LACTIVICIN, A NATURALLY OCCURRING NON-β-LACTAM ANTIBIOTIC HAVING β-LACTAM-LIKE ACTION: BIOLOGICAL ACTIVITIES AND MODE OF ACTION

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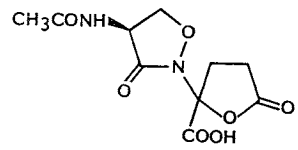
Lactivicin is moderately active against a wide range of Gram-negative bacteria and highly active against Gram-positive bacteria. It shows various biological activities commonly observed with β-lactam antibiotics, such as higher activity against β-lactam hypersensitive mutants than against their parents, sensitivity to β-lactamases, inhibitory activity against β-lactamases and ability to induce β-lactamase activity. The primary lethal target of lactivicin in *Escherichia coli* is highly likely to be penicillin-binding protein (PBP) 1; lactivicin strongly lysed *E. coli* cells with induction of spheroplasts at its MIC, and showed high affinity for PBPs 1A and 1B. At concentrations above×5 MIC, however, lactivicin dominantly exhibited secondary antibacterial action possibly owing to inhibition of crucial SH proteins engaged in the fundamental membrane functions. In contrast, against *Bacillus subtilis*, lactivicin showed the typical β-lactam action under a wide range of concentrations. It showed high affinity for PBPs 1, 2 and 4, the possible lethal targets of β-lactam antibiotics in this organism. In conclusion, lactivicin is the first non-β-lactam antibiotic showing β-lactam action through binding to PBPs.

In previous papers,[1~3] we briefly reported on the producing organisms, the fermentation, isolation and structure elucidation of the novel non-β-lactam antibiotic, lactivicin (Fig. 1), detected in our screening system using β-lactam hypersensitive mutants derived from two Gram-negative bacteria.[4,5] This paper deals with biological activities of lactivicin and its mode of action against *Escherichia coli* and *Bacillus subtilis*.

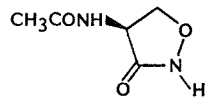
Here we report that lactivicin exhibits biological activities believed to be specific to β-lactam antibiotics, although it does not have a β-lactam ring in its molecule.[3] Furthermore, lactivicin shows the same mode of action through binding to penicillin-binding proteins (PBPs) in microorganisms as do β-lactam antibiotics.

Lactivicin is the first non-β-lactam antibiotic having affinity for microbial PBPs. The discovery of this compound is a breakthrough in that it shows the β-lactam nucleus to be dispensable for exerting β-lactam action.

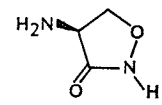
Fig. 1. Structures of lactivicin and D-cycloserine analogues.



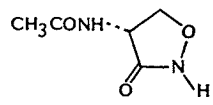
Lactivicin



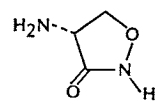
N-Acetyl-L-cycloserine



L-Cycloserine



N-Acetyl-D-cycloserine



D-Cycloserine

Materials and Methods

MIC Determination

MICs were determined by the conventional agar dilution method with DYAB agar[6] for aerobic bacteria and GAM agar (Nissui) for anaerobic bacteria.

Assay of Stability to β-Lactamases

Stability to β-lactamases was assayed on nutrient agar plates seeded with *E. coli* PG-8[4] in the presence or absence of a β-lactamase. Enzymes of *Bacillus cereus* and *E. coli* 205 TEM R^+ (566) are products of Calbiochem Co. (U.S.A.) and Boehringer Mannheim Co. (Germany), respectively. Other enzymes were partially purified from cell lysates as described previously.[7]

Assay of β-Lactamase Inhibitory Activity

β-Lactamase inhibitory activity was determined as described previously.[7] The concentration giving 50% inhibition (I_{50}) was determined from a plot of percentage inhibition against antibiotic concentration.

