BIPHENOMYCINS A AND B*, NOVEL PEPTIDE ANTIBIOTICS

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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Biphenomycin A, $C_{23}H_{28}N_4O_8$, and biphenomycin B, $C_{23}H_{28}N_4O_7$, were isolated from the cultured broth of *Streptomyces griseorubiginosus* No. 43708. The antibiotics are active *in vitro* and *in vivo* against bacteria, and are especially potent against Gram-positive bacteria. The acute toxicity of biphenomycin A is very low in mice.

In the course of screening for antibiotics, we found two new antibiotics, biphenomycins A and B,[1,2] possessing a novel 15-membered cyclic peptide structure including a biphenyl group. In this paper, we describe taxonomic studies on the producing strain, fermentation, isolation procedures and physico-chemical and biological properties of biphenomycins A and B. Details of structure determination are presented in the accompanying paper.[3]

Taxonomic Studies on Strain No. 43708

The strain No. 43708 was isolated from a soil sample obtained from Akashi City, Hyogo Prefecture.

The methods described by SHIRLING and GOTTLIEB[4] were principally employed for the taxonomic studies. Morphological observations were made with light and electron microscopes on cultures grown at 30°C for 14 days on yeast - malt extract agar, oatmeal agar or inorganic salts - starch agar. The mature spores occurred in chains of more than 30 spores forming *Rectiflexibiles* (Fig. 1). The spores were cylindrical and $0.5\sim0.7\times1.3\sim1.5$ μm in size. Spore surfaces were smooth or warty (Fig. 2).

Cultural characteristics were observed on ten kinds of media described by SHIRLING and GOTTLIEB[4] and WAKSMAN.[5] Incubations were made at 30°C for 14 days. The color names used in this study were based on the Color Standard (Nihon Shikisai Co., Ltd.). Colonies were in the gray color series when grown on oatmeal agar, yeast - malt extract agar or inorganic salts - starch agar. Soluble pigment was produced in yeast - malt extract agar and other agars. Results are shown in Table 1. The cell wall analysis was performed by the methods of BECKER *et al.*[6] and YAMAGUCHI.[7] Analysis of whole cell hydrolysates of strain No. 43708 showed that it contained LL-diaminopimelic acid and glycine. Accordingly, the cell wall of the strain was of type I.

* Biphenomycins A and B are originally designated as WS-43708 A (FR 900451) and B.

Fig. 1. Aerial mycelium of strain No. 43708 on yeast extract - malt extract agar (incubated for 14 days at 30°C).

The organism was observed with an optical microscope (×800).

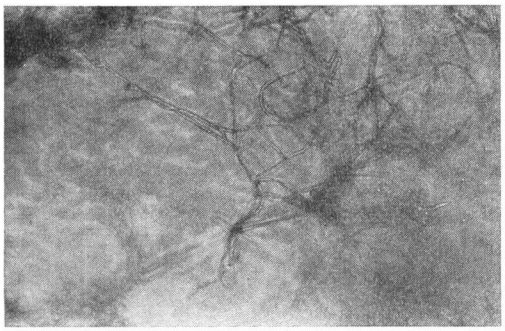
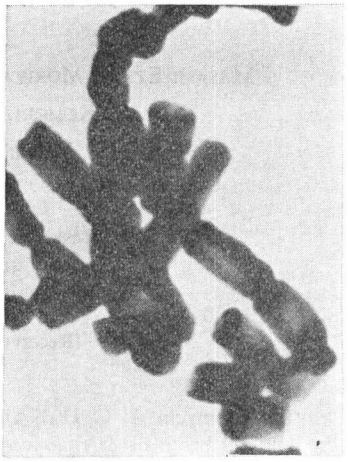


Fig. 2. Electron micrograph of spore chain of strain No. 43708 on yeast extract - malt extract agar, 10 days culture.

Bar represents 1 μm.



Physiological properties of strain No. 43708 are shown in Table 2. Temperature range and optimum temperature for growth were determined on yeast - malt extract agar using a temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.). Temperature range for growth was from 17°C to 41°C with optimum from 29°C to 31°C. Starch hydrolysis, milk peptonization, melanin production, gelatin liquefaction and H_2S production were positive.

Utilization of carbon sources by this strain was examined according to the methods of PRIDHAM and GOTTLIEB.[8] Results are summarized in Table 3. Almost all carbon sources were utilized except cellulose, chitin and sodium acetate.

