SPERABILLINS, NEW ANTIBACTERIAL ANTIBIOTICS WITH POTENT *IN VIVO* ACTIVITY

TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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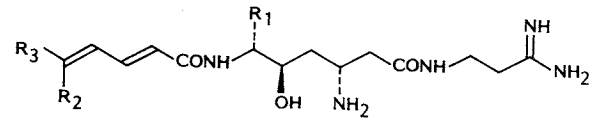
A Gram-negative bacterium was found to produce new antibacterial antibiotics, sperabillins A, B, C and D, and the producing bacterium was characterized and identified as *Pseudomonas fluorescens* YK-437. Sperabillins were isolated by column chromatographies using cation-exchange resins, activated carbon and cation-exchange Sephadex, and preparative reverse-phase HPLC. Sperabillins showed antibacterial activity against Gram-negative and Gram-positive bacteria including antibiotic-resistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Sperabillin A inhibited DNA, RNA, protein, and cell wall biosynthesis in *Escherichia coli*. Sperabillins showed good protective effects in experimentally infected mice.

In the course of our screening program for new antibiotics which show antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, we found that a Gram-negative bacterium, strain YK-437, produces new antibacterial antibiotics named sperabillins A, B, C and D (formerly called TAN-749[1,2]). Sperabillins have unique structures consist of an amino acid to which an unsaturated fatty acid and aminoethylamidine are bound by amide bonds like a peptide (Fig. 1[3]). Sperabillins were effective *in vivo* against infections of Gram-negative and Gram-positive bacteria. These antibiotics are of particular interest, because the pretreatment of mice with sperabillin A protected mice against lethal bacterial infection.

In this paper, we describe the taxonomy of the producing organism and the fermentation, isolation and biological activities of sperabillins.

Fig. 1. Structures of sperabillins A, B, C and D.



Sperabillin A	$R_1=H$	$R_2=CH_3$	$R_3=H$
Sperabillin B	$R_1=CH_3$	$R_2=CH_3$	$R_3=H$
Sperabillin C	$R_1=H$	$R_2=H$	$R_3=CH_3$
Sperabillin D	$R_1=CH_3$	$R_2=H$	$R_3=CH_3$

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Materials and Methods

Taxonomy

Utilization of organic compounds, production of soluble pigments and accumulation of poly-β-hydroxybutyrate were examined by the methods of STANIER *et al.*[4], while the other physiological characteristics were examined by the methods of COWAN and STEEL[5].

Assay of Sperabillin

Sperabillins were detected by their antibacterial activity against *P. aeruginosa* C141[6] and NS[7]. The contents of sperabillins were determined by reverse-phase HPLC using a YMC-Pack A-312 (Yamamura Chem. Lab.) with a mobile phase of 30% acetonitrile - 0.04 M phosphate buffer (pH 3.0) containing 0.02 M 1-octanesulfonic acid.

Incorporation of Radioactive Precursors

Escherichia coli LD-2[8], a lysine and diaminopimelic acid auxotroph, was cultivated for 1 day at 37°C in DYAB medium[8]. Cells were centrifuged, washed twice with M9 medium[9] and suspended to give an absorbance of 0.5 at 600 nm in M9 medium supplemented with 20 μg/ml of each L-threonine, L-leucine, L-lysine and diaminopimelic acid and 2 μg/ml of thiamine. For the protein and the cell wall synthesis assays, L-leucine and diaminopimelic acid were omitted from the medium described above, respectively. An incubation mixture consisting of 0.4 ml of the above cell suspension; 0.05 ml of [*methyl*-^{3}H]thymidine solution (25 Ci/mmol, 10 μCi/ml), [2-^{14}C]uridine solution (51 mCi/mmol, 10 μCi/ml), L-[*U*-^{14}C]leucine solution (10 mCi/mmol, 10 μCi/ml), or DL-[G-^{3}H]diaminopimelic acid (1 Ci/mmol, 100 μCi/ml); and 0.05 ml of antibiotic solution was incubated for 1 hour at 37°C without shaking. After 0.1 ml of 30% TCA was added, the mixture was allowed to stand for 30 minutes in an ice bath. In experiments looking at the incorporation of leucine and diaminopimelic acid, the mixture was further heated at 90°C for 10 minutes. The mixture was centrifuged, and the acid-insoluble precipitate was washed with 0.5 ml of 5% TCA and suspended with 0.1 ml of 0.1 M ammonium acetate. Half of this suspension was transferred to a toluene-based scintillator, and the radioactivity was counted by a scintillation counter.

