STRUCTURE-ACTIVITY RELATIONS OF 5,6-*cis* CARBAPENEM ANTIBIOTICS AND ROLE OF FACTORS DETERMINING SUSCEPTIBILITY OF *ESCHERICHIA COLI* TO β-LACTAM ANTIBIOTICS

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The antibacterial activities of twelve 5,6-*cis* carbapenem antibiotics, including four semi-synthetic derivatives of C-19393 H_2 and S_2, against 15 microorganisms were examined, and their structure-activity relations are discussed in relation to minimum inhibitory concentrations against *Staphylococcus aureus* and *Escherichia coli* as a Gram-positive and a Gram-negative standard strain, respectively. The contribution of chromosomal β-lactamase (*amp C*), permeability barrier, and penicillin-binding protein (PBP) 1B to the resistance of *E. coli* to these carbapenem antibiotics was examined using mutants lacking each of these cellular components. The β-lactamase was not involved in the resistance. These antibiotics easily permeated the outer membrane. A PBP 1B-defective mutant was supersensitive to these carbapenem antibiotics and to other types of β-lactam antibiotics.

Since the discovery of thienamycin by Merck researchers[1], a number of carbapenem antibiotics, such as olivanic acids[2,3], epithienamycins[4], PS-series antibiotics[5,6], C-19393 H_2 and S_2[7] (carpetimycins[5]), C-19393 E_5[9], asparenomycins[10], pluracidomycins[11], and SQ-27860[12], have been discovered from natural sources. On the basis of the stereochemistry of protons at the C-5,6 positions of the carbapenem nucleus, these carbapenem antibiotics, except the asparenomycins with a isopropylidene side chain at C-6 position, are grouped into *cis* compounds having a 5*R*,6*R*-configuration and *trans* compounds having a 5*R*,6*S*-configuration.

We reported that *Streptomyces griseus* subsp. *cryophilus* C-19393 produces eight 5,6-*cis* carbapenem antibiotics[7,9,13] and proposed a possible biosynthetic pathway for these 5,6-*cis* carbapenem antibiotics in this organism[14]. Four 8-dimethyl compounds, C-19393 H_2M1, C-19393 H_2M3, C-19393 S_2M1, and C-19393 S_2M3 (Fig. 1), were derived from C-19393 H_2 and S_2 by chemical modifications[15], although they could not be detected in the culture filtrates of the organism[14].

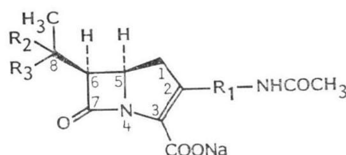
This paper deals with the antibacterial activities of the above twelve structurally related carbapenem antibiotics against 15 microorganisms and their structure-activity relations. In addition, the contribution of *amp C* chromosomal β-lactamase[16], the permeability barrier[17], and penicillin-binding protein (PBP) 1B[18,19] to the resistance of *Escherichia coli* to the carbapenem antibiotics will be presented.

Materials and Methods

Compounds

Eight natural 5,6-*cis* carbapenem antibiotics, *i.e.*, epithienamycins A and B, C-19393 E_5, C-19393 H_2,

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



Hydroxyl compound (R_3=OH)	R_2	R_1	Sulfonyloxy compound (R_3=OSO_3Na)
8-Monomethylhydroxyl compound			8-Monomethylsulfonyloxy compound
Epithienamycin A	H	S-CH_2-CH_2	MM 17880
Epithienamycin B	H	S-CH=CH	MM 13902
C-19393 E_5	H	O ↑ S-CH=CH	MM 4550
8-Dimethylhydroxyl compound			8-Dimethylsulfonyloxy compound
C-19393 H_2M3	CH_3	S-CH_2-CH_2	C-19393 S_2M3
C-19393 H_2M1	CH_3	S-CH=CH	C-19393 S_2M1
C-19393 H_2	CH_3	O ↑ S-CH=CH	C-19393 S_2

MM 17880, MM 13902, MM 4550 and C-19393 S_2 (Fig. 1), were prepared by fermentation of *S. griseus* subsp. *cryophilus* or its mutant strains[9,13]. Four modified compounds, *i.e.* C-19393 H_2M1, H_2M3, S_2M1 and S_2M3 (Fig. 1), were chemically prepared from C-19393 H_2 or S_2[15]. Benzylpenicillin, ampicillin, cephalexin, and cephaloridin were obtained from commercial sources. Mecillinam was a kind gift of Dr. F. LUND of Leo Pharmaceutical Co., Copenhagen, Denmark. Penicillin N, phenoxymethylpenicillin, cephalosporin C, cephamycin C, clavulanic acid, nocardicin A and sulfazecin were fermentation products obtained in our laboratories.

