LONOMYCINS B AND C
TWO NEW COMPONENTS OF POLYETHER ANTIBIOTICS
FERMENTATION, ISOLATION AND CHARACTERIZATION

TAKU MIZUTANI, MICHIO YAMAGISHI, HIROSHI HARA, SADAFUMI ŌMURA and MASAHIRO ŌZEKI

Research Laboratories, Taisho Pharmaceutical Co., Ltd.,
1-403, Yoshino-cho, Omiya-shi, Saitama 330, Japan

KAZUTOSHI MIZOUE, HARUO SETO and NOBORU ŌTAKE

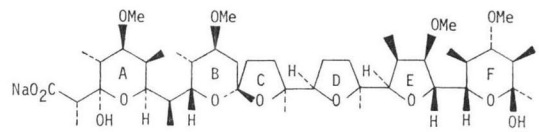
Institute of Applied Microbiology, the University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

(Received for publication May 22, 1980)

Lonomycin B (**II**), $C_{44}H_{75}O_{14}Na$, m.p. 181~182°C, and lonomycin C (**III**), $C_{43}H_{73}O_{14}Na$, m.p. 186~187°C, were isolated as their sodium salts from the fermentation broth of *Streptomyces ribosidificus* TM-481. Their physicochemical properties demonstrated that **II** and **III** are closely related congeners of lonomycin A (**I**). The identical mass spectra of methyl esters of **I** and **II** indicated that **II** is a stereoisomer of **I**. On the other hand, the mass spectrum of a methyl ester of **III** showed a peak at *m/e* 810 due to $M^+ - H_2O$ which is smaller by 14 mass units than the maximum peak at *m/e* 824 due to $M^+ - H_2O$ of the methyl esters of **I** and **II**. This result together with the elemental analysis strongly suggested that **III** is a demethyl derivative of **I** or **II**. **II** and **III** are slightly less active than **I** in their antimicrobial activities.

Lonomycin, a member of the polyether antibiotic family, was previously isolated from the fermentation broth of *Streptomyces ribosidificus* TM-481[1] and its absolute configuration has been determined by an X-ray analysis[2] of its thallium salt as shown in Fig. 1.

Fig. 1. The structure of lonomycin A (**I**) sodium salt.



Lonomycin A

The antibiotic is active against Gram-positive bacteria, mycobacteria, protozoa and, especially, coccidia.[1]

As a result of further screening for concurrent minor components of the antibiotic complex, two minor congeners of lonomycin, *i.e.*, lonomycin B (**II**) and lonomycin C (**III**) have been isolated from the fermentation broth of the same producing organism. Since the isolation of the minor components, the antibiotic previously called lonomycin is hereafter designated lonomycin A (**I**). **II** and **III** are similar to **I** in their physicochemical properties.

This paper deals with the fermentation, isolation, physicochemical and biological properties of lonomycins, and the structural analysis by mass spectrometry.

Fermentation

Streptomyces ribosidificus TM-481 was maintained on an agar slant consisting of 2.0% oat meal and 1.5% agar at 30°C. Two ml of its spore suspension was added to 100 ml of a medium containing 2% oat meal, 1% glucose, 0.3% meat extract, 0.3% NaCl, 0.2% $CaCO_3$, 0.04% $MnCl_2$ and 0.04% $Fe_2(SO_4)_3$, and incubated on a reciprocal shaker at 30°C for 72 hours. Two liters of the resulting culture

was transferred to a 250-liter fermentor containing 200 liters of the same medium, and fermentation was carried out at 30°C for 72 hours with aeration at 200 liters/minute and agitation. In order to measure the total potency, 10 ml of the fermentation broth was extracted with an equal volume of benzene, and antibiotic activities were assayed by a paper disc agar diffusion method using *Staphylococcus epidermidis* TPR-25 as a test organism. The maximum titer corresponding to 250 mcg/ml of **I** was obtained after 72 hours fermentation. TLC analysis (silica gel plate, solvent system: benzene - acetone, 2: 1) of the benzene solution revealed that the production of **II** and **III** increased with the concomitant accumulation of **I** over the whole fermentation period.

