METHYLENOMYCIN A, AN ANTIBIOTIC WITH CHEMICALLY VERSATILE FUNCTIONS

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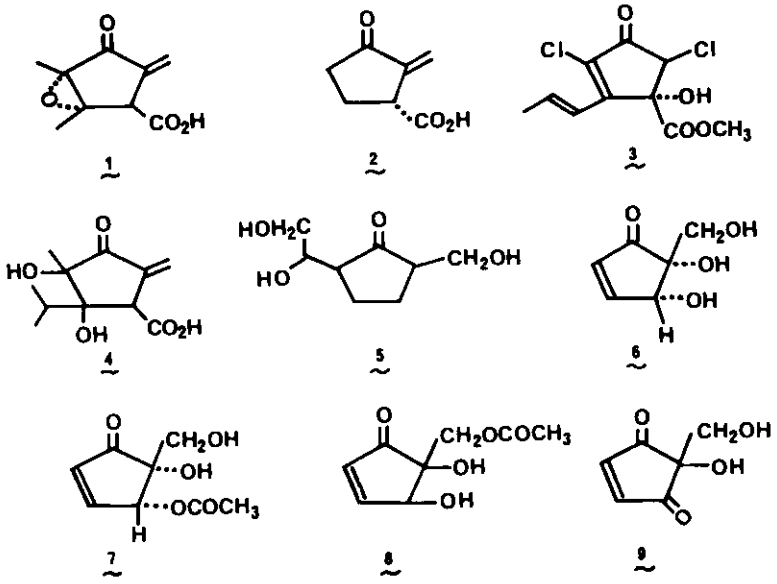
Introduction

Methylenomycin A (1) is an antibiotic obtained in the culture filtrate of a streptomycete identified as Streptomyces violaceus-ruber[1] (formerly the name of this species was described as S. violaceoruber.[2]). The chemical structure (1)[3] of the antibiotic consists of a compact molecule fused with several functions which are assumed to be related to its biological activity. The antibiotic was first discovered by its antibacterial activity.[1]

There exist several antibiotics with cyclopentanone nuclei similar to methylenomycin A. Sarkomycin (2),[4-9] isolated from the culture broth of S. erythrochromogenes by Umezawa et al., possesses antitumor as well as antibacterial activity. Methylenomycin A was primarily thought to have no antitumor activity because of its ineffectiveness to Ehrlich ascites carcinoma in mice. However, later it has been found to be active against Lewis lung carcinoma in mice.[10] Cryptosporiopsin (3),[11-13] produced by Sporornia affinis, Cryptosporiosis sp. and Batula alleghaniensis was isolated by McGahren et al. and is distinguished from other cyclopentanone antibiotics with respect to its fungal origin, existence of chlorine in its molecule and its antifungal activity. Although no antifungal activity was displayed by methylenomycin A itself, some derivatives with its activity have been synthesized from this antibiotic.[14] Xanthocidin (4)[15-16] isolated from the culture filtrate of Streptomyces sp. by Asahi et al. is also closely related to methylenomycin A in its chemical structure, but it has only limited activity against some genera of gram-negative bacteria, such as Xanthomonas oryzae and Escherichia coli. Vertimycin (5)[17] without α,β-unsaturated carbonyl group was isolated from the culture filtrate of Streptomyces



sp. JA 4498 by Straus and is active against gram-positive bacteria, some species of Nocardia and tumors. Pentenomycins I (6) and II (7) isolated from the culture filtrate of S. eurythermus by Umino et al.[18] have moderate antibacterial activity against limited strains of gram-negative bacteria and Mycobacterium tuberculosis H37Rv, and weakly gram-positive bacteria. C2554-I (8)[19] produced by S. lavenduligriseus is also closely related to pentenomycins in its structure. Recently, a new cyclopentane antibiotic G2201-C (9)[20] isolated from the culture filtrate of S. cattleya, thienamycin producing strain, was reported by Noble et al. It also exhibited moderate antimicrobial activity in common to this group of antibiotics, but showed comparatively high toxicity in mice (LD_{50}, 19mg/Kg, i.p.).

As mentioned above these cyclopentanone antibiotics were active against bacteria in general but also some against tumor and other against fungi. However, only sarkomycin was active in vivo and successfully applied to the patients suffered from tumors.

Chemical synthesis of methylenomycin A derivatives was carried out with the aim to strengthen its antibacterial activity and to expose its potential antifungal activity.

Total synthesis of methylenomycin A by chemical procedure was also succeeded by two groups, Hoffman La Roche[21] and Koreeda et al.[22]

One of the most impressive work concerning this antibiotic is the finding of

its plasmid-involved biosynthesis by Hopwood et al.[23-25] Methylenomycin A is the first and still a sole antibiotic whose biosynthesis was adequately documented to be coded by the genes on plasmid in the cells of producing organism.

The present article will provide the review on methylenomycin A from such various points of interest.

Basic studies

1. Production and isolation.[1]

Methylenomycin A was obtained from the culture filtrate of a streptomycete No. 2416 isolated from a soil sample collected at Sagamihara, Kanagawa Prefecture, Japan. The organism was identified as a strain of Streptomyces violaceus-ruber.

The antibiotic was produced in a medium composed of glycerol 2%, soy-bean meal 1%, corn-steep liquor 1%, KH_2PO_4 1% and $CaCO_3$ 0.3%. The pH of the medium was adjusted to 7.2 before sterilization. The potency of the culture broth was determined by cylinder-plate method on 0.5% peptone agar seeded with E. coli NIHJ JC-2 as the test organism. For the highest production of the antibiotic, a spore suspension of strain No. 2416 was used as a seed culture. The spore suspension was prepared by the following method. A two-liter Erlenmeyer flask containing 100g of rice, which had been immersed in water for 30 minutes and filtered to remove the excess of water, was autoclaved for 45 minutes at 120°C. An agar slant of the organism was suspended in sterilized water, inoculated onto this medium and incubated at 28°C under appropriately moistened atmosphere. After two weeks, it was suspended in 500 ml of sterilized water and transferred into 600-liter submerged tank containing 300 liters of the medium described above. Fermentation was carried out at 28°C for 48 to 60 hours under aeration of 300 liters per minute and agitation of 170 r.p.m..

