MUTATIONAL BIOSYNTHESIS OF BUTIROSIN ANALOGS

II. 3′,4′-DIDEOXY-6′-N-METHYLBUTIROSINS, NEW SEMISYNTHETIC AMINOGLYCOSIDES

KATSUO TAKEDA, AKIO KINUMAKI, HARUO HAYASAKA, TOUTARO YAMAGUCHI and YUKIO ITO

Microbiological Research Laboratory, Tanabe Seiyaku Co., Ltd.
Toda, Saitama, Japan

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A pair of new butirosin analogs was isolated from the fermentation broth obtained by cultivating a neamine-negative mutant of the butirosin-producing organism *Bacillus circulans* in the medium supplemented with 6′-N-methylgentamine C_{1a}. These antibiotics were characterized and elucidated as 3′,4′-dideoxy-6′-N-methylbutirosins A and B (DMB-A & DMB-B), by chemical and spectroscopic studies. DMB-A and DMB-B exhibited broad-spectrum antibacterial activities with *in vitro* potency similar to or slightly less than that for the butirosin A, with the exception of strains of *Pseudomonas aeruginosa* and *Serratia marcescens* against which they exhibited activities equal to or slightly greater than that for butirosin A. As expected, they exhibited stronger activities against butirosin-resistant organisms which contain acetylating enzymes AAC(6′)-I and AAC(6′)-IV, and phosphorylating enzyme APH(3′)-II. They were also active against some of the clinical isolates resistant to butirosins, dibekacin and/or gentamicin. The acute intravenous toxicity in mice of the DMB complex (B: 70~80%) was somewhat less than that of the butirosin A.

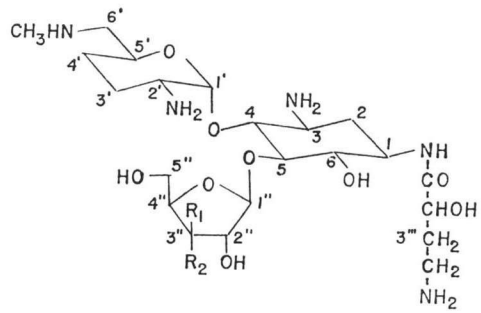
The previous paper[1] dealt with the production of several butirosin analogs from neamine analogs by neamine-negative mutants of the butirosin-producing organism *Bacillus circulans*.

In this paper, the isolation, structural elucidation and biological properties of a pair of new butirosin analogs, 3′,4′-dideoxy-6′-N-methylbutirosins A (DMB-A) and B (DMB-B) derived from 6′-N-methylgentamine C_{1a} are described.

Isolation

A complex of DMB-A and DMB-B (B: 70~80%) was isolated as the major product from the fermentation broth of a neamine-negative mutant of *B. circulans* harvested in the medium supplemented with 6′-N-methylgentamine C_{1a} as described in the experimental section. From the complex, DMB-A and DMB-B were separated and purified.

Fig. 1. Structures of DMB-A and DMB-B



DMB-A: R_1 = OH, R_2 = H

DMB-B: R_1 = H, R_2 = OH

Structures

The molecular compositions of DMB-A and DMB-B were both shown to be $C_{22}H_{43}N_5O_{10}$ by elemental analyses. ^{13}C-NMR spectrum of DMB-B (Table 3) indicated the presence of 22 carbon atoms, supporting the above compositions. The IR spectra (KBr) of DMB-A and DMB-B (Fig. 2)

Table 1. TLC of the acid hydrolyzates of DMB-A, DMB-B and related compounds

Reference compound	TLC(Rf)[a]		Detection of the constituents[b]			
	A	B	DMB-A	DMB-B	MGC	BTN-A
DOS	0.18	0.24	+[c]	+	+	+
AHBA	0.23	0.31	+	+	−	+
6-N-Methyl-purpurosamine C	0.30	0.43	+	+	+	−
Neosamine C	0.20	0.26	−	−	−	+
D-Xylose	0.68	0.64	+	−	−	+
D-Ribose	0.71	0.68	−	+	−	−

Abbreviations: DOS, 2-deoxystreptamine; AHBA, L-(−)-4-amino-2-hydroxybutyric acid; MGC, 6′-N-methylgentamine C_{1a} and BTN-A, butirosin A.