Isolation

The isolation procedures of **I**, **II** and **III** are shown in Chart 1.

After adjusted at pH 9.0 with 1 N NaOH solution, the fermentation broth was centrifuged to separate the mycelium. The supernatant was applied to a column of Amberlite XAD-8 resin. Then, the column was washed with a small volume of water and adsorbed antibiotics were eluted with methanol. The solution was concentrated *in vacuo* until the methanol was removed, and the active substances were

Chart 1. Isolation procedures of lonomycins A (**I**), B (**II**) and C (**III**).

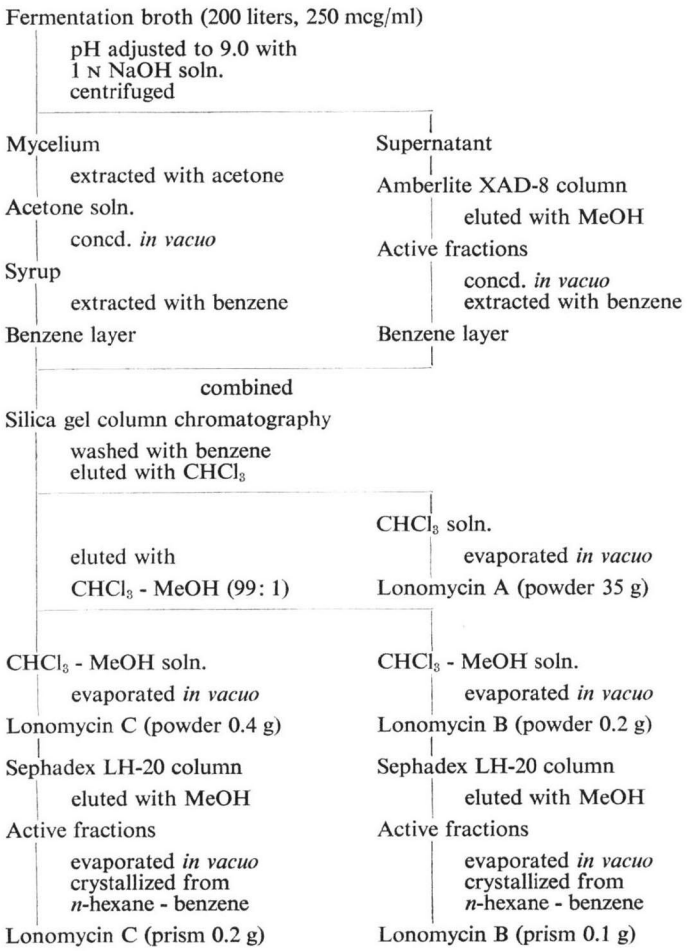


Table 1. Physicochemical properties of lonomycins A (I), B (II) and C (III).

	Lonomycin A		Lonomycin B		Lonomycin C	
Appearance	White prism		White prism		White prism	
Melting point	188~189°C		181~182°C		186~187°C	
Elemental analysis	Found	Calcd.	Found	Calcd.	Found	Calcd.
C:	61.93 %	(62.11)	62.16 %	(62.11)	61.87 %	(61.72)
H:	8.64	(8.84)	8.81	(8.84)	8.86	(8.74)
Na:	2.68	(2.70)	2.68	(2.70)	2.78	(2.74)
Maximum mass number:						
free acid	766 ($M^+-CO_2-H_2O$)		766 ($M^+-CO_2-H_2O$)		752 ($M^+-CO_2-H_2O$)	
methyl ester	824 (M^+-H_2O)		824 (M^+-H_2O)		810 (M^+-H_2O)	
Molecular weight	850		850		836	
Molecular formula	$C_{44}H_{75}O_{14}Na$		$C_{44}H_{75}O_{14}Na$		$C_{43}H_{73}O_{14}Na$	
Specific rotation	+47° (*c* 1.0, MeOH)		+48.2° (*c* 0.5, MeOH)		+49.2° (*c* 0.5, MeOH)	
Rf values of TLC*						
EtOAc	0.49		0.34		0.25	
EtOAc - benzene (1 : 1)	0.23		0.07		0.04	
Benzene - acetone (2 : 1)	0.55		0.39		0.32	
$CHCl_3$ - MeOH (9 : 1)	0.69		0.63		0.54	

*TLC plate: Kieselgel 60 F_{254} (0.25 mm, Merck), Detection; vanillin - H_2SO_4

Fig. 2. The IR spectra of lonomycins A (I), B (II) and C (III) sodium salts (KBr).