Three hundred liters of the culture filtrate were adjusted to pH 2.0 with hydrochloric acid and extracted with an equal volume of ethyl acetate. The concentrated extracts were dissolved in 500 ml of chloroform and passed through 1 Kg of silica gel packed into a column with chloroform and eluted with 5.5 liters of the same solvent. The active fraction was concentrated in vacuo to 100 ml and 175 g of methylenomycin A crystallized upon addition of 500 ml of carbon tetrachloride. The crude crystals were filtered and recrystallized as

colorless needles (140 g) from a mixture of chloroform and carbon tetrachloride. The mother liquor obtained after filtration of the crude crystals of methylenomycin A was adsorbed on a column of silica gel (500 g) packed with benzene and eluted with the same solvent. Seven grams of crude methylenomycin B, co-produced in the culture broth, were thus obtained as slightly yellowish oil. The crude methylenomycin B was dissolved in a mixture of benzene and n-hexane (1:4) and was applied on a column of silica gel (350 g) packed with n-hexane and eluted with a mixture of benzene and n-hexane (1:1). Finally 5.3 g of purified methylenomycin B were obtained as neutral colorless oil.

2. Physico-chemical characterization.[1)]

Methylenomycin A is an acidic (pKa' 3.65), lipophilic, colorless crystalline substance: m.p. 115°C (decomp.), $[\alpha]_D^{20}$ +42.3° (c 1, in $CHCl_3$). It is slightly soluble in n-hexane, carbon tetrachloride and fairly soluble in benzene, chloroform ethyl acetate, acetone, methanol and water. The molecular weight was confirmed to be 182 by the parent peak in its mass spectrum. The elementary analysis and the molecular weight indicate that the molecular formula of methylenomycin A is $C_9H_{10}O_4$. The UV spectrum in methanol exhibited a maximum at 224nm (ε 6300). The IR spectrum indicated two characteristic absorption bands at 1740 and 1720cm^{-1} in the carbonyl region, and a band at 1650cm^{-1} due to unsaturated bond. The PMR spectrum, taken at 100 MHz in $CDCl_3$ using TMS as an internal standard, demonstrated all 10 protons as follows: $\delta^{CDCl_3}_{ppm}$ 9.95 (1H, s), 6.27 (1H,d, J=1.9Hz), 5.65 (1H,d,J=1.68Hz), 3.82 (1H,dd,J=1.9 and 1.68Hz), 1.58 (3H,s) and 1.48 (3H,s).

Methylenomycin B was obtained as a neutral colorless oily substance. It is slightly soluble in n-hexane, petroleum ether and fairly soluble in ether, benzene, chloroform, ethyl acetate, acetone and alcohols. The molecular weight of methylenomycin B was shown to be 138 from the parent peak in its mass spectrum. The elementary analysis and molecular weight indicate that the molecular formula is $C_8H_{10}O_2$. The UV spectrum of methylenomycin B in methanol exhibited a maximum at 240nm (ε 7650) and a shoulder at 270nm. The IR spectrum showed a characteristic absorption band at 1720cm^{-1} in the carbonyl region and another at 1650cm^{-1} due to an unsaturated bond. The PMR spectrum showed 10 protons as follows: $\delta^{CDCl_3}_{ppm}$ 6.03 (1H,d,J=1.45Hz), 5.32 (1H,d,J=1.68Hz), 3.08 (2H,dd,J=1.45 and 1.68 Hz), 2.08(3H,dd,J=0.92 and 1.1Hz) and 1.79 (3H,dd,J=0.92 and 1.1Hz).

3. Biological activities.

(1) Antimicrobial activities. The minimal inhibitory concentrations (MIC) of methylenomycins A and B against various microorganisms were determined by serial two-fold broth dilution and agar dilution methods. The results are presented in Table 1. Methylenomycin A was moderately active against gram-negative bacteria, especially, Proteus vulgaris and Proteus morganii at lower concentrations, and more weakly active against gram-positive bacteria, but inactive against mycoplasma, anaerobic gram-positive and gram-negative bacteria, fungi and yeast.

Table 1. Antimicrobial spectra of methylenomycins A and B.

Test organism	Broth dilution MIC (mcg/ml)		Agar dilution MIC (mcg/ml)	
	A	B	A	B
Staphylococcus aureus FDA 209P JC-1	50	50	200	100
Micrococcus luteus PCI 1001	12.5		25	50
Bacillus subtilis PCI 219			25	
Alcaligenes faecalis	0.78		100	100
Escherichia coli NIHJ JC-2	100	>100	200	200
E. coli CM,TCf	1.56		25	25
Proteus vulgaris 0X19	0.39	1.56	25	50
P. mirabilis	12.5		200	100
P. morganii	0.39	50		
P rettgeri	12.5		50	
Shigella flexneri 2a	25			50
Salmonella typhosa	400			
Klebsiella pneumoniae PCI 602	25	>100	200	400
Pseudomonas aeruginosa	50		50	
Mycobacterium smegmatis ATCC 607	>400			
Mycoplasma gallisepticum			>100	
M. mycoides subsp. mycoides			>100	
M. pulmonis			>100	
Bacteroides fragilis			>100	
Clostridium tetani			>100	
Aspergillus oryzae			>400	
Penicillium chrysogenum			>400	
Pyricularia oryzae			>400	
Trichophyton mentagrophytes			>400	
T. interdigitale			>400	
Candida albicans			>400	

Table 2. Effect of pH on antimicrobial activity.

