LAGUNAMYCIN, A NOVEL 5-LIPOXYGENASE INHIBITOR

I. TAXONOMY, FERMENTATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL CHARACTERISTICS

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(Received for publication December 11, 1992)

Lagunamycin, a novel 5-lipoxygenase inhibitor, was isolated from the culture broth of *Streptomyces* sp. AA0310. This compound showed potent rat 5-lipoxygenase inhibitory activity (IC_{50} 6.08 μM) without lipid peroxidation.

As previously described,[1~3] a 5-lipoxygenase (5-LPO) inhibitor screen which was composed of a rat 5-lipoxygenase assay and an automated HPLC system were successfully employed for analysis of new 5-LPO inhibitory compounds in fermentation broths. In the continued search for 5-LPO inhibitors, *Streptomyces* sp. AA0310 was found to produce a new antibiotic designated lagunamycin. Lagunamycin was produced in good yield by addition of high porous polymer resin in fermentation medium. The active principle was effectively extracted with ethyl acetate from the fermentation broth under acidic conditions and purified by silica gel column chromatography followed by reversed phase silica gel column chromatography. Lagunamycin belonged to a novel family of antibiotics containing a diazo group with potent inhibition against RBL-1 cell's 5-LPO without lipid peroxidation. In this paper, the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties and biological activities of lagunamycin are reported.

Materials and Methods

Taxonomy

Strain AA0310 was isolated from a soil sample collected at College, Laguna, Philippines. The taxonomic studies were carried out mostly according to the procedure of the International Streptomyces Project (ISP) using 16 media as recommended by SHIRLING and GOTTLIEB[4], WAKSMAN[5] and ARAI[6]. Incubation was carried out at 28°C for 27 days. Morphological observations of the culture grown on inorganic salts - starch agar (ISP-4) were recorded with light and scanning electron microscopes. Color assignment was made using the Manual of Color Names (Japan Color Enterprise Co., Ltd., 1987).

Cell wall analysis was performed by the methods of BECKER *et al.*[7,8]. Phospholipid and mycolate compositions were determined by the methods of LECHEVALIER *et al.*[9,10] and MINNIKIN *et al.*[11], respectively. Menaquinone was analyzed by the procedure of COLLINS *et al.*[12]. Fatty acid type was determined by the method of SUZUKI *et al.*[13].

Temperature range for growth was examined on inorganic salts - starch agar (ISP-4) using a temperature gradient incubator TN-3 (Toyo Kagaku Sangyo Co., Ltd.).

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HPLC Analysis

A 3-ml portion of the whole broth was extracted with the same volume of ethyl acetate at pH 2.0 and then centrifuged at 3,000 rpm for 15 minutes. The ethyl acetate extract (1 ml) was concentrated to dryness *in vacuo*. The residue was dissolved in 1 ml of dimethyl sulfoxide and was filtered (Nihon Millipore Kogyo K.K.; FH type; pore size: 0.5 μm). This filtrate (10 μl) was analyzed by HPLC on a COSMOSIL packed column (5C18-AR; Nacalai Tesque, Inc.) using acetonitrile - water (40 : 60; v/v) at a flow rate of 1.0 ml/minute under monitoring at 254 nm.

Physico-chemical Properties

The IR and UV spectra were determined on a JASCO IR-810 and a UVIDEC-610C spectrometer, respectively. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JMN-GX400 with $CDCl_3$ as an internal standard. The MS spectra were obtained with a JEOL JMS-AX505H mass spectrometer. Elemental analysis was carried out with a Perkin-Elmer 240C Elemental Analyzer.

Sample Preparations

Lagunamycin was dissolved in 10% DMSO in water. The small amount of DMSO (final concentration of 1%) employed as a vehicle had no effect on 5-LPO activity and lipid peroxidation. Epocarbazolin A was also dissolved in 10% DMSO in water.