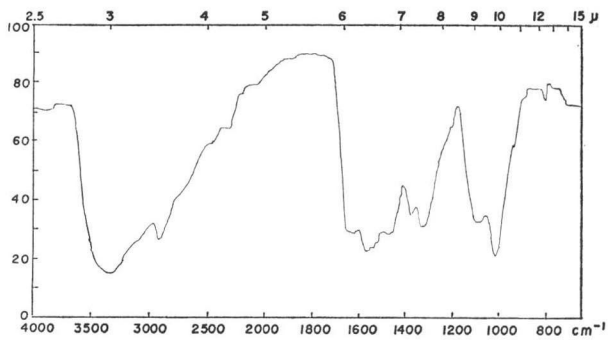
a) Cellulose plate (Merck, Art. 5552); A, AcOEt - pyridine - AcOH - H_2O (4 : 5 : 1 : 3, v/v) and B, *n*-PrOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 10, v/v)

b) Xylose and ribose were detected in the mild acid hydrolyzates (0.4 N HCl at 65°C for 3 hours), whereas the other constituents were detected in the strong acid hydrolyzates (6 N HCl at 100°C for 6 hours on DMB-A, DMB-B and MGC, and for 40 hours on BTN-A).

c) +: detected, −: not detected

were quite similar and showed absorptions at 1630 and 1560 cm^{-1}, indicating the presence of an amide group in their molecules.

Fig. 2. IR Spectrum of DMB-B (KBr)

