MUTATIONAL BIOSYNTHESIS OF BUTIROSIN ANALOGS

III. 6′-N-METHYLBUTIROSINS AND 3′,4′-DIDEOXY-6′-C-METHYLBUTIROSINS, NEW SEMISYNTHETIC AMINOGLYCOSIDES

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Two pairs of butirosin analogs were isolated from the fermentation broths obtained by cultivating a neamine-negative mutant of the butirosin-producing organism *Bacillus circulans* in the medium supplemented with 6′-N-methylneamine and gentamine C_2, respectively. These antibiotics were characterized as 6′-N-methylbutirosins A and B (NMB-A & NMB-B), and 3′,4′-dideoxy-6′-C-methylbutirosins A and B (DCB-A & DCB-B), respectively, by chemical and spectroscopic studies. NMB-A and NMB-B exhibited broad-spectrum antibacterial activities with *in vitro* potency similar to or slightly less than that for butirosin A, but with greater activities against butirosin-resistant strain which contains acetylating enzyme AAC-(6′)-I. The activities of NMB-A and NMB-B against *Pseudomonas aeruginosa* strains were relatively weak. DCB-B had broad-spectrum activity, and exhibited greatly improved activity against butirosin-resistant organisms which contain acetylating enzymes AAC(6′)-I and AAC(6′)-IV, and phosphorylating enzyme APH(3′)-II. These new butirosin analogs were also active against some of the clinical isolates resistant to butirosins, dibekacin and/or gentamicin.

The previous papers[1,2] concerned the production of butirosin analogs from neamine analogs by neamine-negative mutants of the butirosin-producing organism *Bacillus circulans*, and the isolation and characterization of 3′,4′-dideoxy-6′-N-methylbutirosins A (DMB-A) and B (DMB-B) derived from 6′-N-methylgentamine C_{1a}.

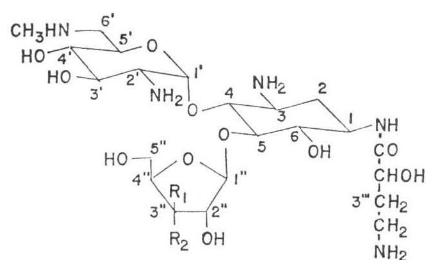
In this paper, the isolation, structural elucidation and antibacterial activities of two pairs of new butirosin analogs 6′-N-methylbutirosins A (NMB-A) and B (NMB-B), and 3′,4′-dideoxy-6′-C-methylbutirosins A (DCB-A) and B (DCB-B), derived from 6′-N-methylneamine and gentamine C_2, respectively, are described.

Isolation

A complex of NMB-A and NMB-B (A: 90~95%) 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-methylneamine as described in experimental part. From the complex, NMB-A and NMB-B were separated and purified. As a minor product, 1-N-(4-amino-2-hydroxybutyryl)-6′-N-methylneamine (AHB-6′-N-methylneamine) was also isolated from the same broth.

Similarly, a complex of DCB-A and DCB-B (B: 80~90%) was isolated from the fermentation broth of the above mutant harvested in the medium supplemented with gentamine C_2 as described in experimental part. From the complex, the major component, DCB-B, was separated and purified.

Fig. 1. Structures of butirosin analogs



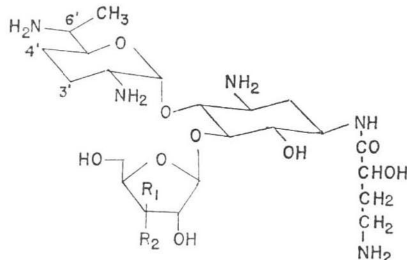


Fig. 2. IR spectrum of NMB-A (KBr)

