DIOXAMYCIN, A NEW BENZ[*a*]ANTHRAQUINONE ANTIBIOTIC

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A new antibiotic, dioxamycin (**1**) was isolated from the culture broth of the strain MH406-SF1, which was closely related to *Streptomyces xantholiticus*. This antibiotic was purified by countercurrent chromatography, column chromatography and preparative HPLC. The molecular formula of **1** was determined to be $C_{38}H_{40}O_{15}$ by HRFAB-MS. The structure was determined by spectral analysis of 2D NMR; ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY, long range ^{13}C-^{1}H COSY (HMBC) and NOESY. The antibiotic is active *in vitro* against Gram-positive bacteria and some tumor cells. Dioxamycin is a benz[*a*]anthraquinone antibiotic related to capoamycin.

In the course of our screening for antibiotics, a new member of the benz[*a*]anthraquinone antibiotic family, dioxamycin (**1**) was found in the fermentation broth of the strain MH406-SF1.

This paper describes the fermentation, isolation, physico-chemical properties, structure elucidation and biological properties of **1**.

Fermentation

The slant culture of the producing organism was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto-Soytone (Difco) 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, and $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The inoculated medium was incubated at 30°C for 5 days on a rotary shaker. Two ml of the seed culture was transferred to a series of 500-ml Erlenmeyer flasks each containing 110 ml of a producing medium. The producing medium was composed of glycerol 1.5%, soluble starch 1.5%, soybean meal 0.5%, fish meal 1.5%, and $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The fermentation was carried out at 27°C for 4 days on a rotary shaker.

Isolation

The culture broth was centrifuged to separate supernatant and mycelium cake. The supernatant (3.91 liters, pH 6.0) was adjusted to pH 5.0 with 2 N HCl and passed through a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited, 300 ml). After washing the column with water and 50% aqueous methanol, the adsorbed material was eluted with methanol. The fractions active against *Staphylococcus aureus* Smith were collected and concentrated *in vacuo* to an aqueous solution. The solution was adjusted to pH 2.0 with 2 N HCl and the antibiotic was extracted with ethyl acetate. The organic layer was evaporated to dryness to give a crude oily material (569 mg). The oily material was subjected to centrifugal partition chromatography (CPC). The chromatography was performed using a CPC apparatus model NMF (Sanki Engineering Limited) with a solvent system of hexane - methanol (2 : 1). The active fractions were collected and dried to give a dark red solid (237 mg). The solid was dissolved in methanol and applied to a Sephadex LH-20 column (610 ml) which was developed with the same solvent to give a red semi-pure solid (151 mg).

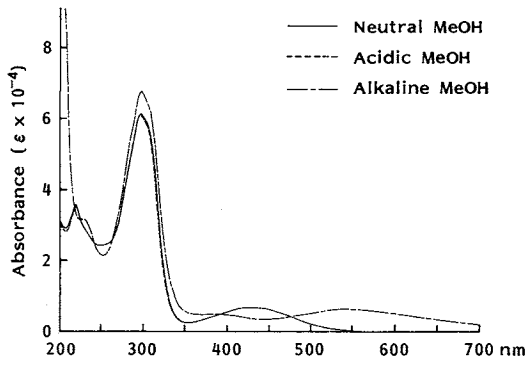
Table 1. Physico-chemical properties of dioxamycin.

MP (dec)	176~178°C	UV λ_{max}^{MeOH} nm (ε)	219 (36,042), 296 (62,060), 428 (6,837)
$[\alpha]_D^{24}$ (MeOH)	+49° (*c* 0.2)	$\lambda_{max}^{0.01\,N\,NaOH\text{-}MeOH}$ nm (ε)	230 (31,898), 296 (68,507), 391 (5,005), 548 (6,278)
Formula	$C_{38}H_{40}O_{15}$	IR ν_{max} (KBr) cm^{-1}	3430, 1720, 1640, 1620, 1260
FD-MS (positive) *m/z*	736 (M^+)	Rf value[a]	0.4
FAB-MS (positive) *m/z*	759 $(M+Na)^+$	HPLC (minutes)[b]	4.9
(negative) *m/z*	736 (M^-)		
HRFAB-MS			
Calcd for $C_{38}H_{40}O_{15}$:	736.2367		
Found:	736.2369 (M^-)		

[a] Silica gel TLC: $CHCl_3$ - MeOH - AcOH - H_2O, 60 : 10 : 1 : 1.
[b] Capcell Pak C_{18}: MeOH-1% citrate - 5% potassium acetate aq (70 : 30).

