BIOLOGICAL CHARACTERIZATION OF DIUMYCIN, A PHOSPHORUS-CONTAINING GLYCOLIPID ANTIBIOTIC

E. MEYERS, D. SMITH SLUSARCHYK, F. E. PANSY, H. I. BASCH, W. H. TREJO and P. J. DEBAECKE

Squibb Institute for Medical Research,
New Brunswick, N. J. 08903, U.S.A.

(Received for publication December 20, 1971)

Diumycin, a phosphorus-containing glycolipid antibiotic, is produced by *Streptomyces umbrinus* ATCC 15972. It is active against gram-positive bacteria and, to a lesser degree, against gram-negative bacteria. Diumycin affects dividing but not resting cells of *Staphylococcus aureus* FDA 209 P. The antibiotic is strongly bound by serum. Although primarily bacteriostatic at levels approximating the minimum inhibitory concentration, it is bactericidal at higher levels. Diumycin is active upon subcutaneous administration to mice infected with *Streptococcus pyogenes* C 203 and *Diplococcus pneumoniae* and, like other members of the phosphorus-containing glycolipid antibiotic group, demonstrates a unique prophylactic activity against these infections. High and prolonged serum levels (lasting several weeks) were achieved in the plasma of dogs and monkeys after a single, parenteral dose of the antibiotic, and small to moderate amounts of bioactivity were recovered in the urine. Toxicity studies indicate that diumycin is well-tolerated in mice, but may cause a transient liver damage at dose levels near the LD_{50}.

The isolation of diumycin, a new member of the group of phosphorus-containing glycolipid antibiotics, has been previously reported[1]. Other members of this group are: prasinomycin[2], moenomycin[3], 11837 R.P.[4], macarbomycin[5], and, presumably, 8036 R.P. (South African patent 65/6204) and 19402 R.P. (Netherlands patent 68, 02093). The structure of the lipid moiety of diumycin, the aggregation behavior, and the probable mode of action of this antibiotic have been the subjects of previous reports[6,7,8]. This manuscript describes our studies on the taxonomic characterization of *Streptomyces umbrinus* ATCC 15972, the organism that produces diumycin and extends our studies on the antimicrobial properties of this antibiotic. Also described are studies on toxicity and blood levels obtained with diumycin.

Experimental

All taxonomic studies were done according to the standard procedures recommended by the Subcommittee on Actinomycetes of the Committee on Taxonomy, American Society for Microbiology[9]. Included in these studies were our soil isolate and an authentic culture of *S. umbrinus*, NRRL B-2572.

Conventional two-fold broth-dilution assays were performed to determine the minimal inhibitory concentration (MIC) of diumycin for most of the test organisms. The susceptibility of *Neisseria gonorrhoeae* was determined by the agar inclusion assay recommended by the United States Public Health Service[10]. A paper-disc agar-diffusion assay with

Staphylococcus aureus FDA 209 P was used to assay potencies of blood and urine samples. The mouse model infection systems described by MIRAGLIA and BASCH[11] were used to evaluate diumycin *in vivo*.

Male, Charles River CD-1 mice were used for acute toxicity studies. They were given food and water *ad libitum*, but were fasted one hour prior to drug administration. A single-dose toxicity study employed both male and female mice. For blood level and urinary excretion studies, three young-adult pure-bred beagle dogs and a young-adult rhesus monkey (*Macaca mulatta*) were used.

All studies, except those for acute toxicity, blood levels and urinary excretion, were done with material of 90~95 % purity (Diumycin lot SQ 20,117). Toxicity, and blood and urine level studies were carried out with a preparation of 80~90 % purity (Diumycin lot XYSCD-23-Cr-1).

Results

Taxonomy

The key taxonomic characteristics of *S. umbrinus* ATCC 15972 involve—

Morphology: The sporophores were in straight chains (Rectus group of PRIDHAM)[12] with no tendency toward flexuousness or spirals. The spores were smooth and cylindric to ovoid.

