ANTIBACTERIAL PROPERTIES OF THE BICYCLIC PYRAZOLIDINONES

N. E. ALLEN, J. N. HOBBS, Jr., D. A. PRESTON, J. R. TURNER
and C. Y. E. WU

Lilly Research Laboratories, Eli Lilly and Company,
Indianapolis, Indiana 46285, U.S.A.

(Received for publication July 22, 1989)

LY173013 and LY186826 are bicyclic pyrazolidinones containing a novel aza-γ-lactam ring structure. The antibacterial properties of these compounds appear to be related to those of β-lactam antibiotics in that both classes of compounds share certain common binding molecules such as β-lactamases and penicillin-binding proteins.

Several reports[1~4] from the Lilly Research Laboratories describe the synthesis of bicyclic pyrazolidinone compounds having broad spectrum antibacterial activity. These bicyclic structures resemble familiar β-lactam antibiotics but they contain a novel aza-γ-lactam ring in place of a β-lactam ring. The bicyclic pyrazolidinones LY173013 and LY186826 (Fig. 1) are substituted with an acylamino side chain at the C-7 position[1]. In this study, we have investigated the antibacterial properties of LY173013 and LY186826 and compared the biological activity of bicyclic pyrazolidinones with that of β-lactams.

Materials and Methods

Bacteria

All strains were from the Lilly Research Laboratories culture collection and are described in Fig. 2. Effects on cell wall formation were determined using *Providencia rettgeri* C24 and *Escherichia coli* K-12.

MIC

MICs were determined by agar dilution testing using Mueller-Hinton agar containing 1% Supplement C (Difco). Petri dishes were surface inoculated with 1.5×10^4 cfu.

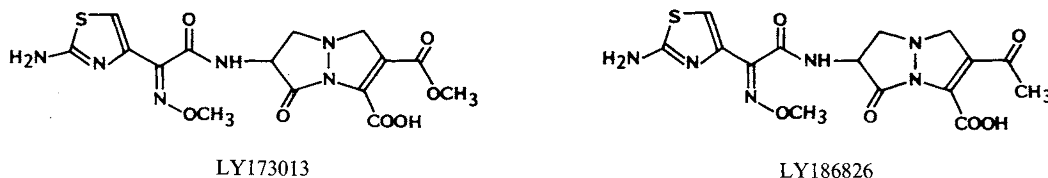
Inactivation by β-Lactamase

Substrate compounds (20 μg) were applied to paper disks and dried. Each disk then received 10 μl of a semi-purified cell-free β-lactamase preparation (prepared as described by MAHONEY *et al.*[5]) and was incubated at 30°C for 24 hours. Inactivation was determined by testing disks for antibacterial activity using *E. coli* ATCC 4157.

Inhibition of β-Lactamase Activity

Inhibition assays were performed as described[5] by incubating serial 5-fold dilutions of the test

Fig. 1. Structures of bicyclic pyrazolidinones.



compound with cell-free preparations of β-lactamases for 10 minutes. Nitrocefin was then added to each of these mixtures. The reciprocal of the highest dilution of test compound allowing β-lactamase activity against nitrocefin was recorded as the end point.

Protein, RNA and DNA Synthesis

Incorporation of ^{14}C-labeled leucine, uracil and thymidine into TCA-insoluble material was measured as described previously[6]. Cells were suspended in phosphate-buffered saline supplemented with 0.1 mM glucose[6].

Cell Wall Synthesis

Incorporation of [^{14}C]diaminopimelic acid into TCA-insoluble material was measured exactly as described[6] in cells suspended in cell wall synthesis medium (CWSM)[7] containing 100 μg/ml chloramphenicol.

Crosslinkage Formation

The extent of crosslinking in *P. rettgeri* following incorporation of [^{14}C]diaminopimelic acid as described above was estimated by two different methods: (i) The ratio of sodium dodecyl sulfate (SDS)-insoluble and TCA-insoluble radioactivity was used to measure crosslinking as described[8,9]. (ii) Extensively washed TCA-insoluble precipitates were dissolved in 5 ml 0.02 M potassium phosphate buffer (pH 6.4) and heated at 90°C for 15 minutes (to destroy autolytic enzymes). This was followed by treatment with 300 μg of *N*-acetylmuramidase (*Streptomyces globisorius* 1829; Miles Laboratories) for 18 hours at 37°C. Muramidase was then removed by extracting with chloroform. The hydrolysates (containing crosslinked and uncrosslinked cell wall fragments) were lyophilized, suspended in water and chromatographed on Whatman 3MM paper for 72 hours in butanol - glacial acetic acid - water (4 : 1 : 5). Radioactivity was located by autoradiography using a Packard Radioactive Strip Scanner and areas corresponding to peptidoglycan monomers and dimers[10,11] were cut, eluted and counted.