5-LPO Inhibitory Assay

The assay method employed in this paper was a slight modification of the original procedure of HOOK *et al.*[1]. RBL-1 cells were grown for 4 days at 37°C and 5% CO_2 in RPMI1640 minimal essential medium (GIBCO) with 20% heat-inactivated fetal bovine serum (GIBCO). They were harvested by centrifugation and washed twice with 28.5 mM PBS, pH 7.0, containing 1 mM EDTA. The cells were resuspended in the same buffer and sonicated. Centrifugation at 13,000 $\times g$ yielded the supernatant, which was stored at -80°C as the assay enzyme. For the routine enzyme assay, the thawed supernatant was diluted to a desired specific enzyme activity with 28.5 mM PBS containing, 1 mM EDTA, 0.9 mM ATP and 0.9 mM glutathione. The enzyme dilution (110 μl) was preincubated at 37°C for 2.5 minutes, followed by addition of 5 μl of substrate ((2 mM arachidonic acid plus 25 mM $CaCl_2$ in ethanol - water (3 : 1)). After incubation for 5 minutes the reaction was terminated by addition of 135 μl of ethanol, and the solution was centrifuged for removal of precipitated protein. The 5-hydroxyeicosatetraenoic acid (5-HETE) in the supernatant was analyzed by HPLC (Ranin Dynamax C18, i.d. 4.6 $\times$ 50 mm) with a solvent of 85% methanol in 29.2 mM lithium acetate buffer, pH 6.3, at a flow rate of 1 ml/minute. Eluate was monitored at 230 nm with a Waters 481 detector and the peak areas were calculated with a Hewlett Packard 3396A integrator.

Iron Dependent Lipid Peroxidation Assay

This assay was performed in a microplate. Reaction mixture (200 μl) consisted of 50 mM Tris-HCl (pH 7.5), 5 mM $FeSO_4$ and a test sample. The reaction was started by addition of 1 μg of arachidonic acid and 0.5% - 2-thiobarbituric acid. After incubation at 37°C for 3 hours, the absorbance was measured at 540 nm.

Antimicrobial activity

Antimicrobial activity of lagunamycin was determined by the serial 2-fold agar dilution method using Nutrient Agar medium (pH 7.0, Eiken). MICs were read in μg/ml after overnight incubation at 32°C.

Results and Discussion

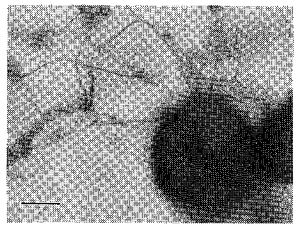
Taxonomy

Morphology

Strain AA0310 formed grayish to olive gray aerial mycelia on various agar media. Aerial mycelium formed sprial chains (*Spirales*; more than 20 spores per chain) and numerous large sclerotia were observed

Fig. 1. Light micrograph of sclerotia (A) and the scanning electron micrograph of a spore chain (B) of strain AA0310 grown on ISP medium No. 4 at 28°C for 27 days.

(A) Bar represents: 1 μm (B) Bar represents: 2.8 μm



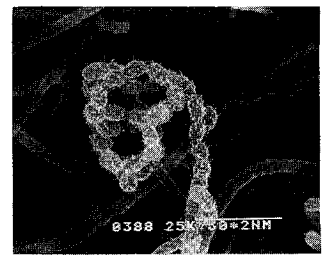


Table 1. Cultural characteristics of strain AA0310.

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose nitrate agar (Waksman med. No. 1)	Grayish brown (120)	Grayish brown (114)	Beige gray (401), cottony, good	None
Glycerol nitrate agar	Dark grayish purple (379)	Reddish black (427)	Reddish gray (409), powdery	None
Glucose asparagine agar (Waksman med. No. 2)	Olive (165)	Olive (167)	Olive gray (411), powdery	None
Yeast extract-malt extract agar (ISP med. No. 2)	Reddish black (427)	Reddish black (427)	Olive gray (411), powdery	Dark grayish brown (123)
Oat meal agar (ISP med. No. 3)	Reddish black (427)	Reddish black (427)	Olive gray (411), powdery, scant	Brownish olive (163)
Inorganic salts-starch agar (ISP med. No. 4)	Reddish black (427)	Reddish black (427)	Grayish olive (168), powdery	Reddish black (427)
Glycerol asparagine agar (ISP med. No. 5)	Reddish black (427)	Reddish black (427)	Olive gray (411), powdery	Reddish black (427)
Tyrosine agar (ISP med. No. 7)	Reddish black (427)	Reddish black (427)	Grayish olive (168), powdery, scant	Reddish black (427)
Nutrient agar (Waksman med. No. 14)	Dull yellow (150)	Dull yellow (150)	None	None~soft yellow (147)
Yeast starch agar	Brownish black (428)	Brownish black (428)	Olive gray (411), powdery, scant	Dark brownish gray (419)
Glucose-Glutamate agar	Greenish black (430)	Greenish black (430)	None	None
Gauze No. 1 agar	Brownish black (428)	Brownish black (428)	Yellowish gray (402), powdery, good	Brownish black (428)
Oatmeal-yeast extract agar	Reddish black (427)	Reddish black (427)	None	None
Peptone corn agar	Brownish black (428)	Olive (167)	Olive gray (410), powdery	Olive (165)
BENNETT's agar	Brownish black (428)	Brownish black (428)	Olive gray (410)~white (398), powdery, scant	Reddish black (427)
Maltose-BENNETT's agar	Reddish black (427)	Reddish black (427)	Olive gray (410), powdery	None

