CEPHABACINS, NEW CEPHEM ANTIBIOTICS OF BACTERIAL ORIGIN

IV. ANTIBACTERIAL ACTIVITIES, STABILITY TO β-LACTAMASES AND MODE OF ACTION

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Cephabacin F group antibiotics with a 7-formylamino substituent showed antibacterial activity against a wide variety of bacteria including β-lactamase-producing clinical isolates and anaerobic bacteria. Cephabacin H group antibiotics without the substituent showed more potent activity against Gram-positive bacteria than cephabacin F group antibiotics, but were not active against Gram-negative bacteria producing β-lactamases. Cephabacin F group antibiotics were highly resistant to hydrolysis by various types of β-lactamases and showed strong inhibitory activity against a cephalosporinase of *Proteus vulgalis* GN 4413 due to the 7-formylamino substituent. Mode of action of cephabacin F_1 and H_1 was examined using *Escherichia coli* and *Bacillus subtilis* as the test organisms. They showed strong lytic activity against these organisms and inhibited their peptidoglycan synthesis. Cephabacin F_1 had the highest affinity for penicillin-binding protein (PBP) 1 in *E. coli* and PBP 4 in *B. subtilis*. Cephabacins showed a protective effect in experimentally infected mice.

We have reported previously[1~3] the discovery, isolation and structures of new cephem antibiotics, cephabacin $F_{1\sim9}$ and $H_{1\sim6}$ (Table 1), produced by new species of *Lysobacter* and *Xanthomonas*. Here we describe their antibacterial activities, stability to β-lactamases and inhibitory activity against β-lactamases, and the mode of action of cephabacin F_1 and H_1.

Materials and Methods

MIC Determination

MICs were assayed by the conventional agar dilution method with DYAB agar[4]. The Mueller-Hinton medium (Difco) was used for an assay against β-lactamase-producing bacteria.

Assay of Stability to β-Lactamases

The rate of hydrolysis by β-lactamases was determined spectrophotometrically by monitoring the decrease of optical density at 265 nm[5]. The differences in molar extinction ($M^{-1}\cdot cm^{-1}$) between antibiotics and the corresponding hydrolyzed compounds (hydrolyzed by the *Pseudomonas aeruginosa* U31 β-lactamase or 0.75 N NaOH) at 265 nm were as follows: Cephabacin F_1 7,640; F_2 8,260; F_3 8,480; H_1 6,520; H_2 5,960; H_3 5,670; 7-formylaminodeacetylcephalosporin C 7,390; deacetylcephalosporin C 6,100; cephamycin C 3,260. For the β-lactamase-resistant substrates, hydrolysis rates were determined microbiologically; an aliquot withdrawn at intervals from the reaction mixture was added to nine volumes of MeOH and the residual amount of antibiotic was assayed by the paper disk method with *E. coli* PG-8[6]. The enzyme activities with benzylpenicillin and cephaloridine as substrates were determined microiodometrically[5].

Assay of β-Lactamase Inhibitory Activity

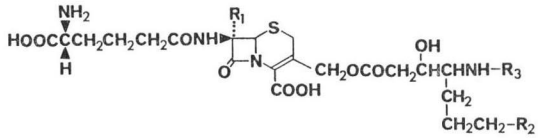
β-Lactamase inhibitory activity was determined as described previously[7]. The percent inhibi-

tion of enzyme activity was calculated against a control reaction in which the inhibitor was replaced by buffer. The concentration giving 50% inhibition (I_{50}) was obtained from a plot of the percentage inhibition against the antibiotic concentration.

Assay of Lytic Activity

Cultures of *E. coli* LD-2 and *B. subtilis* PCI 219 grown in DYAB medium to the exponential phase

Table 1. Structures of cephabacins.