A portion of the solid thus obtained (80 mg) was further purified by preparative HPLC using a reverse phase silica gel column (Shiseido Capcell Pak C-18, 20 × 250 mm) with a solvent system of methanol - aqueous buffer solution containing citric acid 1.0% and potassium acetate 5.0% as a mobile phase (Table 1). The fractions containing dioxamycin (**1**) were concentrated and passed through MCI gel CHP-20P (Mitsubishi Chemical Industries Limited, 2 ml) to remove inorganic salts. The adsorbed material was eluted with methanol and the active fractions were collected and concentrated under reduced pressure. Finally, the concentrated solution was diluted with a small volume of water adjusted to pH 2.0 with 1 N HCl and extracted with ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield a pure orange powder of **1** (49.4 mg).

Fig. 1. UV-VIS spectrum of dioxamycin.



Physico-chemical Properties

The physico-chemical properties of dioxamycin (**1**) are summerized in Table 1. The substance is an orange amorphous powder. It is soluble in methanol, ethanol, and dimethyl sulfoxide, slightly soluble in $CHCl_3$, and insoluble in hexane and water. Dioxamycin gave positive color reactions to sulfuric acid and 2,4-dinitrophenylhydrazine but negative to ninhydrin and Rydon-Smith.

The molecular formula was established to be $C_{38}H_{40}O_{15}$ on the basis of HRFAB-MS (*m/z* 736.2369, M^-). It exhibited characteristic UV spectra, shown in Fig. 1, with maxima at 219 (ε 36,042), 296 (ε 62,060) and 428 nm (ε 6,837) in methanol and 296 (ε 68,507), 391 (ε 5,005), and 548 nm (ε 6,278) in alkaline methanol. The IR spectrum of **1** is shown in Fig. 2. All these physico-chemical properties suggest that **1** is a new antibiotic.

Structure Elucidation

The FAB-MS of dioxamycin (**1**) revealed the molecular ion peak at *m/z* 736 (M^-, negative ion mode) and *m/z* 759 ($(M+Na)^+$, positive ion mode). FD-MS of **1** also revealed a molecular ion at *m/z* 736 (M^+, potitive ion mode). The molecular formula was established as $C_{38}H_{40}O_{15}$ by HRFAB-MS (negative ion mode, Calcd: 736.2367, Found: 736.2369) and NMR studies. The UV-VIS spectrum of **1** suggested that **1** is a benz[*a*]anthraquinone antibiotic related to saquayamycin[1] or sakyomicin[2]. The IR spectrum of **1**

Fig. 2. IR spectrum of dioxamycin (KBr).

