STRUCTURE-ACTIVITY RELATIONSHIPS ON THE TERMINAL D-AMINO ACID MOIETY OF A NOVEL CEPHAMYCIN MT-141

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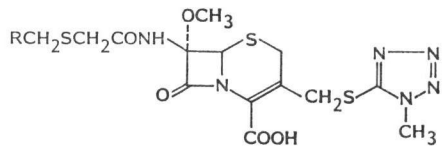
(Received for publication May 16, 1984)

The effect of chemical modification of the D-amino acid function, which represents the C-7β substituent of cephamycin MT-141 on *in vitro* antibacterial activity was examined. MT-141 was more active on Gram-negative organisms than Gram-positive ones. It showed strong bacteriolytic activity on Gram-negative organisms. Lysis of *Escherichia coli* K-12 strain JE1011 treated with a low concentration of this antibiotic was preceded by frequent formation of multiple bulges from the cells. Amidation or decarboxylation, removing the acidic function from the D-amino acid of MT-141, resulted in an increase in activity against Gram-positive bacteria, and a decrease against Gram-negative ones. Cells treated with the amide or the decarboxylate did not form multiple bulges but formed single bulges. *N*-Acetylation of the D-amino acid moiety removing the basic function, caused a marked drop in activity against both Gram-positive and Gram-negative bacteria. The bacteriolytic activity on *E. coli* was reduced, and cells treated with the *N*-acetate became filamentous. Conversion of the D-amino acid function of MT-141 to the L configuration caused a moderate drop in activity against both Gram-positive and Gram-negative organisms. Both bacteriolytic and bactericidal activities against *E. coli* were reduced in the L-congener. Cefoxitin, cefmetazole and latamoxef used as reference antibiotics were less active than MT-141 in the bactericidal activity against *E. coli*, and induced single bulge formation or filamentation of the cells around MIC levels. Cell-surface permeability, stability to β-lactamases, and binding affinity to PBPs of *E. coli* did not differ between MT-141 and its derivatives.

Certain β-lactam antibiotics of natural origin, such as penicillin N, cephamycin C[1], antibiotic SF-1623[2], nocardicin A[3] and sulfazecin[4], possess a D-amino acid function at the terminal position of the side chain. However, the importance of these D-amino acid moieties in terms of the antibacterial activity is not known.

MT-141 is a semisynthetic cephamycin containing a *S*-carbamoylmethyl-D-cysteine moiety as the C-7β substituent[5] (Fig. 1), and is a good model to investigate the role of the D-amino acid on bioactivity. A series of derivatives were prepared by chemical modification of its D-amino acid moiety in order to compare various *in vitro* antimicrobial properties. The derivatives compared were the decarboxylate (MT-116), amide (MT-180), *N*-acetate (MT-199) and the L-amino acid congener (MT-304) (Fig. 1). Their antibacterial spectra, bactericidal and bacteriolytic activities, effects on morphological change and other properties such as cell-surface permeability, β-lactamase stability, and binding affinity to penicillin binding proteins (PBPs) were examined using *Escherichia coli* strains as the main test organism.

Fig. 1. Structures of MT-141 and its derivatives.



MT-141 R= D-CH(NH$_2$)-COOH

MT-116 R= CH$_2$(NH$_2$)-

MT-180 R= D-CH(NH$_2$)-CONH$_2$

MT-199 R= D-CH(NHCOCH$_3$)-COOH

MT-304 R= L-CH(NH$_2$)-COOH

Materials and Methods

Antibiotics and Bacterial Strains

MT-141, MT-116, MT-180, MT-199 and MT-304 were prepared according to the procedures reported[6]. Cefoxitin (Daiichi Seiyaku Co. Ltd., Tokyo), cefmetazole (Sankyo Co. Ltd., Tokyo), latamoxef (moxalactam) and cephalothin (Shionogi & Co. Ltd., Osaka) and [^{14}C]benzylpenicillin potassium salt (51 mCi/mmol, The Radiochemical Centre, Amersham, England) were purchased. Stock cultures from the Central Research Laboratories of Meiji Seika Kaisha, Ltd., and clinical isolates from hospitals in Japan were used as test organisms. *E. coli* K-12 strain JE1011 and novobiocin- and cloxacillin-supersensitive mutants (NS 1, NS 14) derived from it were obtained from the Institute of Applied Microbiology, University of Tokyo[7].