Cephabacin	R_1	R_2	R_3
F_1	$-NHC(=O)H$	$-NHC(=NH)NH_2$	-L-Ala
F_2	〃	〃	-L-Ala-L-Ala
F_3	〃	〃	-L-Ala-L-Ala-L-Ala
F_4	〃	〃	-L-Ser
F_5	〃	〃	-L-Ser-L-Ser
F_6	〃	〃	-L-Ser-L-Ser-L-Ala
F_7	〃	$-CH_2NH_2$	-L-Ser
F_8	〃	〃	-L-Ser-L-Ser
F_9	〃	〃	-L-Ser-L-Ser-L-Ala
H_1	-H	$-NHC(=NH)NH_2$	-L-Ala
H_2	〃	〃	-L-Ala-L-Ala
H_3	〃	〃	-L-Ala-L-Ala-L-Ala
H_4	〃	〃	-L-Ser
H_5	〃	〃	-L-Ser-L-Ser
H_6	〃	〃	-L-Ser-L-Ser-L-Ala

Table 2. Antibacterial activity

Organism	F_1	F_2	F_3	F_4	F_5	F_6	F_7
Escherichia coli NIHJ JC2	12.5	25	25	25	25	50	25
Salmonella typhimurium IFO 12529	12.5	25	25	25	25	50	25
Citrobacter freundii IFO 12681	25	50	100	50	50	100	50
Klebsiella pneumoniae IFO 3317	25	50	50	50	50	50	50
Enterobacter cloacae IFO 12937	25	25	100	25	50	100	50
Serratia marcescens IFO 12648	25	50	50	25	25	50	25
Proteus mirabilis ATCC 21100	12.5	50	25	25	25	25	12.5
P. vulgaris IFO 3988	12.5	50	25	25	25	25	25
P. morganii IFO 3168	25	25	50	25	25	50	50
Pseudomonas aeruginosa IFO 3080	25	100	>100	50	100	>100	100
Alcaligenes faecalis IFO 13111	1.56	6.25	12.5	12.5	6.25	12.5	6.25
Acinetobacter calcoaceticus IFO 12552	0.78	1.56	3.13	1.56	3.13	3.13	1.56
Staphylococcus aureus FDA 209P	50	>100	>100	>100	>100	>100	>100
Bacillus subtilis PCI 219	3.13	12.5	12.5	6.25	12.5	12.5	12.5
B. megaterium IFO 12108	6.25	12.5	12.5	12.5	12.5	12.5	12.5
Brevibacterium thiogenitalis ATCC 19240	3.13	6.25	6.25	3.13	6.25	6.25	6.25

[a] 7-FD, 7-formylaminodeacetylcephalosporin C; DCPC, deacetylcephalosporin C.

were diluted 5-fold with fresh medium. Portions of 4.5 ml of the diluted cultures were delivered into sterilized tubes which were then incubated at 37°C with reciprocal shaking. After 3 hours incubation, 0.5 ml of an antibiotic solution was added to the culture. Growth was followed by measuring the absorbance at 600 nm every hour with a Spectronic 20 colorimeter (Shimadzu, Bausch & Lomb).

Determination of Inhibition of Peptidoglycan Synthesis

Peptidoglycan synthesis was assayed by incorporation of [*G*-^{3}H]diaminopimelic acid (281 mCi/mmol, Amersham) into hot TCA-insoluble fractions using *E. coli* LD-2 and *B. subtilis* PCI 219[4].

Assay of Affinity for Penicillin-binding Proteins (PBPs)

The affinity of PBPs in *E. coli* LD-2 was assayed as described previously[4]. The assay of affinity for PBPs in *B. subtilis* PCI 219 was carried out by the method of GEORGOPAPADAKOU and LIE[8] except for the SDS-PAGE and fluorography procedures. These procedures were those used with *E. coli* LD-2.

Protective Effect in Mice

Mice were infected intraperitoneally with 0.5 ml of a suspension of *E. coli* O-111[9]. Groups of five mice at each dose level were subcutaneously given 0.2 ml of an antibiotic solution immediately after infection. The 50% effective dose (ED_{50} mg/kg) was calculated by the conventional method from the survival rate at 5 days after infection.