Results and Discussion

Discovery and Taxonomy of the Producing Organism

Strain YK-437 was isolated from a plant specimen collected in the Kita-azumi district of Nagano

Table 1. Morphological and physiological characteristics of strain YK-437.

Cell shape	Rods	Urease	+
Size (μm)	0.5~1.0 × 1.5~4.0	Oxidase	+
Flagella	Polar, multitrichous	Catalase	+
Gram stain	Negative	Oxygen demand	Aerobic
Spore formation	−	O-F test	Oxidative
Reduction of nitrate	−	Range of growth	
Denitrification	−	pH	4.1~8.5
Methyl red test	−		(optimum 6.3~8.2)
Voges-Proscauer test	−	temperature (°C)	8~36
Production of indole	−		(optimum 11~24)
Production of H_2S	−	Hydrolysis of starch	−
Utilization of		Liquefaction of gelatin	+
citrate	+	Degradation of Tween 80	+
potassium nitrate	+	Accumulation of	−
ammonium sulfate	+	poly-β-hydroxybutyrate	
Production of fluorescent	+	Arginine dihydrolase	+
pigment		Tolerance to NaCl (%)	5
Growth factor requirement	−	GC content of DNA (%)	66.4±1.5

Table 2. Acids and gas formation from sugars and utilization of organic compounds by strain YK-437.

Compound	Growth	Acid	Gas	Compound	Growth	Acid	Gas
L-Arabinose	+	+	−	Inositol	+	−	−
D-Xylose	+	+	−	Glycerol	+	−	−
D-Glucose	+	+	−	Starch	−	−	−
D-Mannose	+	+	−	2-Ketogluconate	+		
D-Fructose	+	−	−	Geraniol	−		
D-Galactose	+	+	−	L-Valine	+		
Maltose	+	−	−	β-Alanine	+		
Sucrose	+	+	−	DL-Arginine	+		
Lactose	+	−	−	Propionate	−		
Trehalose	+	−	−	Butyrate	−		
D-Sorbitol	+	−	−	Adnitol	−		
D-Mannitol	+	−	−	Propylene glycol	−		

Prefecture, Japan. It was selected as an antibiotic producer based on its antibacterial activity against *P. aeruginosa*. Its colonies were opaque, beige, circular, convex, and entire-edged on nutrient agar. The cultural and physiological characteristics are listed in Tables 1 and 2. The following key characteristics such as Gram-negative rods, polar multitrichous flagella, aerobic, oxidative respiration, growth factor requirement negative, and a mol% G+C of 66.5 indicate that strain YK-437 belongs to the genus *Pseudomonas*. Its characteristics were compared with those of the species of *Pseudomonas* described in BERGEY's Manual of Systematic Bacteriology[10]. Strain YK-437 did not accumulate poly-β-hydroxybutyrate, produced fluorescent pigments and arginine dihydrolase, did not denitrify, and hydrolyzed gelatin. From these characteristics and the pattern of organic compound utilization, strain YK-437 was identified as *Pseudomonas fluorescens* and has been deposited in the Institute for Fermentation, Osaka with the accession number IFO 14446. This strain was also deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan and assigned the accession number FERM BP-1005.

