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CHEMISTRY OF A NEW ANTIBIOTIC: LACTIVICIN

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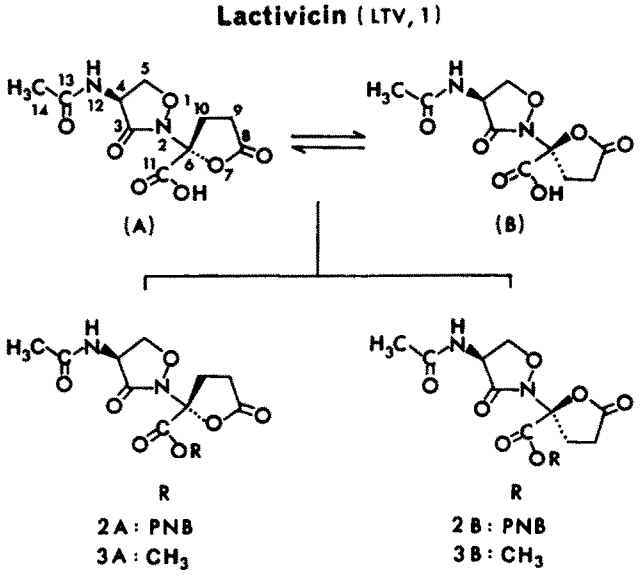
Abstract: A novel antibiotic, lactivicin (LTV), was isolated from the culture filtrates of two bacterial strains by various types of chromatography. LTV exists in aqueous solution as an equilibrium mixture of two epimers in a ratio of about 1:1. The chemical structure of LTV ($C_{10}H_{12}N_2O_7$) was determined to be 2-(4S-acetylamino-3-oxo-2-isoxazolidinyl)-5-oxo-tetrahydrofuran-2-carboxylic acid. The absolute configuration at the C-6 position was elucidated from the CD spectral data and X-ray crystallographic analysis of 4-amino-lactivinic acid obtained by the iminoether method. This compound is useful as a starting material for chemical modification. LTV showed antibacterial activity against Gram-positive and negative bacteria, susceptibility to β-lactamases, and affinity for penicillin-binding proteins. We therefore concluded that LTV is a novel skeleton antibiotic having β-lactam-like activities.

In our screening program for new inhibitors of cell wall synthesis from bacterial strains, we discovered lactivicin (LTV, 1),[1] a novel antibiotic, in culture filtrates of Empedobacter lactamgenus sp. nov. YK-258 and Lysobacter albus sp. nov. YK-422[2]. LTV was purified by the method used to isolate acidic, water-soluble substances. It was obtained as an equilibrium mixture in aqueous solution. The structure of this antibiotic has been determined by chemical degradation and spectroscopic analyses to be a dicyclic dipeptide as shown in Fig. 1.[1] LTV is active against Gram-positive and some Gram-negative bacteria, in vitro and in vivo,[2] and shows biological activities which are similar to those of β-lactam antibiotics in that it is susceptible to β-lactamases and has an affinity for penicillin-binding proteins.[2,3] As LTV gave 4-amino-lactivinic acid (4-ALA) by the iminoether method in good yield, we tried to synthesize more effective and less toxic derivatives of this antibiotic based on the known β-lactam antibiotic chemistry.

This paper deals with the isolation and structure elucidation of lactivicin in detail and preliminary chemical modifications of this compound.

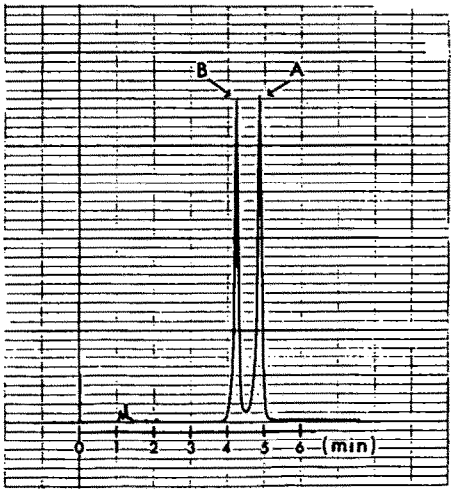
LTV is an acidic, water-soluble antibiotic. It was isolated from the culture filtrate as the sodium salt by column chromatography using anion-exchange resins, anion-exchange Sephadex, an adsorptive resin and activated carbon. In place of QAE-Sephadex^R chromatography, ion-paired extraction using 2% trioctylmethylammonium chloride/dichloromethane solution or preparative reversed-phase HPLC using a octadecyl silicate column/0.01M phosphate buffer (P.B., pH 6.3) could be used. Bioactive fractions were detected by measuring antimicrobial activity using β-lactam-hypersensitive mutants, Pseudomonas aeruginosa C 141 and Escherichia coli PG-8, and by HPLC.

Fig. 1 Structures of lactivicin and its esters



Two close peaks having almost the same heights were observed on HPLC of purified 1 as shown in Fig. 2. Peaks A and B showed the same antimicrobial activity in the diffusion method and were present in a ratio of 53 to 47 (average of five lots). We tried separating these peaks by preparative HPLC followed by desalination using activated carbon chromatography, but we found that the HPLC patterns of the recovered solutions were the same as those of the starting solutions. The

Fig. 2 Typical HPLC pattern of lactivicin



Column: ODS, YMC-Pack A-312(Yamamura Chem. Lab.)
Mobile phase: 4% MeOH/0.01M P.B.(pH 6.3)
Detection: UV absorbance at 214 nm
Flow rate: 2 ml/min

Fig. 3 Equilibrium patterns of lactivicin (A and B) in 0.1 M P.B.(pH 7.0) at 23°C

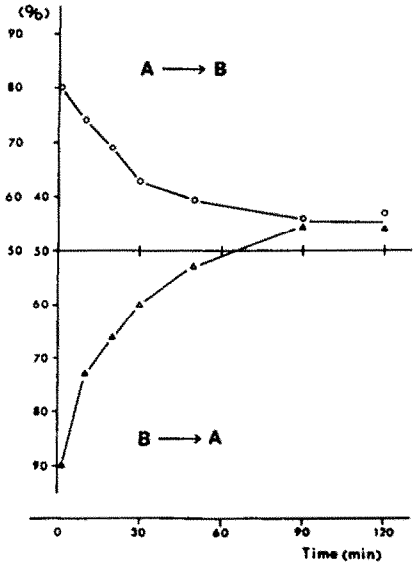


Table 1. Stabilities of lactivicin and naturally occurring β-lactam antibiotics in P.B. (60°C, 100 μg/ml)[a]

Compound	Half-life time (hr)[b] pH 3	pH 5	pH 6	pH 7	pH 8	pH 9
Lactivicin	0.39	1.63		0.45		0.35
Cephabacin H_1[14]	0.85	0.85		0.85		0.58
Cephabacin F_1[14]	1.4	0.73		0.87		0.50
Clavulanic acid[15]		0.70		1.4		0.40
C-19393 E_5[16]		1.5	2.4	1.5	0.50	
Cephalosporin C	1.6	1.5		1.5		0.90
Cephabacin M_1[5]	1.8	1.8		1.5		0.37
Epithienamycin B[17]		0.43	1.1	2.2	4.8	
Epithienamycin A[17]		0.50	1.8	5.1	8.5	
Penicillin N		2.7		8.0		2.0
Deacetylcephalosporin C	1.9	27		29		6.3

a, The samples prepared in our laboratories were used. Underlines express the most stable pH regions. b, The residual amounts were measured by HPLC.

conversion rates from A to B and from B to A starting with samples prepared just after the separation of peaks A and B by preparative HPLC are shown in Fig. 3. Equilibrium was attained in both cases after about one and a half hours at 23°C, pH 3 to 7.

The sodium salt of **1** obtained as a equilibrium mixture gave positive color reactions with potassium permanganate and negative color reactions with Greig-Leaback, Sakaguchi, Ehrlich, Barton and Dragendorff reagents. LTV is readily soluble in water and dimethyl sulfoxide, soluble in methanol, and sparingly soluble in acetone and ethyl acetate. The stability of **1** at 60°C in P.B. of various pHs is shown in Table 1. The stability of **1** at these pH regions was almost the same as those of the cephabacins, clavulanic acid, carbapenems, or cephalosporin C. LTV is most stable at pH 5.

The UV spectrum of **1** contained a shoulder at 216 nm. The CD spectrum of **1** showed a negative Cotton effect at 238 nm and a positive one at 212 nm. The UV absorptions and Cotton effects in the

Table 2. UV and CD spectra of lactivicin and naturally occurring β-lactam antibiotics[a]

Compound	UV: λ_{max} nm (ε)	CD:[θ] nm
Lactivicin	216 (4050, sh)	212 (+18400), 238 (-22200)
Penicillin N	End abs.	232 (+18900)
Clavulanic acid	End abs.	215 (-39800), 238 (+27900)
Sulfazecin[18]	End abs.	236 (+11000)
Cephabacin H_1[14]	260 (9000)	226 (-50700), 258 (+21000)
" M_1[5]	264 (9000)	234 (-58800), 260 (+49100)
" F_1[14]	260 (9520)	228 (-30900), 260 (+29500)
TAN-458[19]	253 (3850)	218 (-12100), 255 (+7510)
C-19393 S_2[20]	244 (16900), 288 (13600)	233 (+27700), 263 (-46200), 300 (-43100)
Formadicin A[21]	224 (24500), 269 (2000)	230 (-113000)

a, The samples were measured at 23-25°C in water by Hitachi Spectrophotometer 320 (UV) and JASCO Automatic Recording Spectropolarimeter J-20A (CD).

