MACROLIDE ANTIBIOTICS M-4365 PRODUCED BY *MICROMONOSPORA*

IV. ANTIMYCOPLASMAL ACTIVITY OF ANTIBIOTIC M-4365 G_2 (DE-EPOXY ROSAMICIN)*

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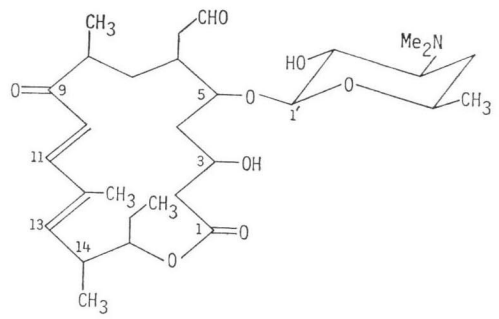
In vitro activities of M-4365 G_2, a new basic 16-membered macrolide antibiotic, against a total 19 strains including human, bovine, porcine, rodent, avian and saprophytic mycoplasmas were compared with those of three other macrolide antibiotics, josamycin, erythromycin and tylosin. M-4365 G_2 exhibited stronger activities than the other macrolide antibiotics against 11 strains of mycoplasma tested. Especially, its higher activities against *M. pneumoniae* Mac and FH, *U. urealyticum* T-960, *M. mycoides* PG-1 and *M. gallisepticum* Kp-13, PG-31 and 9-49A were to be noticed (final minimum inhibitory concentrations: 0.0001~0.049 μg/ml). Higher antimycoplasmal activity of M-4365 G_2 than that of tylosin was also proved in experimental treatment of chickens intranasally inoculated with *M. gallisepticum* Kp-13 by feeding a diet containing the drug. *M. gallisepticum* Kp-13 was not isolated from the infected chickens fed a diet containing 0.0063% or more of M-4365 G_2.

Macrolide antibiotics are known to be highly active against mycoplasmas *in vitro*[1~7]. Moreover, erythromycin and tylosin were reported to be effective in treatments of atypical pneumonia caused by *M. pneumoniae*[8] and chronic respiratory disease of chickens by *M. gallisepticum*[9], respectively.

Antibiotic M-4365 G_2 (de-epoxy rosamicin, Fig. 1) is a new basic 16-membered macrolide antibiotic isolated in our laboratory[10,11] and the activities against Gram-positive and Gram-negative bacteria as well as some strains of mycoplasma were reported in the previous paper[12]. This preliminary experimental result suggested that antimycoplasmal activity of M-4365 G_2 is superior to other macrolide antibiotics. Therefore, we have further evaluated *in vitro* activities of M-4365 G_2 in detail. In this paper, the activities of the antibiotics against 19 strains of the mycoplasma selected from human, bovine, porcine, rodent, avian and saprophytic origins were reported and compared with those of three other macrolide antibiotics, josamycin, erythromycin and tylosin. Furthermore, *in vivo* activities of M-4365 G_2 and tylosin on experimentally infected chickens with *M. gallisepticum* Kp-13 were described. The superiority of M-4365 G_2 to tylosin *in vivo* was discussed in term of their differences in growth inhibitory effect on *M. gallisepticum* Kp-13.

Fig. 1. Structure of M-4365 G_2.



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Materials and Methods

Macrolide Antibiotics

M-4365 G_2 (G_2), josamycin (JM), erythromycin (EM) and tylosin (TS) were used as sulfate salts for the experiments.

Mycoplasma Strains

Mycoplasmas employed in this study included the following strains: *Mycoplasma pneumoniae* Mac and FH, *M. fermentans* PG-18, *M. hominis* PG-21, *Ureaplasma urealyticum* T-960 (human mycoplasmas); *M. mycoides* PG-1 (bovine); *M. hyorhinis* BTS (porcine); *M. arthritidis* PG-6 and *M. pulmonis* PG-22 (rodent); *M. gallisepticum* Kp-13, PG-31, 9-49A and 10-16S and *M. synoviae* 10-18S, 9-34S, 45-5S and 51-1S (avian); and *Acholeplasma laidlawii* PG-9 and PG-10 (saprophytic).