Results

Antibacterial Activities

Cephabacin $F_{1\sim9}$ showed moderate antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria. They were highly active against *Alcaligenes faecalis* and *Acinetobacter calcoaceticus*. On the other hand, cephabacin $H_{1\sim6}$ were less active against Gram-negavite bacteria but more active against Gram-positive bacteria than cephabacin $F_{1\sim9}$ (Table 2).

of cephabacins and related compounds.

MIC (μg/ml) at 10^6 cfu/ml									
F_8	F_9	H_1	H_2	H_3	H_4	H_5	H_6	7-FD[a]	DCPC[a]
25	50	50	50	>100	50	50	>50	100	100
25	50	50	50	100	50	100	>50	50	50
50	100	>100	>100	>100	>100	>100	>50	100	>100
50	50	100	100	100	100	100	>50	50	100
50	100	>100	>100	>100	>100	>100	>50	>100	>100
50	50	>100	>100	>100	>100	>100	>50	50	>100
12.5	50	25	25	25	25	25	25	25	12.5
25	50	50	50	100	>100	>100	50	25	12.5
50	50	>100	>100	>100	>100	>100	>50	100	>100
>100	>100	>100	>100	>100	>100	>100	>50	>100	100
12.5	50	12.5	12.5	12.5	6.25	6.25	12.5	25	12.5
3.13	3.13	50	100	100	25	100	>50	12.5	>100
>100	>100	25	25	12.5	25	50	50	>100	50
25	25	0.78	1.56	1.56	1.56	1.56	1.56	>100	3.13
12.5	25	0.78	1.56	1.56	1.56	1.56	1.56	>100	6.25
6.25	12.5	1.56	3.13	3.13	1.56	3.13	3.13	>100	12.5

Table 3. Antibacterial activity of cephabacins and related compounds against β-lactamase producing bacteria.

Organism	β-Lactamase activity[a] (U/mg d.w.)	MIC (μg/ml) at 10^6 cfu/ml										
		F_1	F_2	F_3	F_4	F_7	H_1	H_2	H_3	7-FD[c]	DCPC[c]	CTM[c]
E. coli J53-2	<0.01	6.25	12.5	25	12.5	12.5	25	50	>100	100	100	≦0.20
E. coli J53-2 (RP4)	11.26	6.25	12.5	25	6.25	12.5	25	100	>100	100	100	0.39
E. coli J53-2 (RGN238)	0.05	12.5	25	25	6.25	12.5	25	100	>100	100	100	≦0.20
E. coli J53-2 (R997)	1.53	6.25	12.5	12.5	6.25	12.5	25	50	>100	100	100	0.78
K. pneumoniae TN 1717	0.02	50	50	50	50	50	>100	>100	>100	100	>100	≦0.20
K. pneumoniae TN 1700	4.56	50	50	50	50	50	>100	>100	>100	100	>100	1.56
K. oxytoca TN 1655	0.02	25	50	50	25	50	50	100	>100	50	100	≦0.20
K. oxytoca TN 1719	4.27	50	12.5	25	50	50	>100	>100	>100	100	>100	3.13
E. cloacae TN 581	0.42[b]	50	50	100	25	50	>100	>100	>100	>100	>100	0.39
E. cloacae TN 2660	10.69	25	25	>100	25	25	>100	>100	>100	100	>100	>100
C. freundii TN 480	0.17[b]	25	25	50	25	25	>100	>100	>100	50	>100	0.78
C. freundii TN 2678	1.76	12.5	25	50	12.5	25	>100	>100	>100	50	>100	100
S. marcescens IFO 12648	0.49[b]	50	100	100	50	50	>100	>100	>100	100	>100	0.78
S. marcescens TN 81	0.96[b]	100	>100	>100	100	100	>100	>100	>100	>100	>100	>100
P. morganii IFO 3168	0.30[b]	50	50	50	25	50	>100	>100	>100	100	>100	≦0.20
P. morganii GN 4738	2.42	25	25	25	12.5	25	>100	>100	>100	100	>100	25
P. vulgaris CS 4035	<0.01[b]	25	25	50	25	25	25	50	100	50	12.5	0.39
P. vulgaris CS 2533	3.20	25	25	50	12.5	25	>100	>100	>100	12.5	>100	>100
P. aeruginosa P 2	<0.01[b]	50	100	100	50	50	>100	>100	>100	100	>100	>100
P. aeruginosa U 31	0.48[b]	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
S. aureus FDA 209P	<0.01[b]	>100	>100	>100	>100	>100	50	50	100	>100	>100	≦0.20
S. aureus 1840	0.60[b]	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	1.56