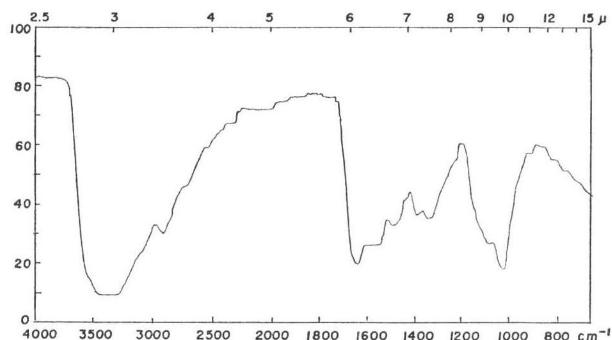
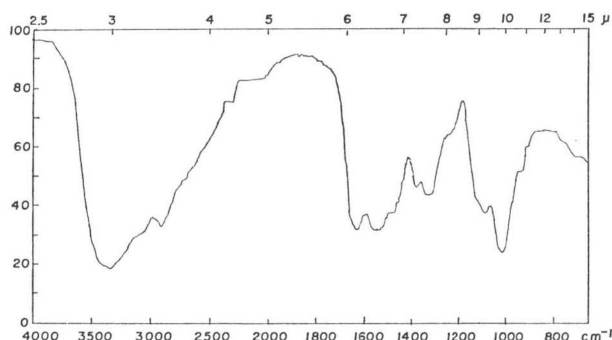


Fig. 3. IR spectrum of DCB-B (KBr)



Structures

The molecular compositions of NMB-A and NMB-B were both shown to be $C_{22}H_{43}N_5O_{12}$ by elemental analyses. ^{13}C-NMR spectrum of NMB-A (Table 2) indicated the presence of 22 carbon atoms, supporting the above compositions. The molecular compositions of DCB complex and DCB-B were equally shown to be $C_{22}H_{43}N_5O_{10}$ by elemental analyses. The IR spectra (KBr) of NMB-A (Fig. 2), NMB-B, DCB complex and DCB-B (Fig. 3) were quite similar to each other and showed absorptions at 1635 and 1560 cm^{-1} for NMB-A and NMB-B, and at 1630 and 1540 cm^{-1} for DCB complex and DCB-B, indicating the presence of an amide group in their molecules.

As shown in Table 1, acid hydrolysis of NMB-A and NMB-B with 6 N HCl (100°C for 40 hours) clearly indicated the presence of 4-amino-2-hydroxybutyric acid (AHBA), 2-deoxystreptamine (DOS) and 6-N-methylneosamine C, as common constituents. Whereas, acid hydrolysis of NMB-A and NMB-B with 0.4 N HCl (65°C for 3 hours) revealed the presence of xylose and ribose, respectively. These data strongly suggested that NMB-A and NMB-B were isomers in the pentose moiety.

Similarly, acid hydrolysis of DCB complex and DCB-B with 6 N HCl (100°C for 6 hours) showed the presence of the common constituents, AHBA, DOS and purpurosamine B. In contrast, acid hydrolysis of DCB complex with 0.4 N HCl (65°C for 3 hours) indicated the presence of two pentoses, ribose (rich) and xylose, whereas that of DCB-B indicated only ribose. These data strongly suggested that a minor component (DCB-A) in the DCB complex was an isomer of DCB-B in the pentose moiety.

Table 1. TLC of the acid hydrolyzates of NMB-A, NMB-B, DCB complex and DCB-B

Reference compounds	TLC(Rf)[a]		Detection of the constituents[b]				
	A	B	NMB-A	NMB-B	DCB complex	DCB-B	BTN-A
DOS	0.18	0.24	+[c]	+	+	+	+
AHBA	0.23	0.31	+	+	+	+	+
6-N-Methylneosamine C	0.27	0.33	+	+	−	−	−
Purpurosamine B	0.34	0.48	−	−	+	+	−
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 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 DCB complex and DCB-B, and for 40 hours on NMB-A, NMB-B and BTN-A).

[c] +: detected, −: not detected.

