TOLYPOMYCIN, A NEW ANTIBIOTIC. V
IN VITRO AND *IN VIVO* ANTIMICROBIAL ACTIVITY

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Tolypomycin-Y and R show a strong *in vitro* antibacterial activity against Gram-positive bacteria and *Neisseria gonorrhoeae*. These antibiotics also inhibit the growth of Gram-negative bacteria to some extent. The antibacterial activities are not influenced by the presence of horse serum in the medium. They are approximately 10~30 times more active at pH 6.0 than at pH 9.0. An enhancement of *in vitro* activity is observed when the bacterial inoculum size is decreased. The rather rapid development of resistance to tolypomycins is shown in the sensitive bacteria by the serial transfer method. The appearance frequencies of one-step resistant strain of *Staphylococcus aureus* to 1~100 mcg/ml of these antibiotics are $2.3\sim5.8\times10^{-6}$. Cross resistance is observed between tolypomycins and rifampicin, but it is not observed between tolypomycins and several other antibiotics tested. These antibiotics are effective against staphylococci isolated from patients at concentrations similar to those needed for the standard laboratory staphylococci. Tolypomycins were demonstrated to have bactericidal activity. Tolypomycins administered by subcutaneous, intraperitoneal and intravenous routes, are effective against experimental infections in mice caused by *Staphylococcus aureus*, *Streptococcus pyogenes* and *Diplococcus pneumoniae* type I. In addition, some activity is also shown by oral route.

Tolypomycin-Y is a new antibiotic obtained from the culture filtrates of *Streptomyces tolypophorus*[1]. The characteristics of the organism were described by SHIBATA *et al.*[2] and the isolation procedures and the physicochemical characteristics of this antibiotic were described by KISHI *et al.*[3] Tolypomycin-R, produced by mild chemical reduction of tolypomycin-Y, is also included in this study.

The present report is mainly concerned with the antimicrobial activity *in vitro*, such as antimicrobial spectrum, the influence of medium pH, serum and inoculum size on activity, the development of resistance, frequency of one step resistance, cross resistance, sensitivity distribution of staphylococci isolated from patients, and the bactericidal activity and stability of the antibiotics. The therapeutic effect against experimental Gram-positive bacterial infections was also studied.

Materials and Methods

Antibiotics: Tolypomycin-Y and R were dissolved in ethanol and then the solutions were diluted with sterile distilled water for studies *in vitro*. For studies *in vivo*, tolypomycin-Y was suspended in 0.2% carboxymethyl cellulose, and the mixture of tolypo-

mycin-R, sodium metabisulfite and sodium araboascorbic acid (1:1:2 in weight) was dissolved in sterile distilled water.

Antimicrobial test: The minimal inhibitory concentration (MIC) of the antibiotics was determined according to the two-fold serial dilution method using Trypticase soy agar (TSA) (BBL) or agar medium plus 10% beef blood as culture media. The test organisms were previously cultivated for 18~24 hours on TSA or blood-TSA, and one loopful of a suspension containing about 10^8 viable units per ml of test organism was streaked on each assay plate. The plates were incubated at 37°C and the antibacterial readings were determined routinely at 18 hours. The minimal inhibitory concentration of the antibiotic was defined as the lowest concentration at which the visible growth of the test organism is prevented.

Development of resistance: The development of bacterial resistance against the antibiotic was studied on *S. aureus* FDA 209P cultivated in Trypticase soy broth (TSB) (BBL). Bacterial transfer from the tube containing the highest concentration of the antibiotic permitting growth was made every 48 hours into the next series of broth tubes containing the same or higher concentrations of antibiotic.

Frequency of resistant mutants: The frequency of resistant strains of *S. aureus* FDA 209P cultivated in TSA to the antibiotics was studied by the modified method of DEMEREC[4]. One-tenth ml of a suspension containing various viable units of bacteria was inoculated on the plates containing various concentrations of the antibiotic. Platings were made in duplicate for each concentration. At 48 hours of incubation at 37°C, colonies on the plates were counted.

