β-LACTAMASE INHIBITORY ACTIVITIES AND SYNERGISTIC EFFECTS OF 5,6-*CIS*-CARBAPENEM ANTIBIOTICS

KENJI OKONOGI, SETSUO HARADA, SUSUMU SHINAGAWA, AKIRA IMADA and MITSUZO KUNO

Applied Microbiology Laboratories, Central Research Division
Takeda Chemical Industries Ltd., Yodogawa-ku, Osaka 532, Japan

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Twelve 5,6-*cis*-carbapenem antibiotics were examined for their β-lactamase inhibitory activities, their types of inhibitions, and their synergistic activities with other β-lactam antibiotics. All the carbapenems inhibited eight types of β-lactamases including cephalosporinases which were insensitive to clavulanic acid and sulbactam. The sulfonyloxy ethyl carbapenems were the most active inhibitors; they inhibited all β-lactamases in a progressive fashion, whereas some of the hydroxyl compounds exerted non-progressive inhibition against several β-lactamases such as those of *Escherichia coli* TN713 and *Proteus vulgaris* GN4413. Several carbapenems were inactivated by the β-lactamases of *Citrobacter freundii* GN1706, *P. vulgaris* GN4413, *E. coli* TN713, and *Klebsiella pneumoniae* TN1698. Most of the carbapenems potentiated the antibacterial activities of ampicillin and cefotiam against β-lactamase-producing bacteria.

Since the discovery of thienamycin by KAHAN *et al.*[1], many new carbapenem antibiotics produced by streptomycetes have been reported[2~8]. The total synthesis and the chemical modification of thienamycin have also been reported[9,10]. They have not only potent antibacterial activities but β-lactamase inhibitory activities as well[3,4,7,11~13]. A combination of classical β-lactam antibiotics and β-lactamase inhibitors represents one of the approaches for treating infections caused by β-lactam antibiotic-resistant bacteria[14~16]. Therefore, it is important to clarify the relation between the structure and the β-lactamase-inhibiting activity of carbapenem antibiotics.

In this paper, we report the inhibitory activities and the types of inhibitions against eight β-lactamases of twelve structurally related 5,6-*cis*-carbapenem antibiotics (Fig. 1). Their synergistic activities with ampicillin and cefotiam against β-lactamase producers are also described.

Materials and Methods

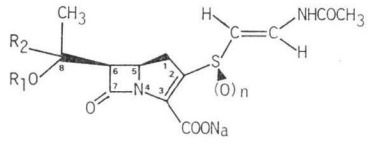
Antibiotics

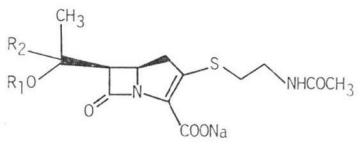
The naturally-occurring 5,6-*cis*-carbapenem antibiotics[17,18] were isolated from the culture filtrate of *Streptomyces griseus* subsp. *cryophilus* C-19393[8] and their derivatives[19] were prepared in our laboratories (Fig. 1). The purities of these compounds were estimated to be more than 70% by TLC, HPLC, UV, IR, and ^{1}H NMR spectra. Clavulanic acid was also prepared in our laboratories. Ampicillin and cefotiam are products of Takeda Chemical Industries, Ltd.. Sulbactam (penicillanic acid sulfone; CP-45,899) was a generous gift from Leo Pharmaceutical Products, Denmark.

Bacterial Strains and β-Lactamases

All strains, except *Escherichia coli* K12 CP13 and *Serratia marcescens* IFO 12648, are clinical isolates. The β-lactamases of *E. coli* TN635 and TN713 are type I penicillinase[20] (TEM-1[21]) and that of *E. coli* TN 649 is a type II penicillinase[20] (OXA-1[21]). The β-lactamases of *Klebsiella pneumoniae* TN1655 and TN1698 are chromosomally mediated constitutive penicillinases. The β-lactamase of *Staphylococcus*

Fig. 1. Chemical structures of 5,6-*cis*-carbapenem antibiotics used in this study.