Media and Culture Conditions

The medium for the growth of all tested mycoplasmas except *M. synoviae, U. urealyticum* and *M. hyorhinis* was CHANOCK's medium[18]. The medium for the growth of *M. hyorhinis* was CHANOCK's medium fortified with 0.5% mucin from porcine stomach. The media for the growth of *M. synoviae* and *U. urealyticum* were FREY's medium[14] and TAYLOR-ROBINSON's medium[15], respectively. In order to test the metabolic activity of the mycoplasmas, 0.02% phenol red and 1% glucose were added to the liquid media. For *M. fermentans* PG-18, *M. hominis* PG-21 and *M. arthritidis* PG-6, glucose was replaced by 1% arginine. Incubation was carried out at 37°C. The colony-forming-unit (CFU) was determined by the method of SMITH[16] and color-changing-unit (CCU) was obtained according to the method of TAYLOR-ROBINSON[17]. Mycoplasma strains other than *M. pneumoniae* Mac and *U. urealyticum* T-960 gave a maximum titer of $10^8 \sim 10^9$ CFU per ml in the liquid media for 2~3 days cultivation. Maximum titers of *M. pneumoniae* Mac (10^7 CFU/ml) and *U. urealyticum* T-960 (10^9 CCU) were obtained after one week and one day incubation, respectively. In the case of the solid media, plates were sealed with adhesive tapes and incubated at 37°C.

Determination of Minimum Inhibitory Concentration (MIC)

(1) Broth dilution method: Each of the antibiotics was dissolved in sterile distilled water to give a concentration of 1000 μg/ml. Then the serial two-fold dilution was prepared with sterile distilled water. Subsequently, 0.2 ml of the drug solution at each concentration was transferred to small test tubes. Then 1.8 ml of the culture diluted with the liquid medium appropriately (usually $10^5 \sim 10^8$ CFU/ml) was added to each of small test tubes. The medium was incubated at 37°C for 7 days. Daily color change of the incubated medium was observed and compared with those of uninoculated and inoculated media containing no macrolide antibiotic. Thus, the lowest concentration of a drug completely inhibiting the color change was regarded as the minimum inhibitory concentration (MIC) of the drug. As the MIC, both of initial MIC (IMIC) and final MIC (FMIC) were described in this paper. IMIC was defined as the observed MIC value when the control culture without antibiotic showed the complete color change. It took 24 and 72 hours for *U. urealyticum* and the other mycoplasmas, respectively. FMIC is the MIC value when no further color change was observed by additional incubation, indicating a complete inhibition of growth. It was generally recorded after 7 days incubation.

(2) Agar dilution method: One ml of the drug solution prepared by the same way as described in the broth dilution method was transferred to a petri-dish (diameter; 9 cm). Then 9 ml of the agar medium was added to each plate. After drying its surface in an incubator at 37°C for 1 hour, 0.01 ml of the culture diluted with the liquid medium to $10^5 \sim 10^6$ CFU/ml was dropped on each plate. The plate was incubated for 7 days and the colony growth on the plate was observed under a microscope (×20). The lowest concentration of a drug that inhibited the formation of colonies completely was regarded as the MIC of the drug.

Determination of Minimum Bactericidal Concentration (MBC)

A loopful culture of 1, 2, 3 and 7 day-incubation was withdrawn and spread over the CHANOCK's agar plate. The test organism was incubated at 37°C for the MIC determination. The plates were incubated for further 7 days and observed for the colony growth. The lowest concentration of a drug that inhibited the formation of colonies completely was regarded as the MBC of the antibiotics.

Bactericidal Curve

A series of antibiotic solutions were prepared by two-fold dilution method as described above. Then, 5 ml of the solution was added to a flask (100 ml) containing 45 ml of liquid culture of *M. gallisepticum* Kp-13 (about 10^5 CFU/ml) in CHANOCK's medium (without glucose) to give final concentrations of 4×10^{-4}, 1.5×10^{-3}, 6.1×10^{-3}, 2.4×10^{-2} and 9.8×10^{-2} μg/ml of an antibiotic. An aliquot was withdrawn from each flask at 0, 24, 48, 72 and 168 hours of incubation at 37°C and the viability of the mycoplasma was determined by the colony counting method.

