PS-5, A NEW β-LACTAM ANTIBIOTIC. III
SYNERGISTIC EFFECTS AND INHIBITORY ACTIVITY AGAINST A β-LACTAMASE*

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PS-5 was shown to have synergistic activity in combination with other β-lactam antibiotics and it markedly decreased the minimum inhibitory concentration values of ampicillin or cephaloridine with a β-lactamase-producing *Proteus vulgaris* strain on agar plates. The synergistic activities were also shown in bactericidal activity in liquid medium. PS-5 was shown to be inhibitory against an extracted β-lactamase of *P. vulgaris*.

PS-5, a new β-lactam antibiotic, was shown to have potent antimicrobial activity against a wide range of Gram-positive and negative organisms and also shown to be bactericidal[1]. The antibiotic was also found to be highly resistant to hydrolysis by some β-lactamases.[2,12] This results in a synergism when PS-5 and other β-lactamase sensitive antibiotics such as ampicillin are used in combination. This paper describes the synergistic antimicrobial activities of PS-5 with ampicillin or cephaloridine and the inhibitory effect of PS-5 against a β-lactamase from *Proteus vulgaris*.

Materials and Methods

Antibiotics

PS-5 (sodium salt) and cephamycin C were prepared in our laboratories. Ampicillin (sodium salt, ABPC) was obtained from Toyo Jozo Co., Ltd., cephaloridine (CER) from Shionogi & Co., Ltd., penicillin G (potassium salt, PCG) from Meiji Seika Kaisha, Ltd., cefazolin (sodium salt, CEZ) from Fujisawa Pharm. Co., Ltd. and methicillin and oxacillin from Banyu Pharm. Co., Ltd.

Bacterial strain

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

Determination of minimum inhibitory concentration (MIC)

A two-fold dilution method in agar medium was used with Heart Infusion (HI) medium (Kyokuto Seiyaku Kogyo). Cultural conditions are the same as given in the previous report[1].

Bactericidal activity in a growing culture

An overnight culture of *Proteus vulgaris* P-5 in Trypto-Soy Broth (TSB) was diluted to 1 : 10 with the same medium and incubated under stationary conditions at 37°C for 3 hours. The culture was then diluted with Heart-Infusion broth to contain 10^5 cells per ml and incubated at 37°C. After 2-hour in-

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cubation, test drugs were added to the culture broth and the viable cell counts were determined by plating at various time intervals. *P. vulgaris* GN76 was grown and the diluted culture of the organism was prepared in the same manner as mentioned above. Drugs dissolved in HI broth were added using one tenth the volume of the diluted culture and viable cell counts were determined at various time intervals at 37°C by plating.

Synergism on agar plates

Qualitative observations of synergism on agar plates were made by the methods reported by KAWAKAMI[3] and SABATH[4].

β-Lactamase inhibition assay

Ampicillin-induced β-lactamase of *Proteus vulgaris* P-5 was extracted by sonication of the cells and purified by CM-Sephadex C-25 chromatography and Sephadex G-10 gel filtration. Suitable dilutions of PS-5 sodium salt were preincubated with the β-lactamase at 26°C for 5 minutes and cephaloridine then added to give a final concentration of 0.1 mM or 0.5 mM. In the determination of *Km* values of cephaloridine and *Ki* values of PS-5, the reaction was started by the addition of the enzyme. Decomposition of cephaloridine was determined from the decrease of absorbance at 255 or 290 nm which was recorded using a Hitachi 200 Spectrophotometer. Initial velocities which occurred during the first 0.5 minute were obtained from the recorded chart and the velocities obtained with PS-5 were compared with those obtained without PS-5.

Results

Synergism on Agar Plates

Two filter paper strips (Toyo Filter Paper No. 2, 8 mm × 45 mm) dipped in 35 μg per ml solution of PS-5 or 10,000 μg/ml solution of penicillin G or cephaloridine were placed as shown in Fig. 1 on agar plates seeded with *Proteus vulgaris* P-5. The plates were incubated at 30°C for 20 hours. Enhanced inhibition zones were observed in the corners formed by the two different antibiotics. PS-5 and penicillin G or cephaloridine were therefore considered to have a synergistic action in combination. A similar phenomenon was observed in disc-plate assays where discs containing PS-5 or cephaloridine were placed closely as shown in Fig. 2.

