AFFINITY OF E1077, A NEW CEPHALOSPORIN, FOR PENICILLIN-BINDING PROTEINS OF *Staphylococcus aureus* AND ITS ANTISTAPHYLOCOCCAL ACTIVITY

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(Received for publication May 17, 1993)

Potent antistaphylococcal activity was conferred on E1077 by the introduction of the propenylammonium group at the 3-position in the cephem nucleus and of the fluoromethoxyimino group in the 7β-side chain. Antistaphylococcal activity was more markedly increased by the former group than by the latter. This effect seemed likely to be due to the increased high affinity for penicillin-binding protein (PBP) 3, which may be one of the essential PBPs of *Staphylococcus aureus*, and secondly for PBP 4. E1077 also showed more potent bactericidal activity than did cefpirome at concentrations above the MICs, although the MICs of E1077 for *S. aureus* were only half those of cefpirome. While cefpirome showed little killing activity within 4 hours at its MIC, the addition of cefoxitin (0.05 μg/ml), a specific inhibitor for PBP 4, enhanced the killing activity of cefpirome to match that of E1077. In addition, peptidoglycan (PG) obtained from cells grown with the subinhibitory E1077 concentration was more susceptible to lytic enzymes than that from untreated cells or cefpirome-treated cells. These results indicated that the increased inhibition of PBPs 3 and 4 by E1077, which was brought about by the introduction of two distinctive functional groups, led to the enhanced antistaphylococcal activity and to the production of poorly cross-linked PG, and thereby to rapid bactericidal activity.

E1077 is a new parenteral cephalosporin with a broad antibacterial spectrum[1]. It possesses two structural features, that distinguish it from other recent cephalosporins, namely, a propenylammonium moiety at the 3-position of the cephem nucleus and a fluoromethoxyimino group in the 7β-side chain. As described previously[2], the introduction of these distinctive functional groups provided E1077 with enhanced antibacterial activity, especially against Gram-positive cocci. However, it had been unknown what this enhanced antibacterial activity resulted from.

In this study, the roles of these groups in the antibacterial activity of E1077 against *Staphylococcus aureus* were investigated. In addition, the potent bactericidal activity of E1077 against *S. aureus* is also analyzed.

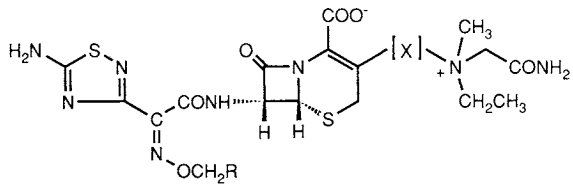
Materials and Methods

Strains and Antibiotics

The strains of *Staphylococcus aureus* used in this study were recent clinical isolates from various hospitals in Japan and were identified as *S. aureus* by using the api STAPH (BIO MÉRIEUX S.A., Mercy-I'Etoile, France). They were susceptible to methicillin, which showed MICs of $\leq$6.25 μg/ml. A laboratory strain 209P JC-1, susceptible to methicillin, was also used. E1077, its three derivatives (Fig. 1), and cefpirome were synthesized (their chemical purities, >95%) at Tsukuba Research Laboratories, Eisai Co. Ceftazidime and cefoxitin were obtained commercially from Nihon Glaxo Co., Tokyo, Japan, and Banyu Pharmaceutical Co., Tokyo, Japan, respectively.

Fig. 1. Chemical structures of E1077 and its derivatives.

"t" in X indicates "*trans*".



Compound	R	X
E1077	F	$-CH\overset{t}{=}CH-CH_2-$
Compound 1	H	$-CH\overset{t}{=}CH-CH_2-$
Compound 2	F	$-CH_2-$
Compound 3	H	$-CH_2-$

Determination of MICs

MICs were determined on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md., U.S.A.) by an agar dilution method. Approximately 5×10^4 CFU per spot were inoculated onto agar plates that contained two-fold serial dilutions of antibiotics. The MIC was considered to be the lowest concentration of antibiotic that completely inhibited visible growth after incubation for 18 hours at 37°C[1].