The mass spectra of NMB-A, NMB-B, DCB complex and DCB-B showed no parent ions, but gave common characteristic fragment ions arising from the pentosyl DOS (*m/e* 323, 305, 295, 277 and 205), DOS (*m/e* 191, 173, 163 and 145), pentose (*m/e* 133) and acyl units (*m/e* 102 and 101)[2]. The spectra of NMB-A and NMB-B gave a prominent ion **a** at *m/e* 175 corresponding to the 6-N-methylneosamine C unit, whereas that of DCB complex and DCB-B gave an ion **b** at *m/e* 143 for the purpurosamine B unit. Ions **a** (NMB-A) and **b** (DCB-B) were analyzed as *m/e* 175.1064 ($C_7H_{15}N_2O_3$, $\Delta = -1.7$ mmu) and *m/e* 143.1181 ($C_7H_{15}N_2O$, $\Delta = -0.1$ mmu), respectively, by high resolution mass spectrometry.

The ^{1}H-NMR spectra (100 MHz, D_2O) of NMB-A, NMB-B and DCB-B were consistent with the proposed structures as shown in Fig. 1. The spectrum of NMB-A sulfate (Fig. 4) indicated the presence of one N-CH_3 group at δ 2.80 (3H, s) and four methylene protons (m, H-2 and H-3‴) in the region δ 1.6~2.4. NMB-A showed two anomeric protons at δ 6.23 (1H, d, J=4.0 Hz, H-1′), indicating that the 6-N-methylneosamine C unit existed in an α-pyranoside form likewise in the precursor, and at δ 5.38 (1H, d, J<1.0 Hz, H-1″), indicating that the D-xylose unit existed in a β-furanoside form. The spectrum of NMB-B sulfate was almost the same as that of NMB-A sulfate, except that the doublet (J=4.0 Hz) anomeric proton of NMB-B appeared at a slightly higher field at δ 6.09.

The spectrum of DCB-B sulfate (Fig. 5) indicated the presence of one C-CH_3 group at δ 1.29 (3H, d, J=6.6 Hz) and eight methylene protons (m, H-2, 3′, 4′ and H-3‴) in the region δ 1.6~2.2. DCB-B showed two anomeric protons at δ 5.97 (1H, d, J=3.5 Hz, H-1′), indicating that the purpurosamine B unit is in an α-pyranoside form as well as in the precursor, and at δ 5.36 (1H, d, J<1.0 Hz, H-1″), indicating that the D-ribose unit existed in a β-furanoside form.

The linkage positions of the acyl and pentosyl units to DOS were confirmed by ^{13}C-NMR studies.

As shown in Table 2, there is an excellent agreement between the chemical shifts of the carbons of the DOS unit (C-1~C-6) in butirosin A and the corresponding carbons of NMB-A, supporting the 1-N-acylated and 4,5-diglycosylated structure for NMB-A. Inspection of the spectra arising from the

Fig. 4. ^{1}H-NMR spectrum (100 MHz, D_2O) of NMB-A sulfate

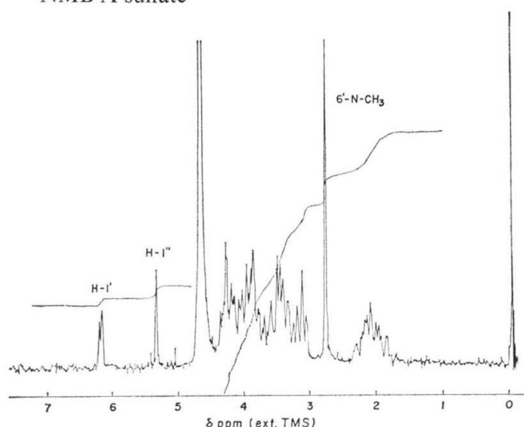
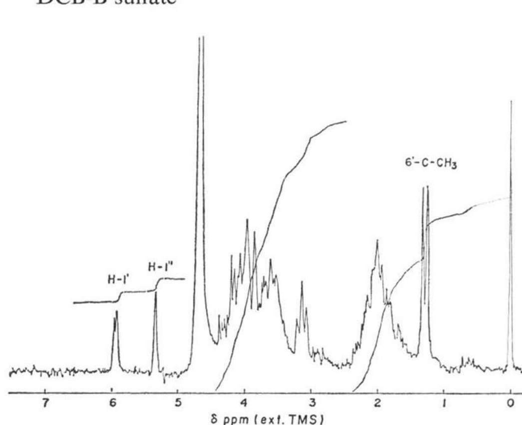