Test organism	MIC (mcg/ml), Müller-Hinton Agar pH				
	5	6	7	8	9
Staphylococcus aureus FDA 209P JC-1	< 0.8	25	200	400	> 400
Micrococcus luteus PCI 1001	< 0.8	12.5	200	> 400	> 400
Alcaligenes faecalis	< 0.8	< 0.8	200	400	> 400
Escherichia coli NIHJ JC-2	6.25	100	400	> 400	> 400
E. coli CM,TCf	< 0.8	3.13	25	200	> 400
Klebsiella pneumoniae 805	25	200	> 400	> 400	> 400
Proteus morganii	< 0.8	12.5	200	> 400	> 400
P. vulgaris OX19	< 0.8	3.13	100	400	> 400
Shigella flexneri 2a	< 0.8	25	200	400	> 400

The antibacterial activity of the antibiotic, however, was dependent on the pH value of the medium tested as shown in Table 2. The antibacterial effect of methylenomycin A was stronger in the acidic range than in the neutral or alkaline pH range.

(2) Antitumor activities. The structure of methylenomycin A was similar to that of antitumor antibiotic sarkomycin (2). The antitumor activities of the antibiotic were examined in our laboratory and later in National Cancer Institute, U. S. A.. It was revealed that methylenomycin A was only active against Lewis lung carcinoma but inactive against all other tumors tested such as Ehrlich ascites carcinoma, L-1210 lymphoid leukemia, B16 melanocarcinoma and P388 lymphocytic leukemia.[10]

Five mice in each group were given subcutaneous inoculation of Lewis lung carcinoma cells Once daily treatment with methylenomycin A via intraperitoneal administration started 24 hours after inoculation, and treatments were continued for nine consecutive days. Elongation of a life span observed in the treated groups and per cent ratios of treated to non-treated (T/C %) were 117, 113, 129 and 107 at doses of 1.25, 2.5, 5.0 and 10 mg/Kg of methylenomycin A, respectively. The animals treated with dose of 5 mg/Kg of the antibiotic for nine days gave more than 125 %, a minimal value of T/C % for effectiveness.

(3) Others. Methylenomycin A was also screened for the other biological activities, such as antifungal activity against Saprolegnia sp. or Fusarium sp., antiviral activity, inhibitory activity of several enzyme systems and some pharmacological activities. It was not shown to possess any activity except an

inhibitory activity of 15-hydroxyprostaglandin dehydrogenase from lung of guinea pig.

The acute toxicity (LD_{50} in mice) of methylenomycin A was 75 mg/Kg intraperitoneally or 1500 mg/Kg orally.

4. Plasmid involvement in methylenomycin A synthesis

In Eubacteria, many characteristics are determined by extragenetic materials called "Plasmid". The well known example of the plasmid is R factor which is transferred among Enterobacteriaceae to endow the recipient cells with resistance to antibiotics. In streptomycetes there are indications that plasmid genes are involved in the production of certain antibiotics, but a direct evidence has been given to none of the cases except methylenomycin A.

The antibiotic isolated from a culture broth of S. coelicolor A3(2) by Wright was identified as methylenomycin A.[23] Much genetic work on streptomycetes has been conducted with strains identified as S. coelicolor but these strains including S. coelicolor A3(2) are described to be more related to S. violaceus-ruber, the original producer of methylenomycin A, than to S. coelicolor.[2]

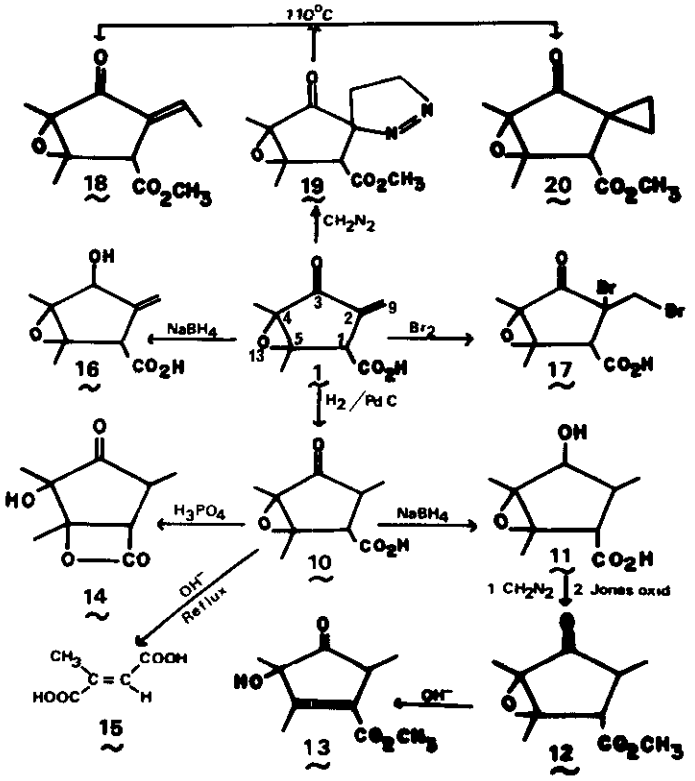
In S. coelicolor A3(2), the ability to produce methylenomycin A, and to be resistant to it, is completely correlated with the presence of SCP1 (S. coelicolor plasmid 1), and this is also true of strains of S. lividans and S. parvulus, which lack SCP1 but to which it can be transferred by mating. More direct evidence that genes coding for biosynthetic enzymes are SCP1 linked comes from the isolation of the mutants lacking SCP1 plasmid which fail to produce the antibiotic. Chemical studies on the biosynthetic pathway of methylenomycin A are so far not presented. However, an immediate precursor of the antibiotic is apparently a compound postulated on mass spectral evidence to be the "des-epoxy" equivalent of the antibiotic.[26] This compound is found, along with methylenomycin A, in SCP1-containing cultures of S. coelicolor A3(2), S. lividans and S. parvulus and is converted to methylenomycin A by a mutant of S. coelicolor A3(2) but not by SCP1-lacking strains. It will be very interesting to know the detailed genetic determination of methylenomycin A biosynthesis, in particular at what point the pathway diverges from primary metabolic reaction determined by chromosomal genes.