(Fig. 1-A). Scanning electron micrographs show that spores are oblong in shape and $0.5 \sim 0.7 \times 0.8 \sim 1.0\ \mu m$ in size with spiny-surface (Fig. 1-B). No sporangia and zoospores were observed.

Cultural characteristics

The characteristics of strain AA0310 on 16 agar media are summarized in Table 1. The strain shows good growth on both synthetic and organic media. Mature aerial mycelia are generally powdery. The color of aerial mass is gray to olive gray (Gray color-series). The colors of vegetative mycelia and colony's reverse sides are reddish black to brownish black. Dark grayish brown, olive or dark brownish gray diffusible pigments are produced in various agar media, but no pH-sensitive. The physiological characteristics and the utilization pattern of carbon sources are listed in Tables 2 and 3, respectively. Strain AA0310 grows at temperatures from 19 to 39°C with an optimum range from 28 to 33°C.

Chemotaxonomy

Analysis of the whole cell hydrolysate of strain AA0310 demonstrated the presence of LL-diaminopimeric acid, ribose, glucose and galactose. Accordingly, the cell wall of this strain was classified as type I, whereas the sugar type was uncharacterizable. Phosphatidyl-ethanolamine and mycolic acids were not detected. Analysis of the menaquinone composition revealed 49% MK-9(H_6), 40% MK-9(H_8) 6% MK-9(H_4), 4% MK-9(H_{10}) and 1% MK-9(H_2). Thus the fatty acid type is of branched chain 2C (Table 4).

The taxonomic properties described above allow to conclude that strain AA0310 belongs to the genus *Streptomyces* Waksman and Henrici, 1943, but are insufficient to determine the species, although similarity to *Streptomyces iakyrus* is recognized.

Table 2. Physiological characteristics of strain AA0310.

Test	Results
Nitrate reduction (Difco, nitrate broth)	Positive
10% skimmed milk (Difco, 10% skimmed milk)	
Coagulation	Positive
Peptonization	Positive
Cellulose decomposition (sucrose nitrate solution with a strip of paper as the sole carbon source)	Negative Growth good
Gelatin liquefaction	
On plain gelatin	Doubtful
On glucose pepton gelatin	Doubtful
Melanin formation	
On ISP med. No. 7	Positive
Temperature range for growth (°C)	19～39
Optimum temperature (°C) (on yeast starch agar)	28～33
pH range for growth	6～9
Optimum pH (on trypticase soy broth, BBL)	7

Table 3. Utilization of carbon sources by strain AA0310.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	+ +
D-Xylose	+ +
Inositol	+
Mannitol	+
D-Fructose	+ +
L-Rhamnose	+
Sucrose	±
Raffinose	+

±, Doubtful; +, weak positive; + +: strong positive (ISP med No. 9, 28°C for 25 days).

Table 4. Fatty acid composition of strain AA0310.

