INTERACTIONS OF FORMYLAMINO- AND METHOXY-SUBSTITUTED β-LACTAM ANTIBIOTICS WITH β-LACTAMASES

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Cephem and nocardicin-type monocyclic β-lactam antibiotics with a formylamino substituent were highly resistant to hydrolysis by both penicillinases and cephalosporinases. Among antibiotics with a methoxy substituent, an *N*-sulfonated monocyclic β-lactam antibiotic, sulfazecin was resistant to β-lactamases, but cephem antibiotics were sensitive to the cephalosporinase of *Enterobacter cloacae*. The resistance of the antibiotics to the β-lactamases depended primarily on the presence of the substituent, but affinity for the β-lactamases was affected not only by the substituent but also by the presence of other side chains. Formylamino compounds and sulfazecin were as good inducers of β-lactamases as semisynthetic 7-methoxycephalosporins, but naturally occurring 7-methoxycephalosporins were poor inducers. The inducer activities of the antibiotics were not necessarily related to their β-lactamase stabilities. The stabilities of the compounds to the β-lactamases were well reflected in their antibacterial activities against β-lactamase producing bacteria.

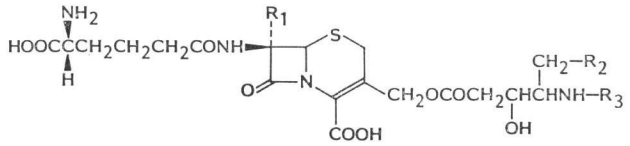
Many β-lactam antibiotics with a formylamino or a methoxy substituent are produced by various microorganisms[1~5]. The cephabacin F group of antibiotics[6] and the formadicins[7] are cephem and nocardicin-type monocyclic β-lactam antibiotics, respectively, having a formylamino group. The cephabacin M group of antibiotics[8] and the cephamycins[4] are 7-methoxycephalosporins, and sulfazecin[5] is an *N*-sulfonated monocyclic β-lactam antibiotic with the methoxy group (Fig. 1). It is known that cephamycins show increased stability to β-lactamases[9]. The introduction of a methoxy group into semisynthetic cephalosporins increases their affinity for some β-lactamases[10,11] and their β-lactamase-inducing activity[10,12], but there is little information on the affinity and the inducing properties of natural cephamycins. Not only their stability to β-lactamases but their affinity for β-lactamases and their β-lactamase-inducing activity affect the antibacterial activity of the antibiotic; antibiotics with a high affinity for β-lactamases, even if they are stable, are not active against some strains of *Enterobacteriaceae* having a nonhydrolytic barrier[13~15], and those with high inducibility antagonize coexisting other β-lactam antibiotics[15~17]. This paper deals with the effects of formylamino and methoxy substituents on the interactions of natural β-lactam antibiotics with β-lactamases.

Materials and Methods

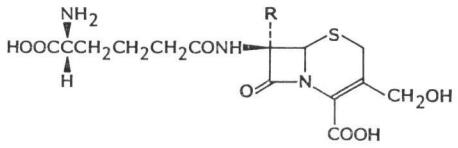
Antibiotics

Cephabacins F_1, M_1, and H_1, 7-formylaminodeacetylcephalosporin C (7-FDCPC), 7-methoxydeacetylcephalosporin C (7-MDCPC), deacetylcephalosporin C (DCPC), formadicins C and D, nocardicin A[18], and sulfazecin were prepared in our laboratories. Benzylpenicillin (Meiji Seika Kaisha, Co.), cephaloridine (Shionogi & Co.), cefazolin (Fujisawa Pharmaceutical Co.), and cefoxitin (Daiichi Seiyaku Co.) were obtained from commercial sources.

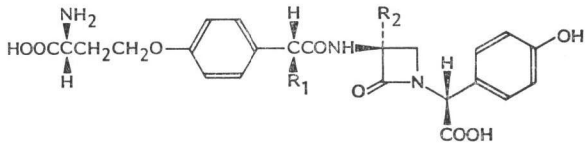
Fig. 1. Structures of the antibiotics.