[a] The β-lactamase activities of penicillinases (*S. aureus*, *E. coli*, *K. pneumoniae* and *K. oxytoca*) were determined with 0.2 mM benzylpenicillin as a substrate and those of cephalosporinases were with 0.2 mM cephaloridine. d.w.: Dry cell weight.

[b] Induced with 1 mg/ml of benzylpenicillin or 1 μg/ml cloxacillin (*S. aureus*).

[c] 7-FD, 7-formylaminodeacetylcephalosporin C; DCPC, deacetylcephalosporin C; CTM, cefotiam.

Table 4. Stability of cephabacins and related compounds to hydrolysis by β-lactamases.

Source of enzyme	Relative rate of hydrolysis[a]										
	F_1	F_2	F_3	F_4	F_7	H_1	H_2	H_3	7-FD[b]	DCPC[b]	CMC[b]
Penicillinase											
S. aureus 1840	<0.01	<0.01	<0.01	<0.01	<0.01	0.05	0.04	0.04	<0.01	0.02	0.02
E. coli TN713	<0.01	<0.01	<0.01	<0.01	<0.01	0.41	0.50	0.55	<0.01	0.12	0.01
E. coli TN649	<0.01	<0.01	<0.01	<0.01	<0.01	3.39	3.10	3.37	<0.01	7.97	0.79
P. aeruginosa GN3407	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	0.02	0.02	<0.01	<0.01	<0.01
K. oxytoca TN1719	<0.01	<0.01	<0.01	<0.01	<0.01	0.84	0.74	0.72	<0.01	0.51	<0.01
Cephalosporinase											
E. cloacae TN1282	<0.01	<0.01	<0.01	<0.01	<0.01	43.1	39.1	37.3	0.02	94.1	28.2
C. freundii GN1706	<0.01	<0.01	<0.01	<0.01	<0.01	33.6	30.0	26.3	0.01	105	29.9
P. aeruginosa U31	<0.01	<0.01	<0.01	<0.01	<0.01	29.3	22.0	20.0	0.02	132	11.8
S. marcescens TN81	<0.01	<0.01	<0.01	<0.01	<0.01	36.7	31.4	31.3	0.02	111	5.38
P. vulgaris GN4413	<0.01	<0.01	<0.01	<0.01	<0.01	21.9	14.4	11.7	<0.01	24.9	<0.01
B. fragilis V284-3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.1	<0.1	<0.1	<0.1	9.7	<0.1

[a] Expressed as relative rate of hydrolysis, taking the rate for benzylpenicillin (penicillinase), or cephaloridine (cephalosporinase) as 100.

[b] 7-FD, 7-formylaminodeacetylcephalosporin C; DCPC, deacetylcephalosporin C; CMC, cephamycin C.

As shown in Table 3, cephabacin F group antibiotics exerted antibacterial activity against β-lactamase-producing clinical isolates as potent as against their non-producing counterparts. Cephabacin F_1 was also active against anaerobic organisms such as *Bacteroides fragilis* (MIC 25 μg/ml) and *Clostridium perfringens* (MIC 25 μg/ml).