Microscopic studies and cell wall composition analysis of the strain indicate that strain No. 43708 is classified in the genus *Streptomyces* Waksman and Henrici 1943. Accordingly, a comparison of this strain was made with the published descriptions[9~12] of various *Streptomyces* species. Strain No. 43708 is considered to resemble *Streptomyces griseorubiginosus* (Ryabova and Preobrazhenskaya 1957) Pridham, Hesseltine and Benedict 1958, *S. phaeopurpureus* Shinobu 1957, and *S. phaeoviridis* Shinobu 1957. Therefore, the cultural characteristics of strain No. 43708 was directly compared with these three species. As shown in Table 1, strain No. 43708 closely resembled *S. griseorubiginosus* IFO 13047. Therefore, further detailed comparison was made with strain No. 43708 and *S. griseorubiginosus* IFO 13047. As shown in Tables 2 and 3, strain No. 43708 is in good agreement with *S. griseorubiginosus* IFO 13047 except for NaCl tolerance and utilization of raffinose and inulin. These differences do not seem to be sufficient to distinguish strain No. 43708 from *S. griseorubiginosus*. Therefore, strain No. 43708 is identified as *Streptomyces griseorubiginosus* No. 43708.

Fermentation

A loopful of the strain No. 43708 from a mature slant was inoculated into each of twenty 500-ml flasks containing 160 ml of the sterile seed medium shown in Table 4. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 30°C for three days. The content of twenty flasks was inoculated into a 200-liter stainless steel jar fermentor containing 160 liters of sterile fermentation medium shown in Table 4. The fermentation was carried out at 30°C for 4 days, with aeration of 160

Table 1. Cultural characteristics of strain No. 43708 and *Streptomyces griseorubiginosus* IFO 13047, *Streptomyces phaeopurpureus* IFO 12899 and *Streptomyces phaeoviridis* IFO 12900.

Medium		No. 43708	IFO 13047	IFO 12899	IFO 12900
Oatmeal agar	G:	Poor	Poor	Moderate	Poor
	A:	Grayish white	Grayish white	Grayish white	Light gray
	R:	Pale pink	Pale pink	Pale cinnamon pink	Colorless
	S:	Pale pink	Pale pink	Pale yellow orange	None
Yeast - malt extract agar	G:	Abundant	Abundant	Abundant	Moderate
	A:	Light gray	Light gray	Light gray	Light gray
	R:	Brown	Brown	Pale reddish brown	Light brown
	S:	Dull reddish orange	Dull reddish orange	Brown	None
Inorganic salts - starch agar	G:	Abundant	Moderate	Moderate	Moderate
	A:	Light gray	Gray	Light gray	White
	R:	Pale yellow orange	Pale yellowish brown	Pale yellowish brown	Pale yellow
	S:	Pale yellow orange	Pale yellow orange	None	None
Glucose - asparagine agar	G:	Moderate	Moderate	Abundant	Moderate
	A:	Light gray	Light gray	Light gray	Gray
	R:	Dull reddish orange	Dull reddish orange	Reddish orange	Pale reddish brown
	S:	Pale pink	Pale pink	Reddish orange	Pale yellow
Glycerol - asparagine agar	G:	Abundant	Abundant	Abundant	Moderate
	A:	Light gray	Gray	Light gray	Gray
	R:	Dull gray	Dull reddish orange	Reddish ogange	Yellowish brown
	S:	Brown	Pale yellow orange	Reddish orange	None
Sucrose - nitrate agar	G:	Abundant	Abundant	Abundant	Moderate
	A:	None	None	None	None
	R:	Brown	Brown	Reddish orange	Colorless
	S:	Pale orange	Pale organge	None	None
Nutrient agar	G:	Moderate	Moderate	Moderate	Poor
	A:	None	None	None	Grayish white
	R:	Colorless	Colorless	Pale yellow brown	Pale yellow
	S:	Pale yellow	Pale yellow	None	None
Potato - dextrose agar	G:	Abundant	Abundant	Moderate	Moderate
	A:	Light gray	Light gray	Light gray to pale yellow orange	Pale yellow orange
	R:	Dark reddish brown	Dark reddish brown	Brown	Pale yellowish brown
	S:	None	None	Brown	Pale yellow
Tyrosine agar	G:	Abundant	Abundant	Abundant	Abundant
	A:	Pale cinnamon pink	Pale cinnamon pink	Gray	Gray
	R:	Black	Black	Black	Dark brown
	S:	Dark brown	Dark brown to black	Black	Dark brown
Peptone - yeast extract - iron agar	G:	Moderate	Moderate	Moderate	Moderate
	A:	None	None	None	None
	R:	Colorless	Colorless	Colorless	Colorless
	S:	Black	Black	Black	Black

Abbreviation: G; Growth, A; aerial mass color, R; reverse side color, S; soluble pigment.

