BINDING OF [^{3}H]TETRAHYDROLEUCOMYCIN A_3 TO *ESCHERICHIA COLI* RIBOSOMES AND THE EFFECT OF 3″-*O*-ACYL DERIVATIVES OF LEUCOMYCINS ON THE BINDING

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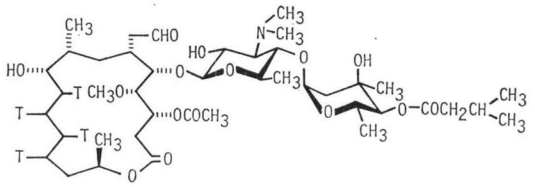
The analysis of [^{3}H]tetrahydroleucomycin A_3 binding to *Escherichia coli* ribosomes are described. The dissociation constant for tetrahydroleucomycin A_3 binding to ribosomes was 1.15×10^{-8} M. One molecule of tetrahydroleucomycin A_3 was bound to each 70 S ribosome (50 S subunit) as reported with erythromycin.

The effect of leucomycins and their 3″-*O*-acyl derivatives on [^{3}H]tetrahydroleucomycin A_3 binding to ribosomes was examined. In general, 3″-*O*-acyl derivatives of leucomycins exhibited stronger antimicrobial activity against Gram-positive bacteria and weaker (or equivalent) activity against *E. coli* than their mother compounds. However, the affinities to ribosomes were approximately equivalent to those of the mother compounds, suggesting that Gram-positive bacterial cells are more permeable to 3″-*O*-acyl derivatives than to the mother compounds.

The mechanism of action of macrolide antibiotics has been studied using erythromycin, the representative of 14-membered macrolides.[1,2] The binding of erythromycin to the 50 S subunit of bacterial ribosome causes an inhibition of protein synthesis. PESTKA *et al.*[3] and ŌMURA *et al.*[4] studied the structure-activity relationship of leucomycins (LMs), the representative of 16-membered macrolides, by comparison of their antimicrobial activities with the affinities to ribosomes that were determined by competition with [^{14}C]erythromycin in binding to *Escherichia coli* ribosomes.[5] In general, the binding of LMs and their derivatives to ribosomes correlates with their antimicrobial activities against Gram-positive bacteria. However, where a discrepancy between antimicrobial activity and affinity to ribosomes exists, other factors such as cellular permeability or modification of the macrolide may be responsible. Some 2′-*O*-acyl derivatives undergo gradual hydrolysis during antimicrobial assays, for their binding to ribosomes is poor compared to their relatively good antimicrobial activities.[4] On the contrary, LM-A_3 *N*-oxide has poor permeability or is inactivated during incubation for determination of antimicrobial activity, since the binding to ribosomes is high compared to the poor antimicrobial activity.[3]

Fig. 1. Structure of [10,11,12,13-^{3}H]tetrahydroleucomycin A_3 ([^{3}H]THLM-A_3).



Recently, [^{3}H]dihydrorosaramicin was synthesized from rosaramicin, a 16-membered macrolide, and their binding to *E. coli* ribosomes was analyzed.[6] However, dihydrorosaramicin is less active than rosaramicin since a hydroxymethyl group is substituted for the aldehyde

group which plays an important role in antimicrobial activity. We attempted the synthesis of [10, 11,12,13-^{3}H]tetrahydroleucomycin A_3 ([^{3}H]THLM-A_3, Fig. 1) from LM-A_3; THLM-A_3 possesses antimicrobial activity similar to that of LM-A_3[7].

In the present paper, we report our analysis of the binding of [^{3}H]THLM-A_3 to *E. coli* ribosomes and the effect of LMs and their 3″-acyl derivatives[8,9] on the binding, and discuss structure-activity relationships.

Materials and Methods

Materials

Ribosomes were prepared by Sephacryl S-300 column chromatography from a 25,000×*g* supernatant fraction obtained by crushing the cells of *Escherichia coli* with alumina as described by JELENC.[10]