Fig. 4 IR spectrum of lactivicin sodim salt(KBr)

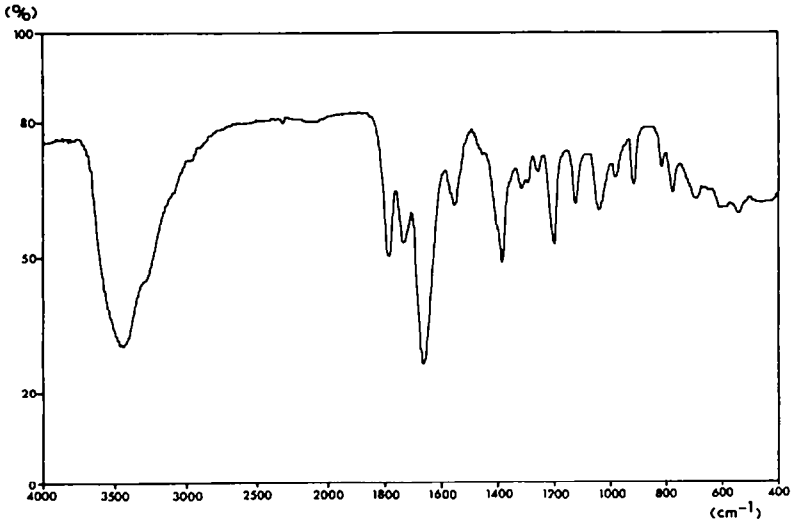
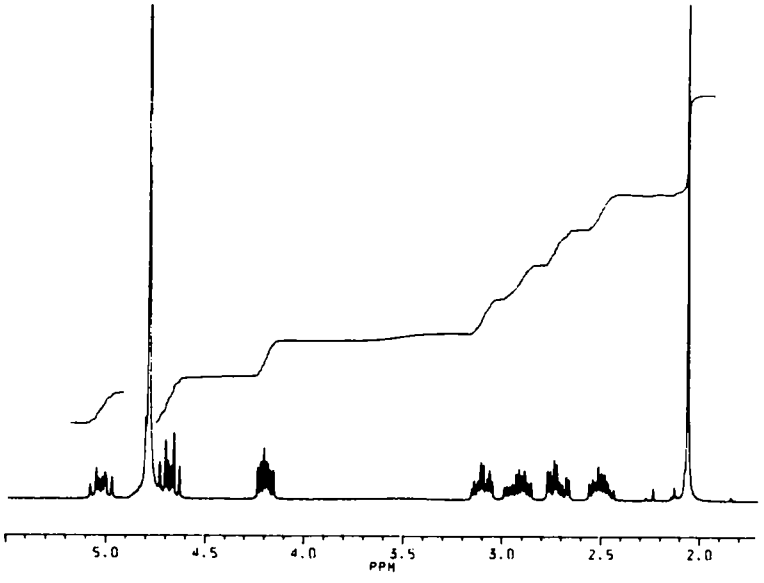


Fig. 5 ^{1}H NMR spectrum of lactivicin [300 MHz, D_2O, Bruker AC-300]



CD spectrum of the known β-lactam antibiotics are shown in Table 2. These characteristic UV and CD spectral data suggested that **1** has different chromophore(s) from those of known β-lactam antibiotics despite the similarity in their biological activities. The IR spectrum of **1** (Fig. 4) was also complicated in the carbonyl region. The IR absorptions at 1780 and 1730 cm^{-1} seemed likely to be β-lactam and ester carbonyls. However, after structure determination of **1**, these absorptions were assigned to γ-lactone and 3-isoxazolidone (γ-lactam) carbonyls, respectively. In the ^{1}H NMR spectrum, all corresponding signals were observed as overlapping signals as shown in Fig. 5.

On acid hydrolysis of **1** in 6N hydrochloric acid, serine was detected in the hydrolysate by amino acid analysis and was found to be the L-form by the modified HPLC method[4] using a chiral mobile phase. Upon treatment with p-nitrobenzyl bromide in dimethylformamide (DMF), **1** gave two p-nitrobenzyl ester epimers (**2A** and **2B**). They were separated in their pure forms by Sephadex LH-20^{R} or silica gel chromatography. When **2B** was hydrogenated using 10 % palladium-carbon to deprotect the

Table 3. ^{13}C NMR spectral data [100 MHz, δ_{ppm}, JEOL GX-400]

Carbon	Compound (Solvent) 1 (DMSO-d_6)	3 A[a] (DMSO-d_6)	3 B (DMSO-d_6)	4 (DMSO-d_6)	8 (D_2O)	10 ($CDCl_3$)
CO	175.84 s	174.27 s	174.39 s		179.62 s	172.80 s
''	172.82, 171.07 s	171.80 s	170.22 s	170.45 s	177.10 s	170.29 s
''	169.68, 169.64 s	169.56 s	169.59 s	169.55 s	175.55 s	170.04 s
''	167.21, 167.02 s	165.54 s	165.41 s		168.42 s	163.28 s
C-6	96.44, 96.28 s	91.48 s	91.38 s		156.19 s	151.86 s
C-5	68.40, 71.07 t	69.52 t	71.75 t	72.48 t	76.77 t	74.86 t
C-4	52.19, 51.93 d	51.60 d	51.02 d	51.07 d	55.73 d	52.60 d
C-9	29.06, 28.82 t[b]	28.31 t[b]	28.28 t[b]		32.84 t	30.15 t
C-10	28.15, 28.12 t[b]	27.17 t[b]	27.11 t[b]		23.56 t	21.03 t
C-14	22.25 q	22.12 q	22.14 q	22.23 q	24.60 q	22.93 q
$COOCH_3$		53.54 q	53.53 q			52.74 q
''						52.60 q
''						51.88 q

a, 67.8 MHz, b, The signals may be reversible.

Table 4. ^{1}H NMR spectral data [400 MHz, δ_{ppm} J(Hz), JEOL GX-400]

Proton	Compound (Solvent) 1 (D_2O)	2A ($CDCl_3$)	2B ($CDCl_3$)
14-H	2.06(s)	2.06(s)	2.06(s)
9-H	2.72(ddd, 4.0, 10.0, 18.1)	2.62(ddd, 4.7, 10.2, 17.9)	2.62(ddd, 5.0, 10.3, 17.8)
	2.91 & 2.93(ddd, 8.3, 10.0, 18.1)	2.86(ddd, 8.1, 10.0, 17.9)	2.86(ddd, 7.6, 10.0, 17.8)
10-H	2.49 & 2.51(ddd, 8.3, 10.0, 14.0)	2.47(ddd, 8.1, 10.2, 14.1)	2.47(ddd, 7.6, 10.3, 14.0)
	3.09 & 3.10(ddd, 4.0, 10.0, 14.0)	3.18(ddd, 4.7, 10.0, 14.1)	3.11(ddd, 5.0, 10.0, 14.0)
5-H	4.19(dd, 8.5, 10.5) & 4.20 (dd, 8.5, 9.0)	4.12(dd, 8.4, 11.0)	4.08(dd, 8.3, 10.4)
	4.66 & 4.70(t, 8.5)	4.81(dd, 8.4, 8.5)	4.88(dd, 8.1, 8.3)
4-H	5.00(dd, 8.5, 10.5) & 5.05 (dd, 8.5, 9.0)	4.68(ddd, 4.9, 8.5, 11.0)	4.95(ddd, 5.2, 8.1, 10.4)
12-H	8.45(d, 8.1) & 8.51(d, 7.8)[a]	5.97(d, 4.9)	6.07(d, 5.2)
PNB[b]			

a, in DMSO-d_6, b, The signals at PNB groups are abbreviated.

PNB group, 1 could only be obtained as the equilibrium mixture. Treatment of 1 with methyl iodide by a similar method afforded a pair of methyl esters (3A and 3B). An equilibrium between the two forms of these esters was not observed in aqueous solutions and only degradation products were found. The ^{13}C NMR spectral data of 1, 3A, and 3B (Table 3) suggested the presence of the following functional groups in 1 ; seryl, acetyl, ethylene, and quarternary carbon. From the coupling constant analysis of the ^{1}H NMR spectrum of 2A (Table 4), the signals at 4.68, 4.12, 4.81 and at 2.62, 2.86, 2.47, 3.18 ppm were assigned to the serine [-O-CH_2-CH(NH)-CO-] and ethylene (-CH_2-CH_2-) moieties, respectively. From these findings, it was clarified that 1 was the equiliblium mixture of the epimers.

The physicochemical and spectral data for 1 and its esters are summarized in Table 5. The