Fig. 5. ^{1}H-NMR spectrum (100 MHz, D_2O) of DCB-B sulfate



remaining carbons of NMB-A and butirosin A also revealed structural identity of NMB-A and butirosin A except for the presence of $N-CH_3$ carbon (δ 34.9 at pD 10.5) in NMB-A. In comparison of NMB-A and butirosin A, NMB-A showed 1.3 ppm upfield shift for C-5′ and 10 ppm downfield shift for C-6′, respectively. These are due to the effect of the $N-CH_3$ group attached at C-6′ in NMB-A as reported for the gentamicin group antibiotics[3~5].

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

Table 2. ^{13}C-Chemical shifts (δ) of NMB-A compared to BTN-A

	NMB-A		BTN-A		$\Delta\delta$*
	pD 10.5	pD 5.2	pD 10.1	pD 4.3	
C-1	50.6	50.2	50.5	50.2	
C-2	36.0	31.5	34.8	31.1	
C-3	51.3	49.8	51.2	49.8	
C-4	82.9	77.0	82.5	76.5	
C-5	85.8	87.4	86.0	87.2	
C-6	75.6	74.9	75.6	74.9	
C-1′	99.9	96.1	99.7	96.2	
C-2′	56.6	54.7	56.6	54.7	
C-3′	74.3	69.2	74.2	69.2	
C-4′	73.1	71.9	72.5	71.8	
C-5′	72.2	70.1	73.5	70.4	−1.3
C-6′	52.6	50.7	42.6	41.4	10.0
6′-NCH_3	34.9	34.8			
C-1″	111.3	113.1	111.4	113.1	
C-2″	81.6	81.9	81.7	81.9	
C-3″	75.6	75.5	75.6	75.6	
C-4″	83.1	84.2	83.1	84.2	
C-5″	61.5	61.7	61.6	61.8	
C-1‴	177.4	176.7	176.9	176.7	
C-2‴	71.0	70.7	70.9	70.8	
C-3‴	36.0	32.0	34.1	32.1	
C-4‴	38.0	37.8	37.8	37.9	

* $\Delta\delta=\delta_{pD\,10.5}$ (NMB-A)$-\delta_{pD\,10.1}$(BTN-A)

Antibacterial Activity

The *in vitro* antibacterial activities of NMB-A, NMB-B and DCB-B and those of the reference antibiotics, butirosin A, DMB-A and DMB-B are shown in Table 3.

NMB-A and NMB-B exhibited broad-spectrum activities with potency similar to or slightly less than that for the butirosin A against butirosin-sensitive bacteria, with the exception of the strains of *Pseudomonas aeruginosa* and *Serratia marcescens.* They showed reduced activities against *P. aeruginosa* strains but exhibited enhanced activities against *S. marcescens* strain as compared with the butirosin A. NMB-A and NMB-B exhibited greater activities against *Escherichia coli* K-12 R 5 which contains 6′-N-acetylating enzyme AAC(6′)-I[6] and *Shigella sonnei* R which is a clinical isolate resistant to butirosins and dibekacin.