Assay of Inhibitory Activity against Macromolecular Synthesis

Cells of *E. coli* LD-2, a lysine and diaminopimelic acid (DAP) auxotroph,[4,6] were grown in DYAB medium overnight without shaking, spun down, washed twice with S medium (described below) lacking leucine and DAP, and suspended in S medium to an extinction of 0.5, measured at 600 nm with a Spectronic 20 colorimeter (Shimadzu Baush & Lomb). An incubation mixture, consisting of 0.4 ml of the above cell suspension; 0.05 ml of [*methyl*-^{3}H]thymidine solution (25 Ci/mmol, 100 μCi/ml), [2-^{14}C]uridine solution (51 mCi/mmol), 10 μCi/ml), L-[*U*-^{14}C]leucine solution (10 mCi/mmol, 10 μCi/ml), or [*G*-^{3}H]DAP solution (1 Ci/mmol, 100 μCi/ml); and 0.05 ml of an antibiotic solution, was incubated at 37°C for 1 hour without shaking. After 0.1 ml of 30% TCA was added, the mixture stood for 1 hour in an ice bath. In the experiment to incorporate leucine and DAP, the mixture was further heated at 90°C for 10 minutes. Acid-insoluble material was spun down, washed once with 0.5 ml of 5% TCA, and suspended with 0.5 ml of 0.1 M ammonium acetate. Its radioactivity was counted in a liquid scintillation spectrometer. S medium contained glucose 2 g, sodium glutamate 1 g, sodium citrate 0.5 g, $(NH_4)_2SO_4$ 1 g, K_2HPO_4 7 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, valine 68 mg, isoleucine 60 mg, methionine 10 mg, proline 110 mg, threonine 29 mg, lysine 45 mg, leucine 88 mg, DAP 50 mg and 1 ml of a vitamin mixture in 1 liter of distilled water. Leucine and DAP were omitted from S medium in experiments to incorporate the labeled compounds. The vitamin mixture consisted of 0.5 mg/ml each of vitamins B_1, B_2, B_6, B_{12}, nicotinic acid, folic acid, choline chloride, *p*-aminobenzoic acid, calcium pantothenate, inositol and biotin. When 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) was added to the incubation mixture, the inhibitory activity was corrected for the effects of these reagents.

Effect on Growth, and Observation of Morphological Changes of Cells

Growth of *E. coli* LD-2 and *B. subtilis* PCI 219 at 37°C was monitored with an automatic growth recorder BIO-LOG II (Jasco Jouan). An antibiotic was added at 1.75 hours for *E. coli* LD-2 and at 3 hours for *B. subtilis* PCI 219. Morphology of the cells was observed by phase-contrast microscopy.

Assay of Inhibitory Activity for Peptidoglycan Synthesis

Peptidoglycan synthesis in *B. subtilis* PCI 219 was assayed by incorporating [*G*-^{3}H]DAP (281 mCi/mmol, 214 μCi/ml) into hot-TCA-insoluble fractions for 2 hours as described previously.[6,8]

Assay of Affinity for PBPs

Affinity for PBPs in *E. coli* LD-2 and *B. subtilis* PCI 219 was assayed by competitive binding with [^{14}C]benzylpenicillin as described previously.[6,8] Briefly, after binding a saturating concentration of [^{14}C]benzylpenicillin to residual PBPs, membrane fractions were solubilized and fractionated by the gel system described previously.[8] The PBPs were quantitated with a TLC scanner (Shimadzu CS910) by densitometry of X-ray films developed from the dried gels. The binding affinity of lactivicin for each PBP is expressed as IC_{50}, a concentration required to reduce binding of [^{14}C]benzylpenicillin by 50%.

Chemicals and Enzymes

All isotopes were products of Amersham Co. Lactivicin and patulin were fermentation products purified in our laboratories. *N*-Acetyl D- and L-cycloserine were prepared in our laboratories. 2-ME, DTT and *N*-ethylmaleimide were products of Wako Pure Chemical Industries, Ltd. (Japan). D- and L-cycloserine and papain (E.C. 3.4.22.2) were products of Sigma Chemical Company (U.S.A.). Ficin (E.C. 3.4.22.3) was a product of Tokyo Kasei (Japan).

Results

Biological Activities of Lactivicin

Lactivicin showed antibacterial activity against a wide range of bacteria but not against some β-lactamase producers (Table 1). Since the partial structure of lactivicin is similar to the structure of D-cycloserine, we also examined antibacterial activity of some D-cycloserine analogues (Fig. 1). As shown in Table 1, *N*-acetyl L- or D-cycloserine, and L-cycloserine were not active against the bacteria tested whereas D-cycloserine showed broad-spectrum activity. However, the antibacterial activity of D-cycloserine was diminished by D-alanine[9] whereas that of lactivicin was not (data not shown).

Table 1. Antibacterial activity of lactivicin and D-cycloserine analogues.