Table 5. Physicochemical properties of lactivicin and its ester derivatives

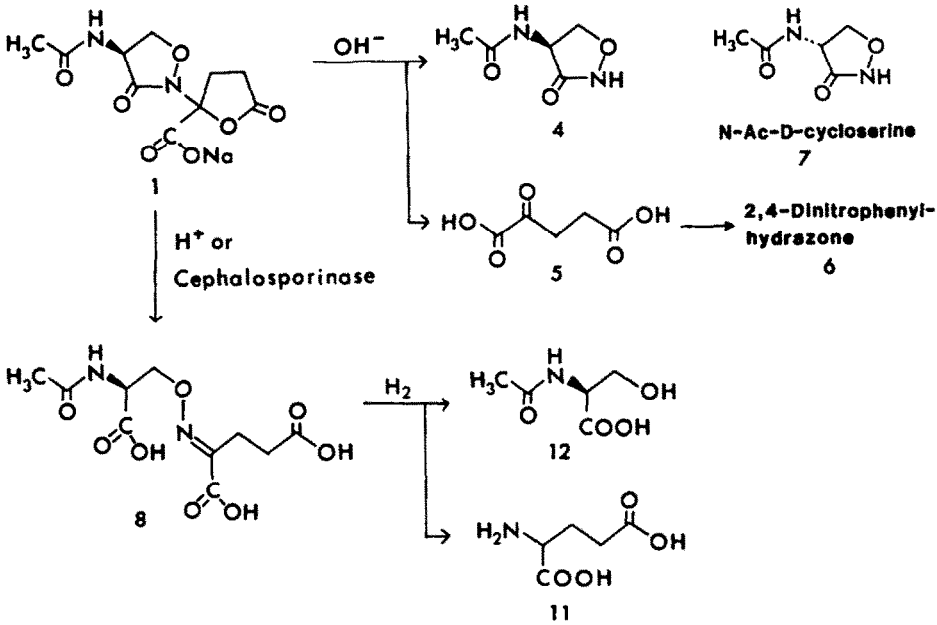
Property	1	3A	3B	2A	2B
Appearance	White powder	Colorless cryst.	Colorless cryst.	White powder	White powder
M.P.		163-166 °C(dec)	180-181 °C(dec)		
$[\alpha]_D$ (c)	-24.1°(0.50)	+76.7°(0.62)	-112°(0.51)	+97.3°(0.48)	-64.5°(0.50)
Sol.[a]	H_2O	$CHCl_3$	$CHCl_3$	$CHCl_3$	$CHCl_3$
SI-MS: m/z	317 $(M+Na)^+$ 295 $(M+H)^+$	287 $(M+H)^+$	287 $(M+H)^+$	408 $(M+H)^+$	408 $(M+H)^+$
Analysis	Found Calcd C,40.12; 39.61 H, 4.17; 3.99 N, 9.25; 9.24 Na; 7.4; 7.58	Found Calcd C,46.29; 46.16 H, 4.78; 4.93 N, 9.74; 9.79	Found Calcd C,45.98; 46.16 H, 4.70; 4.93 N, 9.71; 9.79	Found Calcd C,50.20; 50.13 H, 4.22; 4.21 N,10.13; 10.32	Found Calcd C,50.10; 50.13 H, 4.21; 4.21 N,10.15; 10.32
Molecular formula	$C_{10}H_{11}N_2O_7Na.$ $0.5H_2O$	$C_{11}H_{14}N_2O_7$	$C_{11}H_{14}N_2O_7$	$C_{17}H_{17}N_3O_9$	$C_{17}H_{17}N_3O_9$
UV: $\lambda_{max}^{nm}(\varepsilon)$	216(4050, sh)	216(3720, sh)	217(4000)	214(11300, sh) 262(11400)	214(11400, sh) 262(11500)
Sol.	H_2O	MeOH	MeOH	MeOH	MeOH
CD: [θ](nm)	-22200(238) +18400(212)	-35500(246) +54300(214)	+ 4900(257) -30300(228)		
Sol.	H_2O	MeOH	MeOH		
IR: ν_{max}(KBr) (cm^{-1})	1780, 1730, 1660	1800, 1760, 1750, 1650	1815, 1805, 1760, 1740, 1665	1805, 1775, 1760, 1680, 1610	1805, 1760, 1680, 1610

a, The values of specific optical rotations were measured at 23-25 °C.

specific rotation of 1 had an intermediate value between methyl esters 3A and 3B. The molecular formula of the sodium salt of 1 was determined from elemental analyses and SI-MS data of the ester derivatives to be $C_{10}H_{11}N_2O_7Na$.

Alkaline and acidic hydrolyses gave clear results for the structure determination. When 1 was

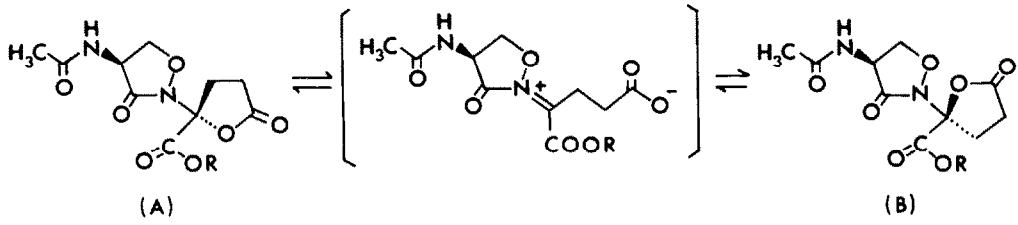
Fig. 6 Degradation patterns of lactivicin



hydrolyzed in 0.1N sodium hydroxide, N-acetyl-L-cycloserine (**4**) and α-ketoglutaric acid (**5**) were obtained as shown in Fig. 6. The latter was converted into the 2,4-dinitrophenyl hydrazone derivative (**6**). The physico-chemical data for **4** were identical with those for the N-acetyl derivative (**7**) synthesized from D-cycloserine, except for the sign of the specific optical rotation. This degradation pattern clearly showed that **1** has 3-isoxazolidone and glutaric acid moieties and the linkage occurs between the nitrogen group in the ring of **4** and one of three carbonyl groups in **5**. Upon treatment with a cation-exchange resin, Dowex 50 W x 2^R (H^+ type), **1** gave a tricarboxylic acid compound (**8**) as the single product, which was converted to a tri PNB ester (**9**) or a trimethyl ester (**10**). In the ^{13}C NMR spectrum of **8** (Table 3), the quaternary carbon signals at δ 96.44 and 96.28 in **1** were absent but a new signal at δ 156.19 was observed. The collapse of these chemical shifts strongly suggested the formation of an oxyimino group [-C=N-O-] in **8**, from a quarternary carbon moiety [-O-C(-CO-)-N-] in **1** which was assumed from the chemical shifts at δ 97.76 to 97.89 in the ^{13}C NMR spectra of cephabacins M_{1-6}[5]. Hydrogenolysis of **8** was carried out using a 10% palladium-carbon catalyst to clarify this assumption. The reaction gave DL-glutamic acid (**11**) and N-acetyl-L-serine (**12**) which was converted into a PNB derivative (**13**). These findings showed that in **8** the linkage occurs between the hydroxy group of the serine moiety and the amino group of the glutamic acid moiety. From the results of the degradation studies, the quarternary carbon should be the α-carbon of glutamic acid. The structure of **1** was finally deduced to be 2-(4S-acetylamino-3-oxo-2-isoxazolidinyl)-5-oxo-tetrahydrofuran-2-carboxylic acid (Fig. 1).

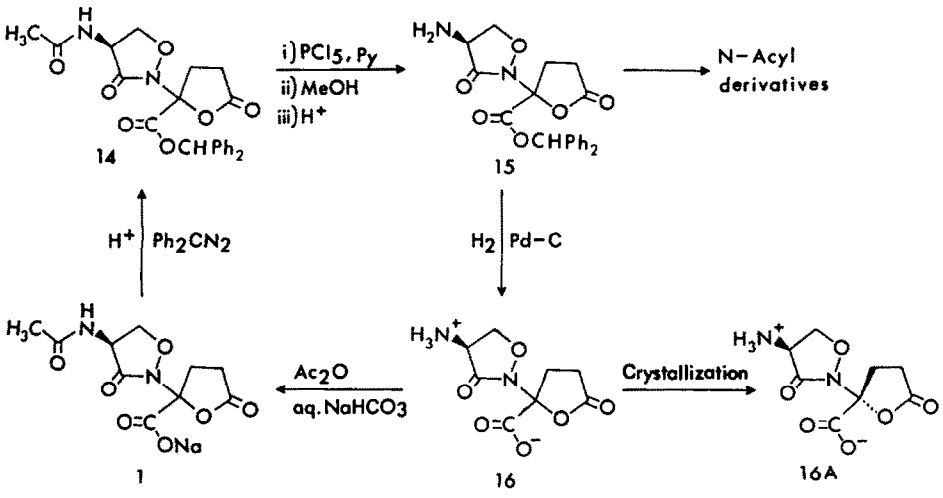
As mentioned above, **1** existed in aqueous solutions as an equilibrium mixture of two epimers in about a 1:1 ratio. The ester derivatives of **1** were isolated as pure epimers and did not isomerize in 50% methanol/0.1M P.B.(pH 3, 5, or 7) for 2 days at room temperature or in methanol when left for 1 week at room temperature. In triethylamine/chloroform at room temperature, **3B** reached an equilibrium of about 1:1 after 22 hours. Treatment of **1** with $H_2{}^{18}O$ at 4°C for 1.5 hours did not increase the molecular weight as detected by the SI-MS measurement. These data indicate that this type of isomerization does not occur by hydrolytic cleavage of the lactone group or by attack of the hydroxyl anion at the C-6 position. The isomerization mechanism of the carboxylates in the aqueous solutions and of the esters in the organic solvents can be explained by the formation of an immonium cation as the intermediate (Fig. 7). The carboxylate compounds may attain equilibrium before decomposition occurs in aqueous solutions, because the carboxyl anion stabilizes the immonium cation.[6] A similar mechanism has been reported in the case of oxapenem.[7]

Fig. 7 Equilibrium mechanism of lactivicin



LTV is an excellent starting material for chemical modification (Fig. 8). LTV benzhydryl ester (**14**) synthesized by diphenyldiazomethane or diphenylmethyl bromide could be deacetylated by the usual iminoether method[8] using phosphorous pentachloride, to afford an amino derivative (**15**). Treatment with trifluoroacetic acid/anisole or hydrogen/10% palladium-carbon to deprotect the carboxyl blocking group produced 4-amino-lactivinic acid (4-ALA, **16**). Preferential crystallization of **16** from water afforded a single epimer (**16 A**). The absolute configuration at the C-6 position of **16 A** was determined as R by X-ray crystallographic analysis.[9] Another partially purified epimer (**16 B**) was obtained from **14 B** in the manner described above. Compound **16** was converted into **1** by acetic

