BIPHENOMYCIN C, A PRECURSOR OF BIPHENOMYCIN A IN MIXED CULTURE

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A precursor of biphenomycin A in mixed culture of *Streptomyces griseorubiginosus* No. 43708 with *Pseudomonas maltophilia* No. 1928 was isolated and characterized. The structure of the precursor, designated biphenomycin C was determined to be a peptide which is composed of biphenomycin A and arginylserine residue (Fig. 1), on the basis of chemical and spectroscopic evidence.

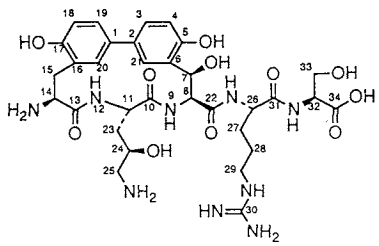
Previously, we reported the isolation of biphenomycins A (**1**) and B (**2**)[1], and the structure determination of them[2,3]. The antibiotics are cyclic tripeptides exhibiting high antibacterial activity. The three-dimentional structure of biphenomycin A was determined by J. C. HEMPEL and F. K. BROWN *et al.*[4,5], and the total synthesis of biphenomycin B was achieved by U. SCHMIDT *et al.*[6]. On the other hand, we reported that biphenomycin A production by *Streptomyces griseorubiginosus* No. 43708 was stimulated by mixed culture with *Pseudomonas maltophilia* No. 1928[7]. In that paper, we showed a hypothesis that strain No. 43708 produced a precursor of biphenomycin A, and that strain No. 1928 converted the precursor to biphenomycin A enzymatically.

In this paper, we have shown about the isolation and structural elucidation of the precursor.

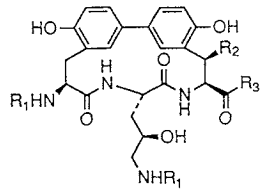
Fermentation

The strain for production of biphenomycin C is *Streptomyces griseorubiginosus* No. 43708 which also produces biphenomycins A and B[1]. The media used for production of biphenomycin C is shown

Fig. 1. Structure of biphenomycin C and derivatives.



Biphenomycin C (**3**)



1 $R_1=H$, $R_2=R_3=OH$ (Biphenomycin A)
2 $R_1=R_2=H$, $R_3=OH$ (Biphenomycin B)
4 $R_1=COOC(CH_3)_3$, $R_2=R_3=OH$
5 $R_1=COOC(CH_3)_3$, $R_2=OH$,
$R_3=NHCHCONHCHCOOCH_3$
$(CH_2)_3$ CH_2OH
$NHCNH_2$
NH

previously[7]. A loopfull of the strain No. 43708 from a mature slant was inoculated into each of four 500-ml flasks containing 100 ml of the seed medium. The flasks were shaken on a rotary shaker (250 rpm, 5 cm stroke) at 30°C for three days. The content of four flasks was inoculated into a 30-liter jar fermentor containing 20 liters of fermentation medium. The fermentation was carried out at 30°C for three days, with aeration of 20 liters/minute, agitation of 250 rpm and inner pressure of 1.0 kg/cm^2. Biphenomycins A and C were monitored by HPLC. (Table 1)

Isolation

The isolation procedure for biphenomycin C is summarized in Fig. 2. The culture broth (20 liters) was filtered with an aid of filter aid (Radiolite No. 600, Showa Chemical Industry). Then, the filtrate (18 liters) was passed through a column of Diaion HP-20 (5 liters, Mitsubishi Chemical Industries Ltd.). The column was washed with water (10 liters) and 50% methanol (10 liters) and then eluted with 50% methanol containing 0.07% aqueous NH_4OH.

Table 1. HPLC of biphenomycins A and C.

Apparatus	Model M6000-A pump, U6K injection, and 440 absorbance detection (Waters Assorciates Ltd.)
Stationary phase	μBondapak C18 (Waters Associates Ltd.)
Length of column	300 mm
Internal diameter of column	7.8 mm
Mobile phase	Acetonitrile - *n*-BuOH - AcOH - SDS - H_2O (35 : 15 : 0.1 : 0.25 : 50)
Column pressure	141 kg/cm^2
Flow rate	2 ml/minute
Detector	Ultraviolet absorption at 260 nm
Retention time (minutes)	Biphenomycin A: 3.7 Biphenomycin C: 10.2

Fig. 2. Purification procedure for biphenomycin C.