Organism	MIC (μg/ml) at 10^6 cfu/ml				
	Lacti-vicin	Acetyl L-CS	Acetyl D-CS	L-CS	D-CS
Escherichia coli NIHJ JC-2	50	>100	>100	>100	25
E. coli LD-2[a]	50	>100	>100	>100	12.5
E. coli CPC-20[a]	50	>100	>100	>100	25
E. coli PG-12[a]	50	>100	>100	>100	25
E. coli PG-8[a]	0.78	>100	>100	>100	12.5
Salmonella typhimurium IFO 12529	50	>100	>100	>100	25
Citrobacter freundii IFO 12681	100	>100	>100	>100	50
Klebsiella pneumoniae IFO 3317	100	>100	>100	>100	100
Enterobacter cloacae IFO 12937	>100	>100	>100	>100	25
Serratia marcescens IFO 12648	50	>100	>100	>100	>100
Proteus mirabilis ATCC 21100	25	>100	>100	>100	100
P. vulgaris IFO 3988	25	>100	>100	>100	100
Morganella morganii IFO 3168	100	>100	>100	>100	>100
Pseudomonas aeruginosa IFO 3080	>100	>100	>100	>100	100
P. aeruginosa C 141[b]	0.2	>100	>100	>100	100
Alcaligenes faecalis IFO 13111	50	>100	>100	>100	>100
Acinetobacter calcoaceticus IFO 12552	50	>100	>100	>100	>100
Staphylococcus aureus FDA 209P	3.13	>100	>100	>100	12.5
Micrococcus luteus IFO 12708	0.39	>100	>100	>100	25
Bacillus subtilis PCI 219	3.13	>100	>100	>100	12.5
Bacteroides fragilis ATCC 2509[c]	12.5	ND	ND	ND	ND
Fusobacterium necrophorum VPI 2891[c]	0.78	ND	ND	ND	ND
Clostridium perfringens PB6K[c]	6.25	ND	ND	ND	ND
Peptostreptococcus anaerobius B-40[c]	3.13	ND	ND	ND	ND

[a] *E. coli* CPC-20 derived from strain LD-2 lacks *amp C* β-lactamase.[4] *E. coli* PG-12 and PG-8 derived from strain CPC-20 are a permeability mutant and a mutant missing PBP 1B, respectively.[4]

[b] *P. aeruginosa* C 141[10] is a β-lactam hypersensitive mutant derived from strain Css[5] originated from strain IFO 3080.

[c] These anaerobic bacteria were grown in a Gas Pak system (BBL).

CS: Cycloserine.

ND: Not determined.

Table 2. Stability of lactivicin to β-lactamases.

Source of enzyme	u/ml	Type of enzyme[a]	Lactivicin (μg/ml)	
			100	1,000
Bacillus cereus	5×10^{-2}	PCase	100	100
Enterobacter cloacae IFO 12937	1×10^{-3}	CPase	0	3
Proteus vulgaris GN 4413	1×10^{-2}	CPase	68	80
Escherichia coli TEM R^+ (566)	1×10^{-3}	CPase	30	30
Pseudomonas aeruginosa U 31	1×10^{-3}	CPase	0	2

Figures in the table are residual lactivicin activity (%).
[a] PCase: Penicillinase, CPase: cephalosporinase.

Table 3. β-Lactamase inhibitory activity of lactivicin and two β-lactam antibiotics.

Compound	I_{50} (μg/ml)[a]			
	Penicillinase		Cephalosporinase	
	Staphylococcus aureus 1840	*Escherichia coli* TN 713	*Enterobacter cloacae* TN 1282	*Proteus vulgaris* GN 4413
Lactivicin	5.1	>100	52	2.4
Deacetylcephalosporin C	>100	>100	66	78
Clavulanic acid	0.04	0.016	>5	0.045

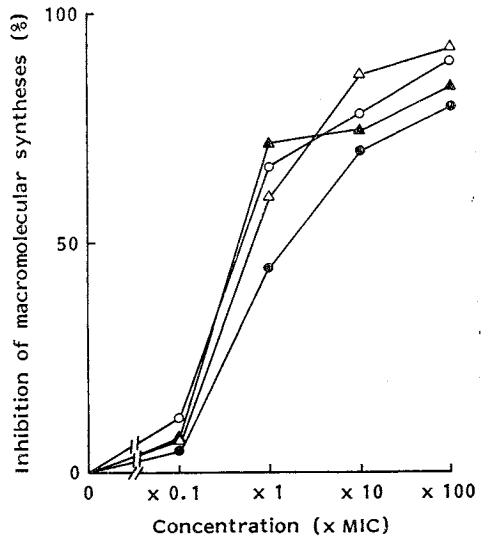
[a] Concentration required to inhibit the enzyme by 50%.

Moreover, it is noteworthy that lactivicin showed much higher activity against β-lactam hypersensitive mutants (PG-8 and C 141) of *E. coli*[4] and *Pseudomonas aeruginosa*[10] than against their parents whereas D-cycloserine showed equivalent activity against the pairs of strains (Table 1). Lactivicin seems to easily permeate the outer membrane of *E. coli* because a permeability mutant (PG-12)[4] and its parent (CPC-20) showed the same sensitivity (Table 1). Lactivicin was also active against anaerobic bacteria (Table 1), but not against mycoplasma and fungi (MICs, >100 μg/ml).