Fermentation

Seed culture for large scale fermentation was initiated by transferring a loopful of cells grown on a nutrient agar slant to a 2-liter Sakaguchi flask containing 500 ml of the following seed medium: glucose 2%, soluble starch 3%, corn steep liquor 0.3%, soy bean flour 1%, Polypepton (Nihon Pharmaceutical Co., Ltd.) 0.5%, NaCl 0.5%, and $CaCO_3$ 0.5% (pH 7.0). The flask was incubated at 24°C for 2 days on a reciprocal shaker. The culture broth was then transferred to a 200-liter fermenter containing 120 liters of the same seed medium supplemented with 0.05% Actcol (an antifoaming agent, Takeda Chem. Ind., Ltd.), and cultivation was carried out at 24°C for 2 days with aeration (120 liters/minute) and agitation (180 rpm). Fifty liters of this culture broth was transferred to a 2,000-liter fermenter containing 1,200 liters of the following production medium: glycerol 3%, glucose 0.1%, Polypepton 0.5%, meat extract 0.5%, NaCl 0.5%, and Actcol 0.05% (pH 7.0). The fermentation was carried out at 24°C for 66 hours with aeration (1,200 liters/minute) and agitation (150 rpm). Under these conditions, the titer of sperabillin A was about 170 μg/ml.

To improve the fermentation conditions, we investigated the effects of the cultivation temperature and medium at a flask level. As shown in Fig. 2, at 28°C, the optimum temperature for sperabillin production, the titer of sperabillin A is about 3 times that at 24°C. The preliminary studies on the fermentation medium revealed that supplementation with the amino acids listed in Table 3 stimulated sperabillin production. Of these amino acids L-valine was the most effective, and the addition of L-valine

Fig. 2. Effect of cultivation temperature on sperabillin production.

Open bars: Sperabillin A, closed bars: sperabillin B. The titer of sperabillins A and B was determined by the HPLC method.

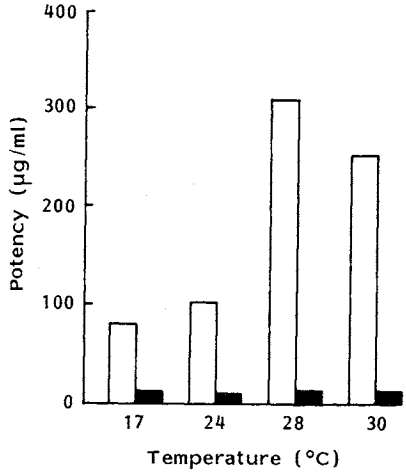


Fig. 3. Time-course of sperabillin production in large scale fermentation.

Sperabillin A (●); determined by the HPLC method. Growth (▲); the DNA content was determined by the method of BURTON[14] after extraction with 5% perchloric acid. pH (■).

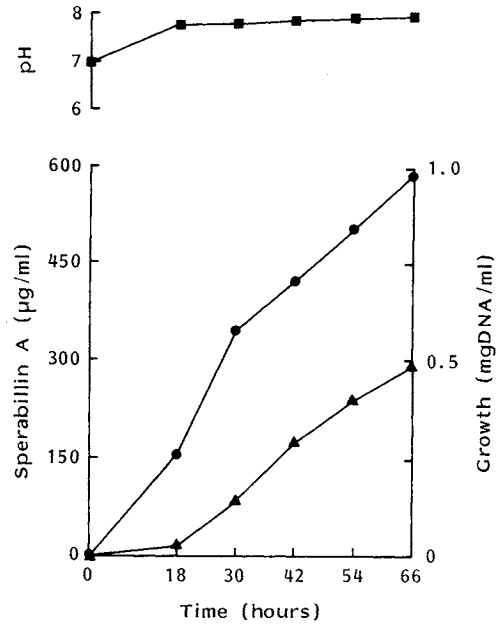


Table 3. Effect of amino acid supplementation on sperabillin production.

