QUATERNARY HETEROCYCLYLAMINO β-LACTAMS

II. THE *IN VITRO* ANTIBACTERIAL PROPERTIES OF L-640,876, A NEW TYPE OF β-LACTAM ANTIBIOTIC

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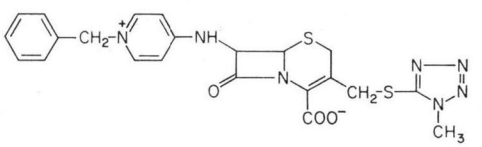
A new semisynthetic cephalosporin antibiotic designated 7-β-(1-benzylpyridinium-4-yl)-amino-3-{[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl}ceph-3-em-4-carboxylate (L-640,876) was compared for antibacterial activity *in vitro* with mecillinam, cefoxitin and cefotaxime. The antibacterial spectrum of L-640,876 and the effect of culture medium composition and inoculum size on activity are most similar to those of mecillinam. In some cases the inoculum effect on MICs correlated with instability of the compound to certain β-lactamases and in others to the presence of ionized compounds such as sodium chloride in the medium. On balance, L-640,876 was superior to mecillinam in potency and breadth of spectrum.

L-640,876 (Fig. 1) is a novel cephalosporin antibiotic belonging to a series of quaternary 7β-heterocyclylaminocephems whose synthesis at the Merck Sharp and Dohme Research Laboratories recently was reported[1]. The new compound most closely resembles mecillinam, a 6β-amidinopenicillanic acid, in that it lacks an acylamino substituent in the 7β-position. The properties of mecillinam such as potent antibacterial activity *in vitro* against *Escherichia coli*[1,2], spectrum limited to certain Gram-negative bacteria[3,4], binding exclusively to penicillin-binding protein 2 and induction of spherical forms in *E. coli*[5] shows that compounds of this type differ fundamentally from other β-lactams. In this paper the results of comparative studies *in vitro* of L-640,876, mecillinam, cefoxitin and cefotaxime are presented. Although L-640,876 and mecillinam share many common characteristics, the new cephalosporin demonstrated a wider spectrum of activity and greater potency against many of the Gram-negative and Gram-positive bacteria tested.

Fig. 1. Chemical structure of L-640,876.



Materials and Methods

Bacterial Cultures

The cultures, other than those specified below, were laboratory strains or recent clinical isolates obtained from a variety of hospitals throughout the United States. The culture of *Escherichia coli* TEM2+, designated MB 4351, was supplied by J. KNOWLES (Harvard University, Cambridge) and those of *Clostridium ramosum* MB 4072, *C. difficile* MB 4073, *Bacteroides distasonis* MB 4361 and *B. asaccharolyticus* MB 4071 were supplied by V. SUTTER (Wadsworth Veterans Administration Hospital Center, Los Angeles). Aerobic cultures were maintained at −70°C on Trypticase soy agar (TSA) slants or in 15% skim milk. Obligate anaerobic cultures were stored at −70°C in brain heart infusion broth (Difco) supplemented with 0.5% yeast extract, 5 μg/ml hemin and 0.5 μg/ml menadione and then diluted 1:1

with skim milk. The aerobic cultures were grown in Trypticase soy broth at 35°C for 18~20 hours and the diluted cultures were used as the inoculum source for antibiotic susceptibility testing. The anaerobic cultures were serially transferred twice in supplemented brain heart infusion broth before final transfer into a formulated thioglycollate broth described by SUTTER *et al.*[6] The anaerobic cultures were routinely incubated for 24 hours at 37°C in an anaerobic chamber charged with 82% nitrogen, 10% hydrogen and 8% carbon dioxide.

Antibiotics

L-640,876 and the sodium salt of cefoxitin were prepared at the Merck Sharp and Dohme Research Laboratories. L-640,876 was synthesized as a golden crystalline product which was stable at room temperature. Mecillinam and cefotaxime were supplied by Leo Pharmaceutical Products (Ballerup, Denmark) and Hoechst-AG (Frankfurt, Germany), respectively. Benzylpenicillin was purchased as the sodium salt from the Upjohn Co. (Kalamazoo).