Fatty acid composition (%)											
Straight chain		Branched chain						Unsaturated chain			
15:0	16:0	*i*-14	*i*-15	*i*-16	*i*-17	*a*-15	*a*-17	*i*-16:1	16:1^9	*i*-17:1	*a*-17:1
1	8	2	9	37	4	11	10	5	6	2	2

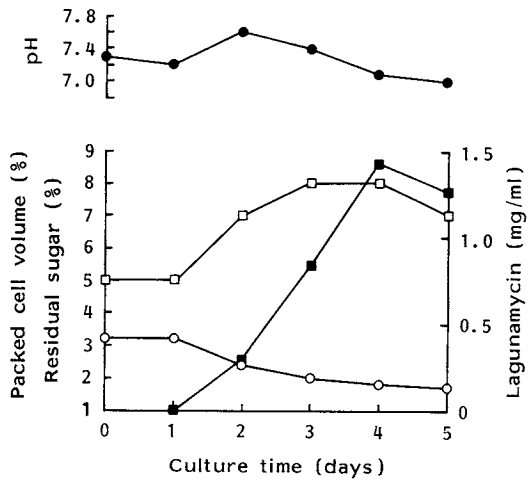
Thus, strain AA0310 is tentatively identified as *Streptomyces* sp. AA0310.

Fermentation

In early stage of experiments for fermentation optimization, *Streptomyces* sp. AA0310 produced 90 to 160 μg/ml of lagunamycin in medium FR-23 composed of soluble starch 3.0%, beet molasses (Nihon Tensai Seito) 2%, Pharmamedia (Trader's Protein) 2% and glucose 0.5% (pH 7.0). Addition of high porous polymer resin Diaion HP-20 (Mitsubishi Kasei Co.) to medium FR-23 significantly enhanced production of lagunamycin. A piece of the mature slant culture of strain AA0310 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium consisting of soluble strach (Nichiden Kagaku) 2.0%, glucose 0.5%, NZ-case (Scheffield) 0.3%, yeast extract (Daigo) 0.2%, fish meal D30X (Banyu Nutrient) 0.5% and $CaCO_3$ 0.3% (pH 7.0) and was incubated at 28°C for 4 days on a rotary shaker (200 rpm). A 5-ml portion of the seed culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of production medium FR-23 containing 1% Diaion HP-20 (pH 7.0). Fermentation was carried out a 28°C for 5 days on a rotary shaker (200 rpm). The antibiotic production in the fermentation broth was monitored by the 5-LPO inhibitory assay and the HPLC analysis. The time course of lagunamycin fermentation in a 500-ml Erlenmeyer flask is shown in Fig. 2. A maximum potency of 1.43 mg/ml was achieved in 4 days.

Fig. 2. Time course of lagunamycin fermentation.

○ Residual sugar, □ packed cell volume, ● pH, ■ lagunamycin.



Isolation

The cultured broth (2.6 liters, pH 7.0) was adjusted to pH 2.0 with 6 N HCl and stirred with ethyl acetate (2 liters) for 2 hours at room temperature. The ethyl acetate layer was separated and concentrated to dryness under reduced pressure at 35°C. The residue was applied onto a silica gel (Kiesel gel 60; 230~400 mesh, Merck) chromatographic column (200 ml). The column was washed first with methylene chloride (1 liter) and then with a mixture of methylene chloride - methanol (200 : 1, 1 liter). Active fractions were eluted with a mixture of methylene chloride - methanol (100 : 1, 3 liters) and then concentrated *in vacuo*. The solid obtained was further applied onto a silica gel column (200 ml). After washing with *n*-hexane (0.3 liter) and a mixture of *n*-hexane - ethyl acetate (4 : 1, 1 liter), the column was eluted stepwise with mixtures of *n*-hexane - ethyl acetate (2 : 1 and 1 : 1, each 2 liters). Active fractions were combined and concentrated *in vacuo*, and the residue was subjected to reversed phase silica gel column chromatography (YMC-ODS-AM 120-S50, YMC Co., Ltd.). The column was developed with a mixture of acetonitrile - water (3 : 2) and active fractions were evaporated to dryness under reduced pressure to yield a pure yellow powder of lagunamycin (680 mg). It was crystallized from aqueous methanol as yellow needles.