Filtrate
↓
Diaion HP-20
↓ 50% MeOH - 0.07% NH_4OH
IRC50 (H^+ form)
↓ 1.4% NH_4OH
DEAE-Sephadex A-25 (OH^-)
↓ 0.2 M NaCl
Diaion HP-20
↓ 50% MeOH - 2.8% NH_4OH
Lobar (LiChroprep RP-8)
↓ 0.1 M K_2HPO_4 - H_3PO_4 buffer (pH 4.8) containing 10% acetonitrile
Silica gel CQ-3
↓ BuOH - EtOH - $CHCl_3$ - 28% NH_4OH (4 : 7 : 2 : 5, v/v)
Diaion HP-20
↓ 50% MeOH - 2.8% NH_4OH
Lyophilized powder
↓ 1 N HCl, pH 2 with 28% NH_4OH
Colorless needles
(Biphenomycin C · 3HCl)

Table 2. Physico-chemical properties of biphenomycin C.

Appearance	Colorless needles	IR ν_{max} (KBr) cm^{-1}	3500~2400, 1650, 1500, 1400, 820
Nature	Amphoteric	Color test:	
MP	195~198°C (dec)	Positive	Ninhydrin, $FeCl_3$, diacetyl
$[\alpha]_D^{20}$	−32.0° (*c* 0.1, 1 N HCl)	Negative	Molish, Dragendorff
FAB-MS	*m/z* 732 $(M+H)^+$	Solubility:	
Molecular formula	$C_{31}H_{45}N_9O_{11} \cdot 3HCl$	Soluble	H_2O
Elemental analysis		Slightly soluble	Acetone, MeOH
Found (%):	C 45.38, H 6.01, N 14.77, C 112.36	Insoluble	*n*-Hexane, benzene, $CHCl_3$, EtOAc
Calcd (%):	C 45.69, H 5.75, N 14.99, C 112.65		
UV $\lambda_{max}^{H_2O}$ nm (ε)	264 (23,700), 287 (sh)		
$\lambda_{max}^{0.1\,N\,HCl}$ nm (ε)	264 (26,600), 287 (sh)		
$\lambda_{max}^{0.1\,N\,NaOH}$ nm (ε)	288 (34,600), 303 (sh)		

The eluate (10 liters) was concentrated *in vacuo* to 2 liters, and adsorbed on a column of Amberlite IRC50 (500 ml, H^+ form, Roam & Haas Co. Ltd.). The column was washed with water (1 liter), and then eluted with 1.4% aqueous NH_4OH. The active fraction (1 liter), was concentrated *in vacuo* to 200 ml and adsorbed on a column of DEAE-Sephadex A 25 (50 ml, OH^- form, Pharmacia Fine Chemicals). The column was washed with water (150 ml) and 0.2 M NaCl (150 ml), and then with 0.5 M NaCl (200 ml). After the elute was neutralized by 6 N HCl, it was adsorbed on a column of Diaion HP-20 (50 ml). The column was washed with water (150 ml) and 50% methanol (200 ml), and then eluted with 50% methanol containing 2.8% aqueous NH_4OH (150 ml). The elute was concentrated to 20 ml *in vacuo* and applied to a Lober column of LiChroprep RP-8 size C (470 ml E. Merk). The column was developed with 0.1 M K_2HPO_4-H_3PO_4 buffer (pH 4.8) containing 10% acetonitrile. Biphenomycin C was eluted from 380 ml to 560 ml. The elute was concentrated to 20 ml *in vacuo* and adsorbed on a column of Silica gel CQ-3 (Fuji Gel Hanbai Co. Ltd. 80 ml). The column was washed with water (100 ml) and then eluted with BuOH - EtOH - $CHCl_3$ - 28%NH_4OH, 4 : 7 : 2 : 5 (200 ml). The eluate was concentrated to 40 ml *in vacuo* and applied to a column of Diaion HP-20 (50 ml). The column was washed with water (200 ml) and eluted with 50% methanol containing 2.8% aqueous NH_4OH (100 ml). The active fraction was lyophilized and pale brown powder was obtained (59 mg). The powder was suspended in 1 N HCl (10 ml). Then, a solution of 28% aqueous NH_4OH was added with stirring at 50°C to adjust to pH 2. The solution was kept overnight at 5°C to produce colorless needles of biphenomycin C·3HCl (11 mg).

Biological Properties

The antimicrobial spectrum of biphenomycin C was determined by the agar dilution method (Table 3). Biphenomycin C is active against Gram-positive bacteria, and the antibacterial activity of it is lower than that of biphenomycin A.