Minimal Inhibitory Concentration (MIC)

Antimicrobial activity was determined by an agar dilution method. This method utilized a multipoint inoculator which delivered approximately 1 μl of inoculum directly onto agar plates containing two-fold dilutions of the antibiotic. Stock solutions of the test antibiotics were made to contain 1.28 mg/ml and further dilutions were made in distilled water. One ml aliquots of each antibiotic-containing solution were mixed with 9 ml of molten agar in 15×100 mm Petri dishes. When testing aerobic bacteria, inoculum densities of 10^4, 10^5 and 10^6 colony forming units (CFU)/spot, were prepared from overnight cultures. Unless otherwise indicated, the MICs of these cultures were determined on TSA or nutrient agar and were incubated at 35°C for 18 to 20 hours.

The anaerobic bacteria were tested on Wilkins-Chalgren agar (Difco) according to the reference dilution method of SUTTER *et al.*[7] The MIC for each organism was defined as the lowest concentration of antibiotic that showed no visible growth or less than 5 colonies/spot.

β-Lactamase Preparations

Strains of bacteria producing both chromosomally mediated (*Klebsiella oxytoca* MB 4354; *Enterobacter cloacae* MB 2646) and R-plasmid mediated enzymes (*E. coli* MB 4351) were grown aerobically to stationary phase at 37°C in trypticase soy broth. The cells were harvested by centrifugation (11,000×*g*) for 20 minutes at 4°C, resuspended in cold 100 mM tris(hydroxymethyl)aminomethane + 1 mM ethylenediaminetetraacetic acid buffer (pH 8.0), and disrupted at 2°C using a Biosonic III ultrasonic disintegrator fitted with a flow-through cooling chamber (Branson Sonic Power Co., Danbury, Conn.). The cellular debris was removed by centrifugation. The supernatant fluid was filtered through an XM-300 ultrafiltration membrane (Amicon), dialyzed at 4°C for 48 hours against 4 changes of 10 mM sodium phosphate buffer (pH 7.4) and concentrated 100-fold with a PM-10 ultrafiltration membrane. The β-lactamase concentrates were purified by diethylaminoethyl-sephacel column chromatography (Pharmacia, Sweden). The fractions containing enzyme activity were combined, dialyzed against phosphate buffer as previously described and stored in the lyophilized state.

An overnight Trypticase soy broth culture of *Staphylococcus aureus* MB 2868 was diluted 100-fold with fresh medium and 10 μg/ml final concentration of benzylpenicillin was added as an inducer. The culture was incubated at 35°C for 8 hours before centrifugation for 30 minutes. The supernatant fluid was filter-sterilized, dialyzed and concentrated as described above.

β-Lactamase Assay

During purification of the enzyme preparations, the activity of the fractions was determined by the nitrocefin-spectrophotometric procedure of O'CALLAGHAN[8]. The relative susceptibility of various β-lactam antibiotics to these enzymes was measured by a UV-spectrophotometric procedure[9]. The substrate and inhibition profiles were used to classify each enzyme according to the scheme of RICHMOND and SYKES[10].

Osmolality and Specific Conductance

Specific conductivity of liquid media was measured at room temperature with a Beckman RC16Ba conductivity bridge (Beckman Instruments) fitted with a 0.1 cm cell. The osmolality of the nutrient broth together with the various additives was calculated using the measurements reported by NEU[16].

Results and Discussion

The antibacterial spectrum of L-640,876 includes both aerobic and anaerobic bacteria (Tables 1 and 2). Exceptions among the former are *Streptococcus faecalis, Pseudomonas aeruginosa* and *Serratia marcescens*. The potency of this new cephem *in vitro* to *Staphylococcus aureus, Streptococcus pyogenes* and *Escherichia coli* isolates was similar to that of cefotaxime (Table 3) with ninety percent of strains inhibited at concentrations ranging from 0.25~2 μg/ml. The rank order of activity of other aerobic bacteria was cefotaxime>cefoxitin>L-640,876>mecillinam. Against strains of clinically significant anaerobic bacteria L-640,876 was as potent as cefoxitin, more potent than cefotaxime, and its spectrum of activity included strains of *Bacteroides* that were resistant to cefoxitin and cefotaxime (Table 2). The antibacterial activity of L-640,876 against anaerobic bacteria appears to be similar to that reported for mecillinam[11].