Bactericidal activity: The viability of staphylococci in the presence of tolypomycin was determined by the plate count technique. An 18-hour culture of *S. aureus* FDA 209P was diluted 10^3 times in TSB and the antibiotic was added to give concentrations of 0.001, 0.01, 0.1 and 1 mcg/ml. Aliquots were withdrawn from each tube prior to incubation and at 2, 4, 6, 8, 24 and 48 hours of incubation at 37°C. Platings were made in duplicate at several dilutions to ensure a reliable count. Colony counts were made after 48 hours.

Therapeutic effect in mice: Male, CF-1/H mice weighing 18~22 g were used. Intraperitoneal infection was made with 0.5 ml of 5% mucin containing 1/10 volume of *S. aureus* 308 A-1 culture (Brain heart infusion broth (BHI) culture), or *S. pyogenes* E-14 suspension (2×10^{-4} mg per ml (blood-TSA culture)), or with 0.5 ml of broth containing *D. pneumoniae* type I (2×10^{-6} mg per ml (blood-TSA culture)). Immediately after challenge, treatment was made either by single subcutaneous, intraperitoneal, intravenous or oral administration of the antibiotic. The 50 per cent effective dose (ED_{50}) was calculated from the survival rate of the animals 7 days later by the method of REED and MUENCH[5].

Results

Antimicrobial Test *in Vitro*

Antibacterial spectrum:

The antibacterial activity of tolypomycin-Y and R against Gram-positive and Gram-negative organisms is summarized in Table 1. Tolypomycin-Y and R show a strong antibacterial activity against Gram-positive bacteria except for *S. viridans* which is moderately sensitive. *S. aureus* 1840, which is resistant to some known antibiotics is also sensitive to these antibiotics. Tolypomycin-Y and R show activity against *N. gonorrhoeae* and *V. cholerae*, but they are very weak or not effective against other Gram-negative bacteria.

Influence of medium pH, addition of serum and inoculum size on the activity of tolypomycin-Y and R:

Table 1. Antibacterial spectrum of tolypomycin-Y, tolypomycin-R and rifamycin group antibiotics

Organism	Medium	Minimum inhibitory concentration (mcg/ml)					
		Tolypomycin-Y	Tolypomycin-R	Rifampicin	Rifamycin-SV	Rifamycin-B	Rifamycin-O
Staphylococcus aureus FDA 209 P	Trypticase soy agar	0.1	0.1	0.05	0.025	1.56	0.78
Staphylococcus aureus Heatley	〃	0.1	0.1	0.025	0.025	1.56	0.39
Staphylococcus aureus 308 A-1	〃	0.1	0.1	0.025	0.025	1.56	0.78
Staphylococcus aureus 1840	〃	0.1	0.1	0.025	0.05	3.125	0.78
Streptococcus pyogenes E-14	Trypticase soy agar +10 % beef blood	0.025	0.0125	0.1	1.56	6.25	3.125
Streptococcus pyogenes Dick	〃	0.025	0.025	0.19	1.56	6.25	3.125
Streptococcus pyogenes S-8	〃	0.0125	0.025	0.05	1.56	3.125	3.125
Streptococcus pyogenes NY-5	〃	0.025	0.025	0.1	1.56	3.125	3.125
Streptococcus viridans sp.	〃	25	50	>100	100	100	100
Diplococcus pneumoniae type I	〃	0.0015	0.0008	0.1	0.39	0.78	0.78
Diplococcus pneumoniae type II	〃	0.06	0.0125	0.1	0.78	0.78	1.56
Diplococcus pneumoniae type III	〃	0.06	0.0125	0.1	0.78	0.78	1.56
Corynebacterium diphtheriae	〃	0.025	0.025	0.006	0.19	6.25	1.56
Bacillus subtilis PCI-219	Trypticase soy agar	1.56	0.78	0.19	3.125	100	25
Neisseria gonorrhoeae	Trypticase soy agar +10 % beef blood	0.39	0.39	0.39	1.56	25	6.25
Shigella flexneri EW-10	Trypticase soy agar	100	100	12.5	50	>100	>100
Shigella sonnei EW-33	〃	100	100	25	100	>100	>100
Salmonella typhosa Boxhill-58	〃	100	100	50	>100	>100	>100
Escherichia coli Umezawa	〃	100	100	50	>100	>100	>100
Vibrio cholerae Inaba	〃	12.5	6.25	3.13	25	>100	>100
Klebsiella pneumoniae	〃	100	100	12.5	50	>100	>100
Proteus vulgaris	〃	100	100	50	100	>100	>100
Pseudomonas aeruginosa	〃	100	100	50	50	>100	>100
Candida albicans	〃	100	100	>100			