Structures of methylenomycins A and B

The structure of methylenomycin A (1) was studied by physico-chemical methods and finally established by X-ray crystallographic analysis as 2-methylenecyclopentane-3-one 4,5-epoxy-4,5-dimethyl-1-carboxylic acid.[3)]

Physico-chemical evidences of each degradation product as shown in chart 1 provided fruitful informations about the structural elucidation of methylenomycin A.

Chart 1



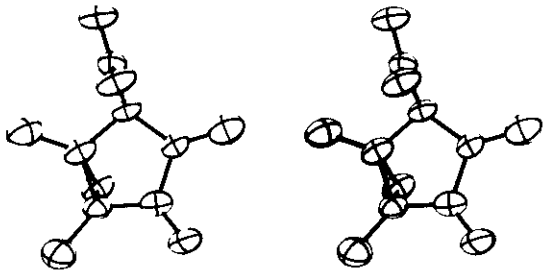
Although the chemical reactions did not give any direct evidence of epoxide function, formation of β-lactone compound 14 and α,β-unsaturated ester compound 13 clearly indicated the presence of a very unique structural character in *methylenomycin A*.

On the other hand, the relative stereochemistry and final structural establishment of methylenomycin A were achieved independently by X-ray crystallographic analysis. The crystal data of methylenomycin A are ; a=7.34, b=10.00,

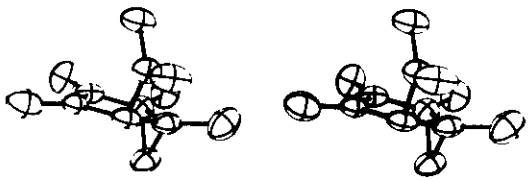
c=12.22 Å, space group $P2_12_12_1$, D obs.=1.34 g/cm^3, D cald.= 1.35 g/cm^3.

Figure 1. (a), (b) shows the stereographic views of methylenomycin A in which the relative configuration between the carboxylic acid group and the epoxide group is trans. Very short intermolecular atomic contact is observed at C9---O13, with a value of 3.01 Å. The dihedral angle between the five membered ring and the epoxide group is 86°.

Fig. 1 Stereodiagram of methylenomycin A

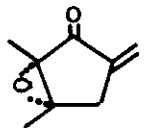


(a) Viewed along the a axis.



(b) Viewed along the c axis.

The structure of unstable congener metabolite methylenomycin B was assigned to 21 in 1973 by the present authors.[3] In 1976, however, the revised structure 22 was proposed with an evidence of total synthesis of methylenomycin B by Jernow et al.[27] The comparative studies and the identification of the synthesized methylenomycin B to the natural methylenomycin B have not been performed yet.



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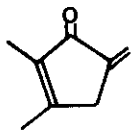
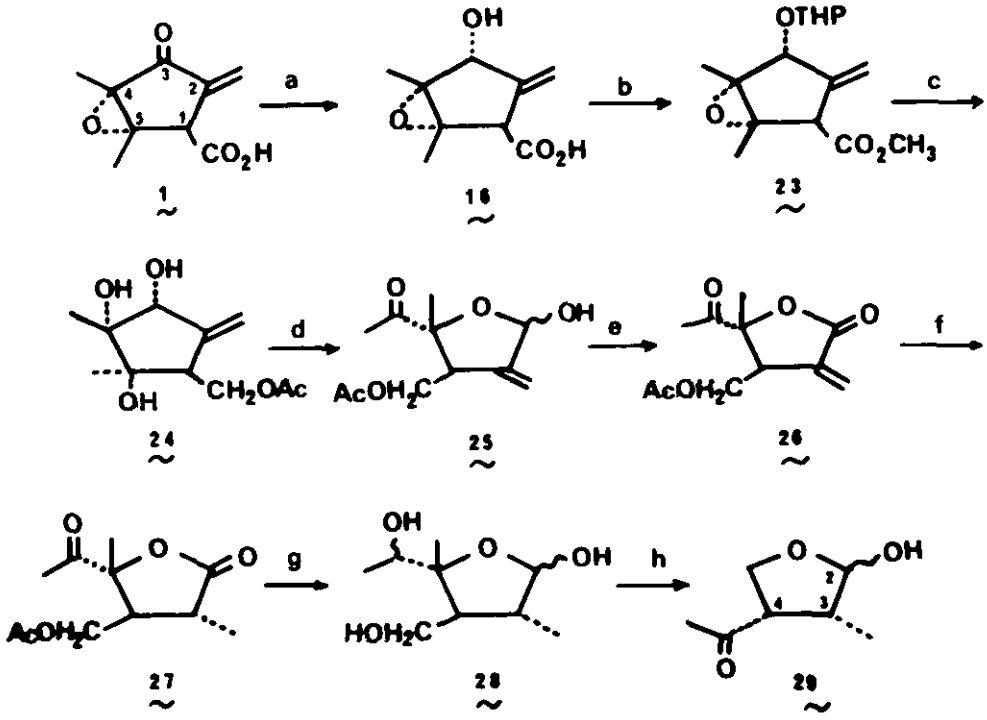


22

The absolute configuration of methylenomycin A was unequivocally established as shown 1 by Sakai et al., in 1979 with the most adroit way.[28] Methylenomycin A was transformed to natural (-)-botryodiplodin (29),[29] an antibiotic isolated from Botryodiplodia theobromae Pat. in eleven steps (Scheme 1).