The 3-side chains of cephabacins clearly had positive effects on their antibacterial activities, that is, cephabacin F and H group antibiotics were more active than their corresponding derivatives without the 3-side chains, namely 7-formylaminodeacetylcephalosporin C and deacetylcephalosporin C, respectively (Tables 2 and 3).

Table 5. β-Lactamase inhibitory activity of cephabacins and related compounds.

Compound	I_{50} (μg/ml)[a]			
	Penicillinase		Cephalosporinase	
	S. aureus 1840	*E. coli* TN 713	*E. cloacae* TN 1282	*P. vulgaris* GN 4413
Cephabacin F_1	>100	>100	>100	0.56
F_2	>100	>100	>100	0.90
F_3	>100	>100	>100	0.90
F_4	>100	>100	>100	1.4
F_7	>100	>100	>100	0.92
H_1	>100	>100	>100	84
H_2	>100	>100	>100	51
H_3	>100	>100	>100	80
7-FD[b]	>100	>100	>100	44
DCPC	>100	>100	66	78
CMC	>100	>100	>100	0.90

[a] A concentration (μg/ml) required to inhibit the enzyme by 50%.
[b] See footnote of Table 4.

Fig. 1. Inhibition of peptidoglycan synthesis in *E. coli* LD-2 (a) and *B. subtilis* PCI 219 (b) by cephabacin F_1 and H_1.
○, Cephabacin F_1; ●, cephabacin H_1. The MICs are indicated by arrows.

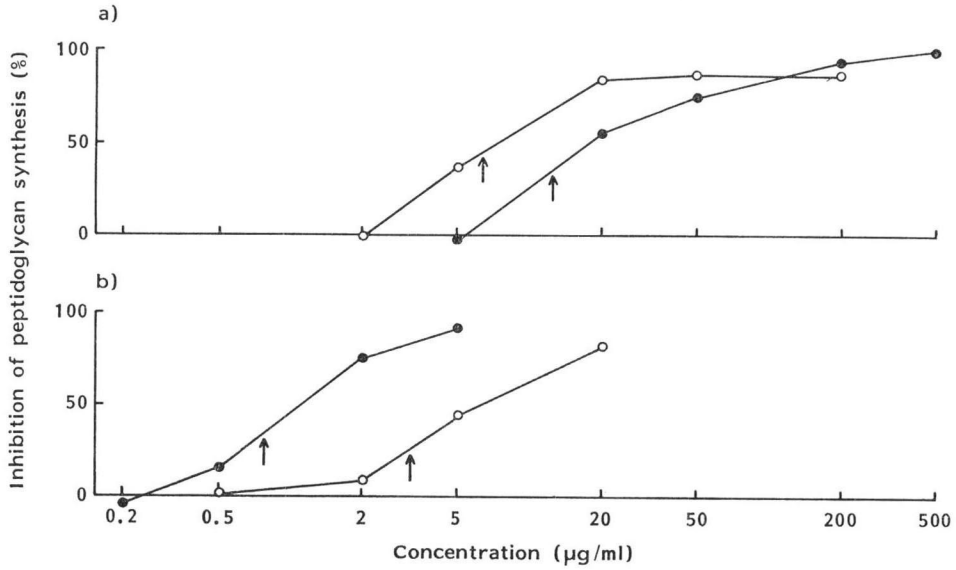
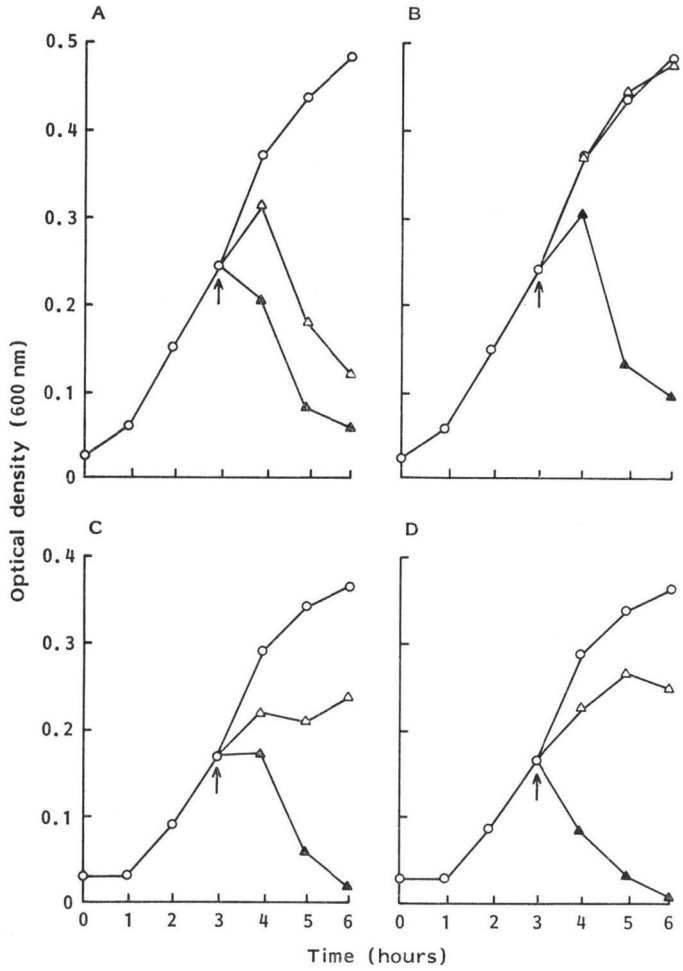