Various β-lactams were tested by the disc-plate method to determine whether they showed synergistic activity with cephaloridine against *Proteus vulgaris* as the test organism. The results are shown

Fig. 1. Synergistic action of PS-5 and PCG or CER.
PS-5(Na): 35 μg/ml, PCG(K): 10,000 μg/ml, CER: 10,000 μg/ml.
Assay organism: *Proteus vulgaris* P-5. Medium: Nutrient agar.

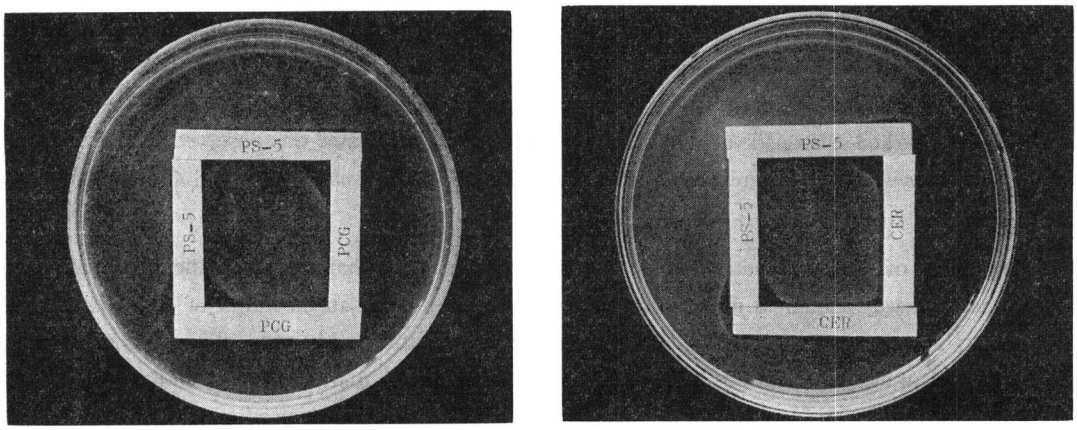
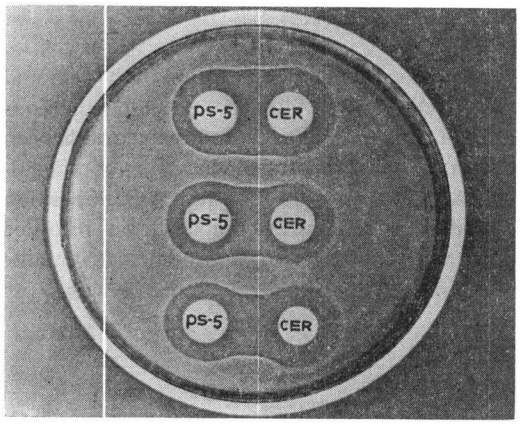


Fig. 2. Synergism on agar plates seeded with *Proteus vulgaris* P-5.

Discs containing 20 μl of PS-5 solution (0.1 mg/ml) or cephaloridine (CER) solution (20 mg/ml) were placed on the agar plate and incubated for 20 hours at 30°C.

(A) Synergism is revealed by merging zones of inhibition.



(B) Each pair shows inhibition but no synergism.

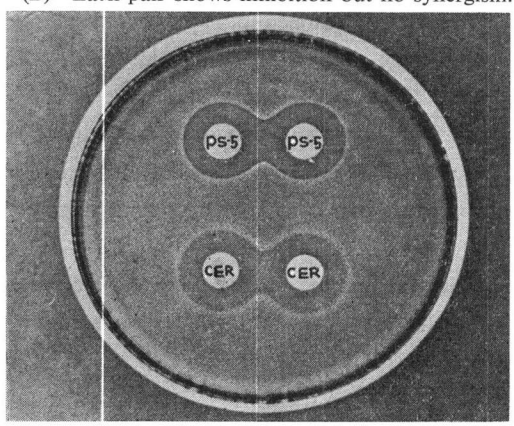


Table 1. Synergistic activity on agar plates.