Enzyme Reaction

A 50 μl of cell free extract from mycelium of *Pseudomonas maltophilia* No. 1928[7] was added to biphenomycin C solution (500 μl, 500 μg/ml in 0.01 M phosphate buffer, pH 7.0). After incubation at 37°C for 3 hours, 500 μl methanol was added. Then, the mixture was boiled for 3 minutes in a water bath, and centrifuged for 5 minutes at 4000 rpm. In the supernatant of the mixture, products of the enzyme reaction were detected on TLC of Silica gel (E. Merk), which was developed in a solvent system of BuOH - EtOH - $CHCl_3$ - 28%NH_4OH, 4 : 7 : 2 : 7. Two spots were found on TLC (Fig. 3). One spot was identical to biphenomycin A and another to authentic arginylserine.

Physico-chemical Properties and Structure Determination

The physico-chemical properties of biphenomycin C (**3**) are summerized in Table 2. The study of the enzyme reaction described in the preceding section suggested that biphenomycin C is a peptide comprised of biphenomycin A and arginylserine. This presumption was supported by the following experimental results. Thus, **3** showed positive color reaction to diacetyl, and by exhaustive acid hydrolysis (6 N HCl, 100°C, 12 hours) affected arginine and serine as degradation products which were identified by conventional amino acid analysis. Furthermore, the ^{1}H-NMR and ^{13}C-NMR spectra of **3** which were summarized in Tables 4 and 5 respectively in comparison with those of biphenomycin A, are quite consistant with the above speculation.

The linkage position of biphenomycin A and arginylserine residue, and the absolute configuration of arginine and serine, were established by the synthesis as described below.

Table 3. Antibacterial spectrum of biphenomycin C.

Test organism	Medium[a]	MIC (μg/ml)
Staphylococcus aureus 209P JC-1	1	4
	2	32
Bacillus subtilis ATCC 6633	2	32
Micrococcus luteus PCI 1001	2	125
Proteus vulgalis IAM 1025	2	>100
Escherichia coli NIHJ JC-2	2	>100
Pseudomonas aeruginosa NCTC 10490	2	>100
Candida albicans	3	>100
Aspergillus niger IAM 2561	3	>100

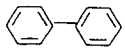
Agar dilution method (1×10^6/ml, 37°C, 20 hours).
[a] Medium 1: Serum agar (20% horse serum, 0.01% $FeSO_4 \cdot 7H_2O$, agar 1.5%), 2: Muller-Hinton agar, 3: Sabouraud's agar.

Table 4. ^{1}H-NMR data of biphenomycins A and C.

Proton	Biphenomycin A (**1**) 270 MHz	Biphenomycin C (**3**) 400 MHz
4-H	6.69, d (8.5)	6.97, d (8.0)
18-H	6.05, d (8.5)	6.96, d (8.0)
20-H	6.86, d (2.5)	7.49, d (2.2)
21-H	7.20, 3H, m	7.03, d (2.2, 8.0)
3-H		7.40, dd (2.2, 8.0)
19-H		7.38, dd (2.2, 8.0)
7-H	5.77, s	5.70, s
11-H	5.02, dd (7.9)	5.10, dd (6.7, 8.0)
8-H	4.55, s	4.90, s
26-H		4.57, dd (6.2, 7.7)
32-H		4.48, br t (4.5)
14-H	3.80, dd (2.5, 8.3)	4.44, dd (3.0, 5.0)
24-H	3.67, m	4.04, m
33-H		3.94, 2H, m
15-H	3.13, dd (8.3, 15.0), 2.91, dd (2.6, 15.0)	3.61, dd (5.0, 15.0), 3.15, dd (3.0, 15.0)
29-H		3.27, 2H, t (7.0)
25-H	2.67, dd (4.4, 13.6), 2.60, dd (7.5, 13.6)	3.18, dd (3.0, 13.2), 2.99, dd (10.0, 13.2)
23-H	2.11, m 1.95, m	2.12, m 1.89, m
27-H		1.99, 2H, m
28-H		1.77, 2H, m

Solvent: Biphenomycin A; D_2O-NaOD, biphenomycin C; CD_3OD-D_2O (1:1).
Chemical shifts are given in ppm. Coupling constants in Hz are given in parentheses.

Table 5. ^{13}C NMR data of biphenomycins A (**1**) and C (**3**) (67.8 MHz).

