NEW AUREOLIC ACID ANTIBIOTICS

I. SCREENING, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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A new antibiotic complex was isolated from the fermentation broth of *Streptomyces aburaviensis* PA-39856. The individual factors, demethylolivomycins A, B and demethylchromomycins A_2, A_3 were separated and purified by preparative HLC. These antibiotics possess high activities against Gram-positive bacteria and P388 leukemia in mice.

A convenient prescreening method for anticancer antibiotics consists of anti-phage and prophage induction tests[1]. We previously reported on anti-phage screening using RNA phage f_2 and *Escherichia coli* K-12[2]. The sensitivity of this screening method was improved using *E. coli* 34S, derived from *E. coli* KL 16 which might be deficient in some components of the outer cell membrane. Screening for anti-phage f_2 substances with this improved method yielded *Streptomyces* sp. PA-39856, which produces a new aureolic acid group of antibiotics designated as demethylolivomycins A, B and demethylchromomycins A_2, A_3.

This paper, outlines the screening method and reports on the isolation, characterization and biological activities of these new antibiotics.

Results

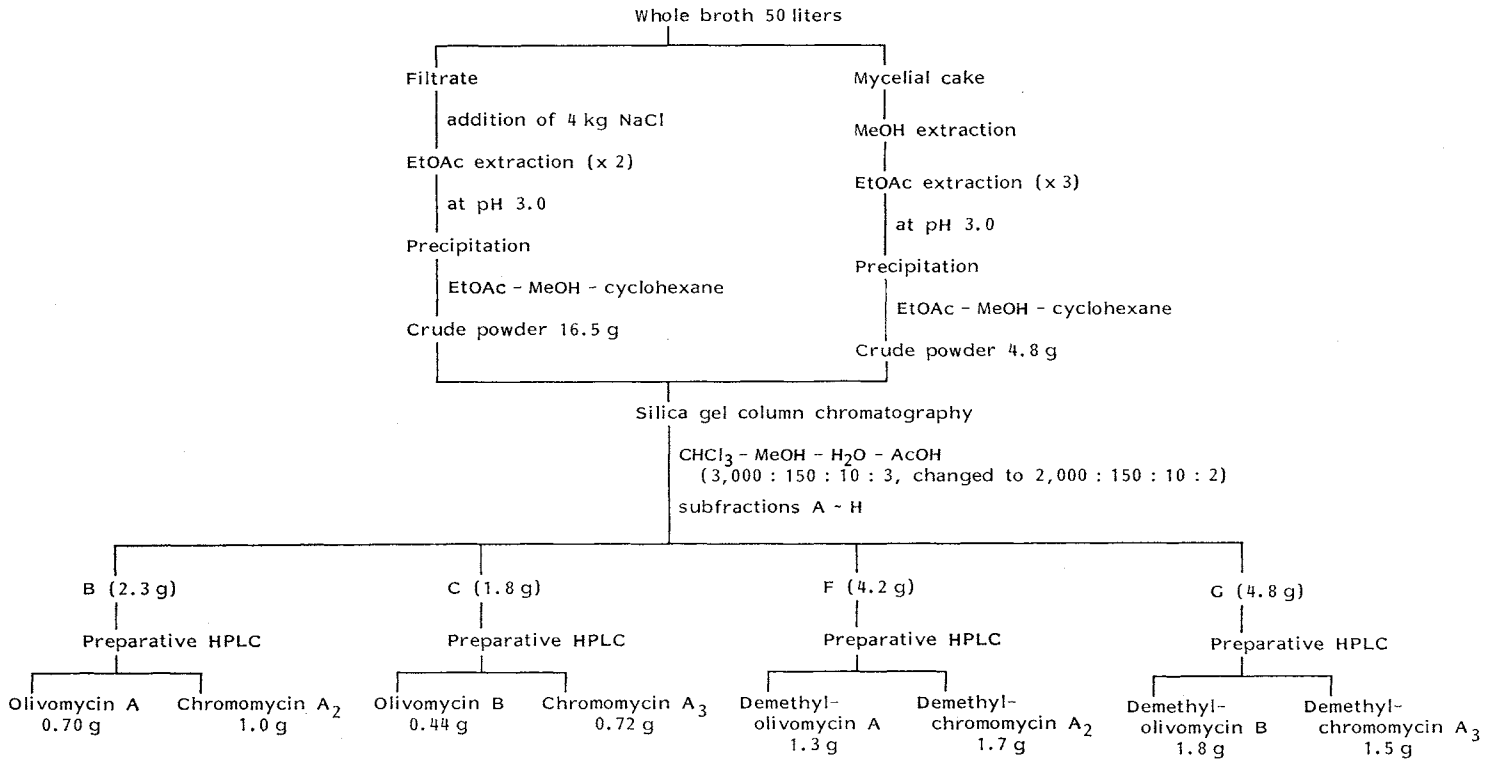
Screening Methods

The screening procedure for anti-phage f_2 was similar to the previous one[2] except for the use of *E. coli* 34S, a mutant derived from *E. coli* KL 16, as the host cell in place of the parent strain. The multiplicity of infection of phage f_2 to *E. coli* KL 16 was 0.002 and that to E. *coli* 34S was a little higher value of 0.005. *E. coli* 34S was almost 10~100 times more sensitive to actinomycin D, rifampicin and erythromycin than wild type strain. The wild type *E. coli* KL 16 is resistant to these antibiotics because the drugs cannot enter the cell. Sensitivity to kanamycin and benzylpenicillin of the mutant was almost unchanged.

