MACROLIDE ANTIBIOTICS M-4365 PRODUCED BY *MICROMONOSPORA*

III. *IN VITRO* ANTIMICROBIAL ACTIVITY OF ANTIBIOTIC M-4365G$_2$ (DE-EPOXY ROSAMICIN)

TOUTARO YAMAGUCHI, HARUO HAYASAKA, HIROTSUGU YOSHIDA, TADAHIRO MATSUSHITA, AKEMI YAMABE and SATOSHI OHSHIMA

Microbiological Research Laboratory, Tanabe Seiyaku Co., Ltd., Toda, Saitama, Japan

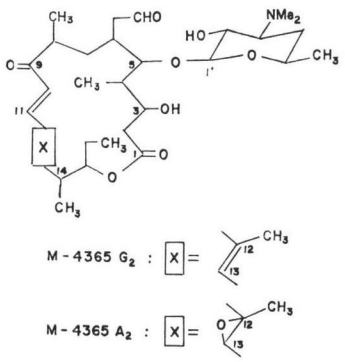
(Received for publication February 17, 1978)

Antibiotic M-4365G$_2$ (de-epoxy rosamicin) produced by *Micromonospora capillata* MCRL 0940 is a new basic 16-membered macrolide antibiotic with activity equal to or superior to erythromycin and josamycin against Gram-positive bacteria. Of interest are the high degree of activity against Gram-negative bacilli and mycoplasmas, and striking inhibitory effects against indole-producing *Proteus* spp. Bactericidal activity of M-4365G$_2$ is also to be noticed.

Antibiotic M-4365G$_2$ (de-epoxy rosamicin) produced by *Micromonospora capillata* MCRL 0940 is a new 16-membered macrolide antibiotic[1,2]. As shown in Fig. 1, M-4365G$_2$ is a de-epoxy compound of M-4365A$_2$ which is co-produced by MCRL 0940 and identified as rosamicin[3] and juvenimicin A$_3$[4].

Succeeding to the previous reports[1,2] dealing with taxonomic studies of the producing organisms, isolation, physicochemical properties and structure of M-4365G$_2$, the present paper concerned the *in vitro* properties of M-4365G$_2$ together with M-4365A$_2$ for comparison. In the paper, antimicrobial spectrum, sensitivity distribution of *Staphylococci, Escherichia coli, Klebsiella pneumoniae* and *Proteus* spp. isolated from patients, influence of the medium pH, serum and inoculum size on activity and bactericidal activity are involved.

Fig. 1. Structures of M-4365



Material and Methods

Antibiotics

M-4365G$_2$ and M-4365A$_2$ were prepared as described in the preceding paper[1]. Erythromycin base, josamycin base and leucomycin base were prepared from the commercial preparations. Antibiotic solutions for the *in vitro* test were prepared by dissolving an antibiotic in ethanol and then diluting with sterile distilled water.

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of the antibiotics was determined by the usual two-fold serial agar dilution technique. Not only the usual strains but also the clinical isolates of *Staphylococci*,

E. coli, K. pneumoniae and *Proteus* spp. kindly supplied by Dr. UMENAI, Central Clinical Laboratory, Tohoku University Hospital were used as the test organisms.

Heart infusion agar (HIA "Eiken") or 10% bovine blood supplemented HIA (blood HIA) was used for the maintenance of microorganisms and also as the test medium. One loopful of a culture suspension of each test organism was streaked on assay plates containing antibiotics and the plates were incubated at 37°C for 18 hours. MIC was determined as lowest concentration at which the visible growth of a test organism is completely inhibited.

Anti-mycoplasma Activity

The anti-mycoplasma activity of the antibiotics was examined against the following five mycoplasma strains: *Mycoplasma pneumoniae* Mac, *M. gallisepticum* Kp-13, *M. gallisepticum* PG-31, *M. pulmonis* mA and *Acholeplasma laidlawii*. These mycoplasmas were cultured in the liquid medium[5] containing 2.1% PPLO broth (Difco) 70 ml, fresh bovine serum 20 ml, thallium acetate 50 mg, penicillin G Na salt 50,000 units in 100 ml (pH 7.6~7.8). To know the growth of mycoplasma, 1 g glucose and 2 mg phenol red were also added to the medium. Incubation at 37°C for about 3 days generally gave a maximum titer of $10^6 \sim 10^7$ colony-forming-unit (CFU) per ml. The anti-mycoplasma activity of the antibiotics was assayed by two fold dilution technique in the PPLO broth by inoculating $10^5 \sim 10^6$ CFU/ml of each mycoplasma.