Antibiotics (μg/ml)		Inhibition zone (mm)	
		Supplementation to an agar plate seeded with *Proteus vulgaris* P-5	
		None	Cephaloridine 50 μg/ml
PS-5(Na)	(70)	13.5	22.5
CER	(10,000)	13.0	13.0
CEZ(Na)	(10,000)	14.0	14.0
PCG(K)	(10,000)	13.0	13.0
ABPC(Na)	(10,000)	11.0	11.0
OXAC(Na)	(10,000)	11.0	11.0
CLXC(Na)	(10,000)	11.0	11.0
METC	(10,000)	11.0	11.0
CEMC	(156)	12.5	12.5

CER, cephaloridine, CEZ; cefazolin, PCG; penicillin G, ABPC; ampicillin, OXAC; oxacillin, CLXC; cloxacillin, METC; methicillin, CEMC; cephamycin C. Disc-plate assay method was employed on agar plates with or without cephaloridine 50 μg/ml. *Proteus vulgaris* P-5 was used as the test organism.

in Table 1. Inhibition zones obtained on an agar plate without supplementation were compared with those obtained on an agar plate containing cephaloridine at the concentration of 50 μg/ml. Marked enhancement of inhibition zone of PS-5 was observed on the cephaloridine-supplemented plate. On the other hand, no other β-lactam antibiotic tested showed enhancement of the inhibition zone.

Inhibition zones of PS-5 on agar plates seeded with *Proteus vulgaris, Enterobacter* sp., *Citrobacter freundii* or *Serratia marcescens* were compared in the presence or absence of cephaloridine (50 μg/ml). The results are shown in Fig. 3. MIC values of cephaloridine with *P. vulgaris, E.* sp., *C. freundii* and *S. marcescens* were 2,500, 800, 2,500 and 5,000 μg/ml respectively. Therefore 50 μg/ml of cephaloridine had no effect on these organisms. Marked synergistic activity between PS-5 and cephaloridine was observed with *P. vulgaris* and *S. marcescens* and also significant synergistic activity with *Enterobacter* sp. and *C. freundii*.

Effect of Combinations on MIC

The effect of the combination of PS-5 and ampicillin or cephaloridine on the MIC values of enteric bacteria was examined in serial dilution tests in agar using an inoculum of 10^8 cells per ml. The results are shown in Table 2. When tested in combination with ampicillin or cephaloridine, PS-5 was present at concentrations of 0.63, 1.25 and 2.0 μg/ml. A marked decrease in the MIC values of both ampicillin and cephaloridine was observed with β-lactamase-producing *Citrobacter* and

Fig. 3. Disc-plate assay of PS-5 on assay plates with or without cephaloridine seeded with *Proteus vulgaris* P-5(a), *Enterobacter* sp. E-16(b), *Citrobacter freundii* E-9(c) or *Serratia marcescens* S-18(d).
Cephaloridine was added to the agar medium at a concentration of 50 μg/ml.

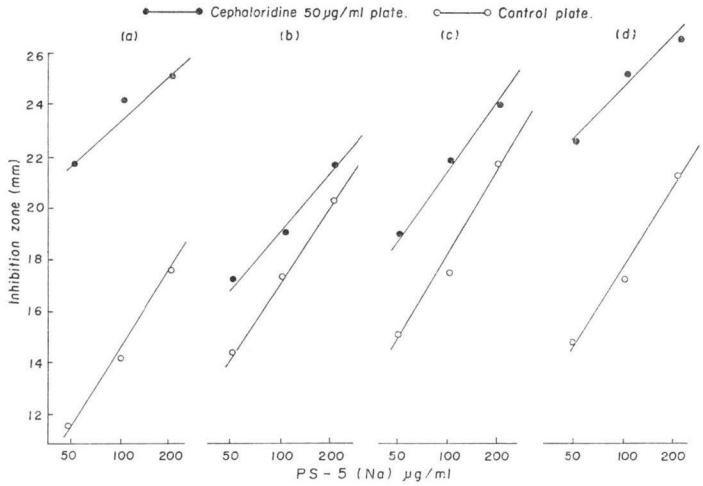


Table 2. Effects of combinations on the MIC (μg per ml).

Microorganisms	PS-5	ABPC	CER	PS-5** +ABPC	PS-5** +CER
Citrobacter freundii E-9*	3.13	1,250	2,500	39.1	78.1
Citrobacter freundii GN346*	3.13	1,250	1,250	39.1	78.1
Proteus vulgaris P-5*	12.5	2,500	2,500	7.8	7.8
Proteus vulgaris GN76*	12.5	2,500	2,500	7.8	7.8
Proteus vulgaris 109	12.5	10,000	5,000	1,000	1,000
Proteus morganii 101	12.5	625	2,500	500	1,000
Serratia marcescens S-18*	6.25	313	5,000	62.5	1,000
Serratia marcescens T55	12.5	156	5,000	125	1,000
Enterobacter cloacae 45	6.25	1,250	5,000	500	1,000
Enterobacter cloacae E-40	6.25	313	1,250	125	125
Enterobacter aerogenes E-34	6.25	2,500	2,500	500	500
Escherichia coli 21*	6.25	10,000	1,250	1,000	500
Klebsiella sp. 118	6.25	10,000	2,500	1,000	125