Affinities for PBPs

The affinities of cephalosporins for penicillin-binding proteins (PBPs) were determined by the conventional competition assay with [^{14}C]-benzylpenicillin (Amersham Japan, Tokyo, Japan; specific activity, 54 mCi/mmol). *S. aureus* cell membranes were prepared as described previously[3], with some modifications. *S. aureus* grown to mid-exponential phase was harvested, and cells were broken by 50 μg/ml lysostaphin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) treatment for 30 minutes at 37°C and sonication for 2 minutes on ice-water. The membrane fractions were obtained by ultracentrifugation (100,000 $\times g$, 1 hour, 4°C), resuspended in 50 mM phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$, and stored at −80°C until used in PBP binding experiments. The competition assay was carried out as described by NOGUCHI *et al.*[4]. Membrane proteins (240 μg in 30 μl) were incubated with various concentrations of antibiotics (3 μl) for 10 minutes at 30°C. [^{14}C]-Benzylpenicillin (0.15 μCi in 3 μl; final concentration, 29 μg/ml) was added, and incubation was continued for an additional 10 minutes. Excess unlabeled benzylpenicillin (final concentration, 3 mg/ml) was added to stop the reaction, and the membranes were solubilized in 1% sodium lauroyl sarcosinate. Sarcosinate-soluble fractions were subjected to 0.1% sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis. For fluorography, gels were incubated for 1 hour in En^{3}Hance (Daiichi Pure Chemicals Co., Tokyo, Japan), soaked in water for 1 hour, dried, and exposed to X-ray film at −80°C for about 1 month. The relative band densities on the fluorograms were determined by using a scanning densitometer with peak integration. The binding affinities of antibiotics for each PBP were expressed in terms of the concentration required to reduce [^{14}C]-benzylpenicillin binding by 50%.

Bactericidal Activity

Time-kill analysis was performed by inoculating exponential-phase cells into prewarmed Mueller-Hinton broth (BBL Microbiology Systems) to an inoculum of about 5×10^5 CFU/ml. Freshly prepared antibiotics were added. At intervals during incubation for 24 hours at 37°C, samples were removed and serially diluted 10-fold. One milliliter of sample dilutions was mixed with melted Mueller-Hinton agar (about 50°C). The CFU was numerated and regarded as viable cells after 18 to 24 hours of incubation at 37°C. The drug carry-over did not affect colony formation since the samples were at least 100-fold diluted. The minimal detection limit was 10 CFU/ml.

Preparation of Peptidoglycan (PG)

PG was prepared by the method of PETERSON *et al.*[5]. Cultures grown in the presence or absence of cephalosporins for 4 hours were used. Cells from 1 liter of exponential-phase cultures were washed and broken by shaking with glass beads (i.d. = 0.10 ~ 0.11 mm) in a Bead-Beater (Biospec Products, Bartlesville, Ok., U.S.A.). Crude cell walls were isolated by differential centrifugation and were washed twice in cold distilled water. The walls were heated in boiling water for 15 minutes to inactivate autolytic enzymes, and were further purified by nuclease and trypsin treatments and by 40%-phenol extraction to remove lipoteichoic acid. PG was obtained by hot trichloroacetic acid treatment of the purified walls and stored in water suspension at −20°C.

Autolysin Extraction

Autolysin extracts from *S. aureus* cells were obtained by the method of BEST *et al.*[6]. Cultures grown to the exponential phase were harvested, and cells were washed twice with 10 mM potassium phosphate buffer (pH 7.0) and suspended in 3 M LiCl at 4°C with stirring for 10 minutes. Cells were removed by centrifugation (25,000 × *g* for 20 minutes at 4°C), and the supernatant was dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.0). Autolysin extracts were stored at −80°C.

Lysis of Whole Cells and PG by Lytic Enzymes

Lysis of PG was measured by the method of QORONFLEH and WILKINSON[7]. PG was suspended in phosphate buffered saline (pH 7.4) for lysozyme (Sigma Chemical) and lysostaphin (Sigma Chemical) and in 10 mM potassium phosphate buffer (pH 7.0) for extracted autolysin, to an absorbance at 580 nm (A_{580}) of 0.4 to 0.6. Enzymes were added, suspensions were incubated at 30°C for autolysin or 37°C for lysozyme and lysostaphin, and A_{580} readings were taken at intervals using a Hitachi 220A spectrophotometer. The final concentrations of the lytic enzymes, lysozyme, lysostaphin, and LiCl-extracted autolysin, that were used for PG lysis, were 25, 0.5, and 50 μg/ml, respectively.