Several reports on the synthesis of optically inactive (±)-botryodiplodin (29) have recently appeared,[30] but sythesis of natural (-)-botryodiplodin (29) has not been reported.

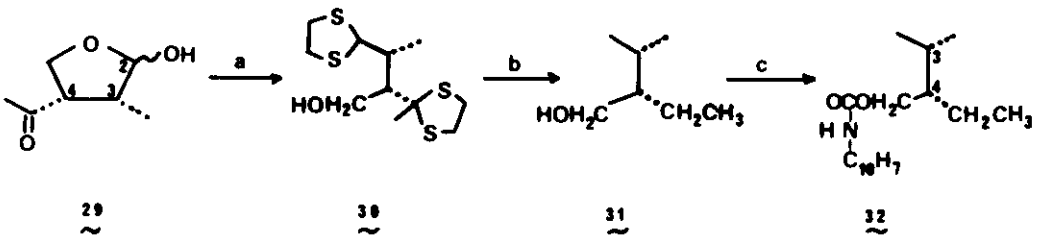
Scheme 1



Reagents: a) $NaBH_4$ b) CH_2N_2, 2H-Pyran /H^+ c) LAH, Ac_2O/Py, aq. AcOH
d) $NaIO_4$ e) Jones oxid. f) Pd-C/H_2 g) DIBAH h) $NaIO_4$

Natural (-)-botryodiplodin (29) derived from *methylenomycin* A gave α-naphthyl urethane derivative (32) via S(-)-2-ethyl-3-methylbutanol (31) whose absolute configuration was already known (Scheme 2).[31] Identity of the α-naphthylurethane to the authentic S(-)-α-naphthylurethane clearly revealed that the absolute configuration of the C3 and C4 positions in (-)-botryodiplodin is 3R, 4S and thus C1 position in(-)-methylenomycin A is 3S.

Scheme 2



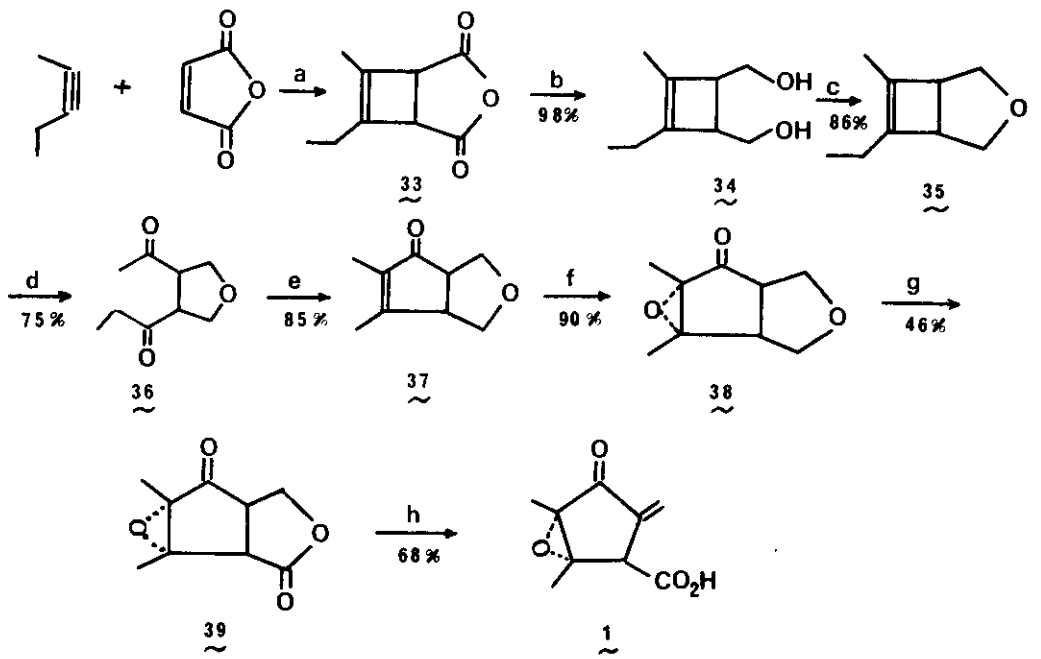
29 30 31 32

Reagents: a) $HS(CH_2)_2SH/BF_3$ b) Raney Ni/H_2 c) α-$C_{10}H_7NCO$

Total synthesis of methylenomycin A

The first total synthesis of methylenomycin A had been performed stereospecifically by Smith et al.[21] Those authors claim that their synthetic route outlined in Scheme 3 is direct and requires no chromatographic separation.