Lactivicin was highly susceptible to various types of cephalosporinases (Table 2). In addition, it was hydrolysed by penicillinases from *Staphylococcus aureus* 1840, *E. coli* TN 713, *E. coli* TN 649 and *Klebsiella pneumoniae* TN 1719 (Dr. K. OKONOGI; personal communication). Lactivicin showed weak inhibitory activity against some β-lactamases (Table 3). Moreover, it was highly active in inducing the β-lactamase of *Bacillus licheniformis* in the assay system developed by SYKES and WELLS[11] (data not shown). The activities of lactivicin described above are quite similar to those observed with typical β-lactam antibiotics.

Fig. 2. Effect of lactivicin on macromolecular synthesis in *Escherichia coli* LD-2.

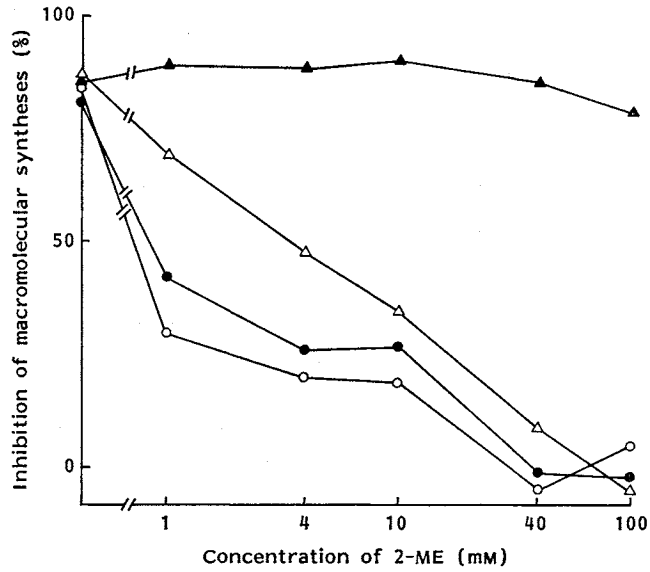
○ DNA, ● RNA, △ protein, ▲ peptidoglycan.



The MIC of lactivicin against *E. coli* LD-2 was 50 μg/ml.

Fig. 3. Effect of 2-mercaptoethanol (2-ME) on inhibitory activity of lactivicin against macromolecular synthesis in *Escherichia coli* LD-2.

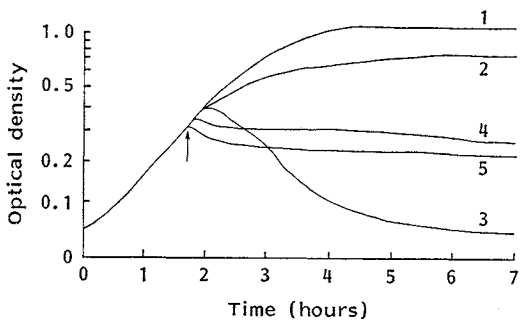
○ DNA, ● RNA, △ protein, ▲ peptidoglycan.



The concentration of lactivicin was ×10 MIC.

Fig. 4. Effect of lactivicin on the growth of *Escherichia coli* LD-2.

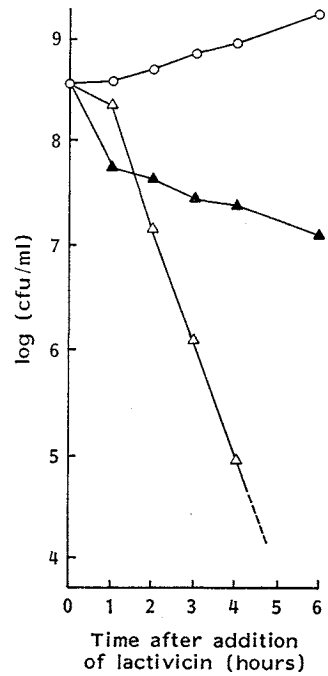
1: Control, 2: ×0.5 MIC, 3: ×1 MIC, 4: ×5 MIC, 5: ×10 MIC.



Lactivicin was added after 1.75 hours of cultivation (shown by an arrow).

Fig. 5. Effect of lactivicin on the viability of *Escherichia coli* LD-2.

○ Control, △ ×1 MIC, ▲ ×10 MIC.



Viable cell counts were determined periodically after the addition of lactivicin on DYAB agar using the culture sample in the experiment in Fig. 4.