Taxonomic Studies

Streptomyces sp. PA-39856 was isolated from a soil sample in May, 1978. Based on its taxonomic character, this strain is considered to be identical with a strain of *Streptomyces aburaviensis*. The strain has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Tokyo, Japan as *S. aburaviensis* PA-39856 with the accession number FERM-P 6129[3].

Fig. 1. Isolation procedure.



Isolation and Chemical Characterization

Demethylolivomycins A, B and demethylchromomycins A_2, A_3 were isolated from the fermentation broth of *S. aburaviensis* PA-39856. The active fractions were detected by bioassay using antiphage activity. The organism also produced a fair amount of chromomycins A_2, A_3[4] and olivomycins A, B[5]. The four demethyl factors were the main products of this strain.

The isolation procedure of the individual factors is shown schematically in Fig. 1 and details are reported in the experimental section. These four factors were observed as yellow orange spots on silica gel TLC plates and their Rf values were summarized in Table 1 together with those of olivomycins A, B, chromomycins A_2, A_3 and mithramycin A. These four factors could be separated well by HPLC on a column of Nucleosil RP-18 using acetonitrile - 30 mM tartrate buffer, pH 3.0 (50 : 45 or 45 : 55). They had similar physico-chemical properties and were assumed to be an aureolic acid group of antibiotics from their UV spectra. Their physico-chemical properties are summarized in Table 2.

Chromomycins A_2, A_3 and olivomycins A, B had two methoxy groups, respectively. The ^{1}H-fourier transformation (FT) NMR spectra of the demethyl factors revealed the presence of only one

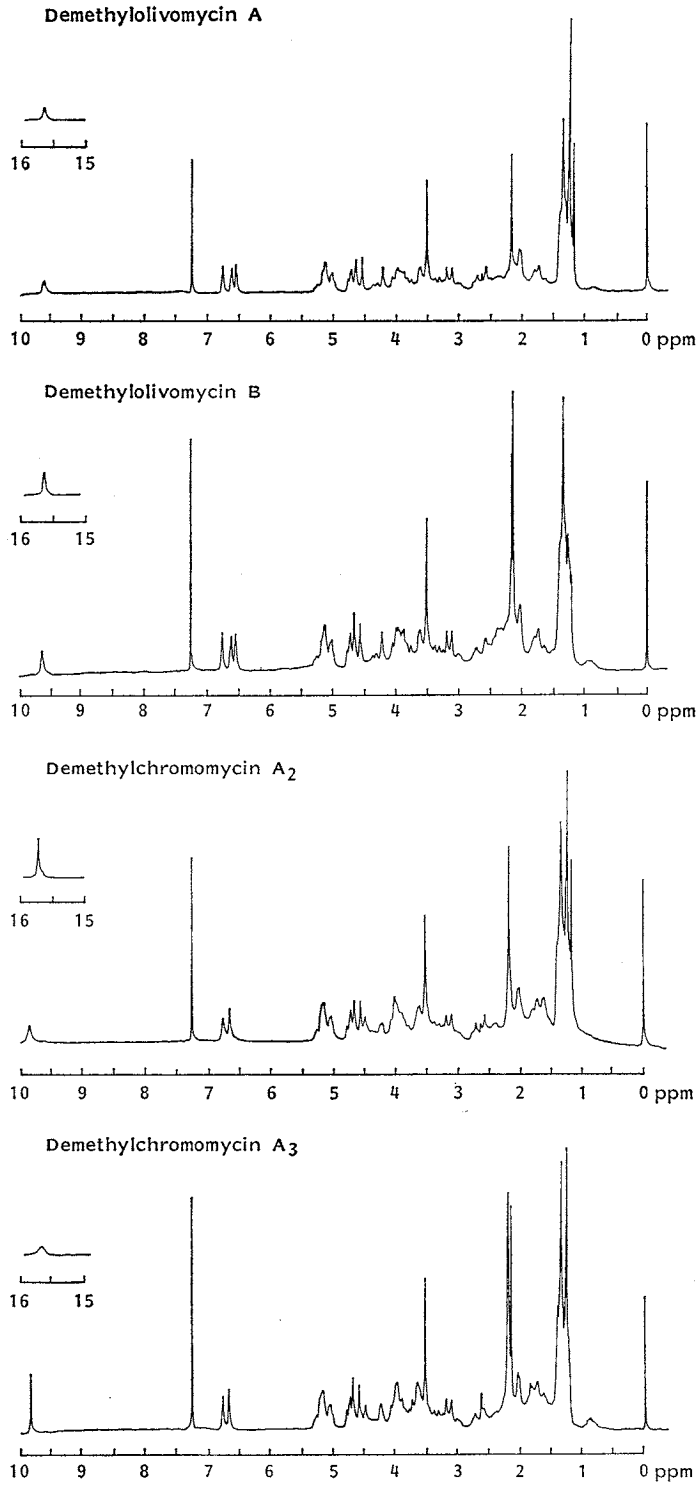
Table 1. Mobilities on TLC and HPLC.