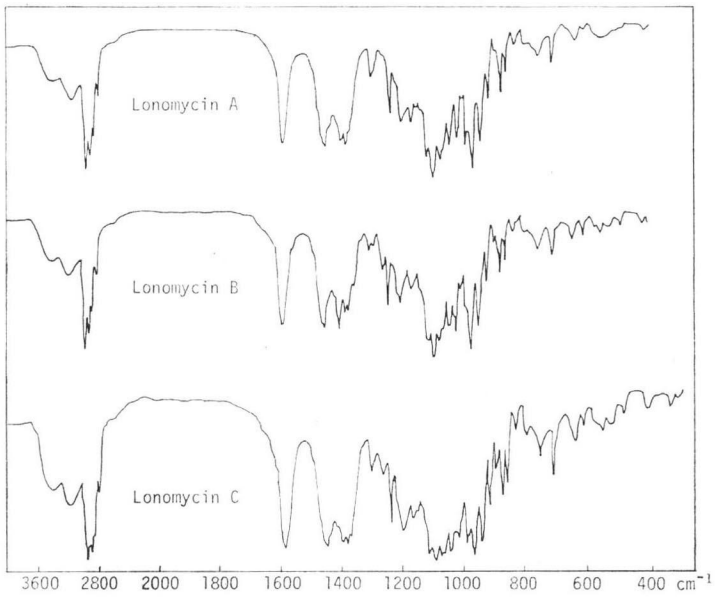
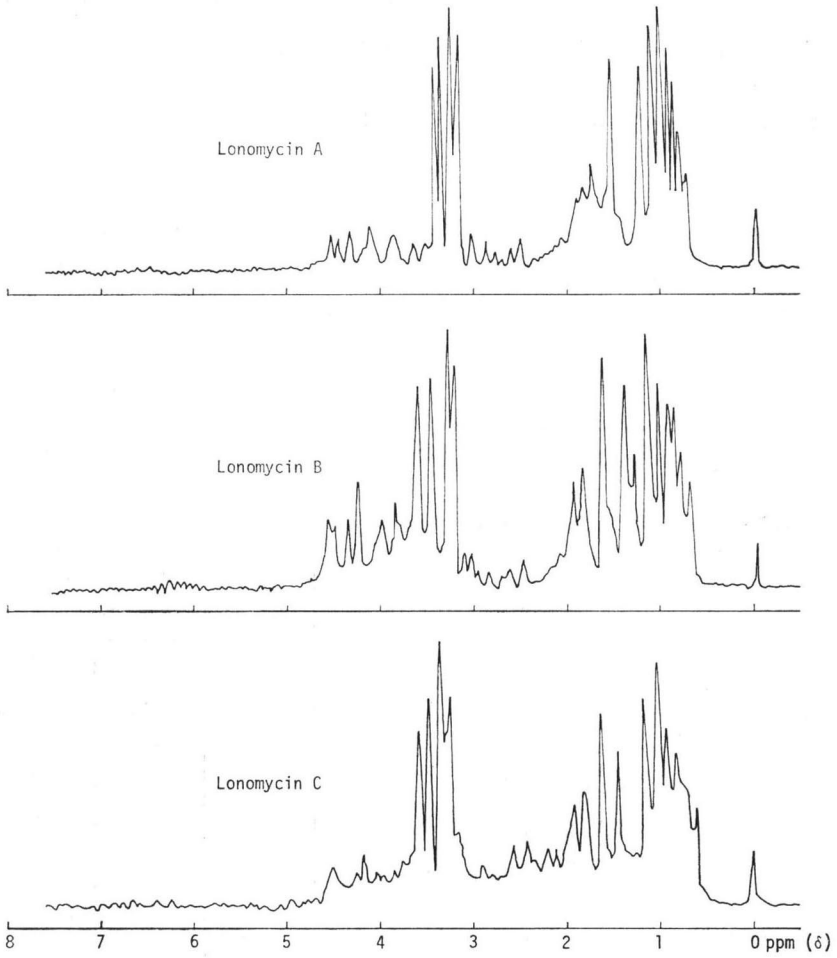


