STUDIES ON T-2636 ANTIBIOTICS. IV

IN VITRO AND *IN VIVO* ANTIBACTERIAL ACTIVITY OF T-2636 ANTIBIOTICS

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(Received for publication November 9, 1970)

T-2636 C shows a strong *in vitro* antimicrobial activity against a variety of Gram-positive bacteria, *Neisseria gonorrhoeae* and *Vibrio cholerae*, while T-2636 A, D and F are relatively weak in this respect. T-2636 A and C are more active *in vitro* at pH 6.0 than at pH 9.0. The antibacterial *in vitro* activity is enhanced by decrease in bacterial inoculum size. Presence of horse serum in the medium results in the decreased activity. The development of resistance of the sensitive bacteria to T-2636 C is demonstrated by exposure according to the serial transfer method. In the cross resistance test using *Staphylococcus aureus* which had been made resistant to T-2636 C or macrolide antibiotics *in vitro*, T-2636 C show a weak activity against microorganisms resistant to spiramycin and triacetyl-oleandomycin, but T-2636 C resistant *S. aureus* was sensitive to macrolide antibiotics. T-2636 C is effective against staphylococci, isolated from patients, at the similar concentration against the standard laboratory staphylococci. T-2636 demonstrated also bacteriostatic activity. The primary active site of T-2636 C on *S. aureus* is inhibition of the protein synthesis. T-2636 A and C are effective against experimental infections in mice by *S. aureus* and *Streptococcus pyogenes*, when administered intraperitoneally or orally. But T-2636 A was less effective by subcutaneous route.

Six antibiotics, named T-2636 A, B, C, D, E and F, have been isolated[1,2] from the culture broth of *Streptomyces rochei* var. *volubilis*. This organism was isolated from a soil sample collected in Osaka Prefecture, Japan[3]. The isolation, chemical structures and mutual interconversions of T-2636 antibiotics were described by HARADA *et al.*[4], KAMIYA *et al.*[5] and FUGONO *et al.*[6].

This communication is concerned with the antimicrobial activity *in vitro*, such as antimicrobial spectrum, influence of pH of the medium, inoculum size, addition of serum, development of resistance, cross resistance, distribution of sensitivity of clinically isolated staphylococci, bacteriocidal activity and chemical stability. The therapeutic effect against experimental staphylococcal and streptococcal infections was also studied.

Materials and Methods

Antibiotics: T-2636 antibiotics were prepared by Takeda Research Laboratories. Macrolide antibiotics such as erythromycin, tryacetyl-oleandomycin, spiramycin and leucomycin,

used for references, were extracted from commercial tablets. The antibiotics were dissolved in methanol and then the solutions were diluted in sterile distilled water for the *in vitro* study. In the *in vivo* study, T-2636 A and C were suspended in 0.2 % carboxymethyl cellulose.

Antimicrobial test: The minimum inhibitory concentration of the antibiotics was determined according to the two-fold serial dilution method using Trypticase soy agar (TSA) (BBL) with or without 10 % ox blood, as culture medium. 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 the test organism was streaked on each assay plate. The plates were incubated at 37°C and the antimicrobial readings were made routinely 18 hours later. The minimal inhibitory concentration of the antibiotic was defined as the prevention of the visible growth of the test organism.

Development of resistance: The development of resistance against the antibiotic was studied on *Staphylococcus aureus* FDA 209 P cultivated in Tripticase soy broth (TSB) (BBL). Transfer of the bacteria from the tube containing the highest concentration of the antibiotic permitting almost similar growth with that of the control tube was made every 48 hours into the next series of broth tube containing the same or higher concentrations of the antibiotics.

Bacteriocidal activity: The viability of staphylococci in the presence of the antibiotics was determined by the plate count technique. An 18-hour culture of the organism was diluted 10^3 time in liquid medium and the antibiotic was added to give concentrations of 0.1, 1, 10 and 100 mcg/ml. Aliquots were taken from each tube prior to incubation and at 2, 4, 6 and 8 hours of incubation at 37°C. Platings were made in duplicate at several dilutions to ensure reliable counts. Colony counts were made after 48 hours.

