STRUCTURE ACTIVITY RELATIONSHIPS IN PIPC-ANALOGUES AGAINST *PSEUDOMONAS AERUGINOSA*

JUNICHI MITSUYAMA, RITSUKO HORI, YUUKO ITO, AKIRA YOTSUJI, TAKASHI YASUDA and ISAMU SAIKAWA

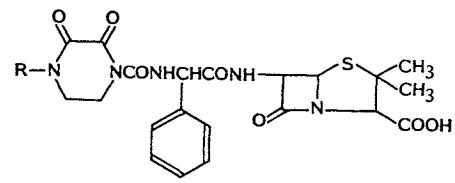
Research Laboratory, Toyama Chemical Co., Ltd., Toyama, Japan

(Received for publication October 8, 1988)

The relationship between the chemical structure and mode of action of piperacillin-analogues (PIPC-analogues) against *Pseudomonas aeruginosa* was investigated. The antibacterial activities of PIPC-analogues became stronger as the chain length of the alkyl group on the N-4 position in 2,3-dioxopiperazine when tested in constitutively β-lactamase-producing strain, but not paralleled in wild and β-lactamase-less strains. The outer membrane permeability was hardly affected by the chain length of the alkyl group at the N-4 position. The stability to β-lactamase was stronger with the increase of the number of the carbon atoms of N-4 position. In the binding-affinities to the lethal penicillin-binding proteins (PBPs), compounds PIPC (C-2), C-3 and C-4 showed lower ID_{50} values than compounds C-1, C-6 and C-8. These results suggested that the stability to β-lactamase was the governing part for the antibacterial activity in constitutively β-lactamase-producing strain, and the binding affinity to lethal PBPs directly contributed to the antibacterial activity in wild and β-lactamase-less strains.

Piperacillin sodium (PIPC: C-2) is a semisynthetic penicillin with broad spectrum activity against Gram-positive and Gram-negative bacteria[1]. We previously reported that the antibacterial activity of piperacillin-analogues (PIPC-analogues) (Fig. 1) became stronger as the increase of chain length of alkyl group on the N-4 position in 2,3-dioxopiperazine moiety in *Escherichia coli* and *Klebsiella pneumoniae*[2], and the main mechanism of antibacterial action was the increased affinities to penicillin-binding proteins (PBPs), especially to PBP 3. However, we found that the antibacterial activities were not always paralleled with the chain length of the compound in *Pseudomonas aeruginosa*. Thus, we considered that the mode of action of these compounds against *P. aeruginosa* differed to *E. coli* and *K. pneumoniae*. In this report, we investigated the mechanisms of action of PIPC-analogues against *P. aeruginosa*.

Fig. 1. Chemical structure of 6-[D(−)α-(4-alkyl-2,3-dioxo-1-piperazinecarboxamide)phenylactamide]-penicillanic acid.



	R	Code No.
C-1	CH_3	T-1187
C-2 (PIPC)	C_2H_5	T-1220
C-3	C_3H_7	T-1224
C-4	C_4H_9	T-1221
C-6	C_6H_{13}	T-1218
C-8	C_8H_{17}	T-1213

Materials and Methods

Organism

The bacterial strains employed in this study were *Pseudomonas aeruginosa* NCTC 10490 stocked in our laboratory. β-Lactamase constitutive and less mutants (*bla*$^+$ and *bla*$^-$ mutants) were derived from *P. aeruginosa* NCTC 10490 as follows. The *bla*$^+$-mutant was spontaneously isolated on the basis of latamoxef-resistance (50 μg/ml). One of the constitutive mutants, NCTC