Determination of MIC Values

Minimum inhibitory concentrations (MICs) were determined by the agar dilution method. Hundred-fold dilutions of overnight cultures in Trypticase soy broth (BBL) were spot-inoculated (5 μl) with a "Microplanter" (Sakuma, Tokyo) onto heart infusion (HI) agar (Difco) containing test antibiotics. The agar plates were incubated at 37°C for 24 hours. The MIC is defined as the lowest antibiotic concentration at which no growth (or less than 3 colonies per plate) was visible. For anaerobes, GAM agar (Nissui, Tokyo) and anaerobic GasPak jars (BBL) were used. The MICs were determined after incubation at 37°C for 48 hours.

The MICs in the presence of ethylenediaminetetraacetic acid (EDTA) were determined using HI agar plates containing the maximum concentrations of EDTA[8] that did not inhibit the growth of test organism. The measurement was performed in duplicate, and average values were recorded.

Cross Streak Test

A growth-inhibition test using a strip of Whatman No. 3 paper and an agar plate streaked with *E. coli* cells was carried out according to the procedure previously reported[9]. One loopful of an overnight culture was streaked on plates containing HI agar. A paper strip, 2 mm wide, was saturated with an aqueous solution of antibiotic and was placed on the plate at a right angle to the streaked cells. The length of the inhibited growth zone was measured after incubation at 37°C overnight.

Viable Cell Count

HI broth (10 ml) inoculated with *E. coli* K-12 JE1011 was pre-incubated at 37°C for 3 hours. Test antibiotic was added, and the mixture was incubated at 37°C with shaking. Serial ten-fold dilutions of the culture fluid (0.1 ml) were examined by the usual plating technique.

Measurement of Cell Density

An automatic biophotometer, MS-2 Research System (Abbott Laboratories, Irving, Texas) was

used with plastic cuvettes of 1 ml capacity to measure growth and lysis of the cells. HI broth (0.9 ml) inoculated with 50 μl of an overnight culture of *E. coli* K-12 strain JE1011 was pre-incubated at 37°C for 5 hours. An aqueous solution of test antibiotic (50 μl) was added, and the cuvettes were incubated at 37°C for 6 hours to record the time course of optical density at 660 nm.

Microscopic Observation

HI agar containing test antibiotic was thinly layered on a sterilized glass slide. Sucrose (20%) was added to prevent cell lysis. A suspension of *E. coli* K-12 strain JE1011 cells in the logarithmic growth phase was smeared over a cover glass. The streaked cover glass was placed on the film of sterile agar and embedded in paraffin. Observation was made with a phase-contrast microscope (Nikon, Tokyo), adjusted at 37°C by connecting with an incubator.

Assay of β-Lactamase Stability

Cells of *Citrobacter freundii* GN-346 and *Bacteroides fragilis* No. 36 were disintegrated by ultrasonication in 0.1 M phosphate buffer (pH 7.0), and centrifuged at 15,000$\times g$. The supernatant fluid that contained β-lactamase was collected. A purified β-lactamase preparation from *C. freundii* was obtained according to the procedure reported by HENNESSEY and RICHMOND[10].

The crude cell extract from *B. fragilis* or the purified β-lactamase solution of *C. freundii* was mixed with 200 μM substrate in 0.05 M phosphate buffer (pH 7.0) in a final volume of 1.0 ml, and incubated at 30°C. The rate of hydrolysis of the β-lactam ring was monitored by the modified NOVICK microiodometric method[11]. Cephalothin was used as the standard, and the relative rates of hydrolysis were arbitrarily defined by comparison to cephalothin.

Measurement of Binding Affinity to PBPs

The assay procedure was carried out as reported previously[12,13]. The binding of [^{14}C]benzylpenicillin was carried out by pre-incubation of cell membranes of *E. coli* K-12 strain JE1011 with unlabeled antibiotic at 37°C for 10 minutes, followed by addition of [^{14}C]benzylpenicillin and incubation at 37°C for 10 minutes. The molar concentration of antibiotic required to inhibit the [^{14}C]benzylpenicillin binding by 50% (I_{50}) was determined from the densitometric tracing of radioactive PBP bands on an X-ray film. Unlabeled benzylpenicillin was used as a reference, and relative values of I_{50} were arbitrarily defined in comparison to that for benzylpenicillin (1.00).