DCB-B exhibited broad-spectrum activity with potency similar to DMB-B against butirosin-sensi-

Table 3. *In vitro* antibacterial activities of NMB-A, NMB-B and DCB-B compared to DMB-A, DMB-B and BTN-A

Organism	Resistance mechanism[6)]	Minimal inhibitory concentration (μg/ml)*					
		NMB-A	NMB-B	DCB-B	DMB-A	DMB-B	BTN-A
Staphylococcus aureus 209P, JC-1		0.4	0.4	0.4	0.4	0.4	0.2
〃 〃 A 20239	APH(3′)-I,II	25	12.5	6.3	6.3	6.3	12.5
Bacillus subtilis ATCC 6633		1.6	1.6	1.6	1.6	1.6	0.8
Escherichia coli K-12		0.4	0.8	0.8	0.4	0.8	0.4
〃 〃 K-12 R 5	AAC(6′)-I	0.2	0.4	6.3	0.4	0.8	100
〃 〃 K-12 ML 1630	APH(3′)-I	1.6	1.6	1.6	1.6	1.6	1.6
〃 〃 JR 35/C 600	APH(3′)-I	0.8	0.8	0.8	0.8	0.8	0.4
〃 〃 JR 66/W 677	APH(3′)-II, ANT(2″)	>100	>100	1.6	0.8	1.6	>100
〃 〃 A 20107	APH(3′)-II	50	100	1.6	1.6	1.6	50
〃 〃 A 20732	ANT(2″)	0.8	0.8	0.8	0.8	0.8	0.8
〃 〃 A 20895	AAC(3)	1.6	1.6	1.6	1.6	1.6	0.8
Klebsiella pneumoniae Type 22♯ 3038	APH(3′)-II, ANT(2″)	>100	>100	1.6	0.8	1.6	>100
Shigella sonnei R[a)]		0.8	0.8	6.3	1.6	1.6	>100
Proteus rettgeri KU-23[b)]		>100	>100	12.5	6.3	12.5	>100
Serratia marcescens OU-29		0.4	0.4	0.8	0.8	0.8	1.6
Providencia stuartii ♯164 A 20894	AAC(2′)-II	>100	>100	12.5	25	25	>100
Pseudomonas aeruginosa A3		3.1	3.1	0.2	0.2	0.2	0.4
〃 〃 No. 12		1.6	1.6	0.2	0.2	0.2	0.2
〃 〃 35-R[c)]		100	100	3.1	3.1	3.1	3.1
〃 〃 TU-412[d)]		>100	>100	0.8	3.1	3.1	>100
〃 〃 GN 315	AAC(6′)-IV	>100	>100	6.3	0.8	0.8	>100

Abbreviations: DMB-A, 3′,4′-dideoxy-6′-N-methylbutirosin A and DMB-B, 3′,4′-dideoxy-6′-N-methylbutirosin B.

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

a) Butirosin, kanamycin, dibekacin, neomycin, ribostamycin and streptomycin-resistant clinical isolate.

b) Butirosin, dibekacin, neomycin, ribostamycin and streptomycin-resistant clinical isolate.

c) Gentamicin, dibekacin, kanamycin, neomycin, ribostamycin and streptomycin-resistant clinical isolate.

d) Butirosin, kanamycin, neomycin, ribostamycin and streptomycin-resistant clinical isolate.

tive organisms, including *Pseudomonas aeruginosa* strains against which it showed activity equal to or slightly greater than that for the butirosin A. DCB-B exhibited improved activity against butirosin-resistant organisms which contain 6′-N-acetylating enzymes AAC(6′)-I and AAC(6′)-IV, and 3′-O-phosphorylating enzyme APH(3′)-II[6)]. DCB-B also exhibited excellent activity against butirosin-resistant clinical isolates such as *P. aeruginosa* TU-412, *S. sonnei* R and *Proteus rettgeri* KU-23, the latter two being resistant to dibekacin[2)]. DCB-B was active against gentamicin-dibekacin-resistant clinical isolate, *P. aeruginosa* 35-R, likewise butirosin A, DMB-A and DMB-B.