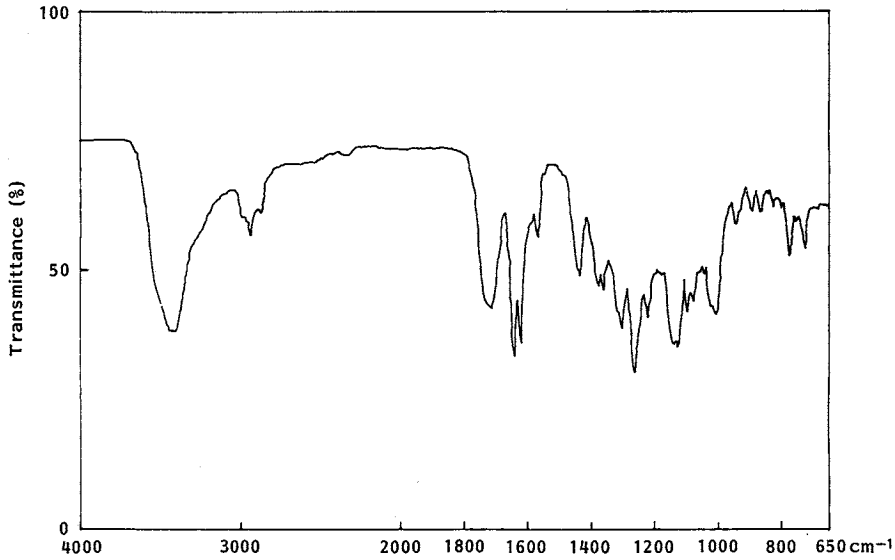
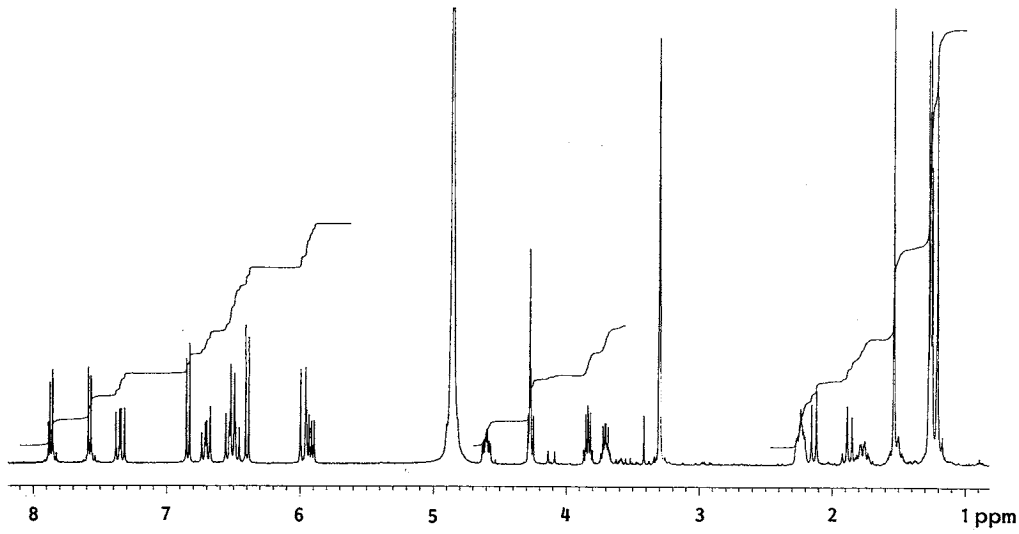


Fig. 3. ^{1}H NMR spectrum of dioxamycin (400 MHz, MeOH-d_4).



contained significant bands attributed to hydrogen bonded OH (3430 cm^{-1}), ester carbonyl (1720 cm^{-1}) and quinone carbonyl (1640 cm^{-1}).

Fig. 3 shows the ^{1}H NMR spectrum of **1** in CD_3OD. Characteristic signals at δ 7.87 (d, J=7.8 Hz, 11-H), 7.58 (d, J=7.8 Hz, 10-H), 6.84 (d, J=9.8 Hz, 6-H), 6.40 (d, J=9.8 Hz, 5-H) as well as 12.28 (not shown, br, 8-OH) in $CDCl_3$ were attributable to the chromophore of **1** and a 2,3-dideoxysugar moiety was suggested by ^{1}H-^{1}H COSY. This partial structure was similar to the published NMR data of saquayamycin except for the D-ring moiety. Chromophore and sugar moiety relationships of **1** were confirmed by ^{13}C-^{1}H COSY and long range ^{13}C-^{1}H COSY (HMBC)[3,4] as illustrated in Fig. 4. The result showed that 2-H (δ 4.28, s) of the D-ring of the chromophore in **1** originated from a hydroxymethine moiety of the same type

Fig. 4. HMBC data for the chromophore and the sugar moiety of dioxamycin.