Influence of Various Factors on MIC

To assess the influence of the pH of the media, pH values of the medium were adjusted by 1 N HCl or 1 N NaOH. The influence of the inoculum size on the *in vitro* activity was evaluated in a tube dilution test by inoculating $10^3 \sim 10^7$ cells/ml of *S. aureus* 209-P JC-1 or *E. coli* NIHJ JC-2 to the media. The influence of serum on MIC was determined by adding increasing concentration of bovine serum to the media.

Bactericidal Activity

An 18-hour culture of *S. aureus* 209-P JC-1 as the test organism was diluted to 1/1,000 with Trypticase Soy Broth (TSB) to give cell suspension containing about 10^6 cells/ml. After 1.5-hour precultivation of the above cell suspension at 37°C, antibiotic solution was added to a tube to give a final concentration of 0.1, 0.5, 1.0, 5.0 or 10 μg/ml of an antibiotic. An aliquot was withdrawn from each tube at 0, 1, 2, 4, 7 and 24 hours after incubation at 37°C, and, after appropriate dilution, spread over on the surface of HIA in a plate. After 24 hours incubation of a plate, the viability of the microorganism was determined by the colony counting technique. Platings were made in duplicate at several dilutions to ensure reliable counts.

Results and Discussion

Antimicrobial Spectrum

Antimicrobial spectra and MICs of M-4365G_2 and M-4365A_2 against Gram-positive bacteria are shown in Table 1. As compared with erythromycin and josamycin, M-4365G_2 and A_2 were active against all of Gram-positive bacteria susceptible to erythromycin and josamycin. Moreover, M-4365G_2 and A_2 were highly active against anaerobic bacteria such as *Clostridium* spp.

However, M-4365G_2 and A_2, like erythromycin and josamycin, were inactive against macrolide-resistant strains of *Staphylococcus aureus* and *Streptococcus faecalis* and also against *Mycobacteria*.

As shown in Table 2, M-4365G_2 and A_2 were proved to be remarkably active against Gram-negative bacteria. Activity against Gram-negative bacilli appears to be considerably greater than that of erythromycin and josamycin. M-4365G_2 was more potent than M-4365A_2 against *Proteus* spp., particularly an indole-producing *Proteus vulgaris*.

The 16-membered macrolide antibiotics are known to inhibit mycoplasma[6] and recommended for infections caused by mycoplasma. As shown in Table 3, M-4365G_2 and A_2 inhibited all

Table 1. Antimicrobial spectra of M-4365 A_2, M-4365 G_2, erythromycin and josamycin against Gram-positive bacteria

Test organisms	MIC (μg/ml)			
	M-4365 A_2	M-4365 G_2	EM	JM
Staphylococcus aureus 209-P, JC-1	0.2	0.2	0.1	0.39
〃 Smith	0.2	0.2	0.1	0.2
〃 Terajima	0.2	0.2	0.1	0.2
〃 252R (TC, AB)	0.2	0.2	0.1	0.39
〃 199R (Mac)	>100	>100	>100	>100
〃 664R (TC)	0.2	0.2	0.78	0.39
Staphylococcus epidermidis 10131R	0.2	0.1	0.05	0.2
〃 Kawamura	0.2	0.2	0.05	0.2
Streptococcus faecalis	0.2	0.39	0.1	0.39
〃 Urayama (Mac-R)	>100	>100	>100	>100
Streptococcus pneumoniae type-1*	0.39	0.39	0.05	0.39
Micrococcus luteus ATCC	0.2	0.2	0.05	0.05
Bacillus subtilis ATCC 6633	0.2	0.2	0.05	0.2
Bacillus anthracis	0.2	0.39	0.78	0.78
Corynebacterium diphtheriae P.W.8*	0.1	0.05	0.1	0.2
*Clostridium tetanii**	0.03	0.03	1.0	0.5
*Clostridium perfringens**	0.1	0.05	0.13	1.0
Mycobacterium tuberculosis $H_{37}Rv$	>100	>100	>100	>100
Mycobacterium smegmatis ATCC 607	>100	>100	>100	>100

Medium: Heart infusion agar (Eiken)
* Heart infusion agar +10% bovine blood

mycoplasma except *Acholeplasma laidlawii* at a low concentration of less than 0.012 μg/ml. M-4365G_2 was 2~3 times more active than M-4365A_2 and far active than leucomycin.

