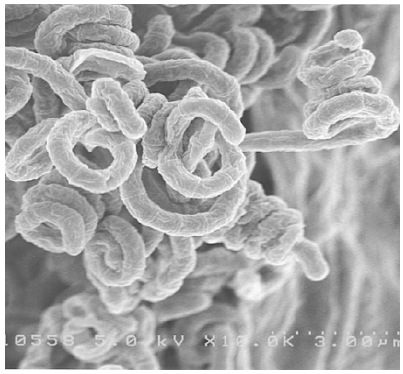

Fig. 2 Scanning electron micrograph of *Streptomyces* sp. TP-A0648.

recorded on a Hitachi U-3210 spectrophotometer. IR spectrum was recorded on a Shimadzu FT-IR-300 spectrophotometer. Optical rotation was measured on a JASCO DIP-1030. Melting point is not corrected.

The Producing Strain

The actinomycete strain TP-A0648 was isolated from a leaf of *Aucuba japonica* Thunb collected in Toyama prefecture, Japan. Isolation procedure was reported previously [4]. A pure culture of strain TP-A0648 was maintained at 23°C for laboratory use as a slant on Bennett's agar. It was also preserved in 20% glycerol at −80°C.

Fermentation

A loopful of a mature slant culture of *Streptomyces* sp. TP-A0648 was inoculated into 500-ml K-1 flasks containing 100 ml of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flask was incubated at 30°C for 4 days on a rotary shaker (200 rpm). Three-ml aliquots of the seed culture were transferred into a thousand 500-ml K-1 flasks each containing 100 ml of the production medium consisting of corn steep liquor 2% (Wako Pure Chemical), maltose 4.5% and HP-20 (Mitsubishi Chemical Co.) 1.0% (pH 7). Fermentation was carried out for 3 days at 30°C on a rotary shaker (200 rpm).

Extraction and Isolation

The fermented whole broth (100 liters) was extracted with EtOAc (100 liters) with stirring the whole mixture vigorously. The organic layer was separated and concentrated *in vacuo* to give an oily residue (8.5 g). The residue was chromatographed on a silica gel column (170 g) with $CHCl_3$ - MeOH (10 : 1～1 : 1). The active fractions (2 : 1～1 : 1) were combined and concentrated *in vacuo* to give brownish solid (750 mg). The solid was further purified by preparative HPLC. The ODS column (300×19 mm, i.d., 7 μm, XTerra™ RP18, Waters) was eluted with 40% CH_3CN in 10 mM NH_4HCO_3 (pH 8) and the fraction of anicemycin was collected. The fraction was concentrated *in vacuo* and the remaining aqueous solution was lyophilized twice to yield anicemycin (3.3 mg).

Biological Assay

The inhibition of anchorage-independent cell growth was measured against human ovarian cancer SKOV-3 and colon cancer DLD-1 cells as previously described [5]. Briefly, SKOV-3 or DLD-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μg/ml kanamycin at 37°C with 5% CO_2. The exponentially growing cells were trypsinized and resuspended in fresh medium and then were inoculated onto polyHEMA-coated (suspension culture) or uncoated (attached culture) plates. The cell growth was determined using MTT.

Results and Discussion

Taxonomy of the Producing Strain

Streptomyces sp. TP-A0648 was isolated from a leaf of *Aucuba japonica* Thunb collected in Toyama prefecture, Japan. Taxonomic characteristics of strain TP-A0648 were determined by cultivation on various media described by Shirling and Gottlieb [6], Waksman [7] and Arai [8]. By scanning electron microscope, spore chains are *Spirales*, and the spore surface is smooth (Fig. 2). The aerial mass color is white to gray and the color of reverse side is pale yellow to dark gray. Diffusible pigments were not formed (Table 1).

Table 1 Cultural characteristics of strain TP-A0648

Medium	Aerial mycelium	Reverse side	Diffusible pigment	Growth
Yeast extract - malt extract agar (ISP med. 2)	Medium gray (406)	Dark gray (421)	None	Good
Oatmeal agar (ISP med. 3)	White (388)	Pale yellow (128)	None	Standard
Inorganic salts - starch agar (ISP med. 4)	Light bluish gray (404)	Brownish gray (428)	None	Good
Glycerol asparagine agar (ISP med. 5)	Medium gray (406)	Dark olive gray (420)	None	Good
Tyrosine agar (ISP med. 7)	Olive gray (410)	Dark gray (417)	None	Good
Pridham - Gottlieb agar (ISP med. 9)	Yellowish white (389)	Yellowish white (393)	None	Poor

The optimum temperature for strain growth is 32~33°C. The strain does not grow below 14°C or above 37°C. Strain TP-A0648 utilizes D-glucose, D-fructose, inositol, D-xylose for growth but does not utilize L-rhamnose, D-mannitol, D-raffinose and sucrose [6]. Whole cell hydrolysates contain LL-diaminopimelic acid, glucose, mannose, xylose and ribose [9, 10]. Sequence data of the full length 16S rDNA [11] indicates 97.42% identity to *Streptomyces thermoviolaceus*. On the basis of these morphological and chemical characteristics, the strain TP-A0648 was identified as *Streptomyces* sp.

