PS-5, A NEW β-LACTAM ANTIBIOTIC. II
ANTIMICROBIAL ACTIVITY*

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PS-5, a new β-lactam antibiotic[1], has relatively potent antimicrobial activity against Gram-positive and Gram-negative bacteria, especially the *Enterobacter* groups, *Serratia marcescens*, the *Proteus* groups and *Klebsiella pneumoniae*. The activity of PS-5 against many β-lactamase-producing organisms is greater than that of cefoxitin or cefazolin. PS-5 has good therapeutic activity in mice infected with *Staphylococcus aureus* Smith or *Enterobacter cloacae* 45.

PS-5, a new β-lactam antibiotic isolated from culture broth of *Streptomyces cremeus* subsp. *auratilis*, strain A271, is active against a variety of Gram-positive and Gram-negative organisms and also shows inhibitory activity against β-lactamases produced by various bacteria[2,3]. The fermentation, isolation and physico-chemical properties of PS-5 have been described by OKAMURA *et al.*[4]. This paper describes the studies carried out on the *in vitro* and *in vivo* antimicrobial activity of PS-5.

Materials and Methods

1. Antibiotics

PS-5 (sodium salt) was prepared in our laboratories. Cefoxitin (sodium salt, CFX) was supplied by Merck Sharp and Dohme. Cefazolin (sodium salt, CEZ) was obtained from Fujisawa Pharm. Co., Ltd., ampicillin (sodium salt, ABPC) from Toyo Jozo Co., Ltd. and cephaloridine (CER) from Shionogi & Co., Ltd.

2. Bacterial strains

The test organisms used are maintained in our laboratories' stock culture collection. Clinical isolates were supplied by Dr. NISHINO, Kyoto College of Pharmacy, Dr. YAMAGISHI, Faculty of Pharmaceutical Sciences, Chiba University and Dr. KAWAGUCHI, Bristol-Banyu Research Institute, Ltd.

3. Determination of minimum inhibitory concentration (MIC)

A two-fold dilution method in agar[5] was used with Heart Infusion (HI) medium (Kyokuto Seiyaku Kogyo). Unless otherwise specified, an overnight culture ($10^8 \sim 10^9$ cells /ml) of each test organism in Trypto-Soy broth (TSB, Eiken Chemi. Co., Ltd.) was diluted with physiological saline to give 10^8 cells/ml and used for the source of inoculum. One loopful of the inoculum-source was streaked onto agar surface for MIC tests. MIC values were determined after 20 hours incubation at 37°C. For *Streptococcus pyogenes* and *Streptococcus pneumoniae*, defibrinated horse blood was added to the medium to give a final concentration of 10% (v/v).

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4. Bactericidal activity in a growing culture

One volume of an overnight culture of *Staphylococcus aureus* Smith or *Proteus vulgaris* 109 in TSB was inoculated into nine volumes of the same medium and then incubated at 37°C for 3 hours. The cells at the logarithmic phase of growth were suspended in HI broth ($10^5 \sim 10^6$ cells per ml) containing PS-5 sodium salt or cefazolin and incubated at 37°C. Viable cell counts were determined by plating at various time intervals during the incubation.

5. Bactericidal activity in the stationary phase

Tests organisms were cultured in HI broth at 37°C for 20 hours and 0.5 ml of PS-5 or cefazolin solution was added into 4.5 ml of the cultured broth and incubated at 37°C. Viable cell counts were determined by plating at various time intervals.

6. Plasma levels in mice

Male ddy-strain mice, aged 5 weeks, weighing 19~21 g, were used in groups of 5 mice each. PS-5 was injected subcutaneously in single dose of 1,600, 400 and 100 mg/kg. Blood was taken from the retroorbital sinus at various time intervals using a heparinized capillary. Each sample was centrifuged at 1,500 *g* for 5 minutes, and the supernatants used for bioassay. The bioassay was carried out using the paper disc-plate method with *Comamonas terrigena* B-996 as the test organism. Known concentrations of PS-5 solutions prepared in normal mouse plasma were used as standards in the bioassay.