Sensitivity of Clinically Isolated Bacteria

The susceptibility pattern of clinically isolated bacteria supplied from the Tohoku University Hospital to M-4365G_2 and A_2 are illustrated in Table 4, Figs. 2, 3, 4 and 5.

Fig. 2. Sensitivity distribution of clinical isolates of *S. aureus* (39 strains, 10^6 cell/ml)

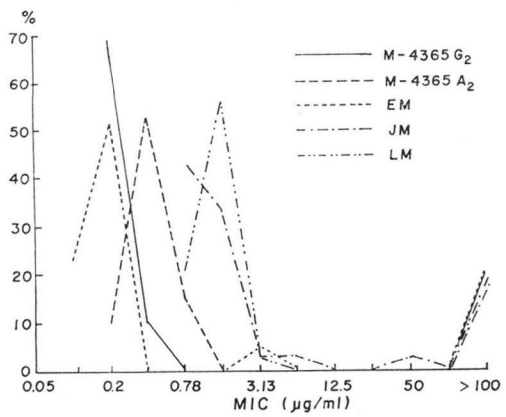


Fig. 3. Sensitivity distribution of clinical isolates of *E. coli* (35 strains, 10^6 cells/ml)

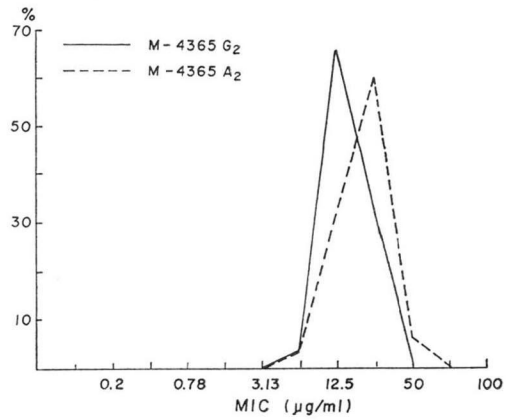


Table 2. Antimicrobial spectra of M-4365 A_2, M-4365 G_2, erythromycin and josamycin against Gram-negative bacteria

Test organisms	MIC (μg/ml)			
	M-4365 A_2	M-4365 G_2	EM	JM
Neisseria gonorrhoeae	0.05	0.05	0.39	0.39
Neisseria meningitidis	0.2	0.1	0.39	0.39
Escherichia coli NIHJ JC-2	12.5	6.25	50	>100
〃 K-12	6.25	6.25	>100	>100
〃 K-12 W3630/R6 GR43	3.13	3.13	—	—
〃 33R (TC, KM, AB)	25	12.5	—	—
〃 6065R (CP, KM, AB)	12.5	12.5	>100	>100
〃 ML-1410 RGN-823	12.5	12.5	50	>100
〃 ML-1630	12.5	12.5	25	>100
〃 JR66/W677	12.5	12.5	12.5	>100
〃 A20892	3.13	0.78	25	>100
Klebsiella pneumoniae PCI-602	25	25	50	>100
〃 5075	25	12.5	100	>100
〃	3.13	3.13	50	>100
Salmonella typhi T-58	12.5	12.5	—	—
Salmonella typhimurium	12.5	6.25	100	>100
Salmonella enteritidis	6.25	6.25	—	—
Shigella flexneri 2a	3.13	3.13	—	—
〃 2a-R (TC, SM, CP)	6.25	6.25	—	—
Shigella sonnei	6.25	6.25	—	—
〃 R (TC, SM, CP, DKB)	6.25	6.25	—	—
Citrobacter freundii 770	50	25	—	—
Enterobacter aerogenes TU-663	100	25	—	—
Enterobacter cloacae KT-19	25	25	—	—
Serratia marcescens 7006	12.5	6.25	50	>100
〃 OU-29	25	12.5	—	—
Proteus vulgaris OX-19	6.25	3.13	—	—
〃 6028	25	3.13	100	>100
Proteus mirabilis TU-1698	25	6.25	>100	>100
Proteus morganii KU-127	100	25	>100	>100
Proteus rettgeri HU-36	100	50	—	—
Proteus inconstans	50	25	—	—
Providencia stuartii A20894	1.56	1.56	100	>100
Pseudomonas aeruginosa No. 12	12.5	12.5	50	>100
〃 PI-67	50	50	100	>100
〃 GN-315	25	25	50	>100