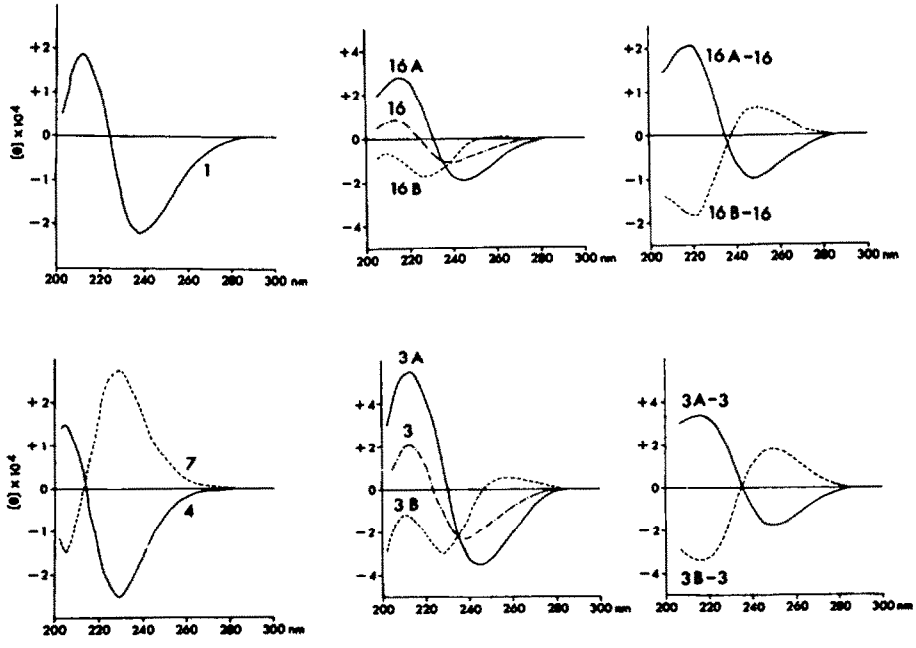
Fig. 8 Procedures of 4-amino-lactivinic acid



anhydride in 2% sodium bicarbonate. Compound **15** was easily acylated and deprotected to give various types of N-acyl derivative of **16**.

CD spectral studies gave important information to determine the absolute configurations at the C-6 position (Fig. 9). LTV and its related compounds, excluding the ester derivatives, occurred in about 1:1 ratios of their epimers in aqueous solutions, which seems to cause the Cotton effects of γ-lactone carbonyl moiety to offset each other. Thus, their CD spectra only reflect the γ-lactam carbonyl moiety. The bathochromic shifts may be due to the N-2 substituent effect, on comparing the CD spectra of **1** and **4**. The residual CD spectra obtained by subtracting the spectra of the mixture

Fig. 9 CD spectral data of lactivicin and related compounds (23-25°C in water)



from those of the isolated epimers may only reflect the γ-lactone carbonyl moiety. The residual spectra of the A-type epimers had a positive Cotton effect at 217-219 nm and a negative one at 248-250 nm. Those of the B-type epimers had typically opposite patterns. CD spectral data in combination with X-ray crystallographic analysis results showed the absolute configurations at the C-6 position to be R for the A-type epimers and S for the B-type ones.

The partial structure of **1** from the acid amide group at the C-13 position to the carboxyl group at the C-11 position through the C-3 amide group including absolute configuration at the C-4 position resembles the active site of the β-lactam antibiotics such as natural penicillins, cephalosporins, or nocardicins. This clearly accounts for the biological activities of **1** being similar to those of β-lactam antibiotics in spite of the absence of the β-lactam ring in the molecule. Enzymatic hydrolysis of **1** by cephalosporinase resulted in cleavage at the γ-lactam group to give compound **8** as with the usual cephalosporins. The hydrolysis rates of **1** by β-lactamases were examined using cephalosporinases from Enterobacter cloacae IFO 12937 and Pseudomonas aeruginosa U 31, and a penicillinase from Escherichia coli 205 TEM R^+(566). As shown in Fig. 10, the hydrolysis rates differed in the presence and absence of the cephalosporinase from E. cloacae IFO 12937 at 4°, 25° and 37°C. These results showed that hydrolysis was not just a chemical reaction but an enzymatic reaction. In these experiments, the two isomers of **1** had different hydrolysis patterns. As the temperature was decreased, the difference between them became greater, as equilibrium between the two isomers is attained slowly at low temperatures; the acquisition time for equilibrium at 4°C was about 20 hours. When a five-fold amount of enzyme was used at 4°C, **1B** was hydrolyzed faster

Fig. 10 Hydrolysis of lactivicin by cephalosporinase (E. cloacae IFO 12937, 0.2 unit/ml)

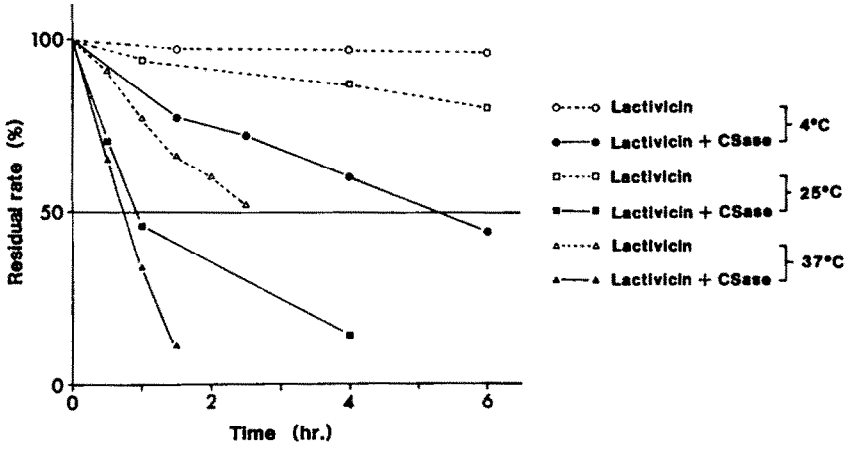
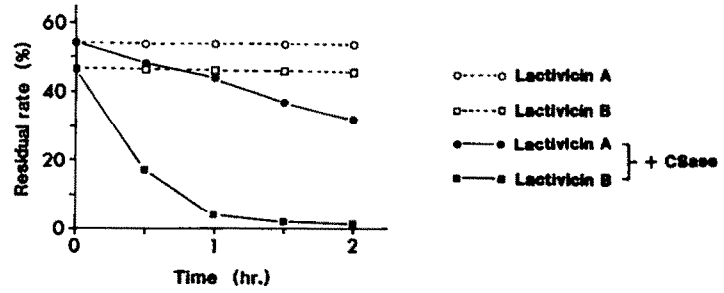


Fig. 11 Hydrolysis of lactivicin by cephalosporinase (E. cloacae IFO 12937, 1.0 unit/ml, 4°C)



than **1A** as shown in Fig. 11. The same results were obtained using a cephalosporinase from P. aeruginosa U31 and a penicillinase from E. coli 205 TEM R^+(566). From these findings, it was assumed that **1B** is more similar to the β-lactam antibiotics than is **1A** in the structural conformation of the active sites. When the crystal conformation of cefmenoxime[10] was overlapped, by the technique of 3-dimensional computer graphics, with the calculated stable-conformations of **1A** and **1B**, **1B** was much more similar to cefmenoxime than was **1A**. These observations are in good agreement with the above assumption.

4-ALA benzhydryl (BH) ester (**15**) was used as the starting material in modification studies of **1**. Commercially available acyl chlorides were used in N,N-dimethylacetamide (DMA) and dichloromethane to acylate **15** (Method I). Free acids were coupled with **15** in the presence of 1-hydroxybenzotriazole (HOBT) and N,N'-dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) (Method II). Formylation was achieved using acetic-formic anhydride (Method III). The benzyloxycarbonyl(Z)-glycyl derivative was prepared by the mixed anhydride method using methyl chloroformate (Method IV). The benzhydryl (BH) group of acylated compounds was removed in the most cases by hydrogenolysis. The reactions were carried out over 10% palladium-carbon under a hydrogen atomosphere for about 50 minutes at room temperature (Method A). The carboxyl group of the DL-phenylmalonyl side chain was protected as a BH ester and thereafter it was also hydrogenolyzed. Under these reaction conditions, the Z group of the amino acid side chain could not be removed and prolonged reaction time led to decomposition. To obtain non-protected phenylglycyl derivatives, we chose a p-methoxybenzyloxycarbonyl(MZ) group for the protection of the amino group. Deprotection of the MZ and BH groups were carried out by the reaction using trifluoroacetic acid (Method B). To obtain effective derivatives for oral administration, N-acyl 4-ALA pivaloyloxymethyl (POM) esters were synthesized by the treatment of the corresponding acid sodium salts with POM chloride. The physico-chemical properties of the derivatives synthesized are shown in Table 6.

Table 7 shows the antibacterial activity of **1** and its derivatives. LTV was weakly active against Gram-positive and some Gram-negative bacteria in vitro. However, **1** showed relatively strong protective effects in experimentally infected mice. The ED_{50} values for administration by subcutaneous injection were 25.4 mg/kg against Staphylococcus aureus 308 A-1, 2.05 mg/kg against Streptococcus pyogenes E-14, and 71.0 mg/kg against Escherichia coli O-111. The preliminary acute toxicities (LD_{50}) of **1** in mice were about 400 mg/kg for subcutaneous administration and more than 4000 mg/kg for oral administration. Among the synthetic derivatives, N-phenylacetyl(**23**), N-phenoxyacetyl(**26**) and N-Z-glycyl(**25**) derivatives had clearly stronger in vitro antibacterial activities than **1**. N-Phenylacetyl(**23**) and N-formyl(**17**) derivatives showed greater protective effects against S. aureus 308 A-1. N-Benzoyl(**21**) , N-p-hydroxy-mandelyl(**29**), N-R-mandelyl(**28**) and N-Z-glycyl(**25**) derivatives had almost the same in vivo efficacy as **1**. Although the D-phenylglycyl group is known to be very effective as a side chain of semi-synthetic β-lactam antibiotics, the corresponding derivative (**31**) had no activity because of its instability in neutral and basic aqueous solutions.