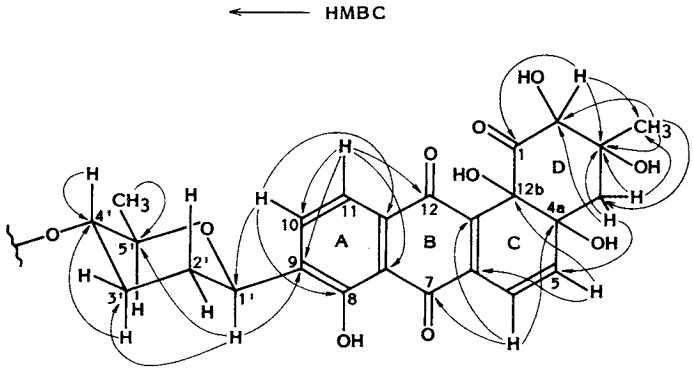
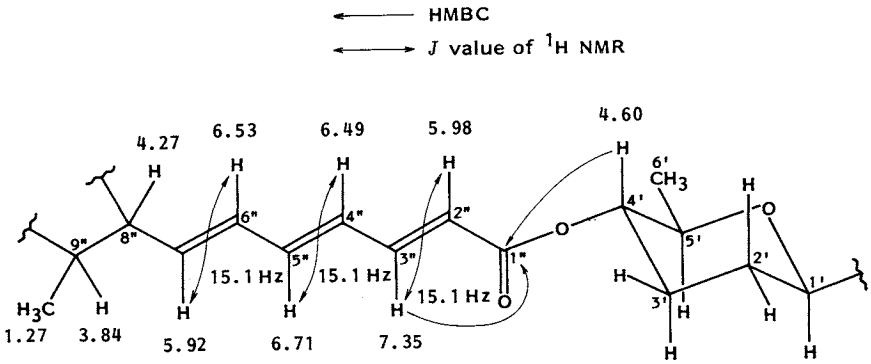


Fig. 5. Triene moiety and its connectivity to the sugar moiety as demonstrated by ^{1}H-^{1}H COSY and HMBC.



as is found in sakyomicin B (Fig. 4).

The sugar moiety was connected to C-9 of chromophore through a C-glycosidic linkage as revealed by the chemical shift of C-1′ at δ 74.5. In the HMBC spectrum, 1′-H correlated with C-9 and 10-H with C-1′.

Considering other parts of this substance, the triene moiety was assigned through the ^{1}H NMR and ^{1}H-^{1}H COSY spectra represented in Fig. 5. This geometry of the olefinic linkages was all *trans* from the J value (15.1 Hz) in the ^{1}H NMR. The chemical shift of 2″-H and C-2″ (δ_H 5.98 d, δ_C 122.4) suggested that it is connected to a carbonyl carbon.

Connectivity of the triene moiety to the sugar moiety was established by HMBC spectroscopy: cross peaks were observed between 4′-H and C-1″ and between 2″-H and 3″-H and C-1″. This triene moiety also assigned to be linked to C-4′ of sugar moiety by an ester bond because of the chemical shifts of C-1″ (δ 167.8) and 4′-H (δ 4.60) and a characteristic absorption peak in the IR spectrum (1720 cm^{-1}).

Upon treatment with 0.2 N HCl-MeOH for an hour at room temperature, **1** gave a mono methyl ester (**2**). Only C-12″ of **2** resonated at higher field shift (δ 170.7) and a methyl signal appeared at δ 52.5 indicating the presence of a $COOCH_3$ group.

The remaining part of the structure of **1**, which represents the most novel features of this antibiotic, is one methyl group (δ_H 1.54, δ_C 23.4, 13″-CH_3), one COOH group (δ_C 173.9, 12″-C) and one quaternary carbon (δ_C 106.3, 11″-C). The presence of a 2,4-methyl 1,3-dioxolan-2-carboxylic acid moiety is consistent with these chemical shifts and multiplicities[5]. This moiety is further supported by the HMBC spectrum in which

cross peaks from 13″-H to C-11″ and C-12″ and NOE's were observed linking 13″-H, 9″-H, 10″-H and 9″-H, 7″-H and 8″-H, respectively (Fig. 6). The planar structure of dioxamycin (**1**) was determined to be as shown in Fig. 7. Table 2 shows the assignments of ^{1}H and ^{13}C signals of **1**.