Mode of Action of Lactivicin against *E. coli*

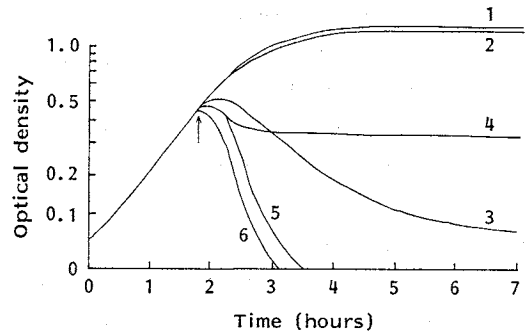
Since lactivicin has a unique structure,[3] we examined its effect on the synthesis of cellular macromolecules (DNA, RNA, protein and peptidoglycan) in *E. coli*. Under our experimental conditions, nalidixic acid, rifampicin, chloramphenicol and ampicillin specifically inhibited the synthesis of DNA, RNA, protein and pep-

tidoglycan, respectively. Contrary to our expectation that lactivicin would specifically inhibit peptidoglycan synthesis, it inhibited all types of macromolecular synthesis (Fig. 2). However, specific inhibition of peptidoglycan synthesis by lactivicin was observed in the presence of SH reagents such as 2-ME and DTT; restoration of the synthesis of intracellular macromolecules (DNA, RNA and protein) was completely dependent on concentration of the SH reagents (Fig. 3).

E. coli showed a peculiar lytic response to lactivicin; although lactivicin strongly lysed cells at its MIC, it brought about growth stasis at concentrations above × 5 MIC (Fig. 4). Under these conditions, viable cell counts rapidly decreased at × 1 MIC and gradually at × 10 MIC (Fig. 5). Simultaneous addition of 2-ME enhanced the lytic activity of lactivicin, especially at higher concentrations of lactivicin (Fig. 6). Lactivicin induced spheroplasts and rabbit ear-like bodies and caused cell lysis at × 1 MIC (Fig. 7) whereas concentrations of lactivicin above × 5 MIC did not induce any distinct morphologi-

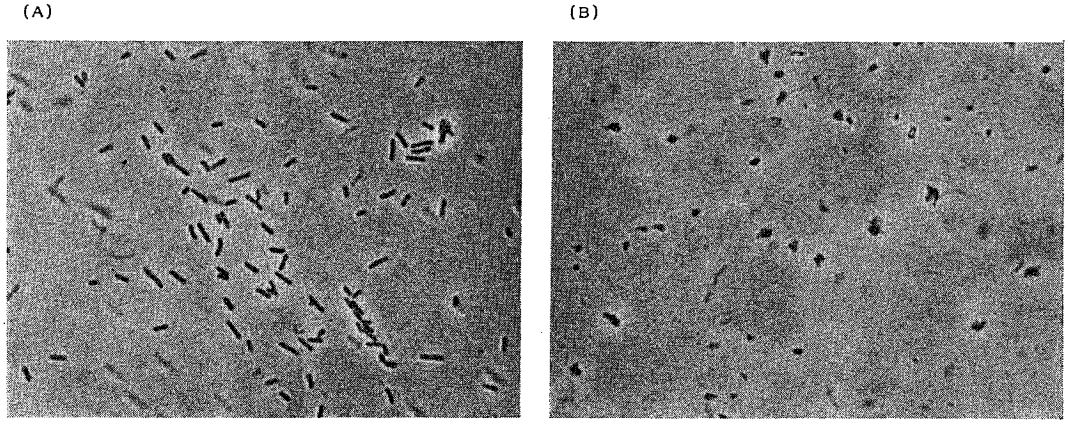
Fig. 6. Effect of 2-mercaptoethanol (2-ME) on the lytic activity of lactivicin against *Escherichia coli* LD-2.

1: Control, 2: 2-ME (5 mM), 3: lactivicin ×1 MIC), 4: lactivicin (×10 MIC), 5: lactivicin (×1 MIC) plus 2-ME (5 mM), 6: lactivicin (×10 MIC) plus 2-ME (5 mM).



Lactivicin and 2-ME were simultaneously added after 1.75 hours of cultivation (shown by an arrow).

Fig. 7. Morphological changes of *Escherichia coli* LD-2 induced by lactivicin.
(A) Control, (B) lactivicin (×1 MIC) treated.



The morphology of cells was observed 1 hour after addition of lactivicin using the culture sample in the experiment in Fig. 4.

Table 4. Affinity of lactivicin for PBPs in *Escherichia coli* LD-2.

Organism	MIC (μg/ml)	IC_{50} (μg/ml)[a]					
		PBP					
		1A	1B	2	3	4	5/6
E. coli LD-2	50	5	14	22	122	160	>500

[a] Concentration required to prevent binding of [^{14}C]benzylpenicillin by 50%.