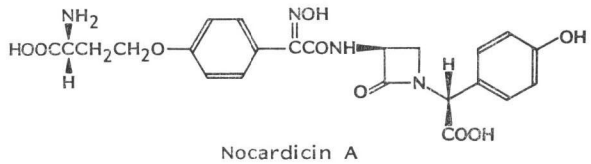
Cephabacin F_1 R_1 = NHC(=O)H, R_2 = $CH_2CH_2NHC(=NH)NH_2$, R_3 = L-Ala
Cephabacin M_1 R_1 = OCH_3, R_2 = $CONH_2$, R_3 = L-Val-α-L-Orn
Cephabacin H_1 R_1 = H, R_2 = $CH_2CH_2NHC(=NH)NH_2$, R_3 = L-Ala



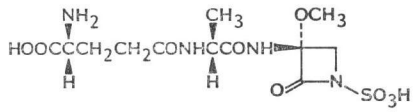
7-Formylaminodeacetylcephalosporin C R = NHC(=O)H
7-Methoxydeacetylcephalosporin C R = OCH_3
Deacetylcephalosporin C R = H



Formadicin C R_1 = OH, R_2 = NHC(=O)H
Formadicin D R_1 = NHC(=O)H, R_2 = H



Nocardicin A



Sulfazecin

Organism

Staphylococcus aureus 1840S lacks the penicillinase plasmid and *S. aureus* 1840-2 produces β-lactamase constitutively. Both strains were obtained spontaneously from *S. aureus* 1840. *Escherichia coli* J53-2 and a plasmid RP4 that mediates TEM-2 penicillinase were generous gifts from Dr. G. A.

JACOBY (Massachusetts Central Hospital, Boston). *Enterobacter cloacae* CS4494, which produces a large amount of β-lactamase constitutively, is a spontaneous mutant of clinically isolated *E. cloacae* GN5788. *E. cloacae* CS4495 is a β-lactamase-deficient mutant of CS4494 obtained by UV irradiation. *Proteus vulgaris* CS4035 and CS4017 are a β-lactamase-deficient and a -constitutive mutant, respectively, obtained from *P. vulgaris* GN4818 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *E. cloacae* GN5797 and *P. vulgaris* GN4421 are clinical isolates.

Preparation of β-Lactamases

The enzymes of *E. cloacae* TN1282 and *P. vulgaris* GN4413 were purified to homogeneity by polyacrylamide gel electrophoresis, and those of *S. aureus* 1840 and *E. coli* TN713 (TEM-1) were purified partially as described previously[19].

Hydrolysis Studies

The rate of hydrolysis by β-lactamases was determined spectrophotometrically by monitoring the decrease in optical density of the antibiotics on a Gilford 250 spectrophotometer. UV spectra were recorded for each antibiotic before and after complete hydrolysis by a selected β-lactamase or by 0.75 M NaOH, and an appropriate wavelength was selected for assay. To analyze at high concentrations of substrate, kinetic studies for cephalosporins were performed at 285 nm, which was not the wavelength associated with the maximum absorbance change in the difference spectra. Kinetic studies for monocyclic β-lactam antibiotics were carried out at 215 nm. Molar extinction coefficients ($\Delta\varepsilon$) calculated from the differential absorbance change at that wavelength were: cephabacin F_1, 1,920; cephabacin M_1, 3,010; cephabacin H_1, 1,550; 7-FDCPC, 1,680; 7-MDCPC, 2,110; DCPC, 1,630; formadicin D, 5,220; nocardicin A, 4,860; and sulfazecin, 1,110. The hydrolysis study for formadicin C was performed microbiologically as described previously[20], since no enzyme could hydrolyze the antibiotic and alkaline hydrolysis of β-lactam antibiotics gave spectra different from those of enzymatically hydrolyzed compounds at wavelengths lower than 240 nm. The rates of hydrolysis of benzylpenicillin and cephaloridine were determined microiodometrically[19].