NOESY experiments on **1** led to a assignment of the relative configuration of the chromophore and sugar moiety as is shown in Fig. 8. The methyl proton (13-H) gave NOE correlation peaks with both 4-H (ax, eq) and 2-H; 4-H (ax) correlated with 2-H; 4-H (eq) correlated with 5-H, respectively. These relationships were also observed by the difference NOE experiments. The results suggest that the C-D ring conformation of the chromo-

Fig. 6. HMBC and NOE data for the dioxolane and the triene moieties in dioxamycin.

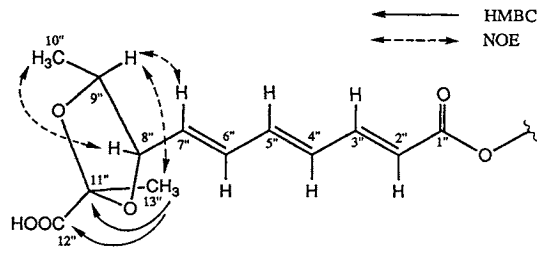


Fig. 7. Planar structure of dioxamycin.

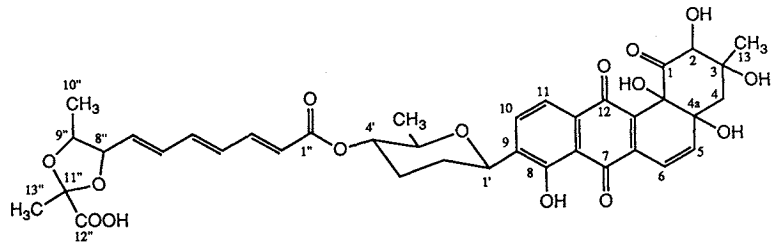
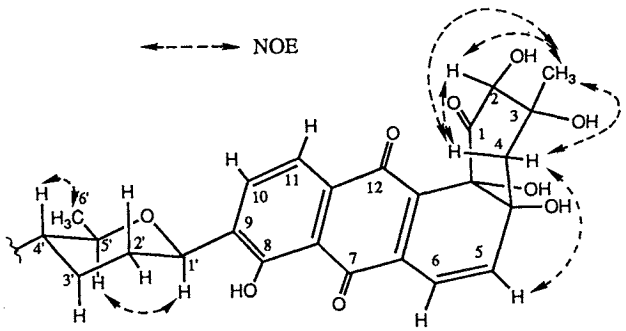


Table 2. ^{13}C and ^{1}H NMR chemical shifts of dioxamycin in CD_3OD.

Position	δ_C (ppm)	δ_H (ppm)	Assignment	Position	δ_C (ppm)	δ_H (ppm)	Assignment
1	206.4		C=O	5′	77.5	3.70 (dq, J=6.4, 9.3 Hz)	CH(O)
2	82.8	4.28 (s)	CH(OH)				
3	77.0		C(OH)	6′	18.7	1.25 (d, J=6.4 Hz)	CH_3
4	45.5	1.87 (d, J=14.7 Hz), 2.14 (d, J=14.7 Hz)	CH_2	1″	167.8		C=O(O)
				2″	122.4	5.98 (d, J=15.1 Hz)	=CH
4a	77.4		C(OH)	3″	146.1	7.35 (dd, J=11.2, 15.1 Hz)	=CH
5	147.6	6.40 (d, J=9.8 Hz)	=CH				
6	117.6	6.84 (d, J=9.8 Hz)	=CH	4″	132.4	6.49 (dd, J=11.2, 15.1 Hz)	=CH
6a	139.4		=C				
7	189.7		C=O	5″	141.0	6.71 (dd, J=10.7, 15.1 Hz)	=CH
7a	115.3		=C				
8	158.9		=C(OH)	6″	134.6	6.53 (dd, J=10.7, 15.1 Hz)	=CH
9	139.7		=C				
10	134.5	7.87 (d, J=7.8 Hz)	=CH	7″	133.9	5.92 (dd, J=7.3, 15.1 Hz)	=CH
11	120.2	7.58 (d, J=7.8 Hz)	=CH				
11a	132.0		=C	8″	85.8	4.27 (dd, J=7.3, 8.3 Hz)	CH(O)
12	183.7		C=O				
12a	139.9		=C	9″	79.2	3.84 (dq, J=6.4, 8.3 Hz)	CH(O)
12b	78.3		C(OH)				
13	21.8	1.21 (s)	CH_3	10″	16.7	1.27 (d, J=6.4 Hz)	CH_3
1′	74.5	4.85[a]	CH(O)	11″	106.3		(O)C(O)
2′	32.5	1.52 (m), 2.25 (m)	CH_2	12″	173.9		C=O(O)
3′	30.6	1.77 (m), 2.23 (m)	CH_2	13″	23.4	1.54 (s)	CH_3
4′	74.7	4.60 (m)	CH(O)				