As **1** showed relatively strong toxicity on parenteral administration, we tested the preliminary acute toxicities of the related compounds of **1** to determine which partial structures have the toxic site. N-acetyl-L-cycloserine did not show any toxicity at a dose of 2,000 mg/kg (ip and sc). Compound **8** was less toxic than **1** when administered parenterally, LD_{50} 500-1000 mg/kg, ip, and >2000 mg/kg, sc. N-Phenylacetyl 4-ALA showed even less toxicity when administered parenterally, LD_{50} 1000-2000 mg/kg (ip and sc). Subcutaneous administration of this compound and oral administration of its POM ester showed almost the same protective effects in mice infected experimentally with S. aureus 308 A-1, S.pyogenes E-14, and E.coli O-111 as shown in Table 8. Furthermore, the POM derivative had no acute toxicity at a dose of 10.0 g/kg by oral administration.

The findings described above gave a great inpact to synthesize lactivicin analogs. After establishment of synthesis for **1**[11], syntheses of various kinds of **16**[12] and tricyclic penams or cephams having a γ-lactone ring[13] have been carried out. These works suggest that it will be possible to synthesize more effective and less toxic lactivicin analogs in the near future.

Table 6. Physico-chemical properties of N-acyl 4-amino-lactivinic acid (* DL-compound)

No.	Acyl	Method & Yield		Molecular formula	Analysis	Calcd	Found	IR:KBr (cm^{-1})	^1H NMR: δ_{ppm} J(Hz) (90 or 100 MHz, D_2O)
17	Formyl	IIIA	58	$C_9H_9N_2O_7Na$ ($1/2H_2O$)	C,	37.38	37.58	1780	2.4-3.4(4H,m), 4.1-4.4(1H,m),
					H,	3.49	3.92	1730	4.6-4.9(1H,m), 5.0-5.3(1H,m),
					N,	9.69	9.36	1670	8.22(1H,s)
18	n-Butyryl	IA	40	$C_{12}H_{15}N_2O_7Na$ ($2/5H_2O$)	C,	43.75	44.01	1790	0.94(3H,t,J=7), 1.64(2H,sex.
					H,	4.83	5.21	1730	J=7), 2.32(2H,t,J=7), 2.4-3.4
					N,	8.50	8.51	1660	(2H,m), 4.22(1H,m), 4.5-5.3
19	Pyruvyl	IIA	25	$C_{11}H_{11}N_2O_8Na$ ($1/2H_2O$)	C,	39.89	39.77	1780	1.59, 2.49(total 3H, s), 2.4-
					H,	3.65	3.68	1730	3.3(4H,m), 4.30(1H,m), 4.76
					N,	8.46	8.50	1660	(1H,m), 5.11(1H,m)
20	Lactyl*	IIA	30	$C_{11}H_{13}N_2O_8Na$ ($1/2H_2O$)	C,	39.65	39.95	1795	1.40(3H,d,J=7), 2.3-3.4(4H,m),
					H,	4.23	4.37	1730	4.2-4.5(2H,m), 4.6-5.3(2H,m)
					N,	8.41	8.49	1660	
21	Benzoyl	IA	28	$C_{15}H_{13}N_2O_7Na$	C,	50.57	50.54	1785	2.4-3.3(4H,m), 4.1-5.6(3H,m),
					H,	3.68	4.72	1730	7.4-7.9(5H,m)
					N,	7.86	6.74	1660	
22	Nicotinyl	IA	25	$C_{14}H_{12}N_3O_7Na$ ($1/2H_2O$)	C,	45.91	45.90	1790	2.4-3.4(4H,m), 4.2-5.5(3H,m),
					H,	3.59	4.20	1730	7.59(1H,dd,J=8,5), 8.24(1H,dt,
					N,	11.47	11.11	1665	J=8,2), 8.72(1H,br.d,J=5),
									8.92(1H,br.s)
23	Phenylacetyl	IA	43	$C_{16}H_{15}N_2O_7Na$ ($1/5H_2O$)	C,	51.40	51.43	1790	2.3-3.3(4H,m), 3.69(2H,s),
					H,	4.15	4.58	1730	4.18(1H,m), 4.5-5.3(2H,m),
					N,	7.49	7.30	1660	7.38(5H,m)
24	Phenyl-malonyl*	IIA	19	$C_{17}H_{14}N_2O_9Na_2$ ($3/5H_2O$)	C,	45.67	45.54	1790	2.4-3.4(4H,m), 4.1-5.3(3H,m),
					H,	3.43	3.63	1730	4.60(1H,s), 7.41(5H,s)
					N,	6.27	6.49	1670	
25	Z-glycyl	IVA	29	$C_{18}H_{18}N_3O_9Na$ (H_2O)	C,	46.86	47.14	1790	2.3-3.4(4H,m), 3.8-5.2(3H,m),
					H,	4.37	4.42	1730	3.91(2H,s), 5.17(2H,s), 7.45
					N,	9.11	9.18	1670	(5H,s)
26	Phenoxy-acetyl	IA	43	$C_{16}H_{15}N_2O_8Na$ ($1/5H_2O$)	C,	49.29	49.25	1790	2.1-3.2(4H,m), 3.7-5.2(3H,m),
					H,	3.98	4.43	1730	4.54(2H,s), 7.00(3H,s), 7.31
					N,	7.19	6.94	1665	(2H,m), 8.67, 8.85(1H,d)
27	S-Mandelyl	IIA	33	$C_{16}H_{15}N_2O_8Na$ ($1/2H_2O$)	C,	48.62	48.46	1785	2.3-3.3(4H,m), 4.28(1H,t,J=9),
					H,	4.08	4.28	1730	4.5-5.3(2H,m), 5.29(1H,s),
					N,	7.09	7.22	1670	7.50(5H,s)
28	R-Mandelyl	IIA	21	$C_{16}H_{15}N_2O_8Na$ ($3/5H_2O$)	C,	48.39	48.46	1785	2.4-3.3(4H,m), 4.23(1H,t,J=9),
					H,	4.11	4.28	1730	4.5-5.3(2H,m), 5.25(1H,s),
					N,	7.05	7.22	1665	7.50(5H,s)
29	p-Hydroxy-mandelyl*	IIA	29	$C_{16}H_{15}N_2O_9Na$ ($1/2H_2O$)	C,	46.72	46.50	1790	2.3-3.4(4H,m), 4.1-4.5(1H,m),
					H,	3.92	4.03	1730	4.5-5.3(2H,m), 5.20,5.22(each
					N,	6.81	6.82	1660	0.5H,s), 6.97(2H,d,J=8), 7.38,
									7.40(each 1H,d)
30	p-Chloro-mandelyl*	IIA	28	$C_{16}H_{14}N_2O_8ClNa(1/2H_2O)$	C,	44.72	44.76	1780	2.3-3.3(4H,m), 4.1-5.3(3H,m),
					H,	3.52	3.54	1730	5.25, 5.28(each 0.5H,s),
					N,	6.52	6.69	1660	7.49(4H,s)
31	R-Phenyl-glycyl	IIB	43	$C_{16}H_{17}N_3O_7$ ($2/5H_2O$)	C,	51.86	52.10	1790	2.3-3.3(4H,m), 3.9-5.3(3H,m),
					H,	4.84	5.10	1730	5.25(1H,s), 7.4-7.7(5H,m)
					N,	11.34	11.48	1670	

Table 7. Antimicrobial activities of lactivicin analogs

Organism	MIC (μg/ml)[a]																
	16	17	1	18	19	20	21	22	23	24	25	26	27	28	29	30	31
S.aureus FDA 209P	100	6.25	6.25	1.56	50	6.25	3.13	3.13	0.2	12.5	0.39	0.39	6.25	1.56	3.13	1.56	>100
" 308 A-1	>100	12.5	25	6.25	>100	50	12.5	12.5	0.78	25	1.56	0.78	50	6.25	12.5	25	>100
" 1840	>100	12.5	25	12.5	>100	50	25	25	1.56	50	3.13	1.56	50	6.25	12.5	25	>100
E.coli NIHJ JC2	>100	25	>100	50	>100	100	>100	100	6.25	50	>100	50	>100	12.5	25	100	>100
" O-111	100	25	100	12.5	>100	100	50	25	3.13	25	25	6.25	100	6.25	12.5	25	>100
" T-7	100	25	100	50	>100	>100	>100	>100	12.5	50	>100	50	>100	12.5	25	>100	>100
C.freundii IFO 12681	>100	50	100	50	>100	>100	>100	100	12.5	50	>100	50	>100	12.5	25	>100	>100
K.pneumoniae DT	>100	50	>100	25	>100	>100	100	50	3.13	50	50	25	100	12.5	25	50	>100
K.oxytoca TN 1711	>100	50	>100	50	>100	>100	>100	100	12.5	50	>100	50	>100	25	50	>100	>100
E.cloacae IFO 12937	>100	100	>100	>100	>100	>100	>100	>100	>100	100	>100	>100	>100	>100	>100	>100	>100
S.marcescens IFO 12648	>100	100	>100	>100	>100	>100	>100	>100	>100	50	>100	>100	>100	50	100	>100	>100
P.vulgaris IFO 3988	>100	50	100	50	>100	>100	>100	>100	6.25	100	25	12.5	>100	12.5	50	>100	>100
P.mirabilis IFO 3849	>100	50	>100	100	>100	>100	>100	>100	25	>100	>100	100	>100	50	100	>100	>100
M.morganii IFO 3168	>100	100	>100	>100	>100	>100	>100	>100	100	50	>100	>100	>100	50	100	>100	>100
P.aeruginosa IFO 3455	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
" U 31	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
" GN 3407	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
A.calcoaceticus IFO 13006	>100	6.25	50	100	>100	50	>100	>100	50	100	>100	>100	>100	>100	100	50	>100
S.aureus FDA 209P	100	3.13	6.25	1.56	50	6.25	3.13	3.13	0.2	6.25	0.39	0.39	6.25	1.56	1.56	1.56	>100
S.pyogenes E-14	50	1.56	1.56	1.56	50	6.25	1.56	0.78	<0.1	12.5	0.39	0.20	3.13	0.39	0.78	0.78	>100
" S-8	50	1.56	1.56	1.56	25	3.13	1.56	0.78	<0.1	12.5	0.78	0.20	3.13	0.39	0.78	0.78	>100
S.mitis America	100	6.25	12.5	6.25	100	25	25	25	0.2	50	3.13	0.39	12.5	1.56	3.13	1.56	>100
E.faecium IFO 3128	>100	50	>100	>100	>100	>100	>100	>100	50	>100	100	50	>100	>100	>100	>100	>100
S.pneumoniae Type I	50	1.56	6.25	1.56	100	12.5	3.13	6.25	<0.1	12.5	0.78	0.39	6.25	0.78	1.56	1.56	>100
C.diphtheriae Tront	25	0.78	0.78	0.20	6.25	1.56	0.39	<0.1	<0.1	3.13	0.39	<0.1	0.78	0.39	0.78	0.78	50
B.bronchiseptica Sagami	>100	50	>100	>100	>100	>100	>100	>100	25	>100	>100	>100	>100	100	100	>100	>100
P.aeruginosa P9	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
ED_{50}(mg/kg)[b], sc	>100	16.0	44.0	>100	>100	>100	28.6	66.0	21.1	>100	44.9	28.1	>100	21.5	14.9	22.3	>100
Control (LTV)	9.92	29.1		55.9	14.5	14.4	29.1	40.5	43.4	50.0	40.5	9.92	50.0	18.9	14.4	14.5	29.1