Results

Antibacterial Spectrum

Table 1 shows the MIC values of MT-141 and its four derivatives against both Gram-positive and Gram-negative aerobes and anaerobes. MT-141 showed moderate activity against *Bacillus* sp. and *Streptococcus pneumoniae* (MIC 0.78~1.56 μg/ml), but weak activity on other Gram-positive aerobic strains (MIC 3.13 μg/ml and up). Its activity against most Gram-negative aerobic strains was much stronger (MIC 0.20 μg/ml and up).

Modification of the D-amino acid moiety of MT-141 caused considerable change in its antibacterial spectrum. MT-116 and MT-180, both lacking the carboxylic acid function, showed a similar (*B. subtilis*) or stronger (other strains) activity than MT-141 on aerobic Gram-positive bacteria, but a much weaker activity against aerobic Gram-negative bacteria. In contrast, MT-199, lacking the basicity due to the amino group, showed a marked decrease in activity against aerobic Gram-positive bacteria. On the contrary, its activity against aerobic Gram-negative bacteria was similar to or slightly higher than the compounds lacking the carboxylic acid function, but lower than MT-141. The activity of MT-304, containing the L-amino acid moiety, was weaker against both Gram-positive and Gram-negative aerobic bacteria than MT-141.

A similar tendency was observed on anaerobes, with some exceptions. Here, the activity of MT-

Table 1. Antibacterial spectra of MT-141 and its derivatives against Gram-positive and Gram-negative aerobes and anaerobes.

Medium: HI agar (aerobic culture) or GAM agar (anaerobic culture). Sensitivity is expressed by MIC value inhibiting 50% of test strains. The number in parenthesis indicates the range of MIC values.