[a] This signal overlapped the solvent signal.

Fig. 8. NOESY results for dioxamycin.



phore of **1** is not the same type observed with sakyomicin B but is of the type of vineomycin A[6]. These inferences are supported by published data. NOE's are observed between 6'-H and 4'-H and between 5'-H and 1'-H. This result and *J* values of 1'-H and 5'-H revealed that the sugar moiety takes a C1 conformation. The planar structure of **1** is related to that of capoamycin[7,8], and the absolute stereochemistry of this antibiotic remains to be studied.

Table 3. Antimicrobial activities of dioxamycin.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	3.12
S. aureus Smith	6.25
Micrococcus luteus IFO 3333	6.25
M. luteus PCI 1001	3.12
Bacillus subtilis PCI 219	12.5
Escherichia coli NIHJ	50
E. coli K-12	>100
Salmonella typhi T-63	>50
Pseudomonas aeruginosa A3	>50
Serratia marcescens	>50
Mycobacterium smegmatis ATCC 607	>100

Biological Effects

Table 3 shows the antimicrobial activities of dioxamycin (**1**) when tested by agar dilution method. The antibiotic was active aginst Gram-positive bacteria mainly and did not inhibited Gram-negative bacteria. The IC_{50} values of **1** for inhibiting the growth of L1210, P388, IMC carcinoma, LX-1 and SC-6 cells were 2.7, 1.4, 6.0, 2.0 and 2.5 μg/ml, respectively. The acute toxicity (LD_{50}, intraperitoneal injection) of **1** in mice was 12.5~25.0 mg/kg.

Experimental

General

MP was determined on a Yamato Scientific Industry melting point apparatus. Optical rotation was measured with a Perkin-Elmer 241 polarimeter. IR spectra were recorded with a Hitachi 260-10 spectrometer. UV spectra were taken on a Hitachi U-3210 spectrometer. ^1H and ^{13}C NMR spectra were recorded in a Jeol GX-400 spectrometer at 400 MHz (^1H) and 100 MHz (^{13}C), respectively. Mass spectra were obtained with a Hitachi M-80H apparatus (FD-MS) and Jeol SX-102 apparatus (FAB-MS and HRFAB-MS).

Mass Spectrometry

The HRFAB-MS of **1** was obtained under the following conditions: ion acceleration voltage-10 kV, resolution power 10,000, xenon gas and *m*-nitrobenzylalcohol matrix. The exact mass number (M^-) of **1** was determined by the use of *m/z* 721.4221 and 765.4484 in polyethylene glycol 1000 as a reference substance. The observed negative ion at *m/z* 736.2369 supported the molecular formula, $C_{38}H_{40}O_{15}$.

NMR Spectrometry

A sample (11.0 mg) of **1** was dissolved in 0.55 ml of CD_3OD. The NOESY spectrum resulted from the following parameters: the spectrum width was 2,900.2 Hz in both dimensions, $2 \times 256 \times 1{,}024$ data matrix, 128 scans per t_1 value, 2.0 s recycle delay, 400 mseconds mixing time and unshifted sine-bell filtering in t_1 and t_2.

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