Fig. 2. Lytic activity of cephabacin F_1 and H_1 against *E. coli* and *B. subtilis*.

Cephabacin F_1 (A, C) or H_1 (B, D) was added after 3 hours of cultivation (indicated by arrows) of *E. coli* LD-2 (A, B) or *B. subtilis* PCI 219 (C, D).

○, Control; △, ×1 MIC; ▲, ×10 MIC.



Stability to β-Lactamases

As shown in Table 4, cephabacin F group antibiotics were highly resistant to hydrolysis by various types of β-lactamases whereas cephabacin H group antibiotics were sensitive, especially to the cephalosporinases. 7-Formylaminodeacetylcephalosporin C was also far more stable to the β-lactamases than deacetylcephalosporin C. The cephalosporins with a 7-formylamino substituent were far more stable to the β-lactamases than cephamycin C, a 7-methoxycephalosporin. The differences in the 3-side chain exhibited no significant effect on the susceptibility of cephabacin antibiotics to β-lactamases.

β-Lactamase Inhibitory Activity

Cephabacin $F_{1\sim4,7}$ showed inhibitory activity against a cephalosporinase of *Proteus vulgalis* GN 4413. This inhibition was 100-fold greater than that shown by cephabacin $H_{1\sim3}$ (Table 5). Cephabacin F group antibiotics appears to be generally more inhibitory to this enzyme than cephabacin H group

Fig. 3. Competition of cephabacin F_1 for [^{14}C]benzylpenicillin to PBPs in *E. coli* and *B. subtilis*.