The reaction mixture consisted of 1.7 ml of 50 mM phosphate buffer (pH 6.9), 0.2 ml of the substrate prepared in phosphate buffer, and 0.1 ml of an enzyme, and was incubated at 30°C. In some experiments, the volume of the reaction mixture was reduced to 0.5 ml without changing the composition, and the reaction was performed in a microcuvette. Kinetic constants were determined from Lineweaver-Burk plots[21] of data of at least five concentrations of substrate spanning the *Km* values where possible. One unit of β-lactamase was defined as the amount of enzyme that hydrolyzed 1 μmol of benzylpenicillin (penicillinase) or cephaloridine (cephalosporinase) per minute at 30°C.

Inhibition Studies

The rates of hydrolysis of ampicillin or cephalothin by penicillinases or cephalosporinases, respectively, in the presence or absence of a test antibiotic were measured microiodometrically, and *Ki* values were determined from Lineweaver-Burk plots.

Induction Studies

The β-lactamases of *P. vulgaris* GN4421 and *E. cloacae* GN5797 grown in Brain Heart Infusion broth (Eiken) at 30°C were induced by the antibiotics for 1 hour as described previously[10]. The enzyme preparations were dialyzed twice against 200 volumes of 50 mM phosphate buffer (pH 6.9) to remove the inducer.

Susceptibility Testing

The minimum inhibitory concentration (MIC) was determined by the microbroth dilution method. One tenth milliliter of Mueller-Hinton broth (Difco) containing two-fold serial dilutions of each antibiotic was prepared in a microtiter plate (96 wells) using a Titertek multichannel pipette. A final inoculum was 10^5 cfu/ml. The plates were incubated overnight at 37°C and the optical density (OD) at 405 nm of each well was measured using a Corona MTP-12 microplate photometer. The growth was defined as negative when the OD was less than 0.050. The MIC was defined as the lowest concentration of antibiotic preventing growth of bacteria.

Results

Stability to β-Lactamases

Tables 1 and 2 show the kinetic constants for hydrolysis of the tested antibiotics by penicillinases and cephalosporinases, respectively. β-Lactam antibiotics with a formylamino substituent attached to the β-lactam ring, cephabacin F_1, 7-FDCPC, and formadicin C, were highly resistant to hydrolysis by both penicillinases and cephalosporinases. Sulfazecin, an *N*-sulfonated monocyclic β-lactam antibiotic with a methoxy substituent was also resistant to β-lactamases. Cephalosporins with a methoxy substituent, cephabacin M_1 and 7-MDCPC, were resistant to the penicillinases and the cephalosporinase

Table 1. Kinetic constants for cephabacins, formadicins, and related antibiotics with penicillinases.

Compound	Kinetic constant for penicillinase from					
	S. aureus 1840			*E. coli* TN713 (TEM-1)		
	Relative Vmax	*Km* (μM)	*Ki* (μM)	Relative Vmax	*Km* (μM)	*Ki* (μM)
Benzylpenicillin	100	8.8	ND[b]	100	20	ND
Cephabacin F_1	<0.01	ND	>200	<0.01	ND	>200
Cephabacin M_1	<0.01	ND	>200	<0.01	ND	>200
Cephabacin H_1	0.18	222	ND	1.27	526	ND
7-FDCPC[a]	<0.01	ND	>200	<0.01	ND	>200
7-MDCPC[a]	<0.01	ND	>200	<0.01	ND	>200
DCPC[a]	0.28	1,350	ND	0.41	400	ND
Formadicin C	<0.01	ND	>200	<0.01	ND	>200
Formadicin D	2.92[c]	>500	ND	6.41[c]	>500	ND
Nocardicin A	<0.01	ND	>200	0.39[c]	>500	>200
Sulfazecin	<0.01	ND	>200	<0.01	ND	>200

[a] 7-FDCPC, 7-formylaminodeacetylcephalosporin C; 7-MDCPC, 7-methoxydeacetylcephalosporin C; DCPC, deacetylcephalosporin C.
[b] ND: Not determined.
[c] Hydrolysis rate at substrate concentration of 500 μM.