Table 2. Physiological properties of strain No. 43708 and *Streptomyces griseorubiginosus* IFO 13047.

	No. 43708	IFO 13047
Temperature range for growth	17~41°C	17~41°C
Optimum temperature	29~31°C	29°C
Nitrate reduction	Negative	Negative
Starch hydrolysis	Positive	Positive
Milk coagulation	Negative	Negative
Milk peptonization	Positive	Positive
Melanin production	Positive	Positive
Gelatin liquefaction	Positive	Positive
H_2S production	Positive	Positive
Urease activity	Negative	Negative
NaCl tolerance (%)	<7%	<10%

Table 3. Carbon sources utilization by strain No. 43708 and *Streptomyces griseorubiginosus* IFO 13047.

	No. 43708	IFO 13047
D-Glucose	+	+
Sucrose	+	+
Glycerol	+	+
D-Xylose	+	+
D-Fructose	+	+
Lactose	+	+
Maltose	+	+
Rhamnose	+	+
Raffinose	±	+
D-Galactose	+	+
L-Arabinose	+	+
D-Mannose	+	+
D-Trehalose	±	±
Inositol	+	+
Mannitol	+	+
Inulin	±	+
Cellulose	−	−
Salicin	+	+
Chitin	−	−
Sodium citrate	+	+
Sodium succinate	+	+
Sodium acetate	−	−

Symbols: +; Utilization, ±; doubtful utilization, −; no utilization.

liters/minute, agitation of 200 rpm and inner pressure of 1.0 kg/cm^2. The progress of the fermentation was monitored by the antibacterial activity using *Staphylococcus aureus* 209P.