7. Therapeutic effect on experimental infection in mice

Male ddy-strain mice, aged 5 weeks, weighing 19~21 g were used in groups of 5 mice each. Each challenge organism was cultured overnight in BHI broth at 37°C and suspended in 5% bacteriological mucin (Wilson Pharmaceutical and Chemical Co.). Thirty to fifty times the LD_{50} were used to infect the mice. A cell suspension of 0.5 ml was injected intraperitoneally and the test antibiotics were given subcutaneously or intravenously in single or multiple doses. The mice were observed for 4 days. The therapeutic effect of the test antibiotics is expressed as CD_{50} (mg/mouse) calculated by the method of LITCHFIELD and WILCOXON.[6]

Results

Agar Dilution MIC

The MIC values of PS-5 sodium salt, cefoxitin and cefazolin for 38 organisms representing 25 species of 17 genera of bacteria are shown in Table 1. PS-5 was more active against all Gram-positive organisms tested than cefoxitin and more active against the majority of organisms than cefazolin. PS-5 also compared favorably against Gram-negative organisms and was especially more active against most of β-lactamase-producing organisms than cefoxitin and cefazolin. Thus, PS-5 inhibited *Enterobacter* and *Citrobacter* at a concentration of 3.13 μg/ml or 6.25 μg/ml while cefoxitin or cefazolin did not inhibit those organisms at the concentrations as high as 100 or 400 μg/ml. Ten strains of *Pseudomonas aeruginosa* were tested for MIC and *P. aeruginosa* IFO 3445 was found to be inhibited by 50 μg/ml PS-5, 100 μg/ml cefoxitin and 100 μg/ml cefazolin. The other strains of *Pseudomonas aeruginosa* were not significantly inhibited by these antibiotics. PS-5 inhibited *Serratia* more strongly than cefoxitin or cefazolin.

Susceptibility of Clinical Isolates in Agar Dilution Tests

The activity of PS-5, cefoxitin and cefazolin against clinical isolates of *Serratia marcescens*, *Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae* is shown in Fig. 1. Ninety per cent of isolates of *Serratia* were inhibited by 6.25 μg/ml of PS-5, while none was inhibited by 6.25 μg/ml of cefoxitin or 400 μg/ml of cefazolin. Ninety per cent of the isolates of *E. coli* were inhibited by 6.25

Table 1. MIC values of PS-5, cefoxitin (CFX) and cefazolin (CEZ).

Microorganisms	PS-5	CFX	CEZ
Staphylococcus aureus FDA 209P	0.025	1.56	0.05
Smith	0.20	3.13	0.20
BX-1633	0.20	1.56	0.20
Russel	0.20	3.13	0.78
(PCG, KM, NM)^r	0.39	3.13	3.13
(EM, OM, PCG, TC)^r	0.39	3.13	12.5
(EM, OM, SM, PCG, TC)^r	0.39	3.13	1.56
(TC, CP, PCG)^r	0.20	3.13	12.5
(PCG)^r	0.20	3.13	0.78
Staphylococcus epidermidis	0.39	3.13	0.10
Bacillus subtilis ATCC 6633	0.05	0.78	0.05
Sarcina lutea S-19	0.10	0.39	0.39
Streptococcus pyogenes NY5*	0.08	0.63	0.008
Streptococcus pneumoniae type III*	0.02	1.25	0.031
Alcaligenes faecalis	0.39	0.78	6.25
Escherichia coli K-12	1.56	1.56	1.56
Enterobacter sp. E-8**	1.56	6.25	1.56
Enterobacter aerogenes E-19**	6.25	>100	>100
Enterobacter cloacae E-16**	3.13	>100	>400
Enterobacter cloacae 45	6.25	>100	>400
Citrobacter freundii E-9**	3.13	>100	>100
Citrobacter freundii GN346**	3.13	>100	>400
Proteus vulgaris P-5**	6.25	12.5	>400
Proteus vulgaris GN76**	12.5	12.5	>400
Proteus vulgaris 109	12.5	6.25	>400
Proteus mirabilis P-6	6.25	6.25	3.13
Proteus rettgeri P-7	6.25	6.25	>400
Proteus sp. P-22	12.5	6.25	>400
Shigella sonnei EW33	1.56	1.56	0.78
Shigella flexneri Komagome	1.56	3.13	0.78
Salmonella enteritidis	3.13	1.56	0.78
Salmonella typhi	0.78	0.78	0.39
Providencia sp. P-8	3.13	6.25	50
Pseudomonas aeruginosa NC5	>100	>100	>400
Pseudomonas aeruginosa IFO3445	50	100	100
Klebsiella pneumoniae K2	3.13	6.25	6.25
Serratia marcescens S-18	3.13	50	>400
Comamonas terrigena B-996	0.013	0.39	0.20

* supplmented with 10% horse blood.
** β-lactamase producing organism.