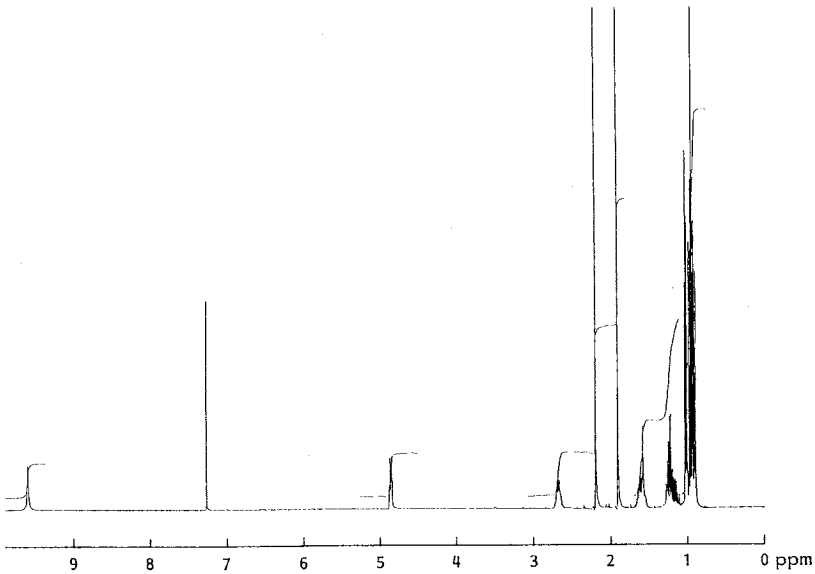
Physico-chemical Properties

Physico-chemical properties are summarised in Table 5. Lagunamycin is obtained as yellow needles which decomposes at 134~136°C. It was soluble in methanol, acetone and ethyl acetate and insoluble in

Table 5. Physico-chemical properties of lagunamycin.

Appearance	Yellow needles
MP (dec)	134~136°C
$[\alpha]_D^{26}$	−33° (*c* 0.2, MeOH)
Molecular formula	$C_{19}H_{21}N_3O_4$
Elementary analysis	
Calcd for $C_{19}H_{21}N_3O_4$:	C 64.21, H 5.96, N 11.82
Found:	C 64.32, H 5.98, N 11.58
FAB-MS (*m/z*)	356 $(M+H)^+$
TLC (Rf value)	0.21 Silica gel plate $60F_{254}$ (Merck, Germany); CH_2Cl_2-MeOH (50:1)
HPLC (Rt)	4.66 minutes YMC-Pack A-301-3 (3 μm, i.d. 4.6×100 mm; YMC Co., Ltd., Japan); CH_3CN-H_2O (6:4); 1.2 ml/minute
UV λ_{max}^{MeOH} nm (ε)	258 (18,300), 302 (15,800), 353 (sh, 3,400), 420 (1,200)
$\lambda_{max}^{MeOH-HCl}$ nm (ε)	258 (19,200), 300 (16,400), 352 (sh, 3,700), 419 (1,200)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	233 (16,300), 264 (sh, 9,500), 284 (9,100), 352 (25,200)
IR ν_{max}^{KBr} cm^{-1}	3430, 2960, 2940, 2875, 2150, 1725, 1680, 1655, 1635, 1390, 1370, 1340, 1320, 1285, 1180, 1160

Fig. 3. ^1H NMR spectrum of lagunamycin (400 MHz, $CDCl_3$).



water. The specific rotation is $[\alpha]_D^{26}$ −33° (*c* 0.2, MeOH). Color reactions were positive to iodine and sulfuric acid and negative to ninhydrin and Dragendorff reagent. The molecular formula was determined as $C_{19}H_{21}N_3O_4$ by elementary analysis. The UV spectrum exhibits maxima at 258, 302, 353 (sh) and 420 nm in neutral solution and at 233, 264 (sh), 284 and 352 nm in alkaline solution. The 400 MHz ^1H NMR (Fig. 3) and ^{13}C NMR spectra (Fig. 4) are shown as more complicated rather than the spectra we expected from its molecular formula. In the course of the structural study, the active principle was pure enough *via* TLC and HPLC experiments, therefore the complexity of the NMR signals was concluded to result from the rotational isomers caused by the bulky side chain against the 3-methyl group of the quinoline plane. The structure of lagunamycin (Fig. 5) will be explained in a separate paper[14].

Fig. 4. ^{13}C NMR spectrum of lagunamycin (100 MHz, $CDCl_3$).