[10,11,12,13-^{3}H]THLM-A_3 was synthesized at The Radiochemical Center, Amersham, and then purified in our laboratory as follows. An ethanol solution of LM-A_3 was stirred under tritium gas atmosphere for one hour in the presence of PtO_2 catalyst to give quantitatively [10,11,12,13-^{3}H]THLM-A_3. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The crude powder was purified by preparative thin-layer chromatography (Merck Kieselguhr 60 F_{254}) using benzene - acetone (1: 1) and then chloroform - methanol - ammonia (20: 1: 0.025) as developing solvents. The Rf values of LM-A_3 and THLM-A_3 were 0.58 and 0.62, respectively, in the former solvent system. A radioactive spot on the plate was detected by autoradiography. The purified [^{3}H]THLM-A_3 gave a single spot in thin-layer chromatography with the above solvent systems and the Rf values were essentially equivalent to those of an authentic sample of non-radioactive THLM-A_3. The purified sample did not exhibited at all the absportion at 232 nm which LM-A_3 had, indicating that it does not contain LM-A_3. The [^{3}H]THLM-A_3 had 4.45 Ci/mmole of specific radioactivity.

LMs were prepared in our laboratory[7] and their 3″-acyl derivatives were synthesized according to SAKAKIBARA *et al.*[8,9]. Each compound was dissolved in 0.01 N HCl to make a stock solution of 10 mM. The solutions were stable in a refrigerator for at least one month. Dilution was made with sterile water.

Determination of [^{3}H]THLM-A_3 Binding to Ribosomes

Binding of [^{3}H]THLM-A_3 to ribosomes was determined by the filter assay method described by PESTKA *et al.*[3] with modification. The reaction mixture contained the following components in a volume of 0.5 ml unless otherwise specified: 5 mM $MgCl_2$, 0.1 M KCl, 0.1 M NH_4Cl, 10 mM tris-HCl (pH 7.2), 5 A_{260} units of ribosomes from *E. coli*, 1.03 μM [^{3}H]THLM-A_3 (58,830 dpm), and a cold antibiotic sample. Reaction was started by adding ribosomes to the reaction mixture and performed at 24°C for 30 minutes. The reaction was stopped by diluting the reaction mixture with 3 ml of cold solution A (5 mM $MgCl_2$, 0.15 M KCl, and 10 mM tris-HCl (pH 7.2)). The diluted reaction mixture was filtered through a 25-mm diameter membrane filter (HAWP, Millipore Co.) and immediately washed ten times with 3 ml of cold solution A. The filter was then dried under an infrared lamp and the radioactivity was determined in a scintillation spectrometer, using a toluene based scintillation fluid.

Analysis of Ribosomes-[^{3}H]THLM-A_3 Complex by Sucrose Density Gradient Centrifugation

A reaction mixture (0.1 ml) containing 0.63 μM [^{3}H]THLM-A_3 (58,200 dpm), 20 A_{260} units of ribosomes (520 pmoles), 5 mM $MgCl_2$, 0.1 M KCl, 0.1 M NH_4Cl and 10 mM tris-HCl (pH 7.2) was incubated at 24°C for 30 minutes. The reaction mixture was cooled on ice and then layered on a linear 5~30% sucrose gradient made in a buffer (pH 7.2) containing 0.5 mM $MgCl_2$, 0.1 M KCl, 0.1 M NH_4Cl and 10 mM tris-HCl. Centrifugation was in an SW41 rotor in a Beckman ultracentrifuge for 4 hours at 4°C and at 40,000 rpm. Gradients were fractionated from the bottom; 0.5-ml fractions were collected, and the radioactivity and A_{260} determined.

Determination of Minimal Inhibitory Concentrations (MICs)

MICs against *Staphylococcus aureus* FDA 209P, *Bacillus subtilis* PCI 219, *Micrococcus luteus* PCI 1001, and *E. coli* NIHJ were determined by the agar dilution method using a medium containing 0.5% peptone and 0.5% meat extract (pH 7.0).

Results and Discussion

Effect of K^+ and Mg^{2+} on [^{3}H]THLM-A_3 Binding to Ribosomes

PESTKA[5] reported that erythromycin binding to *E. coli* ribosomes requires K^+ and Mg^{2+}. Fig. 2 shows the effect of K^+ and Mg^{2+} on [^{3}H]THLM-A_3 binding to *E. coli* ribosomes. Binding of THLM-A_3 to ribosomes was hardly recognized in the absence of K^+, and a strong binding was shown at 50~500 mM concentrations of K^+. A high binding activity was observed even at 1 mM Mg^{2+} which is the lowest concentration in the reaction mixtures tested, and at Mg^{2+} concentrations between 5 and 20 mM a definite activity was shown. It is estimated that binding of [^{3}H]THLM-A_3 to ribosomes would be decreased at lower concentrations of Mg^{2+} as is the binding of erythromycin to ribosomes. In the following experiments, the reaction mixtures contained 100 mM of KCl and 5 mM of $MgCl_2$.