Alanine Release

DD-Carboxypeptidase and transpeptidase activities were estimated by measuring release of [^{14}C]alanine from UDP-*N*-acetylmuramyl-[^{14}C]alanine-pentapeptide (*C*-terminal labeled; prepared as described[10]). Release of [^{14}C]alanine was measured using ether-permeabilized cells following the procedures of MIRELMAN *et al.*[10]. Free [^{14}C]alanine was determined using Dowex cation exchange chromatography of TCA-soluble material.

Penicillin-binding Protein (PBP) Assays

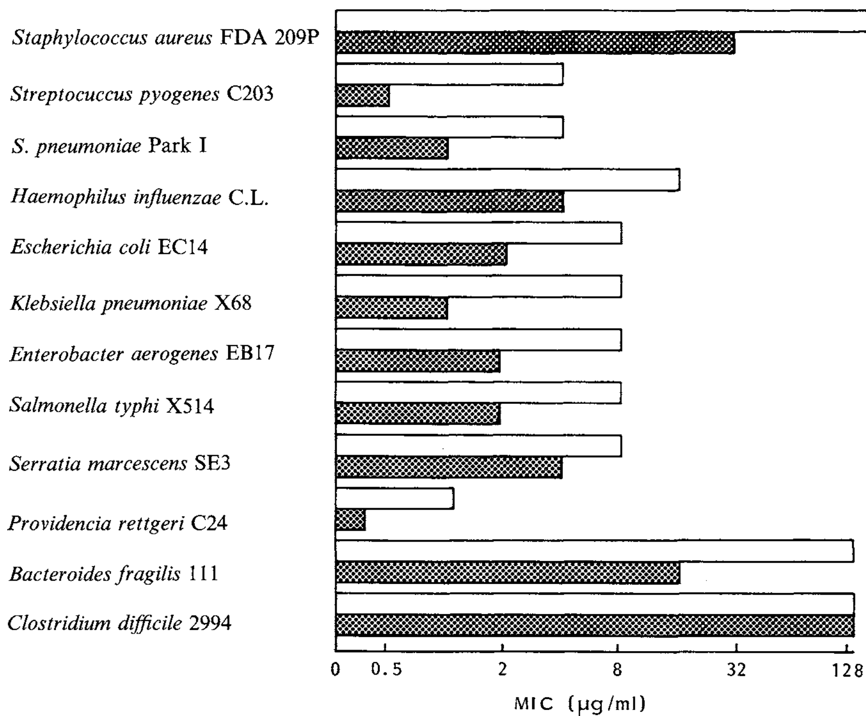
Cells were grown in brain-heart infusion (*P. rettgeri*) or antibiotic medium No. 3 (*E. coli*). Cells were broken in an X-Press (Bios) and membranes were purified by discontinuous sucrose gradient centrifugation. Binding specificity of LY186826 at various concentrations was determined by competition with [^{3}H]benzylpenicillin according to the method of SPRATT[12]. PBPs were separated by SDS-polyacrylamide gel electrophoresis and detected by fluorography.

Results

LY173013 and LY186826 (Fig. 1) contain an aminothiazole methoximino acetic acid side chain at the C-7 position. These two compounds differ with respect to the substitution at the C-3 position. LY173013 contains a carboxymethyl at C-3 whereas LY186826 has an acetyl group at this position. Both compounds inhibited growth of a variety of Gram-positive and Gram-negative bacteria (Fig. 2). LY186826 was more active than LY173013, as indicated by the lower MIC values.