* β-lactamase producing organisms.
** PS-5 was used in concentrations of 0.63 μg/ml (*Citrobacter*), 1.25 μg/ml (*Serratia*) and 2 μg/ml (other organisms).
Incubation: at 37°C for 20 hours.
Inoculum: 10^8 cells/ml.

Proteus and *S. marcescens* S-18. Two or five fold decreases in MIC values were observed with *Enterobacter* in combination with a small amount of PS-5.

Bactericidal Activity of Combinations

The bactericidal activity of ampicillin or cephaloridine against a growing culture of *Proteus vulgaris* was examined in the presence or absence of PS-5. The results are shown in Figs. 4 and 5.

Fig. 4. Effect of PS-5 alone and in combination with ABPC on a growing culture of *Proteus vulgaris* GN 76.
Medium: Heart Infusion broth
Incubation temperature: 37°C

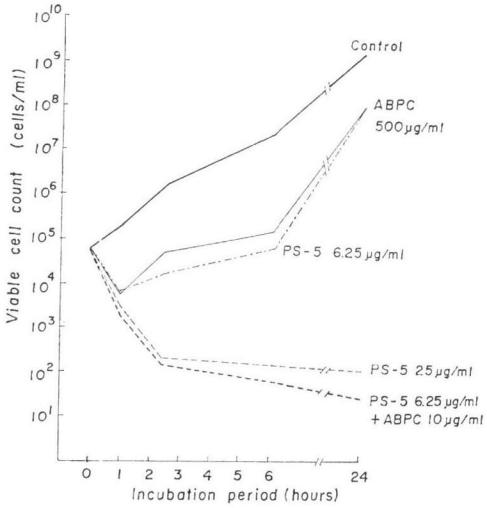
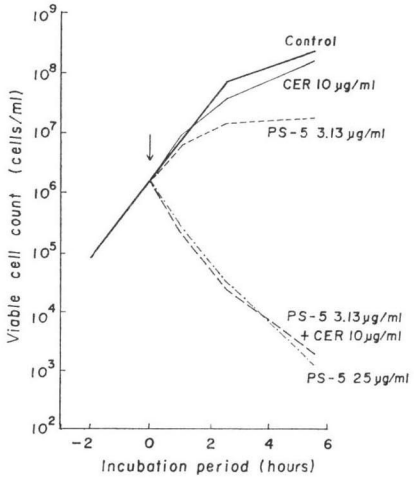


Fig. 5. Effect of PS-5 alone and in combination with CER on a growing culture of *Proteus vulgaris* P-5.
Medium: Heart Infusion broth
Incubation temperature: 37°C
Drugs were added at the time indicated by the arrow.



Five hundred μg ampicillin per ml resulted in a temporary decrease in the viable cell count of *P. vulgaris* GN76 and thereafter a great increase in viable cell count occurred. On the other hand, 25 μg PS-5 per ml and combinations containing 6.25 μg PS-5 per ml and 10 μg ampicillin per ml showed more than a 2 log decrease (>99%) in counts in less than 3 hours.

Fig. 6. LINEWEAVER-BURK plots of PS-5 inhibition.
Reaction mixtures contained cephaloridine (0.05~0.6 mM), phosphate buffer (100 mM, pH 7.0) and PS-5 (0.115 μM, 0.521 μM or none).
The reaction was started by the addition of 3.9 units of *P. vulgaris* β-lactamase (total volume 3 ml).
The reactions were carried out at 26°C throughout.

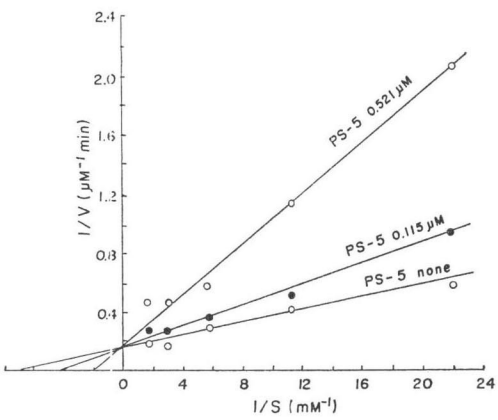


Table 3. Inhibition of β-lactamase from *Proteus vulgaris* strain P-5 by PS-5.