Whole cells were washed once in 10 mM potassium phosphate buffer (pH 7.0), suspended in the same buffer to an A_{580} of 0.5 to 0.6, and incubated with shaking at 30°C. A_{580} readings were taken with a Hitachi spectrophotometer at intervals during incubation.

Results

Antistaphylococcal Activity of E1077 and Its Derivatives

One derivative of E1077, compound 1, possesses a methoxyimino group in place of the fluoromethoxyimino group in the 7β-side chain. Compound 2 is a derivative with a methylammonio substituent replacing the propenylammonio moiety at the 3-position of the cephem nucleus. In compound 3, the third derivative, both of these substitutions are present (Fig. 1). The activity of E1077 and its derivatives against 29 methicillin-susceptible clinical isolates of *S. aureus* is shown in Table 1. The MICs of E1077 for 50% of the isolates (MIC_{50}) and for 90% of the isolates (MIC_{90}) were 0.39, and 0.78 μg/ml, respectively. This activity was 2, 8, and 16 times higher than the respective activities of compounds 1, 2, and 3. This indicated that antistaphylococcal activity was increased eight-fold by the introduction of the propenylammonium group into the 3-position, and doubled by the introduction of fluorine into the 7β-methoxyimino group.

To investigate the relationship between the activity of and the substituents in E1077 derivatives, the PBP binding affinities of these compounds were estimated by using membranes isolated from *S. aureus* E31295, a clinical isolate included in Table 1. Affinities were determined by a series of competition assays with [^{14}C]-benzylpenicillin. The results indicate that E1077 and compound 1, qualitatively, bind well to all of the PBPs, and that compounds 2 and 3 do not bind as well to PBP 3 as do E1077 and compound 1. The amount of unlabeled antibiotic which reduces binding to a PBP by 50% when radiolabeled

Table 1. Antibacterial activity of E1077 and its derivatives against 29 strains of *Staphylococcus aureus*[a].

Compound	MIC (μg/ml)		
	Range	50%	90%
E1077	0.20~0.78	0.39	0.78
Compound 1	0.39~1.56	0.78	0.78
Compound 2	1.56~6.25	3.13	6.25
Compound 3	1.56~12.5	6.25	12.5
Cefpirome	0.20~3.13	0.78	1.56
Ceftazidime	3.13~25	12.5	12.5

[a] Methicillin-susceptible strains with methicillin MICs of ≤6.25 μg/ml were used.

Table 2. Affinities of E1077 and its derivatives for PBPs of *Staphylococcus aureus* E31295.

Compound	IC_{50} (μg/ml) with PBP:				MIC (μg/ml)
	1	2	3	4	
E1077	0.05	0.20	0.39	0.10	0.39
Compound 1	0.10	0.20	0.39	0.20	0.78
Compound 2	0.10	0.20	6.25	0.78	3.13
Compound 3	0.20	0.39	25	1.56	6.25

benzylpenicillin is added (IC_{50}) was estimated (Table 2). No significant differences were observed between the affinities of the compounds tested for PBPs 1 and 2. However, E1077 and compound 1 had IC_{50}s for PBP 3 that were 16- to 64-fold lower than those of compound 2 and compound 3, which have no propenylammonium group. In addition, the affinities for PBP 4 were increased eight-fold by the introduction of the propenylammonium group. This affinity profile of E1077 for *S. aureus* PBPs was confirmed in the strain 209P JC-1 other than the strain E31295. The MIC value of E1077 for the strain 209P JC-1 was 0.20 μg/ml, and the IC_{50}s of E1077 for PBPs 1, 2, 3, and 4 of 209P JC-1 were 0.05, 0.20, 0.20, and 0.05 μg/ml, respectively. The effect of fluorine on the affinities for PBPs was less significant than that of the 3-propenylammonium group, and the introduction of fluorine increased only twice the affinities for PBPs 1 and 4. For *S. aureus*, the MICs seemed to correlate roughly with the compounds' respective affinities for PBPs 3 and 4.