Medium: Heart infusion agar (Eiken)

In the case of *S. aureus* (Fig. 2), josamycin and leucomycin inhibited growth at concentrations of 0.78~3.13 μg/ml. However, M-4365G_2 and A_2 as well as erythromycin inhibited at lower concentrations, 0.1~1.56 μg/ml. Therefore, M-4365G_2 and A_2 were approximately 4~8 times more active than josamycin and leucomycin, and comparable to erythromycin.

M-4365G_2 and A_2 possessed marked antimicrobial activity against strains of *Enterobacteriaceae*.

Table 3. Antimicrobial activity of M-4365 A_2, M-4365 G_2 and leucomycin against *Mycoplasma*

Test organisms	MIC (μg/ml)		
	M-4365 A_2	M-4365 G_2	LM
Mycoplasma pneumoniae Mac	0.012	0.006	0.78
Mycoplasma gallisepticum Kp-13	0.003	0.0015	0.2
Mycoplasma gallisepticum PG-31	0.006	0.003	0.2
Mycoplasma pulmonis mA	0.006	0.003	0.2
Acholeplasma laidlawii	1.56	0.78	3.13

Serial dilution in PPLO broth "Eiken", 37°C, 20 hours.
Inoculum size: $10^6 \sim 10^7$ CFU/ml.

Fig. 4. Sensitivity distribution of clinical isolates of *K. pneumoniae* (40 strains, 10^6 cells/ml)

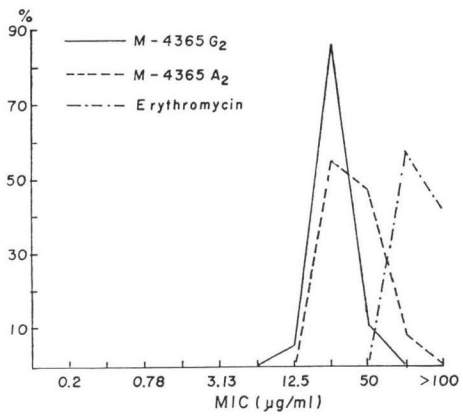
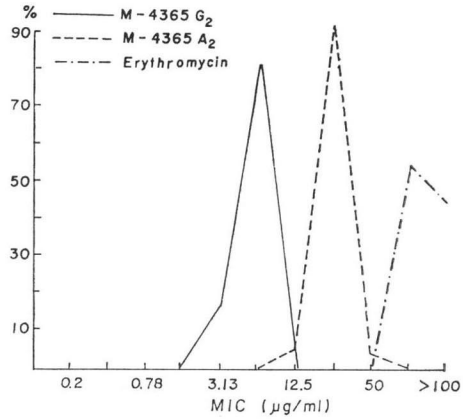


Fig. 5. Sensitivity distribution of clinical isolates of *Pr. vulgaris* (40 strains, 10^6 cells/ml).



About 60% of *E. coli* were susceptible to M-4365G_2 at a concentration of 12.5 μg/ml, and it was two times more effective than M-4365A_2 (Fig. 3). Against *K. pneumoniae*, M-4365G_2 and A_2 were active at concentration of less than 50 μg/ml and superior to erythromycin (Fig. 4).

Recently, an indole-positive *Proteus* has been increasingly isolated as a pathogenic microbe. This organism was reported to be highly resistant to β-lactam antibiotics and other chemotherapeutics, except for the aminoglycoside antibiotics. Against *Proteus vulgaris* (Fig. 5), M-4365G_2 was active at a concentration of less than 6.25 μg/ml, and approximately 4 to 8 times more active than M-4365A_2 and 32 times more active than erythromycin.

