BAGOUGERAMINES A AND B, NEW NUCLEOSIDE ANTIBIOTICS PRODUCED BY A STRAIN OF *BACILLUS CIRCULANS*

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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Bagougeramines A and B obtained as sulfates were soluble in water and positive to Sakaguchi, chlorine-tolidine and ninhydrin color reactions. Their structures were determined by acid hydrolysis and spectroscopic analysis. Structurally they were closely related to gougerotin and they contained the guanidino-D-alanine instead of the serine residue in gougerotin. Bagougeramine B had the spermidine instead of the 6'-NH_2 in structure of bagougeramine A.

In the preceding paper[1], we reported that bagougeramines A and B which had antimicrobial and anti-mite activities were produced by *Bacillus circulans* TB-2125. The producer had unique physiological properties and the moving colonies on agar plate as described as "swarming".

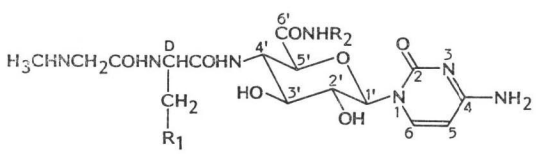
In this report, we describe the physico-chemical properties and structural elucidation of bagougeramines A and B.

Results and Discussion

Physico-chemical Properties of Bagougeramines A and B

As summarized in Table 1, the sulfates of bagougeramines A (**1**) and B (**2**) isolated from the fermentation broth of *B. circulans* TB-2125[1] were white hygroscopic powders which were soluble in water and positive to Sakaguchi and chlorine-tolidine reactions. UV spectra (Fig. 2) of antibiotics showed the presence of N^1-substituted cytosine nucleus in their structures. The IR spectra were shown in Fig. 3. Molecular formulas of bagougeramines A (**1**) and B (**2**) were determined as $C_{17}H_{28}N_{10}O_7$ and

Fig. 1. Structures of bagougeramines and gougerotin.



Bagougeramine A (**1**)	R_1: $NHC(=NH)NH_2$	R_2: H
Bagougeramine B (**2**)	R_1: $NHC(=NH)NH_2$	R_2: $CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2NH_2$
Gougerotin	R_1: OH	R_2: H

Table 1. Physico-chemical properties of the sulfates of bagougeramines A and B.

	Bagougeramine A	Bagougeramine B
Appearance	White hygroscopic powder	White hygroscopic powder
MP	230°C (dec)	237°C (dec)
Optical rotation	+34.1° (*c* 0.5, 27°C)	+22.1° (*c* 0.5, 26°C)
UV_{max} ($E^{1\%}_{1cm}$) nm		
Neutral	232 (119), 265 (121)	232 (101), 265 (102)
0.1 N HCl	274 (171)	274 (143)
0.1 N NaOH	235 (s), 265 (122)	235 (s), 265 (106)
MW, SI-MS	*m/z* 485 (MH^+)	*m/z* 613 (MH^+)
Molecular formula	$C_{17}H_{23}N_{10}O_7 \cdot 1\frac{1}{2}H_2SO_4 \cdot 3H_2O$	$C_{24}H_{44}N_{12}O_7 \cdot 2H_2SO_4 \cdot 6H_2O$
Elemental analyses		
Found:	C 29.83, H 5.54, N 20.19, S 7.04	C 31.92, H 6.47, N 17.71, S 7.55
Calcd:	C 29.78, H 5.40, N 20.44, S 7.07	C 31.75, H 6.50, N 18.52, S 7.06
Solubility		
Soluble:	H_2O	H_2O
Insoluble:	MeOH, EtOH, acetone	MeOH, EtOH, acetone
Color reaction		
Positive:	Sakaguchi, chlorine-tolidine, ninhydrin*	Sakaguchi, chlorine-tolidine, ninhydrin
Negative:	Anisaldehyde-H_2SO_4	Anisaldehyde-H_2SO_4
Rf value on cellulose TLC** (5718, Merk)	0.24	0.15
High voltage paper electrophoresis*** (Rm)	1.62 (Ala 1.00)	1.89 (Ala 1.00)

* Weakly positive.
** Solvent; PrOH - pyridine - AcOH - H_2O (15: 10: 3: 12).
*** Formic acid - AcOH - H_2O (1: 3: 36), 3,000 V, 20 minutes.

Fig. 2. UV spectra of the sulfates of bagougeramines A and B.