PS-5 (mM)	Enzyme activity	
	No preincubation*	5-Minute preincubation**
0	100%	100%
5.20×10^{-4}	43.7	38.0
1.04×10^{-3}	29.5	7.6
2.08×10^{-3}	16.8	3.6

Reaction mixtures contained 0.2 ml of the β-lactamase solution (3.9 units), 0.1 ml of PS-5 solution, 1.7 ml of M/10 sodium phosphate buffer (pH 7) and 1 ml of 0.3 mM cephaloridine in M/10 sodium phosphate buffer (pH 7). One unit of enzyme hydrolyses 1 μmole of cephaloridine per hour at pH 7 and 26°C. Enzyme activity was determined from the initial rate of decrease of absorbance at 255 nm. Reactions were carried out at 26°C.

* The reaction was started by adding the enzyme solution.

** The reaction was started by adding cephaloridine solution after 5-minute preincubation.

Against *Proteus vulgaris* P-5, 25 μg PS-5 per ml and combinations of 3.13 μg PS-5 per ml and 10 μg cephaloridine per ml showed a 2 log decrease in counts of *P. vulgaris* P-5 in 3 hours. Ten μg cephaloridine per ml alone had no effect on the growth because the MIC values of cephaloridine against the *Proteus* strain was 2,500 μg/ml. One fourth the MIC concentration of PS-5 (3.13 μg/ml) slightly repressed the growth of the *Proteus*. Thus PS-5 and ampicillin or cephaloridine showed synergistic antimicrobial activities against a growing culture of *Proteus vulgaris*.

Inhibition of β-Lactamase by PS-5

The inhibition of β-lactamase of *Proteus vulgaris* P-5 by PS-5 was investigated. The kinetic parameters of the enzyme activity were obtained from LINEWEAVER-BURK plots. The *Km* value with cephaloridine as a substrate was $1.0 \sim 1.5 \times 10^{-4}$ M. The *Ki* value for PS-5 determined with cephaloridine as a substrate was 2.1×10^{-7} M (mean) (Fig. 6). The enzyme was greatly inactivated by low levels of PS-5. The inactivation of the enzyme by PS-5 was progressive with time and 5-minute preincubation of the enzyme with PS-5 gave maximal inactivation.[5] Table 3 shows the per cent of inhibition without preincubation and with 5-minute preincubation of the enzyme with PS-5. Further detailed kinetics of PS-5 inhibition will be presented in a separate paper.

Discussion

New types of β-lactam antibiotics, clavulanic acid[6], olivanic acid derivatives[7] and thienamycin[8] were reported to be resistant to β-lactamases and to have inhibitory activity against β-lactamase. Clavulanic acid and olivanic acid derivatives cause synergistic activity in combination with other β-lactam antibiotics. PS-5 was found to have similarity in structure to these new types of β-lactam antibiotics[9], and to have inhibitory activity against a β-lactamase from *Proteus vulgaris*.

Clavulanic acid was reported to inhibit a wide range of β-lactamase and to give very marked improvement of activity for bacterial strains that owe their resistance to β-lactamase-production, especially *Staphylococcus aureus* Russell, *Klebsiella aerogenes* and *Proteus mirabilis*[10]. PS-5 showed marked improvement of the activity of β-lactam antibiotic against β-lactamase producing *Proteus vulgaris* and *Citrobacter freundii*. These organisms are known to produce Class I β-lactamase[11]. PS-5 inhibited a β-lactamase from *Proteus vulgaris* P-5 which was shown to produce Class I β-lactamase[12]. On the other hand, clavulanic acid is known to show good inhibitory activity against TEM-β-lactamase[13,14].

Clavulanic acid and olivanic acid derivatives were suggested to act as alkylating agents,[14] and the inhibition of clavulanic acid is time-dependent, which may be caused by irreversible slow inactivation of β-lactamases.

It can be concluded that there are two stage of inhibition by PS-5[5]. In the first stage, PS-5 competitively inhibits a β-lactamase of *P. vulgaris* and in the second stage subsequently shows a progressive inhibition which reaches the maximal inhibition after a few minute-preincubation with the enzyme.

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