It is interesting to note here that DCB-B possessing a free amino group at C-6′ position (Fig. 1) was active against the 6′-N-acetylating strains [AAC(6′)-I and AAC(6′)-IV], though the activity was less than that of the structural isomer, DMB-B, which possesses a methylamino group at C-6′ position[2)]. This fact suggested that the presence of the 6′-C-methyl group in DCB-B might protect DCB-B from 6′-N-acetylation by the inactivating enzyme AAC(6′) as in the case of gentamicin C_2.[7,8)] It is also interesting to note here that DCB-B showed somewhat greater activity than DMB-B against *Providencia stuartii* ♯164 A20894 which contains 2′-N-acetylating enzyme AAC(2′)-II[6)].

Experimental

Instrumental analyses and fermentation were performed as previously reported[1,2)]. *Bacillus*

circulans MCRL 5003, a neamine-negative mutant, was used in this study. Precursors were added at 100 μg/ml after 24 hours of fermentation.

Preparation of precursors

Gentamine C_2 was prepared by methanolysis of gentamicin followed by chromatography as described for the gentamicin C complex[9].

6′-N-Methylneamine (I) was prepared by essentially the same method described by UMEZAWA *et al.*[10], starting from 6′-N-benzyloxycarbonylneamine (II). To improve the solubility of **II** in THF, the following modification was made. The compound (**II**) was converted to 1,2′,3-tri-N-benzylidene-6′-N-benzyloxycarbonylneamine, which was further reduced with $LiAlH_4$ in THF to give the protected 6′-N-methylneamine derivative. Removal of the protecting group and purification on a CM-Sephadex C-25(NH_4^+) column afforded a pure **I** (total yield 45.2%), m.p. 210~214°C (dec.); $[\alpha]_D^{24}$ +105.0° (*c* 1.0, H_2O); mass, *m/e* 337 for $(M+1)^{+\cdot}$. The sulfate of **I** was obtained from I, m.p. 225~230°C (dec.); $[\alpha]_D^{23}$ +70.0° (*c* 0.5, H_2O); ^{1}H-NMR, δ 1.8~2.8 (2H, m, H-2), 2.92 (3H, s, N-CH_3) and 6.17 (1H, d, J=4.0 Hz, H-1′).

Anal. Calcd. for $C_{13}H_{28}N_4O_6 \cdot 2H_2SO_4 \cdot 2H_2O$: C 27.46, H 6.38, N 9.85
Found: C 27.49, H 6.22, N 9.93

After this work was completed, we found that essentially the same procedure had been reported in a patent literature[11].

Isolation and separation of NMB-A, NMB-B and AHB-6′-N-methylneamine

From the fermentation broth (10 liters) supplemented with 6′-N-methylneamine, a crude mixture (2.8 g) containing NMB-A and NMB-B was isolated by the procedure described previously[2]. 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 (NH_4^+, 20 ml). After washing with water, the column was eluted stepwise with 400 ml of 0.1 N, 500 ml of 0.15 N and 1.5 liters of 0.175 N NH_4OH. A crude mixture (326 mg) containing NMB-A, NMB-B and AHB-6′-N-methylneamine was obtained from the 0.175 N NH_4OH eluate, whereas the precursor was recovered from the 0.15 N NH_4OH eluate. The crude mixture was dissolved in 5 ml of water, and the solution was passed through a column of Dowex 1×2 (OH^-, 30 ml). The column was eluted with water. The eluate was collected in portions of 10 ml. Tubes No. 11~26 gave a purified complex of NMB-A and NMB-B (270 mg, A: 90~95%). Whereas, tubes No. 5~8 gave purified AHB-6′-N-methylneamine (45 mg); m.p. 185~190°C (dec.); $[\alpha]_D^{22}$ +42.2 (*c* 0.6, H_2O); IR (KBr), 1635 and 1560 cm^{-1} (amide); ^{1}H-NMR (sulfate), δ 1.8~2.6, (4H, m, H-2 and β-methylene of the acyl unit), 2.94 (3H, s, N-CH_3) and 6.16 (1H, d, J=4.0 Hz, H-1′).