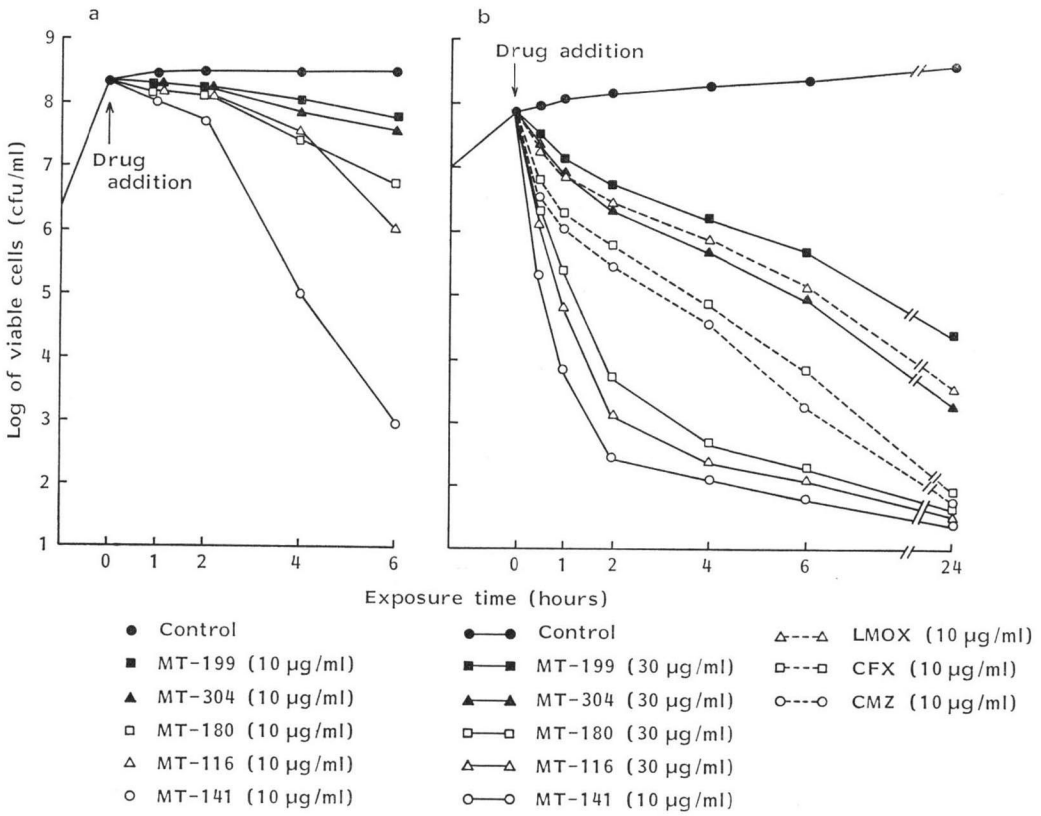
Organism (No. of strains)	MIC (μg/ml)				
	MT-141	MT-116	MT-180	MT-199	MT-304
Staphylococcus aureus (4)	6.25 (6.25~12.5)	1.56 (0.78~1.56)	1.56 (1.56)	25 (12.5~50)	12.5 (12.5~25)
S. epidermidis (3)	25 (12.5~25)	1.56 (1.56~3.13)	3.13 (3.13~6.25)	100 (50~>100)	25 (25~50)
Streptococcus pneumoniae (6)[a]	1.56 (1.56)	0.78 (0.78)	0.78 (0.78)	12.5~25 (12.5~25)	6.25 (1.56~6.25)
S. pyogenes (5)[a]	3.13 (3.13~6.25)	0.78 (0.78~1.56)	0.78 (0.39~0.78)	25 (12.5~50)	12.5 (1.56~12.5)
S. faecalis ATCC8043	50	12.5	25	>100	100
Bacillus subtilis ATCC6633	1.56	1.56	1.56	25	25
B. anthracis No. 119	0.78	0.39	0.39	50	3.13
Escherichia coli (7)	0.39 (0.20~0.39)	3.13 (1.56~3.13)	1.56 (0.78~1.56)	1.56 (0.39~3.13)	1.56 (0.78~3.13)
Klebsiella pneumoniae (4)	0.39 (0.20~0.39)	3.13 (3.13~6.25)	1.56 (1.56~3.13)	0.78~1.56 (0.78~1.56)	3.13 (1.56~3.13)
Proteus morganii (3)	1.56 (0.78~1.56)	25 (12.5~25)	25 (12.5~50)	12.5 (6.25~12.5)	12.5 (12.5~25)
P. vulgaris (3)	0.20 (0.20)	12.5 (12.5)	6.25 (6.25~12.5)	3.13 (3.13~6.25)	6.25 (6.25~12.5)
Salmonella sp. (3)	0.39 (0.20~0.39)	3.13 (1.56~3.13)	1.56 (1.56)	1.56 (0.78~1.56)	3.13 (1.56~6.25)
Shigella dysenteriae Shigae	0.20	1.56	0.78	0.78	1.56
Citrobacter freundii GN346/16	0.78	3.13	3.13	1.56	3.13
Peptococcus variabilis ATCC14955	0.78	0.20	0.39	1.56	0.78
Clostridium difficile No. 51	1.56	6.25	6.25	50	12.5
Other Gram-positive anaerobes (14)	0.78 (0.05~12.5)	0.39 (0.025~50)	0.39 (0.05~50)	3.13 (0.10~>100)	1.56 (0.10~50)
Bacteroides fragilis GM-7000	0.78	3.13	6.25	6.25	6.25
Other Gram-negative anaerobes (8)	0.78 (0.20~3.13)	3.13 (1.56~25)	6.25 (3.13~100)	100 (6.25~>100)	6.25 (6.25~50)

[a] HI agar supplemented with 5% horse blood.

141 against Gram-positive anaerobes was relatively strong compared with Gram-positive aerobes. In particular, its potent activity against *Clostridium difficile*, to which most cephem antibiotics are much less active, was surprising.

Neutralization of the acidic function, as seen in MT-116 and MT-180, resulted in an increase of activity against Gram-positive anaerobes except *C. difficile* and a decrease of activity against Gram-negative anaerobes. On the other hand, neutralization of the basic function (MT-199) caused an enormous decrease in activity on both Gram-positive and Gram-negative anaerobes. Conversion of the D-amino acid to the L-congener (MT-304) also caused a marked decrease in activity against Gram-negative anaerobes and the Gram-positive *C. difficile*.

Fig. 2. Time course of bactericidal effects of MT-141, its derivatives, cefoxitin (CFX), cefmetazole (CMZ) and latamoxef (LMOX) against *E. coli* K-12 strain JE1011.
Cells were incubated in HI broth at 37°C with gentle shaking.