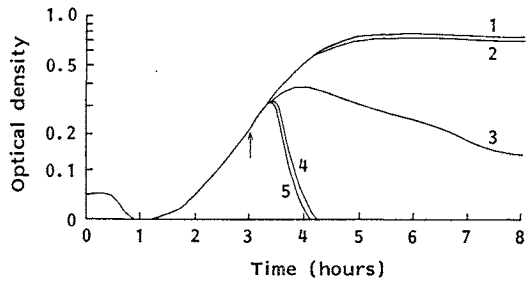
cal change. The higher concentration of lactivicin also completely protected cell lysis by cephaloridine. This lysis-protecting activity was also observed with inhibitors of SH enzymes, such as *N*-ethylmaleimide[12] and patulin[13] (data not shown).

Lactivicin exhibited the affinity for PBPs in *E. coli* typical of β-lactam antibiotics.[2] Table 4 shows that the highest affinity was for PBP 1 (1A and 1B), with high affinity also for PBP 2 among the lethal targets (PBPs 1, 2 and 3) in this organism.[14] This result agrees well with the spheroplast-inducing activity of lactivicin against *E. coli* shown in Fig. 7. The binding affinity of lactivicin for PBPs 4~6, D-alanine carboxypeptidases,[14] was very low, compared with those of typical β-lactam antibiotics (Table 4).

From all of these results, it is clear that lactivicin has at least two activities against *E. coli*; one is that of β-lactam antibiotics (specific inhibitors of peptidoglycan synthesis) and the other may be inhibition of SH proteins involved in fundamental membrane functions; the primary mode of action of lactivicin is due to the former.

Fig. 8. Effect of lactivicin on the growth of *Bacillus subtilis* PCI 219.

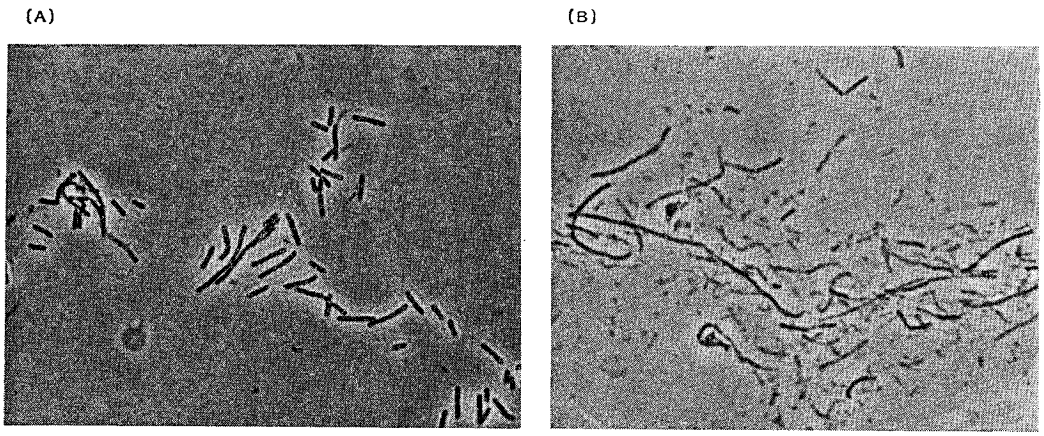
1: Control, 2: ×0.5 MIC, 3: ×1 MIC, 4: ×5 MIC, 5: ×10 MIC.



Lactivicin was added after 3 hours of cultivation (shown by an arrow). The MIC of lactivicin against *B. subtilis* PCI 219 was 3.13 μg/ml.

Fig. 9. Morphological changes of *Bacillus subtilis* PCI 219 induced by lactivicin.

(A) Control, (B) lactivicin (×1 MIC) treated.



The morphology of cells was observed 2 hours after the addition of lactivicin using the culture sample in experiment in Fig. 8.

Table 5. Affinity of lactivicin for PBPs in *Bacillus subtilis* PCI 219.

Organism	MIC (μg/ml)	IC_{50} (μg/ml)[a]				
		PBP				
		1	2	3	4	5
B. subtilis PCI 219	3.13	0.28	1.0	12	0.05	120

[a] See the legend to Table 4.

Mode of Action of Lactivicin against *B. subtilis*

In contrast to the results with *E. coli*, lactivicin showed dose-dependent lytic activity against *B. subtilis* similar to typical β-lactam antibiotics (Fig. 8). Adding 5 mM 2-ME to the culture did not have any significant effect on growth in the presence or the absence of lactivicin. Lactivicin induced filamentous cells of this organism at its MIC (Fig. 9) and strongly lysed the cells at concentrations above × 5 MIC (Fig. 8).