LY173013 and LY186826 were inactivated by β-lactamases (Table 1); however, only LY186826 was inactivated by the *Enterobacter* enzyme. This is consistent with the observation that LY186826 was more active against a non β-lactamase producing *Enterobacter* than against a β-lactamase-producing strain (data

Fig. 2. MICs of LY173013 (open) and LY186826 (closed).



not shown). LY173013 did not show this differential activity. Furthermore, cefoxitin induced resistance in *Enterobacter* to LY186826 but did not induce resistance to LY173013 (unpublished observations). The data in Table 2 show that LY186826 but not LY173013 is an inhibitor of the Ia enzyme from *Enterobacter*. Since LY173013 is neither a substrate for nor an inhibitor of the *Enterobacter* β-lactamase, a critical factor for recognition of the pyrazolidinones by this enzyme appears to reside in the substitution at the C-3 position.

Table 1. Inactivation by β-lactamases.

Enzyme	Inactivation[a]	
	LY173013	LY186826
Ia (*Enterobacter cloacae* 265A)	−	+
Id (*Pseudomonas aeruginosa* PS185)	−	−
IIIa (*Escherichia coli* W3110 R$^+$)	−	−
IVc (*Klebsiella pneumoniae* 1082E)	+	+
I and II (*Bacillus cereus* 569H)	+	+

[a] Loss of antibacterial activity (see Materials and Methods).

P. rettgeri was used to examine the mechanism of action of LY186826. This compound inhibited the incorporation of [^{14}C]diaminopimelic acid into cell wall, but had no dose-related effect on the incorporation of radiolabeled precursors into RNA, DNA or protein in this organism (Table 3). The fact that LY186826 and the β-lactam cefamandole were relatively weak inhibitors in this system necessitated the use of higher concentrations of inhibitors in subsequent experiments using *P. rettgeri*.

By two different methods, LY186826 was shown to affect the formation of crosslinkages in cell wall. LY186826 reduced the ratio of SDS/TCA-insoluble incorporation of [^{14}C]diaminopimelic acid into cell wall (Table 4). LY186826 also reduced the extent of crosslinkage formation when the amount of crosslinked fragments was compared with the amount of uncrosslinked fragments by chromatography following hydrolysis of cell wall (Table 5). Cefamandole had a similar effect in both experiments, and inhibition

Table 2. Inhibition of β-lactamase activity[a].

Compound	Enzyme type[b]				
	Ia	Id	IIIa	IVc	*Staphylococcus aureus*
LY173013	5	5	5	5	5
LY186826	15,625	125	5	625	5
Cefotaxime	15,625	625	5	5	5

[a] Values are reciprocals of highest dilution showing β-lactamase activity (see Materials and Methods).
[b] Enzyme sources given in Table 1; *S. aureus* enzyme from strain 13136.

Table 3. Effect of LY186826 on macromolecular biosynthesis in *Providencia rettgeri*.

Antibiotic	Inhibition (%)[a]															
	Cell wall				Protein				RNA				DNA			
	500	100	10	1	500	100	10	1	500	100	10	1	500	100	10	1
LY186826	80	54	38	36	<0	<0	5	<0	<0	33	13	<0	<0	<0	<0	<0
Cefamandole	68	27	<0	6												
Chloramphenicol					98	88	48	6								
Rifampicin									79	68	54	43				
Nalidixic acid													100	94	75	57

Compounds were tested at 500, 100, 10 and 1 μg/ml.
[a] <0: Stimulation.

Table 4. Effects of LY186826 on crosslinkage formation[a].

Compound	Concentration (μg/ml)	SDS-Insoluble incorporation		TCA-Insoluble incorporation		SDS/TCA
		dpm	Inhibition (%)	dpm	Inhibition (%)	
None	—	11,157	—	12,073	—	0.92
LY186826	250	2,004	82	3,668	69	0.54
	50	5,527	50	7,994	34	0.69
Cefamandole	25	5,302	52	8,393	30	0.63
	5	6,474	42	9,892	18	0.65

[a] Effects on crosslinkage formation were estimated by measuring SDS-insoluble and TCA-insoluble incorporation of [^{14}C]diaminopimelic acid (Table 3) and comparing SDS/TCA incorporation ratios (see text).

Table 5. Effect of LY186826 on percent crosslinkages.