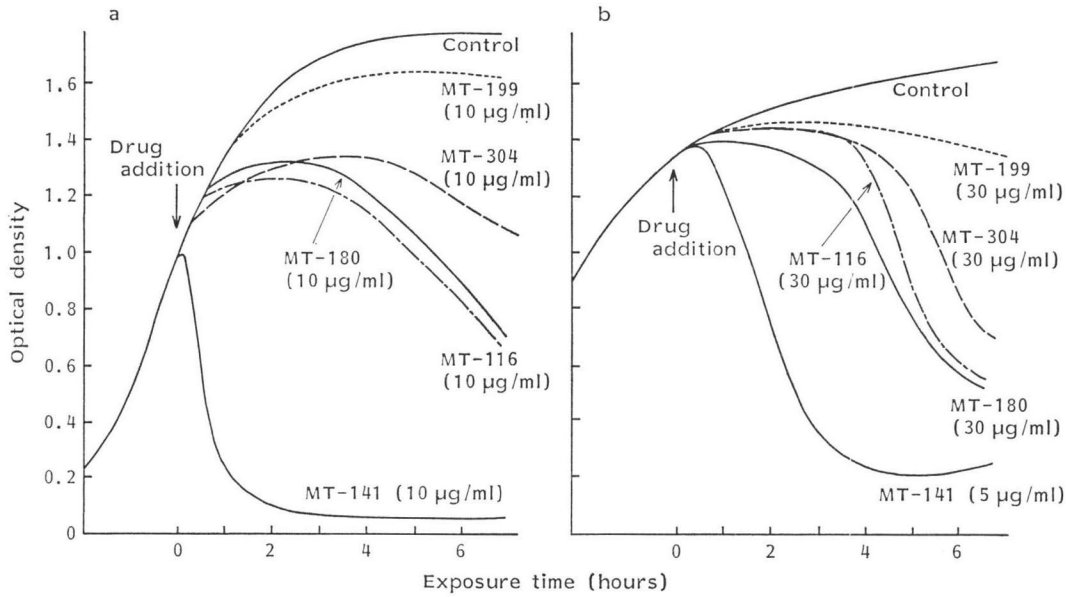
Bactericidal Activity

Fig. 2 shows the time course of killing action of MT-141 and its derivatives on *E. coli* K-12 strain JE1011. A large inoculum size (10^8 cfu/ml) was used in order to show distinct differences between MT-141 and its derivatives. When the bactericidal activity was compared at a concentration of 10 μg/ml (Fig. 2a), MT-141 decreased the number of viable cells most markedly and killed more than 10^5 cells in a 6-hour exposure. MT-116 and MT-180 showed less activity, killing 90% to 99% of the cells in 6 hours. The killing abilities of MT-304 and especially MT-199 were very weak.

Bactericidal activities of the derivatives at a higher concentration (30 μg/ml) were also compared with MT-141 (10 μg/ml), to compensate for the higher MIC values of the derivatives as compared to MT-141 (0.39 μg/ml for MT-141, and 3.13 μg/ml for its four derivatives) (Fig. 2b). Under these conditions, MT-141, MT-180 and MT-116 showed similar killing activities, while MT-199 and MT-304 showed much less killing activity on exposure up to 24 hours. In this experiment, the cell density of the culture was lower than that in Fig. 2a, which caused more rapid decrease of the viable cell number after addition of drugs.

For comparison, activities of cefoxitin, cefmetazole and latamoxef (10 μg/ml) were measured in the same series of experiments (Fig. 2b). The activity of MT-141 was more rapid and more potent than those of the reference antibiotics.

Fig. 3. Time course of bacteriolytic effects of MT-141 and its derivatives against *E. coli* K-12 strain JE1011. Cells were incubated in HI broth at 37°C with gentle shaking. Bacteriolysis was measured with an automatic biophotometer.



Bacteriolytic Activity

Fig. 3 shows the bacteriolysis curves of *E. coli* K-12 strain JE1011 measured with an automatic biophotometer. When the bacteriolysis was compared at 10 μg/ml (Fig. 3a), MT-141 caused the most rapid decrease in optical density, which fell below 0.1 after a 2-hour exposure. On the contrary, MT-199 caused only a slight decrease in optical density during 6 hours. MT-116, MT-180 and MT-304 caused intermediate decreases in optical density.

This tendency also was observed when the concentration of MT-141 was decreased to 5 μg/ml and that of its derivatives was increased to 30 μg/ml (Fig. 3b). MT-141 at 5 μg/ml reduced optical density of the cells at a rate similar to that at 10 μg/ml, whereas MT-116, MT-180, MT-304 and MT-199 at 30 μg/ml increased the bacteriolytic activity as compared to 10 μg/ml, but were definitely weaker in activity than MT-141 even at 5 μg/ml.

