ARUGOMYCIN, A NEW ANTHRACYCLINE ANTIBIOTIC

I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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Arugomycin (AGM) is a new anthracycline antibiotic produced by strain No. 1098-AV_2 which was identified as *Streptomyces violaceochromogenes*. AGM was isolated by solvent extraction, silicic acid chromatography and Sephadex LH-20 column chromatography. Acid treatment of AGM gave the chromophore, named arugorol, which was identified as 4′-*epi*-nogalarol, and sugar moieties. AGM inhibited the growth of Gram-positive bacteria and showed antitumor activity against sarcoma S-180 and Ehrlich ascites tumors.

During the course of our screening program for new antitumor antibiotics, the cultured broth of a strain No. 1098-AV_2, which was isolated from a soil sample collected at Motoyama, Saga, Japan, was found to induce the differentiation of M1 cells and to show antimicrobial activities against Gram-positive bacteria. The organism produced a new anthracycline antibiotic named arugomycin (AGM)[1]. This paper describes the taxonomy of the producing organism, and the production, isolation and physico-chemical properties of AGM.

Taxonomy

Taxonomic studies were carried out following the procedures of the International Streptomyces Project[2].

Morphological Characteristics

The aerial mycelium branched monopodially. Sporophores formed spore chains with 10 to 25 spores per chain. The morphology of spore chains was terminal compact spiral (2~3 μm in diameter, 1~4 turns). The spores were cylindrical or ellipsoidal (0.7~0.9×0.5~0.6 μm) with smooth surface. On nutrient agar medium, spores were also formed on substrate mycelium singly or in short chain (2~15 spores per chain) which curved irregularly. The other special morphology were not observed. Whole cell hydrolysate of the strain 1098-AV_2 contained L,L-diaminopimelic acid which suggests that the strain belongs to cell wall type I.

Cultural and Physiological Characteristics

The cultural and physiological characteristics are shown in Tables 1 and 2, respectively. The description in Table 1 follows the color standard "Color Harmony Manual" published by Container Corporation of America, U.S.A.

The Properties of Strain 1098-AV_2

The properties of strain 1098-AV_2 such as the presence of L,L-diaminopimelic acid in the cell wall

Table 1. Cultural characteristics of *Streptomyces violaceochromogenes* 1098-AV_2.

Glycerol - asparagine agar	AM :	Grey
	RC :	Pale orange to light yellow orange
	SP :	Pale orange to brownish white
Inorganic salts - starch agar	AM :	Grey to red
	RC :	Pale yellow orange
	SP :	None
Yeast extract - malt extract agar	AM :	Grey
	RC :	Light yellow to orange dark orange
	SP :	Pale brown to light orange
Oatmeal agar	AM :	Grey to red
	RC :	Pale orange to light yellow orange
	SP :	Pale orange to light yellow orange

AM: Aerial mass color, RC: reverse color of colony, SP: soluble pigments.

and of the spore chains with more than 10 spores suggested that the strain 1098-AV_2 belongs to the genus *Streptomyces*. The following characteristics of the strain 1098-AV_2 were almost the same as those of *Streptomyces violaceochromogenes*[3].

a) Spore chain was terminal compact spiral. b) Spore surface was smooth. c) Color of colony was grey or red color-series. d) Reverse side of colony had no distinctive pigments (pale orange, bright yellow or dark orange). e) Melanoid pigments were produced.

Although the carbon utilization of the strain 1098-AV_2 was slightly different from that of *S. violaceochromogenes* (with regard to L-rhamnose and raffinose), the strain 1098-AV_2 was identified as a new strain of *S. violaceochromogenes*.

Table 2. Physiological characteristics of *Streptomyces violaceochromogenes* 1098-AV_2.

Temperature range for growth	20~40°C
Optimum growth temperature	27~37°C
Liquefaction of gelatin	+
Hydrolysis of starch	+
Coagulation of milk	−
Peptonization of milk	+
Production of melanoid pigments:	
Tyrosine agar	+
Peptone - yeast - iron agar	+
Tryptone - yeast broth	+
Carbon utilization:	
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
Inositol	+
L-Rhamnose	−
Raffinose	±
D-Mannitol	+

+: Positive, ±: doubtful, −: negative.