Amino acid (0.5%)	A	B
	μg/ml	
L-Valine	639	27
L-Threonine	423	27
L-Alanine	301	45
L-Aspartic acid	459	32
L-Glutamic acid	405	31
None	283	27

Basal medium: Glycerol 3%, glucose 0.1%, Polypepton 0.5%, meat extract 0.5%, NaCl 0.5%, pH 7.
Cultivation temperature: 28°C.

resulted in an approximately 2.3-fold increase in sperabillin production. The addition of L-valine may cause a change in nitrogen compounds metabolism, but the reason for this stimulation of sperabillin production is not clear. According to these results, the cultivation temperature was changed to 28°C, and L-valine (0.5%) was added to the basal production medium. The titer of sperabillin A in a large-scale fermentation under these improved culture conditions was about 590 μg/ml (Fig. 3).

Isolation

The culture broth of strain YK-437 (1,150 liters, pH 6.5) was filtered through Hyflo-Super Cel (John's Manvile Prod.). The filtrate was adjusted to pH 6.2 (1,220 liters) and applied to a column of Amberlite IRC-50 (Na^+ type, 20 liters, Rohm & Haas Co.), and the active substance was eluted with 0.5 N HCl (200 liters). The eluate was adjusted to pH 5.6, applied to a column of Diaion SP-207 (20 liters, Mitsubishi Kasei Co.), and eluted with water (120 liters). The active fractions were combined and concentrated (2 liters). The concentrate was chromatographed on Amberlite CG-50 (NH_4^+ type, 3 liters) and eluted with 0.4 M NaCl (40 liters) and 0.6 M NaCl (40 liters). The former eluate containing a mixture of sperabillins A, B, C and D and the latter eluate containing sperabillin A were separately desalted with activated

carbon chromatographies eluting with 8% isobutyl alcohol (4 liters and 10 liters, respectively). The eluate containing only sperabillin A was concentrated and freeze-dried to give a white powder of sperabillin A (2HCl, 47.5 g). The eluate containing the mixture of sperabillins A ~ D (from 3 batches) was concentrated, and the concentrate was again chromatographed on Amberlite CG-50 (NH_4^+ type, 3 liters). Stepwise elution with 0.5 M NaCl (40 liters) and 0.8 M NaCl (40 liters) afforded two fractions. The latter fraction was desalted, concentrated and lyophilized to give a white powder of sperabillin A (20 g) containing a small amount of sperabillin C. After desalination with activated carbon chromatography, the former fraction was purified by a column chromatography using CM-Sephadex C-25 (Na^+ type, 1 liter, Pharmacia) and eluting with 0.2 M NaCl. The concentrate of the eluate containing sperabillins B and D was loaded onto a preparative HPLC column of YMC-Pack S-30 ODS (Yamamura Chem. Lab.) with a mobile phase of 5% MeOH - 0.02 M phosphate buffer (pH 3.0). The fraction containing only sperabillin B was applied to a column of CM-Sephadex C-25 and desalted with activated carbon to afford a white powder of sperabillin B (3.05 g). The fraction containing both sperabillins B and D was again purified by preparative HPLC. The fraction containing only sperabillin D was passed through a column of Amberlite IRA-402 (Cl^- type, 10 ml). The effluent was desalted by carbon chromatography and concentrated to give white powder of sperabillin D (15.5 mg). The powder of sperabillin A (3 g) containing a small amount of sperabillin C was repeatedly chromatographed on CM-Sephadex C-25, Amberlite CG-50 and activated carbon by the above-mentioned methods and concentrated to give a powder. The powder was twice treated by preparative HPLC and the above-described chromatographies to give a white powder of sperabillin C (2HCl, 20.2 mg).

Sperabillin·2HCl salts show positive color reactions to Ehrlich, dimethylaminobenzaldehyde and potassium permanganate reagents and negative color reactions to ninhydrin, Greig-Leaback, Sakaguchi and Dragendorff reagents. They are highly soluble in water or dimethyl sulfoxide, soluble in methanol and sparingly soluble in acetone or ethyl acetate. The results of a stability test of sperabillin A at 80°C in phosphate buffer of various pH's indicated that this compound is relatively unstable in the basic pH region.