Cultural Characteristics: Growth was good on tomato paste-oatmeal and yeast-malt extracts agars; vegetative growth was rapid, producing a characteristic chocolate to mahogany brown vegetative mycelium; an abundant aerial mycelium was produced in approximately 14 days on both of the above media and was assignable to the olive-buff color series of PRIDHAM[12]; the color matched chips 3 ba to 3 cb in the Color Harmony Manual[13]; melanoid pigment was produced on organic media. Table 1 summarizes a comparison of the taxonomic characteristics of *S. umbrinus* ATCC 15972 and an authentic reference culture *S. umbrinus* NRRL B-2572.

Table 1. Comparison of the taxonomic characteristics of *S. umbrinus* ATCC 15972 and *S. umbrinus* NRRL B-2572

Character	*S. umbrinus* ATCC 15972	*S. umbrinus* NRRL B-2572
Morphology group	RF	RF
Spores	Smooth	Smooth
Color series	Buff	Buff
Reverse color	Reddish brown	Reddish brown
Chromogenicity	Melanin produced	Melanin produced
Other pigments	Quinoid	Quinoid
NO_3 reduction	+	+
Carbon utilization pattern		
Basal minimal control	−	−
Glucose	+	+
Mannitol	+	+
Inositol	+	+
Sorbitol	−	−
Xylose	+	+
Arabinose	+	+
Rhamnose	+	+
Fructose	+	+
Raffinose	+	+
Galactose	+	+
Trehalose	+	+
Melibiose	+	+
Sucrose	+	+
Lactose	+	−

In Vitro

The sensitivity of various microorganisms to diumycin is shown in Tables 2 and 3. These data, as extensions of the spectrum given in our previous report[1], indicate that diumycin was quite inhibitory to gram-positive bacteria. Gram-negative bacteria were also inhibited by this antibiotic, but

Table 2. Antimicrobial spectrum of diumycin

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209 P	0.06
Streptococcus pyogenes C 203	0.001
Diplococcus pneumoniae Type 3 ATCC 6303	0.30
Bacillus cereus ATCC 10876	0.05
Escherichia coli SC 8294*	50.0
Pseudomonas aeruginosa SC 8329*	31.2
Proteus vulgaris SC 8504*	37.5
Pasteurella multocida SC 8739*	0.59
Trichophyton mentagrophytes SC 2637*	>100.0
Trichomonas vaginalis SC 8560*	>100.0

* Squibb Culture Collection

Table 3. Antimicrobial activity of diumycin against clinical isolates of *Neisseria gonorrhoeae*

N. gonorrhoeae	MIC (μg/ml)*		
	Diumycin	Penicillin G	Tetracycline
SC 8586**	1.0	0.08	0.25
SC 8587**	1.0	0.03	0.25
SC 8590**	1.5	0.25	0.5
SC 8591**	1.5	0.01	<0.13
SC 8593**	2.0	0.4	1.0

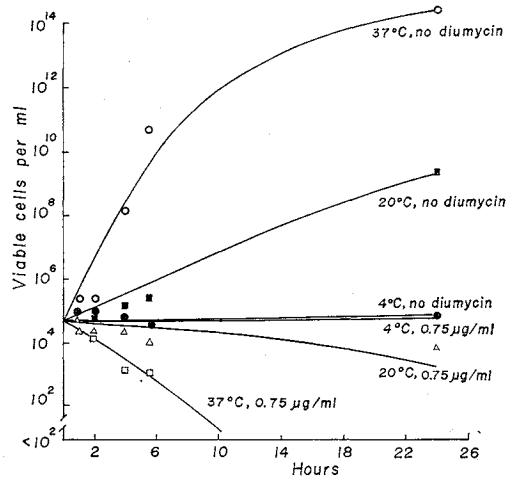
* Determined by an agar-inclusion plate method.
** Squibb Culture Collection.