Experimental Treatment of Mycoplasma Infected Chickens with Antibiotics

White Leghorns (GOTO 205) which had been regarded as free from *M. gallisepticum* on the basis of the results of clinical examination and serological test (agglutination test), were purchased from a commercial raiser and used for experiment. Liquid culture of *M. gallisepticum* Kp-13 was dropped into the nasal cavity of one-week-old chickens, the total amount of inoculation being 0.2 ml per head. Pure powder of the antibiotics was mixed with the diet in such manner that it was contained at the ratio of $0.2\sim0.0016\%$ of the diet. Antibiotic treatment was begun simultaneously with the inoculation of the liquid culture. All chickens were sacrificed 7 days after infection. For isolation of *M. gallisepticum*, mucus was collected from the intraorbital sinus, trachea and air sac and cultured in CHANOCK's broth and PPLO enrichment agar (Eiken)[18]. Colonies on the agar medium were conducted hemadsorption tests according to the technique described by SATO[19]. For evaluating antibodies, agglutination tests were performed by the whole blood plate test, as described by ANDO[20], using antigen purchased from Kyoto Biken Lab., Kyoto.

Results and Discussion

Sensitivity of Mycoplasmas to G_2 and the Other Macrolide Antibiotics

The sensitivity of various mycoplasmas to G_2 and the other macrolide antibiotics, as determined by the broth dilution method, are shown in Table 1.

G_2 showed the superior activity to the other macrolide antibiotics against human mycoplasmas tested. Especially, the activity of G_2 was remarkable against *M. pneumoniae* Mac and FH and *U. urealyticum* T-960 (FMICs: $0.0001\sim0.049$ μg/ml). As compared with the MICs of EM, G_2 may be potential use in treatment of atypical pneumonia caused by *M. pneumoniae*.

G_2 also exhibited much stronger activity than the other antibiotics tested against *M. mycoides* PG-1 (bovine), *M. hyorhinis* BTS (porcine) and *M. arthritidis* PG-6 (rodent). Against *M. pulmonis* PG-22, TS exerts much better activity than the others. In contrast, EM was found to be ineffective even at much higher concentrations of 50 μg/ml against *M. hyorhinis* BTS, *M. pulmonis* PG-22 and *M. arthritidis* PG-6.

Moreover, G_2 was proved to have much potent activity against some of the avian mycoplasma strains. Among the 4 strains of *M. gallisepticum* tested, G_2 showed stronger activity (FMICs: 0.0061 μg/ml) than the others against three strains except *M. gallisepticum* 10-16S, which was considered to be a macrolide resistant strain. Against the latter strain, TS was most active (FMIC: 3.13 μg/ml) and G_2 was moderately active (FMIC: 12.5 μg/ml), while EM was substantially inactive (FMIC: >50 μg/ml).

As for the 4 strains of *M. synoviae*, TS was most effective at low concentrations of $0.049\sim0.098$ μg/ml. G_2 was also effective at relatively low concentrations of $0.39\sim1.56$ μg/ml. These strains were all resistant to EM.

Against saprophytic mycoplasmas, EM showed superior activity to the others (FMICs: $0.024\sim0.098$ μg/ml).

Among 19 strains of mycoplasma tested, EM inhibited the growth of 8 strains of mycoplasma at the

Table 1. Sensitivity of mycoplasmas to M-4365 G_2 and the other macrolide antibiotics (Broth dilution method).