μg/ml of PS-5, while 60% of the isolates were inhibited by the same concentration of cefoxitin and only 25% of the isolates by the same concentration of cefazolin.

Eighty five per cent of the isolates of *Klebsiella* were inhibited by a concentration of 6.25 μg/ml of PS-5 and cefoxitin respectively. Only 25% of the isolates were inhibited by the same concentration of cefazolin.

PS-5 inhibited 100% of the isolates of *Enterobacter cloacae* at a concentration of 12.5 μg/ml, while only one strain among them was inhibited by 100 μg/ml of cefoxitin and none was inhibited by 400 μg/ml of cefazolin. Thus PS-5 showed higher activity against these enteric pathogens than cefoxitin and cefazolin.

Effect of pH on MIC

Effect of pH on the MIC of PS-5, cefoxitin and cephaloridine was examined in HI broth at pH 6, 7 and 8 by the two-fold serial dilution method with *E. coli* and *Serratia marcescens* (Table 2). No change in MIC was observed for cefoxitin with *E. coli*. Cephaloridine showed a two fold increase in MIC values at pH 6 with *E. coli*.

Fig. 1. Cumulative percentage of clinical isolates of *Enterobacter cloacae* (17), *Klebsiella pneumoniae* (12), *Serratia marcescens* (11) and *Escherichia coli* (14) inhibited by PS-5, cefoxitin (CFX) or cefazolin (CEZ).

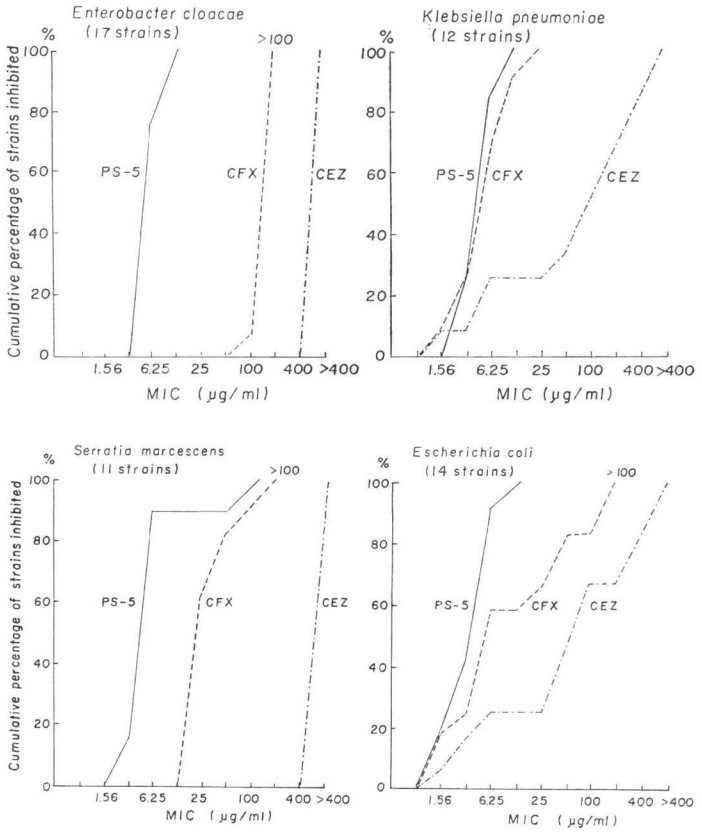


Table 2. Effect of pH on activity of PS-5 *in vitro*.