From the NMB complex (250 mg), homogeneous NMB-A (149 mg) and NMB-B (15.3 mg) were obtained by repeated column chromatography on CM-Sephadex C-25 (NH_4^+, 70~90 ml) eluted with 0.125 N NH_4OH. The homogeneous NMB-A (149 mg) in 5 ml of water was adsorbed on a column of CM-Sephadex C-25 (NH_4^+, 4 ml). After washing with 40 ml of 0.05 N NH_4OH, the column was eluted with 40 ml of 0.5 N NH_4OH. The active eluate was concentrated *in vacuo* and lyophilized to give 125 mg of pure NMB-A carbonate as white amorphous powder, m.p. 188~192°C (dec.); $[\alpha]_D^{25}$ +24.3° (*c* 0.7, H_2O).

Anal. Calcd. for $C_{22}H_{43}N_5O_{12} \cdot 2H_2CO_3 \cdot 2H_2O$: C 39.51, H 7.05, N 9.60
Found: C 39.62, H 6.58, N 9.71

Similarly, from the homogeneous NMB-B, 10.5 mg of pure NMB-B carbonate was obtained as white amorphous powder, m.p. 192~197°C (dec.); $[\alpha]_D^{25}$ +32.0° (*c* 0.2, H_2O).

Anal. Calcd. for $C_{22}H_{43}N_5O_{12} \cdot 2H_2CO_3 \cdot 2H_2O$: C 39.51, H 7.05, N 9.60
Found: C 39.32, H 7.44, N 9.45

Isolation and separation of DCB complex and DCB-B

From the fermentation broth (10 liters) supplemented with gentamine C_2, a crude mixture (2.4 g) containing DCB-A and DCB-B was isolated by the procedure described previously[2]. The mixture was adsorbed on a column of Amberlite CG-50 (NH_4^+, 30 ml). After washing with water, the column was eluted stepwise with 600 ml of 0.1 N, 1 liter of 0.3 N and 4 liters of 0.35 N NH_4OH. A crude mixture (172 mg) containing DCB-A, DCB-B and AHB-gentamine C_2 was obtained from the 0.35 N

NH_4OH eluate, whereas the precursor was recovered from the 0.3 N NH_4OH eluate. The crude mixture was purified by a column chromatography on Dowex 1×2 (OH^-, 30 ml) eluted with water. The eluate was collected in portions of 10 ml. Tubes No. 3~5 gave a mixture (33 mg) containing DCB-A, DCB-B and AHB-gentamine C_2. Whereas, tubes No. 6~40 gave a purified complex of DCB-A and DCB-B (121 mg, B: 80~90%), m.p. 180~190°C (dec.).

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 41.15, H 6.93, N 9.82

From the DCB-complex (100 mg), homogeneous DCB-B (25 mg) was separated by a column chromatography on Amberlite CG-50 (NH_4^+, 10 ml) eluted with 0.3 N NH_4OH. The homogeneous DCB-B was purified on a column of CM-Sephadex C-25 (NH_4^+, 1 ml). The column was washed with 50 ml of 0.1 N NH_4OH and DCB-B (19.6 mg) was eluted with 40 ml of 0.5 N NH_4OH. The purified DCB-B was further passed through a column of Dowex 1×2 (OH^-, 0.25 ml) and eluted with water. The active eluate was concentrated *in vacuo* and lyophilized to give 18.3 mg of pure DCB-B carbonate as white amorphous powder; m.p. 188~192°C (dec.); $[\alpha]_D^{25}$ +23.0°C (*c* 0.3, H_2O).

Anal. Calcd. for $C_{22}H_{43}N_5O_{10}\cdot H_2CO_3\cdot H_2O$: C 44.73, H 7.67, N 11.34
Found: C 44.44, H 7.27, N 11.29

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