Killing Activity of E1077

The killing activities of E1077 and cefpirome, which had comparable MICs against *S. aureus*, were compared. E1077 showed rapid killing activity against a strain E31295, decreasing the number of viable cells by two to three orders of magnitude within 2 hours at concentrations above the MIC (0.39 μg/ml) (Fig. 2A). The killing activity of cefpirome during the initial 2 and 4 hours, by contrast, was slower even at concentrations above the MIC (0.78 μg/ml), the viable cell count being reduced by only one order of magnitude. With the addition of 0.05 μg of cefoxitin per ml, a selective inhibitor for PBP 4[8], cefpirome showed a killing activity as rapidly as did E1077 not only at concentrations above the MIC but also at 1/2 MIC (Fig. 2B). No significant effect of cefoxitin addition was observed on the killing activity of E1077 at any concentrations tested. Cefoxitin alone showed no bactericidal activity at 0.05 μg/ml (data not shown).

The affinities of cefpirome for PBPs 1, 2, and 3 were as high as those of E1077. However, the affinity of cefpirome for PBP 4 (IC_{50}, >12.5 μg/ml) was at least two orders of magnitude lower than that of E1077 (IC_{50}, 0.10 μg/ml). With the addition of 0.05 μg of cefoxitin per ml, cefpirome showed almost the same profile of PBP inhibition as did E1077 with or without cefoxitin (Table 3).

Against a strain E31308 of *S. aureus*, E1077 again showed rapid killing at concentrations above the MIC (0.78 μg/ml), but the killing activity of cefpirome was lower at concentrations above the MIC (1.56 μg/ml) (Fig. 3A). However, a paradoxical effect on killing activity against this strain was observed at concentrations above the MIC: E1077 showed less killing activity at higher concentrations. This phenomenon may be identical to that described by EAGLE and MUSSELMAN[9]. Interestingly, this effect was not observed with cefpirome alone but was seen when cefpirome was used with cefoxitin (0.05 μg/ml),

Fig. 2. Bactericidal activity of E1077 and cefpirome against *Staphylococcus aureus* E31295 in the absence (A) and in the presence (B) of 0.05 μg cefoxitin per ml.

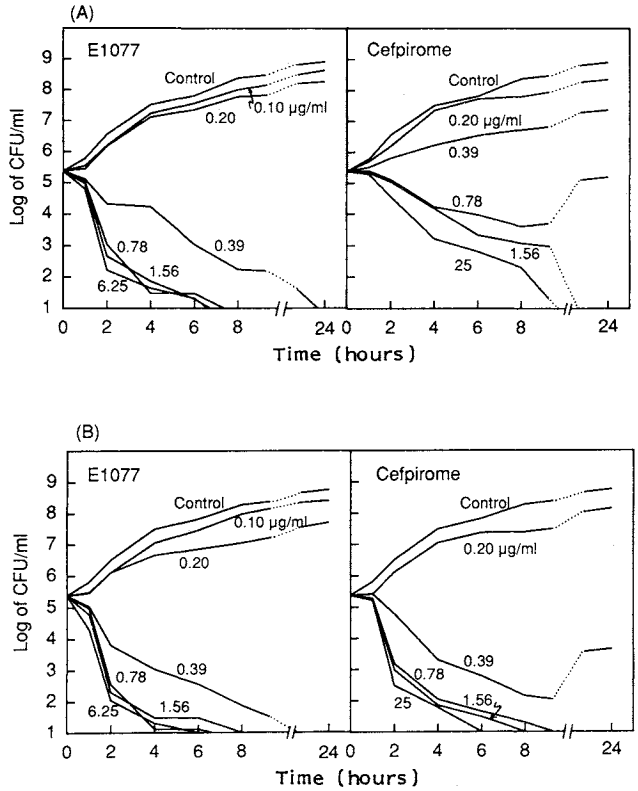


Table 3. Affinities of E1077 and cefpirome for PBPs of *Staphylococcus aureus* E31295 in the presence or absence of 0.05 μg cefoxitin per ml.