Compound	R_1	R_2	n
C-19393 S_2	SO_3Na	CH_3	1
C-19393 S_2M_1	SO_3Na	CH_3	0
MM 4550	SO_3Na	H	1
MM 13902	SO_3Na	H	0
C-19393 H_2	H	CH_3	1
C-19393 H_2M_1	H	CH_3	0
C-19393 E_5	H	H	1
Epithienamycin B	H	H	0

Compound	R_1	R_2
C-19393 S_2M_3	SO_3Na	CH_3
MM 17880	SO_3Na	H
C-19393 H_2M_3	H	CH_3
Epithienamycin A	H	H

aureus 1840 is an inducible penicillinase. The enzymes of *Citrobacter freundii* GN1706 and TN515, *S. marcescens* TN81 and IFO12648, *Proteus vulgaris* GN4413 and GN4815 and *Enterobacter cloacae* TN 1282 are inducible cephalosporinases. The enzymes used were purified as described previously[12]. The β-lactamases of *P. vulgaris* GN4413 and *E. cloacae* TN1282 were homogeneous in the sodium dodecylsulfate polyacrylamide gel electrophoresis. Other enzymes were partially purified preparations.

Determination of β-Lactamase Inhibitory Activity

β-Lactamase activity was determined microiodometrically[22] using ampicillin as a substrate for the penicillinase assay and cephalothin for the cephalosporinase assay. β-Lactamase inhibitory activity was determined as described previously[12]. Percentage inhibition of enzyme activity was calculated against a control reaction where the inhibitor was replaced by buffer. The concentration of the inhibitor giving 50% inhibition (I_{50}) was obtained from a plot of the percentage inhibition against the inhibitor concentration.

Assay for Inactivation of C-19393 H_2 by a *P. vulgaris* β-Lactamase

One milliliter of reaction mixture containing 10 μg (27.5 nmole) of C-19393 H_2, 0.27 to 5.4 nmole of the *P. vulgaris* GN4413 β-lactamase and 50 mM phosphate buffer (pH 6.9) was incubated at 30°C. At intervals, 50 μl aliquots of the reaction mixture were withdrawn and added to 150 μl of methanol to stop the reaction. The amount of residual C-19393 H_2 was measured by the paper disk method with *E. coli* CP13 as a test organism.

Test for Synergistic Activity

The potentiation of the antibacterial activities of ampicillin and cefotiam by carbapenem antibiotics was examined by the agar dilution method. Serial two-fold dilutions of the β-lactam antibiotics were prepared in Mueller-Hinton agar (Difco), with or without the addition of carbapenem antibiotics. Test organisms were grown overnight in Mueller-Hinton broth (Difco) at 37°C. About 5 μl of bacterial suspension containing approximately 10^7 colony-forming units per ml was inoculated onto agar plates with a multiple inoculator (Sakuma, Tokyo). Plates were incubated at 37°C for 18 hours, and the minimum inhibitory concentration (MIC) was taken as the lowest concentration of drug that inhibited the visible growth of bacteria.