Scheme 3



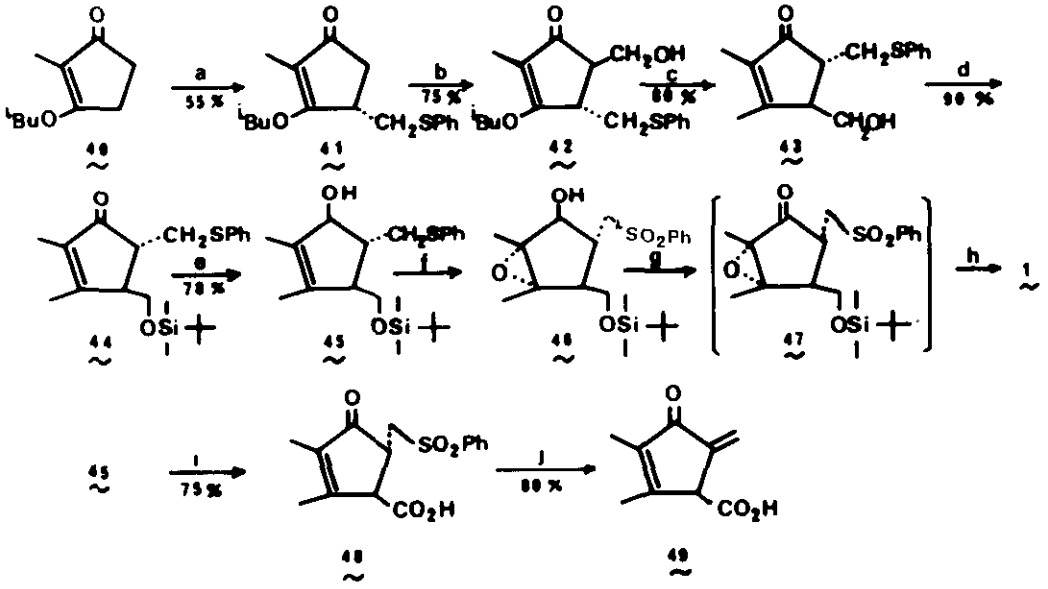
33 34 35 36 37 38 39 1

Reagents: a) hν b) LAH c) TsCl/Py d) O_3 e) base
f) H_2O_2/base g) $RhO_2/NaIO_4$ h) CH_3SLi/HMPA

The structure and stereochemistry of key intermediate 39 was confirmed by X-ray crystallography. The final step of this synthesis is the most crucial stage. Initially, a direct retrolactonization process induced via the ketone enol or enolate was envisioned, but all attempts to effect either acid or base promoted ring opening of 39 led only to recover starting material or to become complex mixtures. As a second approach, a nucleophilic cleavage of the alkyl-oxygen bond of the lactone by a nonbasic nucleophile was chosen. After an exhaustive examination on a wide variety of reaction conditions, success was finally achieved by the use of lithium methyl mercaptide.

The another total synthesis of methylenomycin A (1) and desepoxy-4,5-didehydromethylenomycin A (49) has been reported recently by Koreeda et al.[22] The synthetic route achieved by them is also highly stereo- and regio-selective (Scheme 4). The most fascinating point of the synthesis is the regioselective hydroxymethylation under basic condition to form the key intermediate 42. The stereoselective epoxidation of allylic alcohol 45 was performed with t-BuOOH in the presence of $VO(acac)_2$.

Scheme 4

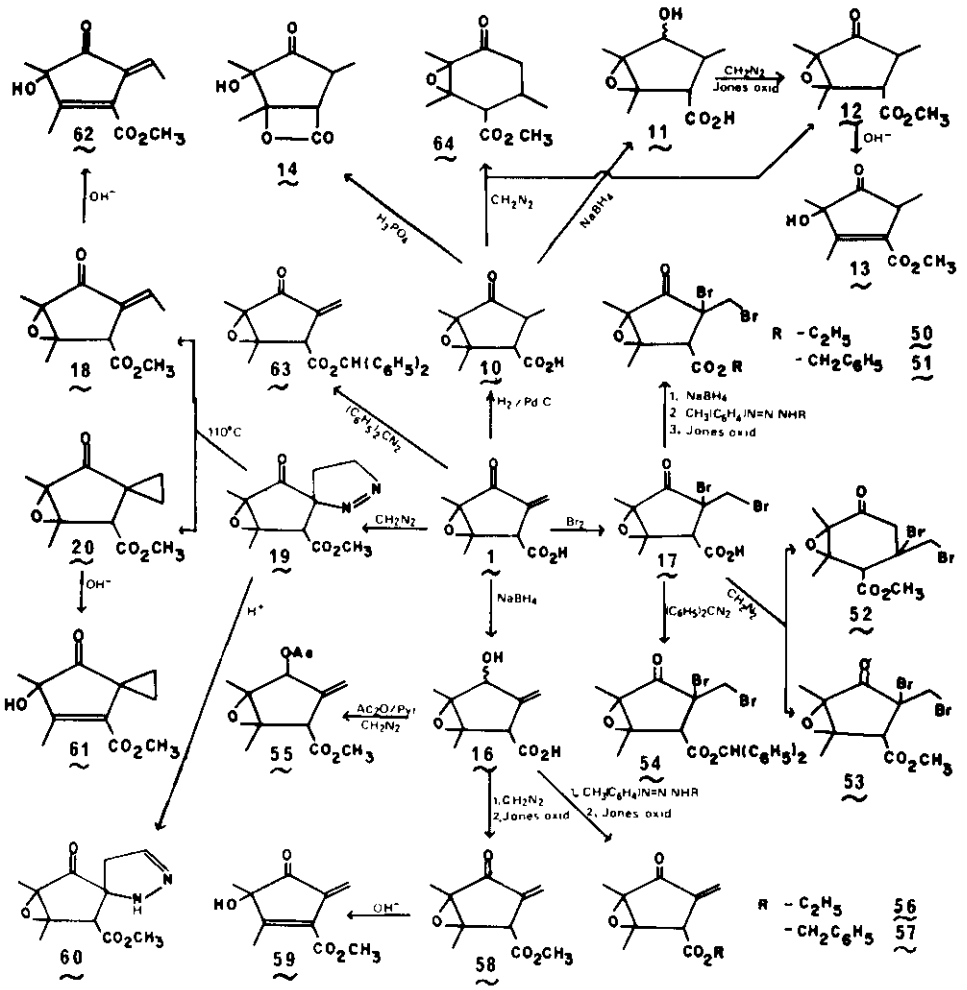


Reagents: a) $(Me_3Si)_2NLi/C_6H_5SCH_2I$ b) LAD/HCHO c) MeLi, H^+
d) t-Bu$(Me)_2$SiCl/Imidazole e) DIBAH f) $VO(acac)_2$/t-BuOOH
g) AcOH:THF:H_2O (3:1:1), Jones oxid. h) $NaHCO_3/\Delta$
i) Jones oxid. j) $NaHCO_3/\Delta$

Chemical modification of methylenomycin A

Methylenomycin A consists of a compact molecule fused with several functions which are assumed to be related to the biological activity. The preliminary chemical modification of methylenomycin A aiming to obtain stronger antibiotic activity as well as to define the basic structural requirements for methylenomycin activity was carried out by Haneishi et al.[14] Various kinds of derivatives (Chart 2) were synthesized.