Influence of Medium pH, Inoculum Size and Serum on the Activity

The effect of pH on the antimicrobial activity of M-4365G_2, A_2, erythromycin and josamycin in HIA is shown in Table 5. The MICs are dependent upon the pH of the test medium and substantially more active at an alkaline pH. The effect of alkalinization was especially remarkable on *E. coli*. This phenomenon has already been demonstrated on rosamicin[7,8] and other macrolide antibiotics. As suggested by ZINNER *et al.*[9] and considering from the present *in vitro* data, M-4365G_2 may be of potential use in treatment of urinary tract infections caused by selected Gram-negative bacilli. So long as tested on *S. aureus* 209-P JC-1 and *E. coli* NIHJ JC-2, changes in inoculum size showed no significant effect on the activities of M-4365G_2 and A_2 as well as erythromycin and josamycin. How-

Table 4. Distribution of susceptibilities of clinical isolates to M-4365 A_2, M-4365 G_2 and other macrolide antibiotics*

Microorganisms	Antibiotics	No. of strains showing MIC (μg/ml) of:											
		>100	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1
S. aureus	M-4365 A_2	8								6	21	4	
(39 strains)	M-4365 G_2	8									4	27	
	EM	8						2				20	9
	JM	7					1	1	13	17			
	LM	7		1				1	22	8			
E. coli	M-4365 A_2			2	21	11	1						
(35 strains)	M-4365 G_2				11	23	1						
C. freundii	M-4365 A_2	3		2	1								
(6 strains)	M-4365 G_2		1	3	1	1							
K. pneumoniae	M-4365 A_2		3	15	22								
(40 strains)	M-4365 G_2			4	34	2							
	EM	17	23										
Enterobacter spp.	M-4365 A_2	3	3	1		1							
(8 strains)	M-4365 G_2		1	5	1	1							
P. vulgaris	M-4365 A_2			1	37	2							
(40 strains)	M-4365 G_2						33	7					
	EM	18	22										
P. mirabilis	M-4365 A_2	8	24	3									
(35 strains)	M-4365 G_2		1	8	26								

* Conditions: Heart infusion agar, stamp method, 37°C, 20 hours, 10^6 cells/ml.

Table 5. Influence of various factors on the antimicrobial activity of M-4365 A_2, M-4365 G_2 and other macrolide antibiotics

Factor	*S. aureus* 209-P JC-1				*E. coli* NIHJ JC-2		
	M-4365 A_2	M-4365 G_2	EM	JM	M-4365 A_2	M-4365 G_2	EM
pH 5.0	0.39	0.39	0.39	0.39	50	25	100
6.0	0.39	0.39	0.39	0.39	50	25	100
7.0	0.39	0.39	0.2	0.39	12.5	6.25	100
7.4	0.2	0.2	0.1	0.2	12.5	6.25	50
8.2	0.1	0.1	0.1	0.2	3.13	3.13	6.25
Inoculum size							
10^7	—	—	—	—	25	6.25	100
10^6	0.2	0.2	0.1	0.39	12.5	6.25	100
10^5	0.1	0.05	0.03	0.39	6.25	0.78	25
10^4	0.05	0.03	0.02	0.39	1.56	0.78	25
10^3	0.03	0.03	0.02	0.39	1.56	0.78	12.5
Serum (%)*							
0	0.78	0.78	0.78	0.78	12.5	6.25	50
10	0.78	0.78	0.78	0.78	12.5	6.25	50
25	0.78	0.78	0.78	0.78	12.5	6.25	50
50	0.78	0.78	0.78	0.78	12.5	6.25	50

* Bovine serum

MIC (μg/ml)

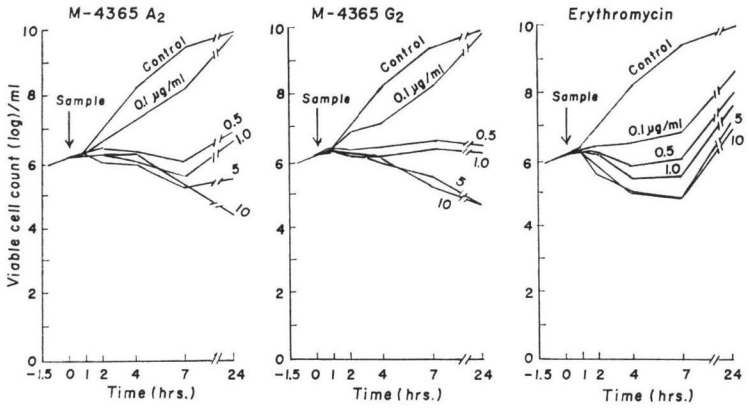
ever, the MICs of these antibiotics tended to be higher as the inoculum size increased. In *E. coli* NIHJ JC-2, the MIC of M-4365G_2 fell dramatically when the inoculum size was decreased.