Fig. 10. Inhibitory activity of lactivicin against peptidoglycan synthesis in *Bacillus subtilis* PCI 219.

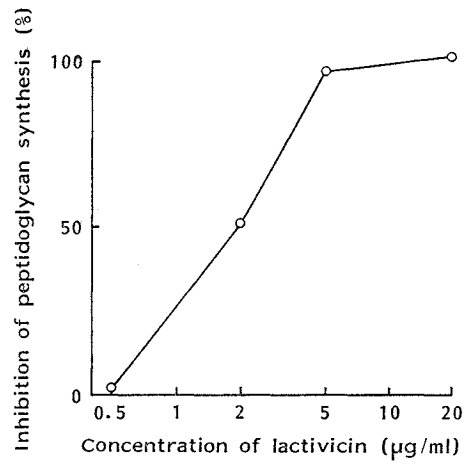


Fig. 10 shows that lactivicin potently inhibited peptidoglycan systhesis in *B. subtilis*. Lactivicin showed high affinity for PBPs 1, 2 and 4 (Table 5),[2] the possible lethal targets of β-lactam antibiotics in this organism.[14] In contrast, it showed poor affinity for PBP 5, D-alanine carboxypeptidase,[14] as in the case of *E. coli*.

The results indicate that lactivicin impedes the growth of *B. subtilis* through the same action as typical β-lactam antibiotics.

Discussion

Here we have reported that the non-β-lactam antibiotic, lactivicin, shows β-lactam action. Lactivicin showed high affinity for bacterial PBPs, much higher activity against β-lactam hypersensitive mutants than against their parents, high sensitivity to various types of β-lactamases, inhibition of β-lactamases and induction of β-lactamases. All of these activities were believed to be specific to β-lactam antibiotics. Although part of the structure of lactivicin resembles that of D-cycloserine, the mode of action differs completely; lactivicin has typical β-lactam action whereas D-cycloserine inhibits D-alanine racemase and D-alanine: D-alanine ligase involved in the primary stages of bacterial cell wall synthesis in the cytoplasm.[9] The mode of action of lactivicin is consistent with the finding that a section of its stereochemical structure from the acid amide group to the carboxyl group is quite similar to that of the functional region of β-lactam antibiotics.[3]

As observed in *E. coli* with β-lactam antibiotics,[14] lactivicin showed high affinity for PBP 1 and induced spheroplasts and cell lysis. Although its primary target is considered to be PBP 1, it also behaves like inhibitors of membrane SH proteins in *E. coli* at concentration above × 5 MIC. This is based on two observations: That the inhibition of synthesis of intracellular macromolecules (DNA, RNA and protein) by lactivicin completely disappeared in the presence of SH reagents without affecting the inhibition of peptidoglycan synthesis (Fig. 3); and that these reagents stimulated the lytic activity of lactivicin and brought about the dose-dependent lytic action of lactivicin (Fig. 6). These results may be explained on the basis that the autolysis of *E. coli* cells requires unbalanced growth,[15] namely specific inhibition of peptidoglycan synthesis without affecting the synthesis of intracellular macromolecules. Since the secondary action of lactivicin was also observed with *Proteus mirabilis*, the target SH proteins (enzymes?) seem to be commonly present in Enterobacteriaceae.

In general, high concentration seems to be required for SH inhibitors, such as *N*-ethylmaleimide and patulin, to exert their antibacterial activity; sufficient binding of the SH inhibitor molecules to the sulfhydryl groups of target proteins may be required to completely prevent the function of the proteins. In fact, in an *in vitro* assay system, high concentrations of lactivicin or *N*-ethylmaleimide inhibited the activity of two SH enzymes, papain[16] and ficin,[17] and the inhibitory activity completely

disappeared in the presence of equimolar 2-ME (data not shown). Sulfhydryl groups and disulfide bridges of SH enzymes in bacterial membranes play an important role in the structural organization, integrity and assembly of membranes.[18] In addition, it is well known that SH inhibitors such as *N*-ethylmaleimide and patulin react covalently with free sulfhydryl groups[12,13] of SH enzymes of membranes and disturb a variety of membrane functions of bacteria.[19,20] The disturbance of functions essential for bacterial metabolism can easily lead to inhibition of the synthesis of intracellular macromolecules.

In contrast, lactivicin impeded the growth of *B. subtilis* in an identical manner to typical β-lactam antibiotics; the lytic activity of lactivicin against this organism paralleled its concentration (Fig. 8) and was not influenced by adding SH reagents. Lactivicin induced filamentous cells of *B. subtilis* at its lowest effective concentration (Fig. 9). It remains to be determined which of the essential PBPs are involved in the cell division process in this organism.