Fermentation

Growth from a well-grown agar slant of *S. violaceochromogenes* 1098-AV_2 was used to inoculate the seed medium shown in Table 3. This was incubated at 27°C for 72 hours on a rotary shaker (200 rpm). The vegetative culture was inoculated to a 50-liter jar fermentors containing 25 liters of the production medium. Culture conditions are shown in Table 3. The cultured broth exhibited the maximum accumulation of AGM after 96 hours of fermentation. A typical time course of fermentation is shown in Fig. 1.

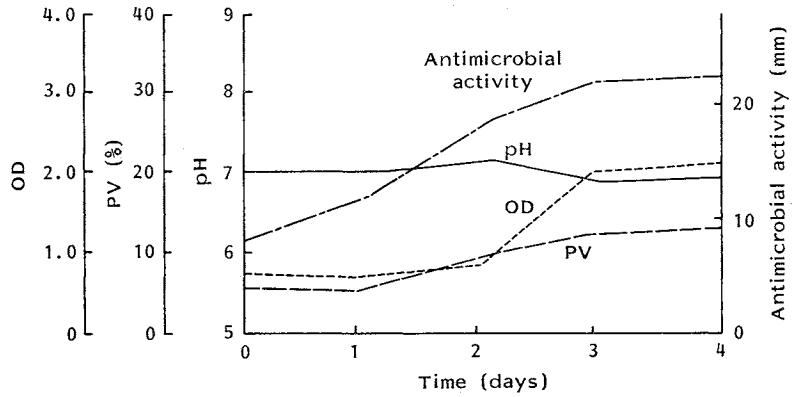
Purification of AGM

AGM was purified to microcrystalline powder by the procedures illustrated in Fig. 2. The cultured broth filtrate (25 liters) was adjusted to pH 2.0 and applied to a Diaion HP-20 column. AGM was eluted with methanol after washing the column with water. The eluate was concentrated to a small volume *in vacuo* followed by extraction with chloroform. The solvent layer was evaporated to give crude AGM which was further purified by chromatography on a silicic gel column with chloro-

Table 3. Culture conditions for production of arugomycin.

Seed culture				
Medium:				
pH		7.3		
Glucose (%)		0.4		
Malt extract (%)		1.0		
Yeast extract (%)		0.4		
Vitamin complex* (ml/liter)		10		
*Vitamin complex (mg/10 ml)				
Thiamine - HCl	0.5		Ca-pantothenate	0.5
Riboflavin	0.5		*p*-Aminobenzoic acid	0.5
Niacin	0.5		Biotin	0.25
Pyridoxine - HCl	0.5		Inositol	0.5
Condition:	27°C, 72 hours, 200 rpm			
Production culture				
Medium:				
pH		7.0		
Starch (%)		2.5		
Soybean meal (%)		1.5		
Dry yeast (%)		0.2		
$CaCO_3$ (%)		0.4		
Condition:	27°C, 96 hours, 200 rpm			

Fig. 1. Fermentation process of arugomycin by *Streptomyces violaceochromogenes* 1098-AV_2.
PV: Packed volume 3,000 rpm×10 minutes. OD: Optical density at 476 nm. Antimicrobial activity: Activity against *Bacillus subtilis* indicated in diameter.



form - methanol (20 : 1). The active fraction was applied to a Sephadex LH-20 column and developed with chloroform - methanol (1 : 1). The eluate was concentrated *in vacuo* to give an orange powder (2.5 g) of pure AGM.

Physico-chemical Properties

Physico-chemical properties of AGM are summarized in Table 4. The UV and visible spectra showed absorption maxima in a methanol solution at 235, 258, 292 and 476 nm, the last one shifted to 550 nm in an alkaline solution. The UV and visible spectra are similar to those of nogalarol.

The IR spectrum (KBr) of AGM indicated the presence of hydroxyl (3430 cm^{-1}), hydroxyanthraquinone (1660 and 1640 cm^{-1}) and ester carbonyl (1740 cm^{-1}) moieties.