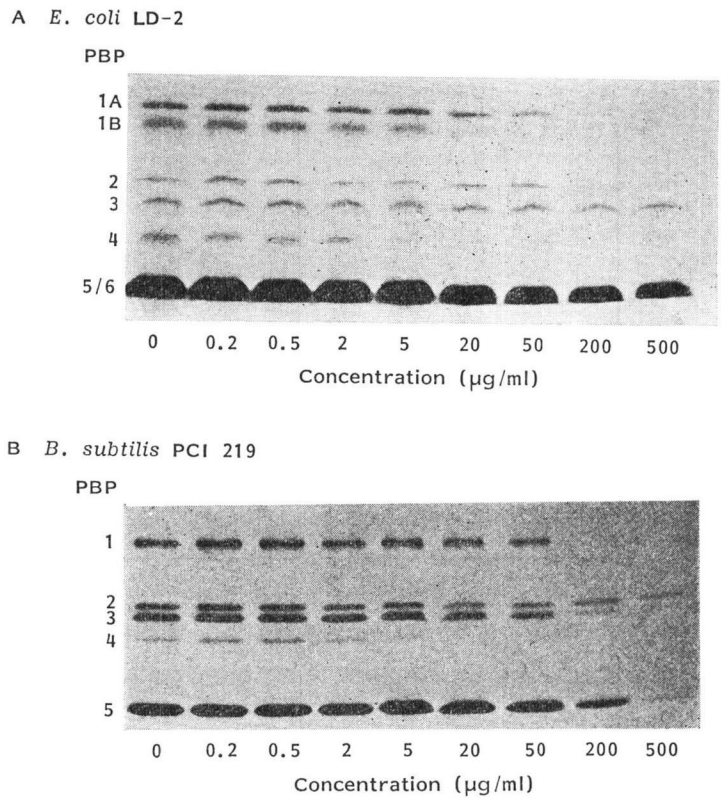


Table 6. Affinities of cephabacin F_1 for each PBP in *E. coli* and *B. subtilis*.

Organism	MIC (μg/ml)	I_{50}[a] (μg/ml)							
		PBP							
		1	1A	1B	2	3	4	5/6	5
E. coli LD-2	6.25		46	15	>500	>500	57	+[b]	
B. subtilis PCI 219	3.13	28			7.4	30	1.8		*ca.* 100

[a] The I_{50} for each PBP was quantitated with the fluorograms shown in Fig. 3.
[b] The affinity for PBP 5/6 was positive but not quantitated.

antibiotics because the former antibiotics showed far more inhibitory activity than the latter antibiotics in the convenient assay system, *i.e.*, the St/Si system described in the preceding paper[1] (data not shown). However, cephabacins did not show inhibitory activity against β-lactamases of *S. aureus*, *E. coli* (TEM-1) and *Enterobacter cloacae*.

Mode of Action

In order to elucidate the mode of action of cephabacins, various biological activities of cephabacin F_1 and H_1 were examined using *E. coli* LD-2 and *B. subtilis* PCI 219 as the test organisms. Cephabacin F_1 and H_1 specifically inhibited peptidoglycan synthesis among intracellular synthesis of macromolecules, *i.e.*, DNA, RNA, protein and peptidoglycan in *E. coli* LD-2 (data not shown). The in-

hibitory activity of cephabacin F_1 for peptidoglycan synthesis was more potent than that of cephabacin H_1 in *E. coli* LD-2 whereas the results were *vice versa* in *B. subtilis* PCI 219 (Fig. 1); these relationships paralleled the antibacterial activities against these organisms (MICs shown in Fig. 1 by the arrows). At the equivalent doses, cephabacin F_1 showed higher lytic activity against *E. coli* LD-2 than cephabacin H_1 whereas they showed similar activity against *B. subtilis* PCI 219 (Fig. 2).

Table 7. Protective effects of cephabacins in mice infected with *E. coli* O-111.

Compound	MIC[a] (μg/ml)	ED_{50} (mg/kg)
Cephabacin F_1	25	18.2
F_2	25	17.2
F_3	25	12.5
F_4	25	16.2
F_7	50	16.2
H_1	50	9.9
H_2	100	21.0
H_3	>100	25.0
7-FD	100	59.5

[a] Against *E. coli* O-111 at 10^9 cfu/ml.