Strains		M-4365 G_2		Josamycin		Erythromycin		Tylosin	
		IMIC	FMIC	IMIC	FMIC	IMIC	FMIC	IMIC	FMIC
Human	*M. pneumoniae* Mac	ND	0.0008*	ND	0.049	ND	0.012	ND	0.012
	M. pneumoniae FH	ND	≦0.0001	ND	0.024	ND	0.0061	ND	0.024
	M. fermentans PG-18	0.024	0.098	0.39	3.13	>50	>50	0.098	0.39
	M. hominis PG-21	0.098	0.39	0.39	0.39	>50	>50	12.5	25
	U. urealyticum T-960	0.024	0.049	1.56	3.13	6.25	25	25	25
Bovine	*M. mycoides* PG-1	≦0.0001	0.0004	0.049	0.2	0.0061	0.024	0.012	0.024
Porcine	*M. hyorhinis* BTS	0.39	0.78	0.78	1.56	>50	>50	1.56	6.25
Rodent	*M. pulmonis* PG-22	0.78	6.25	6.25	25	>50	>50	0.098	0.39
	M. arthritidis PG-6	0.049	0.2	0.39	0.39	>50	>50	3.13	6.25
Avian	*M. gallisepticum* Kp-13	0.0004	0.0061	0.024	0.098	0.024	0.024	0.024	0.024
	M. gallisepticum PG-31	0.0004	0.0061	0.049	0.2	0.012	0.049	0.024	0.049
	M. gallisepticum 9-49A	0.0015	0.0061	0.098	3.13	0.049	0.39	0.098	0.39
	M. gallisepticum 10-16S	6.25	12.5	25	25	>50	>50	1.56	3.13
	M. synoviae 10-18S	0.2	0.39	0.78	1.56	25	50	0.049	0.049
	M. synoviae 9-34S	0.2	0.39	1.56	1.56	50	50	0.049	0.098
	M. synoviae 45-5S	0.098	0.2	0.78	1.56	25	50	0.049	0.049
	M. synoviae 51-1S	0.39	1.56	1.56	6.25	25	>50	0.049	0.098
Saprophytic	*A. laidlawii* PG-9	0.78	3.13	3.13	3.13	0.049	0.098	0.098	0.78
	A. laidlawii PG-10	0.39	1.56	0.39	1.56	0.012	0.024	0.098	0.2

ND: Not determined.
* μg/ml.

low concentrations of less than 0.39 μg/ml, however, it was ineffective even at much higher concentrations against the other strains; FMICs were 25 μg/ml and over. Such great variability in sensitivity to EM among the species of mycoplasma have long been noted by some researchers[21,22] and our present result described above agreed with the former findings.

In conclusion, G_2 is considered to be a most interesting macrolide antibiotic, in respect of its broad and strong inhibitory activity against mycoplasmas. So far as EM resistant strains concerned, the order of the activity of the macrolide antibiotics is considered to be as follows: G_2>TS>JM>EM. As for the EM sensitive strains, the following order may also be possible: G_2>EM≒TS>JM.

Influence of Medium, Inoculum Size and Incubation Time

(1) Medium

In this experiment, MICs were also determined by the agar dilution method against some of mycoplasmas tested. Though not dealt in detail in this paper, it was found that the MICs obtained by the agar dilution method ranged from IMICs to FMICs which resulted from the broth dilution method. OGATA *et al.* have reported that the MICs obtained by two methods against some of mycoplasmas were largely agreed within five-fold variations in approximately 90% of the specimens tested[21].

(2) Inoculum size

In order to evaluate the effect of inoculum size on the sensitivity of mycoplasmas, the MICs against

M. mycoides PG-1, *M. pulmonis* PG-22, *M. gallisepticum* PG-31 and Kp-13 and *A. laidlawii* PG-10 were examined by means of the broth dilution method with increased inoculum size, 10^2 CFU/ml, and compared with those obtained by using the standard inoculum size ($10^5 \sim 10^6$ CFU/ml). In most cases, the IMICs and FMICs tended to be higher as the inoculum size increased and the differences in these values were found to be within four-fold variation. However, exceptionally larger differences over eight-fold were observed in the following cases: FMIC of G_2 against *M. mycoides* PG-1 and *M. pulmonis* PG-22; FMICs of EM against *M. mycoides* PG-1 and *M. gallisepticum* Kp-13. However, the reason for these abnormality was not further investigated. It has been reported that the MICs of macrolide antibiotics against some strains of *M. pneumoniae*[4] or *Staphylococcus*[23] were not significantly dependent upon the inoculum size.