Mode of action: *S. aureus* FDA 209 P was cultivated in TSB at 37°C in shaking culture. The culture of the microorganism at the point of half maximum growth was divided into three portions: one was used as a control, other two were added with the antibiotic to give concentrations of 10 and 100 mcg/ml. The cells were harvested 30, 60 and 120 minutes after the addition. Cell growth was followed by measuring the optical density of the bacterial suspension at 650 mμ by Coleman Universal Spectrophotometer. Aliquots of 25 ml of culture were centrifuged and cells were washed twice with water. The protein fraction of the organisms was isolated as described by PARK and HANCOCK[7], and was determined by the method of LOWREY *et al.*[8] Extraction of N-acetylamino sugar from the organisms was performed as described by STROMINGER[9]. The N-acetylamino sugar was determined by the method of REISSING *et al.*[10] Neucleic acid from *S. aureus* was isolated according to the method of SCHNEIDER[11]. DNA and RNA were determined with the diphenylamine reagent and by the orcinol method, respectively.

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 *Streptococcus pyogenes* E-14 suspension in the dose of 2×10^{-4} mg per ml (blood-TSA culture). Immediately after challenge, treatment was made either by single subcutaneous, intraperitoneal or oral administration of the antibiotic. The 50 % effective dose (ED_{50}) was calculated from the survival rate of the animals at 7 days later by the method of REED and MUENCH[12].

Results

Antimicrobial Test *In Vitro*

1. Antimicrobial Spectrum of T-2636 Antibiotics

The antibacterial activity of T-2636 antibiotics against Gram-positive and Gram-negative organisms is summarized in Table 1.

Table 1. Antibacterial spectrum of T-2636 antibiotics and macrolide antibiotics

Organism	Medium	MIC in mcg/ml							
		T-2636 A	T-2636 C	T-2636 D	T-2636 F	Erythromycin	Leucomycin	Spiramycin	Triacetyl-oleandomycin
Staphylococcus aureus FDA 209 P	Trypticase soy agar	100	6.25	>100	>100	0.39	1.56	12.5	6.25
Staphylococcus aureus Heatley	〃	100	6.25	>100	>100	0.78	1.56	12.5	6.25
Staphylococcus aureus 308 A-1	〃	100	6.25	>100	>100	0.39	3.12	12.5	6.25
Staphylococcus aureus 1840	〃	100	6.25	>100	>100	0.39	1.56	12.5	6.25
Streptococcus pyogenes E-14	Trypticase soy agar +10% ox blood	6.25	0.39	50	6.25	0.1	0.78	0.39	3.12
Streptococcus pyogenes Dick	〃	12.5	0.78	50	12.5	0.2	0.78	0.78	3.12
Streptococcus pyogenes S-8	〃	12.5	0.78	50	6.25	0.1	0.78	0.39	3.12
Streptococcus pyogenes NY-5	〃	6.25	0.39	25	3.125	0.1	0.39	0.39	3.12
Streptococcus viriduns sp.	〃	25	3.125	>100	>100	0.025	0.39	0.39	3.12
Diplococcus pneumoniae type-I	〃	25	3.125	>100	>100	0.05	0.39	0.1	3.12
Diplococcus pneumoniae type-II	〃	12.5	0.78	>100	>100	0.025	0.39	0.2	3.12
Diplococcus pneumoniae type-III	〃	25	3.125	>100	>100	0.025	0.39	0.2	1.56
Corynebacterium diphtheriae	〃	0.19	0.1	3.125	0.78	0.05	0.39	0.39	0.39
Bacillus subtilis PCI-219	Trypticase soy agar	>100	>100	>100	>100	0.39	1.56	1.56	6.25
Neisseria gonorrhoeae	Trypticase soy agar +10% ox blood	6.25	0.39	50	6.25	0.39	0.78	0.78	
Shigella flexneri EW-10	Trypticase soy agar	>100	50	>100	>100	25	>100	>100	>100
Shigella sonnei EW-33	〃	>100	>100	>100	>100	>100	>100	>100	>100
Salmonella typhosa	〃	>100	>100	>100	>100	>100	>100	>100	>100
Escherichia coli Umezawa	〃	>100	>100	>100	>100	>100	>100	>100	>100
Vibrio cholerae Inaba	〃	6.25	0.2	25	6.25	3.12	3.12	12.5	>100
Klebsiella pneumoniae	〃	>100	>100	>100	>100	100	>100	>100	>100
Proteus vulgaris	〃	>100	>100	>100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa	〃	>100	>100	>100	>100	>100	>100	>100	>100

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

T-2636 A and C were active mainly against Gram-positive bacteria except *B. subtilis*. Both antibiotics showed stronger activity against *S. pyogenes*, *S. viridans*, *Diplococcus pneumoniae* and *Corynebacterium diphtheriae* than *S. aureus*. T-2636 A and C were also active against *Neisseria gonorrhoeae* and *Vibrio cholerae*. While, these antibiotics were not active against other Gram-negative bacteria. The antimicrobial activity of T-2636 C was stronger than that of T-2636 A.