Compound	Cefoxitin	IC_{50} (μg/ml) with PBP:				MIC (μg/ml)
		1	2	3	4	
E1077	None	0.05	0.20	0.39	0.10	0.39
	Added[a]	0.05	0.20	0.39	≤0.025	0.39
Cefpirome	None	≤0.05	0.20	0.78	>12.5	0.78
	Added	≤0.05	0.20	0.78	≤0.05	0.78
Cefoxitin	None	1.56	0.78	1.56	0.012	3.13

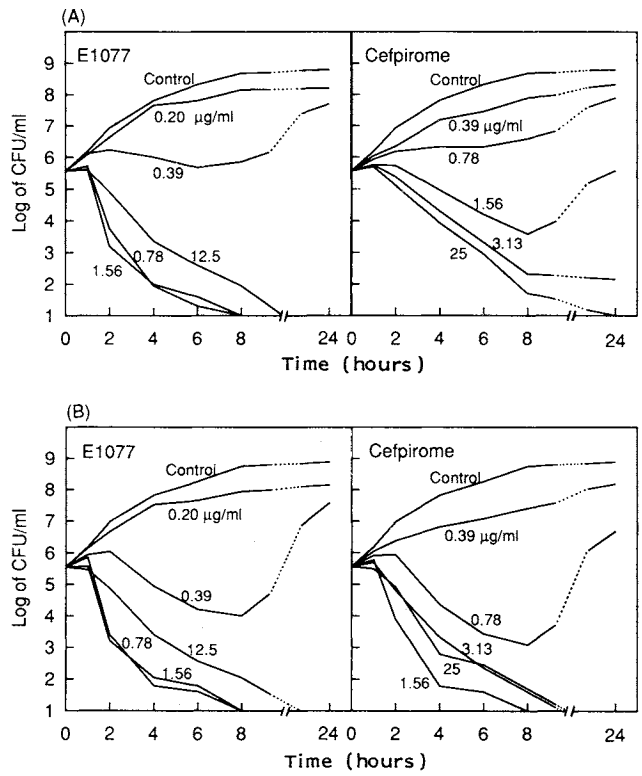
[a] Membrane proteins were incubated with 0.05 μg of cefoxitin per ml and the various concentrations of the competing cephalosporins for 10 minutes at 30°C. The following procedure was the same as that for the usual competition assay.

although the killing activity of cefpirome was increased by cefoxitin (Fig. 3B).

Lysis of Whole Cells and PG

Since cefoxitin, which specifically inhibits PBP 4, has been shown to lead to hypo-cross-linked PG[7,10], the cell walls of *S. aureus* grown in the presence of E1077 were expected to be more susceptible to lytic enzymes. Since cefpirome caused cell lysis at 0.39 μg/ml (1/2 MIC) in the presence of 0.05 μg of cefoxitin per ml, the susceptibility to lytic enzymes of the walls from cells grown in the presence of 1/4

Fig. 3. Bactericidal activity of E1077 and cefpirome against *Staphylococcus aureus* E31308 in the absence (A) and in the presence (B) of 0.05 μg cefoxitin per ml.