Table 2 Physico-chemical properties of anicemycin

Appearance	White powder
Melting point	>200°C (dec)
$[\alpha]_D^{20}$	+19.2 (*c* 0.17, methanol)
HRFAB-MS	Found: 620.3776 $[M+H]^+$
	Calcd: 620.3772 (for $C_{30}H_{50}N_7O_7$)
Molecular formula	$C_{30}H_{49}N_7O_7$
UV λ_{max}^{MeOH} nm (ε)	205 (22,800), 264 (13,230)
IR ν_{max} (cm^{-1})	3300, 1625

Structure Determination of Anicemycin

Anicemycin was obtained as a white powder with the melting point of 200°C (decomposition). The IR spectrum indicated the presence of an amide (1625 cm^{-1}) and hydroxyl (3300 cm^{-1}) groups. The UV-vis spectrum of anicemycin [λ_{max} (ε) 205 (22,800), 264 (13,230) in MeOH] showed a high similarity to that of spicamycin, suggesting the presence of an adenine-like chromophore. The molecular formula was determined as $C_{30}H_{49}N_7O_7$ on the basis of the high resolution FABMS which gave a $[M+H]^+$ ion at *m*/*z* 620.3776 (Δ+0.4 mmu; calcd for $C_{30}H_{50}N_7O_7$) and the NMR data.

The aromatic protons were observed at δ 8.07 (s) and 8.25 (s). The carbon signals due to the adenine moiety were not observed in the ^{13}C NMR spectrum except for the peak at δ 153.0, but the remaining carbons could be assigned by the analysis of HMQC and HMBC spectra. The HMQC spectrum correlated the proton signals at δ 8.25 and 8.07 to carbon signals at δ 153.0 and 142.5, respectively. HMBC correlations were observed from the proton at δ 8.25 to the carbons at δ 152.4 and 154.2 and from the proton at δ 8.07 to the carbons at δ 119.0 and 152.4.

The anomeric proton signal was observed at δ 5.63, five methine signals at δ 4.13 (H-4′), 3.99 (H-2′), 3.75 (H-3′), 3.74 (H-6′) and 3.68 (H-5′), and methylene signals at δ 3.75 and 3.64. Connectivities between these signals were confirmed by DQF-COSY and HMBC experiments. H-4′ signal was long-range coupled to the carbonyl carbon at δ 172.5. The configuration of the sugar moiety was confirmed, based on the coupling constants ($J_{3',4'}=J_{4',5'}=$ 10 Hz, $J_{2',3'}=2$ Hz), except for the C-6′ stereochemistry (Fig. 3). The axial configuration of the anomoric proton H-1′ was confirmed by a NOESY correlation between H-1′ and H-5′ observed in the tetra-*O*-acetyl derivative which was prepared by the treatment of anicemycin with acetic anhydride in pyridine. The absolute configuration of the sugar moiety is not determined.

Methylene signals of the glycine moiety were observed at δ 3.85 and 3.89. These signals had HMBC correlations with carbonyl carbons at δ 177.0 (C-1‴) and 172.5 (C-1″).

The fatty acid moiety was identified as 14-methyl-*cis*-9-pentadecenoate based on the NMR analysis. The DQF-COSY spectrum showed a coupling of the methyl signal at δ 0.87 (6H, d, J=6.6 Hz) to a methine proton at δ 1.52, which, in turn, was coupled to a methylene group at δ 1.19, indicating the presence of an *iso*-butyl group. The signal at δ 1.19 showed a ^1H-^1H correlation to another methylene at δ 1.35 and an HMBC correlation to a methylene carbon (δ_C 28.7, δ_H 2.02). ^1H-^1H correlations were observed

Table 3 NMR data for anicemycin in CD_3OD

Position	δ_C	δ_H (integral, mult., *J* Hz)
Adenine moiety		
2	153.0	8.25 (1H, s)
4	152.4	
5	119.0	
6	154.2	
8	142.5	8.07 (1H, s)
Sugar moiety		
1′	80.7	5.63 (1H, br.s)
2′	71.7	3.99 (1H, d, 2)
3′	73.8	3.75 (1H, m)
4′	50.6	4.13 (1H, t, 10)
5′	78.8	3.68 (1H, m)
6′	73.4	3.74 (1H, m)
7′	64.0	3.64 (1H, dd, 6.8, 11.5)
		3.75 (1H, dd, 3.2, 11.5)
Glycine moiety		
1″	172.5	
2″	43.8	3.85 (1H, d, 16.6)
		3.89 (1H, d, 16.6)
Fatty acid moiety		
1‴	177.0	
2‴	36.9	2.27 (2H, t, 7.8)
3‴	26.8	1.63 (2H, m)
4‴	30.8	1.28∼1.38 (2H, m)
5‴	30.4[a]	1.28∼1.38 (2H, m)
6‴	30.4[a]	1.28∼1.38 (2H, m)
7‴	30.2[a]	1.28∼1.38 (2H, m)
8‴	28.4[b]	2.02 (2H, m)
9‴	130.8[c]	5.34 (1H, m)
10‴	130.9[c]	5.34 (1H, m)
11‴	28.7[b]	2.02 (2H, m)
12‴	28.1[a]	1.35 (2H, m)
13‴	39.8	1.19 (2H, m)
14‴	29.1	1.52 (1H, m)
15‴	23.0	0.87 (3H, d, 6.8)
16‴	23.0	0.87 (3H, d, 6.8)