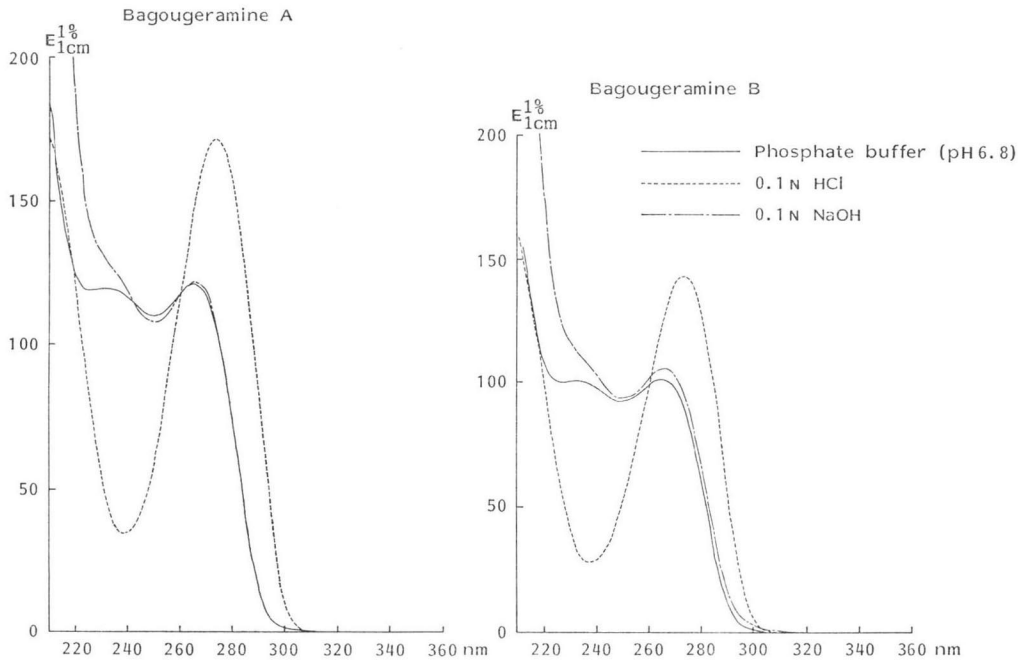


Fig. 3. IR spectra of the sulfates of bagougeramines A and B (KBr).

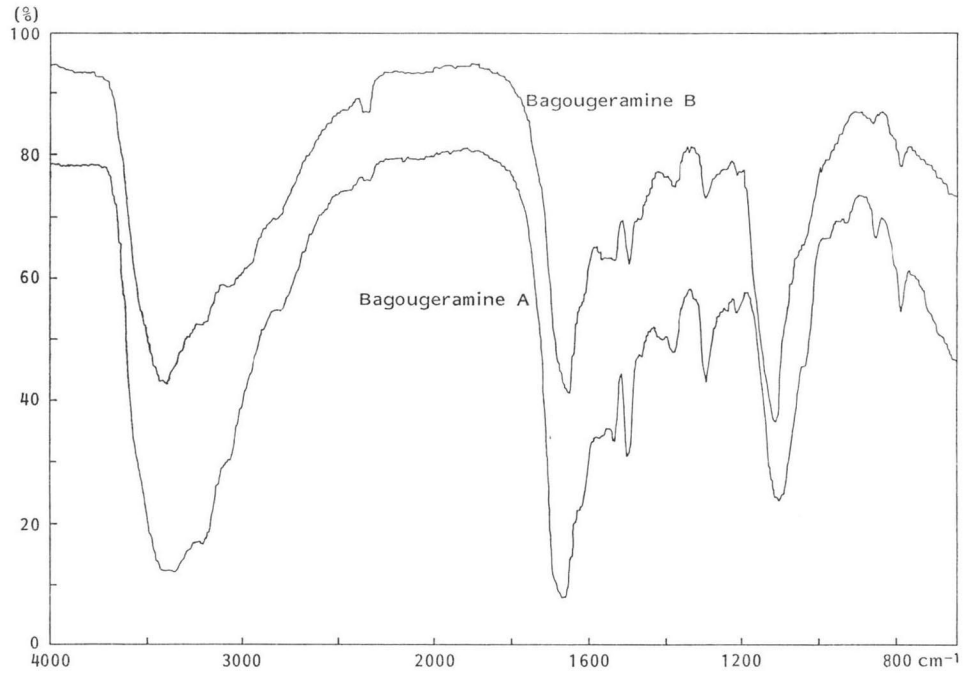


Table 2. ^{1}H NMR data of bagougeramine A (**1**), bagougeramine B (**2**) and *C*-substance (**3**) in D_2O.