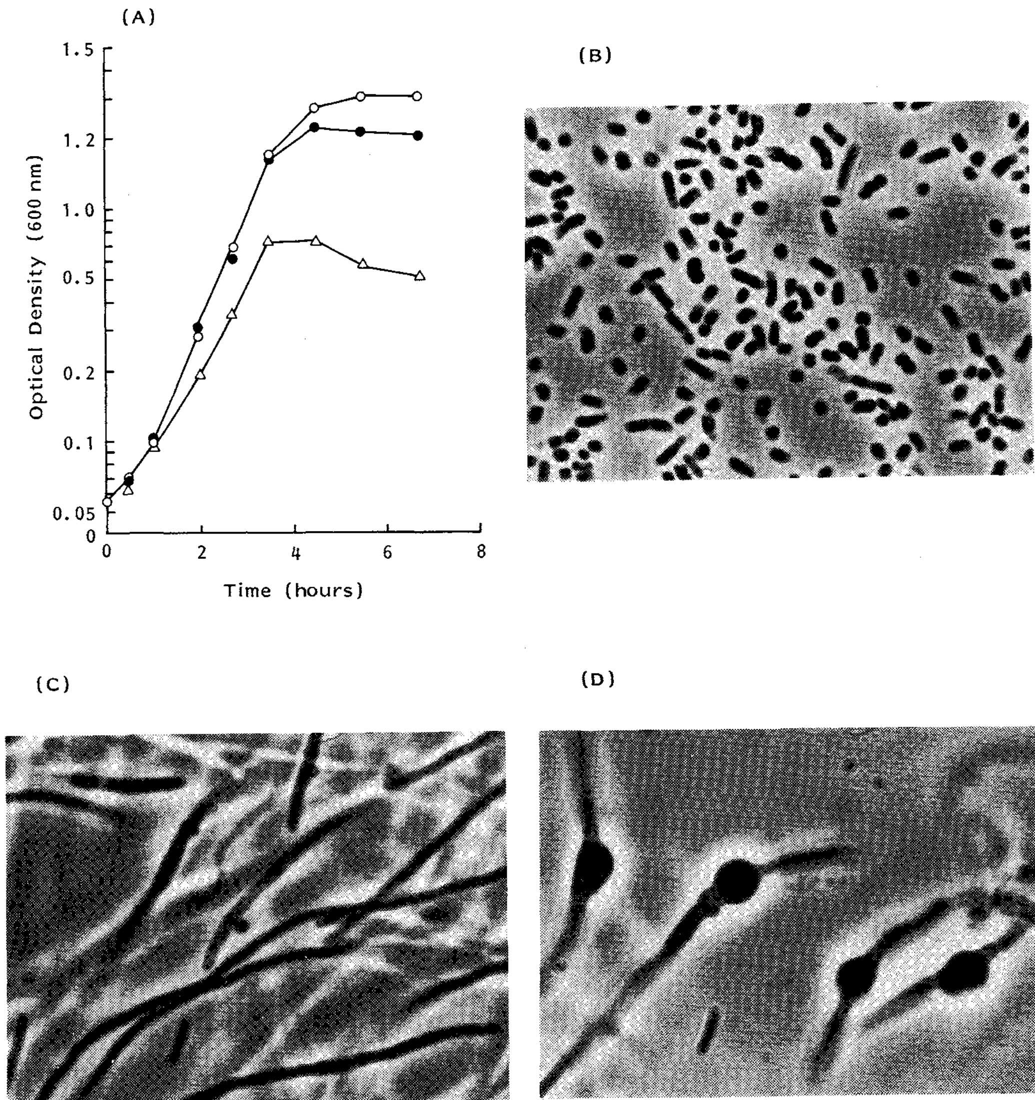
Compound	Concentration (μg/ml)	Radioactivity (dpm) in cell wall fragments[a]		Percent crosslinkages[b]
		Crosslinked	Uncrosslinked	
None	0	2,368	9,517	20
LY186826	500	525	6,363	8
	100	2,952	10,150	23
Cefamandole	500	1,051	6,511	14
	100	3,333	9,632	26

[a] Cell wall was hydrolyzed and crosslinked and uncrosslinked fragments were quantitated by paper chromatography as described in Materials and Methods.
[b] Calculated as: $\frac{\text{dpm in crosslinked fragments}}{\text{Total dpm}} \times 100$

Fig. 3. Effects of LY186826 on cell morphology.

(A) Optical density of *Providencia rettgeri* grown in trypticase soy broth in the presence (● 10 μg/ml, △ 100 μg/ml) and absence (○) of LY186826.