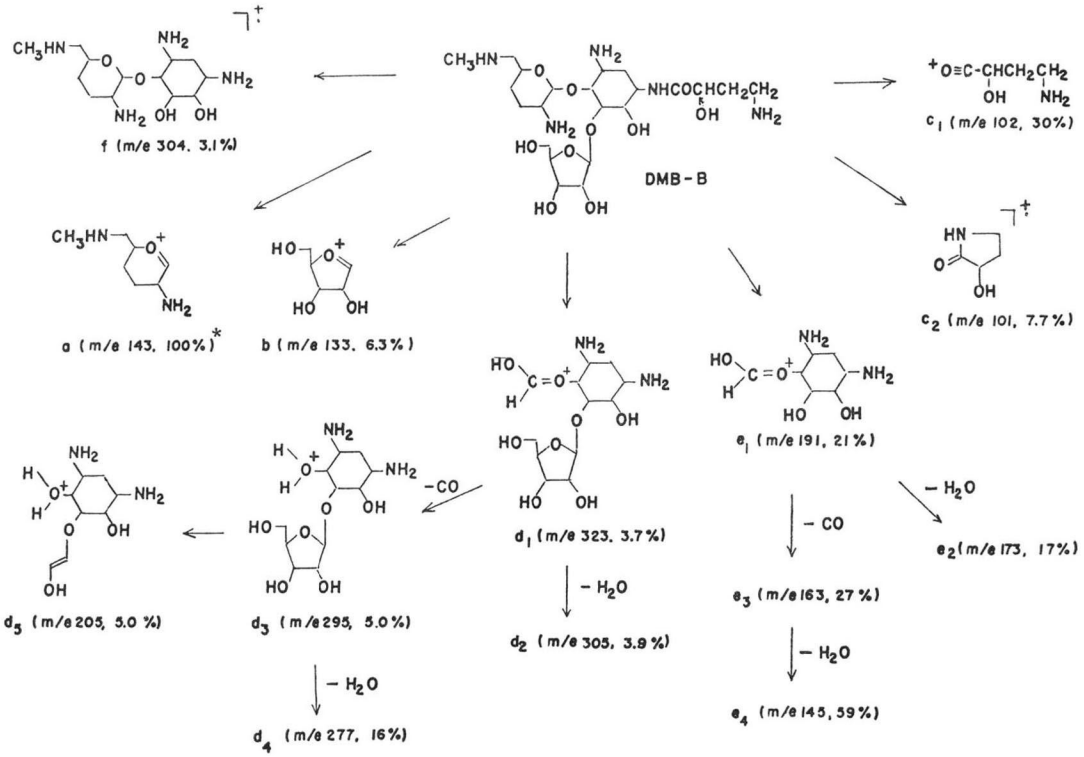


As shown in Table 1, acid hydrolysis of DMB-A and DMB-B with 6 N HCl (100°C for 6 hours) clearly indicated the presence of 4-amino-2-hydroxybutyric acid, 2-deoxystreptamine (DOS) and 6-N-methylpurpurosamine C as the common constituents. DOS and 6-N-methylpurpurosamine C were also found in the hydrolyzate of the precursor, 6′-N-methylgentamine C_{1a}. Furthermore, acid hydrolysis of DMB-A and DMB-B with 0.4 N HCl (65°C for 3 hours) revealed the presence of xylose and ribose, respectively. These data strongly suggested that DMB-A and DMB-B were isomers in the pentose unit.

Further evidence of the presence of the above constituents in DMB-A and DMB-B was obtained by mass spectrometry. The mass spectral pattern of DMB-A and DMB-B were identical except for minor differences in intensities. They gave no parent ions, but showed the prominent fragment ions expected for the structures as explained in Chart 1.

The spectra of DMB-A and DMB-B exhibited an ion **a** at *m/e* 143, indicating the presence of the 6-N-methylpurpurosamine C unit in the molecules[2]. Glycosyl cleavage of the pentose unit gave rise to an ion **b** at *m/e* 133. The characteristic ions $\mathbf{c}_1$ and $\mathbf{c}_2$ formed by cleavage of the 4-amino-2-hydroxybutyryl(AHB) unit were observed at *m/e* 102 and 101, respectively[3,4]. The ion $\mathbf{c}_2$ was predominant when measured at lower temperature (130 °C). Two series of characteristic ions were observed in DMB-A and DMB-B; ions $\mathbf{d}_1$, $\mathbf{d}_2$, $\mathbf{d}_3$, $\mathbf{d}_4$ and $\mathbf{d}_5$ at *m/e* 323, 305, 295, 277 and 205, respectively, indicated

Chart 1. Prominent mass spectral fragment ions of DMB-B



Mass measurement was carried out at 50 eV with a sample temperature of 190°C.
* The base peak was arbitrarily selected from the peaks above *m/e* 100.

the presence of the pentosyl DOS unit, and ions $\mathbf{e}_1$, $\mathbf{e}_2$, $\mathbf{e}_3$ and $\mathbf{e}_4$ at *m/e* 191, 173, 163, and 145, respectively, indicated the presence of the DOS unit[5]. The fragment corresponding to the 6′-N-methylgentamine C_{1a} was also present as an ion **f** at *m/e* 304.

The compositions of the above ions were determined on DMB-B by high resolution mass spectrometry as shown in Table 2.

The ^{1}H-NMR spectrum (100 MHz, D_2O) of DMB-B sulfate (Fig. 3) indicated the presence of one N-CH_3 group at δ 2.76 (3H, s) and eight methylene protons (m, H-2, 3′,4′ and H-3″) in the region δ 1.2~2.4. DMB-B showed two anomeric protons at δ 5.92 (1H, d, J=3.2 Hz, H-1′), indicating that the 6-N-methylpurpurosamine C unit existed in an α-pyranoside form as well as in the precursor, and at δ 5.35 (1H, d, J<1.0 Hz, H-1″), indicating that the D-ribose unit existed in a β-furanoside form[6]. The spectrum of DMB-A sulfate was almost the same as that of DMB-B sulfate, except that the doublet (J=3.2 Hz) anomeric proton of DMB-A appeared at a slightly lower field at δ 6.07.