Medium pH	Antibiotic	MIC* (μg/ml)	
		Escherichia coli K12	*Serratia marcescens* S-18
6.0	PS-5	5.0	12.5
7.0		2.5	3.13
8.0		5.0	3.13
6.0	Cefoxitin	2.5	100
7.0		2.5	50
8.0		2.5	50
6.0	Cephaloridine	5.0	>100
7.0		2.5	>100
8.0		2.5	>100

* Conditions were same as in Materials and Methods. The cell concentration of the source of inoculum was 10^8 cells/ml.

Table 3. Effect of horse serum on activity of PS-5 *in vitro*.

Horse serum (%)	Antibiotic	MIC* (μg/ml)	
		Escherichia coli K12	*Serratia marcescens* S-18
0	PS-5	1.25	3.13
10		1.25	3.13
50		2.5	3.13
0	Cefoxitin	1.25	50
10		1.25	50
50		1.25	50
0	Cefazolin	2.5	>100
10		2.5	>100
50		2.5	>100

* Conditions were same as in Table 2.

PS-5 showed a two fold increase in MIC values at pH 6 and 8 with *E. coli*. PS-5 was more effective at pH 7 or 8 than at pH 6 (four fold decrease in MIC values) with *Serratia marcescens*. Cefoxitin also showed slightly higher activity against *Serratia marcescens* at pH 7 or 8 than at pH 6.0.

In view of the above results, effects of pH on MIC values of the antibiotics were not considered to be significant except that PS-5 showed higher activity against *Serratia marcescens* at pH 7 and 8 than at pH 6.

Effect of Addition of Serum

Horse serum was added to HI broth to give a final concentration of 10% or 50% and MIC values of PS-5, cefoxitin and cefazolin were determined with *E. coli* and *Serratia marcescens*. The results are shown in Table 3. Horse serum had little or no influence on the MIC values of these antibiotics. There were no more than two fold variations in the MIC values.

Effect of Inoculum Size

The effect of inoculum size on MIC values of PS-5 was examined using final cell concentrations of $10^8 \sim 10^4$ cells per ml with *Staphylococcus aureus* FDA 209P, *E. coli* K12 and *E. coli* ML1410 RGN823 (Table 4).

Table 4. Effect of inoculum size on activity of PS-5 *in vitro*.

Organisms	Inoculum* (cells/ml)	MIC (μg/ml)		
		PS-5	Cefoxitin	Cefazolin
Staphylococcus aureus FDA 209P	10^8	0.025	1.56	0.19
	10^7	0.025	1.56	0.19
	10^6	0.025	1.56	0.10
	10^5	0.013	0.78	0.05
	10^4	0.013	0.78	0.025
Escherichia coli K12	10^8	1.56	3.13	0.78
	10^7	1.56	3.13	0.78
	10^6	1.56	3.13	0.78
	10^5	1.56	3.13	0.78
	10^4	1.56	1.56	0.39
Escherichia coli ML1410 RGN823	10^8	1.56	1.56	50
	10^7	1.56	1.56	12.5
	10^6	1.56	1.56	6.25
	10^5	1.56	1.56	6.25

* Each concentration of culture broth served as the source of inoculum. The other conditions were same as in Materials and Methods.

PS-5 and cefoxitin showed no significant difference in MIC values with the organisms over this range of inocula. MIC values of cefazolin were affected by the inoculum size with *S. aureus* FDA 209P and *E. coli* ML1410 RGN823.

Bactericidal Activity

The bactericidal activity of PS-5 and cefazolin is shown in Figs. 2 and 3. Against actively growing cells PS-5 at a concentration of 25 μg/ml resulted in a decrease of 3 logs in the viable count of *Proteus vulgaris* 109 in 2 hours. Cefazolin showed a similar decrease at a concentration of 500 μg/ml. However, with cefazolin the viable count gradually increased after 2 hours of incubation. Whereas this did not occur with PS-5.

Using logarithmic phase cells of *Staphylococcus aureus* Smith PS-5 at a concentration of 0.5 μg/ml resulted in a decrease of >3 logs in the viable count in 4 hours whereas under the same conditions cefazolin resulted in a decrease of no more than 1 log. Both PS-5 and cefazolin showed a slower bactericidal effect against resting cells of *S. aureus*.