Morphological Changes

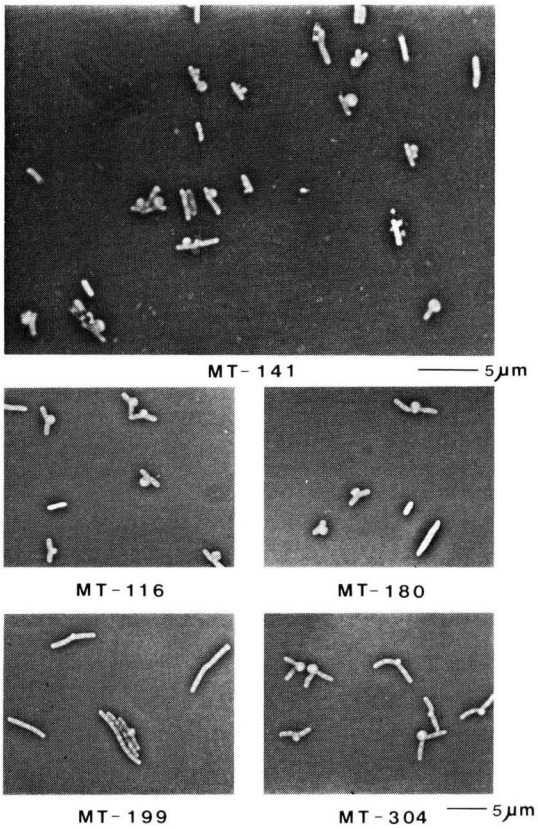
Fig. 4 shows the morphological changes of *E. coli* K-12 strain JE1011 cells when treated with MT-141 and its derivatives. Treatment with 10 μg/ml of MT-141 for 1 hour caused formation of both single and multiple bulges without forming filamentous cells. The cells with bulges lysed after extended exposure of the cells to antibiotic. Multiple bulges were frequently formed even by treatment with as low a concentration as 0.39 μg/ml (MIC) of MT-141 for 2 hours.

Treatment with 30 μg/ml of MT-116, MT-180 and MT-304 for 1.5 hours induced formation of only single bulges at the center of the cells, multiple bulges not being formed even by prolonged treatment. On the other hand, MT-199 caused filamentation, and bulges were scarcely formed. Thus, modification of the terminal D-amino acid moiety of MT-141 brought about considerable effects on the cellular morphology of *E. coli* K-12.

Cefoxitin (3.13 μg/ml, MIC) and cefmetazole (1.56 μg/ml, 2×MIC) induced the formation of

single bulges, whereas latamoxef (0.39 μg/ml, 4×MIC) induced the filamentation under similar conditions (data not shown).

Fig. 4. Phase-contrast micrographs of *E. coli* K-12 strain JE1011 exposed to MT-141 (10 μg/ml for 1 hour) and its derivatives (30 μg/ml for 1.5 hours). The change of cell morphology was observed in HI agar containing 20% sucrose.



Cell-surface Permeability

The ability of MT-compounds to penetrate the bacterial cell-surface was examined using cell-surface defective mutants on one hand and using EDTA on the other. *E. coli* K-12 strain NS 1 and NS 14 are highly susceptible to novobiocin and cloxacillin and are supposed to be deep-rough mutants with defective lipopolysaccharides[7]. Table 2 shows the susceptibilities of these mutant strains to MT-141 and its derivatives as compared to the parent strain JE1011. As judged by the increase in the size of the inhibition zone, susceptibilities of the mutant strains increased only slightly for all the MT-compounds. The increase in susceptibility may indicate only a small extent of permeability change in the cell-surface of the mutants.

Table 3 shows MIC values of the MT-compounds against eight Gram-negative bacteria as measured in the presence (A) and absence of EDTA (B). EDTA was expected to increase the permeability of the cell-surface[14,15]. The apparent increase in antibacterial activity due to EDTA was expressed by the ratio B/A. Except for *C. freundii* GN-346 and two *Pseudomonas* strains, the ratios B/A of MT-compounds were in the range of 1~8, indicating no large difference among MT-compounds.

Stability to β-Lactamase

Table 4 shows the relative rates of hydrolysis (V_{max}) of five MT-compounds and three reference

Table 2. Susceptibility of *E. coli* K-12 and its cell-surface defective mutants to MT-141, its derivatives, novobiocin and cloxacillin.