Binding of [^{3}H]THLM-A_3 to Ribosomes and Their Subunits

When a reaction mixture containing 1.04 μM [^{3}H]THLM-A_3 and 0.26 μM ribosomes was incubated at 24°C for 30 minutes, about 90% of ribosomes bound to [^{3}H]THLM-A_3 (Fig. 3). From the Scatchard plot (Fig. 3-right), the association constant, dissociation constant and number of bound THLM-A_3 molecules per ribosome were calculated to be 8.68×10^7 M^{-1}, 1.15×10^{-8} M and 0.91, respectively. These values indicate that THLM-A_3 binds to ribosomes at single binding site per ribosome as reported with chloramphenicol[11], erythromycin[5] and rosaramicin[6]. The above association constant and dissociation constant are similar to those reported for erythromycin (9.9×10^7 M^{-1} and 1.0×10^{-8} M, respectively), indicating that THLM-A_3 and erythromycin have a similar affinity to ribosomes. Furthermore, the result of analysis by sucrose density gradient centrifugation of ribosomes incubated with [^{3}H]THLM-A_3 (Fig. 4) showed that the labeled compound bound to 50S subunits but not to 30S subunits of ribosomes.

The affinity of *E. coli* ribosomes for 16-membered macrolide antibiotics has been determined by examining the effect of these antibiotics on [^{14}C]erythromycin binding to ribosomes[3,4]. The above results indicate that [^{3}H]THLM-A_3 instead of [^{14}C]erythromycin can be used for direct determination of the affinity of 16-membered macrolides to ribosomes.

Fig. 2. Effect of K^+ and Mg^{2+} on [^{3}H]THLM-A_3 binding to ribosomes.

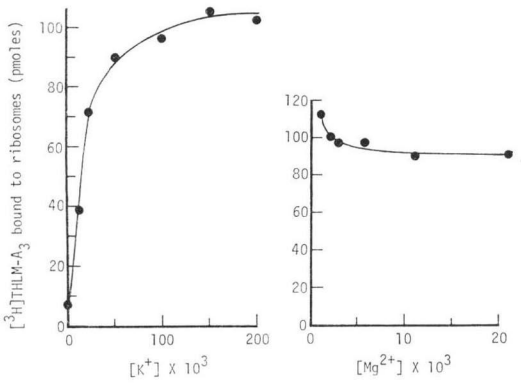


Fig. 3. Binding of [^{3}H]THLM-A_3 to ribosomes as a function of [^{3}H]THLM-A_3 concentrations.
r: Moles of [^{3}H]THLM-A_3 bound per mole of ribosomes.
A: The concentration of free [^{3}H]THLM-A_3.

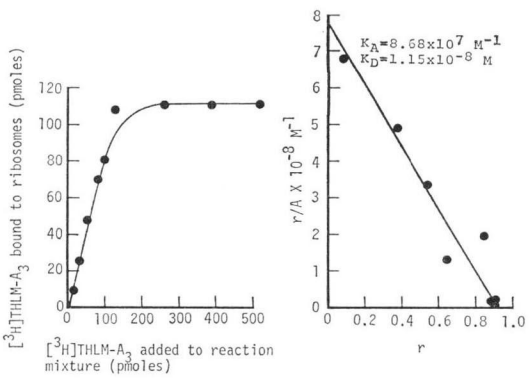
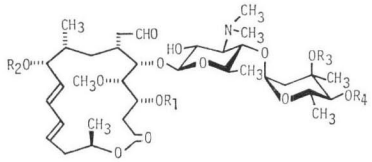


Table 1. Affinities to ribosomes and antimicrobial activities of leucomycins and their *O*-acyl derivatives. Concentration (ID_{50}) of a compound for 50% inhibition of [^{3}H]THLM-A_3 binding to ribosomes and the minimal inhibitory concentration (MIC) were assayed as described in the text.