10490 *bla*$^+$-R3, was then treated with 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanigine (NTG; Nakarai Chemical Co., Kyoto) in nutrient broth for 1 hour at 37°C and washed with 0.147 M sodium chloride. After an appropriate dilution, 0.1 ml of the cell suspension forming 100 to 200 colonies per plate was spread on nutrient agar plates. The colonies formed after incubation overnight at 37°C were overlayed with soft agar containing 5 ml of Difco agar 1%, 1 ml of KI 10 mg/ml and iodine 4 mg/ml, 0.5 ml of soluble starch 0.5% and 0.5% of benzylpenicillin (PCG) 10%. Colonies which did not form halos around them on agar were picked up and purified and referred to as *bla*$^-$ mutants. One of them is mutant NCTC 10490 *bla*$^-$-L2. For the measurement of outer membrane permeability, plasmid RP-4, specifying TEM-1 type β-lactamase was transferred to the strain NCTC 10490, mutants *bla*$^+$-R3 and *bla*$^-$-L2 by conjugation or transformation.

Antibiotics

PIPC-analogues (C-1, C-3, C-4, C-6 and C-8) were synthesized in our laboratory. PCG (Nippon Merck-Banyu Co., Ltd., Osaka, Japan) and cefmetazole (CMZ) (Sankyo Co., Ltd., Tokyo, Japan) were commercially available. The hydrophilic character of the drugs was expressed as the Rf value which was measured by reverse-phase TLC reported previously[2]. The polar mobile phase was acetate-veronal buffer (pH 7.0) - MeOH (3 : 2). Merck TLC Silica gel 60 F_{254} siliconized plate was used as the nonpolar stationary phase. The sample for examination was dissolved in the acetate-veronal buffer to give a concentration about 3 mg/ml, and 1 to 2 μl of the solution was then located on the TLC plate. After development at room temperature, the antibiotics were detected on the plate by using UV light. [^{14}C]PCG (specific activity, 58.9 mCi/mmol) was purchased from the Radiochemical Center, Amersham, England.

Measurement of Bacterial Susceptibility

The susceptibility of bacteria was measured by agar dilution method, and the susceptibility was expressed as the MIC. An overnight culture of bacterial strain in peptone broth (Polypeptone, 10 g, NaCl 5 g/liter) was diluted to give a final concentration of 10^6 cells/ml, and one loopful (about 5 μl) of each culture was inoculated on heart infusion agar (Eiken, Tokyo) plates using a replicating device (Microplanter; Sakuma Factory, Tokyo, Japan). The MICs were determined after overnight incubation at 37°C.

Outer Membrane Proteins

Outer membrane proteins were prepared from overnight cultures in heart infusion broth by differential solubilization of the cytoplasmic membrane with sodium lauryl sarcosinate after ultrasonic treatment[3]. Membranes were harvested by centrifugation at 100,000$\times g$ for 30 minutes at 4°C. Prior to electrophoresis, samples were boiled for 5 minutes at 100°C in 60 mM Tris-HCl buffer, adjusted to pH 6.8, containing glycerol 20%, 2-mercaptoethanol 5%, sodium dodecyl sulfate 2% and bromophenol blue 0.01%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels were prepared by the method of LUGTENBERG *et al.*[4] with 10% acrylamide in the separating gel. A 20-μg portion of protein from each sample was applied to the gel and subjected to electrophoresis at 25 mA until the dye front reached the bottom of the gel. The gels were stained with Coomassie brilliant blue R250.

Assay of Outer Membrane Permeability

The assay of outer membrane permeability was carried out using the method of SAWAI *et al.*[5], as expressed by the parameter "C" (cm^3/minute/μg of dry cell)[6].

Assay of PBPs

Strain NCTC 10490 was used for the PBPs assay. PBPs of *P. aeruginosa* were detected according to the method of SPRATT[7] with slight modifications. The concentration of the drugs required to inhibit the [^{14}C]PCG binding by 50% (ID_{50}) was determined from the densitometric tracing of radioactive PBP bands on an X-ray film.