The physico-chemical properties of sperabillins are summarized in Table 4. The molecular formulae were determined from elemental analyses, molecular ion peaks in SI-MS and carbon numbers in ^{13}C NMR spectrometry to be $C_{15}H_{27}N_5O_3 \cdot 2HCl$ for sperabillins A and C and $C_{16}H_{29}N_5O_3 \cdot 2HCl$ for sperabillins B and D. The UV, IR and ^{13}C NMR spectra of sperabillin A are shown in Figs. 4, 5 and 6, respectively.

Table 4. Physico-chemical properties of the hydrochlorides of sperabillins A, B, C and D.

Property	A		B		C		D	
Appearance	White powder		White powder		White powder		White powder	
$[\alpha]_D$ (c)[a]	−11° (1.06)		+56° (1.0)		−11° (0.68)		+30° (0.5)	
SI-MS: $(M+H)^+$	326		340		326		340	
Molecular formula	$C_{15}H_{27}N_5O_3$ 2HCl (H_2O)		$C_{16}H_{29}N_5O_3$ 2HCl ($1\frac{1}{2}H_2O$)		$C_{15}H_{27}N_5O_3$ 2HCl ($\frac{1}{2}H_2O$)		$C_{16}H_{29}N_5O_3$ 2HCl ($\frac{1}{2}H_2O$)	
Analysis	Calcd:	Found:	Calcd:	Found:	Calcd:	Found:	Calcd:	Found:
C	43.27	43.61	43.94	43.62	44.23	44.35	45.61	45.15
H	7.50	7.37	7.37	7.53	7.42	7.83	7.65	7.98
N	16.82	17.01	16.01	16.06	17.19	17.28	16.62	16.44
O	15.37		16.46		13.75		13.29	
Cl	17.03	16.40	16.21	16.31	17.41	17.59	16.83	16.59
UV λ_{max} nm (ε)[a]	266 (27,400)		264 (28,400)		262 (27,600)		262 (27,500)	

[a] Measured at 23 ~ 25°C in water.

Sperabillins showed similar UV spectra one another and their absorption maxima were observed at 262~266 nm (ε about 28,000). The IR spectra of sperabillins appeared a typical pattern of peptide-like compounds. The chemical characterization and structure elucidation of sperabillins will be reported elsewhere.

Biological Activity

Sperabillins showed antibacterial activity against some species of *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, and *Staphylococcus* (Table 5). Sperabillins B

Fig. 4. UV spectrum of sperabillin A hydrochloride (in H_2O).

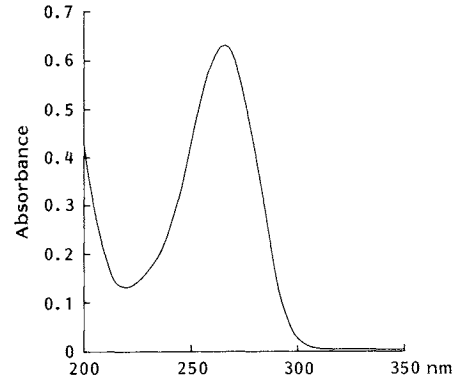


Fig. 5. IR spectrum of sperabillin A hydrochloride (KBr).