Photomicrographs of cells exposed for 4 hours to (B) 0 μg/ml, (C) 10 μg/ml, and (D) 100 μg/ml LY186826.



required high concentrations of both inhibitors. The fact that SDS/TCA ratios (Table 4) were affected by antibiotic concentrations lower than those required to inhibit total crosslinkages estimated chromatographically (Table 5) may be due to a preferential inhibition of crosslinks involved in attaching newly synthesized peptidoglycan polymer to preexisting wall[11]. The findings are consistent with inhibition of crosslinkage formation[8~11].

Ether-permeabilized cells of *P. rettgeri* were used to measure inhibition of release of [^{14}C]alanine from UDP-*N*-acetylmuramyl-[^{14}C]alanine-pentapeptide by LY186826 (Table 6). LY186826 inhibited alanine

Table 6. Effect of LY186826 on [^{14}C]alanine release[a].

Compound	Concentration (μg/ml)	−UDP-GlcNAc		+UDP-GlcNAc		Difference[d]	Inhibition (%)
		pmol[b]	Inhibition (%)	pmol[c]	Inhibition (%)		
None	—	2,231	—	2,940	—	709	—
LY186826	500	1,148	49	1,445	51	297	58
	100	1,222	45	1,588	46	366	48
	10	1,744	22	2,230	24	484	32
Cefamandole	10	1,860	17	2,096	29	236	67

[a] See Materials and Methods.
[b] Estimate of DD-carboxypeptidase activity.
[c] Estimate of DD-carboxypeptidase + transpeptidase activities.
[d] Estimate of transpeptidase activity; Difference = c − b.

Fig. 4. Binding of LY186826 to PBPs of *Providencia rettgeri*.

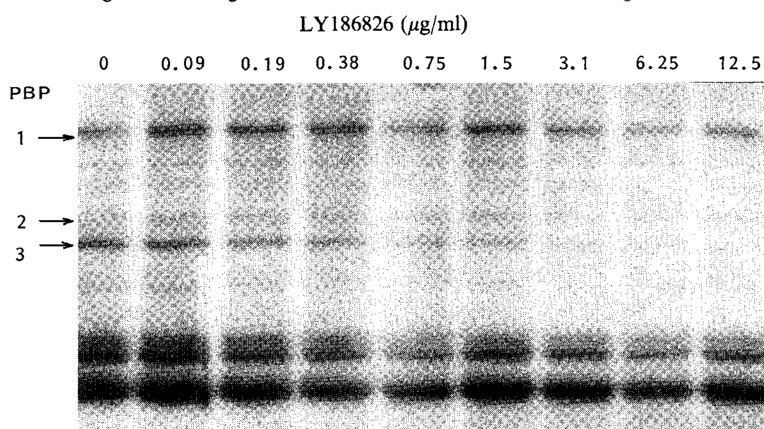
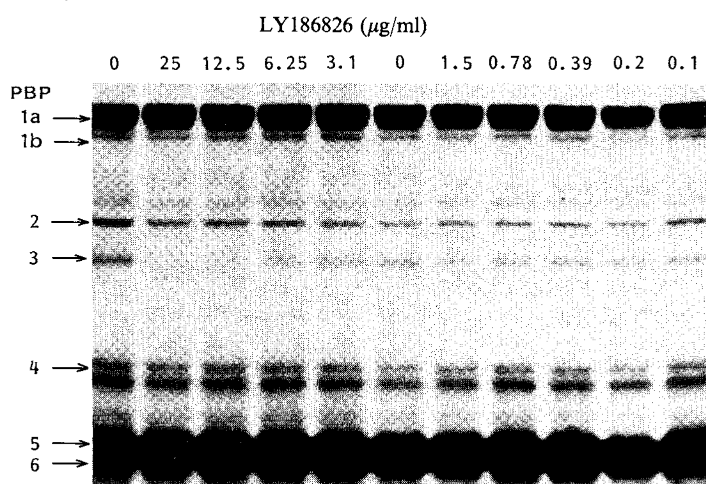


Fig. 5. Binding of LY186826 to PBPs of *Escherichia coli*.



(i) PBP 1b is not visible in the lane corresponding to 0.2 μg/ml LY186826 because less protein was added to this lane. A faint band was visible in the original gel.