T-2636 D and F showed low antimicrbial activity against microorganisms under this test condition.

2. Characteristics of *In Vitro* Antimicrobial Effect of T-2636 A

Influence of medium pH, inoculum size and addition of serum on the activity of T-2636 A: The minimum inhibitory concentration of T-2636 A against *S. pyogenes* E-14 and S-8, *D. pneumoniae* type I, II and III were observed under various conditions of cultivating medium or inoculum size.

The minimum inhibitory concentration of the antibiotic against test organisms cultivated on the medium ranging from pH 6.0 to 9.0 is shown in Table 2. The antibacterial activity of T-2636 A against *S. pyogenes* and *D. pneumoniae* was slightly affected by medium pH. The minimum inhibitory concentration of T-2636 A at pH 6.0 was one-half to one-fourth as that at pH 9.0.

Table 2. Effect of medium pH on antibacterial activity of T-2636 A

Organism	M.I.C. in mcg/ml			
	pH 6.0	pH 7.0	pH 8.0	pH 9.0
Strept. pyogenes E-14	3.125	3.125	6.25	12.5
Strept. pyogenes S-8	3.125	3.125	6.25	12.5
D. pneumoniae type I	50	50	100	100
D. pneumoniae type II	25	25	50	100
D. pneumoniae type III	25	25	50	50

Inoculum size: One loopful of bacterial suspension (10^8 V.U./ml).
Medium: Tripticase soy agar plus 10% ox blood.

Table 4. Effect of horse serum concentration in medium on antibacterial activity of T-2636 A

Organism	M.I.C. in mcg/ml			
	0%	10%	20%	50%
Strept. pyogenes E-14	3.125	6.25	12.5	12.5
Strept. pyogenes S-8	3.125	6.25	12.5	12.5
D. pneumoniae type I	1.56	100	>100	>100
D. pneumoniae type II	1.56	100	>100	>100
D. pneumoniae type III	1.56	50	50	100

Inculum size: One loopful of bacterial suspension (10^8 V.U./ml).
Medium: Brain heart Infusion broth.

Table 3. Effect of inoculum size on antibacterial activity of T-2636 A

Medium	Organism	M.I.C. in mcg/ml			
		10^4	10^5	10^6	10^7
Tripticase soy agar plus 10 % ox blood	*Strept. pyogenes* E-14	1.56	3.125	3.125	3.125
	Strept. pyogenes S-8	1.56	3.125	3.125	6.25
	D. pneumoniae type I	25	25	25	—
	D. pneumoniae type II	12.5	12.5	25	—
	D. pneumoniae type III	12.5	12.5	25	—
Brain heart infusion broth	*Strept. pyogenes* E-14	1.56	1.56	3.125	6.25
	Strept. pyogenes S-8	1.56	1.56	3.125	6.25
	D. pneumoniae type I	25	25	50	—
	D. pneumoniae type II	25	50	50	—
	D. pneumoniae type III	25	25	25	—

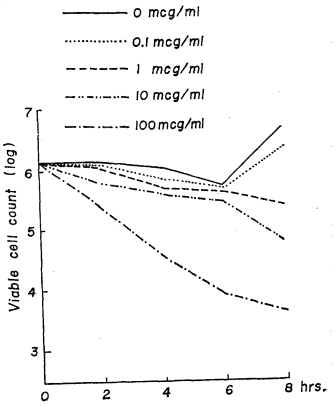
Inoculum size: One loopful of bacterial suspension.

Effect of inoculum size on the activity of T-2636 A is demonstrated in Table 3. Slight increase in antibacterial activity of the antibiotic is attained by the reduction in the inoculum size.

Effect of the addition of horse serum to the medium on the activity of T-2636 A is shown in Table 4. Antimicrobial activity of this antibiotic against *S. pyogenes* shows a slight decrease by the addition of serum. The activity of the antibiotic against *D. pneumoniae* markedly decreased by the presence of serum in the concentration of above 10 %.