Preparation and Measurement of β-Lactamase

Enzyme was extracted by sonication of harvested logarithmic phase cells of NCTC 10490 *bla*$^+$-

R3 which had been grown in heart infusion broth at 37°C. Sonicates were cleared of cell debris by ultracentrifugation (100,000×*g*, 30 minutes). Nucleic acid contamination of the crude enzyme was reduced by adding streptomycin sulfate (Banyu Pharmaceutical Co., Ltd., Japan) to a final concentration of 4 mg/ml. The obtained supernatant was dialyzed for 18 hours against 1,000-fold excess of 50 mM phosphate buffer (pH 7.0) at 4°C and stored at −20°C until use. The protein content of the enzyme was determined by the method of LOWRY *et al.*[8]. β-Lactamase activity was measured by the micro-iodometric method of NOVICK[9] with slight modifications. Vmax and *Km* values were obtained from Lineweaver-Burk plots of initial hydrolysis velocities (v) at the various substrate concentrations used. Physiological efficiency was defined as the ratio Vmax/*Km*[10].

Induction of β-Lactamase

Eight ml of heart infusion broth were inoculated with 1 ml of overnight cultures of the parent and mutants which had been grown in the same broth at 37°C with agitation. After 3 hours incubation, inducers were added to final concentrations of 100 μg/ml (CMZ) or at MIC (PIPC-analogues). After a further 1 hour incubation, the cells were harvested, washed and resuspended in 5 ml of 50 mM phosphate buffer (pH 7.0), then disrupted by ultrasonication. Supernatant which was cleared of cell debris by ultracentrifugation (100,000×*g*, 30 minutes) was used for β-lactamase assay.

Results

Antibacterial Activity of PIPC-analogues

Table 1 shows the MIC values against parent NCTC 10490, mutant *bla*$^+$-R3 and *bla*$^-$-L2 and hydrophilicities of the PIPC-analogues. The hydrophobicity became stronger with increase in the carbon number of the compounds. The antibacterial activity of mutant *bla*$^+$-R3, which is constitutive β-lactamase producer, became stronger with increase in the carbon number at the N-4 position of 2,3-dioxopiperazine. However, the antibacterial activities against parent and mutant *bla*$^-$-L2 of compounds C-2, C-3 and C-4 were 2- to 4-fold lower than C-1, C-6 and C-8 and not paralleled to the carbon number of the compounds.

Outer Membrane Permeability of PIPC-analogues

The outer membrane protein patterns of the parent, mutants *bla*$^+$-R3 and *bla*$^-$-L2 showed almost similar profiles in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The penetration rates of PIPC-analogues were measured with parent NCTC 10490, mutants *bla*$^+$-R3 and *bla*$^-$-L2 (Table 2). Although the increased carbon number at the N-4 position of 2,3-dioxopiperazine increased hydrophobicity of the compound, the penetration rates of these compounds in these strains showed almost constant levels regardless of their hydrophobicities.

Table 1. MICs of PIPC-analogues against *Pseudomonas aeruginosa* NCTC 10490 and mutant strains.

Compound	Hydrophilicity[a]	MIC (μg/ml)		
		Parent	*bla*$^-$-L2	*bla*$^+$-R3
C-1	0.69	0.2	0.1	200
C-2 (PIPC)	0.63	0.1	0.05	100
C-3	0.55	0.1	0.05	50
C-4	0.44	0.1	0.05	50
C-6	0.21	0.2	0.1	25
C-8	0.09	0.39	0.2	25

[a] Given as Rf value of reverse-phase TLC.