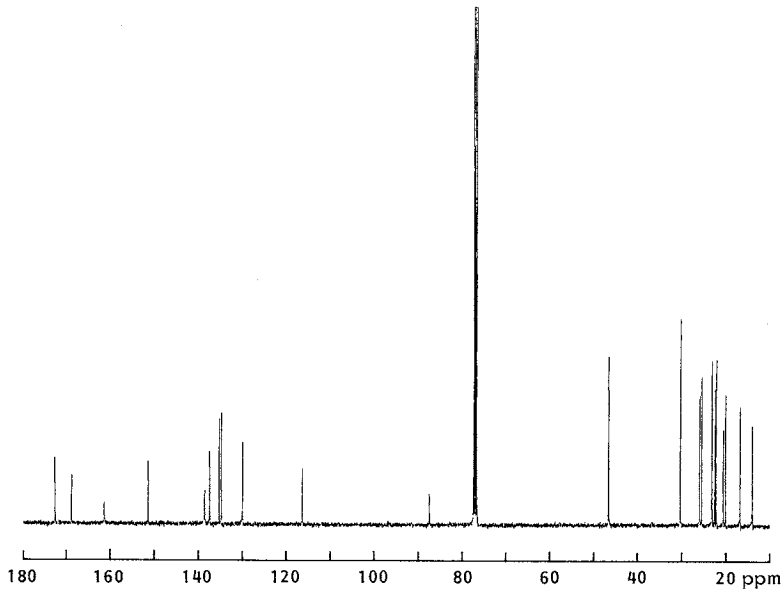


Fig. 5. Structure of lagunamycin.

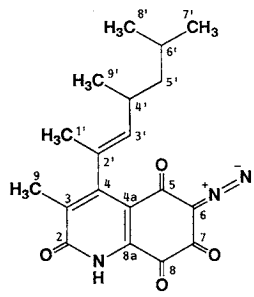


Table 6. Comparison of lagunamycin and known inhibitors in 5-lipoxygenase and lipid peroxidation inhibitions.

Sample	IC_{50} (μM)	
	5-LPO	Peroxidation
Epocarbazolin A	2.41	0.35
Epocarbazolin B	3.60	0.44
Carbazomycin B	4.94	20.5
Lagunamycin	6.08	>200

Table 7. Antimicrobial activities of lagunamycin.

Test organism	MIC (μg/ml)
Staphylococcus aureus 6538P	3.1
S. aureus Smith	6.3
S. aureus Sa-247 (MRSA)	3.1
Enterococcus faecalis A9808	6.3
Micrococcus luteus ATCC 9341	6.3
Bacillus subtilis ATCC 6633	3.1
Escherichia coli Juhl	>100
E. coli 255	>100
Klebsiella pneumoniae ATCC 10031	>100
Proteus mirabilis IFO 3849	>100
P. vulgaris Pv-44	>100
Serratia marcescens Sm-237	>100
Pseudomonas aeruginosa A9843A	>100

Biological Properties

Lagunamycin inhibits 5-LPO with an IC_{50} of 6.08 μM, but shows no effect on the lipid peroxidation (Table 6). Carbazomycin B and epocarbazolins A and B which were reported as 5-lipoxygenase inhibitors[1,2], inhibited not only 5-LPO (IC_{50}; 2.41~4.94 μM) but also lipid peroxidation (IC_{50}; 0.35~20.5 μM). The experimental results reveal that the 5-LPO inhibitory activity of lagunamycin does not depend on the antioxidative activity like epocarbazolins.

Lagunamycin shows moderate activity against Gram-positive bacteria, but is inactive against Gram-negative bacteria as shown in Table 7. When tested in mice by intraperitoneal administration,

lagunamycin was found to have an LD_{50} of 15 mg/kg.

Discussion

In the continued search for 5-LPO inhibitors from microbial metabolites, carbazomycins[1], epocarbazolins[2] and nitrosoxacins[3] and nitrosoxacins[3] were found to show 5-LPO inhibitory activities. In addition to the above antibiotics, lagunamycin was found to have a diazo-tetraoxoquinoline nucleus in the molecule. Lagunamycin showed strong 5-LPO inhibitory activities without peroxidation activity. Since desdiazo derivatives of lagunamycin were equipotent to lagnamycin in the 5-LPO assay[14], the diazo group in the molecule seems to have no essential roles for 5-LPO activity. Therefore, the current interest is focused on the *in vivo* anti-chronic skin inflammation activity of this compound.

Acknowledgments

The authors wish to express their thanks to Prof. M. OHASHI of the University of Electrocommunications for valuable discussion. They are also indebted to the Analytical Group of their institute for the spectral analysis.

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