Table 4. Serum binding of diumycin *in vitro*

Organism	MIC (μg/ml)	
	No serum	50% Human serum
Staphylococcus aureus FDA 209P	0.02	28.0
Streptococcus pyogenes C 203	0.001	4.2
Escherichia coli SC 2927*	31.0	>1000.0

* Squibb Culture Collection

Fig. 1. Effect of diumycin on dividing and nondividing cells of *Staphylococcus aureus* FDA 209 P



higher concentrations were required. Recent clinical isolates of *Neisseria gonorrhoeae* were susceptible to the action of diumycin, including two isolates with increased resistance to penicillin (Table 3). Though cross-resistant with prasinomycin, a closely related but chemically distinguishable antibiotic, diumycin was not cross-resistant with a variety of chemically unrelated antibiotics, *e.g.*, penicillin, tetracycline, streptomycin, erythromycin, novobiocin, and thiostrepton. Diumycin was bound to a significant extent by serum, as indicated by a 1,000~ 4,000-fold increase in MIC values obtained in the presence of 50% human serum in the medium, as compared with the MIC values obtained in the assay medium devoid of serum (Table 4).

Like prasinomycin, diumycin appears to kill only those cells of *Staphylococcus aureus* FDA 209 P that are actively dividing. Thus, cells of *S. aureus* FDA 209 P incubated under conditions allowing rapid division were quickly killed in the presence of diumycin ; however, cells of *S. aureus* FDA 209 P kept from multiplying by incubation at 4°C were not killed (Fig. 1). To determine whether a correlation existed between the rate of growth and the degree of inhibition of growth by diumycin, counts of viable cells were made at intervals during incubation at 37°C, 20°C and 4°C in the presence and absence of the antibiotic, 0.75 μg/ml. At 37°C, rapid growth occurred in the absence of diumycin, whereas cells were killed rapidly in its presence. Growth at 20°C in the absence of the antibiotic was slower than at 37°C, and the rate

of loss of cell viability in the presence of diumycin was also slower than at 37°C. At 4°C, no changes in viable cell numbers in the absence or in the presence of the antibiotic were noted. The results of this experiment are in complete agreement with those previously obtained with prasinomycin[14].

Diumycin, like prasinomycin, was either bactericidal or bacteriostatic for *S. aureus*

Fig. 2. Bactericidal and bacteriostatic action of diumycin against *Staphylococcus aureus* FDA 209 P

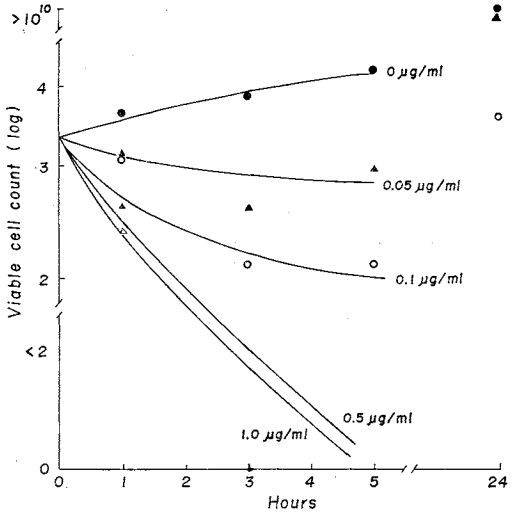


Fig. 3. Bactericidal and bacteriostatic action of diumycin against *Streptococcus pyogenes* C 203

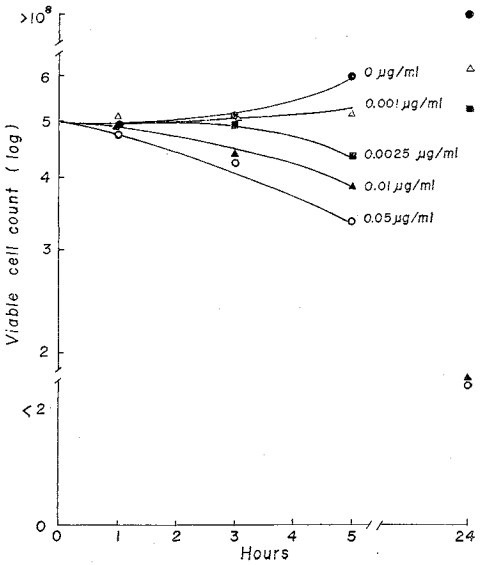


Fig. 4. Effect of 50 % human serum on the bactericidal and bacteriostatic action of diumycin against *Staphylococcus aureus* FDA 209 P