In conclusion, lactivicin is the first non-β-lactam antibiotic showing affinity for PBPs in microorganisms. Its discovery has demonstrated that β-lactam nuclei are not essential to exhibit the so-called β-lactam action against pathogenic bacteria.

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References

1) OKAZAKI, H.: Lactivicin, a new type of antibiotic having β-lactam-like activity. *In* Frontiers of Antibiotic Research. *Ed.*, H. UMEZAWA, pp. 303~311, Academic Press Japan, Inc., Tokyo, 1987
2) 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
3) HARADA, S.; S. TSUBOTANI, T. HIDA, H. ONO & H. OKAZAKI: Structure of lactivicin, an antibiotic having a new nucleus and similar biological activities to β-lactam antibiotics. Tetrahedron Lett. 27: 6229~6232, 1986
4) NOZAKI, Y.; S. HARADA, K. KITANO & A. IMADA: Structure-activity relations of 5,6-*cis* carbapenem antibiotics and role of factors determining susceptibility of *Escherichia coli* to β-lactam antibiotics. J. Antibiotics 37: 218~226, 1984
5) KITANO, K.; K. KINTAKA, S. SUZUKI, K. KATAMOTO, K. NARA & Y. NAKAO: Screening of microorganisms capable of producing β-lactam antibiotics. J. Ferment. Technol. 53: 327~338, 1975
6) NOZAKI, Y.; A. IMADA & M. YONEDA: SCE-963, a new potent cephalosporin with high affinity for penicillin-binding proteins 1 and 3 of *Escherichia coli*. Antimicrob. Agents Chemother. 15: 20~27, 1979
7) OKONOGI, K.; Y. NOZAKI, A. IMADA & M. KUNO: C-19393 S_2 and H_2, new carbapenem antibiotics. IV. Inhibitory activity against β-lactamases. J. Antibiotics 34: 212~217, 1981
8) NOZAKI, Y.; K. OKONOGI, N. KATAYAMA, H. ONO, S. HARADA, M. KONDO & H. OKAZAKI: Cephabacins, new cephem antibiotics of bacterial origin. IV. Antibacterial activities, stability to β-lactamases and mode of action. J. Antibiotics 37: 1555~1565, 1984
9) NEUHAUS, F. C.: D-Cycloserine and *O*-carbamyl-D-serine. *In* Antibiotics I. *Eds.*, D. GOTTLIEB & P. D. SHAW, pp. 40~83, Springer Verlag, Berlin, 1967
10) ONO, H.; Y. NOZAKI, N. KATAYAMA & H. OKAZAKI: Cephabacins, new cephem antibiotics of bacterial origin. I. Discovery and taxonomy of the producing organisms and fermentation. J. Antibiotics 37: 1528~1535, 1984
11) SYKES, R. B. & J. S. WELLS: Screening for β-lactam antibiotics in nature. J. Antibiotics 38: 119~121, 1985
12) COHEN, L. A.: Group-specific reagents in protein chemistry. Annu. Rev. Biochem. 37: 695~725, 1968
13) ASHOOR, S. H. & F. S. CHU: Inhibition of alcohol and lactic dehydrogenases by patulin and penicillic acid *in vitro*. Fd. Cosmet. Toxicol. 11: 617~624, 1973
14) WAXMAN, D. J. & J. L. STROMINGER: Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. Annu. Rev. Biochem. 52: 825~869, 1983

15) TOMASZ, A.: From penicillin-binding proteins to the lysis and death of bacteria: A 1979 view. Rev. Infect. Dis. 1: 434~467, 1979
16) ANDERSON, B. M. & E. C. VASINI: Nonpolar effects in reactions of the sulfhydryl group of papain. Biochemistry 9: 3348~3352, 1970
17) LIENER, I. E.: A study of the number and reactivity of the sulfhydryl groups of ficin. Biochim. Biophys. Acta 53: 332~342, 1961
18) AZOCAR, O. & E. MUNOZ: Molecular organization in bacterial cell membranes. Sulfhydryl groups and disulfide bridges in *Streptomyces albus* and *Escherichia coli* K-12 cytoplasmic membranes. Eur. J. Biochem. 68: 245~254, 1976
19) DOSKOCIL, J.: The role of thio groups in nucleoside transport. Mol. Cell. Biochem. 10: 137~143, 1976
20) JANICK, P. A.; G. B. GRUNWALD & J. M. WOOD: The effects of *N*-ethylmaleimide on active amino acid transport in *Escherichia coli*. Biochim. Biophys. Acta 646: 328~337, 1977