Fig. 3. The ^{1}H-NMR spectra of lonomycins A (I), B (II) and C (III) sodium salts taken in $CDCl_3$ at 100MHz.



extracted with benzene three times. The mycelial cake was extracted with acetone, and the solution was evaporated until the acetone was removed, and the resulting aqueous phase was extracted with benzene three times. The solvent layers from the supernatant and the mycelial cake were combined and, after drying over Na_2SO_4, concentrated *in vacuo* to give a solid which was applied to a silica gel column (15 cm×100 cm) packed with benzene. Then, the column was washed with benzene, and then with chloroform to elute all of **I**. Subsequently, the column was developed with chloroform - methanol (99: 1) to elute **II** first and then **III**. Three active fractions were separately concentrated *in vacuo*. Each of the resulting solids was further purified by gel filtration on a Sephadex LH-20 column using methanol as a eluant. The active fractions were combined and evaporated *in vacuo* to give a white powder. Crystallization from *n*-hexane - benzene (4: 1) gave colorless prisms of **I**, **II** and **III** sodium salts.

Physicochemical and Biological Properties

I, **II** and **III** thus obtained were very similar in their physicochemical properties. They were

soluble in lower alcohols, acetone, ethyl acetate, benzene, chloroform and ethyl ether, and slightly soluble in *n*-hexane and petroleum ether, but insoluble in water. They give positive color reactions with *p*-anisaldehyde, antimonytrichloride and vanillin-sulfuric acid, but negative for MOLISCH, ninhydrin and ferric chloride.

The physicochemical properties of **I**, **II** and **III** sodium salts are summarized in Table 1.

Spectral Evidences of Structural Analysis

The antibiotics in methanol solution show end absorption in the U.V. spectra.

Comparison of the IR and ^{1}H-NMR spectra of **II** and **III** with those of **I** (as depicted in Figs. 2 and 3) demonstrate the structural similarities of those compounds. Their IR spectra show characteristic carboxylate bands of 1580 cm^{-1}.

Their ^{1}H-NMR spectra taken in $CDCl_3$ show four characteristic singlets at δ H 3.25~3.52 ppm. The known polyether antibiotics possessing four methoxyl groups in their structures are lonomycin A[1], DE-3936[3], emericid[4], A-218[5], A-28695A[6] and septamycin[7]. However, they can be differentiated

Table 2. Antimicrobial spectra of lonomycins A (**I**), B (**II**) and C (**III**).

Microorganisms	MIC (mcg/ml)			
	Lonomycins			Medium
	A	B	C	
Staphylococcus aureus FDA 209P	3.13	6.25	6.25	1
Staphylococcus aureus Smith	3.13	50	50	1
Staphylococcus aureus TPR-18 (SA-, PC-, TC-, KM-, CP- & Mac-R)	6.25	25	25	1
Staphylococcus aureus TPR-23 (SA-, PC-, TC-, SM-, CP- & Mac-R)	6.25	25	25	1
Staphylococcus aureus TPR-26 (SA-, PC-, TC-, SM-, CP- & Mac-R)	6.25	25	25	1
Staphylococcus aureus TPR-27 (SA-, PC-, TC-, SM-, KM-, CP- & Mac-R)	6.25	25	25	1
Staphylococcus epidermidis TPR-13 (SA-, PC-, CP-, EM- & OM-R)	6.25	25	25	1
Staphylococcus epidermidis TPR-14 (PC- & CP-R)	6.25	12.5	12.5	1
Staphylococcus epidermidis TPR-16 (SA-, PC-, TC- & CP-R)	3.13	12.5	12.5	1
Staphylococcus epidermidis TPR-25 (SA-, PC-, TC-, SM-, KM-, CP- & Mac-R)	6.25	12.5	12.5	1
Staphylococcus epidermidis TPR-28 (SA-, PC-, TC-, SM-, KM-, CP- & Mac-R)	6.25	12.5	12.5	1
Bacillus subtilis PCI 219	3.13	12.5	12.5	1
Micrococcus luteus NIHJ	6.25	12.5	25	1
Escherichia coli O-55	>50	>50	>50	1
Proteus vulgaris HX 19	>50	>50	>50	1
Pseudomonas aeruginosa P-32	>50	>50	>50	1
Aspergillus niger	>50	>50	>50	2
Trichophyton asteroides	>50	>50	>50	2
Candida albicans	>50	>50	>50	2
Saccharomyces cerevisiae	>50	>50	>50	2