Determination of Minimum Inhibitory Concentration (MIC)

MICs were determined by the two-fold agar dilution method as described previously[20].

Organisms

Authentic cultures were obtained from the Institute for Fermentation, Osaka. Mutants derived from *E. coli* LD-2 (a lysine and 2,6-*meso*-diaminopimelic acid auxotroph[20]), which lacked β-lactamase (*amp C*), the permeability barrier or PBP 1B[21], were used to examine the role of these cellular components in the antibiotic resistance of *E. coli*.

Results

Antibacterial Activities and Structure-activity Relations of 5,6-*cis* Carbapenem Antibiotics

The antibacterial activities of the 5,6-*cis* carbapenem antibiotics against 15 microorganisms are shown in Table 1. For convenience, the antibiotics are grouped into four types according to the combination of the side chains at position C-8.

All the antibiotics, except MM 4550 and the three 8-dimethylsulfonyloxy compounds, showed superior antibacterial activities. The 8-monomethyl compounds were generally more potent than their corresponding 8-dimethyl ones. However, C-19393 H_2 was far more potent than its corresponding monomethyl compound, C-19393 E_5. On the other hand, the 8-hydroxyl compounds were more active than their corresponding 8-sulfonyloxy compounds. But, against two exceptional species, *Klebsiella pneumoniae* IFO 3317 and *Pseudomonas aeruginosa* IFO 3080, MM 17880 and MM 13902 were more active than their hydroxyl derivatives, epithienamycins A and B, respectively.

Table 1. Antibacterial activities of twelve 5,6-*cis* carbapenem antibiotics.

Organism	MIC (μg/ml)											
	8-Monomethyl-hydroxyl compound			8-Monomethyl-sulfonyloxy compound			8-Dimethyl-hydroxyl compound			8-Dimethyl-sulfonyloxy compound		
	Epi A (s*)	Epi B (u*)	E_5 (uo*)	MM 17880 (s)	MM 13902 (u)	MM 4550 (uo)	H_2M3 (s)	H_2M1 (u)	H_2 (uo)	S_2M3 (s)	S_2M1 (u)	S_2 (uo)
Staphylococcus aureus 209P	0.1	0.1	0.78	0.39	0.2	6.25	1.25	1.25	0.625	20	10	6.25
Micrococcus luteus IFO 12708	0.05	0.05	0.2	0.39	0.2	3.13	0.625	0.313	0.313	20	5	12.5
Bacillus sphericus IFO 12622	0.1	0.1	1.56	0.78	0.2	12.5	>5	2.5	2.5	>40	20	>50
Escherichia coli NIHJ JC-2	0.1	0.2	1.56	0.2	0.2	12.5	0.313	0.313	0.156	20	5	12.5
Salmonella typhimurium IFO 12529	0.2	0.2	1.56	0.2	0.05	12.5	0.625	0.313	0.156	20	5	12.5
Citrobacter freundii IFO 12681	1.56	1.56	3.13	3.13	6.25	100	0.313	1.25	0.625	20	5	12.5
Klebsiella pneumoniae IFO 3317	3.13	6.25	12.5	0.1	0.05	12.5	0.313	0.313	0.625	20	5	25
Enterobacter cloacae IFO 12937	3.13	6.25	50	12.5	6.25	>100	2.5	5	1.25	40	20	25
Serratia marcescens IFO 12648	3.13	3.13	12.5	1.56	3.13	50	1.25	1.25	0.625	20	20	40
Proteus vulgalis IFO 3988	0.2	1.56	3.13	0.39	0.78	25	2.5	2.5	1.25	40	40	50
Proteus mirabilis ATCC 21100	0.2	0.78	1.56	0.2	0.2	12.5	2.5	1.25	1.25	40	20	>50
Pseudomonas aeruginosa IFO 3080	25	25	12.5	0.78	3.13	100	>5	>10	10	>40	>40	>50
Comamonas terrigena IFO 13299	0.2	0.2	1.56	0.39	0.2	12.5	0.625	0.156	0.313	40	5	25
Alcaligenes faecalis IFO 13111	0.39	0.39	3.13	1.56	0.2	25	1.25	1.25	1.25	>40	20	>50
Acinetobacter calcoaceticus IFO 12552	1.56	1.56	6.25	12.5	25	50	5	5	1.25	>40	>40	50

Antibiotics: Epi A, epithienamycin A; Epi B, epithienamycin B; E_5, C-19393 E_5; H_2M3, C-19393 H_2M3; H_2M1, C-19393 H_2M1; H_2, C-19393 H_2; S_2M3, C-19393 S_2M3; S_2M1, C-19393 S_2M1; S_2, C-19393 S_2.