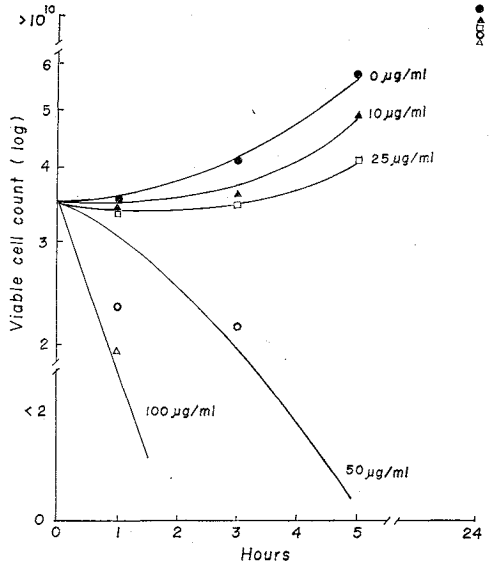
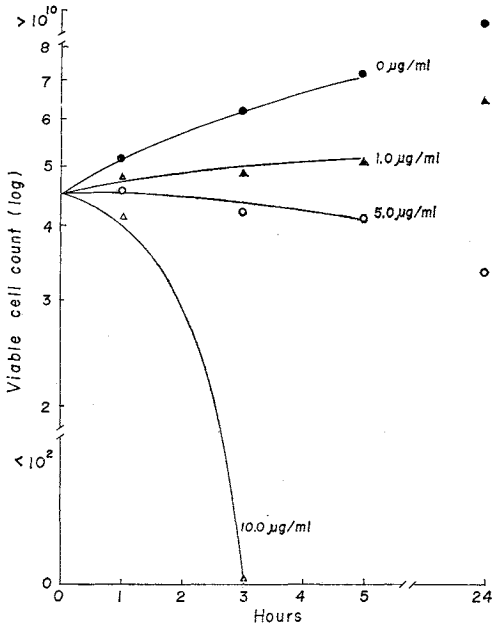


Fig. 5. Effect of 50 % human serum on the bactericidal and bacteriostatic action of diumycin against *Streptococcus pyogenes* C 203



FDA 209 P and *Streptococcus pyogenes* C 203, depending on the concentration of the antibiotic and the duration of contact between the antibiotic and the test organism (Figs. 2 and 3). It must be noted that the concentrations of antibiotic employed in the experiment reflect the sensitivities of these two organisms to diumycin. Diumycin, at a concentration of 0.5 μg/ml, was bactericidal to *S. aureus* FDA 209 P, whereas only 0.01 μg/ml was needed for a bactericidal effect on *S. pyogenes* C 203. At these levels, no outgrowth of *S. aureus* FDA 209 P or *S. pyogenes* C 203 was observed after 24 hours of incubation.

Because of the marked binding of diumycin by serum (Table 4), the bactericidal-bacteriostatic activities of diumycin were determined in the presence of 50 % human serum. These data are shown in Figs. 4 and 5. In the presence of 50 % serum, a diumycin concentration of 50 μg/ml effected a marked reduction in the number of viable cells of *S. aureus* FDA 209 P within 5 hours; however, many viable cells were present after 24 hours of incubation. Even at a concentration of 100 μg/ml, sterilization of the culture was not realized. With *S. pyogenes* C 203, the concentration of diumycin needed to cause a rapid decrease in the number of viable cells was 10 μg/ml, and at this level, no viable cells were found after 24 hours of incubation.

In Vivo

Diumycin was highly effective in protecting mice against death from lethal infections of *S. pyogenes* C 203 and *Diplococcus pneumoniae* Type 3. The PD_{50} values (the minimum dose of antibiotic that protects 50 % of the infected mice against death) for diumycin in these infection models were 0.22 and 5.06 mg/kg, respectively, when the antibiotic was administered subcutaneously.