Proton	Chemical shifts (δ value in ppm) and coupling constants (Hz)				
	1		**2**		**3**
	pD 5.0	pD 9.2	pD 5.0	pD 8.8	
5	6.14 (d, J=7.7)	6.13	6.15 (d, J=7.2)	6.15	6.11 (d, J=7.4)
6	7.83 (d, J=7.7)	7.81	7.81 (d, J=7.2)	7.82	7.74 (d, J=7.4)
1′	5.76 (d, J=9.3)	5.75	5.73 (d, J=9.3)	5.73	5.73 (d, J=9.6)
2′	3.92 (m)	3.90	3.93 (m)	3.93	3.82 (t, J=9.6, 10.1)
3′	3.86 (m)	3.86	3.90 (m)	3.88	3.88 (t, J=10.1, 10.1)
4′	4.12 (t, J=10.7, 10.7)	4.11	4.09 (t, J=10.3, 10.4)	4.11	3.31 (t, J=10.1, 11.5)
5′	4.20 (d, J=10.7)	4.20	4.23 (d, J=10.4)	4.21	4.20 (d, J=11.5)
Sarcosine					
2	4.01 (ABq)	3.41	4.05 (ABq)	3.54	
NCH_3	2.82 (s)	2.38	2.82 (s)	2.45	
G-Ala[a]					
2[b]	4.78 (dd, J=6.0, 7.0)	4.78	4.78 (dd, J=5.2, 8.0)	4.78	
3-a	3.56 (dd, J=7.0, 15.0)	3.52	3.54 (dd, J=8.0, 14.7)	~3.54	
3-b	3.68 (dd, J=6.0, 15.0)	3.68	3.70 (dd, J=5.2, 14.7)	3.70	
Spermidine					
1-a			3.20 (dt, J=6.9, 13.9)	3.22	
1-b			3.36 (dt, J=6.9, 13.9)	3.33	
2			1.91 (m)	1.87	
3, 5, 8			3.07 (m)	3.01	
6, 7			1.78 (m)	1.75	

[a] Guanidino-D-alanine.
[b] Measured at 40°C.

$C_{24}H_{44}N_{12}O_7$, respectively, by elemental analyses and secondary ion mass spectra of their sulfates. Their ^1H and ^{13}C NMR data were listed in Tables 2 and 3.

Structures of Bagougeramines A and B

In the ^1H NMR spectrum (Table 2) of bagougeramine B (**2**) (sesquisulfate, D_2O), two doublets at δ 7.81 and 6.15 were easily recognizable to be due to the cytosine nucleus. An anomeric proton was observed at δ 5.73 (d, J=9.3 Hz). *N*-Methyl

Table 3. ^{13}C NMR data for bagougeramine A (**1**) and bagougeramine B (**2**).

Carbon*	Chemical shift (ppm)	
	1 (pD 5.0)	**2** (pD 5.0)
2	158.2 or 158.7 s**	158.2 or 158.6 s
4	167.1 s	167.0 s
5	98.1 d	98.1 d
6	142.7 d	143.0 d
1′	84.3 d	84.7 d
2′	72.2 d	72.0 d
3′	74.3 d	74.4 d
4′	54.3 d	54.3 d
5′	76.8 d	77.3 d
6′	167.7 s	167.7 s
Sarcosine		
1	171.6 or 172.8 s	170.4 or 171.6 s
2	50.6 t	50.6 t
NCH_3	34.1 q	34.1 q
G-Ala		
1	171.6 or 172.8 s	170.4 or 171.6 s
2	53.3 d	53.5 d
3	43.1 t	43.3 t
NCN ‖ N	158.2 or 158.7 s	158.2 or 158.6 s
Spermidine		
2, 6, 7		23.4 t, 25.0 t, 26.2 t
1, 3, 5, 8		37.2 t, 39.8 t, 46.1 t, 47.9 t

* Assignments of carbons were based on comparison of their chemical shifts with those of gougerotin in the literature[10].

** Multiplicity.

Fig. 4. Acid hydrolysis of bagougeramine B (**2**).