a, Inoculum size; 10^8 cfu/ml, Medium; Trypticase soy agar (TSA)-Upper column, or 10% Horse serum/TSA-Lower column.
b, Intraperitoneal infection with S. aureus 308 A-1.

Table 8. Protective effects of lactivicin analogs in infected mice

Antibiotic	Organism	MIC (μg/ml)	ED_{50} (mg/kg)[a]
Lactivicin (Na)	S. aureus 308A-1	25	25.4 (sc)
	S. pyogenes E-14	1.56	2.05 (sc)
	E. coli O-111	50	71.0 (sc)
N-Phenylacetyl-4ALA (Na)	S. aureus 308A-1	0.78	21.1 (sc)
	S. pyogenes E-14	<0.1	0.65 (sc)
	E. coli O-111	3.13	17.7 (sc)
N-Phenylacetyl-4ALA pivaloyloxymethylester	S. aureus 308A-1	-	17.7 (po)
	S. Pyogenes E-14	-	1.39 (po)
	E. coli O-111	-	40.4 (po)

a, Intraperitoneal infection.

Experimental

The specific optical rotations, UV and CD (JASCO J-20A with DP-501N) spectra were measured at 23-25°C in water unless otherwise stated. The IR spectra were measured in KBr pellets. The δ values in the ^1H NMR (90 MHz) spectra were recorded in ppm downfield from TMS using a Varian EM-390 spectrometer unless otherwise stated. The SI-mass spectra (MS) were measured with a Hitachi M-80 A mass spectrometer with xenon ion beam source. The samples were suspended in glycerol.

Isolation of 1: The culture broth of E. lactamgenus YK-258 was filtered at below 5°C through Hyflo-Super Cel^R. The filtrate (4360 liters) was loaded onto a column of Amberlite IRA-402^R (Cl^- type, 400 liters) and the active substance was eluted with 2% NaCl (2000 liters). The eluate was applied to activated carbon chromatography (160 liters) eluting with 8% iso-BuOH (1640 liters). The eluate was chromatographed on IRA-68^R (Cl^- type, 40 liters) and active fractions were eluted with 1% NaCl (2000 liters). The eluate was desalted with activated carbon (80 liters) and concentrated. The concentrate was lyophilized to give a powder containing 57% of **1** (620 g). The powder (5 g) was dissolved in water, loaded onto QAE-Sephadex^R (Cl^- type, 200 ml) and eluted with 0.03M NaCl. The active fractions were desalted with activated carbon (500 ml), concentrated and freeze-dried to give the sodium salt of **1** (2.5 g) as a white powder.

p-Nitrobenzylation of 1 (2A and 2B): To a solution of **1** (400 mg) in DMF (4 ml) was added Et_3N (0.1 ml) and p-nitrobenzyl bromide (800 mg). The resulting solution was stirred for 3 hours at room temperature. The reaction mixture was diluted with AcOEt, washed with 0.01M P.B.(pH 6.3) and water, evaporated and treated with AcOEt-petroleum benzin to give a mixture of **2A** and **2B**. Chromatography on Sephadex LH-20^R, using AcOEt-MeOH (19:1) as an eluant, gave **2A** (105 mg), **2B** (67 mg) and a mixture (280 mg).

Methylation of 1 (3A and 3B): To a solution of the sodium salt of **1** (2.0 g) in DMF (10 ml) was added MeI (0.8 ml) and the mixture was stirred for 5 hours at room temperature. The concentrated residue was suspended in water and extracted with AcOEt and CH_2Cl_2. The organic layer was concentrated and pulverized from AcOEt-ether to give **3** (1.36 g). The mixture (1.0 g) was chromatographed on silica gel (50 g) using $CHCl_3$-MeOH (50:1 to 20:1) as a developing solvent. The pure fractions containing **3B** were concentrated and then crystallized from AcOEt to give **3B** (81 mg) as colorless crystals. The fractions containing **3A** were concentrated and crystallized from EtOH-ether to give **3A** (277 mg) as colorless crystals. The purity of this sample was 85 % by HPLC. A mixture of **3A** and **3B** was also obtained (246 mg).

Alkaline hydrolysis of 1 (4-6): A solution of **1** (1.0 g) in 0.1N NaOH (48 ml) was stirred for 2 hours at room temperature. After addition of 0.1N NaOH (7 ml), the solution was stirred for a further 1 hour at room temperature. The reaction mixture was diluted with water (250 ml) and chromatographed on QAE-Sephadex A-25^{R} (AcO^{-} type, 100 ml) using 0.02-0.03M $AcONH_4$ as the eluant. The fractions giving a single peak by HPLC were collected and passed through Dowex 50W x 2^{R} (50-100 mesh, H^{+} type, 150 ml). The effluent was concentrated and freeze-dried to give **4** (280 mg) as a white powder. An analytical sample was obtained by crystallization from EtOH, mp 174-175°C (dec), $[\alpha]_D$ -70.1°(c 0.50). SI-MS: m/z 145 $(M+H)^{+}$, UV : 225 nm (ε3700), IR: 1710, 1665, 1565 cm^{-1}, ^1H NMR (DMSO-d_6): δ 1.83 (3H, s), 3.87 (1H, dd, J=8, 9), 4.46 (1H, t, J=8), 4.72 (1H, m), 8.35 (1H, d, J=7), 11.3 (1H, br). Anal. Calcd. for $C_5H_8N_2O_3$: C, 41.67; H, 5.59; N, 19.44; O, 33.30. Found: C, 41.73; H, 5.56; N, 19.16.

A solution of **1** (1.0 g) in 0.1N NaOH (42 ml) was stirred for 1 hour at room temperature. After addition of 0.1N NaOH (10 ml), the solution was again stirred for 1 hour at room temperature. The reaction mixture was adjusted to pH 7.6, concentrated and freeze-dried to give crude **5** as a powder (1.17 g). To a solution of crude **5** (390 mg) in 95% EtOH (10 ml) was added 2,4-dinitrophenyl hydrazine (400 mg) in H_3PO_4-EtOH (3:2, 8 ml). The resulting solution was allowed to stand for 30 min at room temperature and was then concentrated. The concentrate was diluted with H_2O and extracted with AcOEt. The organic layer was extracted with 2% $NaHCO_3$. After adjustment to pH 1.5, the aqueous layer was re-extracted with AcOEt. The extract was washed with brine and concentrated to afford **6** as a white powder (180 mg). An analytical sample was obtained by crystallization from AcOEt-Et_2O, mp 215-216°C (dec). IR: 1720, 1620, 1500, 1410, 1340 cm^{-1}, ^1H NMR (CD_3OD): δ 2.70 (2H, m), 2.97 (2H, m), 8.08 (1H, d, J=9), 8.36 (1H, dd, J=3,9), 9.00 (1H, d, J=3). Anal. Calcd. for $C_{11}H_{10}N_4O_8$: C, 40.50; H, 3.09; N, 17.17; O, 39.24. Found: C, 40.29; H, 2.81; N, 17.12. The physico-chemical data were identical with those of the authentic sample, synthesized from α-ketoglutaric acid.