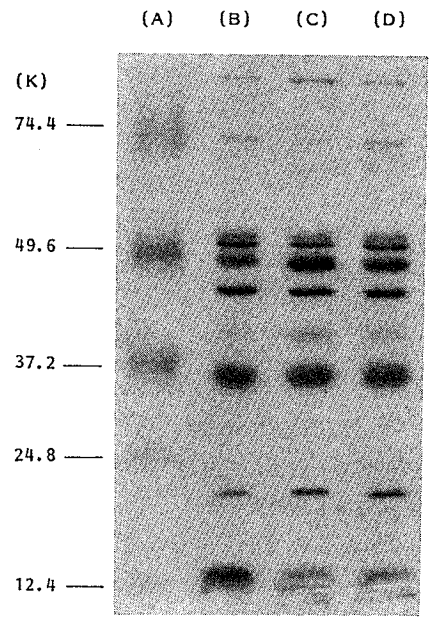
β-Lactamase Production

β-Lactamase activities of parent and mutant strains were shown in Table 3. By the addition of inducer (100 μg/ml of CMZ), β-lactamase production has been increased up to forty times higher in parent when compared with the growth in normal growth conditions, but not in mutants *bla*$^+$-R3 and *bla*$^-$-L2. Both mutants *bla*$^+$-R3 and *bla*$^-$-L2 seem to be lacking in inducibility. Furthermore, no inducible production of β-lactamase was seen by the addition of PIPC-analogues at MIC (data not shown).

Stability to β-Lactamase

β-Lactamase prepared from mutant *bla*$^+$-R3 was used to determine kinetic data for the test compounds. The results (Table 4) show the apparent *Km* and Vmax values for six of the test compounds. Although the Vmax values were not changed in all compounds, the *Km* values were increased with the chain length of the compounds. Thus, the Vmax/*Km* values, which was the indication of stability to β-lactamase, were decreased with the chain length of the compounds.

Fig. 2. SDS-PAGE of the outer membrane proteins.



Proteins were prepared from sarcosyl-treated cell envelopes as described in the text and analyzed on SDS-10% polyacrylamide slab gel. The standards were cytochrome c hexamer (74.4 K), tetramer (49.6 K), trimer (37.2 K), dimer (24.8 K) and monomer (12.4 K), purchased from Oriental Yeast Co., Ltd.
(A) Standard proteins, (B) *Pseudomonas aeruginosa* NCTC 10490, (C) *P. aeruginosa* NCTC 10490 *bla*$^-$-L2, (D) *P. aeruginosa* NCTC 10490 *bla*$^+$-R3.

Table 2. Outer membrane permeability of PIPC-analogues.

Strain	Permeability coefficient ($\times 10^{-4}$ cm^3/minute/μg of dry cell)					
	C-1	C-2 (PIPC)	C-3	C-4	C-6	C-8
Parent (NCTC 10490)	0.95	1.07	0.78	1.35	1.02	1.03
bla$^-$-L2	0.78	0.91	1.25	1.12	0.89	0.95
bla$^+$-R3	1.01	1.07	0.78	1.35	1.02	1.03

Table 3. β-Lactamase production in *Pseudomonas aeruginosa* NCTC 10490 and their mutant strains.

Strain	β-Lactamase activity ($\times 10^{-3}$ u/mg protein)		Induced/non-induced ratio
	Non-induced	Induced	
Parent (NCTC 10490)	5.68	253	44.5
bla$^-$-L2	0.90	0.86	0.96
bla$^+$-R3	2,620	2,650	1.01

β-Lactamase was induced by the addition of 100 μg/ml CMZ, and further incubated for 1 hour at 37°C.

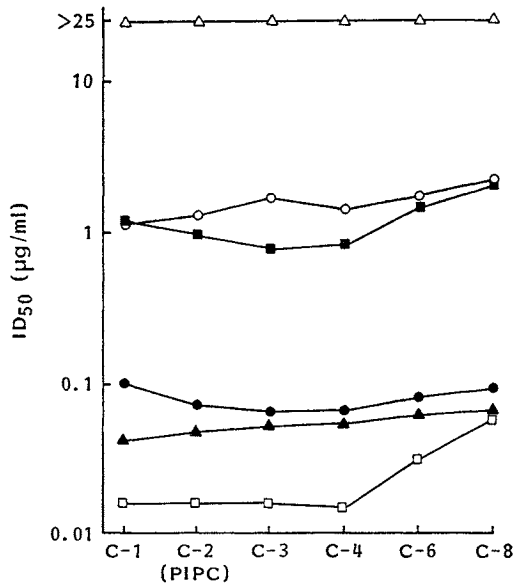
Table 4. Hydrolysis kinetics of PIPC-analogues for the chromosomal β-lactamase from strain *bla*$^+$-R3.