Biphenomycin A	Biphenomycin C	Assignments
174.0 s	174.1 s	
173.2 s	173.7 s	–CONH–
168.6 s	173.1 s	or
	173.0 s	–COOH–
	168.7 s	
	158.0 s	>C=NH
154.2 s	155.0 s	
152.8 s	153.6 s	
133.0 s	133.8 s	
132.9 s	133.6 s	
130.6 d	131.1 d	
127.9 s	129.1 s	
127.6 d	127.6 d	
127.2 d	127.5 d	
126.2 d	126.7 d	
120.3 s	120.9 s	
116.9 d	117.0 d	
116.4 d	116.6 d	
65.2 d	65.6 d	
64.4 d	62.2 t	–C(–)–OH
	58.8 d	
57.4 d	56.0 d	
55.0 d	55.5 d	
50.9 d	54.3 d	N–CH(–)–CO
	51.3 d	
	49.8 d	
44.9 t	45.5 t	
37.9 t	41.7 t	
30.4 t	38.6 t	
	31.0 t	$-CH_2-$
	29.8 t	
	25.5 t	

Solvent: Biphenomycin A; D_2O-DCl, biphenomycin C; D_2O-CD_3OD (1:1).

Biphenomycin A (**1**) was treated with di-*tert*-butyl dicarbonate and triethylamine to yield diBoc biphenomycin A (**4**). On the other hand, methyl arginyl-serinate was prepared from Z-Arg-SerOMe by reduction with 10% Pd-C in 4.4% HCOOH-MeOH. Di-HCl salt of methyl arginyl-serinate was coupled with **4** using dicyclohexylcarbodiimide (DCC) as a condensation reagent in pyridine to lead to protected peptide **5**. The HCl salt of **5** was then hydrolyzed with 1 N NaOH followed by treatment with TFA-anisole to afford **3** as the TFA salt. The tri-HCl salt of this synthetic compound was identical with biphenomycin C·3HCl in all respects. Consequently, the structure of biphenomycin C was determined as **3**.

Experimental

Infrared spectra were recorded on a JASCO A-102 infrared spectrophotometer. UV spectra were measured on a Hitachi 220A double beam spectrophotometer. ^1H and ^{13}C NMR spectra were recorded by using JEOL JNM-GX400 and JEOL JNM-FX270 spectrophotometer. FAB-MS and SI-MS spectra were determined with JEOL JMS-DX300 and Hitachi M-80 mass spectrometers respectively.

DiBoc Biphenomycin A (**4**)

To a soln of biphenomycin A (**1**, 1 g) and triethylamine (1.15 ml) in H_2O (15 ml), was added a soln of di-*tert*-butyl carbonate (1.79 g) in dioxane (15 ml). The resulting mixture was stirred at room temperature for 30 minutes and concd to dryness under reduced pressure to give a residue, which was chromatographed on Toyopearl HW-40S eluting with MeOH to afford **4** (1.1 g) as a powder: IR (Nujol) 3300, 1650, 1610, 1500, 1250, 1160, 1050, 820 cm^{-1}; ^1H NMR (CD_3OD) δ 7.59 (1H, dd, J=2.5 Hz), 7.22 (1H, dd, J=2.5 and 8.5 Hz), 7.17 (1H, dd, J=2.5 and 8.5 Hz), 7.02 (1H, d, J=2.5 Hz), 6.80 (1H, d, J=8.5 Hz), 6.78 (1H, d, J=2.5 Hz), 5.88 (1H, br s), 4.96 (1H, m), 4.62 (1H, br s), 4.41 (1H, dd, J=2.6 and 5.5 Hz), 3.81 (1H, m), 3.44 (1H, dd, J=5.5 and 14 Hz), 3.12 (2H, m), 2.91 (1H, dd, J=2.6 and 14 Hz), 2.00 (1H, m), 1.80 (1H, m), 1.44 (18H, s); SI-MS, m/z 689 $(M+H)^+$.

Methyl L-Arginyl-L-Serinate

To a stirred soln of Z-Arg-SerOMe (1 g) in 4.4% HCOOH-MeOH (50 ml) was added a suspension of 10% Pd-C (1 g) and 4.4% HCOOH-MeOH (50 ml) under nitrogen atmosphere. The resulting mixture was stirred at room temperature for 5 hours under nitrogen atmosphere. After removal of the catalyst by filtration, the filtrate was concd under reduced pressure to give a residue, to which a dioxane soln (5 ml) containing 10% 1 N HCl was added. The mixture was concd to dryness under reduced pressure to afford the H-Arg-SerOMe·2HCl (653 mg) as a powder: ^1H NMR (D_2O) δ 4.15 (2H, m), 3.96 (2H, m), 3.77 (3H, s), 3.27 (2H, t, J=7 Hz), 1.90 (4H, m); SI-MS, m/z 276 $(M+H)^+$.