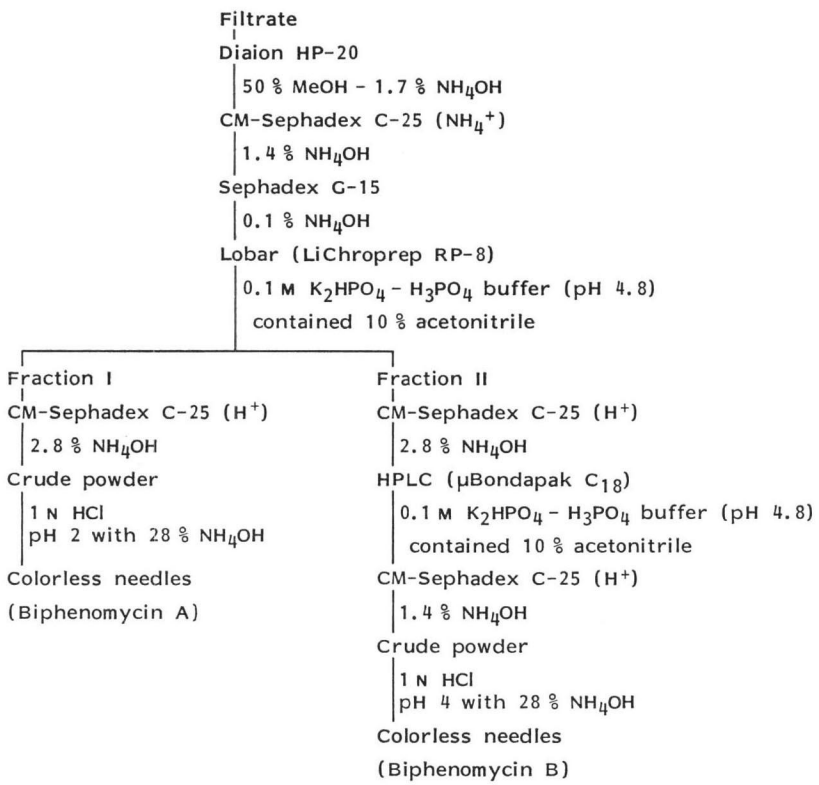
Isolation

The isolation procedure for biphenomycins A and B is summarized in Fig. 3. The cultured broth (160 liters) was filtered with the aid of filter aid (Radiolite). The filtrate (150 liters) was passed through a column of Diaion HP-20 (60 liters, Mitsubishi Chemical Industries Ltd.). The column was washed with water (120 liters) and 50% aqueous methanol (120 liters) and then eluted with 50% aqueous methanol containing 1.7% aqueous ammonium hydroxide. The eluate (300 liters) was concentrated *in vacuo* to 2 liters, and adsorbed on a column of CM-Sephadex C-25 (12 liters, NH_4^+ form, Pharmacia Fine Chemicals). The column was washed with water (12 liters) and 1 M NaCl solution (12 liters), and then eluted with 1.4% aqueous ammonium hydroxide. The active fraction (12 liters) was concentrated *in vacuo* to 200 ml and adsorbed on a column of Sephadex G-15 (1 liter, Pharmacia Fine Chemicals). The column was washed with water (6 liters) and continually eluted with 0.1% aqueous ammonium hydroxide. The active fraction (1 liter) was concentrated to 5 ml *in vacuo*, ad-

Table 4. Media used for production of biphenomycins A and B.

Seed medium	% (w/v)	Production medium	% (w/v)
Corn starch	1.0	Sucrose	5.0
Glycerol	1.0	Dried yeast	0.5
Glucose	0.5	$(NH_4)_2SO_4$	0.5
Cotton seed flour	1.0	K_2HPO_4	0.1
Dried yeast	0.5	$MgSO_4 \cdot 7H_2O$	0.1
Corn steep liquor	0.5	NaCl	0.1
$CaCO_3$	0.2	$CaCO_3$	0.5
pH 6.5		$FeSO_4 \cdot 7H_2O$	0.001
		pH 7.0	

Fig. 3. Purification procedure for biphenomycins A and B.



justed to pH 2 with 1 N HCl and applied to a Lobar column of LiChroprep RP-8 size C (470 ml, Merck). The column was developed with 0.1 M K_2HPO_4 - H_3PO_4 buffer (pH 4.8) containing 10% acetonitrile. Biphenomycins A and B were eluted from 60 ml to 80 ml (fraction I), and from 85 ml to 120 ml (fraction II) respectively.

Fraction I was desalted with CM-Sephadex C-25 (H^+ form, 1 liter, Pharmacia Fine Chemicals). The active fraction (400 ml) was dried *in vacuo* to give a pale brown powder (30 mg). The powder was suspended in 1 N HCl (10 ml). Then, a solution of 28% aqueous ammonium hydroxide 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 A (20 mg).

Fraction II was desalted with CM-Sephadex C-25 (H^+ form, 1 liter) and then concentrated *in vacuo* to 2 ml. Further purification was achieved by a high performance liquid chromatography on μBondapak C_{18} (7.8 mm × 300 mm, Waters Associates Ltd.) using 0.1 M K_2HPO_4 - H_3PO_4 buffer (pH 4.8) containing 10% acetonitrile, with pressure of 141 kg/cm², flow rate at 2 ml/minute. Under these conditions, retention time of biphenomycins A and B were 4.2 minutes and 4.8 minutes, respectively. The active fraction containing biphenomycin B was also desalted with CM-Sephadex C-25 (H^+ form). According to the same procedure for crystallization as biphenomycin A, colorless needles of biphenomycin B (2 mg) were obtained.

Physico-chemical Properties

The physico-chemical properties of biphenomycins A and B are summarized in Table 5. Their

Table 5. Physico-chemical properties of biphenomycins A and B.