Table 2. Kinetic constants for cephabacins, formadicins, and related antibiotics with cephalosporinases.

Compound	Kinetic constant for cephalosporinases from					
	E. cloacae TN1282			*P. vulgaris* GN4413		
	Relative Vmax	*Km* (μM)	*Ki* (μM)	Relative Vmax	*Km* (μM)	*Ki* (μM)
Cephaloridine	100	551	ND[b]	100	118	ND
Cephabacin F_1	<0.01	ND	>200	<0.01	ND	2.1
Cephabacin M_1	20.7	1,000	ND	<0.01	ND	14.8
Cephabacin H_1	75.0	910	ND	44.9	87.0	ND
7-FDCPC[a]	0.02[c]	ND	>200	<0.01	ND	39.1
7-MDCPC	74.1	1,450	ND	<0.01	ND	>200
DCPC	120	400	ND	97.6	167	ND
Formadicin C	<0.01	ND	>200	<0.01	ND	>200
Formadicin D	0.11	400	ND	15.5	280	ND
Nocardicin A	0.02[c]	ND	>200	16.6	280	>200
Sulfazecin	0.01	9.5	4.7	0.08	1,700	>200

[a] See footnote of Table 1.
[b] ND: Not determined.
[c] Hydrolysis rate at substrate concentration of 500 μM.

Table 3. Antibacterial activities of cephabacins, formadicins, and related antibiotics against β-lactamase-producing and -nonproducing bacteria.

Organism[a]	β-Lactamase activity[b] (U/mg d.w.)	MIC (μg/ml)											
		Cephabacins			7-FDCPC[c]	7-MDCPC	DCPC	Formadicins		Nocardicin A	Sulfazecin	Benzylpenicillin	Cephaloridine
		F_1	M_1	H_1				C	D				
S.a. 1840S	<0.01	400	400	100	>800	>800	400	>800	>800	400	800	0.02	0.02
S.a. 1840-2	1.14	400	800	200	>800	>800	800	>800	>800	>800	800	50	0.39
E.c. J53-2	<0.01	3.13	12.5	12.5	50	400	50	100	400	50	50	25	3.13
E.c. J53-2 (RP4)	11.26	6.25	25	50	100	800	100	200	>800	400	50	>800	100
E.cl. CS4495	<0.01	25	100	100	50	200	200	200	>800	200	12.5	12.5	3.13
E.cl. CS4494	4.92	50	>800	>800	100	>800	>800	400	>800	>800	200	>800	800
P.v. CS4035	<0.01	12.5	50	50	25	100	25	25	50	3.13	25	6.25	3.13
P.v. CS4017	2.44	12.5	50	400	25	100	200	25	800	200	50	>800	800

[a] Abbreviations: *S.a.*, *Staphylococcus aureus*; *E.c.*, *Escherichia coli*; *E.cl.*, *Enterobacter cloacae*; *P.v.*, *Proteus vulgaris*.

[b] The activity was determined with 0.2 mM benzylpenicillin for the β-lactamases of *S. aureus* and *E. coli*, and with 0.2 mM cephaloridine for the enzymes of *E. cloacae* and *P. vulgaris*.

[c] See footnotes of Table 1.

of *P. vulgaris*, but were sensitive to the cephalosporinase of *E. cloacae*. Antibiotics without the substituent on the nucleus, cephabacin H_1, DCPC, and formadicin D, were sensitive to the four enzymes. Nocardicin A was more resistant to hydrolysis by the penicillinases and the cephalosporinase of *E. cloacae* than was formadicin D in spite of the structural similarity of the two antibiotics.

Affinity for β-Lactamases

The antibiotics tested in this study had relatively high *Km* and *Ki* values for the β-lactamases, indicating low affinity of the antibiotics for the enzymes (Tables 1 and 2). Only cephabacin F_1 and sulfazecin showed high affinities for the β-lactamases of *P. vulgaris* and *E. cloacae*, respectively. Cefoxitin and cefmetazole, both semisynthetic 7-methoxycephalosporins, have high affinities for the β-lactamases of *E. cloacae*[10], but cephabacin M_1 and 7-MDCPC showed very low affinities for this enzyme. The unique 3-side chains of cephabacins increased the affinities of the antibiotics for the β-lactamase of *P. vulgaris*.