In the ^{13}C-NMR spectrum of DMB-B, the signals of 22 carbons were compared with the constitutional compounds 6′-N-methylgentamine C_{1a}, D-ribofuranose[7], and 4-amino-2-hydroxybutyric acid as well as the related compounds ribostamycin[8] and butirosin A[9].

As shown in Table 3, comparison of the chemical shifts at pD *ca.* 5, where their amino groups were protonated, showed good additivity of each component in DMB-B except for the carbons involved in binding of these components. The chemical shifts of carbons binding the D-ribofuranosyl and AHB units to DOS in DMB-B well agreed with the corresponding carbons of ribostamycin and butirosin A.

Table 2. High resolution mass spectrometry of characteristic ions of DMB-B

Obsd.(*m/e*)	Composition	Error (mmu)
101.0485	$C_4H_7NO_2$	0.9
102.0549	$C_4H_8NO_2$	−0.4
133.0495	$C_5H_9O_4$	−0.4
143.1192	$C_7H_{15}N_2O$	0.8
145.0978	$C_6H_{13}N_2O_2$	0.1
163.1100	$C_6H_{15}N_2O_3$	1.8
173.0955	$C_7H_{13}N_2O_3$	3.0
191.1033	$C_7H_{15}N_2O_4$	0.3
205.1199	$C_8H_{17}N_2O_4$	1.1
277.1401	$C_{11}H_{21}N_2O_6$	0.2
295.1505	$C_{11}H_{23}N_2O_7$	0.1
304.2106	$C_{13}H_{28}N_4O_4$	−0.2
305.1326	$C_{12}H_{21}N_2O_7$	−2.0
323.1496	$C_{12}H_{23}N_2O_8$	4.3

Mass measurement was carried out at 70 eV with a sample temperature of 180°C.

Fig. 3. ^{1}H-NMR Spectrum (100 MHz·D_2O) of DMB-B sulfate

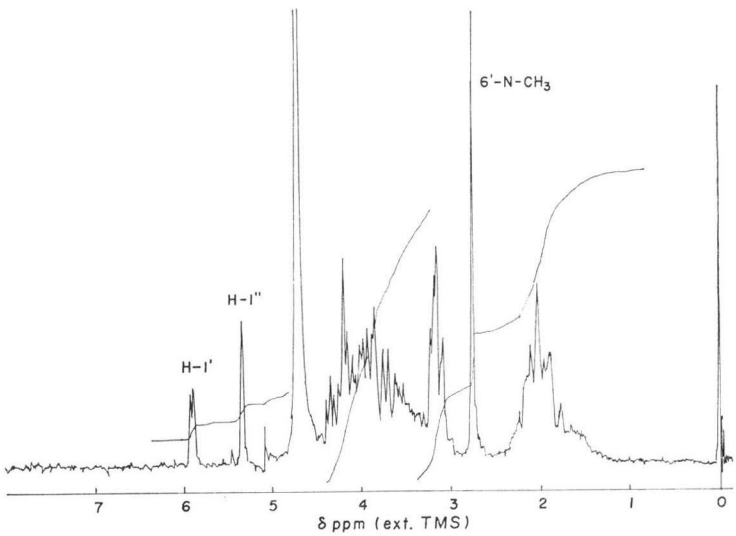


Table 3. ^{13}C-Chemical shifts of DMB-B compared to MGC, D-ribofuranose* (Rib), AHBA, ribostamycin (RM) and BTN-A