As with other members of the phosphorus-containing class of glycolipid antibiotics[14,15,16], a single subcutaneous dose of diumycin provided prolonged protection to mice infected with *S. pyogenes* C 203 and *D. pneumoniae* Type 3. In Table 5 are presented data showing the relationship between the PD_{50} values and the period of time elapsed between drug administration and challenge with the infecting organism. To our knowledge, no antibiotics other than members of this group have been reported to possess this prolonged duration of activity.

Table 5. Effect of time of administration of diumycin on effectiveness in protecting mice against infection

Infecting organism	Time of administration of antibiotic	PD_{50}, s.c.** (mg/kg)
Streptococcus pyogenes C 203	−8 weeks*	62.0
	−6 weeks	27.8
	−4 weeks	16.2
	−2 weeks	9.7
	−1 day	0.5
	+1 hour	0.22
Diplococcus pneumoniae Type 3 ATCC 6303	−6 weeks	299.0
	−4 weeks	181.0
	−2 weeks	93.4
	−1 week	45.6
	−20 hours	6.85
	+1, +5 hours	5.06

* The plus and minus signs denote the time lapse between antibiotic treatment and infection. A minus sign indicates that the antibiotic was given *prior to challenge*: a plus sign indicates that the antibiotic was given after challenge.

** s.c.=subcutaneous route

Oral administration of high doses of diumycin protected mice against an otherwise lethal infection with *S. pyogenes* C 203. When diumycin was given 1 hour after challenge with the infecting organism, the value was 470 mg/kg, a value 2,136 times greater than the PD_{50} value after subcutaneous dosing. The oral route cannot, therefore, be considered of practical value for the administration

of diumycin.

Toxicity

By both intravenous and intraperitoneal routes of administration to mice, the LD_{50} for diumycin was 590 mg/kg and the LD_2 was 455 mg/kg. Deaths occurred 1~4 days after dosing and were generally preceded by ataxia and weight-loss. No overt effects, other than weight-loss, were observed in animals dosed intravenously with 125 or 250 mg/kg. Representative mice that had received single, intravenous doses of 500, 250 or 125 mg/kg were necropsied 8 days after dosing. Independent of the dosage at these drug levels, the livers showed microscopic evidence of cell damage which was evidenced by multifocal areas of hepatocyte necrosis with acute inflammatory cell infiltration perivascularly and in portal zones. There were numerous mitotic figures indicative of liver cell regeneration. Animals necropsied 20 or 30 days after dosing showed no liver cell necrosis; however, there was some residual inflammatory reaction in one high dose animal at each time period, suggesting a transient pathogenic process. No other significant gross or microscopic changes were seen.

Plasma Level and Decay; Urinary Excretion

The half-life of diumycin in plasma was approximately 4 days in dogs given an intravenous or intramuscular injection of 20 or 40 mg/kg. After an intramuscular dose of 20 mg/kg, maximum bioactivity in plasma was attained at about 2.25 hours (Fig. 6). In the monkey, maximal bioactivity in plasma was attained 24 hours after an intramuscular injection of 40 mg/kg, and activity continued at that level for 7 more days (Fig. 7). Diumycin activity was still detectable in dog and monkey plasma 23 and 21

Fig. 6. Plasma decay curves of diumycin in dogs given a single intravenous or intramuscular injection.

The activity is expressed in units, whereby one unit equals 1.25 μg of the preparation used for the injection.