The cross streak test was conducted on HI agar streaked with an overnight culture. A paper strip wetted with antibiotic solution (MT-compounds, 1 mg/ml; novobiocin, 20 mg/ml; cloxacillin, 10 mg/ml), was placed at right angles to the streaked cells.

Strain	Inhibited growth zone (mm)						
	MT-141	MT-116	MT-180	MT-199	MT-304	Novobiocin	Cloxacillin
JE1011	31	21	22	19	25	6	3
NS 1[a]	34	26	27	22	27	28	24
NS 14[a]	33	24	26	24	27	29	32

[a] Novobiocin, cloxacillin-supersensitive mutants derived from *E. coli* K-12 JE1011.

Table 3. Effect of EDTA on MIC values of MT-141
A, MIC in the presence of EDTA; B, MIC in the absence of EDTA; B/A, enhancement ratio of

Organism	EDTA added (μmol/ml)	MT-141			MT-116		
		A	B	B/A	A	B	B/A
E. coli No. 29	2.5	0.39	0.78	2	1.56	3.13	2
S. typhimurium LT-2	10	0.20	0.39	2	3.13	6.25	2
K. pneumoniae 22#3038	5	0.20	0.78	4	3.13	6.25	2
P. morganii Kono	5	0.39	1.56	4	25	50	2
C. freundii GN346	5	25	>100	>4	12.5	25	2
S. marcescens MB3848	20	0.78	6.25	8	12.5	25	2
P. aeruginosa E-2	10	6.25	>100	>16	3.13	>100	>32
P. cepacia M-0527	10	0.39	12.5	32	3.13	12.5	4

drugs as catalysed by β-lactamases of *C. freundii* GN-346 and *B. fragilis* No. 36. MT-141, its derivatives and the reference antibiotics (cefoxitin and cefmetazole) were all stable to the *B. fragilis* β-lactamase, but were slightly sensitive to the *C. freundii* β-lactamase to different extents.

Table 4. Stability of MT-141, its derivatives, cefoxitin and cefmetazole against β-lactamases of *C. freundii* GN-346 and *B. fragilis* No. 36.
Rate of hydrolysis, relative to an arbitrary value of 100 for cephalothin, was determined by the micro iodometric method.

	Relative rate of hydrolysis (V_{max})	
	C. freundii[a]	*B. fragilis*[b]
MT-141	0.20	<0.01
MT-116	0.03	<0.01
MT-180	0.12	<0.01
MT-199	0.06	<0.01
MT-304	0.09	<0.01
Cefoxitin	0.14	<0.01
Cefmetazole	0.14	<0.01
Cephalothin	100	100

[a] Purified enzyme. [b] Crude enzyme.

Binding Affinity to PBPs

Table 5 shows the relative binding affinities of MT-141 and its derivatives to the PBPs of *E. coli* K-12 strain JE1011. All of the MT-compounds showed similar binding patterns. The similarity in binding, especially to high molecular weight PBPs (which are important for the cell cycle process, *i.e.*, PBP-1A, 1Bs, 2 and 3) was noted, because it did not seem to reflect the large disparities between MT-compounds in the bactericidal and bacteriolytic activities and the mode of morphological alteration which they caused.

Table 5. Competition of MT-141 and its derivatives with ^{14}C-labeled benzylpenicillin for binding to PBPs of *E. coli* K-12 JE1011.

PBP	Relative ratio of 50% inhibition concentration[a]				
	MT-141	MT-116	MT-180	MT-199	MT-304
1A	0.8	0.8	0.7	0.5	0.6
1Bs	1.4	0.9	1.1	1.2	1.4
2	160	39	110	260	150
3	3.2	4.1	3.0	4.2	3.8
4	<1.0	<1.0	<1.0	1.5	<1.0
5/6	0.1	0.07	0.1	0.02	0.03

[a] Relative value of the molar concentration of MT-compound required for 50% inhibition of binding of labeled benzylpenicillin to that of unlabeled benzylpenicillin, which was arbitrarily assigned as 1.0.

and its derivatives against 8 Gram-negative bacteria.
activity by the addition of EDTA. Medium, HI agar; inoculum size, 10^8 cfu/ml.