* Type of C-2 side chain: s, saturated; u, unsaturated; uo, unsaturated and oxidated.

The following structure-activity relations emerged using *Staphylococcus aureus* 209P and *E. coli* NIHJ JC-2 as a Gram-positive and a Gram-negative standard strain, respectively.

Dehydrogenation of a Cysteamyl Side Chain at Position C-2

Table 2A shows the effect of this type of structural change on four combinations of carbapenem antibiotics. The dehydrogenation of the cysteamyl side chain hardly affected the antibacterial activity against either organism.

S-Oxidation in C-2 Side Chain

As shown in Table 2B, the effect on the activity of *S*-oxidation in the C-2 side chain greatly differed between 8-monomethyl and 8-dimethyl compounds; it greatly decreased the activities of the former, especially the sulfonyloxy-type, whereas it showed little effect on the activities of the latter.

C-Methylation at Position C-8

C-Methylation at position C-8 of the 8-monomethyl compounds had a negative effect on activity except the change of C-19393 E_5 to C-19393 H_2, and MM 4550 to C-19393 S_2 (Table 3A). It is noteworthy that C-19393 H_2 was much more active against *E. coli* NIHJ JC-2 than was C-19393 E_5.

Sulfation at Position C-8

Generally, sulfation of 8-hydroxyl compounds resulting in 8-sulfonyloxy derivatives decreased the antibacterial activities (Table 3B). This negative effect was remarkable with 8-dimethyl compounds.

The above conclusions apply to other microorganisms with two exceptions, *Klebsiella pneumoniae* IFO 3317 and *Pseudomonas aeruginosa* IFO 3080.

Contribution of Three Cellular Components to the Resistance of *E. coli* to 5,6-*cis* Carbapenem Antibiotics

We have isolated a series of β-lactam-sensitive mutants of *E. coli*. One mutant, strain CPC 20 derived from strain LD-2, lacks a chromosomally mediated β-lactamase coded for by the *amp C* gene. Further mutation of strain CPC 20 gave two types of β-lactam supersensitive mutants. These mutants designated PG 12 and PG 8 were defective in the permeability barrier and PBP 1B, respectively[20,21].

Comparison of the sensitivities to a given antibiotic between a mutant and its parent will clarify the contribution of the defective cellular component to the resistance of *E. coli* to the antibiotic.

Table 2. Effects of single structural changes in the C-2 side chain of 5,6-*cis* carbapenem antibiotics on antibacterial activity.

A) Dehydrogenation

Organism	$-\log_2$ (MIC ratio)			
	Epi B / Epi A	MM 13902 / MM 17880	H_2M1 / H_2M3	S_2M1 / S_2M3
S. aureus 209P	0	1	0	1
E. coli NIHJ JC-2	−1	0	0	2

B) *S*-Oxidation

Organism	$-\log_2$ (MIC ratio)			
	E_5 / Epi B	MM 4550 / MM 13902	H_2 / H_2M1	S_2 / S_2M1
S. aureus 209P	−3	−5	1	1
E. coli NIHJ JC-2	−3	−6	1	−1

Abbreviations of the antibiotics are shown in the legend to Table 1.

Table 3. Effects of single structural changes at the C-8 position of 5,6-*cis* carbapenem antibiotics on antibacterial activity.

A) Methylation

Organism	−log$_2$ (MIC ratio)					
	H_2M3 / Epi A	H_2M1 / Epi B	H_2 / E_5	S_2M3 / MM 17880	S_2M1 / MM 13902	S_2 / MM 4550
S. aureus 209P	−4	−4	0	−6	−6	0
E. coli NIHJ JC-2	−2	−1	3	−7	−5	0

B) Sulfation

Organism	−log$_2$ (MIC ratio)					
	MM 17880 / Epi A	MM 13902 / Epi B	MM 4550 / E_5	S_2M3 / H_2M3	S_2M1 / H_2M1	S_2 / H_2
S. aureus 209P	−2	−1	−3	−4	−3	−3
E. coli NIHJ JC-2	−1	0	−3	−6	−4	−6

Abbreviations of the antibiotics are shown in the legend to Table 1.

Table 4. Contribution of the three cellular components to the resistance of *E. coli* to β-lactam antibiotics.