Protected Peptide (**5**)

To a stirred soln of **4** (300 mg) and H-Arg-SerOMe·2HCl (174 mg) in pyridine (5 ml) was added DCC (110 mg). The resulting mixture was stirred at room temperature for 63 hours under nitrogen atmosphere. The resulting dicyclohexylurea was removed by filtration and the filtrate was concd under reduced pressure to give a residue, which was dissolved in a minimum quantity of MeOH. Addition of Et_2O precipitated the HCl salt of **5** (342 mg) as a powder: IR (Nujol) 3150, 1725, 1655, 1520, 1410, 1255, 1165, 820 cm^{-1}; ^1H NMR (CD_3OD) δ 7.50 (1H, d, J=2.5 Hz), 7.24 (1H, dd, J=2.5 and 8.5 Hz), 7.16 (1H, dd, J=2.5 and 8.5 Hz), 6.81 (1H, d, J=8.5 Hz), 6.78 (1H, d, J=8.5 Hz), 5.76 (1H, s), 5.04 (1H, m), 4.78 (1H, s), 4.60 (2H, m), 4.39 (1H, m), 3.92 (2H, m), 3.86 (1H, dd, J=5 and 17 Hz), 3.75 (3H, s), 3.40 (1H, m), 3.23 (2H, m), 3.12 (1H, m), 2.94 (1H, m), 2.00~1.70 (6H, m), 1.44 (9H, s), 1.43 (9H, s), SI-MS, m/z 947 $(M+H)^+$.

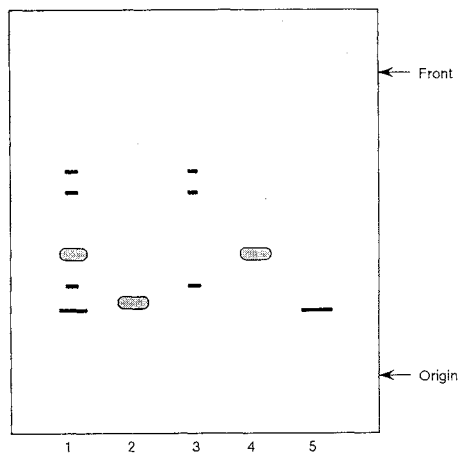
Biphenomycin C (**3**)

To the HCl salt of **5** (40 mg) was added 1 N NaOH (8 ml) and the mixture was stirred at room temperature for 30 minutes. The reaction mixture was neutralized with 1 N HCl and applied to a column of Diaion HP-20 (20 ml). The column was eluted with H_2O (50 ml) and then with MeOH (50 ml). The fraction eluted with MeOH was concd *in*

Fig. 3. TLC of the product of enzyme reaction.

Closed: Ninhydrin, others: UV and ninhydrin.

1: Biphenomycin C treated by cell free extract of strain No. 1928, 2: biphenomycin C, 3: cell free extract of strain No. 1928, 4: biphenomycin A, 5: arginyl serine.



vacuo to give a residue, which was dissolved in a soln of TFA (1 ml) and anisole (0.2 ml) at 0°C. The resulting soln was stirred at 0°C for 20 minutes and then concd under reduced pressure to give a residue, which was triturated with Et_2O. The resulting powder was dissolved in a minimum quantity of aqueous HCl soln (pH 2.0). Standing the soln in refrigerator overnight afforded tri-HCl salt of biphenomycin C in IR, NMR, UV and $[\alpha]_D$.

Discussion

Biphenomycin C, the precursor of biphenomycin A in mixed culture of *Streptomyces griseorubiginosus* No. 43708 and *Pseudomonas maltophilia* No. 1928, was isolated from the culture filtrate of strain No. 43708, and the structure was determined to be a novel peptide antibiotic which comprised of biphenomycin A and arginylserine. Antibacterial activity of biphenomycin C is rather lower than that of biphenomycin A (Table 3). So, we could not detect the antibacterial activity of biphenomycin C in the culture broth but could find and isolate it as the precursor of biphenomycin A in mixed culture.

Biphenomycin C was hydrolyzed to biphenomycin A and arginylserine by cell free extract of strain No. 1928 (Fig. 3). It becomes clear that biphenomycin A is accumulated by the hydrolysis of biphenomycin C in mixed culture[7], and that this hydrolysis is catalyzed by the enzyme of strain No. 1928.

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