Compound	Vmax (relative)	*Km* (μM)	Physiological efficiency[a] (relative)
C-1	93.4	9.7	135
C-2 (PIPC)	100	14.0	100
C-3	110	25.2	61.3
C-4	110	30.9	49.9
C-6	106	41.9	35.4
C-8	121	50.0	33.9

[a] Vmax/*Km*.

Fig. 3. Competitions of PIPC-analogues to penicillin binding proteins of *Pseudomonas aeruginosa* NCTC 10490.

● PBP 1A, ○ PBP 1B, ■ PBP 2, □ PBP 3, ▲ PBP 4, △ PBP 5.



Binding Affinities of PIPC-analogues to *P. aeruginosa* PBPs

The ID_{50} values of PIPC-analogues to parent NCTC 10490 PBPs were shown in Fig. 3. In PBPs 1A and 2, the compounds C-2, C-3 and C-4 showed lower values than the other compounds. In PBP 3, the ID_{50} values were almost constant levels within the compounds C-1 to C-4, but increased drastically in the compounds C-6 to C-8. In PBPs 1B and 4, the ID_{50} values were increased moderately with the increase in the carbon number of the compounds. In PBP 5, the ID_{50} values were over 25 μg/ml in all compounds. The binding affinities for the PBPs in mutant *bla*$^-$-L2 were also shown in a similar manner (data not shown).

Discussion

In this work, we investigated the antibacterial activity of PIPC-analogues against *P. aeruginosa*. The MIC patterns of PIPC-analogues against *P. aeruginosa* were independent to the carbon number of the compounds distinct from that of *E. coli* and *K. pneumoniae* reported previously[2].

Permeability of the outer membrane has been proposed as a major contributing part in the intrinsic resistance of this species[11~14]. However, our permeability data strongly suggested that the outer membrane permeability of PIPC-analogues had no contribution on their antibacterial activities. Similar conclusions have been observed previously for *E. coli* and *K. pneumoniae*[2]. The outer membrane permeability of PCG and ampicillin, which have no antipseudomonal activity, showed similar values to PIPC-analogues (data not shown), suggesting that, in penicillins at least, the antibacterial activities are hardly affected by the outer membrane permeability.

The β-lactamase inducibility and the stability to β-lactamase are also known to play an important role in resistance to β-lactam antibiotics[15]. However, induction of the chromosomal enzyme had little, if any, effect on the differences of the antibacterial activity in PIPC-analogues, since the MIC pattern of mutant *bla*$^-$-L2 which hardly produce β-lactamase, was similar to those of parent NCTC 10490. Furthermore, parent NCTC 10490 hardly induced chromosomal β-lactamase at or near the MIC in PIPC-analogues. JACOBS *et al.*[16] also reported that the ureido-type penicillin were poor enzyme inducer at or below MIC. The specific activity of *bla*$^-$-L2 and *bla*$^+$-R3 remained at the uninduced levels, even in the presence of inducer (Table 3). These results suggested that β-lactamase inducibility were not correlated to their antibacterial activity in parent and mutant strains.

The lethal targets of PBPs in *P. aeruginosa* were PBPs 1A, 2 and 3[17]. In parent and mutant *bla*$^-$-L2, the MIC patterns were directly correlated to the ID_{50} value, especially to PBP 3. It can be expected that PBP 3 was affected primarily in intact cell because of PBP 3 was the most sensitive enzyme against PIPC-analogues.