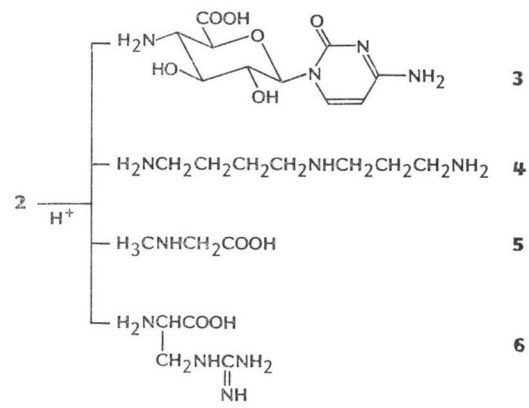
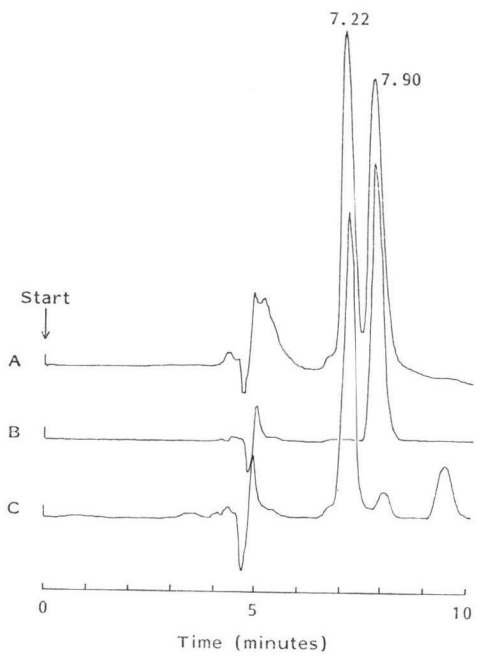


Fig. 5. HPLC of diastereomeric thiourea derivatives of guanidinoalanine with GITC* as a chiral reagent.

A: Guanidino-DL-alanine synthesized according to the method described by TAKAGI *et al.*[5], B: guanidino-L-alanine (Sigma), C: acid hydrolysis product (**6**, guanidino-D-alanine).

Column: SSC-ODS-276 (6×200 mm). Mobile phase: MeOH - 10 mM phosphate buffer, pH 2.8 (45: 55). Flow rate: 1.0 ml/minute. Detection: UV 250 nm.

* 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate[9].



protons at δ 2.82 and isolated methylene protons at δ 4.05 (ABq) indicated that **2** had a sarcosyl residue. The presence of a spermidine moiety was indicated by methylene signals at δ 1.78 (4H, m), 1.91 (2H, m), 3.07 (6H, m), 3.20 and 3.36 (each 1H, dt). Spin decoupling experiment indicated that signals at δ 3.20 and 3.36 were 1-CH_2 protons of the spermidine moiety linked to the 6′-CO group through the amide bond. Furthermore, signals at δ 4.78 (1H, dd), 3.54 (1H, dd, ABX) and 3.70 (1H, dd, ABX) indicated the presence of guanidinoalanyl residue (α-amino-β-guanidinopropionyl residue).

Acid hydrolysis of **2** (hydrochloride) with 6 N HCl at 100°C, followed by purification of its hydrolysate by column chromatographies on Sephadex LH-20 and Amberlite CG-50 (NH_4^+) gave compounds **3**, **4**, **5** and **6** (Fig. 4).

A ninhydrin-positive compound **3** was obtained as needles: $[\alpha]_D^{28}$ +4° (H_2O); SI-MS m/z 287 (MH^+). The ^1H NMR spectrum of **3** (Table 2) revealed that **3** was identical with *C*-substance[2,3] which was a degradation product of gougerotin.

Compound **4** was identical with spermidine by SI-MS m/z 146 (MH^+), ^1H and ^{13}C NMR. This was confirmed by a direct comparison with an authentic sample of spermidine on TLC.

All physico-chemical data of **5** were consistent with those of sarcosine.

Compound **6**, which was positive to Sakaguchi and ninhydrin tests, was obtained as a hygroscopic hydrochloride: $[\alpha]_D^{23}$ −12.5° (H_2O), SI-MS m/z 147 (MH^+). In the ^1H NMR spectrum, a methine proton at δ 3.99 (t) and methylene proton at δ 3.77 (d) were observed. The ^{13}C NMR spectrum of **6** showed four carbon signals at δ 172.3 (C=O), 158.2 (NC(=NH)NH_2), 54.6 (CHN) and 42.5 (CH_2N). From these data, compound **6** was determined as guanidino-D-alanine. An authentic sample of guanidino-L-alanine hydrochloride (Sigma Chem., Co.) showed $[\alpha]_D^{22}$ +14.6° (H_2O). The absolute structure of compound **6** was confirmed by HPLC for enantiomeric resolution of amino acid[4] in comparison with synthetic guanidino-DL-alanine[5] and authentic L-enantiomer. Fig. 5 indicates that the peak of compound **6** (7.22 minutes) was superimposable with that of D-enantiomer while an authentic sample of L-enantiomer migrated at 7.90 minutes.