	Biphenomycin A	Biphenomycin B
Appearance	Colorless needles	Colorless needles
Nature	Amphoteric	Amphoteric
MP (°C, dec)	205~209	206~209
$[\alpha]_D^{20}$ (*c* 0.1, 1 N HCl)	−22.5°	−10.6°
FAB-MS (*m/z*)	489 (M^++1)	473 (M^++1)
Molecular formula	$C_{23}H_{28}N_4O_3 \cdot 2HCl \cdot H_2O$	$C_{23}H_{28}N_4O_7 \cdot 2HCl$
Anal (%): Found	C 47.80, H 5.85, N 9.75, Cl 12.11	C 50.15, H 5.68, N 10.08, Cl 12.77
Calcd	C 47.65, H 5.62, N 9.67, Cl 12.23	C 50.65, H 5.54, N 10.27, Cl 13.00
UV: $\lambda_{max}^{H_2O}$ nm (ε)	264 (18,600), 287 (sh)	264 (23,500), 287 (sh)
$\lambda_{max}^{0.1N\ HCl}$ nm (ε)	264 (18,550), 287 (sh)	264 (25,100), 287 (sh)
$\lambda_{max}^{0.1N\ NaOH}$ nm (ε)	288 (24,000), 303 (sh)	288 (31,100), 303 (sh)
Color test: Positive	Ninhydrin, $FeCl_3$	Ninhydrin, $FeCl_3$
Negative	Molish, Dragendorff, diacetyl	Molish, Dragendorff, diacetyl
Solubility: Soluble	H_2O	H_2O
Slightly soluble	Acetone, MeOH	Acetone, MeOH
Insoluble	*n*-Hexane, benzene, $CHCl_3$, EtOAc	*n*-Hexane, benzene, $CHCl_3$, EtOAc

Fig. 4. UV spectra of biphenomycin A.

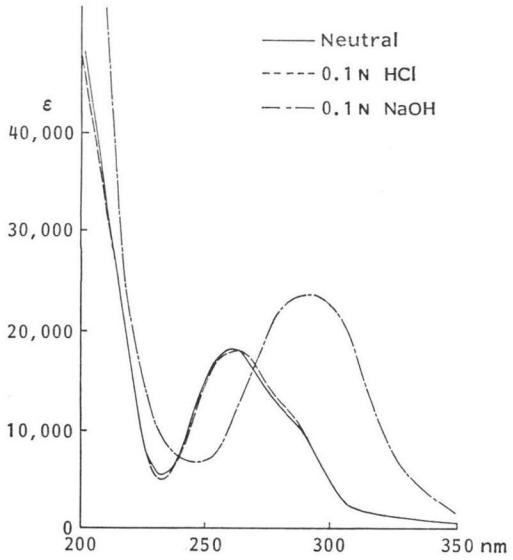
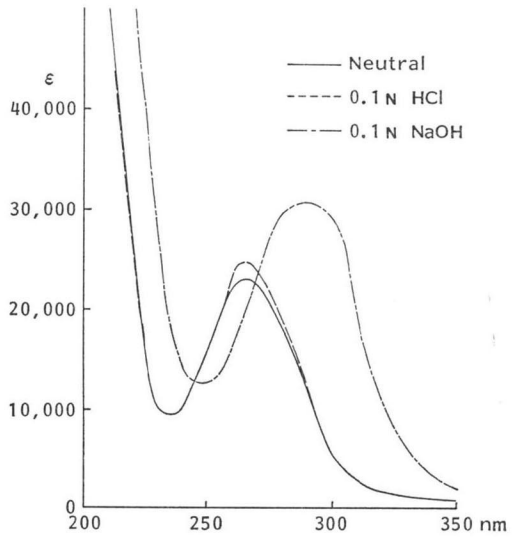


Fig. 5. UV spectra of biphenomycin B.



UV and IR spectra are shown in Figs. 4~7. These data indicate that the two compounds have similarities in most of their physico-chemical properties. They however, differ in their specific optical rotation values and molecular formulae. Thus, biphenomycin B is possibly a deoxy-derivative of biphenomycin A. In the structure of each component, there may be present a phenolic moiety and a peptide function judging from their UV, IR spectra and color tests.

Biological Properties

The antimicrobial spectra of biphenomycins A and B determined by the agar dilution method are shown in Tables 6 and 7. Biphenomycins A and B are active against Gram-positive bacteria, weakly active against Gram-negative bacteria, and inactive against fungi.

Fig. 6. IR spectrum of biphenomycin A.