During the course of antibacterial testing, it was found that the MICs of L-640,876 against Gram-negative bacteria but not *S. aureus*, were significantly lower (apparent greater activity) when determined using nutrient agar than TSA (Table 4). The 2 to >2,000-fold MIC shifts were similar to those seen with mecillinam against Gram-negative bacteria.

In order to investigate the reason(s) for these MIC shifts, nutrient agar used as a basal medium was supplemented with substances known to selectively affect the specific conductivity and/or osmolality of the culture medium (Table 5). Using representative species from four genera of *Enterobacteriaceae* (*E. coli, Salmonella typhimurium, Klebsiella pneumoniae, Proteus vulgaris*), it was determined that increases in conductivity effected by NaCl, Na_2SO_4 and $MgSO_4$, but not osmolality, adversely influence the antibacterial activity of L-640,876 in a manner similar to that of mecillinam[12,13].

Table 1. Comparison of the antibacterial activity of L-640,876, mecillinam, cefoxitin and cefotaxime against selected strains of aerobic bacteria.

Organism	MIC (μg/ml)[a]			
	L-640,876	Mecillinam	Cefoxitin	Cefotaxime
Staphylococcus aureus MB 2868	16	128	2	1
S. aureus MB 2865	0.25	8	2	0.5
Streptococcus faecalis MB 2864	128	>128	>128	32
Escherichia coli MB 4351	<0.06	32	2	<0.06
E. coli MB 2891	<0.06	<0.06	64	4
Salmonella typhimurium MB 2860	0.5	0.125	2	0.125
Enterobacter cloacae MB 2646	0.5	0.125	>128	128
E. cloacae MB 2647	<0.06	<0.06	16	<0.06
E. aerogenes MB 2828	2	64	>128	0.125
Klebsiella oxytoca MB 4354	16	>128	2	1
K. pneumoniae MB 4005	8	16	2	<0.06
Proteus morganii MB 2833	32	>128	4	0.5
P. vulgaris MB 2829	<0.06	0.5	2	<0.06
P. mirabilis MB 2830	32	>128	4	<0.06
Pseudomonas aeruginosa MB 3350	>128	>128	>128	64
P. aeruginosa MB 2835	>128	>128	>128	16
Serratia marcescens MB 2840	128	>128	32	8

[a] Agar dilution test, inoculum 10^4 CFU/spot, Trypticase soy agar, incubation at 35°C for 18~20 hours.

Table 2. Antibacterial activity of L-640,876, cefoxitin and cefotaxime against selected anaerobic bacteria.

Organism	MIC (μg/ml)[a]		
	L-640,876	Cefoxitin	Cefotaxime
Actinomyces naeslundii MB 4053	2	<0.06	<0.06
Eubacterium limosum MB 3344	4	2	<0.06
Propionibacterium acnes MB 2249	0.125	0.125	<0.06
Peptostreptococcus anaerobius MB 3282	2	<0.06	0.25
Clostridium perfringens ATCC 13124	2	2	0.25
C. ramosum MB 4072	8	4	0.25
C. difficile MB 4073	64	64	64
Bifidobacterium dentium MB 4427	>128	64	16
Bacteroides fragilis MB 4324	8	8	1
B. fragilis MB 3214	16	16	>128
B. fragilis ATCC 25285	4	16	2
B. distasonis MB 4361	8	32	64
B. distasonis MB 3445	128	128	64
B. ovatus MB 3248	8	32	32
B. thetaiotaomicron ATCC 29741	8	64	16
B. thetaiotaomicron MB 4362	64	64	32
B. asaccharolyticus MB 4071	1	<0.06	<0.06
Fusobacterium mortiferum ATCC 25557	32	64	64
Veillonella alcalescens MB 1952	8	1	<0.06

[a] A standardized agar dilution test described by SUTTER *et al.*[7] was used.

Table 3. Susceptibility distribution of clinical isolates of bacteria to L-640,876, mecillinam, cefoxitin and cefotaxime.