Inoculum size: One loopful of bacterial suspension (1 mg/ml)

The minimum inhibitory concentration of tolypomycin-Y and R against *S. aureus* FDA 209P, Heatley, 308 A-1 and 1840 was observed under various conditions of medium or inoculum size.

Table 2 demonstrates the minimum inhibitory concentration of these antibiotics against test organisms cultivated on media ranging pH 6.0 to 9.0. The antibacterial activity of these antibiotics is influenced by the pH of the medium. The minimum inhibitory concentration of tolypomycin-Y at pH 6.0 is one-tenth of that at pH 9.0 and that of tolypomycin-R at pH 6.0 is one-thirtieth of that at pH 9.0. Table 3 indicates that the addition of 50 % horse serum to the medium does not influence the activity. Results shown in Table 4 demonstrate that the antibacterial activity of the antibiotic is dependent on the inoculum size of the test organisms. The influence of the inoculum size on the antibacterial activity is more marked in TSB than in TSA.

Development of resistance:

The development of resistance of *S. aureus* FDA 209P to tolypomycin-Y, R and dihydrostreptomycin was compared. The rapidity and degree of resistance to the three antibiotics are shown in Fig. 1. A high bacterial resistance to tolypomycin-Y

Table 2. Effect of medium pH on antibacterial activity of tolypomycin-Y and tolypomycin-R against *S. aureus* strains

Medium pH	Minimum inhibitory concentration (mcg/ml)							
	Tolypomycin-Y				Tolypomycin-R			
	209 P	Heatley	308 A-1	1840	209 P	Heatley	308 A-1	1840
6.0	0.0125	0.025	0.025	0.025	0.003	0.025	0.0125	0.006
7.0	0.0125	0.0125	0.0125	0.0125	0.0125	0.025	0.025	0.025
8.0	0.025	0.05	0.05	0.025	0.025	0.025	0.1	0.05
9.0	0.1	0.2	0.1	0.1	0.1	0.39	0.39	0.39

Inoculum size: One loopful of bacterial suspension (10^6 viable units/ml)
Medium: Trypticase soy agar

Table 3. Effect of horse serum on antibacterial activity of tolypomycin-Y and tolypomycin-R against *S. aureus* strains

Horse serum concentration	Minimum inhibitory concentration (mcg/ml)							
	Tolypomycin-Y				Tolypomycin-R			
	209 P	Heatley	308 A-1	1840	209 P	Heatley	308 A-1	1840
5 %	0.2	0.2	0.39	0.2	0.2	0.2	0.2	0.2
10	0.39	0.2	0.39	0.1	0.1	0.2	0.2	0.2
20	0.2	0.2	0.78	0.2	0.2	0.2	0.2	0.2
50	0.39	0.2	0.78	0.2	0.1	0.2	0.2	0.2

Inoculum size: 0.1 ml of bacterial suspension (10^5 viable units/ml)
Medium: Trypticase soy broth

Fig. 1. Patterns of development of resistance of *S. aureus* FDA 209 P.