MIC (μg/ml)								
MT-180			MT-199			MT-304		
A	B	B/A	A	B	B/A	A	B	B/A
1.56	3.13	2	3.13	3.13	1	3.13	3.13	1
3.13	3.13	1	0.78	1.56	2	1.56	3.13	2
3.13	6.25	2	0.78	1.56	2	1.56	3.13	2
50	50	1	12.5	12.5	1	25	25	1
25	>100	>4	25	>100	>4	25	100	4
25	50	2	6.25	50	8	6.25	12.5	2
3.13	>100	>32	25	>100	>4	12.5	>100	>8
3.13	12.5	4	1.56	12.5	8	3.13	12.5	4

Discussion

It is obvious from our study on the structure-activity relationships that the terminal D-amino acid function of MT-141 at its 7β-side chain exerts a considerable effect on antibacterial activity. First of all, this can be seen in the antibacterial spectrum, showing a high degree of activity against Gram-negative bacteria. This observation is consistent with previously obtained knowledge that similar, naturally occurring β-lactam antibiotics, such as cephamycin C[1], antibiotic SF-1623[2], nocardicin[3] and sulfazecin[4] also are more active on Gram-negative bacteria than on Gram-positive ones.

The modification in the D-amino acid moiety was accompanied by changes in the antibacterial spectrum: neutralization of the acidic function shifted the spectrum from Gram-negative to Gram-positive bacteria, whereas neutralization of the basic function caused a marked drop in activity against both Gram-positive and Gram-negative bacteria. Thus, a carboxyl group appears to favor the anti-Gram-negative activity, and an amino group anti-Gram-positive and anti-Gram-negative activities.

Based on the resemblance of the cephem nucleus to the D-alanyl-D-alanine moiety of the cell wall[16], the 7β-side chain of MT-141 may constitute an extended resemblance to the *meso*-diaminopimelyl-D-alanyl-D-alanine moiety of the peptidoglycans of Gram-negative and Gram-positive bacilli, and that of MT-116 to the L-lysyl-D-alanyl-D-alanine moiety of Gram-positive cocci. In this connection, it is of interest that MT-141 was more active on bacilli containing *meso*-diaminopimelic acid in their cell wall peptidoglycans, whereas MT-116 was more active on cocci containing L-lysine.

The D-amino acid moiety in the 7β-side chain exhibited profound effects on the lysis and morphological change of *E. coli* K-12. MT-141 caused rapid lysis with frequent formation of multiple bulges on the cells, but this property was lost by neutralization of the acidic and basic functions or optical conversion. Especially, *N*-acetylation caused a significant decrease in the lytic activity so that the cells treated with the *N*-acetate almost did not from bulges but became filaments.

Cefoxitin, cefmetazole and latamoxef were less active than MT-141 in bactericidal and bacteriolytic[17] activities against *E. coli* K-12, and induced single bulge formation as seen in MT-116 and MT-180, or filamentation of the cells as seen in MT-199.

Recently, cellular alteration of *E. coli* exposed to β-lactam antibiotics has been correlated with binding affinities to PBPs[18]: cell lysis associated with bulge formation is ascribed to the binding to PBP-1A and -1Bs, and the formation of filamentous cells to that of PBP-3. However, we could not find any correlations between the binding affinity of MT-compounds to the above PBPs and their bacteriolytic activity or ability to induce formation of filamentous cells.

The binding affinities of cefoxitin[19] and cefmetazole[20] determined under similar conditions were also similar to those of MT-compounds. In contrast, the binding affinity of latamoxef seemed to be different from those of MT-compounds, as it was reported to have the highest affinity to PBP-3[21].

Furthermore, we saw no positive contribution of the D-amino acid moiety to β-lactamase stability or the cell-surface permeability, which, together with the binding affinity to PBPs, have been reported

to be important for the antibacterial activity of β-lactam antibiotics[22].

Summarizing the results obtained, we conclude that the D-amino acid moiety of MT-141 plays an important role in enhancing the antibacterial activity especially against Gram-negative bacteria, frequently causing multiple bulges on the cells followed by rapid lysis.

The bioactivity of β-lactam antibiotics has been correlated mainly with the chemical reactivity of the β-lactam ring[23]. However, it is hard to accept that the terminal amino acid moiety in the 7β-side chain of a cephem nucleus could affect the reactivity of the β-lactam ring, since X-ray analysis[24,25] indicates that the amino acid moiety located at the end of the extended side chain is remote from the β-lactam ring. Accordingly, the role of the D-amino acid function in terms of antibacterial activity may not be primarily concerned with the known mechanism of action of β-lactam antibiotics, but may be involved in a new mechanism. This problem is under active investigation, and the results will be reported at a later date.

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