Antibiotic	Method		
	TLC, silica gel, Rf value ($CHCl_3$ - MeOH - H_2O, 160 : 40 : 2)	HPLC, Nucleosil 10 C_{18}, retention time (minutes) (CH_3CN - H_2O - AcOH, 50 : 50 : 0.1)	HPLC, Nucleosil 10 C_{18}, retention time (minutes) (MeOH - H_2O - AcOH, 80 : 20 : 0.1)
Demethylolivomycin A	0.42	6.12	5.86
Demethylchromomycin A_2	0.42	9.03	9.40
Demethylolivomycin B	0.38	4.05	4.66
Demethylchromomycin A_3	0.38	5.40	6.80
Olivomycin A	0.54	8.25	6.77
Chromomycin A_2	0.54	12.87	10.80
Olivomycin B	0.49	5.10	5.20
Chromomycin A_3	0.50	7.15	7.60
Mithramycin A	0.23	3.47	7.07

Table 2. Physico-chemical properties of demethylolivomycins A, B and demethylchromomycins A_2, A_3.

Property	Factor			
	Demethyl-olivomycin A	Demethyl-olivomycin B	Demethyl-chromomycin A_2	Demethyl-chromomycin A_3
MP (°C, dec)	163.4~165.1	167.5~169.0	171.5~172.4	175.5~176.3
$[\alpha]_D^{21}$ (*c* 1.0, EtOH)	−33.6±0.7°	−36.4±0.7°	−52.6±0.9°	−50.6±0.9°
Molecular formula	$C_{57}H_{82}O_{26}$	$C_{55}H_{78}O_{26}$	$C_{58}H_{84}O_{26}$	$C_{56}H_{80}O_{26}$
Elemental analysis	C H	C H	C H	C H
Found:	57.94, 7.16	57.36, 7.05	58.16, 7.25	57.60, 7.10
Calcd:	57.86, 6.99	57.18, 6.81	58.18, 7.07	57.52, 6.90
MW	1,183	1,155	1,197	1,169
UV-visible spectrum $\lambda_{max}^{95\%\ EtOH}$ nm (log ε)	228 (4.33), 276 (4.67), 319 (3.72), 333 (3.60), 408 (4.08)	228 (4.32), 276 (4.66), 319 (3.71), 333 (3.58), 406 (4.07)	230 (4.41), 281 (4.72), 318 (3.95), 332 (3.85), 416 (4.08)	230 (4.38), 280 (4.70), 319 (3.90), 333 (3.81), 416 (4.04)

Fig. 2. ^{1}H-FT NMR spectrum of demethylolivomycins A, B, demethylchromomycins A_2 and A_3 in $CDCl_3$ at 30°C.



methoxy signal (δ 3.52~3.53) besides hydroxyl signals and at least one acetyl signal (δ 2.13~2.19). The ^1H spectra are shown in Fig. 2. Gas chromatography of demethylolivomycin A and demethylchromomycin A_2 showed peaks assignable to an equimolar amount of acetic acid and isobutyric acid, while the hydrolysate of demethylolivomycin B and demethylchromomycin A_3 showed the peak of acetic acid but not that of isobutyric acid. Thus, the isobutyryl group and/or the acetyl group is present in the demethyl-factors.