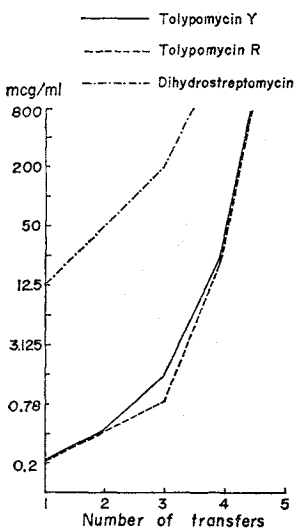


Fig. 2. Survival curves for *S. aureus* FDA 209 P plated on Trypticase soy agar containing various concentrations of tolypomycin-Y. The three curves represent results of three independent experiments.

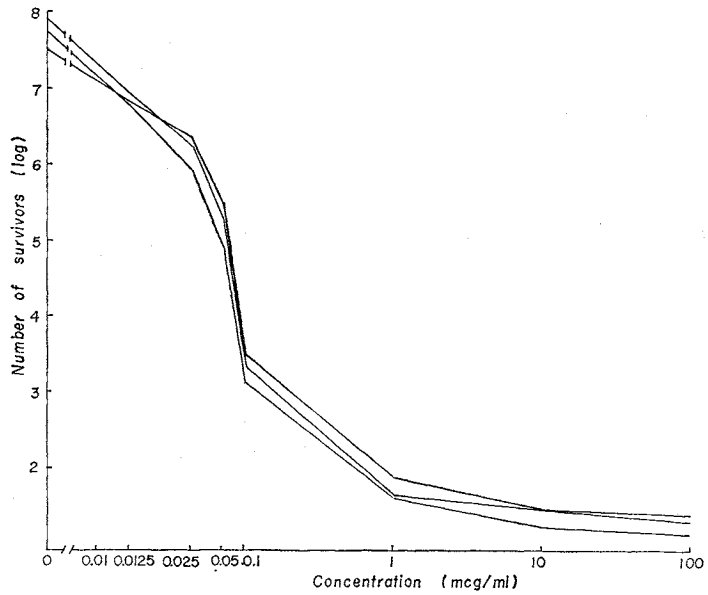


Table 4. Effect of inoculum size of *S. aureus* strains on antibacterial activity of tolypomycin-Y and tolypomycin-R

Trypticase soy agar

Inoculum size (V.U./ml)	Minimum inhibitory concentration (mcg/ml)							
	Tolypomycin-Y				Tolypomycin-R			
	209 P	Heatley	308 A-1	1840	209 P	Heatley	308 A-1	1840
10^3	0.006	0.006	0.0125	0.003	0.006	0.0125	0.006	0.006
10^4	0.006	0.0125	0.0125	0.0125	0.0125	0.025	0.0125	0.0125
10^5	0.0125	0.025	0.025	0.025	0.025	0.025	0.025	0.025
10^6	0.025	0.025	0.05	0.025	0.025	0.05	0.05	0.05
10^7	0.025	0.025	0.05	0.025	0.05	0.05	0.05	0.05
10^8	0.05	0.05	0.1	0.1	0.2	0.2	0.2	0.5

Trypticase soy broth

Inoculum size (V.U./ml)	Minimum inhibitory concentration (mcg/ml)							
	Tolypomycin-Y				Tolypomycin-R			
	209 P	Heatley	308 A-1	1840	209 P	Heatley	308 A-1	1840
10^1	0.05	0.025	0.025	0.025	0.05	0.025	0.05	0.05
10^2	0.1	0.05	0.05	0.025	0.05	0.1	0.1	0.05
10^3	0.1	0.1	0.1	0.05	0.1	0.1	0.2	0.1
10^4	0.1	0.1	0.39	0.1	0.1	0.1	0.2	0.2
10^5	0.39	0.2	0.39	0.2	0.2	0.2	0.39	0.2
10^6	3.125	0.2	1.56	0.39	12.5	0.39	25	0.39
10^7	>100	>100	25	3.125	100	6.25	50	12.5
10^8	>100	>100	>100	25	100	>100	>100	>100

Inoculum size: 0.1 ml of bacterial suspension

and R developed after 3~4 transfers. The rapidity of development of resistance against the two antibiotics is aprroximately equal.