	DMB-B			MGC	Rib	AHBA	RM	BTN-A		
	pD 10.2	pD 5.1	$\Delta\delta^{\beta}$**	pD 4.9	pD 5.1	pD 5.1	pD 5.1	pD 10.1	pD 4.3	$\Delta\delta^{\beta}$**
C-1	50.6	50.3		51.0			51.1	50.5	50.2	
C-2	35.4	31.1	4.3	29.4			29.4	34.8	31.1	3.7
C-3	51.2	49.8		49.9			49.7	51.2	49.8	
C-4	81.9	76.5	5.4	76.4			76.5	82.5	76.5	6.0
C-5	86.3	86.9		78.0			86.0	86.0	87.2	
C-6	75.6	74.9		73.7			73.7	75.6	74.9	
C-1′	99.2	95.7	3.5	96.2			96.6	99.7	96.2	3.5
C-2′	55.0	53.3		53.3			54.8	56.6	54.7	
C-3′	25.2	21.6	3.6	21.6			70.6	74.2	69.2	5.0
C-4′	28.6	26.8		26.8			71.8	72.5	71.8	
C-5′	68.1	66.8	1.3	66.7			70.4	73.5	70.4	3.1
C-6′	50.6	49.8		49.9			41.4	42.6	41.4	
6′-NCH_3	34.8	34.8		34.7						
C-1″	110.3	111.5			102.0		111.4	111.4	113.1	
C-2″	76.2	76.5			76.4		76.5	81.7	81.9	
C-3″	70.8	70.8			71.5		69.2	75.6	75.6	
C-4″	83.4	83.3			83.5		83.7	83.1	84.2	
C-5″	62.9	62.2			63.6		61.9	61.6	61.8	
C-1‴	177.1	176.7				181.1		176.9	176.7	
C-2‴	70.8	70.2				71.5		70.9	70.8	
C-3‴	34.6	32.0	2.6			32.4		34.1	32.1	2.0
C-4‴	37.8	37.9				38.2		37.8	37.9	

* Signals attributable to D-ribofuranose were taken from the measured spectrum for mutarotated D-ribose according to the data reported by BREITMAIER and HOLLSTEIN[7].

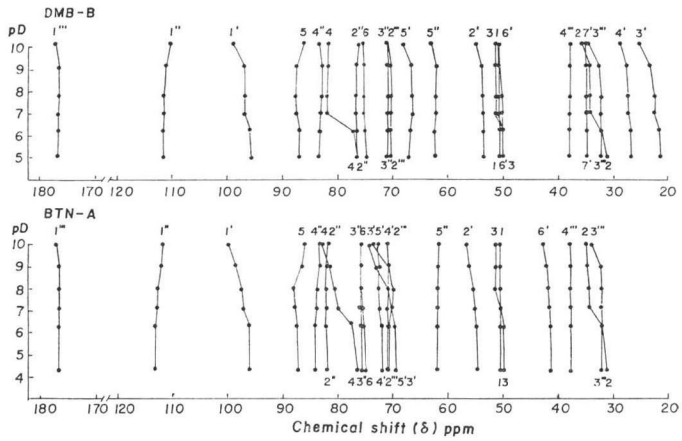
** $\Delta\delta^{\beta}=\delta$(pD 10.2)$-\delta$(pD 5.1) for DMB-B, and $\Delta\delta^{\beta}=\delta$(pD 10.1)$-\delta$(pD 4.3) for BTN-A.

The pH-titration study allowed assignment of carbon β to amino groups and the linkage position of the AHB unit in DMB-B. The pD-profiles of DMB-B are shown in Fig. 4 and the β-shift values ($\Delta\delta^{\beta}$) of DMB-B are given in Table 3 together with those of butirosin A.

DMB-B showed the expected β-shifts at 6 carbons, C-2, 4, 1′, 3′, 5′ and C-3‴. Among them, C-2, 4, 1′ and C-3′ showed relatively large $\Delta\delta^{\beta}$ ($\geqq$3.5 ppm) which are normal values reported for the other aminoglycoside antibiotics[8,10,11]. In contrast, the $\Delta\delta^{\beta}$ (1.3 ppm) at C-5′ in DMB-B was rather small as compared with that of butirosin A (3.1 ppm). This might reflect the presence of a disubstituted amine structure at C-6′ in DMB-B. Also, relatively small $\Delta\delta^{\beta}$ (2.6 ppm) at C-3‴ in DMB-B was observed as well as that of butirosin A (2.0 ppm).