	MIC (μg/ml)				−log$_2$ (MIC ratio)		
	Strain*				(A)	(B)	(C)
	LD-2	CPC 20	PG 12	PG 8	CPC 20 / LD-2	PG 12 / CPC 20	PG 8 / CPC 20
Epithienamycin A	0.1	0.1	0.1	<0.00625	0	0	>4
Epithienamycin B	0.1	0.1	0.1	0.0125	0	0	3
C-19393 E_5	0.39	0.39	0.78	0.025	0	−1	4
MM 17880	0.1	0.1	0.1	0.025	0	0	2
MM 13902	0.39	0.2	0.2	0.025	1	0	3
MM 4550	1.56	3.13	3.13	0.2	−1	0	4
C-19393 H_2M3	0.156	0.156	0.313	0.039	0	−1	2
C-19393 H_2M1	0.156	0.156	0.156	0.039	0	0	2
C-19393 H_2	0.078	0.078	0.156	0.039	0	−1	1
C-19393 S_2M3	10	10	10	1.25	0	0	3
C-19393 S_2M1	5	2.5	2.5	0.625	1	0	2
C-19393 S_2	6.25	6.25	6.25	0.78	0	0	3
Benzylpenicillin	50	12.5	0.39	1.56	2	5	3
Phenoxymethylpenicillin	>100	100	0.78	25	>0	7	2
Penicillin N	100	6.25	12.5	0.78	4	−1	3
Ampicillin	3.13	1.56	0.2	0.39	1	3	2
Cephalosporin C	50	12.5	25	0.2	2	−1	6
Cephamycin C	12.5	12.5	6.25	0.78	0	1	4
Cephaloridine	3.13	1.56	1.56	0.1	1	0	4
Cephalexin	6.25	3.13	3.13	0.78	1	0	2
Clavulanic acid	50	25	25	12.5	1	0	1
Nocardicin A	50	50	50	0.78	0	0	6
Sulfazecin	100	50	50	12.5	1	0	2

* *E. coli* CPC 20 derived from *E. coli* LD-2 lacks *amp C* chromosomal β-lactamase. *E. coli* PG 12 and PG 8 derived from *E. coli* CPC 20 harbor defects of permeability barrier and PBP 1B, respectively[20,21].

Chromosomal β-Lactamse

It is thought that the chromosomal β-lactamase coded for by the *amp C* gene does not contribute to the resistance of *E. coli* to penicillins and cephalosporins[22]. We examined the effect of the mutational loss of this enzyme on the sensitivity of *E. coli* to the 5,6-*cis* carbapenem antibiotics and other types of β-lactam antibiotics. As shown in column (A) of Table 4, the *amp* C^{+} parent (LD-2) and its mutant harboring the *amp* C^{-} mutation (CPC 20) showed no difference in their sensitivities to most β-lactam antibiotics except some natural compounds, namely benzylpenicillin, penicillin N and cephalosporin C. The sensitivities of *E. coli* LD-2 and CPC 20 to all 5,6-*cis* carbapenem antibiotics tested were also almost the same, indicating that this enzyme does not contribute to the tolerance level of *E. coli* to the carbapenem antibiotics.

Permeability Barrier

It is known that the rate of diffusion of the β-lactams tends to decrease with an increase in the lipophilic character of the side chains[23]. Data shown in the column (B) of Table 4 agree with this observation; the antibacterial activities of penicillins with lipophilic side chains against the permeability mutant (PG 12) and its parent (CPC 20) differed greatly, although cephalosporins and penicillin N with a hydrophilic side chain showed similar activity against these strains.

All of the 5,6-*cis* carbapenem antibiotics tested were equally active against PG 12 and CPC 20, indicating that these carbapenem antibiotics as well as cephalosporins, penicillin N, clavulanic acid and monocyclic β-lactams (nocardicin A and sulfazecin) easily permeated the membrane of the *E. coli* cells.

PBP 1B

It is well known that the lack of PBP 1B in *E. coli* results in supersensitivity to the β-lactam antibiotics[18,19,21]. The data in column (C) of Table 4 shows that the PBP 1B-defective mutant (PG 8) is far more sensitive to the carbapenem antibiotics than its parent (CPC 20) as is the case with other types of β-lactam antibiotics.