Frequency of resistant mutants to tolypomycin-Y:

Frequency of resistant mutants was investigated using *S. aureus* FDA 209P. Results are shown in Table 5 and Fig. 2. Colonies highly resistant to tolypomycin-Y are detected by the one step selection of test cocci at concentrations ranging from 2.3 to 5.8×10^{-6}. The tolypomycin-Y resistance of each isolated colony was tested by the agar dilution method. All of the colonies isolated from the plates in the presence of 10 and 100 mcg/ml of the antibiotic proved to be resistant to concentrations more than 100 mcg/ml.

Table 5. The isolation of tolypomycin-resistant strains from the large population of sensitive *S. aureus* FDA 209 P cultivated on Trypticase soy agar containing tolypomycin-Y

Concentration of tolypomycin-Y (mcg/ml)	No. of plates	Exp. 1	Exp. 2	Exp. 3
0	1	8.4×10^8	2.3×10^8	9.3×10^8
	2	8.8×10^8	4.0×10^8	4.7×10^8
0.0125	1	9.5×10^7	8.4×10^7	5.4×10^7
	2	4.7×10^7	5.8×10^7	6.9×10^7
0.025	1	1.3×10^7	7.5×10^6	1.9×10^7
	2	1.6×10^7	8.8×10^6	3.1×10^7
0.05	1	3.9×10^6	1.0×10^6	1.9×10^7
	2	3.7×10^6	1.0×10^6	3.1×10^7
0.1	1	3.0×10^5	1.8×10^4	1.8×10^5
	2	3.3×10^5	1.2×10^4	3.3×10^5
1	1	8.6×10	3.8×10	5.4×10
	2	7.8×10	5.4×10	4.1×10
10	1	3.6×10	1.6×10	3.8×10
	2	2.9×10	2.1×10	2.7×10
100	1	1.8×10	1.4×10	3.0×10
	2	2.1×10	1.3×10	1.9×10

Cross-resistance:

Cross-resistance was studied with S. *aureus* FDA 209P which had been made resistant respectively to tolypomycin-Y, R, chlortetracycline, chloramphenicol, peni-

Table 6. Cross-resistance test among tolypomycin-Y, tolypomycin-R, rifampicin, penicillin G, streptomycin, erythromycin, novobiocin, chlortetracycline and chloramphenicol

Organisms	Minimum inhibitory concentration (mcg/ml)								
	Tolypomycin-Y	Tolypomycin-R	Rifampicin	Penicillin G	Streptomycin	Erythromycin	Novobiocin	Chlortetracycline	Chloramphenicol
S. aureus FDA 209 P (parent)	0.025	0.0125	0.01	0.0125	12.5	0.39	0.78	0.78	3.125
R-Tolypomycin-Y	50	50.	>100	0.0125	6.25	0.39	1.56	0.78	3.125
R-Tolypomycin-R	>100	>100	>100	0.025	6.25	0.39	0.78	0.78	3.125
R-Rifampicin	>100	>100	>100	0.025	6.25	0.39	0.78	0.78	3.125
R-Chlortetracycline	0.025	0.0125	0.01	0.025	3.13	0.39	1.56	12.5	6.25
R-Chloramphenicol	0.025	0.0125	0.01	0.0125	3.13	1.56	0.39	1.56	50
R-Penicillin G	0.025	0.0125	0.01	50	3.13	0.39	1.56	0.78	3.125
R-Streptomycin	0.025	0.0125	0.01	0.0125	>100	0.39	0.1	0.78	3.125
R-Erythromycin	0.05	0.0125	0.01	0.003	3.13	>100	0.2	0.78	6.25
R-Novobiocin	0.025	0.0125	0.01	0.0125	3.13	0.2	>100	0.78	3.125

Inoculum size: One loopful of bacterial suspension (10^6 viable units/ml)

Table 7. Distribution of sensitivity of *Staphylococcus* strains against tolypomycin and other antibiotics