Results

β-Lactamase Inhibitory Activities

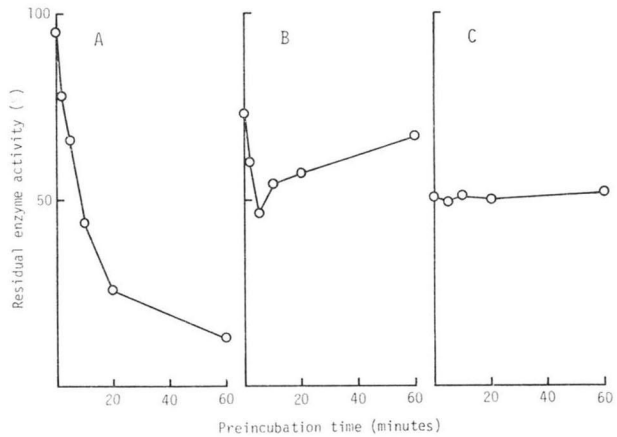
Table 1 shows the inhibitory activities of 12 carbapenem antibiotics against 8 β-lactamases. The activities of clavulanic acid and sulbactam were also shown in the table. Every carbapenems inhibited all the enzymes. The cephalosporinases of *E. cloacae* TN1282, *S. marcescens* TN81, and *C. freundii* GN1706, which were insensitive to clavulanic acid[12,23], and the penicillinase of *E. coli* TN649 were very sensitive to all of the carbapenems tested. Among the 12 carbapenems MM 4550 and MM 13902 were

Table 1. β-Lactamase inhibitory activities of carbapenem antibiotics.

Compound	I_{50} (ng/ml)							
	Penicillinase of				Cephalosporinase of			
	S. aureus 1840	*E. coli* TN713	*E. coli* TN649	*K. pneumoniae* TN1698	*E. cloacae* TN1282	*S. marcescens* TN81	*C. freundii* GN1706	*P. vulgaris* GN4413
C-19393 S_2	210	0.27	2.5	5.2	43	55	16	0.37
C-19393 S_2M_1	2,400	25	7.7	40	2.4	39	2.8	4.4
C-19393 S_2M_3	2,300	42	18	34	5.8	290	6.2	9.0
MM 4550	3.0	0.037	0.35	0.19	1.2	0.58	2.5	0.059
MM 13902	8.2	2.9	0.71	2.6	0.11	0.35	0.20	0.23
MM 17880	21	4.7	0.66	1.0	0.29	1.7	0.33	0.38
C-19393 H_2	43	3.0	1.4	5.2	34	0.50	15	0.60
C-19393 H_2M_1	600	9.6	11	300	5.4	0.85	5.4	6.0
C-19393 H_2M_3	560	4.5	17	250	5.4	7.0	13	11
C-19393 E_5	0.32	27	0.21	0.42	15	0.29	15	12
Epithienamycin B	12	320	3.6	200	0.98	0.63	1.3	300
Epithienamycin A	37	690	2.7	290	2.7	3.5	2.5	580
Clavulanic acid	40	16	300	12	>5000	>5000	>5000	45
Sulbactam	1,400	320			>5000			110

Fig. 2. Effect of preincubation of β-lactamases with C-19393 H_2 on enzyme activity.

Each enzyme was incubated with C-19393 H_2 for the time indicated, prior to the addition of substrate. (A) *E. cloacae* TN1282 β-lactamase (0.003 U/ml), 30 ng/ml of C-19393 H_2. (B) *P. vulgaris* GN4413 β-lactamase (0.003 U/ml), 0.6 ng/ml of C-19393 H_2. (C) *E. coli* TN713 β-lactamase (0.002 U/ml), 3 ng/ml of C-19393 H_2.



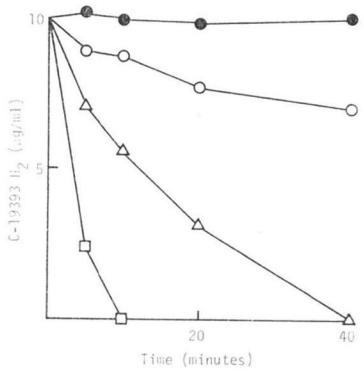
the most active: their I_{50}s were below 10 ng/ml against all the enzymes. C-19393 S_2M_1, C-19393 S_2M_3, C-19393 H_2M_1, C-19393 H_2M_3, epithienamycin A, and epithienamycin B were relatively weak in inhibiting certain β-lactamases such as those of *S. aureus* 1840 and *K. pneumoniae* TN1698.