(3) Incubation time

In this report, MICs were reported as both IMIC and FMIC. As described in the experimental section, IMICs were determined after one day or 3 days incubation and FMICs were reported at 7 days after incubation.

As can be seen in Table 1, FMICs were generally two to four times higher than IMICs, however, considerable great differences between FMICs and IMICs were observed for some cases: G_2 against *M. pulmonis* PG-22, *M. gallisepticum* Kp-13 and PG-31; JM against *M. fermentans* PG-18 and *M. gallisepticum* 9-49A; EM against *M. gallisepticum* 9-49A; TS against *A. laidlawii* PG-9. Since the IMICs were determined by observing the pH changes in the incubated media, IMICs might be largely dependent upon the metabolic activity of each strain producing acid or base and not strictly correlated with the complete inhibition of the living cells. In this respect, it seems probable that IMICs reflect the bacteriostatic activity. In contrast, FMICs may correspond to the bactericidal activity (MBC).

As seen in Table 2, the MIC of G_2 against *M. gallisepticum* Kp-13 after 3 days incubation was extremely lower than the MBC, but that after 7 days was close to MBC. Therefore, it was suggested IMIC determined at 3 days after the incubation demonstrated the bacteriostatic activity of G_2. Of course, such great difference between IMIC and FMIC might also caused by an inactivation of G_2 during the prolonged incubation, however, this possibility was denied, since G_2 solution kept in a CHANOCK's broth at 37°C for 7 days showed the same activity as that of G_2 solution freshly prepared.

Table 2. Relationship of MIC and MBC against *M. gallisepticum* Kp-13.

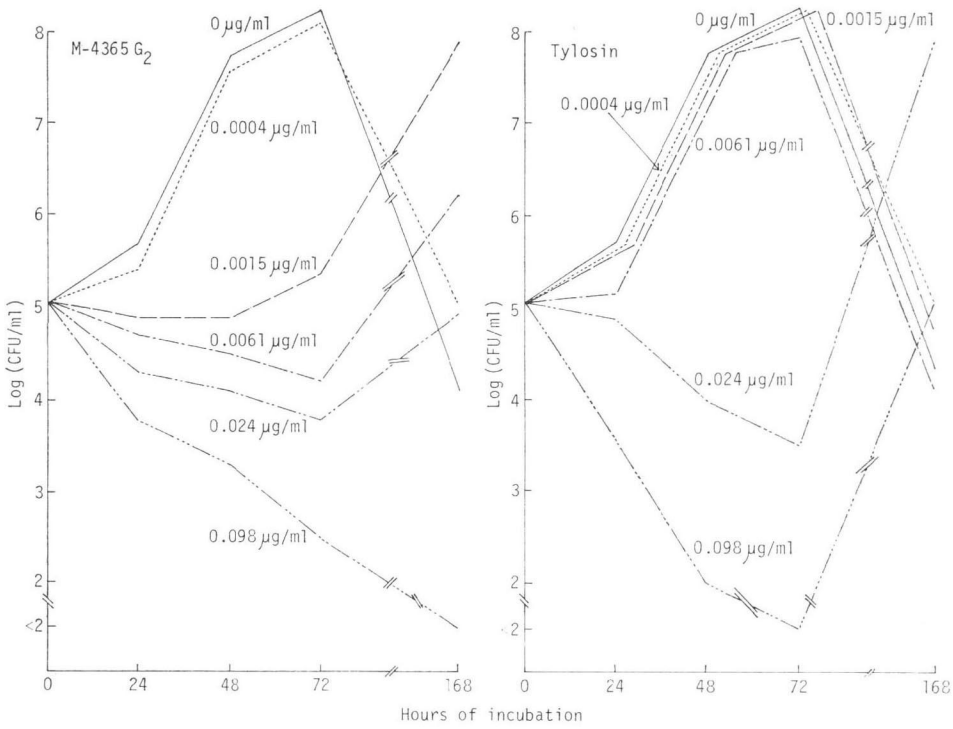
Day	M-4365 G_2		Tylosin	
	MIC	MBC	MIC	MBC
1	ND	0.024	ND	0.049
2	ND	0.024	ND	0.049
3	0.0001*	0.024	0.012	0.024
7	0.0031	0.024	0.024	0.024

ND: Not determined.
* μg/ml.