Organism	Number of strains	MIC required to inhibit (μg/ml)[a]							
		L-640,876		Mecillinam		Cefoxitin		Cefotaxime	
		50% of strains	90% of strains	50% of strains	90% of strains	50% of strains	90% of strains	50% of strains	90% of strains
S. aureus	20	1	1	64	128	2	2	2	2
S. pyogenes	10	0.06	0.5	1	16	0.5	4	<0.01	0.5
E. coli	30	0.13	0.25	0.25	2	4	8	0.06	0.25
K. pneumoniae	15	4	16	>128	>128	4	16	0.06	0.13
K. oxytoca	15	2	128	>128	>128	4	8	0.06	0.13
Yersinia enterocolitica	10	0.6	4	0.5	>128	4	8	0.03	0.06
Salmonella sp.	10	0.5	8	1	2	2	4	0.13	0.25
Shigella sp.	10	0.5	1	4	>128	4	4	0.06	0.13
P. vulgaris	10	128	>128	>128	>128	8	8	0.5	4
P. mirabilis	5	16	32	128	>128	4	8	0.03	0.06
B. fragilis	33	8	64	NT[b]	NT	16	32	8	>128

[a] Aerobic bacteria: agar dilution test, inoculum 10^5 CFU/spot, Trypticase soy agar, incubation at 35°C for 18~20 hours. *Bacteroides*: agar dilution method of SUTTER *et al.*[7].

[b] NT=Not tested.

Table 4. The effect of culture medium and inoculum density on the antibacterial activity of L-640,876 and mecillinam.

Organism	β-Lactamase[b]	MIC (μg/ml) at inoculum level (CFU)[a]							
		L-640,876				Mecillinam			
		Nutrient agar		Trypticase soy agar		Nutrient agar		Trypticase soy agar	
		10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6
S. aureus MB 2865	−	0.25	0.5	0.25	0.5	16	16	8	8
E. coli MB 2891	+	<0.06	<0.06	<0.06	8	<0.06	0.06	<0.06	>128
S. typhimurium MB 3860	−	0.06	0.125	0.5	16	0.06	0.125	0.125	64
E. cloacae MB 2647	−	0.06	0.125	<0.06	0.125	0.06	0.125	<0.06	0.25
E. aerogenes MB 2828	+	0.125	8	4	>128	0.25	>128	64	>128
K. pneumoniae MB 4005	−	<0.06	0.06	8	32	0.06	0.25	16	>128
P. morganii MB 2833	+	0.06	8	16	>128	8	>128	>128	>128
P. vulgaris MB 2829	+	<0.06	<0.06	0.125	>128	0.06	0.5	0.5	>128
P. mirabilis MB 2830	+	0.06	8	32	>128	64	>128	>128	>128
S. marcescens MB 2840	+	0.5	1	>128	>128	1	>128	>128	>128

[a] CFU=colony-forming units.

[b] β-Lactamase production was determined by the nitrocefin assay of O'CALLAGHAN, *et al.*[8].

Significant MIC shifts as a function of inoculum size were also noted for L-640,876 and mecillinam when tests with Gram-negative organisms were conducted in the same culture medium (Table 4). An analysis of the ability of the organism to produce β-lactamase suggested that these shifts in MICs are related to lack of β-lactamase stability (Table 6) of the two compounds. The rate of hydrolysis of L-640,876 by the β-lactamase from *E. coli* RP4$^+$ was greater than that seen with cefoxitin and cefotaxime and yet the MICs were similar. In this instance it is possible that the greater inhibitory activity of L-640,876 may be due to a low physiological efficiency of the β-lactamase which may be characterized by a *Vmax*/*Km* similar to that of mecillinam[14] or to a greater penetration rate into the periplasm. Mecillinam was the most potent agent against *Enterobacter cloacae* P-99, probably because it was the most resistant of the β-lactams to the β-lactamase produced by the organism. The addition of sodium chloride or magnesium sulfate (final concentration 0.5%) to the β-lactamase reaction mixtures had little or no effect on the specific activity of cell-free preparations; furthermore, the "salt effect" was demonstrated with non β-lactamase producing strains of Gram-negative bacteria. These observations were similar to those of BONGAERTS and BRUGGEMAN-OGLE[15] with mecillinam and indicate that the salt-dependent increase in MIC of both compounds is probably not due to salt-enhanced hydrolysis nor to stimulation of β-lactamase production.