Acid hydrolysis of 1 (8): A solution of **1** (400 mg) in H_2O (100 ml) was passed through Dowex 50W x 2^{R} (50-100 mesh, H^{+} type, 40 ml). The effluent was concentrated and freeze-dried to give **8** as a white powder (396 mg), $[\alpha]_D$ +17.3°(c 0.52). SI-MS: m/z 291 $(M+H)^{+}$, UV: 220 nm (ε5,300, sh), IR: 1735, 1625, 1545 cm^{-1}, ^1H NMR (400 MHz, D_2O): δ 2.05 (3H, s), 2.63 (2H, t, J=7.3), 2.81 (2H, t, J=7.3), 4.60 (2H, d, J=4.9), 4.77 (1H, t, J=4.9). Anal. Calcd. for $C_{10}H_{14}N_2O_8$: C, 41.38; H, 4.86; N, 9.65; O, 44.11. Found: C, 41.51; H, 4.96; N, 9.24.

p-Nitrobenzylation of 8 (9): To a solution of **8** (100 mg) in DMF (5 ml) were added Et_3N (100 μl) and p-nitrobenzyl bromide (300 mg) and the mixture was stirred for 5 hours at room temperature. The reaction mixture was diluted with AcOEt, washed with water and then concentrated. The residue was treated with petroleum benzine to give crude **9** (200 mg). This was chromatographed on silica gel (20 g) using $CHCl_3$:MeOH (49:1) as the eluant. The pure fractions were concentrated to afford **9** as a hygroscopic powder (170 mg), $[\alpha]_D$ -9.6°(c 0.50, $CHCl_3$). UV (CH_3CN): 214 nm (ε27,600), 266 nm (31,600), IR: 1745, 1685, 1610, 1525 cm^{-1}, ^1H NMR (400 MHz, $CDCl_3$): δ 2.01 (3H, s), 2.65 (2H, t, J=7.1), 2.81 (1H, dt, J=13.8, 7.1), 2.88 (1H, dt, J=13.8, 7.1), 4.58 (1H, dd, J=3.3, 11.7), 4.75 (1H, dd, J=3.3, 11.7), 4.95 (1H, dt, J=8.1, 3.3), 6.65 (1H, d, J=8.1) and PNB signals. Anal. Calcd for $C_{31}H_{29}N_5O_{14}.1/2H_2O$: C, 52.84; H, 4.29; N, 9.94; O, 32.93. Found: C, 52.99; H, 4.22; N, 9.82.

Methylation of 8 (10): To a solution of **8** (150 mg) in MeOH (5 ml) was added a solution of CH_2N_2/Et_2O and the mixture was allowed to stand for 30 min at room temperature. After concentration, the residue was diluted with 2% $NaHCO_3$ (30 ml) and extracted with AcOEt (60 ml). The extract was washed with water and concentrated to give **10** as a white powder (167 mg). An analytical sample was crystallized from Et_2O, mp 81-82°C, $[\alpha]_D$ +56.0°(c 0.51, $CHCl_3$). UV (MeOH): 223 nm(ε8800), IR: 1745, 1735, 1650, 1555 cm^{-1}, ^1H NMR ($CDCl_3$): δ 2.05 (3H, s), 2.55 (2H, m), 2.82 (2H, m), 3.65 (3H, s), 3.75 (3H, s), 3.82 (3H, s), 4.47 (1H, dd, J=3, 12), 4.70 (1H, dd, J=3, 12), 4.87 (1H, m), 6.73 (1H, d, J=8). Anal. Calcd for $C_{13}H_{20}N_2O_8$: C, 46.99; H, 6.07; N, 8.43; O, 38.52. Found: C, 46.92; H,

6.15; N, 8.34.

Hydrolysis of 1 by β-lactamase (8, 9): To a solution of **1** (100 mg) in 0.01M P.B.(pH 6.8, 100 ml) was added a partially purified cephalosporinase prepared from Enterobacter cloacae IFO 12937 (21 unit/ml, 10 ml) and the solution was allowed to stand for 15 hours at 4°C. The reaction mixture was desalted with carbon (30 ml) to give crude **8** (122 mg). Crude **8** was esterified as described above and afforded **9** (150 mg), which was identical with the product obtained by acid hydrolysis of **1**.

Hydrogenolysis of 8 (11, 12, 13): Compound **8** (300 mg) was hydrogenated over Pt-black (150 mg) in 50% aq. AcOH (10 ml). The catalyst was filtered off and the filtrate was evaporated to dryness. The residue dissolved in water (50 ml) was applied to a column of Dowex 50W x 2^R (50-100 mesh, H^+ type, 30 ml) and eluted with 0.5% NH_4OH. The fractions monitored by TLC were combined, concentrated and freeze-dried to yield a white powder of **11** (136 mg). An analytical sample was obtained by crystallization from water, mp 186-187°C (dec), $[\alpha]_D$ +0.9°(c 1.0, 6N HCl) [cf. L-Glu:$[\alpha]_D$ +31.4°(6N HCl)]. IR: 3070, 1640, 1515 cm^{-1}, ^1H NMR (D_2O): δ 2.37 (2H, m), 2.67 (2H, m), 4.00 (1H, t, J=6). Anal. Calcd. for $C_5H_9NO_4$: C, 40.82; H, 6.17; N, 9.52; O, 43.50. Found: C, 40.66; H, 6.16; N, 9.61. The Rf values of TLC, mp, IR and ^1H NMR spectra were identical with those of the authentic sample.

The effluent was freeze-dried to give crude **12** as a powder (184 mg). To a solution of crude **12** in DMF (1 ml) was added Et_3N (350 μl) and p-nitrobenzyl bromide (540 mg). The mixture was stirred for 3 hours at room temperature. The reaction mixture was diluted with H_2O and extracted with AcOEt. The extract was washed with 2% $NaHCO_3$ and brine and evaporated to dryness. The residue was subjected to column chromatography on silica gel using a mixture of AcOEt-acetone (19:1) as an eluant. The fractions containing the desired compound were combined and evaporated to afford **13** as a white powder (199 mg). An analytical sample was obtained by crystallization from AcOEt-Et_2O-petroleum ether, mp 86-89°C, $[\alpha]_D$ -15.9°(c 0.49, MeOH)[cf. synthetic sample: $[\alpha]_D$ -15.6°(c 0.57, MeOH)]. IR: 1770, 1740, 1655, 1625, 1520 cm^{-1}, ^1H NMR (DMSO-d_6): δ 1.87 (3H, s), 3.70 (2H, m), 4.40 (1H, m), 5.03 (1H, t, J=5), 7.62 (2H, d, J=8), 8.22 (3H, d, J=8). Anal. Calcd. for $C_{12}H_{14}N_2O_6$: C, 51.06; H, 5.00; N, 9.92; O, 34.01. Found: C, 51.11; H, 4.81; N, 9.71. The Rf values of TLC, mmp, IR and ^1H NMR spectra were identical with those of the authentic sample synthesized from L-serine.

Benzhydryl ester of 1 (14): To a suspension of **1** (31 g) in THF (150 ml) was added the solution of diphenyl diazomethane[10] in THF (150 ml). Into the solution cooled to 0°C was added dropwise 2N HCl (60 ml) and the mixture was stirred for 1 hour at room temperature. After addition of 2N HCl (10 ml), the reaction mixture was stirred for 1 hour and added into CH_2Cl_2 (3 liters). After washing the solution was concentrated and pulvirized from Et_2O to give **14** as a white crystalline powder (28 g, a mixture of A and B), mp 153-155°C(dec), $[\alpha]_D$ +9.2°(c 0.52, $CHCl_3$). UV (MeOH): 220 nm(ε12,500), 250-260(1,200), IR: 1800, 1780, 1755, 1705, 1690, 1540 cm^{-1}, ^1H NMR ($CDCl_3$): δ 1.97 (3H, s), 2.1-3.5 (4H, m), 3.8-4.2 (1H, m), 4.5-5.1 (2H, m), 6.1-6.4 (1H, br), 6.97 (1H, s), 7.3-7.4 (10H, m). Anal. Calcd for $C_{23}H_{22}N_2O_7$: C, 63.01; H, 5.06; N, 6.39; O, 25.54. Found: C, 62.83; H, 5.32; N, 6.28.

The mixture (1.8 g) was chromatographed on a column of silica gel (180 ml) using $CHCl_3$:MeOH (97:3) as an eluant. The fractions were separately concentrated to afford colorless crystals of **14 A** (433 mg), **14 B** (400 mg) and a mixture of **14 A** and **B** (476 mg). **14 A**: mp 97-135°C (gradually foamed and decomposed), $[\alpha]_D$ +44.2°(c 0.505, $CHCl_3$). EI-MS: m/z 438 (M^+), UV (MeOH): 220 nm (ε12,700, sh) and 250-260 (1310, sh), IR: 1800, 1780, 1760, 1685 cm^{-1}, ^1H NMR (100 MHz, $CDCl_3$-DMSO-d_6): δ 1.98 (3H, s), 2.2-3.4 (4H, m), 4.10 (1H, dd, J=8, 10), 4.4-5.0 (2H, m), 6.93 (1H, s), 7.3-7.5 (10H, m), 8.27 (1H, d, J=7). Anal. Calcd. for $C_{23}H_{22}N_2O_7$: C, 63.01; H, 5.06; N, 6.39; O, 25.54. Found: C, 62.62; H, 5.06; N, 6.32. **14 B**: mp 157-160°C (dec), $[\alpha]_D$ -28.8°(c 0.5, $CHCl_3$). EI-MS: m/z 438 (M^+), UV (MeOH): 220 nm (ε13,100, sh) and 250-260 (1,140, sh), IR: 1815, 1780, 1735, 1705 cm^{-1}, ^1H NMR (100 MHz, $CDCl_3$): δ 1.98 (3H, s), 2.2-3.4 (4H, m), 4.03 (1H, dd, J=8,10), 4.6-5.2 (2H, m), 6.32 (1H, d, J=5), 6.96 (1H, s), 7.2-7.5 (10H, m). Anal. Calcd for $C_{23}H_{22}N_2O_7$: C, 63.01; H, 5.06; N, 6.39; O, 25.54. Found: C, 63.11; H, 5.13; N, 6.30.