Plasma Levels in Mice

The mean plasma levels of PS-5 in 5 mice after single subcutaneous injection are shown in Table 5. The plasma levels were observed to decrease after 1/4 hour for all doses and the expected dose response relationship were observed.

Fig. 2. Effects of PS-5 and cefazolin (CEZ) on *Proteus vulgaris* 109 in a growing or stationary phase.
Medium: Heart Infusion broth.
Incubation temperature: 37°C.
CEZ(Na): 500 μg/ml, PS-5(Na): 25 μg/ml.

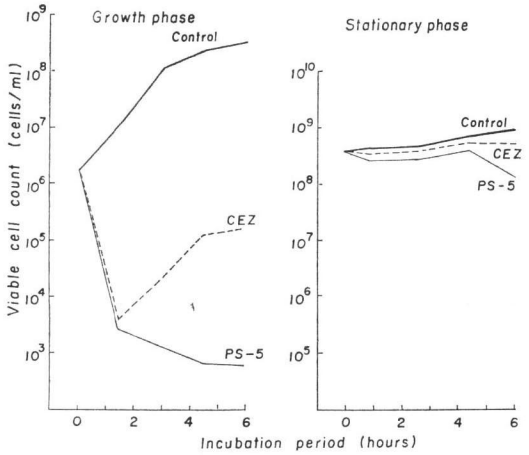


Fig. 3. Effects of PS-5 and cefazolin (CEZ) on *Staphylococcus aureus* Smith in a growing or stationary phase.
Medium: Heart Infusion broth.
Incubation temperature: 37°C.
CEZ(Na): 0.5 μg/ml, PS-5(Na): 0.5 μg/ml

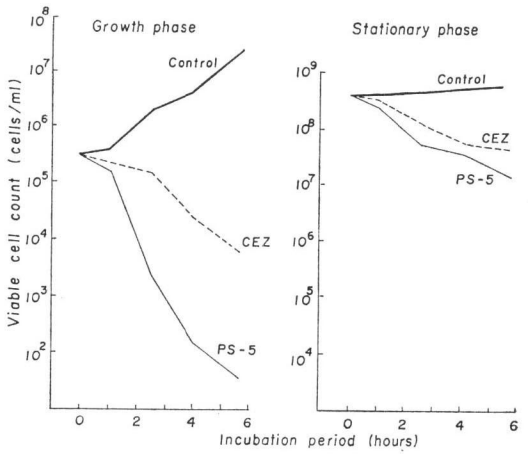


Table 5. Plasma level of PS-5 in mice after single subcutaneous injections of 100, 400 and 1,600 mg/kg.

Dose (mg/kg)	Mean blood level (μg/ml), N=5 (mean±S.D.)					
	1/4 hr	1/2 hr	1 hr	2 hr	4 hr	6 hr
1,600	477±52.0	348±40.3	132±15.7	3.6±2.3	0	0
400	116±39.8	50±21.7	4.5±3.1	0	0	0
100	30.4±10.7	8.8±3.6	0	0	0	0

Therapeutic Effect against Experimental Infection in Mice

The subcutaneous CD_{50} of PS-5 after single or multiple administration in mice using *S. aureus* Smith as the challenge organism was found to be 0.18 mg/mouse and 0.062 mg/mouse respectively by the method of LITCHFIELD and WILCOXON[6] from the data in Table 6. Table 7 compares the activity of PS-5 and cefazolin given subcutaneously or intravenously in mice infected intraperitoneally with *Enterobacter cloacae* 45. The antibiotics were given as a single or multiple dose. PS-5 was effective against this organism resistant to cefazolin and multiple dosage with PS-5 showed good therapeutic effects.

Table 6. Protective effect of PS-5 in mice infected intraperitoneally with *Staphylococcus aureus* Smith.

Subcutaneous injection	Dose (mg/mouse)	Survival/ total
Single dosing*	2.0	5/5
	0.5	4/5
	0.13	2/5
	0.031	0/5
Multiple dosing**	0.13×4	5/5
	0.031×4	4/5
	0.008×4	1/5
	0.002×4	0/5

* 2 hours after challenge.
** Four doses at hourly intervals commencing 2 hours after challenge.
Inoculum size: $30 \times LD_{50}$.

Table 7. Protective effect of PS-5 in mice infected with *Enterobacter cloacae* 45.