The mechanism by which increased conductivity of the culture medium protects certain Gram-negative bacteria from the antibacterial action of mecillinam or L-640,876 has not been adequately deduced and several reasons for its action may exist. For example, the salt-mediated effect may be due to an alteration in the cell envelope which is a selective diffusion barrier[15,16]. This may explain why this

Table 5. The effect of specific conductance and osmolality of growth medium on the antibacterial activity of L-640,876 and mecillinam.

Medium addition[b] (% w/v)	Specific conductivity[c] (mmho cm^{-1})	Osmolality ($mOsmKg^{-1}$)	MIC (μg/ml)[a]							
			E. coli MB 2891		*S. typhimurium* MB 3860		*K. pneumoniae* MB 4005		*P. vulgaris* MB 2829	
			L-640,876	Mecillinam	L-640,876	Mecillinam	L-640,876	Mecillinam	L-640,876	Mecillinam
None	1.2	38	0.03	0.06	0.5	0.125	0.03	0.25	0.015	0.5
Glycerol (3)	1.1	375	0.03	0.06	1	0.25	0.03	0.25	0.03	0.25
Ethylene glycol (2)	1.2	368	0.03	0.03	0.5	0.125	0.015	0.25	0.015	0.25
NaCl (0.25)	4.7	118	4	2	32	16	8	64	>128	>128
NaCl (1)	13.5	357	>128	>128	>128	>128	>128	>128	>128	>128
$MgSO_4$ (3.5)	11.5	363	>128	>128	>128	>128	>128	>128	>128	>128
Na_2SO_4 (2)	18	364	>128	>128	>128	>128	>128	>128	>128	>128

[a] Agar dilution test, inoculum 10^6 CFU, nutrient agar, incubation at 35°C for 18~20 hours.
[b] Basal medium was nutrient agar.
[c] All conductivity determinations were made at 24°C.

Table 6. Effect of inoculum size and β-lactamase stability on antibacterial activity of L-640,876, mecillinam, cefoxitin and cefotaxime.

Organism	MB	Enzyme[a] class	MIC at inoculum level (CFU)[b]								Relative rate of hydrolysis (%)[c]			
			L-640,876		Mecillinam		Cefoxitin		Cefotaxime					
			10^4	10^6	10^4	10^6	10^4	10^6	10^4	10^6	L-640,876	Mecillinam	Cefoxitin	Cefotaxime
S. aureus	2868	penase[d]	16	16	>128	>128	2	4	0.5	1	0	0.6	0	0
E. coli RP4+	4351	III	0.007	0.03	4	>128	1	2	0.007	0.015	24	42	0.5	3
E. cloacae P-99	2646	I	0.5	2	0.06	0.125	>128	>128	>128	>128	3	0.01	0.5	0.5
K. oxytoca	4354	IV	0.25	>128	8	>128	4	8	4	>128	118	13	0.7	54

[a] Enzyme classification according to RICHMOND and SYKES[10].
[b] Agar dilution test, nutrient agar, incubation at 35°C for 18~20 hours.
[c] Hydrolysis rate relative to that of benzylpenicillin.
[d] penase: Penicillinase.

effect was demonstrated only in Gram-negative microorganisms. Moreover, in the case of L-640,876 the chemical structure was unaffected by 0.6% NaCl in solution based on similarities in NMR spectra of the compound when tested in D_2O in the presence and absence of salt, nor was the compound destroyed as was suggested for mecillinam[18]. Finally, specific binding targets of L-640,876 and mecillinam may be altered by the ionic changes, thus creating a need for a greater β-lactam concentration to inhibit growth. Although L-640,876 and mecillinam are structurally different, their antibacterial properties are strikingly similar. This suggests a similarity in their mode of action. However, our studies have shown that morphological changes in *E. coli* induced by L-640,876 among others were different from those seen with mecillinam. This topic will be the subject of the next report.

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