Fig. 2 shows that demethylolivomycin A has the three ^1H signals of the aromatic protons [*i.e.* δ 6.56 (1H, d, J=2.0 Hz), 6.64 (1H, d, J=2.0 Hz) and 6.78 (1H, s)]. Demethylolivomycin B also has the three corresponding signals [*i.e.* δ 6.56 (1H, d, J=2.0 Hz), 6.65 (1H, d, J=2.0 Hz) and 6.78 (1H, s)]. These results were consistent with the fact that these two factors possessed the olivomycinone moiety as an aglycone[6]. Each two protons among them had a typical *meta* coupling constant, J= 2.0 Hz. The spectrum of demethylchromomycin A_2 showed two signals of the aromatic protons at δ 6.66 (1H, s) and 6.76 (1H, s) and the spectrum of demethylchromomycin A_3 had them at δ 6.65 (1H, s) and 6.75 (1H, s), respectively. These results revealed that demethylchromomycins A_2 and A_3 had the chromomycinone moiety as an aglycone[7]. These data made it evident that the demethyl factors were new aureolic acid group antibiotics. The details of their structural elucidation are described in next papers[8,9].

Biological Properties

The biological properties of demethylolivomycins A, B and demethylchromomycins A_2, A_3 were compared with those of olivomycin A, chromomycin A_3 and mithramycin A[10].

Table 3 shows the anti-phage activities of these antibiotics. Demethylchromomycin A_3 and chromomycin A_3 displayed strong activities for RNA phage f_2. Demethylolivomycin A and olivomycin A were slightly less active than demethylchromomycin A_3 and chromomycin A_3. In the latter case, the growth of the host cell, *E. coli* 34S, was not as good as that with the former. Mithramycin A showed no activity for phage f_2.

Compared with chromomycin A_3, demethylolivomycin A, demethylchromomycin A_2 and A_3 were equivalent or a little less potent against the susceptible bacteria. Demethylolivomycin B was less active than chromomycin A_3. These demethyl factors were not as potent against Gram-negative bacteria, fungi and yeasts as chromomycin A_3 (Table 4).

Table 3. Comparison of anti-phage f_2 activity.

Antibiotic	Activity	
	Concentration	
	100 μg/ml	300 μg/ml
Chromomycin A_3	23.4 (0)	27.6 (15.3)
Olivomycin A	25.9 (18.5)	31.1 (23.4)
Mithramycin A	0 (11.5)	0 (17.4)
Demethylolivomycin A	27.7 (19.0)	30.6 (23.5)
Demethylchromomycin A_2	27.5 (19.9)	30.6 (24.5)
Demethylolivomycin B	20.5 (14.6)	25.0 (20.5)
Demethylchromomycin A_3	23.3 (0)	27.7 (16.5)

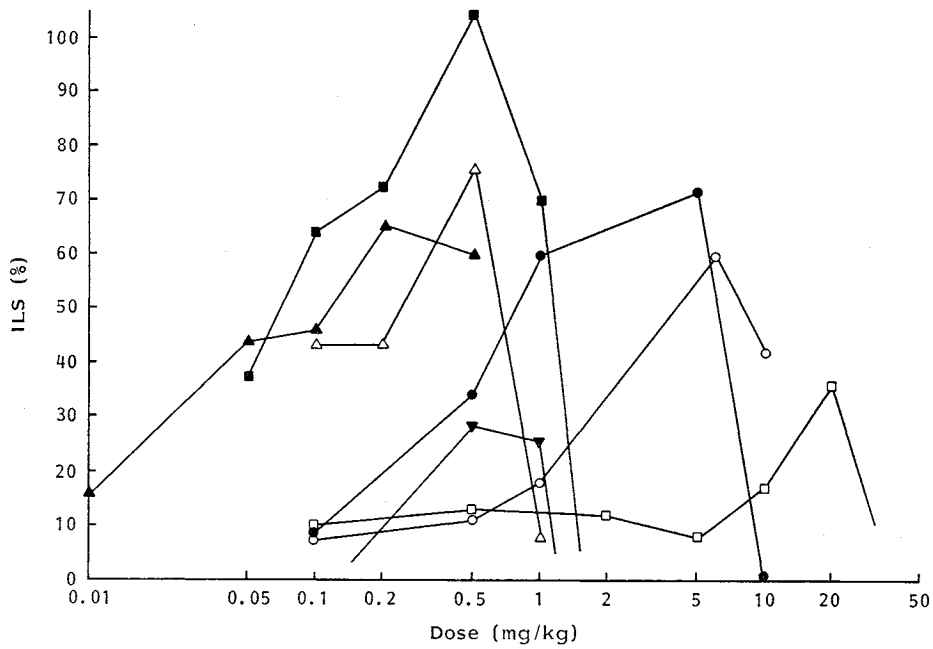
Paper disks (ϕ 6.0 mm) were used in this assay system.
Growth inhibition zone (ϕ mm) of *Escherichia coli* 34S is given in parenthesis.