No.	Compound*	R_1	R_2	R_3	R_4	ID_{50} (μM)	MIC (μg/ml)**			
							BS	SA	ML	EC
1	Leucomycin (LM)-V	H	H	H	H	4.0	2.5	1.3	0.16	50
2	4''-*O*-Ac-3''-*O*-Bu-LM-V	H	H	Bu	Ac	1.0	0.08	0.16	<0.01	50
3	9,4''-Di-*O*-Ac-3''-*O*-Bu-LM-V	H	Ac	Bu	Ac	1.3	0.16	0.31	0.04	50
4	9,4''-Di-*O*-Ac-3''-*O*-*i*Va-LM-V	H	Ac	*i*Va	Ac	1.4	0.16	0.63	<0.01	100
5	LM-A_7	H	H	H	Pr	2.7	0.78	0.78	<0.1	25
6	3''-*O*-Pr-LM-A_7	H	H	Pr	Pr	0.9	0.16	0.16	<0.02	>10
7	9-*O*-Ac-3''-*O*-Bu-LM-A_7	H	Ac	Bu	Pr	1.2	0.16	0.08	0.08	50
8	9-*O*-Pr-3''-*O*-Bu-LM-A_7	H	Pr	Bu	Pr	1.4	0.16	0.16	0.02	>100
9	Midecamycin	Pr	H	H	Pr	—	0.31	0.31	0.04	50
10	9,3''-Di-*O*-Ac-midecamycin	Pr	Ac	Ac	Pr	1.6	0.16	0.08	0.02	50
11	LM-A_5	H	H	H	Bu	1.2	0.16	0.16	0.02	13
12	3''-*O*-Ac-LM-A_5	H	H	Ac	Bu	1.3	0.08	0.04	<0.01	13
13	3''-*O*-Pr-LM-A_5	H	H	Pr	Bu	1.1	0.04	0.08	<0.01	25
14	3''-*O*-Bu-LM-A_5	H	H	Bu	Bu	1.3	0.16	0.08	0.02	25
15	9,3''-Di-*O*-Ac-LM-A_5	H	Ac	Ac	Bu	1.8	0.16	0.31	0.02	25
16	9-*O*-Ac-3''-*O*-Pr-LM-A_5	H	Ac	Pr	Bu	1.2	0.08	0.04	0.02	25
17	9-*O*-Ac-3''-*O*-Bu-LM-A_5	H	Ac	Bu	Bu	1.0	0.08	0.08	<0.01	100
18	LM-A_4	Ac	H	H	Bu	1.3	0.31	0.31	0.04	25
19	3''-*O*-Ac-LM-A_4	Ac	H	Ac	Bu	1.3	0.08	0.16	0.04	25
20	9-*O*-Ac-LM-A_4	Ac	Ac	H	Bu	1.8	0.16	0.16	0.04	50
21	9,3''-Di-*O*-Ac-LM-A_4	Ac	Ac	Ac	Bu	1.3	0.04	0.16	<0.01	100
22	LM-A_1	H	H	H	*i*Va	1.1	0.16	0.08	0.04	13
23	3''-*O*-Ac-LM-A_1	H	H	Ac	*i*Va	1.3	0.16	0.08	0.02	25
24	3''-*O*-Pr-LM-A_1	H	H	Pr	*i*Va	0.9	0.16	0.08	<0.02	>10
25	LM-A_3	Ac	H	H	*i*Va	1.8	0.31	0.31	0.04	25
26	3''-*O*-Ac-LM-A_3	Ac	H	Ac	*i*Va	1.7	0.16	0.08	0.02	50
27	10,11,12,13-Tetrahydro-LM-A_3	Ac	H	H	*i*Va	1.8	0.31	0.4	<0.1	100

* Ac, acetyl ($COCH_3$); Pr, propionyl ($COCH_2CH_3$); Bu, butyryl ($COCH_2CH_2CH_3$); *i*Va, *iso*-valeryl ($COCH_2CH(CH_3)_2$).

** BS, *Bacillus subtilis* PCI 219; SA, *Staphylococcus aureus* FDA 209P; ML, *Micrococcus luteus* PCI 1001; EC, *Escherichia coli* NIHJ.

Competition Experiments Using [^{3}H]THLM-A_3 as a Marker

For the comparison of the affinities of LM-A_3 and THLM-A_3 for *E. coli* ribosome, competition experiments were performed using [^{3}H]THLM-A_3 as a marker. As shown in Fig. 5, when an excess of unlabeled LM-A_3 or THLM-A_3 was added to the reaction mixture, the [^{3}H]THLM-A_3 binding to ribosomes was almost completely inhibited. The 50% inhibition dose (ID_{50}) of LM-A_3 for the binding was cal-

Fig. 4. Analysis of ribosome-THLM-A_3 complex by sucrose density gradient centrifugation.