Type of Inhibition

The β-lactamase inhibitory activity of certain inhibitors, such as clavulanic acid, increases when the inhibitor and the enzyme are incubated before the addition of substrate[24,25]. This type of inhibition is called "progressive". C-19393 S_2 inhibited the β-lactamase of *E. coli* TN713 in a progressive fashion[12], whereas C-19393 H_2 did not (Fig. 2 C). Our previous study revealed that the progressive inhibition was irreversible whereas the non-progressive inhibition was reversible[12]. Another type of inhibition was observed with the combination of the *P. vulgaris* GN4413 β-lactamase and C-19393 H_2 (Fig. 2 B). In this case, the inhibition, once attained, was relieved by prolonged incubation. This relief can be explained by the experiment shown in Fig. 3. C-19393 H_2 was inactivated by the *P. vulgaris* β-lactamase at the rate of 1.1 mole per minute per mole of the enzyme. Under the same condition, the rate of cephaloridine hydrolysis was 1,400 mole per minute per mole of the enzyme (data not shown).

Fig. 3. Inactivation of C-19393 H_2 by *P. vulgaris* GN4413 β-lactamase.

C-19393 H_2 (10 μg/ml, 27.5 μM) was incubated with 0.27 μM (○), 1.35 μM (△) and 5.4 μM (□) of the *P. vulgaris* GN4413 β-lactamase and with a phosphate buffer (●) at 30°C. The amount of residual C-19393 H_2 was determined microbiologically as described in Materials and Methods.



We examined the type of inhibition of various β-lactamases by the 12 carbapenems. The results

Table 2. Type of inhibition of β-lactamases by carbapenem antibiotics.

Compound	Inhibition type of β-lactamase* from							
	S. aureus 1840	*E. coli* TN713	*E. coli* TN649	*K. pneumoniae* TN1698	*E. cloacae* TN1282	*S. marcescens* TN81	*C. freundii* GN1706	*P. vulgaris* GN4413
C-19393 S_2	A	A	A	A	A	A	A	A
C-19393 S_2M_1	A	A	A	A	A	A	A	A
C-19393 S_2M_3	A	A	A	A	A	A	A	A
MM 4550	A	A	A	A	A	A	B	A
MM 13902	A	A	A	A	A	A	B	A
MM 17880	A	A	A	A	A	A	B	A
C-19393 H_2	A	C	A	A	A	A	A	B
C-19393 H_2M_1	A	B	A	A	A	A	B	B
C-19393 H_2M_3	A	B	A	A	A	A	B	C
C-19393 E_5	A	C	A	B	A	A	A	B
Epithienamycin B	C	C	A	B	A	A	B	B
Epithienamycin A	A	C	A	C	A	A	B	B

* Experimental procedure is described in Fig. 2 legend. The type of inhibition was categorized as follows. A: Inhibited in a progressive fashion as Fig. 2 A. B: Inhibited in a progressive fashion first, then the enzyme activity recovered (Fig. 2 B). C: Inhibited in a non-progressive fashion as Fig. 2 C.

are summarized in Table 2. The carbapenems with a sulfonyloxy group at position 8 (C-19393 S_2, C-19393 S_2M_1, C-19393 S_2M_3, MM 4550, MM 13902, and MM 17880) inhibited all enzymes in a progressive fashion. On the other hand, inhibition of β-lactamases such as those of *E. coli* TN713 and *P. vulgaris* GN 4413 by several carbapenems with a hydroxyl group instead of the sulfonyloxy group was non-progressive. The β-lactamases of *E. coli* TN649, *E. cloacae* TN1282, and *S. marcescens* TN81 were inhibited in a progressive fashion by all carbapenems. The inhibition of the *C. freundii* β-lactamase by MM 4550, MM 13902, MM 17880, C-19393 H_2M_1, C-19393 H_2M_3, epithienamycin A, and epithienamycin B was relieved by prolonged incubation.