Koch *et al.*[10] reported that the $\Delta\delta^{\beta}$ (8.1 ppm) at C-2 in DOS was nearly twice that of C-4 or C-6 (each 5.4 ppm) in view of the former carbon being placed between two amino groups. However, the $\Delta\delta^{\beta}$ (4.3 ppm) at C-2 in DMB-B was one-half of the expected value, suggesting that the linkage position of the AHB unit in DMB-B was an amino group either at C-1 or C-3 in DOS. Lemieux and Koto[12] reported that kanamycin derivative(BB-K8) having the AHB unit at C-1 showed β-shift at C-4 but not at C-6, whereas the derivative (BB-K29) having the AHB unit at C-3 gave β-shifts both at C-4 and C-6. On acidification, β-shift of DMB-B was observed at C-4 but not at C-6. Therefore, it could be concluded that the linkage of the AHB unit to DOS in DMB-B was an amino group at C-1.

Fig. 4. pD profiles of ^{13}C-chemical shifts of DMB-B and BTN-A



The chemical shifts of C-4, 5 and C-6 in many aminoglycoside antibiotics (free base) having 4,6-disubstituted DOS moiety have been observed at δ 88$\pm$2, 75$\pm$1 and 87$\pm$2, respectively[11~14]. On the other hand, in 4,5-disubstituted derivatives, the chemical shifts for C-4, 5 and C-6 have been reported to be δ 82$\pm$1, 85$\pm$2 and 77$\pm$2, respectively[8,12]. If DMB-B is a 4,5-diglycosylated derivative, the chemical shift at C-6 would have appeared in a region δ 69~77, because upfield shift (4$\pm$2 ppm)[8,12] at C-6 might be caused by the acyl substitution of the amino group at C-1. The chemical shifts at C-4, 5 and C-6 of DMB-B (pD 10.2) were observed at δ 81.9, 86.3 and 75.6, respectively, and hence it could be concluded that the linkage of the D-ribofuranosyl unit to DOS in DMB-B was at C-5. The resonance position of C-1″ (δ 111.5) of DMB-B corresponded well with that of ribostamycin (δ 111.4), indicating the β-linkage of the D-ribofuranoid in DMB-B. ^{13}C-NMR spectrum of butirosin A have been reported but complete assignment of the full carbons remained unsolved[9]. As shown in Table 3, all the chemical shifts of butirosin A could be assigned by the same technique described in DMB-B.

The linkage position of each component in DMB-B was finally substantiated by periodate oxidation study. The oxidation of DMB-B with excess periodate, followed by hydrolysis with 6 N HCl afforded a hydrolyzate in which DOS was detectable. In contrast, when alkaline-hydrolyzed DMB-B (acyl

free DMB-B) was subjected to oxidation and subsequent hydrolysis, DOS could not be detected in the hydrolyzate. These results were consistent with the presence of the acyl amide linkage at C-1 and the glycosidic linkages at C-4 and C-5 in DMB-B[15].

Based upon the above findings, the structures of DMB-A and DMB-B were elucidated as shown in Fig. 1.

Biological Properties

The *in vitro* antibacterial activities of DMB-A and DMB-B, and those of the reference antibiotics, butirosin A, kanamycin, dibekacin and gentamicin are shown in Table 4. DMB-A and DMB-B exhibited broad-spectrum activities with potency similar to or slightly less than that for the butirosin A against butirosin-sensitive bacteria, with exception of strains of *Pseudomonas aeruginosa* and *Serratia marcescens* against which they exhibited activities equal to or somewhat greater than that of the butirosin A. As expected, they exhibited stronger activities against butirosin-resistant organisms which contain aminoglycoside-inactivating enzymes such as 6′-N-acetylating enzymes AAC (6′)-I and AAC(6′)-IV, and 3′-O-phosphorylating enzyme APH(3′)-II[16]. They were also active against butirosin-resistant clinical isolates such as *P. aeruginosa* TU-412, *Proteus rettgeri* KU-23 and *Shigella sonnei* R, the latter two being resistant to dibekacin. They were active against gentamicin-dibekacin-resistant clinical isolate, *P. aeruginosa* 35-R, as was butirosin A. It is interesting to note here that DMB-A and DMB-B exhibited improved activities against *Providencia stuartii* #164 A 20894 which contains 2′-N-acetylating enzyme AAC(2′)-II.