MIC (mcg/ml)	Tolypomycin-Y	Tolypomycin-R	Rifampicin	Penicillin G	Dihydrostreptomycin	Kanamycin	Erythromycin	Novobiocin	Chloramphenicol	Chlortetracycline
>100					16	2	27		1	36
100					9	1			2	
50				4			1	1	3	5
25				6	3		1	1		1
12.5				9	1				1	1
6.25				7	4				48	2
3.125				6	50	14		1	35	2
1.56				2	6	63	2			38
0.78				9	1	5				5
0.39				22		5	25	64		
0.2	1	4		14			34	23		
0.1	35	22		4						
0.05	44	57	6	7						
0.025	10	7	79							
0.0125			5							

Inoculum size: One loopful of bacterial suspension (10^6 viable units/ml)

cillin G, streptomycin, erythromycin or novobiocin by serial subcultures in TSB containing increasing concentrations of each antibiotic. The data in Table 6 are obtained by the agar dilution method.

Tolypomycins exert their full activity against microorganisms resistant to other antibiotics and the tolypomycin-Y or R-resistant organisms are also sensitive to other antibiotics. But mutual cross-resistance of bacteria is observed between both tolypomycins.

Sensitivity of the staphylococcal strains isolated from patients:

The data in Table 7 indicate that tolypomycins at concentrations of 0.025~0.2 mcg/ml are effective against clinically isolated staphylococci*, and that 79 of 90 strains show growth inhibition at minimum concentrations ranging from 0.05 to 0.1 mcg/ml,

* The cultures were kindly supplied by Miss SHIMIZU of Central Clinical Laboratory, Osaka University Hospital.

Fig. 3. Effect of different concentration of tolypomycin-Y on viable count of *S. aureus* FDA 209 P.

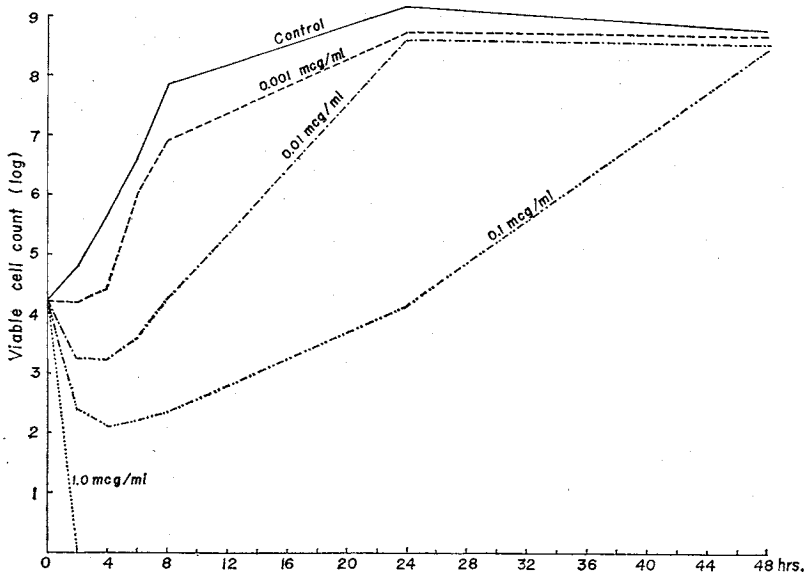
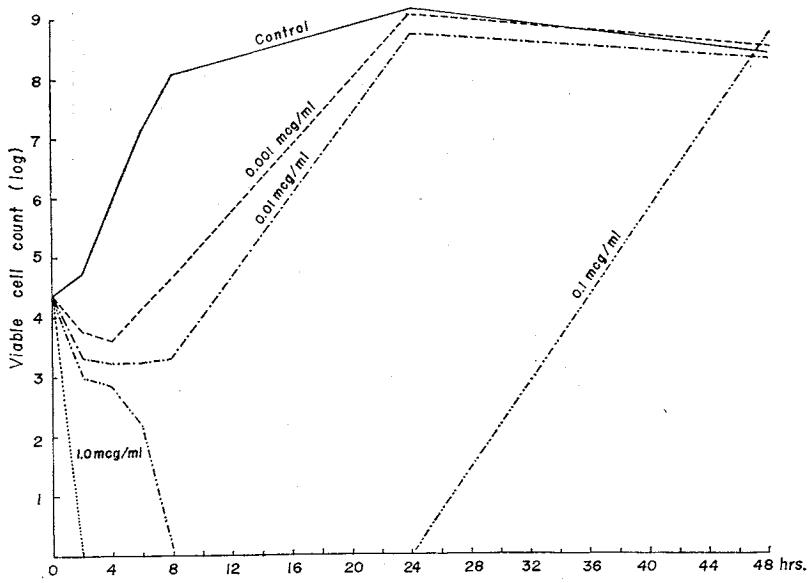