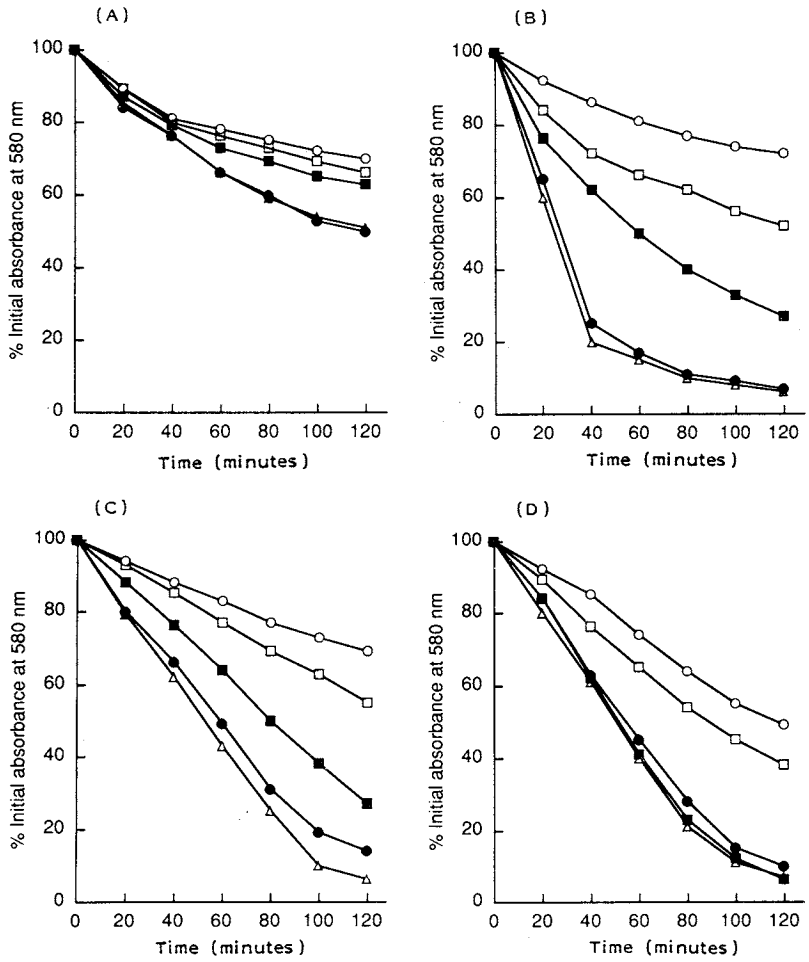
MIC of each cephalosporin was investigated. Strain E31295 cells, grown for 4 hours in the presence of 0.10 μg of E1077 per ml and in the presence of 0.20 μg/ml cefpirome plus 0.05 μg/ml cefoxitin, autolyzed more rapidly, though its difference might be slight, than they did when grown in the presence of cefpirome alone (0.20 μg/ml) or in the absence of antibiotics (Fig. 4A). No differences between the amount or activity of autolysin extracted by LiCl from cells grown in the presence of E1077 or cefpirome, or grown in the absence of antibiotics were observed (data not shown).

PG isolated from cells grown in the presence of E1077 was more susceptible to the lytic enzymes lysozyme (Fig. 4B), lysostaphin (Fig. 4C), and LiCl-extracted autolysin (Fig. 4D), than that isolated from cells grown in the presence of cefpirome or in the absence of antibiotics. PG isolated from cells grown in the presence of cefpirome plus cefoxitin was also more susceptible to the lytic enzymes than that isolated from cells grown in the presence of cefpirome alone, and the suceptibility of the former was increased to almost the same extent as that of PG isolated from cells grown in the presence of E1077. PG isolated from cells grown in the presence of cefoxitin was also more susceptible to the lytic enzymes that that isolated from cells grown in the presence of cefpirome or in the absence of antibiotics, but was less susceptible to lysozyme and lysostaphin but not to autolysin than that isolated from cells grown in the presence of E1077 or in the presence of cefpirome plus cefoxitin.

These experiments provide evidence that the increased susceptibility of cell walls to lytic enzymes is due to the reduction of PG cross-linking by the inhibition of PBP 4.

Fig. 4. Autolysis of whole cells of strain E31295 (A) and lysis of peptidoglycan from strain E31295 by lysozyme (B), lysostaphin (C), and LiCl-extracted autolysin (D).

Peptidoglycan was isolated from *Staphylococcus aureus* E31295 cells grown for 4 hours in the absence of antibiotics (○), in the presence of 0.10 μg/ml E1077 (●), in the presence of 0.20 μg/ml cefpirome (□), in the presence of 0.05 μg/ml cefoxitin (■), and in the presence of 0.20 μg/ml cefpirome plus 0.05 μg/ml cefoxitin (△).