		Antibiotic	Dose (mg/mouse)	Survival/Total
Subcutaneous injection	Single dosing (2 hours after challenge)	PS-5	32	4/5
			8	1/5
		Cefazolin	32	0/5
			8	1/5
	Multiple dosing (Four doses at hourly intervals commencing 2 hours after challenge)	PS-5	8 ×4	5/5
			2 ×4	2/5
			0.5×4	1/5
		Cefazolin	8 ×4	1/5
			2 ×4	1/5
			0.5×4	0/5
Intravenous injection	Single dosing (2 hours after challenge)	PS-5	32	4/4
			8	1/5
			2	0/5
			0.5	1/4
		Cefazolin	32	0/4
			8	0/5
			2	1/5
			0.5	0/4

Inoculum size: $50 \times LD_{50}$

Effect of PS-5 on the Morphology of *E. coli* K12

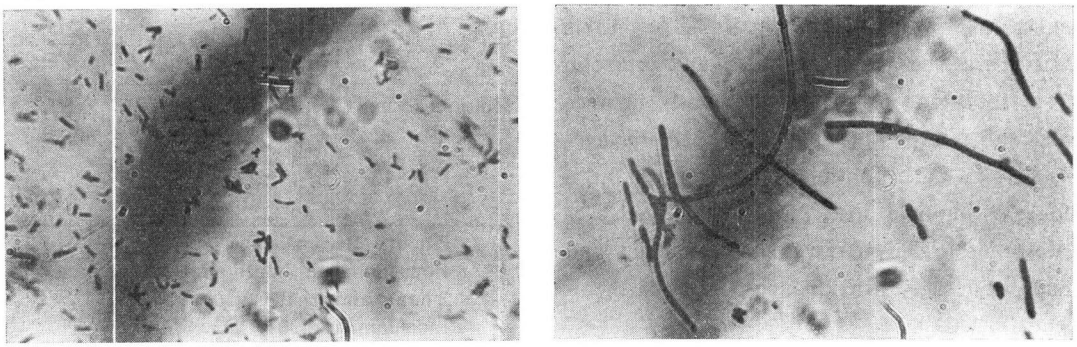
E. coli K12 was grown overnight at 37°C in TSB broth. The culture broth (0.05 ml) was inoculated into 50 ml HI broth and cultured until the cell concentration reached 10^7 cells/ml. PS-5 or ampicillin (ABPC) was added and the cell morphology was observed under phase-contrast microscopy after a further 60 minutes cultivation.

In the presense of 1/2~10 MIC ABPC (1 MIC=1.56 μg/ml), the cells became filamentous after 60 minutes.

In the presense of 1/2 MIC PS-5, the cells became filamentous (Fig. 4). In 1 MIC (1.56 μg/ml) PS-5, the cells became filamentous and began to lyse. Above 2 MIC PS-5, the cells had lysed after 60 minutes.

Fig. 4. Effect of PS-5 on the morphology of *E. coli* K12.
Medium: Heart Infusion broth.

(a) Control (b) PS-5 0.78 μg/ml



Discussion

PS-5 has similarity in structure to clavulanic acid[7], olivanic acid derivatives[8,9] and thienamycin[10]. Clavulanic acid was reported to show a broad antimicrobial spectrum but the activity level is relatively low[7]. According to data on antimicrobial activities of olivanic acid derivatives which were given in the patent specification, MM 13902[11] and MM 17880[12] show potent activities against selected Gram-positive and negative microorganisms. MM 4550 was described to have moderate activity against Gram-positive and negative bacteria[7,13].

Thienamycin was reported to inhibit many strains of *Escherichia coli* and *Pseudomonas aeruginosa* at 8 μg or less per ml, and *Serratia marcescens* and *Proteus* species at 16~32 μg/ml[14]. Thienamycin has also potent antistaphylococcal activity[14]. PS-5 inhibited 90% of given *Serratia marcescens*, *Klebsiella pneumoniae* and *Escherichia coli* strains at 6.25 μg/ml and had potent antistaphylococcal activity which could be comparable with thienamycin, however, thienamycin seems to be more potent against *Pseudomonas aeruginosa* than PS-5.

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