Antibacterial Activity

Table 3 shows the antibacterial activities of the antibiotics against β-lactamase-producing bacteria and their β-lactamase-deficient isogenic strains. β-Lactamase-constitutive strains were used. The antibacterial activities of cephabacin F_1, 7-FDCPC, and formadicin C were as potent against β-lactamase-producers as against nonproducers. Other antibiotics showed higher MICs against producers of the β-lactamase capable of hydrolyzing the antibiotic than against nonproducers (see Tables 1 and 2).

Table 4. Induction of β-lactamases in *E. cloacae* GN5797 and *P. vulgaris* GN4421 by cephabacins, formadicins, and related compounds.

Inducer	β-Lactamase activity (U/mg d.w.)[a] of							
	E. cloacae GN5797 at inducer concentration (μg/ml) of				*P. vulgaris* GN4421 at inducer concentration (μg/ml) of			
	1	10	100	1,000	1	10	100	1,000
None	0.002				0.001			
Cephabacin F_1	0.304	1.108	2.420	1.996	0.078	0.034	0.032	0.048
Cephabacin M_1	0.017	0.051	0.293	1.311	0.101	0.383	0.403	0.502
Cephabacin H_1	0.017	0.160	1.177	ND[b]	0.021	0.339	0.296	ND
7-FDCPC[c]	0.039	0.668	1.488	2.615	0.113	0.342	0.171	0.274
7-MDCPC	0.014	0.015	0.029	0.174	0.004	0.069	0.828	0.762
DCPC	0.025	0.087	0.370	1.297	0.172	0.683	0.870	1.146
Formadicin C	0.018	0.199	1.050	2.865	0.180	1.019	1.602	1.413
Formadicin D	0.013	0.088	0.487	ND	0.005	0.305	0.693	ND
Nocardicin A	0.018	0.062	0.145	0.711	0.008	0.287	0.602	1.000
Sulfazecin	0.400	1.039	1.092	1.381	0.140	0.940	1.327	1.478
Benzylpenicillin	ND	0.002	0.043	1.391	ND	0.369	1.007	1.444
Cefazolin	ND	0.317	1.230	1.491	ND	0.671	1.041	1.356
Cefoxitin	0.055	1.323	2.002	1.269	ND	1.199	1.436	0.789

[a] The β-lactamase of cells growing logarithmically in BHI broth at 30°C was induced by the β-lactam antibiotic for 1 hour, and the enzyme activity was determined microiodometrically with 0.2 mM cephaloridine after dialysis.

[b] ND: Not determined.

[c] See footnotes of Table 1.

Induction of β-Lactamase

Since the semisynthetic 7-methoxycephalosporin analogues, cefoxitin, cefmetazole, cefotetan, and latamoxef, are resistant to hydrolysis by β-lactamases[22~25] and are good inducers of β-lactamases[10,12], we examined the relation between β-lactamase stability and inducer activity of naturally occurring β-lactam antibiotics with or without a substituent (Table 4). In *E. cloacae* GN5797, the inducer activities of formylamino-substituted compounds, cephabacin F_1, 7-FDCPC, and formadicin C, were about 10 times those of the corresponding nonsubstituted compounds, cephabacin H_1, DCPC, and formadicin D, and were comparable to the activity of cefoxitin. Formadicin C showed potent activity for inducing the β-lactamase of *P. vulgaris* GN4421, too. The β-lactamase activity of *P. vulgaris* induced by cephabacin F_1 and 7-FDCPC, which inhibit this enzyme[20], were weak even after extensive dialysis of the enzyme preparations. Sulfazecin showed a potent inducer activity comparable to that of cefoxitin, but contrary to our expectation cephabacin M_1 and 7-MDCPC showed rather weak activities.