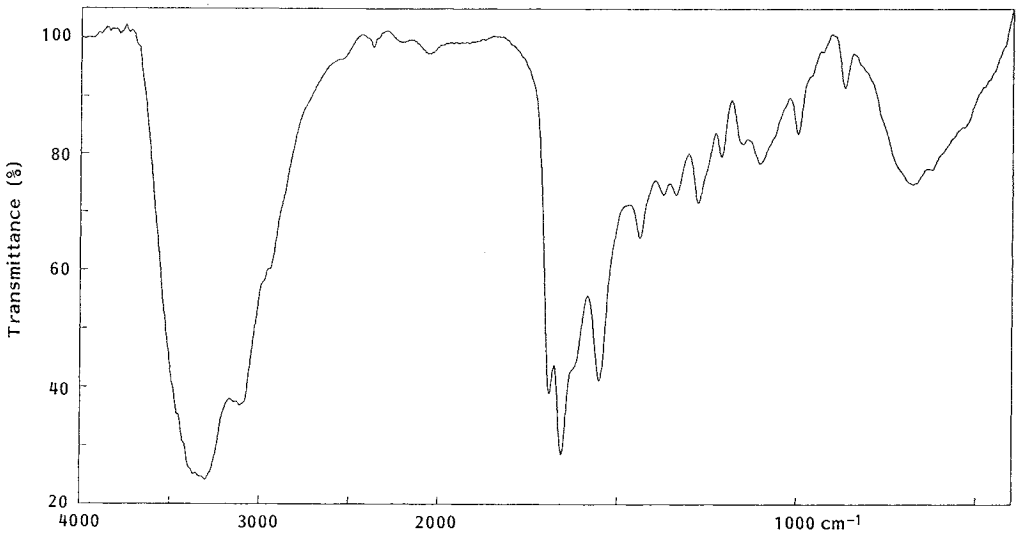


Fig. 6. ^{13}C NMR spectrum of sperabillin A hydrochloride (75 MHz, in H_2O).

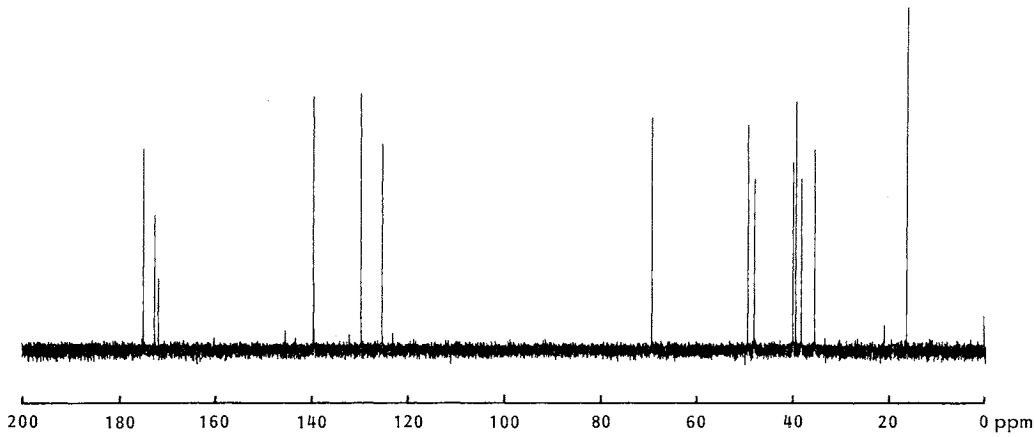


Table 5. Antibacterial activity of sperabillins A, B, C and D.

Organism	MIC (μg/ml) at 10^6 cfu/ml			
	A	B	C	D
Staphylococcus aureus FDA 209P	50	12.5	>100	50
S. epidermidis IFO 3762	>100	>100	>100	>100
Bacillus subtilis NIHJ PCI 219	>100	>100	>100	>100
Escherichia coli NIHJ JC-2	>100	>100	>100	>100
Serratia marcescens IFO 12648	>100	>100	>100	>100
Klebsiella pneumoniae IFO 3317	>100	100	>100	>100
Proteus mirabilis ATCC 21100	>100	100	>100	>100
P. vulgaris IFO 3988	100	25	100	100
Pseudomonas aeruginosa IFO 3080	25	50	50	100
Alcaligenes faecalis IFO 13111	3.13	6.25	12.5	6.25
Acinetobacter calcoaceticus IFO 12552	12.5	3.13	25	3.13

MIC values were determined by an agar dilution method using DYAB medium.

Table 6. Susceptibility of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to sperabillin A.