The strong bacteriostatic activity of G_2 was further proved by the examination of bactericidal curve. From Fig. 2, complete bactericidal concentration of G_2 was estimated to be 0.098 μg/ml. In the concentrations between 0.0015 μg/ml and 0.024 μg/ml, G_2 may act bacteriostatically and over these concentrations, G_2 appeared to inhibit the growth of the strain.

Consequently, the great difference between the IMIC and FMIC of G_2 against *M. gallisepticum* Kp-13 found in this experiment may be explained in term of the great bacteriostatic activity of this antibiotic. In contrast, as is evident from Table 2 and Fig. 2, MICs of TS at 3 and 7 days were almost the same and they were close to the MBC of TS. Therefore, TS seems to be essentially bactericidal antibiotic.

Fig. 2. Effect of M-4365 G_2 and tylosin on the growth of *M. gallisepticum* Kp-13.



In connection to the MIC determination, it was noticed that the growth rate of mycoplasma is slower than that of common bacteria. For instance, generation time of *M. gallisepticum* Kp-13 can be estimated about 200 minutes from Fig. 2. For such slow growing microorganism, MIC seems to be affected by the incubation time. Nevertheless, the incubation times reported in literatures, have varied from person to person. Moreover, some researchers expressed MICs as both of IMICs and FMICs[4,8,24], but others expressed them as only either of the two. As described above, present experimental results suggested that the expression in both of IMICs and FMICs is meaningful to know whether the antimycoplasmal activity is bacteriostatic or bactericidal.

In Vivo Activity of G_2 against *M. gallisepticum* Kp-13

As shown in Table 1, *in vitro* activity of G_2 against some strain of *M. gallisepticum* was found to be superior to that of TS which has been extensively utilizing for the control of mycoplasma infection in domestic animals and chickens.

To confirm the superiority of G_2 *in vivo* as well, the activity of G_2 was compared with that of TS in treatment of chickens infected *M. gallisepticum* Kp-13. The results are shown in Table 3. After the treatments of G_2 and TS, *M. gallisepticum* Kp-13, of which colonies were adsorbed by chicken red blood cells, could be isolated only from the intraorbital sinus except one case from trachea. In all chickens, no clinical signs were observed. The amounts of the diet ingested and the rates of increase of the body weights were almost the same in all chickens. To eliminate *M. gallisepticum* Kp-13 from the infected flock, about 16 times more quantity of TS than that of G_2 was needed. From the data in Table 2, it was found that MBCs of G_2 and TS were almost the same (0.012 μg/ml and 0.024 μg/ml, respectively) and

these values were not so much affected by incubation time. Therefore, at least a part of the superior activity of G_2 *in vivo* must be ascribable to the stronger bacteriostatic activity of G_2. As can be seen in Fig. 2, G_2 showed strong bacteriostatic activity at low concentrations between 0.0015 μg/ml and 0.024 μg/ml, while TS was devoid of such remarkable bacteriostatic activity. To support this possibility, the influence of some other factor(s) affecting *in vivo* activity: distribution and/or adsorption property of G_2 *etc.*, should be compared with those of TS and the study along this line is currently under investigation in experimental animals.

Table 3. Protecting effects of M-4365 G_2 and tylosin against infection of chickens with *M. gallisepticum* Kp-13.

Concentration of antibiotics (% in food)	Isolation of *M. gallisepticum* Kp-13 from intraorbital sinus	
	M-4365 G_2	Tylosin
0.2	0/5*	0/5
0.1	0/5	0/5
0.05	0/10	1/10
0.025	0/10	4/10
0.013	0/10	5/10
0.0063	0/5	2/5
0.0031	4/5	2/5
0.0016	5/5	3/5
0	10/10	
Non-infected control	0/10	

* No. positive/No. examined.

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