Fig. 4. Effect of different concentration of tolypomycin-R on viable count of *S. aureus* FDA 209 P.



whereas the same inhibition of the standard laboratory staphylococci is obtained with a minimum concentration of 0.1 mcg/ml. This inhibitory pattern of tolypomycins was same with rifampicin. On the other hand, it shows a sharp contrast with the other antibiotics which present a relatively wide range of minimum inhibitory concentrations against clinically isolated staphylococci.

Bactericidal activity:

The viability of *S. aureus* FDA 209P cultured in TSB with various concentra-

tions of tolypomycin-Y and R was determined by plate counting and the result are shown in Figs. 3 and 4. The logarithm of the viable count is plotted against time of exposure to the antibiotic. Immediately after the addition of the antibiotics a marked bactericidal action is shown by both antibiotics at concentrations of 1 mcg/ml and also at 0.1 mcg/ml for tolypomycin-R. At concentrations of 0.1 mcg/ml or lower, a weak bactericidal activity or prolonged lag phase is observed. At these concentrations the growth of the bacteria 24 and 48 hours later is similar to that of the control.

Antibacterial stability of tolypomycin-Y:

Tolypomycin-Y solution in distilled water, in simulated gastric juice, at pH 1.2, in simulated intestinal juice and at pH 8.3 were kept at 37°C and the growth inhibitory activity against *S. aureus* FDA 209P was observed 0.5, 1, 2 and 4 hours later. As shown in Figs. 5 and 6, tolypomycin-Y is relatively stable in distilled water but unstable in the test solutions.

Fig. 5. Stability of tolypomycin-Y in distilled water, simulated gastric juice and pH 1.2 solution.

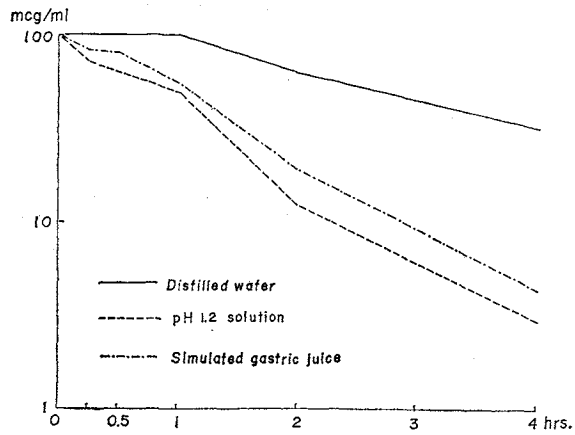
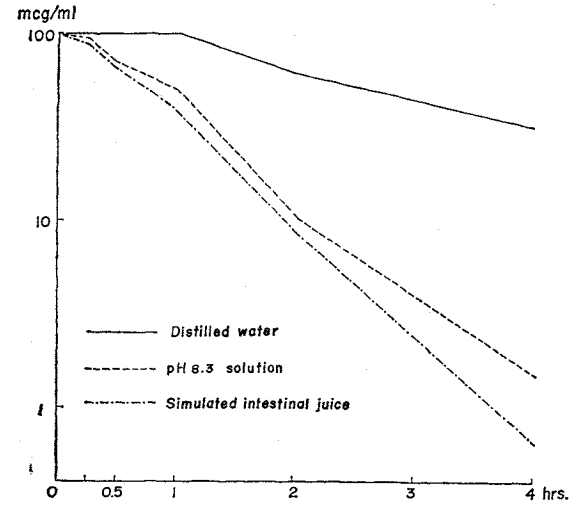


Fig. 6. Stability of tolypomycin-Y in distilled water, simulated intestinal juice and pH 8.3 solution.