In contrast, the MIC patterns of *bla*$^+$-R3 was correlated to the stability to β-lactamase of PIPC-analogues. This result presumably suggested that the stability to β-lactamase was the rate-limiting step to determine their antibacterial activities when the enzymes already existed abundantly in periplasmic space.

References

1) UEO, K.; Y. FUKUOKA, T. HAYASHI, T. YASUDA, H. TAKI, Y. WATANABE, I. SAIKAWA & S. MITSUHASHI: In vitro and in vivo antibacterial activity of T-1220, a new semisynthetic penicillin. Antimicrob. Agents Chemother. 12: 455~460, 1977
2) MITSUYAMA, J.; M. TAKAHATA, T. YASUDA & I. SAIKAWA: The mechanism of action of piperacillin-analogues *in vitro*; effect of the carbon number at the N-4 position of 2,3-dioxopiperazine on the outer membrane permeability, stability to β-lactamase and binding affinity to penicillin-binding proteins. J. Antibiotics 40: 868~872, 1987
3) FILIP, C.; G. FLETCHER, J. L. WULFF & C. F. EARHART: Solubilization of cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl-sarcosinate. J. Bacteriol. 115: 717~722, 1973
4) LUGTENBERG, B.; J. MEIJERS, R. PETERS, P. VAN DER HOEK & L. VAN ALPHEN: Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. FEBS Lett. 58: 254~258, 1975
5) SAWAI, T.; R. HIRUMA, N. KAWANA, M. KANEKO, F. TANIYASU & A. INAMI: Outer membrane permeation of β-lactam antibiotics in *Escherichia coli*, *Proteus mirabilis*, and *Enterobacter cloacae*. Antimicrob. Agents Chemother. 22: 585~592, 1982
6) YAMAGUCHI, A.; N. TOMIYAMA, R. HIRUMA & T. SAWAI: Difference in pathway of *Escherichia coli* outer membrane permeation between penicillins and cephalosporins. FEBS Lett. 181: 143~148, 1985
7) SPRATT, B. G.: Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. 72: 341~352, 1977
8) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265~275, 1951
9) NOVICK, R. P.: Micro iodometric assay for penicillinase. Biochem. J. 83: 236~240, 1962
10) POLLOCK, M. R.: Purification and properties of penicillinases from two strains of *Bacillus licheniformis*; a chemical, physiochemical, and physiological comparison. Biochem. J. 94: 666~675, 1965
11) LEIVE, R.: The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. 235: 109~125, 1974
12) NIKAIDO, H. & T. NAKAE: The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. 20: 163~250, 1979
13) ZIMMERMANN, W.: Penetration of β-lactam antibiotics into their target enzymes in *Pseudomonas aeruginosa*: Comparison of a highly sensitive mutant with its parent strain. Antimicrob. Agents Chemother. 18: 94~100, 1980
14) GODFREY, A. J. & L. E. BRYAN: Resistance of *Pseudomonas aeruginosa* to new β-lactamase-resistant β-lactams. Antimicrob. Agents Chemother. 26: 485~488, 1984
15) MINAMI, S.; N. MATSUBARA, A. YOTSUJI, Y. WATANABE, T. YASUDA, I. SAIKAWA & S. MITSUHASHI: Antibacterial activity of cefoperazone alone and in combination against cephalosporinase-producing *Enterobacter cloacae*. Antimicrob. Agents Chemother. 24: 123~125, 1983
16) JACOBS, J. Y.; D. M. LIVERMORE & K. W. M. DAVY: *Pseudomonas aeruginosa* β-lactamase as a defence against azlocillin, mezlocillin and piperacillin. J. Antimicrob. Chemother. 14: 221~229, 1984
17) NOGUCHI, H.; M. MATSUHASHI & S. MITSUHASHI: Comparative studies of penicillin-binding proteins in *Pseudomonas aeruginosa* and *Escherichia coli*. Eur. J. Biochem. 100: 41~49, 1979