(ii) Two controls without LY186826 were included in this experiment: One at the far left and one in the center lane.

release in the presence and absence of added UDP-*N*-acetylglucosamine (UDP-GlcNAc). Alanine released in the absence of UDP-GlcNAc estimates DD-carboxypeptidase activity whereas alanine released when UDP-GlcNAc is present estimates DD-carboxypeptidase plus transpeptidase activities[10]. The results in Table 6 suggest that LY186826 may inhibit both enzymes.

Exposoure of *P. rettgeri* to 10 μg/ml LY186826 resulted in the formation of long filaments (Fig. 3C) with little change in optical density (Fig. 3A). At 100 μg/ml, cells appeared as shorter filaments with bulges (Fig. 3D). Filament formation implies an effect on elongation and possible interaction with PBP 3[13]. In PBP binding experiments, LY186826 competed with [^{3}H]benzylpenicillin for binding to PBP 3 in membranes isolated from *P. rettgeri* (Fig. 4). Binding to PBP 2 could not be completely ruled out in this organism; however, LY186826 clearly showed preferential binding to PBP 3 in *E. coli* membranes (Fig. 5).

Discussion

The bicyclic pyrazolidinones LY173013 and LY186826 share structural similarities with many β-lactam antibiotics. For example, these agents contain a bicyclic ring system, have a lactam bond and carry an aminothiazole methoximino acetic acid side chain. These structural features are characteristic of many β-lactam compounds[14]. The novel feature of LY173013 and LY186826 is the presence of an aza-γ-lactam ring instead of a classic β-lactam ring.

In spite of the fact that β-lactam antibiotics comprise a wide array of structural modifications, they all share the ability to acylate certain cytoplasmic membrane proteins and inhibit cell wall assembly[15]. However, the structural diversity in this class of compounds has been limited to modifications in the fused secondary ring and side chain substitutions. Until now, expansion of the β-lactam ring by one atom to produce a γ-lactam has resulted in biologically inactive molecules[1]. The apparently unique interaction between the aza-γ-lactam nucleus and the appended substituents in the bicyclic pyrazolidinones provided impetus to compare the mechanisms of antibacterial activity of these compounds with those of the penicillins, cephalosporins and other β-lactam antibiotics.

The study reported here did not reveal any major qualitative differences between β-lactam and γ-lactam antibacterials with respect to their mechanisms of action. LY173013 and LY186826 have broad spectrum antibacterial activity. Both were susceptible to inactivation by β-lactamases and LY186826 inhibited the Ia enzyme from *Enterobacter* to the same extent as did cefotaxime. LY186826 inhibited the incorporation of radiolabeled diaminopimelic acid into peptidoglycan by interfering with enzymes that catalyze crosslinkage formation. Relatively high concentrations of LY186826 were required for inhibition in these *in vitro* systems, but the same was found to be true for cefamandole. LY186826 was competitive with benzylpenicillin for binding to PBPs and showed a strong preference for PBP 3 which was consistent with the observation of filament formation[13]. Other studies[16] have shown that LY173013 binds to PBP 3 of *E. coli.*

NOZAKI *et al.*[17,18] have described a natural product lactam-containing antibacterial agent (lactivicin) that binds to PBPs yet lacks a classic β-lactam ring. These reports and the findings from the present study suggest that the lactam bond itself, rather than the β-lactam ring, may be the common structural feature that determines the common mechanism of action exhibited by all of these compounds[19].

Acknowledgments

We thank M. ANDONOV-ROLAND, L. HATFIELD, L. JUNGHEIM and F. MERTZ for assistance and encouragement with this work.

References

1) JUNGHEIM, L. N. & S. K. SIGMUND: 1,3-Dipolar cycloaddition reactions of pyrozolidinium ylides with acetylenes. Synthesis of a new class of antibacterial agents. J. Org. Chem. 52: 4007∼4013, 1987
2) JUNGHEIM, L. N.; S. K. SIGMUND & J. W. FISHER: Bicyclic pyrazolidinone, a new class of antibacterial agent based