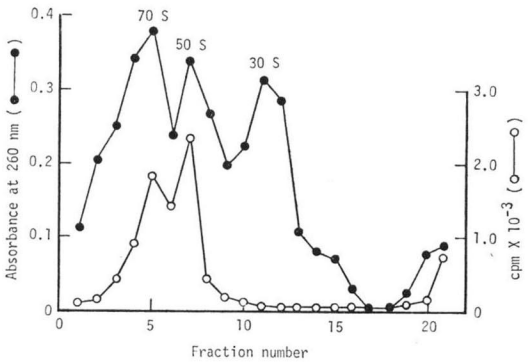
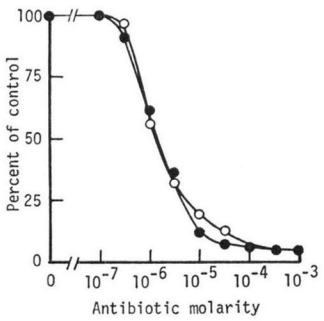


Fig. 5. Effect of LM-A_3 (●) and THLM-A_3 (○) on [^{3}H]THLM-A_3 binding to ribosomes.



culated from the results shown in Fig. 5 to be 1.8 μM and shown to be equivalent to that for THLM-A_3.

The ID_{50} value is similar level to those assayed using [^{14}C]erythromycin by PESTKA *et al.*[3] (4.2 μM) and ŌMURA *et al.*[4] (1.2 μM). THLM-A_3 and LM-A_3 also exhibited approximately the same antimicrobial activity against Gram-positive bacteria (Table 1 and Reference 7). Thus, the new method for direct determination of ribosome affinities of 16-membered macrolide antibiotics was established.

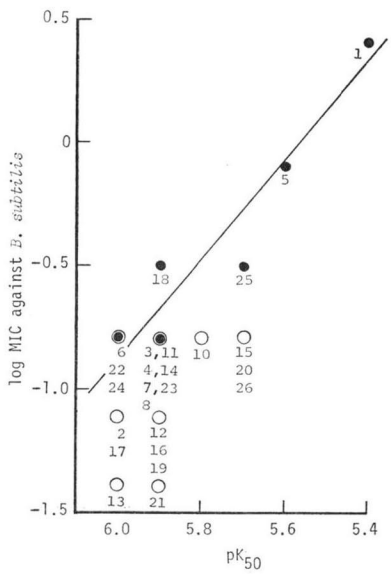
Correlation of the Affinities to Ribosomes of 3″-*O*-Acyl Derivatives of LMs with Their Antimicrobial Activities

The ID_{50} of LMs and 3″-*O*-acyl derivatives and the MICs against *B. subtilis*, *S. aureus*, *M. luteus*, and *E. coli* are summarized in Table 1. As shown in Table 1, 3″-*O*-acyl derivatives of LM-A_5, LM-A_4, and LM-A_3 (3″-*O*-acetyl-LM-A_5, 3″-*O*-propionyl-LM-A_5, 3″-*O*-acetyl-LM-A_4, and 3″-*O*-acetyl-LM-A_3) exhibited stronger antimicrobial activity against Gram-positive bacteria and weaker (or equivalent) activity against Gram-negative bacteria than their mother compounds. However, the affinities of the 3″-*O*-acyl derivatives to ribosomes were approximately equivalent to those of the mother compounds. This suggests that Gram-positive bacterial cells are more permeable to 3″-*O*-acyl derivatives than to the mother compounds. On the other hand, with a compound possessing a longer acyl group than butyryl, such as LM-A_1, 3″-*O*-acylation has little effect. 3″-*O*-Propionyl-LM-A_7 exhibited both a stronger antimicrobial activity and a higher affinity to ribosomes than LM-A_7. This good correlation suggests that the increase in the

Fig. 6. Log(MIC) as a function of pK_{50} of [^{3}H]-THLM-A_3 binding to ribosomes for LMs (●) and their 3″-*O*-acyl derivatives (○).

The MIC values against *B. subtilis* (Table 1) was used. The pK_{50} is the negative log of ID_{50} (Table 1).

The numbers in the figure refer to the compounds as numbered in Table 1.



affinity to ribosomes resulted in the increase in the antimicrobial activity.