Comparing the ^1H NMR spectrum of **2** with that of **3**, H-4′ signal in **2** shifted downfield from the corresponding signal in **3** (from δ 3.31 to 4.09, Table 2). The ^1H NMR spectra of **2** in D_2O at pD 8.8 showed that the *N*-methyl and methylene signals in a sarcosyl moiety shifted upfield by 0.37 and 0.51 ppm, respectively, compared with those at pD 5.0 (Table 2). Therefore, compound **2** incorporated a sarcosyl (guanidino-D-alanyl) residue at the 4′-amino group.

Thus, the structure of bagougeramine B was determined to be **2** as shown in Fig. 1.

By refluxing in 6 N HCl, compound **1** afforded three fragments, *C*-substance (**3**), sarcosine (**5**), and guanidino-D-alanine (**6**). The ^1H and ^{13}C NMR spectra of **1** were quite similar to those of **2** except for the lack of signals for the spermidine moiety. Thus, bagougeramine A was determined as shown in Fig. 1.

These structural studies of bagougeramines A and B revealed that they were new cytosine-nucleoside antibiotics with an unusual amino acid, guanidino-D-alanine, in their structures and were structurally related to gougerotin[6~8] (Fig. 1).

Experimental

Melting points were determined with a Yazawa melting point aparatus and were uncorrected. Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. IR spectrum was re-

corded with a Hitachi 260-10 infrared spectrophotometer. The ^{1}H and ^{13}C NMR spectra were measured with Jeol JNM-GX400 spectrometer. TLC was performed on a silica gel (Kieselgel 60 F_{254}, Merck) developed with a mixture of BuOH - AcOH - H_2O (2: 1: 1).

Acid Hydrolysis of **2**

A solution of **2** (hydrochloride, 82 mg) in 8 ml of 6 N HCl was refluxed for 6 hours. The solution was diluted with 72 ml of H_2O and neutralized with Amberlite IRA-45 (OH^-). After removal of solid, the aqueous solution was concentrated under reduced pressure to give a pale yellow solid. The residue was dissolved in 1 ml of 50% aq MeOH and applied to a column of Sephadex LH-20. The column was developed with 50% aq MeOH. The eluate was collected in 2.5 ml fractions. Fractions (Nos. 52~58) were combined and concentrated to give a solid (HL-A). The same procedures of fractions (Nos. 59~63) gave a solid (HL-B). A solution of HL-A was passed through a column of Amberlite CG-50 (NH_4^+, 5 ml). The column was developed with H_2O (2 ml fractions). Fractions (Nos. 3~5) were combined and concentrated to dryness. The residue was applied again to a column of Sephadex LH-20 and eluted with 50% aq MeOH (1.25 ml fractions). Fractions (Nos. 90~93) were combined and concentrated to give a colorless solid, which was recrystallized from H_2O - MeOH (1: 1) to afford needles of compound **3** (*C*-substance): MP 235°C (dec); $[\alpha]_D^{28}$ +4° (*c* 0.47, H_2O) (literature, $[\alpha]_D^{25}$ +6°[3], $[\alpha]_D^{20}$ +2°[2]); SI-MS *m/z* 287 (MH^+); TLC Rf 0.18. The ^{1}H NMR (400 MHz, D_2O) spectrum was shown in Table 1.

Fractions (Nos. 94~98) were combined and concentrated to give a compound **5**: SI-MS *m/z* 90 (MH^+); TLC Rf 0.31. All physico-chemical data of **5** were identical with those of sarcosine.

After elution of *C*-substance and sarcosine through a column of Amberlite CG-50 with H_2O, the column was subsequently washed with 1 N NH_4OH and H_2O, then developed with 1 N HCl to afford compound **4**. Fractions containing **4** were collected and concentrated to dryness. The residue was desalted on a column of Sephadex LH-20 with 50% aq MeOH to give a hydrochloride of **4**: SI-MS *m/z* 146 (MH^+); TLC Rf 0.05. Compound **4** was identical with spermidine in all respects.

A solid, HL-B was applied to a column of Sephadex LH-20. The column was developed with 50% aq MeOH (2.5 ml fractions) and fractions (Nos. 61 and 62) were combined and concentrated to give a hygroscopic hydrochloride of **6**: $[\alpha]_D^{23}$ −12.5° (*c* 0.18, H_2O); SI-MS *m/z* 147 (MH^+); ^{1}H NMR (400 MHz, D_2O), δ 3.99 (1H, t, J=5.5 Hz), 3.77 (2H, d, J=5.5 Hz); ^{13}C NMR (100 MHz, D_2O), δ 172.3, 158.2, 54.6, 42.5; TLC Rf 0.24. HPLC was shown in Fig. 5. Compound **6** was determined as guanidino-D-alanine.

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