on the β-lactam model. Tetrahedron Lett. 28: 285~288, 1987

3) JUNGHEIM, L. N.; S. K. SIGMUND, N. D. JONES & J. K. SWARTZENDRUBER: Bicyclic pyrazolidinones, steric and electronic effects on antibacterial activity. Tetrahedron Lett. 28: 289~292, 1987
4) TERNANSKY, R. J.; S. E. DRAHEIM, A. J. PIKE & J. L. OTT: Pyrazolidinone antimicrobial agents. Synthesis and in vitro activity of new, highly potent analogues. Program and Abstracts of the 27th Intersci. Conf. on Antimicrob. Agents Chemother., No. 1213, p. 309, New York, Oct. 4~7, 1987
5) MAHONEY, D. F.; G. A. KOPPEL & J. R. TURNER: Substrate inhibition of beta-lactamases, a method for predicting enzymatic stability of cephalosporins. Antimicrob. Agents Chemother. 10: 470~475, 1976
6) ALLEN, N. E.; J. N. HOBBS, Jr. & W. E. ALBORN, Jr.: Inhibition of peptidoglycan biosynthesis in gram-positive bacteria by LY146032. Antimicrob. Agents Chemother. 31: 1093~1099, 1987
7) LUGTENBERG, E. J. J. & P. B. DEHAAN: A simple method for following the fate of alanine-containing components in murein synthesis in *Escherichia coli.* Antonie van Leeuwenhoek 37: 537~552, 1971
8) MIRELMAN, D. & Y. NUCHAMOWITZ: Biosynthesis of peptidoglycan in *Pseudomonas aeruginosa*. 1. The incorporation of peptidoglycan into the cell wall. Eur. J. Biochem. 94: 541~548, 1979
9) ZAFRIRI, D.; E. ROSENBERG & D. MIRELMAN: Mode of action of *Myxococcus xanthus* antibiotic TA. Antimicrob. Agents Chemother. 19: 349~351, 1981
10) MIRELMAN, D.; Y. YASHOUV-GAN & U. SCHWARZ: Peptidoglycan biosynthesis in a thermosensitive division mutant of *Escherichia coli.* Biochemistry 15: 1781~1790, 1976
11) MIRELMAN, D.; Y. YASHOUV-GAN & U. SCHWARZ: Regulation of murein biosynthesis and septum formation in filamentous cells of *Escherichia coli* PAT 84. J. Bacteriol. 129: 1593~1600, 1977
12) SPRATT, B. G.: Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. 72: 341~352, 1977
13) SPRATT, B. G.: Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. 72: 2999~3003, 1975
14) NEU, H. C.: β-Lactam antibiotics: Structural relationships affecting *in vitro* activity and pharmacologic properties. Rev. Infect. Dis. 8: 5237~5259, 1986
15) WAXMAN, D. J. & J. L. STROMINGER: β-Lactam antibiotics: Biochemical modes of action. *In* Chemistry and Biology of β-Lactam Antibiotics. Vol. 3, Biochemistry. *Eds.*, R. B. MORIN & M. GORMAN, pp. 209~285, Academic Press, 1982
16) WU, C. Y. E.; W. E. ALBORN, Jr., J. E. FLOKOWITSCH & D. A. PRESTON: Studies of penicillin binding protein (PBP) affinities and beta-lactamase stabilities of bicyclic pyrazolidinones. Program and Abstracts of the 27th Intersci. Conf. on Antimicrob. Agents Chemother., No. 1215, p. 309, New York, Oct. 4~7, 1987
17) NOZAKI, Y.; N. KATAYAMA, H. ONO, S. TSUBOTANI, S. HARADA, H. OKAZAKI & Y. NAKAO: Binding of a non-β-lactam antibiotic to penicillin-binding proteins. Nature 325: 179~180, 1987
18) NOZAKI, Y.; N. KATAYAMA, S. HARADA, H. ONO & H. OKAZAKI: Lactivicin, a naturally occurring non-β-lactam antibiotic having β-lactam-like action: Biological activities and mode of action. J. Antibiotics 42: 84~93, 1989
19) ALLEN, N. E.; D. B. BOYD, J. B. CAMPBELL, J. B. DEETER, T. K. ELZEY, B. J. FOSTER, L. D. HATFIELD, J. N. HOBBS, Jr., W. J. HORNBACK, D. C. HUNDEN, N. D. JONES, M. D. KINNICK, J. M. MORIN, Jr., J. E. MUNROE, J. K. SWARTZENDRUBER & D. G. VOGT: Molecular modeling of γ-lactam analogues of β-lactam antibacterial agents: Synthesis and biological evaluation of selected penem and carbapenem analogues. Tetrahedron 45: 1905~1928, 1989