Fig. 2. Isolation procedure of arugomycin.

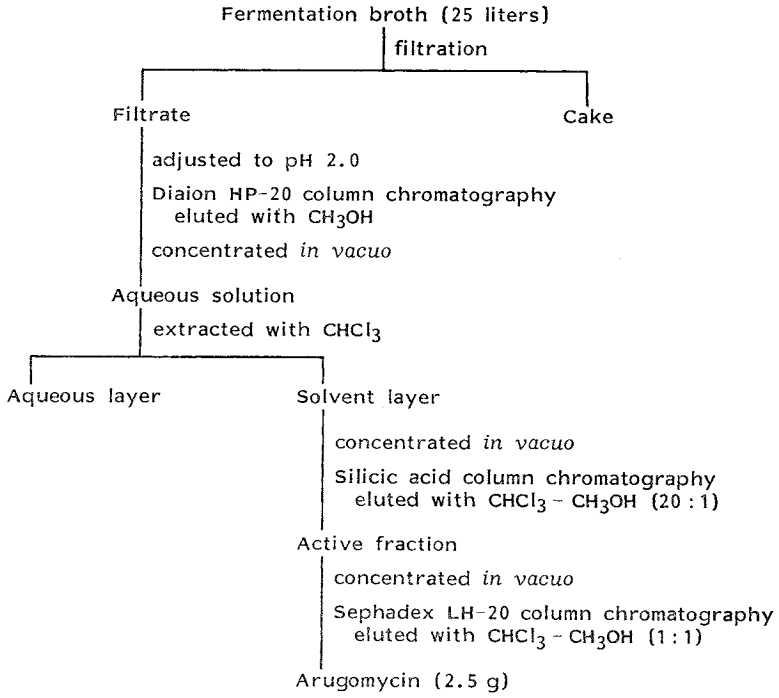


Table 4. Physico-chemical properties of arugomycin.

Appearance	Orange powder
Molecular formula	$C_{80}H_{112}O_{37}N_2$
MW (SI-MS)	*m/z* 1,694 $(MH)^+$
Elemental analysis	
Found:	C 56.22, H 6.85, N 1.78, O 35.14.
Calcd for:	C 56.71, H 6.67, N 1.65, O 34.97.
MP (°C)	208~212
$[\alpha]_D^{25}$	+112° (*c* 0.1, $CHCl_3$ - CH_3OH, 9 : 1)
UV λ_{max} nm ($E_{1cm}^{1\%}$)	
MeOH	235 (363), 258 (167), 292 (61), 476 (104)
MeOH+0.1 N HCl	235 (387), 258 (159), 292 (61), 468 (110)
MeOH+0.1 N NaOH	239 (302), 294 (41), 543 (88)
IR ν_{max} (KBr) cm^{-1}	3430, 1740, 1660, 1640

SI-MS: Secondary ion mass spectrum.

The ^1H NMR spectrum shown in Fig. 3 (400 MHz, in pyridine-d_5) indicated the presence of one carbomethoxy group (δ_H 3.6), four methoxy groups (δ_H 3.2~3.4), and one dimethyl amino group (δ_H 2.7).

Treatment of AGM (200 mg) with 40% formic acid (40 minutes, 85°C) gave a mixture of the aglycone and sugar moieties. The aglycone was separated from the sugar moieties by adsorbing on a Diaion HP-20 column. It was further purified by preparative silica gel TLC and Sephadex LH-20 column chromatography to give 42 mg of arugorol (AGR).

The physico-chemical properties of AGR are summarized in Table 5.

Fig. 3. ^{1}H NMR spectrum of arugomycin (in pyridine-d_5).