Deacetylation of **14** (15): To a suspension of **14** (657 mg) in CH_2Cl_2 (60 ml) were added pyridine (1.46 ml) and PCl_5 (936 mg) at -20°C and the solution was stirred for 50 min at -15°- 10°C. After cooling to -40°C, MeOH (9 ml) was added to the reaction mixture and the solution was stirred for 30 min at -15°- -10°C and for 1 hour at room temperature. To the reaction mixture was added N HCl (30 ml) and the solution was stirred for 45 min at room temperature. After adjustment to pH 7.9, the reaction mixture was diluted with water, extracted with CH_2Cl_2 and the extract was concentrated. The residue was treated with Et_2O to give **15** (506 mg), $[\alpha]_D$ -15.2°(c 0.50, $CHCl_3$). UV (MeOH): 220 nm (ε13,300), 250-260 (1,300), IR: 1800, 1780, 1740, 1600 cm^{-1}, ^1H NMR ($CDCl_3$): δ 2.2-3.5 (4H, m), 3.7-4.0 (2H, m), 4.4-4.6 (1H, m), 6.97 (1H, s), 7.2-7.4 (10H, m). Anal. Calcd. for $C_{21}H_{20}N_2O_6$: C, 63.63; H, 5.09; N, 7.07; O, 24.21. Found: C, 63.63; H, 5.05; N, 7.02.

The compounds **15 A** (340 mg) and **15 B** (200 mg) were prepared in the same way as described above from **14 A** (657 mg) and **14 B** (657 mg), respectively. **15 A**: $[\alpha]_D$ +30.8°(c 0.50, $CHCl_3$). UV (MeOH): 218 nm (ε13,700), 250-260 (1,300), IR: 1800, 1765, 1740, 1600 cm^{-1}, ^1H NMR ($CDCl_3$):δ2.2-3.5 (6H, m, 2H disappeared after addition of D_2O), 3.6-4.1 (2H, m), 4.3-4.6 (1H, m), 6.97 (1H, s), 7.2-7.3 (10H, m). Anal. Calcd. for $C_{21}H_{20}N_2O_6$: C, 63.63; H, 5.09; N, 7.07; O, 24.22. Found: C, 62.97; H, 4.87; N, 6.68. **15 B**: $[\alpha]_D$ -37.4°(c 0.50, $CHCl_3$). UV (MeOH) : 218 nm (ε14,300), 250-260 (1,400), IR:ν 1800, 1770, 1735, 1600 cm^{-1}, ^1H NMR ($CDCl_3$): δ 2.2-3.5 (6H, m, 2H disappeared after addition of D_2O), 3.6-4.1 (2H, m), 4.3-4.6 (1H, m), 6.97 (1H, s), 7.2-7.3 (10H, m). Anal. Calcd for $C_{21}H_{20}N_2O_6$: C, 63.63; H, 5.09; N, 7.07; O, 24.22. Found: C, 63.24; H, 5.09; N, 6.92.

Debenzhydration of **15** (16): To a suspension of **15** (396 mg) in CH_2Cl_2 (10 ml) were added anisole (434 µl) and CF_3COOH (924 µl) at -20°C and the solution was stirred for 40 min at -20°- -10°C. The reaction mixture was diluted with CH_2Cl_2 (100 ml) and extracted with 0.1M P.B.(pH 7.3, 100 ml). The aqueous layer was concentrated and adjusted to pH 6.0. The solution was chromatographed on carbon (50 ml) using 8% iso-BuOH as the eluant. The fractions were concentrated and freeze-dried to give **16** as a white powder (143 mg), $[\alpha]_D$ -11°(c 0.1). FD-MS: 231 $(M+H)^+$, UV: 221 nm (ε3,700), IR: 1800, 1760, 1740, 1670, 1580 cm^{-1}, ^1H NMR (400 MHz, D_2O): δ 2.52 (1H, m), 2.72 (1H, m), 2.91 (1H, m), 3.08 (1H, m), 4.35 (1H, m), 4.56 (1H, m), 4.80 (1H, m). Anal. Calcd. for $C_8H_{10}N_2O_6 \cdot 1/2H_2O$: C, 40.17; H, 4.64; N, 11.71; O, 43.48. Found: C, 40.42; H, 4.36; N, 11.65.

When **16** was crystalized from water, **16 A** was obtained as colorless needles, mp 177-181°C(dec), $[\alpha]_D$ +124°(c 0.1). UV: 221 nm (ε3,500), IR: 1800, 1735, 1660, 1580 cm^{-1}, ^1H NMR (400 MHz, D_2O): δ 2.52 (1H, m), 2.72 (1H, m), 2.91 (1H, m), 3.08 (1H, m), 4.34 (1H, m), 4.55 (1H, m), 4.78 (1H, m). Anal. Calcd. for $C_8H_{10}N_2O_6$: C, 41.75; H, 4.38; N, 12.17; O, 41.71. Found: C, 41.57; H, 4.39; N, 12.11.

The compound **15 B** (180 mg) was hydrogenated over 10% Pd-C (90 mg) in THF:H_2O (1:1, 18 ml). The catalyst was filtered off and the filtrate was washed with Et_2O. The aqueous layer was concentrated and freeze-dried to give **16 B** as a white powder (77 mg), $[\alpha]_D$ -97°(c 0.2). The purity of **16 B** was 80% by HPLC. UV: 221 nm (ε3,200), IR: 1800, 1760, 1670, 1570 cm^{-1}, ^1H NMR (400 MHz, D_2O): δ 2.52 (1H, m), 2.72 (1H, m), 2.90 (1H, m), 3.08 (1H, m), 4.44 (1H, m), 4.68 (1H, m), 4.86 (1H, m).

N-Acylation:

Method I: To a suspension of the amino derivative **15** (1.0 g, 2.53 mmole) in CH_2Cl_2 (20 ml) were added DMA (1.0 ml, 10.8 mmole) and n-butyryl chloride (265 µl, 2.53 mmole) and the mixture was stirred for 30 min at 0°C. Into the reaction mixture was added CH_2Cl_2 (200 ml) and the organic layer was washed with 2% $NaHCO_3$ and concentrated to give N-n-butyryl 4-ALA BH ester (860 mg) as a white powder.

Method II: To a suspension of **15** (1.5 g, 3.79 mmole) in DMF (15 ml) were added HOBT (525 mg, 3.89 mmole), MZ-D-phenylglycine (1.20 g, 3.81 mmole) and DCC (792 mg, 3.84 mmole). The reaction mixture was stirred for 30 min at 0°C and then concentrated. The residue was suspended in AcOEt (75 ml) and the precipitate was filtered off. The filtrate was washed with 0.1N HCl and 5% $NaHCO_3$. The organic layer was concentrated and chromatographed on silica gel. Elution with AcOEt-hexane (1:1) gave N-D-phenylglycyl 4-ALA BH ester (1.77 g) as a white powder.

Method III: To a solution of **15** (800 mg, 2.02 mmole) in CH_2Cl_2 (40 ml) were added pyridine (1.60 ml, 19.8 mmole) and acetic-formic anhydride (1.0 ml). The reaction mixture was stirred for 1.5 hours at 0°C and washed with N HCl and 2 % $NaHCO_3$. The solution was dried and concentrated to give N-formyl 4-ALA BH ester (720 mg) as a white powder.

Method IV: To a solution of methyl chloroformate (196 µl, 2.54 mmole) in THF (20 ml), cooled to -10°C, was added dropwise a soln of Z-glycine (531 mg, 2.54 mmole) and Et_3N (357 µl, 2.56 mmole) in THF (10 ml) during 20 min. Then, **15** (1.0 g, 2.53 mmole) suspended in THF (20 ml) was added slowly. The reaction mixture was stirred for 2 hr at -10°C. After concentration, the residue was dissolved in AcOEt (100 ml) and washed with 0.1N HCl and 5% $NaHCO_3$. The solution was dried and concentrated to give N-Z-glycyl 4-ALA BH ester (1.24 g) as a white powder.

Deprotection of BH ester

Method A: A solution of N-n-butyryl 4-ALA BH ester (760 mg, 1.68 mmole) in THF (30 ml) and 0.1M P.B. (pH 7.5, 20 ml) was hydrogenated over 10% Pd-C (760 mg) under a hydrogen atmosphere for 50 min at room temperature. The filtered reaction mixture was concentrated to remove THF and washed with ether. The aqueous layer was desalted with Diaion HP-20 (100 ml) and freeze-dried to give N-n-butyryl 4-ALA sodium salt (295 mg) as a white powder.

Method B: To a solution of N-D-phenylglycyl 4-ALA BH ester (1.0 g, 1.44 mmole) in CH_2Cl_2 (50 ml) were added anisole (940 µl, 8.65 mmole) and trifluoroacetic acid (2.0 ml, 26 mmole) and the reaction mixture was stirred for 6.5 hours at -10°C. The reaction mixture was extracted with 0.1M P.B. (pH 7.3, 250 ml) and the aqueous layer was adjusted to pH 5.3. The solution was desalted with HP-20 and freeze-dried to give N-D-phenylglycyl 4-ALA sodium salt (410 mg) as a white powder.

N-Phenylacetyl 4-ALA POM ester: To a solution of N-phenylacetyl 4-ALA sodium salt (3.5 g, 9.46 mmol) in DMF (10 ml) was added pivaloyloxymethyl chloride (2.73 ml, 18.9 mmol). The reaction mixture was stirred for 22 hours at room temperature and then concentrated. The residue was diluted with EtOAc (200 ml) and washed with water. The solution was dried and concentrated. The residue was pulverized from n-hexane to give N-phenylacetyl 4-ALA POM ester (3.5 g) as a white powder. IR: 1785, 1750, 1660 cm^{-1}, EI-MS: m/z 462 (M^+), ^1H NMR ($CDCl_3$): δ 1.20 (9H, s), 2.2-3.4 (4H, m), 3.60 (2H, s), 3.8-4.2 (1H, m), 4.5-5.0 (2H, m), 5.82 (2H, s), 6.0-6.3 (1H, m), 7.30 (5H, m) ppm. Anal. Calcd. for $C_{22}H_{26}N_2O_9$: C, 57.14; H, 5.67; N, 6.06. Found: C, 56.98; H, 5.73; N 6.12.

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