Medium 1: heart infusion agar, Medium 2: SABOURAUD agar
Abbreviations: Mac: macrolide, R: resistant strain

Fig. 4. The mass spectral fragmentations of lonomycin A (I) methyl ester in the higher mass region.

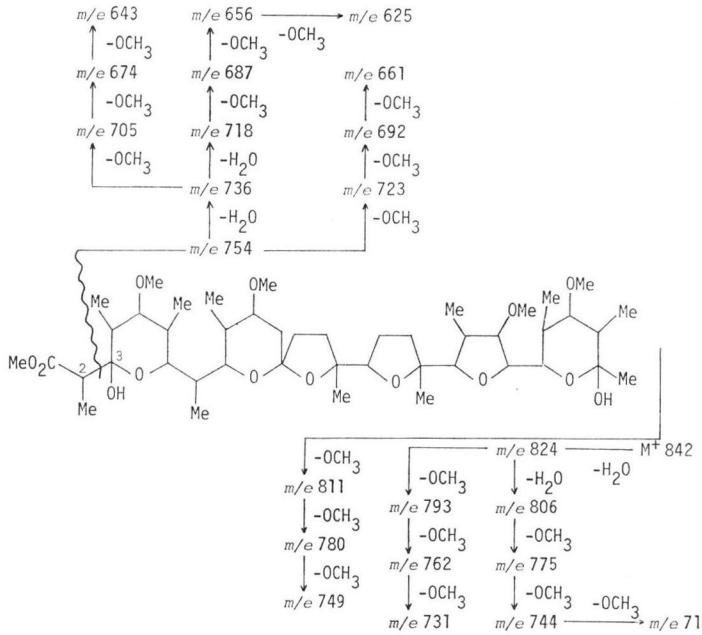
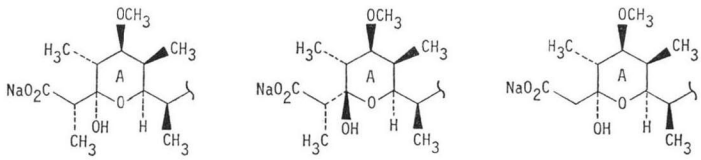


Fig. 5. The partial structure of lonomycins A (I), B (II) and C (III) sodium salts.



from II and III by Rf values of thin-layer chromatography and other physicochemical properties. Therefore, II and III can be concluded to be new members of polyether antibiotic family.

The microbial spectra of I, II and III are shown in Table 2. These antibiotics are active against Gram-positive bacteria but inactive against Gram-negative bacteria and fungi. It is noticeable that II and III show slightly lower activities than I.