Discussion

A formylamino substituent endowed both cephem and monocyclic β-lactam antibiotics with high resistance to hydrolysis by β-lactamases. The ability of the formylamino group to protect β-lactam antibiotics from the attack of β-lactamases was greater than that of the methoxy group; methoxy-substituted β-lactam antibiotics were sensitive to some cephalosporinases. Formadicin D, a compound with a formylamino group on the side chain, was sensitive to β-lactamases, indicating that the substituent must be located on the β-lactam nucleus to protect the antibiotic from the β-lactamase attack. Cephabacin F_1 showed low affinity for the β-lactamases of *S. aureus*, *E. coli*, and *E. cloacae*, but showed high affinity for that of *P. vulgaris*. Moreover, it seemed to inhibit the *P. vulgaris* β-lactamase irreversibly as discussed below. These results suggest that there is more than one mechanism for the resistance of the antibiotic to β-lactamase hydrolysis. The reaction of β-lactamase proceeds through the formation of an enzyme-substrate complex (Michaelis complex), formation of an acyl enzyme intermediate, and deacylation, followed immediately by release of the product from the enzyme[26]. Cephabacin F_1 seems to readily form an acyl intermediate with the *P. vulgaris* β-lactamase. Deacylation may be a rate-limiting step. On the other hand, binding may be a rate-limiting step for the β-lactamases of *S. aureus*, *E. coli*, and *E. cloacae*. Though the stability of the β-lactam antibiotics tested to β-lactamases was directly related to the presence or absence of substituents on the β-lactam nuclei, their affinity for β-lactamases was not. The affinities of β-lactam antibiotics for β-lactamases are determined by the entire structure.

The β-lactamase stabilities of the tested compounds were well reflected in their antibacterial activities against β-lactamase-producing bacteria. The activities of formylamino-substituted compounds were almost the same against both producers and nonproducers of β-lactamase, whereas the activities of methoxy-substituted or nonsubstituted compounds were impaired against some strains producing β-lactamase. Formylamino-substituted compounds were active against *E. cloacae* CS4494 which produced large amounts of β-lactamase constitutively. In this strain, a nonhydrolytic barrier mechanism[13~15] in which antibiotics having affinities for the β-lactamase are prevented from reaching the targets, is considered to be operating. The low affinity for and the high stability to the β-lactamase are responsible for the potent antibacterial activity of these compounds against *E. cloacae* CS4494. The precise characteristics of this strain will be published elsewhere.

Although all of the semisynthetic cephamycin group antibiotics induce β-lactamases well[10,12], the naturally occurring 7-methoxycephalosporins tested in this study induced poorly. This result indicates that the potent β-lactamase-inducing activity does not result from the methoxy group alone but from its association with appropriate side chains. Although the inducer activities of natural com-

pounds are potent, those of formylamino-substituted compounds may be reduced by changing side chains. GOOTZ and SANDERS reported that the inducer activities of β-lactam antibiotics were directly related to their β-lactamase stabilities[27]. This was true when the inducer activities of antibiotics with a formylamino substituent and their nonsubstituted counterparts were compared, but was not true when other antibiotics were included in the comparison. For example, nocardicin A, which was as stable as 7-FDCPC to the *E. cloacae* β-lactamase, was a poorer inducer in this organism than was 7-FDCPC, and 7-MDCPC, which was more stable than DCPC to the *P. vulgaris* β-lactamase, was a poorer inducer than DCPC. Naturally occurring 7-methoxycephalosporins were poorer inducers than semisynthetic ones, though both were equally resistant to hydrolysis by the β-lactamase of *P. vulgaris*. Cephabacin F_1 and 7-FDCPC induced appreciable amounts of the *P. vulgaris* β-lactamase at the lowest concentration but small amounts at higher concentrations. This phenomenon might be due to the β-lactamase inhibitory properties of these compounds. The activities of enzyme preparations of *P. vulgaris* induced by higher concentrations of cephabacin F_1 and 7-FDCPC did not change even after extensive dialysis, suggesting that these compounds inhibited the β-lactamase irreversibly.

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