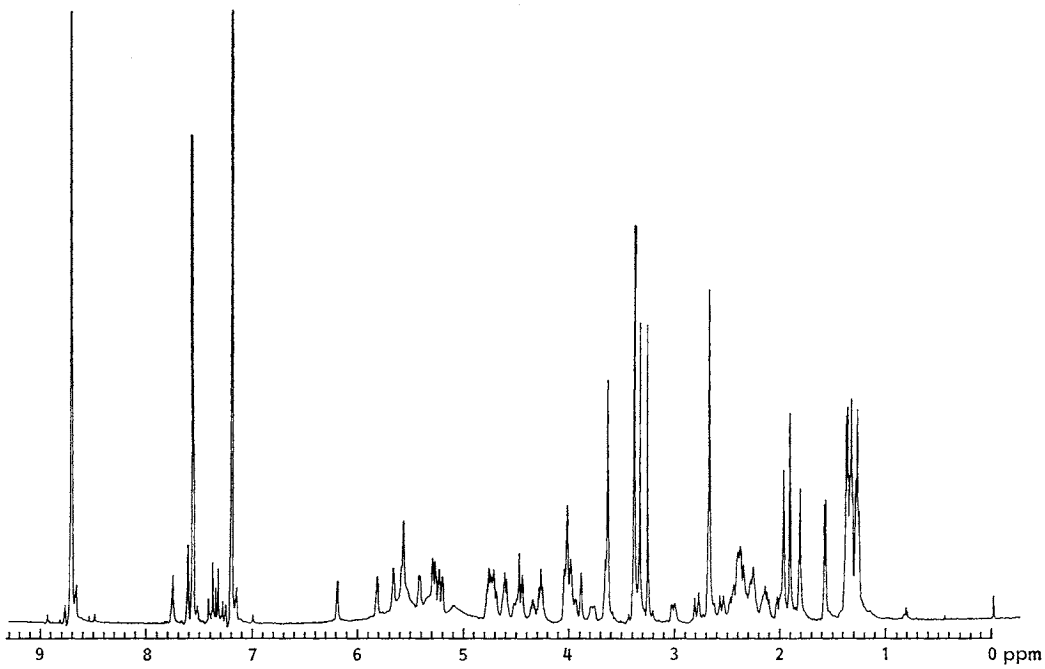
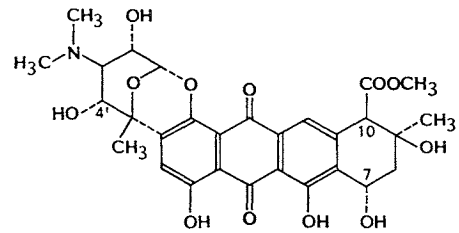


Table 5. Physico-chemical properties of arugorol.

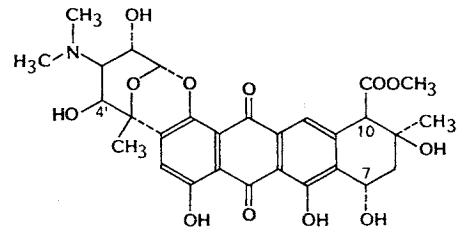
Molecular formula	$C_{29}H_{31}O_{12}N$
MW (SI-MS)	*m/z* 586 $(MH)^+$
MP (°C)	210~213
$[\alpha]_D^{21}$	+424° (*c* 0.1, MeOH)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	234 (734), 258 (342), 288 (128), 475 (219)
IR ν_{max} (KBr) cm^{-1}	3430, 1730, 1660, 1620

The ^{13}C NMR spectrum of AGR (Table 6) is very similar to that of nogalarol[4] (NOG) except for the signals due to the amino sugar moiety directly fused to the aglycone [C-2′ to C-6′ and $N(CH_3)_2$]. The large upfield shift of C-2′ (δ_C 73.5 in NOG to δ_C 67.4 in AGR) is clearly ascribed to the stereochemical change of C-4′ (γ-effect)[5]. In agreement with this, the ^{1}H NMR coupling constants of AGR indicated that the amino sugar moiety was 3,6-dideoxy-3-dimethylaminogalactopyranose with axial configurations for 2′-H ($J_{1',2'}$=3.0 Hz and $J_{2',3'}$=11.0 Hz) and 3′-H ($J_{2',3'}$=11.0 Hz and $J_{3',4'}$=2.6 Hz), and an equatorial configuration for 4′-H ($J_{3',4'}$= 2.6 Hz). On the other hand, the ^{1}H NMR coupling constants of NOG were reported to be as follows; for 2′-H ($J_{1',2'}$=3.3 Hz and $J_{2',3'}$=

Fig. 4. Structures of nogalarol and arugorol.