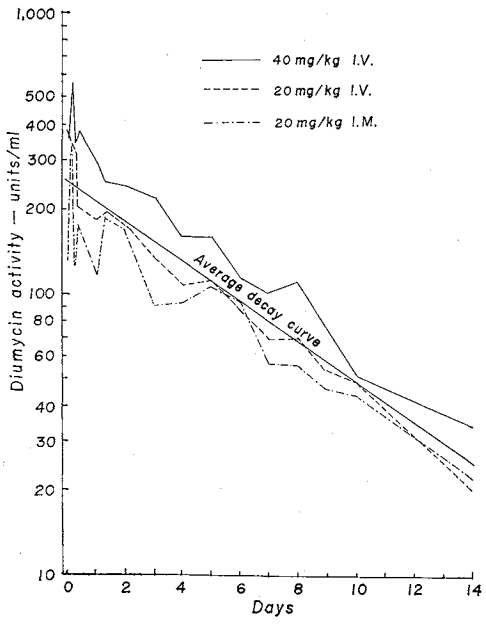
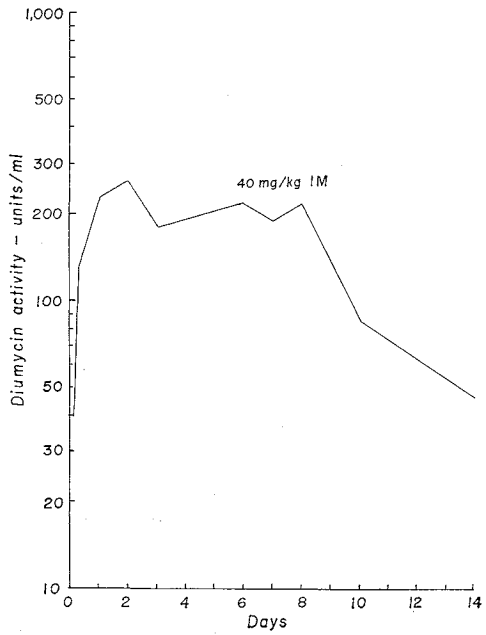


Fig. 7. Plasma decay curve of diumycin in a rhesus monkey given a single intramuscular injection

The activity is expressed in units, whereby one unit equals 1.25 μg of the preparation used for the injection.



days, respectively, after drug administration. The studies were then discontinued. The apparent volume of distribution of diumycin in the dog, estimated by extrapolation of the average plasma decay curve (Fig. 6) to zero-time, was 75 ml/kg or 7.5 % of the body weight.

The cumulative recovery of diumycin in the urine 9 days after intravenous or intramuscular dosing averaged 4.6 % of the administered dose in dogs and 20 % in the monkey. Maximal recovery of the antibiotic was obtained between 30 and 48 hours after drug adminstration to dogs and approximately 4 days after drug administration to the monkey.

Discussion

The mode of action of diumycin was reported to involve inhibition of the biosynthesis of the cell wall[8]; the exact locus at which this occurs is still unreported. The relatively low toxicity of this antibiotic, however, is in keeping with its probable action as an inhibitor of cell wall synthesis. Prasinomycin and moenomycin, two related phosphorus-containing glycolipid antibiotics were also reported to inhibit the synthesis of bacterial cell walls[8,17,18,19].

The prolonged protective action conferred by a single, parenteral dose and the corresponding long duration of demonstrable blood levels and the apparent volume of distribution in the plasma compartment of the dog, make this antibiotic highly unusual. It is conceivable that the marked binding of diumycin to serum could play a role in the prolonged body retention of the compound, as the complex could act as a reservoir, releasing diumycin over a relatively long period of time.

Whether the phenomenon of aggregation[7] plays a role in long duration of activity is also speculative. Since aggregation was not favored at low concentrations of antibiotic, however, the role of this phenomenon might be of limited importance.

Acknowledgements

We thank Mr. W. P. JAMBOR and Mrs. J. MURPHY for technical assistance, Dr. D. ISAACSON for the bioassay of blood and urine samples and Dr. E. BERNAL for the histological examination of the mouse tissues.