The intravenous acute toxicity in mice of DMB complex (B: 70~80%) was somewhat less than

Table 4. *In vitro* antibacterial activities of DMB-A and DMB-B compared to BTN-A, kanamycin (KM), dibekacin (DKB) and gentamicin (GM)

Organism	Resistance mechanism[16]	Minimal inhibitory concentration (μg/ml)*					
		DMB-A	DMB-B	BTN-A	KM	DKB	GM
Staphylococcus aureus 209P, JC-1		0.4	0.4	0.2	0.2	0.05	0.01
〃 〃 A 20239	APH(3′)-I,II	6.3	6.3	12.5	>100	0.8	0.1
Bacillus subtilis ATCC 6633		1.6	1.6	0.8	0.8	0.1	0.05
Escherichia coli K-12		0.4	0.8	0.4	0.4	0.2	0.1
〃 〃 K-12 R-5	AAC(6′)-I	0.4	0.8	100	100	12.5	0.1
〃 〃 K-12 ML 1630	APH(3′)-I	1.6	1.6	1.6	>100	0.8	0.2
〃 〃 JR 35/C 600	APH(3′)-I	0.8	0.8	0.4	>100	0.4	0.1
〃 〃 JR 66/W 677	APH(3′)-II, ANT(2″)	0.8	1.6	>100	>100	25	12.5
〃 〃 A 20107	APH(3′)-II	1.6	1.6	50	>100	0.8	0.2
〃 〃 A 20732	ANT(2″)	0.8	0.8	0.8	100	100	25
〃 〃 A 20895	AAC(3)	1.6	1.6	0.8	0.8	0.8	12.5
Klebsiella pneumoniae Type 22#3038	APH(3′)-II, ANT(2″)	0.8	1.6	>100	>100	50	6.3
Shigella sonnei R		1.6	1.6	>100	>100	100	0.4
Proteus rettgeri KU-23		6.3	12.5	>100	1.6	12.5	3.1
Serratia marcescens OU-29		0.8	0.8	1.6	1.6	3.1	0.2
Providencia stuartii #164 A 20894	AAC(2′)-II	25	25	>100	3.1	25	6.3
Pseudomonas aeruginosa A_3		0.2	0.2	0.4	12.5	0.1	0.1
〃 〃 No. 12		0.2	0.2	0.2	12.5	0.1	0.1
〃 〃 4-R		1.6	1.6	1.6	>100	25	3.1
〃 〃 35-R		3.1	3.1	3.1	>100	50	25
〃 〃 TU-412		3.1	3.1	>100	>100	1.6	0.8
〃 〃 GN 315	AAC(6′)-IV	0.8	0.8	>100	>100	25	0.2

* Agar dilution method: Heart infusion agar (Eiken Chemical Co., Ltd.), 37°C for 18 hours.

that of the butirosin A. The intravenous maximum tolerated dose was determined to be 720 mg/kg for the sulfate of DMB complex and 600 mg/kg for the sulfate of butirosin A[17].

Experimental

Instrumental analyses

Melting points were uncorrected. IR spectra were determined on a Hitachi EPI-32 infrared spectrometer. ^{1}H-NMR spectra (100 MHz) were obtained in D_2O with a JEOL PS-100 spectrometer, using TMS as an external standard. ^{13}C-NMR (25.2 MHz) was measured in D_2O with a JEOL PS-100 spectrometer by FOURIER transform with a JEOL EC-100 computer and chemical shifts were reported in δ (ppm) downfield from TMS as an external standard. Low and high mass spectra were obtained on a Hitachi RMU-6M spectrometer and a Hitachi RMU-7M spectrometer, respectively. Optical rotations were measured with JASCO DIP-180 automatic polarimeter.