Organism	Remarks[a]	MIC (μg/ml)[b]
Staphylococcus aureus TN2613	Methicillin-resistant	6.25
S. aureus TN2629	KM-resistant	6.25
S. aureus TN2636	SM, KM-resistant	3.13
S. aureus TN2647	KM, GM-resistant	6.25
S. aureus TN2684	EM, OL-resistant	6.25
S. aureus TN2687	EM, LM, LCM-resistant	6.25
S. aureus TN2688	EM-resistant	6.25
Pseudomonas aeruginosa PM3	Outer membrane mutant	6.25
P. aeruginosa PM3 (RPL11)	SM-resistant	6.25
P. aeruginosa POA1		100
P. aeruginosa POA1 (R2)	SM, KM-resistant	100
P. aeruginosa POA1 (RP1)	KM-resistant	100
P. aeruginosa POA1 (pMG90)	SM, GM-resistant	100
P. aeruginosa POA1 (RIP64)	GM-resistant	100
P. aeruginosa POA1 (RPL11)	SM-resistant	100

[a] KM: Kanamycin, SM: streptomycin, GM: gentamicin, EM: erythromycin, OL: oleandomycin, LM: kitasamycin, LCM: lincomycin.

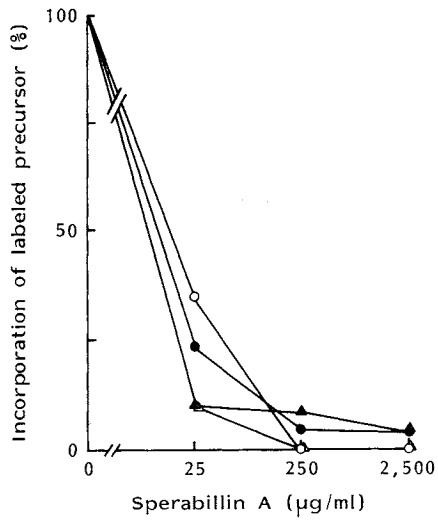
[b] MIC values were determined by an agar dilution method using Mueller-Hinton agar. Inoculum size was 10^6 cfu/ml.

and D tended to be more active than their demethyl counterparts, sperabillins A and C, respectively, and sperabillins A and B which have *cis* configuration were more active than sperabillins C and D having *trans* configuration, respectively (Table 5). Sperabillin A was also active against clinical isolates of *S. aureus* and *P. aeruginosa* including the methicillin-, aminoglycoside- or macrolide-resistant strains (Table 6, K. OKONOGI; unpublished data).

We examined the effect of sperabillin A on the incorporation of radioactive precursors into the macromolecules in *E. coli* LD-2. As shown in Fig. 7, sperabillin A strongly inhibits DNA, RNA, protein and peptidoglycan synthesis at the same level in this microorganism. Similar results were obtained in experiments using *P. aeruginosa* and *S. aureus* as test strains (data not shown). Sperabillin A and negamycin[11] have the same amino acid in their structures. Although negamycin was reported to inhibit

Fig. 7. Effect of sperabillin A on macromolecule synthesis in *Escherichia coli*.

DNA (●), RNA (○), protein (△) and peptidoglycan (▲).



protein synthesis[12,13], sperabillin A showed mode of action different from that of negamycin.

The antibacterial activity of sperabillin A was affected by the assay media (Table 7). Although, in the nutrient broth, sperabillin A showed good antibacterial activity, it was only weakly active in the other media. Investigating the difference between the nutrient broth and the other media, we found that the conductivity of the nutrient broth was quite low compared to that of the other media (Table 7). When we added salt to the nutrient broth to elevate the conductivity to the level of the other media, the antibacterial activity of sperabillin A decreased to the level obtained in the other media (Table 8). In the assay media, sperabillins are positively charged, and there are negatively charged compounds such as phospholipid in the bacterial cell membrane.

Table 7. Influence of assay medium on the antibacterial activity of sparabillin A.