Discussion

The various types of structural change of the carbapenem antibiotics gave almost the same effects on activities against *S. aureus* and *E. coli* which represented a Gram-positive and a Gram-negative pathogenic bacterium, respectively. The following could be concluded from the results. (1) Dehydrogenation of the C-2 cysteamyl side chain did not affect antibacterial activities; (2) *S*-oxidation in the C-2 side chain decreased the activities of the 8-monomethyl compounds but not those of the 8-dimethyl compounds; (3) methylation at the C-8 position decreased the activities of compounds other than C-19393 E_5 and MM 4550; and (4) sulfation at the C-8 position slightly decreased the activities of the 8-monomethyl compounds and greatly decreased those of the 8-dimethyl compounds. Observations on other bacteria confirmed these conclusions with few exceptions.

Beecham researchers have recently reported the effect of deacetylation of carbapenem antibiotics on antibacterial activities. NA 26978, a deacetylated derivative of epithienamycin A, showed much improved activity against *P. aeruginosa* and *E. coli* producing R-TEM β-lactamase; that is, it had an antibacterial spectrum similar to that of thienamycin[24].

Recently, OKONOGI *et al.*[25] reported that, of the 5,6-*cis* carbapenem antibiotics used in this study, the 8-dimethylsulfonyloxy compounds were the most active inhibitors of various types of β-lactamases. However, we demonstrated that the 8-dimethylsulfonyloxy compounds showed the weakest antibacterial activity. The facts indicate that the binding affinities of the carbapenem antibiotics for target enzymes (PBPs) may greatly differ from those of the β-lactamases.

The tolerance level of microorganisms toward β-lactams is believed to be determined by (1) the

amounts and specificities of β-lactamases[16,22,26], (2) the diffusion rates of the β-lactams through the permeability barrier[17,23], and (3) the affinities of the β-lactams for PBPs[27].

The mode of action of carbapenem antibiotics, such as thienamycin[28] and C-19393 S_2 and H_2[29], has been reported; the primary lethal effect is caused by prevention of the function of PBP 2 of *E. coli*, leading to the formation of ovoid cells. Thus, the affinities of several carbapenem antibiotics for the targets (PBPs) have been elucidated, but the contribution of a chromosomal β-lactamase and the permeability barrier to the resistance to carbapenem antibiotics has not been reported.

E. coli produces a small amount of a chromosomal β-lactamase coded for by *amp C* in the periplasmic space of cells. This enzyme activity is, however, so low that it does not contribute to the penicillin and cephalosporin tolerance level of this organism[16,22,26]. In this study, we have demonstrated that the twelve 5,6-*cis* carbapenem antibiotics were equally active to the *amp C* β-lactamase-negative mutant and its parent, indicating that this enzyme plays little or not part in the resistance of *E. coli* to the carbapenem antibiotics.

RICHMOND *et al.*[30] have developed an indirect, but very convenient, method for assessing the penetration of β-lactams. It only involves measuring the MIC values against a permeability mutant of *E. coli* (DO 2 or DO 3) and its wild-type parent (UB 1005). The permeability of many penicillin and cephalosporin derivatives have been measured by the method[30,31,32]. In this study, we assayed the permeability of the carbapenem antibiotics by the same method using a similar type of permeability mutant (PG 12) and its parent (CPC 20) and demonstrated that these antibiotics easily permeate across the outer membrane of *E. coli*, which is believed to be a permeability barrier to various antibiotics.

TAMAKI *et al.*[18] and SUZUKI *et al.*[19] isolated PBP 1B-negative mutants of *E. coli* that are supersensitive to β-lactam antibiotics. The supersensitivity can be explained by the fact that PBP 1A, a detour enzyme of PBP 1B, shows a much higher affinity for β-lactam antibiotics than does PBP 1B. As shown in column (C) of Table 4, our PBP 1B-negative mutant (PG 8)[21] was also more sensitive to β-lactam antibiotics, including the carbapenem antibiotics, than was its parental strain (CPC 20). However, the ratios of the MIC values of the twelve carbapenem antibiotics against the mutant and the parent differed in the order of $\times 2^1$ to $\times 2^4$ probably because of the differences of their affinities for the residual essential PBPs (PBP 1A, 2 and 3). By the use of this mutant as a detector strain for β-lactam antibiotics in our screening program, we found novel β-lactam antibiotics of natural origin[7,9,33].

Generally, the antibacterial activity of β-lactam antibiotics against *E. coli* depends on binding affinities for the lethal targets (PBPs) located in the inner membrane, the permeability through the outer membrane, and the resistance to a β-lactamase located in the periplasm. We here demonstrated that the last two factors are negligible in explaining the activity of the 5,6-*cis* carbapenem antibiotics, and therefore, the antibacterial activity of these compounds seems to be reflect their affinities for essential PBPs in *E. coli* (PBP 1A, 1B, 2 and 3).

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