Table 4. Antimicrobial activity.

Organisms	MIC (μg/ml)				
	Chromomycin A_3	Demethylolivomycin A	Demethylchromomycin A_2	Demethylolivomycin B	Demethylchromomycin A_3
Staphylococcus aureus FDA 209P JC-1	0.2	0.39	0.1	0.78	0.2
S. aureus Smith	0.78	1.56	0.2	3.13	0.2
S. aureus S-41	0.39	0.78	0.2	3.13	0.2
S. epidermidis ATCC 14990	0.78	0.78	0.2	3.13	0.39
Bacillus subtilis PCI 219	0.1	0.39	0.05	0.39	0.1
Streptococcus pyogenes C-203	0.2	0.2	0.2	0.78	0.1
S. faecalis CN 478	0.39	0.39	0.39	1.56	0.39
S. pneumoniae type 1	0.2	0.2	0.2	0.78	0.1
Escherichia coli NIHJ JC-2	≧100	≧100	≧100	≧100	≧100
Pseudomonas aeruginosa ATCC 9721	≧100	≧100	≧100	≧100	≧100
Candida albicans M-9	>100	>100	>100	>100	>100

Fig. 3. Activity against P388 leukemia.

● Olivomycin A, ○ demethylolivomycin A, □ demethylolivomycin B, ■ chromomycin A_3, ▲ demethylchromomycin A_2, △ demethylchromomycin A_3, ▼ mithramycin A.



Ascites tumor cells (10^6) were implanted ip into BDF_1 mouse. Single ip treatment began 24 hours after implantation. The parameter is the percentage increased life span (ILS). Results are averages of seven experiments.

Fig. 3 shows the antitumor activities of these antibiotics *in vivo* against P388 leukemia. Demethylolivomycin A and demethylchromomycins A_2 and A_3 significantly increased the mouse life span. However, chromomycin A_3 was the most potent against this experimental cancer. Mithramycin A showed markedly weak activity but strong toxicity in this system.

Discussion

Demethylolivomycins A, B and demethylchromomycins A_2, A_3 were isolated in an anti-RNA phage f_2 screening program using a mutant of *E. coli* KL 16, *E. coli* 34S, which is sensitive to many types of antibiotics. Demethylchromomycins A_2 and A_3 were almost as potent *in vitro* as chromomycin A_3 against Gram-positive bacteria.

HARUNA *et al.* found that chromomycin A_3 does not inhibit RNA-dependent RNA polymerase in the RNA phage $Q\beta$[11]. This has been explained as being due to the fact that the compound binds to only DNA and not to RNA. However, the four demethyl antibiotics and chromomycin A_3 exhibited significant anti-phage activity. It is interesting that mithramycin A has no anti-phage activity for RNA phage f_2 and weak activity for P388 leukemia. The more potent anti-phage f_2 factors, such as demethylchromomycins A_2, A_3 and demethylolivomycin A showed stronger anti-leukemic activity against P388 in mouse than the less potent ones such as demethylolivomycin B and mithramycin A.

Materials and Methods

Chemicals and Organisms

Actinomycin D, rifampicin, chromomycin A_3 and olivomycin A were purchased from Calbiochem-Behring Corporation, La Jolla, California, U.S.A. Mithramycin A was isolated and purified from the fermentation broth of *Streptomyces plicatus* ATCC 12957. IR spectrum (in KBr), UV spectrum (in MeOH), elemental analysis and mp data of these substances were identical with those reported for mithramycin A[10]. *E. coli* Hfr KL 16 and phage f_2 were kindly supplied by Professor H. SAITO, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.