Chart 2



The antibacterial and antifungal activities of those derivatives were tested (Table 3). Taking these results into consideration, it is obvious that the carbonyl group at C3 and the terminal methylene at C2 are essential for the activity of methylenomycin A, but an ethylidene group can be replaced by the terminal methylene. Therefore, it was concluded that the existence of an α,β - unsaturated carbonyl function was most important for the antibacterial activity of methylenomycins and that epoxide and cyclopentane ring were also essential for the activity.

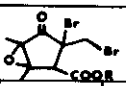
Table 3. Antimicrobial activities (MIC) of methylenomycin derivatives

Compound	MIC (mcg/ml)			Compound	MIC (mcg/ml)		
	Staphylococcus aureus FDA 209P JC-1	Proteus vulgaris OX19	Trichophyton interdigitale		Staphylococcus aureus FDA 209P JC-1	Proteus vulgaris OX19	Trichophyton interdigitale
1	200	25	>400	51	6.3	25	25
10	>400	>400	>400	52	>400	>400	400
11	>400	>400	>400	53	6.3	3.1	6.3
12	>400	>400	>400	54	6.3	25	25
13	>400	>400	>400	55	>400	>400	>400
14	>400	>400	>400	56	12.5	25	25
16	>400	>400	>400	57	12.5	50	>400
17	50	3.1	>400	58	12.5	6.3	6.3
18	200	50	50	59	>400	>400	>400
19	>400	>400	100	60	>400	>400	200
20	400	200	100	62	400	>400	>400
21	100	50	>400	63	6.3	50	>400
50	12.5	25	12.5	64	>400	>400	>400

All of the compounds with antifungal activity retained C1 carboxyl ester group. Among them, the methyl ester derivative was found to be most active, as shown in Table 3. Some differences in structure-activity relationship were observed between an antibacterial and antifungal activities of methylenomycin A derivatives. The antibacterial and antifungal activities of the ethyl ester derivatives were remarkably depressed comparing with those of methyl ester. The antifungal activity significantly decreased with an increase in the length of the carbon chain of aliphatic groups. Aromatic esters were all inactive except for dibromomethylenomycin A diphenyl ester (54). These facts clearly indicated that acyl groups useful for antifungal activity were restricted to aliphatic types,

and especially to methyl ester of methylenomycin A (58). The α,β-unsaturated carbonyl group was not critical for antifungal activity because C2-cyclopropane methyl ester derivative (20) possessed weak antifungal activity. The carbonyl group at C3 position was a basic requirement for the antifungal activity since the C3-hydroxy methyl ester derivatives were inactive against fungi. The epoxide and cyclopentane ring were also assumed to be essential factors for the antifungal activity because cyclohexane and epoxide-disrupted derivatives were all inactive.

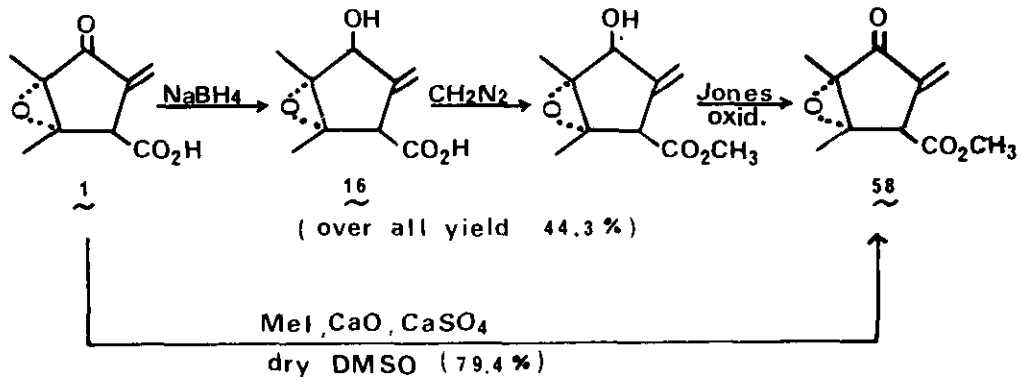
Table 4. Comparison of MIC and toxicity of selected derivatives of methylenomycin A and dibromomethylenomycin A.