Synergistic Activity

The twelve carbapenems were highly active in inhibiting various β-lactamases, and synergistic activities with other β-lactams sensitive to β-lactamase hydrolysis was expected. Therefore, we tested the synergistic activities of the carbapenems with ampicillin against penicillinase producers and with cefotiam against cephalosporinase producers and compared them with the activities of clavulanic acid and sulbactam (Tables 3 and 4).

Most carbapenems acted synergistically with the other β-lactams against many organisms. C-19393 S_2, for example, was very active in potentiating the antibacterial activities of ampicillin and cefotiam against *S. aureus, E. coli, K. pneumoniae, P. vulgaris*, and *S. marcescens*, but was not active against *E. cloacae* and *C. freundii*. None of carbapenems exhibited strong synergistic activity against *E. cloacae*

Table 3. Potentiation of antibacterial activity of ampicillin against penicillinase producers by carbapenem antibiotics.

Addition (μg/ml)	MIC of ampicillin (μg/ml)			
	S. aureus 1840	*E. coli* TN635	*E. coli* TN649	*K. pneumoniae* TN1655
None	100	800	800	100
C-19393 S_2 (0.1)	12.5	3.13	100	6.25
〃 (0.5)	3.13	1.56	12.5	1.56
C-19393 S_2M_1 (0.1)	50	100	100	25
〃 (0.5)	12.5	12.5	12.5	12.5
MM 4550 (0.2)	1.56	200	200	25
MM 13902 (0.1)	0.39	200	400	12.5**
〃 (0.5)	≦0.20	—*	—	—
MM 17880 (0.1)	1.56	200	25	1.56
C-19393 H_2 (0.1)	3.13	50	≦0.20**	3.13
〃 (0.5)	≦0.20	—	—	—
C-19393 H_2M_1 (0.1)	25	50	25	50
〃 (0.5)	3.13	—	—	—
C-19393 E_5 (0.1)	12.5	800	200	50
〃 (0.5)	3.13	800	100	25
Epithienamycin B (0.1)	0.78	400	—	100
〃 (0.5)	≦0.20**	400	—	100
Clavulanic acid (0.1)	6.25	50	200	6.25
〃 (1.0)	1.56	6.25	50	1.56
Sulbactam (1.0)	6.25	400	800	100

* Organism was inhibited by a carbapenem antibiotic alone.
** Organism was inhibited partially by a carbapenem antibiotic alone.

Table 4. Potentiation of antibacterial activity of cefotiam against cephalosporinase producers by carbapenem antibiotics.

Addition (μg/ml)	MIC of cefotiam (μg/ml)			
	P. vulgaris GN4815	*S. marcescens* IFO12648	*E. cloacae* TN1282	*C. freundii* TN515
None	400	25	200	200
C-19393 S_2 (0.1)	0.78	12.5	200	200
〃 (0.5)	0.78	6.25	200	100
C-19393 S_2M_1 (0.1)	1.56	6.25	100	100
〃 (0.5)	0.78	3.13	50	50
MM 4550 (0.2)	6.25	12.5	50	200
MM 13902 (0.1)	0.78	12.5	100	200
〃 (0.5)	—*	6.25	50	100
MM 17880 (0.1)	0.78	12.5	100	200
C-19393 H_2 (0.1)	0.78	1.56	200	200
〃 (0.5)	≦0.20**	—	200	200
C-19393 H_2M_1 (0.1)	25	6.25	100	200
〃 (0.5)	≦0.20**	0.39**	100	100
C-19393 E_5 (0.1)	400	25	200	200
〃 (0.5)	200	25	100	100
Epithienamycin B (0.1)	400	12.5	200	200
〃 (0.5)	200	12.5	100	50
Clavulanic acid (0.1)	3.13	25	200	200
〃 (1.0)	1.56	25	200	200
Sulbactam (1.0)	3.13	12.5	200	200

* Organism was inhibited by a carbapenem antibiotic alone.
** Organism was inhibited partially by a carbapenem antibiotic alone.

and *C. freundii*, though they inhibited the β-lactamases of these organisms. The synergistic activities of most carbapenems were superior to that of sulbactam and were of the same order as or slightly stronger than that of clavulanic acid.