Antimicrobial Test *in vivo*

Therapeutic effects of tolypomycin-Y and R against experimental infections of mice produced by strains of *Staph. aureus* 308 A-1, *Strept. pyogenes* E-14 and *D.*

Table 8. Effect of tolypomycin-Y and tolypomycin-R against Gram-positive bacterial infection in CF-1 mice infected intraperitoneally

Organisms		*Staph. aureus* 308 A-1		*Strept. pyogenes* E-14		*D. pneumoniae* type I	
Antibiotic		Tolypomycin-Y	Tolypomycin-R	Tolypomycin-Y	Tolypomycin-R	Tolypomycin-Y	Tolypomycin-R
In vitro sensitivity (mcg/ml)		0.1	0.1	0.025	0.0125	0.0015	0.0008
Administration route and ED_{50} (mg/kg)	SC	0.073	0.0625	0.048	0.0193	0.14	0.0919
	IP	0.002	0.0017	0.004	0.00022	0.005	0.00088
	IV	0.137	0.0743	0.125	0.0312	0.6	0.217
	Oral	14.5	14.14	2.0	1.54	7.1	11.22

pneumoniae type I are shown in Table 8. Against Gram-positive bacterial infection, tolypomycin-Y and R are very effective by subcutaneous, intraperitoneal and intravenous administration, and the therapeutic effect is similar to tetracycline by oral administration.

As can be seen in the table, therapeutic activity of both antibiotics against Gram-positive bacterial infections is approximately equal.

Discussion

The antibacterial spectrum of tolypomycins was determined by the usual agar dilution methods. Tolypomycins are growth-inhibitory against Gram-positive bacteria and *N. gonorrhoeae*. These antibiotics show stronger activity against Gram-positive bacteria than most antibiotics. The activity of these antibiotics against Gram-positive bacteria was approximately equivalent to that of structurally related rifampicin. *S. aureus* 1840, which is resistant to several antibiotics, is also sensitive to the tolypomycins.

The development of resistance to tolypomycin is very rapid *in vitro*. In the course of 3 to 4 cultivating transfers, *S. aureus* FDA 209P become resistant to 1,000-times the previous concentration. In *S. aureus* FDA 209P, the frequency of resistant mutants to 1, 10 and 100 mcg/ml of tolypomycin-Y is approximately 10^{-6}. These frequencies are similar to those of rifamycin-group antibiotics[6~8]. Resistant strains of *S. aureus* FDA 209P against tolypomycin-Y and R are still sensitive to chlortetracycline, chloramphenicol, benzylpenicillin, dihydrostreptomycin, erythromycin and novobiocin. The strains resistant to these known antibiotics are sensitive to the tolypomycins. This finding is confirmed by the presence of the antibacterial activity in tolypomycin-Y and R against clinically isolated staphylococci. The fact that the viable units are diminished by the addition of 1 mcg/ml of these antibiotics shows that tolypomycin-Y and R have bactericidal action.

A remarkable therapeutic effect is observed against Gram-positive bacterial infection in mice. Tolypomycin show very strong therapeutic activity in mice experimentally infected with Gram-positive bacteria when they are given subcutaneously, intraperitoneally and intravenously. By oral administration, therapeutic activity of these antibiotics is moderate, but is similar to that of known antibiotics, *i. e.* chlortetracycline and chloramphenicol. The lower therapeutic effect of tolypomycins by oral adminisration may partly be related to their unstable character in simulated gastric and intestinal juices.

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