The acylation at 9 position of 4''-*O*-acetyl-3''-*O*-butyryl LM-V, 3''-*O*-acetyl-LM-A_5, 3''-*O*-propionyl-LM-A_5, 3''-*O*-butyryl-LM-A_5, LM-A_4, and 3''-*O*-acetyl-LM-A_4 gave less effect on the ID_{50} and antimicrobial activity against Gram-positive bacteria, but decreased slightly the antimicrobial activity against *E. coli*.

As reported by PESTKA *et al.*[3] and ŌMURA *et al.*[4], the substitution of the 4'' position of LM-V with propionyl, butyryl, and *iso*-valeryl groups increased both affinity to ribosomes and antimicrobial activity, but the acetylation at the 3 position (LM-A_5→LM-A_4, LM-A_1→LM-A_3) decreased them.

Correlation between the concentration (pK_{50}) for 50% of [^{3}H]THLM-A_3 binding to ribosomes and log (MIC) values for the acyl derivatives is summarized in Fig. 6. There was a good correlation between the pK_{50} and log (MIC) values for LMs (solid circles, Fig. 6). On the other hand, most of 3''-*O*-acyl derivatives (open circles, Fig. 6) exhibited relatively high antimicrobial activity compared with binding activity to ribosomes, suggesting that the 3''-*O*-acyl derivatives are more permeable than their mother compounds. These results might provide significant hints for synthesizing more active derivatives. Recently, among these 3''-*O*-acyl derivatives, 3''-*O*-propionyl-LM-A_5 was found to exhibit the highest antimicrobial activity *in vitro* against Gram-positive bacteria and the highest serum level in dogs after oral administration[9]. Development of the compound for medicinal use is now in progress.

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References

1) CORCORAN, J. W.: Erythromycin and the Bacterial Ribosome. *in* "Drug Action and Drug Resistance in Bacteria", *ed.* S. MITSUHASHI, p. 177~200, Tokyo Press, 1971
2) OLEINICK, N. L.: The Erythromycins. *in* "Antibiotics. III" *ed.* J. W. CORCORAN & F. E. HAHN, p. 396~419, Springer Verlag, 1975
3) PESTKA, S.; A. NAKAGAWA & S. ŌMURA: Effect of leucomycins and analogues on binding [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicr. Agents Chemoth. 6: 606~612, 1974
4) ŌMURA, S.; A. NAKAGAWA, H. SAKAKIBARA, O. OKEKAWA, R. BRANDSCH & S. PESTKA: Structure-activity relationships among the *O*-acyl derivatives of leucomycin. Correlation of Minimal inhibitory concentrations with binding to *Escherichia coli* ribosomes. J. Med. Chem. 20: 732~736, 1977
5) PESTKA, S.: Binding of [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicr. Agents Chemoth. 6: 474~478, 1974
6) SIEGRIST, S.; J. LAGOUARDT, N. MOREAU & F. LE GOFFIC: Mechanism of action of a 16-membered macrolide. Binding of rosaramicin to the *Escherichia coli* ribosome and its subunits. Eur. J. Biochem. 115: 323~327, 1981
7) ŌMURA, S.; M. KATAGIRI, I. UMEZAWA, K. KOMIYAMA, T. MAEKAWA, K. SEKIKAWA, A. MATSUMAE & T. HATA: Structure-biological activities relationships among leucomycins and their derivatives. J. Antibiotics 21: 532~538, 1968
8) SAKAKIBARA, H.; O. OKEKAWA, T. FUJIWARA, M. OTANI & S. ŌMURA: Acyl derivatives of 16-membered macrolides. I. Synthesis and biological properties of 3''-*O*-propionylleucomycin A_5 (TMS-19-Q). J. Antibiotics 34: 1001~1010, 1981
9) SAKAKIBARA, H.; O. OKEKAWA, T. FUJIWARA, M. AIZAWA & S. ŌMURA: Acyl derivatives of 16-membered macrolides. II. Antibacterial activities and serum levels of 3''-*O*-acyl derivatives of leucomycin. J. Antibiotics 34: 1011~1018, 1981
10) JULENC, P. C.: Rapid purification of highly active ribosomes from *Escherichia coli*. Anal. Biochem. 105: 369~374, 1980
11) PESTKA, S.: Antibiotics as probes of ribosome structure: Binding of chloramphenicol and erythromycin to polyribosomes; effect of other antibiotics. Antimicr. Agents Chemoth. 5: 255~267, 1974