Discussion

The mode of action of β-lactam antibiotics against some Gram-negative bacteria has been elucidated, and so the biological role of each PBP has been clarified. However, in *S. aureus*, it is as yet unknown which of the four PBPs is the target for the lethal action of β-lactam antibiotics or what role each PBP plays, since no distinct morphological changes of this organism are induced by β-lactam antibiotics. PBP 2 and PBP 3 have been reported to possess transpeptidase activity and to be the primary target of β-lactams[11,12]. It has been also reported that PBP 1 and PBP 4 are probably non-essential for cell growth, since mutants lacking PBP 1 or PBP 4 grow normally[13,14]. However, BEISE *et al.* and REYNOLDS have recently indicated that inhibition of PBP 1 is likely to be important for the antibacterial activity of β-lactam antibiotics against *S. aureus*[8,15].

In this study, it was evident that the introduction of the propenylammonium group in place of the methylammonium group at the 3-position of cephem nucleus enhanced the antistaphylococcal activity. This increased activity was due to the increased inhibition of PBP 3 of *S. aureus*, confirming that PBP 3

is a one of the essential PBPs of *S. aureus*[11,12,15]. However, the detailed mechanism of PBP 3 inhibition by the propenyl side chain is not yet known. The introduction of the propenylammonium group also increased the affinity for PBP 4. Although the increased inhibition of PBP 4 by this group also correlated with the MIC, the inhibition of PBP 4 is unlikely to lead to higher antistaphylococcal activity since no significant correlations were observed between the MICs of and the PBP 4 inhibition by cefpirome or cefoxitin.

The susceptibility to lytic enzymes of peptidoglycan (PG), which was isolated from cells grown with a subinhibitory concentration of E1077, corresponding to the IC_{50} with PBP 4, was increased, suggesting that E1077 decreased the cross-linking of PG. The same effect was observed on the PG isolated from cells grown in the presence of cefpirome plus cefoxitin or in the presence of cefoxitin, a selective inhibitor for PBP 4[7,10]. Thus, the inhibition of PBP 4 function, a secondary transpeptidase activity which was responsible for the high degree of PG cross-linking[10], is confirmed to lead to the increased susceptibility of PG to lytic enzymes. Therefore, the rapid killing activity of E1077 against *S. aureus* was probably due mainly to inhibition of the secondary cross-linking function of PBP 4.

However, one may doubt whether its rapid killing activity is due to only the inhibition of PBP 4, because other PBPs were also fully or to some extent inhibited even at subinhibitory concentrations of E1077. This question may be resolved by the finding that the killing activity of cefpirome was enhanced by a low concentration of cefoxitin. It should be also noted that the rapid killing activity of E1077 was observed only at concentrations above the MICs. This can perhaps be explained as follows. Since essential PBP(s) may not have been fully inhibited and autolysin activity may not have been expressed in spite of the decreased degrees of PG cross-linking at concentrations less than the MICs, no killing activity of E1077 was observed. In contrast, at concentrations above the MICs, E1077 exhibited rapid killing activity, since PG cross-linking may have decreased and autolysin activity may perhaps be expressed together with the inhibition of essential PBP(s). The killing activity of cefpirome is likely to appear more gradually than that of E1077, since autolysins are expressed but PBP 4 is not inhibited and PG cross-linking is not decreased even at concentrations above the MICs.

Although QORONFLEH and WILKINSON reported that whole cells treated with cefoxitin for 18 hours autolyzed at the same rate as did those untreated with antibiotics[7], our results indicated that the autolysis rate of whole cells treated with E1077 for 4 hours was more rapid than that of those treated with cefpirome or untreated with antibiotics. This discrepancy seems to result from the cultivation time. No significant differences between autolysis rates of whole cells grown with or without antibiotics for 18 hours were observed, and the autolysin activity extracted from cells grown for 18 hours was about one-tenth of that from cells grown for 4 hours (data not shown). Stationary-phase cells have increased the *O*-acetylation of PG[16,17], and so the susceptibility of PG to lytic enzymes may be decreased. It seems likely that the prolonged cultivation time was responsible for the decreased autolysin activity and the decreased autolysis rate of whole cells.

In strain E31308, paradoxically reduced activity was observed with E1077 or with cefpirome plus cefoxitin but not with cefpirome alone, although this effect seems to be strain-specific. This may suggest that inhibition of PBP 4 regulates the expression of essential PBP(s) or autolysin activity. Further studies on this effect are warranted.

Acknowledgment

We thank K. KAMIYA, T. NAITO, and N. SATO for the supply of cephalosporins.

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