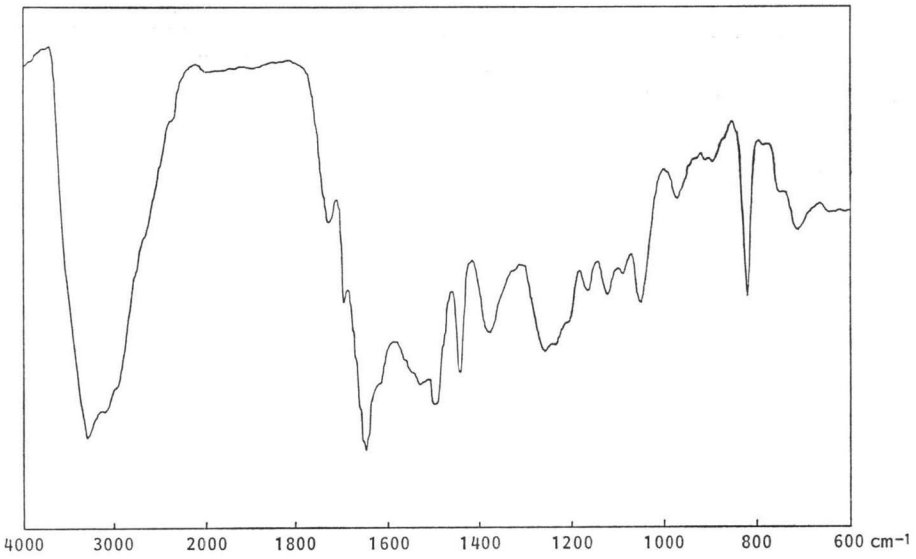
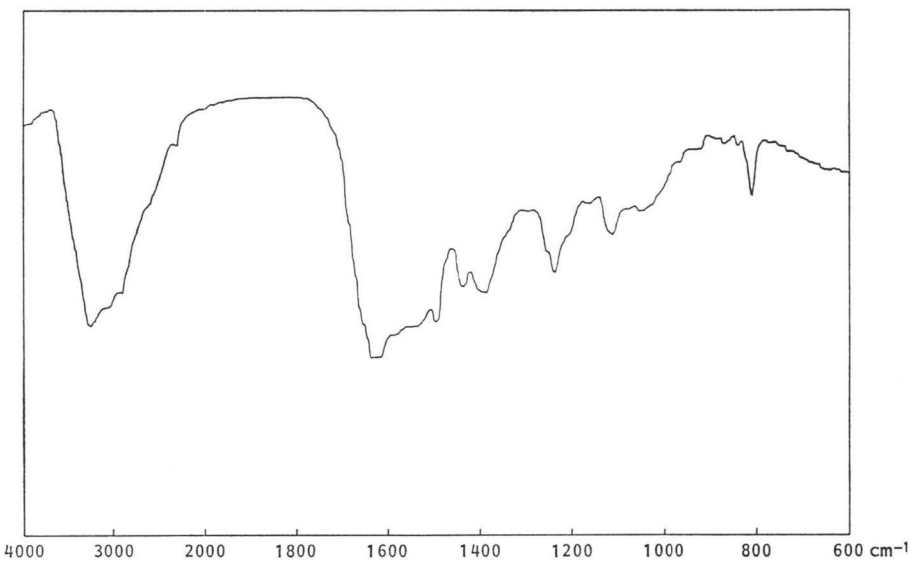


Fig. 7. IR spectrum of biphenomycin B.



A single intravenous administration of 500 mg/kg of biphenomycin A into ICR mice did not result in any toxic symptoms for 1 week after injection.

The *in vivo* activity of biphenomycin A against experimental infections due to bacteria was examined. Mice were challenged intraperitoneally with clinical isolates of *S. aureus* and other organisms. The *in vivo* activity of biphenomycin A against these infections is expressed in terms of the ED_{50} values. The results are shown in Table 8. Biphenomycin A has protective efficacy against infections by various strains of Gram-positive bacteria. It is to be noticed that the antibiotic is active *in vitro* and *in vivo* against *S. aureus* 2508 and 2485 which are highly resistant to ampicillin.

Table 6. Antimicrobial spectra of biphenomycins A and B.

Test organism	Medium*	MIC (μg/ml)	
		Biphenomycin A	Biphenomycin B
Staphylococcus aureus 209P JC-1	1	0.25	0.25
	2	8	16
	3	125	125
Bacillus subtilis ATCC 6633	1	2	4
	2	16	32
	3	125	125
Micrococcus luteus PCI 1001	2	1	8
Escherichia coli NIHJ JC-2	1	4	4
	2	64	64
	3	64	64
Proteus vulgaris IAM 1025	1	4	4
	2	>100	>100
	3	>100	>100
Pseudomonas aeruginosa NCTC 10490	2	>100	>100
Candida albicans	4	>100	>100
Aspergillus niger IAM 2561	4	>100	>100

Agar dilution method (1×10^6/ml, 37°C, 20 hours).