Mass Spectral Evidences of Structural Analysis

The structural elucidation of I, II and III by mass spectrometry was carried out with their methyl esters because the mass spectra of their sodium salts did not show any significant peaks in the high mass area.*

In the mass spectrum of I methyl ester, the molecular ion peak (M^+) was absent and the highest ion peak was observed at *m/e* 824 corresponding to $M^+ - H_2O$. Main fragmentation peaks in the high

* The E-I mass spectra of the sodium and the potassium salts of DE-3936 (identical with lonomycin A) were reported[8] to show molecular ions at *m/e* 850 and 866, respectively. However, the authors could observe no molecular ions in the E-I mass spectra of the sodium and the potassium salts of lonomycin A.

mass region were due to the loss of one to three methoxyls accompanied by one or two dehydrations from M^+, whereas the other main peaks corresponded to the loss of one to two water molecules and/or one to three methoxyls after β-cleavage between C2 and C3 as shown in Fig. 4.

The mass spectrum of **II** methyl ester showed the same fragmentation patterns in the whole region as that of **I** methyl ester. The same was true with the mass spectra of free acids and sodium salts of **I** and **II**. These results together with the identical elemental analysis of **I** and **II** (see Table 1) suggested that **II** is a stereoisomer of **I**.

In the mass spectra of **I** and **III** methyl esters the fragments shown in Fig. 4 resulting from dehydration and demethoxylation of the molecular ion occurred 14 mass units lower in the spectrum of **III** methyl ester. The ion resulting from dehydration and demethoxylation of the fragment formed by loss of the C-3 substituent occurred at the same mass in both spectra. The same relationships were observed in the spectra of the free acids of **I** and **III**. These results in conjunction with the elemental analysis of **I**, **II** and **III** suggested that the methyl substituent at C2 in **I** or **II** was absent in **III**.

The stereochemistry of **II** and **III** could not be determined by mass spectrometry. Further study of the structural elucidation of **II** and **III** was carried out by ^{13}C-NMR spectrometry and their structures have been determined as shown in Fig. 5, including the stereochemistry. A preliminary communication of the structural elucidation of **II** and **III** has been published.[8]

References

1) ŌMURA, S.; M. SHIBATA, S. MACHIDA, J. SAWADA & N. ŌTAKE: Isolation of a new polyether antibiotic, lonomycin. J. Antibiotics 29: 15~20, 1976
2) ŌTAKE, N.; M. KOENUMA, H. MIYAMAE, S. SATO & Y. SAITO: Studies on the ionophorous antibiotics. III. The structure of lonomycin, a polyether antibiotic. Tetrahed. Lett. 1975: 4147~4150, 1975
3) OHSHIMA, M.; N. ISHIZAKI, K. ABE, M. UKAWA, Y. MARUMOTO, K. NAKATSUKA, T. HORIUCHI, Y. TONOOKA, S. YOSHINO & N. KANDA: Antibiotic DE-3936, a polyether antibiotic identical with lonomycin. Taxonomy, fermentation, isolation, and characterization. J. Antibiotics 29: 354~365, 1976
4) NINET, L.; F. BENAZET, H. DEPARIRE, J. FLORENT, J. LUNEL, D. MANCY, A. ABRAHAM, J. R. CARTIER, N. DE CHEZELLES, C. GODARD, M. MOREAU, R. TISSIER & J. Y. LALLEMAND: Emericid, a new polyether antibiotic from *Streptomyces hygroscopicus* (DS 24369). Experientia 32: 319~321, 1976
5) TSUJI, N.; K. NAGASHIMA, M. KOBAYASHI, Y. WAKISAKA, Y. KAWAMURA, S. KŌZUKI & M. MAYAMA: Two new antibiotics, A-218 and K-41. Isolation and characterization. J. Antibiotics 29: 10~14, 1976
6) HAMILL, R. L. & M. M. HOEHN: Anticoccidial method. U. S. Patent 3,839,559, Oct. 1, 1974
7) KELLER-JUSLÉN, C.; H. D. KING, Z. L. KIS & A. VON WARTBURG: Septamycin, a polyether antibiotic. Taxonomy, fermentation, isolation and characterization. J. Antibiotics 28: 854~859, 1975
8) SETO, H.; K. MIZOUE, N. ŌTAKE, M. YAMAGISHI, T. MIZUTANI, H. HARA & S. ŌMURA: Studies on the ionophorous antibiotics. 17. The structures of lonomycins B and C. J. Antibiotics 31: 929~932, 1978