Medium	Conductivity (mS/cm)	MIC (μg/ml) at 10^6 cfu/ml	
		Pseudomonas aeruginosa IFO 3080	*Escherichia coli* NIHJ JC-2
Nutrient broth	1.5	1.56	12.5
DYAB	11.8	100	>100
Heart infusion broth	12.6	50	>100
Trypticase soy broth	11.2	25	>100
Mueller-Hinton broth	11.9	100	>100
Synthetic medium AOAC	11.5	50	>100

MIC values were determined by a microdilution method.
Conductivity was measured by a conductivity meter (Model SC82, Yokogawa Electric Co.).
DYAB medium was prepared as described earlier[8] and Trypticase soy broth was purchased from BBL and the other media were purchased from Difco.

Table 8. Relationship between conductivity of the medium and antibacterial activity of sperabillin A.

Medium	Conductivity (mS/cm)	MIC (μg/ml) at 10^6 cfu/ml	
		Pseudomonas aeruginosa IFO 3080	*Escherichia coli* NIHJ JC-2
Nutrient broth (NB)	1.5	1.56	12.5
NB+100 mM NaCl	11.7	50	>100
NB+100 mM KCl	13.8	50	>100
NB+ 50 mM $MgCl_2$	11.2	50	>100
NB+ 50 mM $CaCl_2$	10.2	6.25	>100

MIC values were determined by a microdilution method.
Conductivity was measured by a conductivity meter (Model SC82, Yokogawa Electric Co.).

Table 9. Protective effect of sperabillins in experimentally infected mice.

Microorganism	Sperabillin	MIC (μg/ml)[a]	ED_{50} (mg/kg)[b]	Route
Staphylococcus aureus 308A-1	A	12.5	1.31	sc
	B	3.13	0.35	sc
	C	>100	12.5	sc
	D	25	1.82	sc
	A	12.5	17.7	po
	B	3.13	16.2	po
Streptococcus pyogenes E-14	A	3.13	8.13	sc
	B	6.25	8.84	sc
Escherichia coli O-111	A	>400	67.2	sc
	B	50	27.3	sc
	C	>100	50	sc
	D	>100	55.9	sc
Pseudomonas aeruginosa P9	A	100	31.0	sc
	B	50	61.4	sc

[a] MIC values were determined by an agar dilution method using Trypticase soy agar. Inoculum size was 10^8 cfu/ml.
[b] Mice were infected intraperitoneally with 0.5 ml of a suspension of bacteria (10^8 cfu/ml). Groups of five mice at each dosage level were given 0.2 ml of an antibiotic solution immediately after infection. The ED_{50} was calculated from the survival rate 5 days after infection.

Therefore, sperabillins may bind to the bacterial cell membrane electrostatically. This may be necessary for sperabillins to show strong antibacterial activity, because high ionic strength disturbs electrostatic binding. Since polymyxin B, a well known inhibitor of membrane function, inhibited all types of macromolecular synthesis as sperabillin A did (data not shown), sperabillin A may inhibit membrane function.

Sperabillins showed good protective effects against Gram-negative and Gram-positive bacteria in experimentally infected mice (Table 9). Moreover, the pretreatment of mice with sperabillin A protected mice against lethal infection with *Serratia marcescens*, even though sperabillin A was not detected in the blood on the day of infection. When the cell suspension of *S. marcescens* TN66 (1.3×10^7 cfu) was injected intraperitoneally to mice, all of ten untreated mice died within 7 days of infection and the mean survival time of them was about 2 days. On the other hand nine of ten mice survived for 7 days after infection, when sperabillin A was injected subcutaneously to mice at a dose of 2 mg/kg/day 1, 4 and 7 days before the infection. Monitoring the amount of sperabillin A by the HPLC method, sperabillin A rapidly disappeared from plasma samples in mice. The half life of sperabillin A in mice was about 20 minutes. To understand the mechanism of this protective effect, further study is necessary. The preliminary acute toxicity (LD_{50}) of sperabillin A in mice was about 200 mg/kg upon intraperitoneal injection and about 500 mg/kg upon subcutaneous injection.

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