Nogalarol



Arugorol

Table 6. ^{13}C NMR chemical shifts of arugorol (AGR), the aglycone part of arugomycin (AGM) and nogalarol (NOG).

Carbon	AGR in CD_3OD	AGM in pyridine-d_5	NOG[4] in DMF-d_7
1	147.5	147.9	148.0
2	138.6	140.1	138.1
3	123.8	122.3	125.2
4	157.0	156.5	155.7
4a	115.7	115.3	115.4
5	191.1	191.8	192.3
5a	114.5	114.8	114.6
6	161.4	162.2	161.6
6a	133.4	131.2	131.3
7	63.8	70.6	63.0
8	40.9	39.9	40.9
9	70.5	69.3	69.7
10	59.0	59.0	56.9
10a	143.8	144.4	144.0
11	120.3	119.8	119.8
11a	133.9	131.2	134.1
12	180.1	180.3	179.8
12a	117.8	117.8	117.2
13	30.1	30.0	29.7
1′	97.5	97.9	97.8
2′	67.4	67.4	73.5
3′	64.7	62.5	66.8
4′	73.2	81.6	70.8
5′	77.5	77.6	76.0
6′	22.6	23.6	24.2
NCH_3	43.1	44.3	41.4
COO	172.3	171.9	171.9
OCH_3	52.9	52.0	52.4

The ^{13}C NMR chemical shifts are indicated in ppm from internal TMS.

Table 7. Antimicrobial activity of arugomycin.

Organisms	MIC (μg/ml)
Bacillus subtilis PCI 219P	12.5
Staphylococcus aureus FDA 209P	12.5
Micrococcus luteus ATCC 9341	12.5
Pseudomonas aeruginosa NCTC 10490	>100
Salmonella typhimurium IFO 12529	>100
Escherichia coli NIHJ JC-2	>100
Saccharomyces cerevisiae No. Yu 1200	>100
Candida albicans IFO 0396	>100
Aspergillus fumigatus IFO 4400	>100
Penicillium chrysogenum ATCC 10002	>100

Table 8. Antitumor activity of arugomycin against sarcoma S-180 ascites and Ehrlich ascites tumors.

Dose (mg/kg)	Sarcoma S-180[a]		Ehrlich ascites[b]
	T/C (%)	Survivors on day 60	T/C (%)
0.063	93	0/8	96
0.13	126	0/8	107
0.25	199	0/8	154
0.50	>457	2/8	189
1.0	>464	3/8	119
2.0	79	0/8	—

[a] Treatment: 1, 5 days, ip; tumor inoculum, 1.0×10^6 ascites cells implanted ip; host, ICR female mice.

[b] Treatment: 1, 3, 5 days ip; tumor inoculum, 1.0×10^6 ascites cells implanted ip; host, *dd*Y female mice.

10.5 Hz), 3′-H ($J_{2',3'}$=10.5 Hz and $J_{3',4'}$=10.5 Hz) and 4′-H ($J_{3',4'}$=10.5 Hz)[4]. Thus AGR is the 4′-epimer of NOG, the chromophore of nogalamycin[4], as shown in Fig. 4 with uncertainty about the absolute configuration. Comparison of the ^{13}C NMR of AGM and AGR revealed the glycosidation shift of C-7 from δ_C 63.8 to 70.6 and C-4′ from δ_C 73.2 to 81.6. These results suggested the attachment of sugar chains to C-7 and C-4′ of the chromophore of AGM.

Biological Activities

The antimicrobial activity of AGM is summarized in Table 7. It inhibited the growth of Gram-positive bacteria. LD_{50} of AGM in mice was 1.75 mg/kg by intraperitoneal injection. The activities against sarcoma S-180 and Ehrlich carcinoma are shown in Table 8.

Further structural studies of AGM will be reported in the accompanying paper.

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