Bacteriocidal activity: The viability of *S. pyogenes* E-14 cultivated in BHI containing in various concentrations of T-2636 A was determined by plate count technique, as illustrated in Fig. 1.

Fig. 1. Bactericidal activity of T-2636 A on *Streptococcus pyogenes* E-14.



The logarithm of the viable count was plotted against exposure time to the antibiotic. When the antibiotic was added simultaneously, the bacteriocidal action was demonstrated at the concentration of 100 mcg/ml of T-2636 A. At concentrations of 10 mcg/ml and 1 mcg/ml, the bacteriocidal action was relatively weak. The increase in viable cell count was observed at the concentration of 0.1 mcg/ml of T-2636 A as well as control culture.

3. Characteristics of *In Vitro* Antimicrobial Effect of T-2636 C

Influence of medium pH, inoculum size and addition of serum on the activity of T-2636 C: The minimum inhibitory concentration of T-2636 C against *S. aureus* FDA 209 P, Heatley, 308 A-1 and 1840 was observed under various conditions of cultivating medium or inoculum size.

Tbale 5. Effect of medium pH on antibacterial activity of T-2636 C

Organism	M.I.C. in mcg/ml			
	pH 6.0	pH 7.0	pH 8.0	pH 9.0
Staph. aureus FDA 209 P	1.56	6.25	12.5	100
Staph. aureus Heatley	1.56	6.25	12.5	100
Staph. aureus 308 A-1	1.56	6.25	25	100
Staph. aureus 1840	1.56	6.25	12.5	100

Inoculum size: One loopful of bacterial suspension (10^8 V.U./ml).
Medium: Trypticase soy agar.

Table 6. Effect of inoculum size on antibacterial activity of T-2636 C

Medium	Organism	M.I.C. in mcg/ml					
		10^3	10^4	10^5	10^6	10^7	10^8
Tripticase soy agar	*Staph. aureus* FDA 209 P	0.2	0.78	0.78	1.56	1.56	3.125
	Staph. aureus Heatley	0.39	0.78	1.56	1.56	3.125	3.125
	Staph. aureus 308 A-1	0.39	0.78	1.56	1.56	6.25	12.5
	Staph. aureus 1840	0.39	0.39	0.78	0.78	1.56	3.125
Tripticase soy broth	*Staph. aureus* FDA 209 P	0.78	0.78	1.56	1.56	1.56	6.25
	Staph. aureus Heatley	1.56	1.56	1.56	1.56	3.125	6.25
	Staph. aureus 308 A-1	0.78	1.56	1.56	3.125	3.125	6.25
	Staph. aureus 1840	1.56	1.56	1.56	3.125	3.125	6.25

Inoculum size: One loopful of bacterial suspension.

The minimum inhibitory concentration of the antibiotic against test organisms cultivated on the medium ranging from pH 6.0 to 9.0 is shown in Table 5. The antibacterial activity of T-2636 C against *S. aureus* depended to a certain extent on medium pH. The minimum inhibitory concentration of T-2636 C at pH 6.0 was one-sixty-fourth as that at pH 9.0.

Effect of inoculum size on the activity of T-2636 C is demonstrated in Table 6. The antibacterial activity of the antibiotic was dependent on the inoculum size of the test organisms. A decrease in minimum inhibitory concentration of the antibiotic followed by decrease in size of the test organism. Influence of the inoculum size on antibacterial activity was more remarkable on TSA than on TSB.

Effect of the addition of horse serum to the medium on the activity of T-2636 C is shown in Table 7. Antimicrobial activity of this antibiotic was found to be slightly decreased in the presence of 50 % serum.