The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

References

1) MEYERS, E.; D. SMITH SLUSARCHYK, J. L. BOUCHARD & F. L. WEISENBORN: The diumycins, new members of an antibiotic family having prolonged *in vivo* activity. J. Antibiotics 22: 490～493, 1969
2) WEISENBORN, F.L.; J. L. BOUCHARD, D. SMITH, F. PANSY, G. MAESTRONE, G. MIRAGLIA & E. MEYERS: The prasinomycins: antibiotics containing phosphorus. Nature 213: 1092～1094, 1967
3) HUBER, G.; U. SCHACHT, H. L. WEIDENMÜLLER, J. SCHMIDT-THOMÉ, J. DUPHORN & P. TSCHESCHE: Moenomycin, a new antibiotic. II. Characterization and chemistry. Antimicr. Agents & Chemoth. -1965: 737～742, 1966
4) MANCY, D.; L. NINET, J. PREUD'HOMME, Y. CHARPENTIE, J. RENAUT & V. VUILLEMIN: Un nouvel antibiotique à longue durée d'action. Le 11837 R.P. préparation et propriétés physicochimiques. Abst. Int. Congr. Microbiol., 9th Moscow p. 165, 1966
5) TAKAHASHI, S.; A. OKANISHI, R. UTAHARA, K. NITTA, K. MAEDA & H. UMEZAWA: Macarbomycin, a new antibiotic containing phosphorus. J. Antibiotics 23: 48～50, 1970
6) SLUSARCHYK, W. A.; J. A. OSBAND & F.L. WEISENBORN: The structure of a novel lipid from the antibiotic diumycin. J. Amer. Chem. Soc. 92: 4486～4488, 1970
7) KIRSCHBAUM, J.; W. A. SLUSARCHYK & F. L. WEISENBORN: Intermolecular bonding of the antibiotic diumycin. J. Pharm. Sci. 59: 749～751, 1970

8) LASKIN, A. I. & W. MAY CHAN : The effects of prasinomycin and diumycin on some parameters related to cell wall biosynthesis. Progress in Antimicrobial and Anticancer Chemotherapy. Proceedings of the 6 th International Congress of Chemotherapy. Univ. Tokyo Press, Tokyo Vol. 1. pp. 223～225, 1970
9) SHIRLING, E. B. & D. GOTTLIEB : Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bacteriol. 16 : 313～340, 1966
10) U. S. Dept. of Health, Education and Welfare. U. S. Public Health Service Publication #499. Gonococcus-procedures for isolation and identification. (Rev.) 1962
11) MIRAGLIA, G. J. & H. I. BASCH : Activity of selected penicillins *in vitro* and in experimental bacterial infections in mice. Appl. Microbiol. 15 : 566～568, 1967
12) PRIDHAM, T. G.; C. W. HESSELTINE & R. G. BENEDICT : A guide for the classification of streptomycetes according to selected groups. Appl. Microbiol. 6 : 52～79, 1958
13) KELLY, K. L. & D. B. JUDD : The ISCC-NBS method of designating colors and a dictionary of color names. Natl. Bur. Std. U. S. Circ. 533, 1955
14) MEYERS, E.; G. J. MIRAGLIA, D. A. SMITH, H. I. BASCH, F. E. PANSY, W. H. TREJO & R. DONOVICK: Biological characterization of prasinomycin, a phosphorus-containing antibiotic. Appl. Microbiol. 16 : 603～608, 1968
15) WASIELEWSKI, E. V.; R. MUSCHAWECK & E. SCHÜTZE : Moenomycin, a new antibiotic. III. Biological properties. Antimicr. Agents & Chemoth. -1965 : 743～748, 1966
16) BENAZET, F.; C. COSAR, L. JULOU & D. MANCY : Un nouvel antibiotique à longue durée d'action: Le 11837 R. P. activité antimicrobienne *in vitro*, activité sur les infections experimentales et tolérance chez l'animal. Abst. Int. Congr. Microbiol., 9 th Moscow p. 166, 1966
17) LASKIN, A. I.; W. MAY CHAN, D. A. SMITH & E. MEYERS : Mode of action of prasinomycin. Antimicr. Agents & Chemoth. -1967 : 251～256, 1968
18) HUBER, G. & G. NESEMANN : Moenomycin, an inhibitor of cell wall synthesis. Biochem. Biophys. Res. Commun. 30 : 7～13, 1968
19) LUGTENBERG, E. J. J.; A. VAN SCHIJNDEL-VAN DAM & T. H. M. VAN BELLEGEM : *In vitro* and *in vivo* action of new antibiotics interfering with the utilization of N-acetylglucosamine-N-acetylmuramyl-pentapeptide. J. Bact. 108 : 20～29, 1971