As shown in Fig. 3A, cephabacin F_1 showed the highest affinity for PBP 1 in *E. coli* LD-2 among the lethal targets (PBP 1, 2 and 3) in this organism[10]. On the other hand, cephabacin F_1 showed the highest affinity for PBP 4 among the possible lethal targets (PBP 1, 2 and 4) in *B. subtilis*[11,12] (Fig. 3B). The concentrations of cephabacin F_1 required to inhibit the binding of [^{14}C]benzylpenicillin by 50% to PBP 1A and 1B in *E. coli* LD-2 and to PBP 4 in *B. subtilis* PCI 219 were consistent with the MICs against these organisms (Table 6).

To obtain information on the effects of the 7-formylamino substituent and the 3-side chains of cephabacins on the affinities for PBPs in *E. coli*, we compared the affinities of cephabacins F_1 and H_1, 7-formylaminodeacetylcephalosporin C and deacetylcephalosporin C. The results led to the conclusion that the complex 3-side chains increases the affinity for PBPs although the 7-formylamino substituent does not significantly affect the affinity for PBPs (data not shown).

Protective Effect in Mice Infected with *E. coli* O-111

Cephabacins protected mice from intraperitonial infection by *E. coli* O-111 (Table 7).

Discussion

Based on the data on the antibacterial activities and the stability to β-lactamases of cephabacins, the following conclusions on the structure-activity relationships of cephabacin antibiotics have been drawn: 1) The 7-formylamino substituent is the only factor of cephabacin F group antibiotics necessary for the stability to β-lactamases and seems to be a more efficient substituent for confering the stability to cephem antibiotics than the 7-methoxy one (Table 4), 2) the 7-formylamino substituent increases the antibacterial activity against Gram-negative bacteria while it decreases that against Gram-positive bacteria (Table 2), 3) the presence or absence of the guanidyl group and the difference between L-alanine and L-serine residues in the 3-side chains do not influence the activity, and 4) the greater the number of L-alanine or L-serine residues in the 3-side chain is, the weaker are the antibacterial activities of cephabacins (Tables 2 and 3).

The inhibitory activities of cephabacin F group antibiotics against the *P. vulgaris* GN 4413 β-lactamase were 100-fold stronger than those of cephabacin H group antibiotics, but that of 7-formylaminodeacetylcephalosporin C was almost the same as those of cephabacin H group antibiotics and deacetylcephalosporin C (Table 5). This observation leads to the conclusion that both the 7-formylamino substituent and the 3-side chains of cephabacin F group antibiotics contribute to the potent inhibitory activity against this enzyme.

Cephabacin F_1 showed the highest affinity for PBP 1 among the lethal targets, *i.e.*, PBP 1, 2 and

3, in *E. coli* (Table 6). Furthermore, its affinities for PBP 2 and 3 were quite low; to our knowledge, it is the most specific inhibitor of PBP 1 in *E. coli* among β-lactam antibiotics tested in their binding affinities. These results agree well with its potent lytic activity (Fig. 2) without inducing filamentous or ovoid cells and its inhibitory activity on peptidoglycan synthesis in *E. coli* (Fig. 1).

Cephabacin F_1 also showed good affinity for PBP 4 and 5/6 in *E. coli*, D-alanine carboxypeptidases[13,14], as did 7-methoxycephalosporins[15,16]; this seems to be a common property of cephalosporins with the formylamino or the methoxy substituent at the 7-position.

Comparison of the affinities of cephabacin F_1 and 7-formylaminodeacetylcephalosporin C, and those of cephabacin H_1 and deacetylcephalosporin C demonstrated that the complex 3-side chains of cephabacins clearly increased the affinity for PBPs in *E. coli*.

STROMINGER *et al.* suggested that PBP 1, 2 and 4 could be lethal targets in *B. subtilis*[11,12]. Among these PBPs in *B. subtilis*, cephabacin F_1 bound PBP 4 with the highest affinity (Table 6). However, discussion of the mode of action of cephabacins in this organism requires further study of the functions of these PBPs.

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