Against *S. aureus*, *K. pneumoniae* and *P. vulgaris*, the synergistic activities of carbapenems except MM 4550 were well correlated with their β-lactamase inhibitory activities. C-19393 S_2 was the only carbapenem that exerted good synergism against *E. coli* TN635. The synergistic activity of carbapenems against *E. coli* TN649 was weak in spite of the sensitivity of its enzyme.

Discussion

All of the twelve 5,6-*cis*-carbapenem antibiotics tested inhibited 8 types of β-lactamases including those which were insensitive to clavulanic acid and sulbactam. There were certain general rules in the structure-activity relation. The presence of a sulfoxide in the 2-side chain increased inhibitory activities against penicillinases and the *P. vulgaris* GN4413 cephalosporinase, but decreased, or did not affect, the activities against other cephalosporinases (C-19393 S_2 *vs.* C-19393 S_2M_1, MM 4550 *vs.* MM 13902, C-19393 H_2 *vs.* C-19393 H_2M_1, C-19393 E_5 *vs.* epithienamycin B). Whether the 2-side chain was saturated or not, scarcely affected the inhibitory activity (C-19393 S_2M_1 *vs.* C-19393 S_2M_3, MM 13902 *vs.* MM 17880, C-19393 H_2M_1 *vs.* C-19393 H_2M_3, epithienamycin B *vs.* epithienamycin A). Carbapenems with a methyl group at position 8 were more active inhibitors than those with dimethyl group in the case of the 8-sulfonyloxy compounds (MM 4550 *vs.* C-19393 S_2, MM 13902 *vs.* C-19393 S_2M_1, MM 17880 *vs.* C-

19393 S_2M_3). But in the case of antibiotics with a hydroxyl group instead of a sulfonyloxy group at position 8, the effects of the number of methyl group on the inhibitory activities were variable (C-19393 E_5 *vs.* C-19393 H_2, epithienamycin B *vs.* C-19393 H_2M_1, epithienamycin A *vs.* C-19393 H_2M_3). Against all enzymes, MM 17880 was a more active inhibitor than epithienamycin A, and MM 13902 was more active than epithienamycin B. MM 4550 was more active in inhibiting the β-lactamases of *E. coli* TN713, *E. cloacae* TN1282, *C. freundii* GN1706, and *P. vulgaris* GN4413 than was C-19393 E_5, but was less active in inhibiting the *S. aureus* β-lactamase than was C-19393 E_5. In these 8-monomethyl carbapenems, the presence of a sulfonyloxy group increased the inhibitory activity with some exceptions. In the case of the 8-dimethyl compounds, the effects of the sulfonyloxy group on the inhibitory activity were not so large and variable.

C-19393 S_2, S_2M_1, and S_2M_3 inhibited all enzymes in a progressive fashion. A previous study revealed that C-19393 S_2, which inhibited the *E. coli* TN713 β-lactamase in a progressive fashion was an irreversible inhibitor of this enzyme[12]. Therefore, these three antibiotics are presumed to be irreversible inhibitors (or inactivators) of β-lactamases. Similarly, β-lactamases of *E. coli* TN649, *E. cloacae* TN1282, and *S. marcescens* TN81 were considered to be inhibited irreversibly by all carbapenems tested. A previous study also revealed that C-19393 H_2, a non-progressive inhibitor of the *E. coli* TN713 β-lactamase, inhibited this enzyme reversibly[12]. The mechanism of inhibition of the *E. coli* TN713 enzyme by C-19393 E_5, epithienamycin A and epithienamycin B are presumed to be similar to that by C-19393 H_2. There were no reversible inhibitors among the 8-sulfonyloxy carbapenems, but many of the 8-hydroxyl compounds were reversible inhibitors of the *E. coli* TN713 β-lactamase.