Effects of the presence of bovine serum on the antimicrobial activity were shown in Table 5. Presence of bovine serum in the medium at a final concentration of 50% did not affect the activities of M-4365G_2 and others.

Bactericidal Activity

The bactericidal activity of M-4365G_2, A_2 and erythromycin against *S. aureus* 209-P JC-1 are shown in Fig. 6, in which the viable counts in a logarithmic scale are plotted against the time of exposure to the antibiotics. Weak bactericidal activity was demonstrated on all antibiotics at concentrations of 5 and 10 μg/ml. However, in contrast to others, even at the concentration of 0.5 μg/ml and 1.0 μg/ml of M-4365G_2, viable units did not vary for 24 hours.

Fig. 6. Bactericidal effect of M-4365 A_2, M-4365 G_2 and erythromycin on *S. aureus* 209P JC-1.



In conclusion, M-4365G_2 is considered to be a most interesting macrolide antibiotic, in respect of its broad spectrum, strong inhibitory activity especially against Gram-negative bacteria including an indole-producing *Proteus* spp. and mycoplasma and also of its bactericidal property.

In the preliminary infection test with mice challenged with *S. aureus* Smith, M-4365G_2 showed the protective effect as M-4365A_2 and josamycin by intramuscular administration. By oral route, the therapeutic activity of M-4365G_2 was similar to erythromycin and surpassed those of M-4365A_2 and josamycin. Details of the *in vivo* tests against Gram-positive and -negative bacteria together with concentration and distribution in blood and other organs will be reported later.

Acknowledgments

We are indebted to Drs. H. UMEZAWA, Institute of Microbial Chemistry, H. KAWAGUCHI, Bristol Banyu Research Institute, Ltd., and S. MATSUURA, Shionogi Research Laboratory, Shionogi & Co., Ltd., for supplying some of the test organisms used in this study. We wish to also thank Dr. T. OKUDA in this company for his encouragement and helpful advices throughout this work.

References

1) FURUMAI, T.; I. MAEZAWA, S. YANO, T. YAMAGUCHI, K. TAKEDA & T. OKUDA: Macrolide antibiotics M-4365 produced by *Micromonospora*. I. Taxonomy, production, isolation, characterization and proper-

ties. J. Antibiotics 30: 443~449, 1977
2) KINUMAKI, A.; K. HARADA, T. SUZUKI, M. SUZUKI & T. OKUDA: Macrolide antibiotics M-4365 produced by *Micromonospora*. II. Chemical structures. J. Antibiotics 30: 450~454, 1977
3) REIMANN, H. & R. S. JARET: Structure of rosamicin, a new macrolide *Micromonospora rosaria*. J. Chem. Soc., chem. Commun. 1972: 1270, 1972
4) KISHI, T.; S. HARADA, H. YAMANA & A. MIYAKE: Studies on juvenimicin, a new antibiotic. II. Isolation, characterization and structures. J. Antibiotics 29: 1171~1181, 1976
5) CHANOCK, R. M.; L. HAYFLICK & M. F. BARILE: Grown on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Nat. Acad. Sci., U.S. 48: 41~49, 1962
6) ŌMURA, S.; Y. HIRONAKA, A. NAKAGAWA, I. UMEZAWA & T. HATA: Antimycoplasma activities of macrolide antibiotics. J. Antibiotics 25: 105~108, 1972
7) WAITZ, J. A.; C. G. DRUBE, E. L. MOSS, Jr. & M. J. WEINSTEIN: Biological studies with rosamicin, a new *Micromonospora*-produced macrolide antibiotic. J. Antibiotics 25: 647~652, 1972
8) CROWE, C. C. & W. E. SANDERS, Jr.: Rosamicin: Evaluation *in vitro* and comparison with erythromycin and lincomycin. Antimicr. Agents & Chemoth. 5: 272~275, 1974
9) ZINNER, S. H.; L. D. SABATH, J. I. CASEY & M. FINLAND: Erythromycin plus alkalinization in treatment of infection. Antimicr. Agents & Chemoth. 1969: 413~416, 1970