Test organism	MIC (mcg/ml)					
						
	-H 1	$-CH_3$ 58	$-CH(C_6H_5)_2$ 63	-H 17	$-CH_3$ 53	$-CH(C_6H_5)_2$ 54
Staphylococcus aureus FDA 209P JC-1	200	12.5	6.3	50	6.3	6.3
S. aureus 56	400	50	25	100	25	100
Micrococcus luteus PCI 1001	25	25	6.3	25	6.3	6.3
Bacillus subtilis PCI 219	25	25	12.5	50	6.3	200
Alcaligenes faecalis	100	50	6.3	25	6.3	12.5
Escherichia coli NIHJ JC-2	200	50	>400	100	12.5	>400
E. coli K-12	400	200	>400	>400	50	>400
E. coli CM,TCf	25	12.5	50	25	3.1	400
Proteus vulgaris OX19	25	6.3	50	25	3.1	25
P. mirabilis	200	50	>400	100	25	>400
P. rettgeri	50	12.5	50	12.5	6.3	100
Klebsiella pneumoniae PCI 602	200	50	25	200	6.3	100
Pseudomonas aeruginosa	50	50	50	200	25	50
Mycobacterium smegmatis ATCC 607	>400	>400	>400	>400	>400	>400
Aspergillus oryzae	>400	400	>400	>400	400	25
Penicillium chrysogenum	>400	400	>400	>400	400	>400
Candida albicans	>400	100	>400	>400	50	>400
Trichophyton mentagrophytes	>400	12.5	>400	>400	25	50
T. interdigitale	>400	6.3	>400	>400	6.3	25
T. rubrum	>400	3.1	>400	>400	3.1	12.5
Epidermophyton floccosum	>400	3.1	>400	>400	6.3	12.5
Blastomyces brasiliensis	>400	3.1	>400	>400	6.3	25
Pyricularia oryzae	>400	50	>400	>400	50	200
Toxicity in mice (i.p.) LD_{50} (mg/kg)	75	250	>400	175	<25	>200

As shown in Table 4, the biological activities of derivatives of methylenomycin A (1) and dibromo-methylenomycin A (17) are compared. Methylenomycin A or dibromo-methylenomycin A was active against gram-positive and gram-negative bacteria, especially active against Proteus, but inactive against fungi. On the other hand, the methyl ester derivatives of these two compounds mentioned above indicated broadened and increased antibiotic activity. They were active against some species of fungi as well as gram-positive and gram-negative bacteria. The biological activities of their ethyl and benzyl esters were weaker than their parent compounds but had a similar tendency to their corresponding methyl and diphenyl ester derivatives. In the cases of diphenyl ester derivatives, antimicrobial activity increased against gram-positive bacteria, but decreased against gram-negative bacteria, and completely lost against fungi. The toxicities (LD_{50}) of those derivatives in mice were examined by intraperitoneal injection as shown in Table 4. The methyl ester of methylenomycin A was only slightly toxic and had stronger antibiotic activity. The methyl ester of dibromo-methylenomycin A (53) showed the strongest antibiotic activity, but also the highest toxicity among all the biologically active derivatives. The low toxicity of the diphenyl esters was assumed to depend on their solubilities because they were almost insoluble in many organic solvents or water.

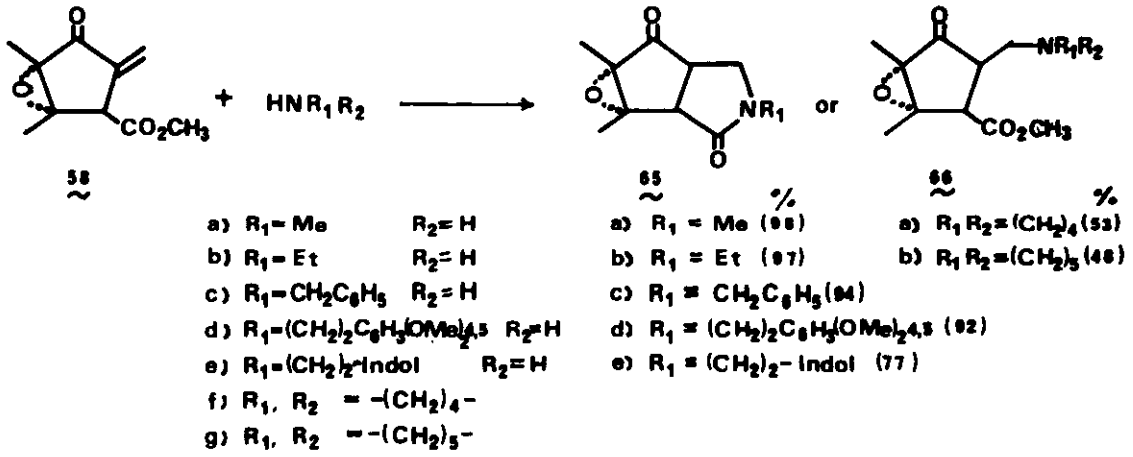
The other chemical reactivity of methylenomycin A methyl ester toward to a nucleophilic reagents had been studied by Kametani et al.[32]

Chart 3



They developed more effective esterification method than original procedure. Their esterification method of methylenomycin A consists with methyl iodide, calcium oxide and calcium sulfate in a dry dimethylsulfoxide, but any nucleophilic addition to the α,β-unsaturated carbonyl group was observed under this conditions (Chart 3).

Chart 4



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a) R_1 = Me R_2 = H
b) R_1 = Et R_2 = H
c) R_1 = $CH_2C_6H_5$ R_2 = H
d) R_1 = $(CH_2)_2C_6H_3(OMe)_{2,4,5}$ R_2 = H
e) R_1 = $(CH_2)_2$-indol R_2 = H
f) R_1, R_2 = $-(CH_2)_4-$
g) R_1, R_2 = $-(CH_2)_5-$

65

a) R_1 = Me (98 %)
b) R_1 = Et (97)
c) R_1 = $CH_2C_6H_5$ (94)
d) R_1 = $(CH_2)_2C_6H_3(OMe)_{2,4,5}$ (92)
e) R_1 = $(CH_2)_2$-indol (77)

66

a) R_1R_2 = $(CH_2)_4$ (53 %)
b) R_1R_2 = $(CH_2)_5$ (46)

Treatment of methylenomycin A methyl ester with various amines as nucleophile afforded lactam derivatives and Michael addition products in good yield (Chart 4). All of these derivative had been tested for antibacterial and antifungal activity as well as pharmacological screening, but none of them revealed any significant activity.

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