Organism and cultivation

Bacillus circulans MCRL 5003, a neamine-negative mutant, was used in this study. Fermentation was carried out as previously reported[1]. 6′-N-Methylgentamine C_{1a} was added at 100 μg/ml after 24 hours of fermentation.

6′-N-Methylgentamine C_{1a} was prepared from 6′-N-methylneamine by applying essentially the same method described by JIKIHARA *et al.*[18]

Isolation and separation of DMB-A and DMB-B

The unfiltered harvested broth (11 liters) was diluted with three volumes of water and stirred for 1 hour with Amberlite IRC-50 (NH_4^+, 400 ml). After washing well with water, the resin was packed into a column and eluted with 1.0 N NH_4OH. The active eluate (*ca.* 4 liters) was concentrated *in vacuo* and lyophilized to give a crude mixture (2.8 g) containing DMB-A and DMB-B. The crude mixture was dissolved in 200 ml of water. The solution was adjusted to pH 7.0 with 2 N H_2SO_4 and passed through a column of Amberlite CG-50 (HN_4^+, 20 ml). After washing with 200 ml of 0.2 N NH_4OH, the column was eluted with 0.4 N NH_4OH. The eluate was collected in portions of 35 ml. Tubes No. 6~24 gave a crude mixture containing DMB-A and DMB-B (530 mg, B: 70~80%), whereas the precursor was recovered from tubes No. 2~4. The crude mixture was dissolved in 10 ml of water and the solution was passed through a column of Amberlite CG-50 (NH_4^+, 60 ml). After washing with water, the column was eluted with 0.25 N NH_4OH. The eluate was collected in portions of 70 ml. Tubes No. 40~60 gave crude DMB-B (48 mg) and tubes No. 61~70 gave homogeneous DMB-B (125 mg). Whereas, tubes No. 71~80 gave a complex of DMB-A and DMB-B (99 mg, A : B=1 : 3) and tubes No. 81~121 gave another complex of DMB-A and DMB-B (123 mg, A : B=1 : 1). The homogeneous DMB-B was purified on a column of CM-Sephadex C-25 (NH_4^+, 4 ml). The column was washed with 100 ml of 0.1 N NH_4OH and DMB-B (106 mg) was eluted with 40 ml of 0.5 N NH_4OH. The purified DMB-B was further passed through a column of Dowex 1×2 (OH^-, 4 ml) and eluted with water. The active eluate was concentrated *in vacuo* and lyophilized to give 99 mg of pure DMB-B carbonate as white amorphous powder, m.p. 190~195°C (dec.); $[\alpha]_D^{25}$ +25.2° (*c* 0.5, H_2O).

Anal. Calcd. for $C_{22}H_{43}N_5O_{10}\cdot 2H_2CO_3\cdot 2H_2O$: C 41.32, H 7.37, N 10.04
Found: C 41.10, H 6.90, N 9.73

DMB-A was obtained as follows: The complex of DMB-A and DMB-B obtained from above tubes No. 81~121 (123 mg) was dissolved in 3 ml of water and the solution was passed through a column of Amberlite CG-50 (NH_4^+, 24 ml). After washing with water, the column was eluted stepwise with each 1 liter of 0.1 N, 0.15 N and 3 liters of 0.175 N NH_4OH. The eluate was collected in portions of 90 ml. Tubes No. 22~38 gave homogeneous DMB-B (43 mg) and tubes No. 39~50 gave a mixture of DMB-A and DMB-B (30 mg, A : B=1 : 1), whereas tubes No. 51~80 gave homogeneous DMB-A (44 mg). The homogeneous DMB-A was further purified by the procedure described for DMB-B and 32 mg of pure DMB-A carbonate was obtained as white amorphous powder, m.p. 175~180°C (dec.); $[\alpha]_D^{25}$ +20.7° (*c* 0.3, H_2O).

Anal. Calcd. for $C_{22}H_{43}N_5O_{10}\cdot 2H_2CO_3\cdot 2H_2O$: C 41.32, H 7.37, N 10.04
Found: C 41.08, H 6.98, N 10.01

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