between the signal at δ 1.38 and 1.63, and δ 1.63 and 2.27 in the DQF-COSY spectrum, and a long-range coupling was detected from the proton at δ 2.27 to the carbonyl carbon at δ 177.0. The olefinic protons at δ 5.34 (2H, m) were coupled to the methylene signals at δ 2.02 (4H, m, δ_C 28.7, δ_C 28.4) and the HMBC correlation was detected from the proton signal at δ 1.19 to the carbon at δ 28.7. The geometry of the olefin was determined to be *Z* by comparing with the ^{13}C NMR data of unsaturated fatty acids. For example, the allylic carbons of elaidic acid (*trans*-9-octadecenoic acid) appear at δ 32.5 and 32.6 whereas those of oleic acid (*cis*-9-octadecenoic acid) appear at δ 27.1 and 27.2 in $CDCl_3$ [12]. This chemical shift difference is rationalized by the electron repulsion between two *cis*-allylic methylene groups which induces the higher electron density on the allylic carbons in oleic acid than in elaidic acid. In case of anicemycin, two allylic carbons were observed around δ 28∼29 that were characteristic chemical shifts observed in *cis*-unsaturated fatty acids.

The structure of anicemycin was thus established as depicted in Fig. 1. Anicemycin consists of four partial structures, adenine, aminosugar, glycine, and fatty acid, and is structurally related to spicamycin [13] and septacidin [14], antitumor antibiotics from *Streptomyces*. The sugar moiety of spicamycin and septacidin has an L-pyranose configuration and their difference lies in the orientation of the hydroxyl group at C-2′. The relative configuration of the pyranose moiety of anicemycin is same with that of spicamycin. Spicamycin was isolated together with several congeners of varying fatty acid chains but none of them contained an unsaturated fatty acid like anicemycin. On the other hand, the production of spicamycin and septacidin by strain TP-A0648 was not detected in the LC-MS analysis.

Biological Activity of anicemycin

IC_{50} values of anicemycin against SKOV-3 and DLD-1 cells under the anchorage-independent condition were 0.015 and 0.029 μM, respectively, while those under the anchorage-dependent condition were 0.045 and 0.068 μM, respectively. Anicequol is the most selective anchorage-independent growth inhibitor we previously isolated from a fungus [5]. It inhibits the anchorage-independent growth of DLD-1 cells with an IC_{50} of 1.6 μM without showing growth inhibition to the cells under anchorage-dependent condition at the same concentration. In spite of the low selectivity, anicemycin is expected as a lead molecule for anchorage-independent growth inhibitors owing to its quite low IC_{50} values. Structure-activity relationship studies are in progress to improve the selectivity.

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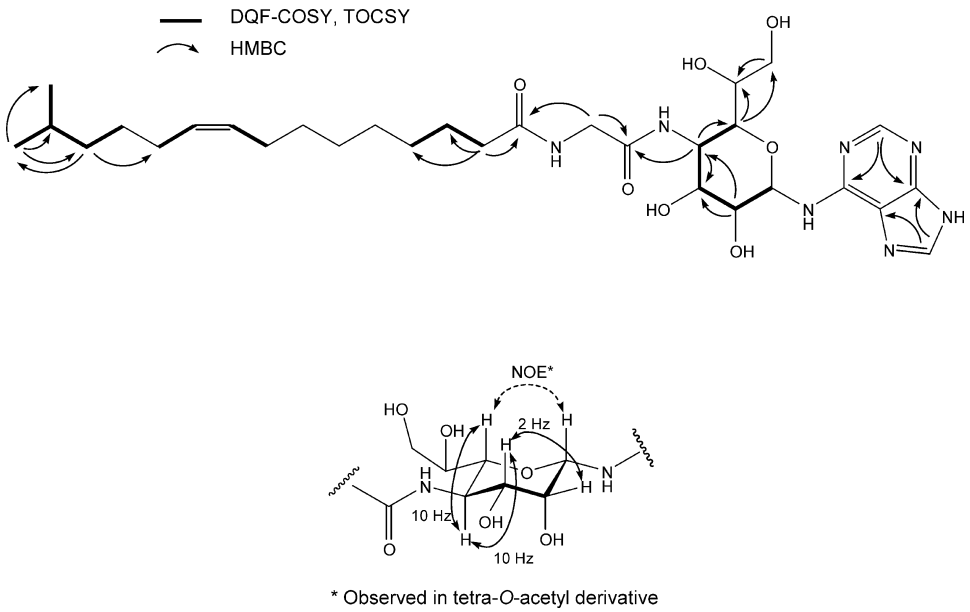

Fig. 3 NMR analysis of anicemycin.

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