C-19393 H_2 was inactivated by the *P. vulgaris* β-lactamase. The rate of inactivation was 1.1 mole per minute per mole of the enzyme, and was 0.08% of that of cephaloridine hydrolysis by the same enzyme. Slow inactivation of C-19393 H_2 would have resulted in the complex pattern of inhibition of the *P. vulgaris* β-lactamase; the inhibition once attained was releaved by prolonged incubation. A similar pattern of time dependent relief of inhibition of β-lactamase was demonstrated with clavulanic acid[24] and PS-5[26]. The inhibitions of the *P. vulgaris* β-lactamase by C-19393 H_2M_1, C-19393 E_5, epithienamycin A, and epithienamycin B were also relieved as in the case of inhibition by C-19393 H_2, and these carbapenems were presumed to be slowly hydrolyzed by the *P. vulgaris* enzyme. Seven carbapenems including MM 4550, MM 13902, and MM 17880 were also considered to be substrates for the *C. freundii* β-lactamase.

The twelve carbapenems acted synergistically with ampicillin and cefotiam against β-lactamase-producing organisms and the synergistic activities of some carbapenems were superior to those of clavulanic acid and sulbactam. The synergistic activities against *P. vulgaris* GN4815 were the most remarkable. Against this organism, 0.1 μg per ml of C-19393 S_3, C-19393 H_2, MM 13902, and MM 17880 reduced the MIC of cefotiam from 400 μg/ml to 0.78 μg/ml, which was the MIC level of cefotiam against a β-lactamase-deficient mutant of this strain (unpublished observation). C-19393 S_2 and MM 17880, even at 0.01 μg/ml, exhibited a 250-fold reduction in the MIC of cefotiam against this organism (data not shown). The synergistic activities of carbapenems against *S. aureus* were also remarkable and were well correlated with the β-lactamase inhibitory activities. In this case, the consistency between synergy and β-lactamase inhibitory activity is well understood because the enzyme of *S. aureus* is excreted into the surrounding medium and there is no barrier between β-lactamase and the inhibitor. The synergistic activities of carbapenems against *E. coli, K. pneumoniae*, and *S. marcescens* were slightly weaker than those against *S. aureus* and *P. vulgaris*. The outer membrane might act to some extent as a permeability barrier for carbapenems in these organisms. MM 4550 was not so active in potentiating the antibacterial activities of β-lactam antibiotics in spite of its strong inhibition of β-lactamases. The lability of MM 4550 may be a major reason for this inconsistency[2,3].

The twelve carbapenems scarcely acted synergistically with cefotiam against *E. cloacae* TN1282 and *C. freundii* TN515. There are three possible explanations for this ineffectiveness: 1) the permeability barrier, not β-lactamase, is a major factor for the cefotiam-resistance, 2) carbapenems are inactivated by the bacterial enzyme, 3) carbapenems are unable to penetrate the outer membrane. Good correlations between resistance levels and β-lactamase activities in *E. cloacae* and *C. freundii* (unpublished observations) suggest that β-lactamase is a major factor for cefotiam-resistance. Many carbapenems were in-

activated by the *C. freundii* β-lactamase. Therefore, the inactivation by the enzyme may be one reason for the ineffectiveness in synergy against *C. freundii*. On the other hand, none was inactivated by the *E. cloacae* β-lactamase. *E. cloacae* might produce an enzyme, other than β-lactamase, that inactivates carbapenems. An alternative explanation for the ineffectiveness in synergy against *E. cloacae* is that carbapenems are unable to penetrate the outer membrane.

The present study indicates that the combination of carbapenems with β-lactam antibiotics may be a promising approach for treating infections caused by β-lactamase-producing strains such as *S. aureus*, *K. pneumoniae*, *E. coli*, and *P. vulgaris*.

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