Bacterial Strain

E. coli 34S was derived from *E. coli* KL 16 by the usual nitrosoguanidine treatment and subsequent replication of colonies on the assay medium, with and without 2 μg of rifampicin per ml. Among the colonies which failed to grow on the supplemented medium, a strain *E. coli* 34S was picked up and purified.

Analysis by Gas Liquid Chromatography

Each factor (8 mg) was saponified with 5% CH_3ONa in MeOH (2 ml) for 5 minutes at 60°C. The reaction mixture was acidified (pH 3.0) with 5% HCl - MeOH. The hydrolysate was extracted with an equal volume of ether and analyzed by gas liquid chromatography (20% PEG 20 M, 1 μm, at 140°C). Both demethylolivomycin A and demethylchromomycin A_2 had an equimolar amount of acetic acid (retention time, 6.02 minutes) and isobutyric acid (8.97 minutes). Both demethylolivomycin B and demethylchromomycin A_3 had only acetic acid (6.02 minutes).

Fermentation

One loop of a slant culture of *S. aburaviensis* PA-39856 was transferred to 100-ml of seed medium in a Sakaguchi flask and shaken for 2 days at 28°C on a reciprocal shaker at 180 rpm. The seed medium (pH 7.0) contained soluble starch 0.5%, glucose 0.5%, Polypeptone 0.5%, meat extract 0.5%, yeast extract 0.25% and NaCl 0.25%. The resulting seed culture (32 ml) was then transferred into a 2-liter Erlenmeyer flask containing 800 ml of the seed medium and the flask was shaken under the above conditions. After inoculation of 800 ml of the secondary culture into a 30-liter jar fermentor containing 20 liters of fermentation medium, fermentation was conducted at 28°C for 3 days with aeration at 20 liters per minute, back pressure at 0.3 kg/cm^2 and agitation at 200 rpm. The fermentation medium (pH 7.0) was composed of soybean meal 1.5%, corn steep liquor 0.5%, glucose 2.0%, glycerol 0.5%, NaCl 0.3% and $CaCO_3$ 0.3%.

Isolation and Purification

The broth was separated from mycelium by centrifugation. Four kg of NaCl was added to the broth which was adjusted to pH 3.0. The antibiotic complex was isolated from the supernatant and the mycelium by extraction with EtOAc and MeOH, respectively. The MeOH extract from the

mycelium was concentrated to an oily residue which was again extracted at pH 3.0 with EtOAc. Both EtOAc extracts were washed twice with a small volume of water and then separately concentrated to oily residues.

Each oily residue was dissolved in a small volume of methanol and the precipitate of the antibiotics was obtained by adding EtOAc and cyclohexane. Yields from the supernatant and the mycelium of 16.5 g and 4.8 g, respectively, were combined to obtain the partially purified antibiotic complex.

The complex was further separated into its subfraction by repeated chromatography on Silica gel (Kiesel gel 60 reinst, Merck) containing 10% of water with $CHCl_3$ - MeOH - H_2O - AcOH (3,000 : 150 : 10 : 3, changed to 2,000 : 150 : 10 : 2). The following eight subfractions were obtained and pooled. Fractions A and H were the first and last eluted from the column (2.3 g of B, 1.8 g of C, 4.2 g of F, 4.8 g of G, traces of A, D, E and H). Subfractions F and G were each loaded on a preparative HPLC column of Lichroprep RP-18 and eluted with acetonitrile - 30 mM tartrate buffer, pH 3.0 (50 : 45 and 45 : 55, respectively). The fractions giving a single peak were, separately collected, concentrated and washed with an excess volume of water. Each factor was finally purified by Sephadex LH-20 and precipitation from MeOH - EtOAc - cyclohexane. Demethylolivomycin A (1.3 g) and demethylchromomycin A_2 (1.7 g) were obtained from fraction F. Demethylolivomycin B (1.8 g) and demethylchromomycin A_3 (1.5 g) were obtained from fraction G. These factors were yellow-orange amorphous powders. Similarly olivomycin A (0.70 g) and chromomycin A_2 (1.0 g) were separated from subfraction B, and olivomycin B (0.44 g) and chromomycin A_3 (0.72 g) from subfraction C.

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