* Medium 1: serum agar (20% horse serum, 0.01% $FeSO_4\cdot7H_2O$, agar 1.5%), 2: Mueller-Hinton agar, 3: bouillon agar, 4: SABOURAUD's agar.

Table 7. Antimicrobial spectrum of biphenomycin A against clinical isolates.

Test organism	Medium*	MIC (μg/ml)		
		Biphenomycin A	Ampicillin	Cefazolin
Staphylococcus aureus 47	1	0.1	0.39	0.78
S. aureus 2508	1	0.1	25	1.56
S. aureus 2485	1	0.2	50	6.25
S. aureus 2536	1	0.1	ND	ND
Streptococcus faecalis 50	1	0.05	0.05	0.025
S. pyogenes FP 1302	2	6.25	≦0.025	≦0.025
S. pneumoniae III FP 166	2	0.2	≦0.025	≦0.025
Escherichia coli 22	1	6.25	1.56	1.56
Proteus vulgaris 8	1	3.13	25	6.25
P. mirabilis 3002	1	3.13	0.39	6.25

Agar dilution method (1×10^6/ml, 37°C, 20 hours). ND: Not determined.

* Medium 1: serum agar, 2: Mueller-Hinton agar containing 5% horse blood.

Table 8. Protective effect of biphenomycin A against experimental infection in mice.

Challenged organism* (challenged size, cfu/mouse)		ED_{50}** (mg/kg, sc)		
		Biphenomycin A	Ampicillin	Cefazolin
Staphylococcus aureus 47	(7.4×10^7)	0.11	12.3	2.03
S. aureus 2508	(4.0×10^8)	0.21	40	40
S. aureus 2485	(1.4×10^8)	0.56	80	320
S. aureus 2536	(5.4×10^8)	0.07	ND	ND
Streptococcus faecalis 50	(4.8×10^8)	1.64	144	320
S. pyogenes FP 1302	(3.7×10^3)	6.47	0.08	0.27
S. pneumoniae III FP 166	(6.0×10^6)	9.83	6.96	7.14
Escherichia coli 22	(6.2×10^6)	80	16.9	80
Proteus vulgaris 8	(1.3×10^8)	36.8	160	160
P. mirabilis 3002	(1.0×10^8)	40	17.6	ND

* Ten mice (ICR strain, 4 weeks, male) of each group were challenged intraperitoneally with 0.5 ml of overnight culture.

** The antibiotics were administered subcutaneously once 1 hour after the challenge.
ND: Not determined.

Discussion

We have described in this paper that biphenomycins A and B are amphoteric, water-soluble, peptide antibiotics. Furthermore, the biphenomycins are novel cyclic peptides containing a biphenyl moiety included in a 15-membered ring as described in succeeding papers.[2,3] It was reported by MARTIN *et al.*,[13] that *S. filipinensis* produced two new, water-soluble, basic antibiotics, LL-AF 283 α and β which exhibited *in vitro* and *in vivo* antimicrobial activities. LL-AF 283 α and β resemble the biphenomycins in physico-chemical properties (UV, IR and solubility). However, the molecular weights of biphenomycins A and B (488 and 472, respectively) are distinct from those of LL-AF 283 β (MW 433). The elemental analysis and $[\alpha]_D$ of the biphenomycins are also distinct from those of LL-AF 283 α and β.

Biphenomycins A and B showed intense antibacterial activity in the serum medium containing 20% horse serum, but poor activity was observed in complex media such as Mueller-Hinton medium and bouillon medium (Table 6). Remarkably the antibacterial activity was inhibited when peptone was added to the serum medium (unpublished data). This suggests that the activity of biphenomycins A and B may be competitively inhibited by peptides in assay medium.

Biphenomycin A is effective against bacterial infections in mice, and such efficacy *in vivo* exceeds our expectation presumed from the activity *in vitro*. Although the reason for the superior efficacy *in vivo* is not yet known, it may be due to the fact that the antibiotic shows intense antibacterial activity in the serum medium.

Our experiments in this paper show the possibility that these compounds may be clinically useful antibiotics of a new type.

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