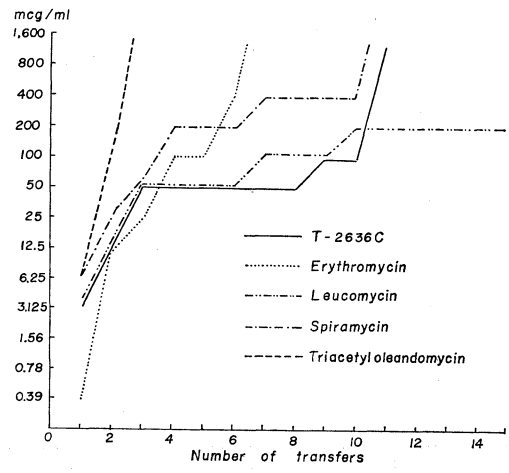
Development of resistance: The development of resistance of *S. aureus* FDA 209 P to T-2636 C and several other macrolide antibiotics was compared. The progression and degree of resistance to five antibiotics are shown in Fig. 2. The minimum inhibitory concentration of T-2636 C increased from 3.125 mcg/ml to 50 mcg/ml only by 3 transfers. Thereafter, the minimum inhibitory concentration was not further affected over a period of 5 serial transfers. Progress of development of resistance against T-2636 C, erythromycin, leucomycin and spiramycin was approximately equal. Development of resistance against triacetyl-oleandomycin was more rapid than those against other four antibiotics.

Table 7. Effect of horse serum concentration in medium on antibacterial activity of T-2636 A

Organism	M.I.C. in mcg/ml			
	0%	10%	20%	50%
Staph. aureus FDA 209 P*	3.125	3.125	3.125	6.25
Staph. aureus Heatley*	3.125	3.125	3.125	25
Staph. aureus 308 A-1*	3.125	3.125	3.125	25
Staph. aureus 1840*	3.125	3.125	6.25	25
Strept. pyogenes E-14**	0.1	0.2	0.2	0.39
D. pneumoniae type I**	0.78	1.56	1.56	3.125

Inoculum size: One loopful bacterial suspension (10^8 V.U./ml).
Medium: *=Tripticase soy broth.
**=Brain heart Infusion broth.

Fig. 2. Patterns of development of resistance of *Staph. aureus* FDA 209P to T-2636 C and macrolide antibiotics.



Cross resistance: Cross resistance was studied with *S. aureus* FDA 209 P which had been made resistant to T-2636 C, erythromycin, leucomycin, spiramycin or triacetyl-oleandomycin respectively by serial subcultures in TSB containing increasing concentrations of each antibiotic. Table 8 shows the data obtained by the agar dilution method. T-2636 C exerted its full activity against microorganisms resistant to erythromycin and leucomycin, but not against microorganisms resistant to spiramycin and

Table 8. Cross resistance test among T-2636 C and macrolide antibiotics

Organism	M. I. C. in mcg/ml				
	T-2636 C	Erythromycin	Leucomycin	Spiramycin	Triacetyl-oleandomycin
Staph. aureus FDA 209 P	6.25	0.39	1.56	3.12	3.12
R-T-2636 C	>100	0.78	6.25	6.25	12.5
R-Erythromycin	6.25	>100	50	>100	>100
R-Leucomycin	6.25	>100	>100	>100	>100
R-Spiramycin	50	3.12	50	>100	25
R-Triacetyl-oleandomycin	25	>100	50	>100	100

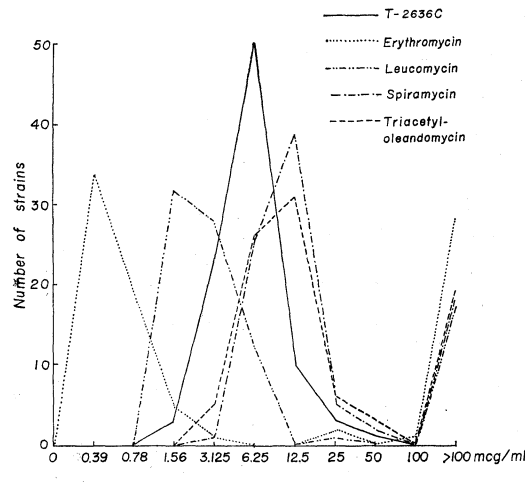
triacetyl-oleandomycin.

T-2636 C resistant *S. aureus* was sensitive to other antibiotics. But cross resistance was observed among macrolide antibiotic resistant organisms.

Sensitivity of the staphylococcus strains isolated from patients: Distribution of minimum inhibitory concentrations of T-2636 C against clinically isolated staphylococci* is illustrated in Fig. 3. Moreover, the minimum inhibitory concentrations of T-2636 C and macrolide antibiotics against macrolide antibiotic resistant strains in clinically isolated *S. aureus* are shown in Table 9 and the relationship between T-2636 C and each macrolide antibiotic is illustrated in Figs. 4~7.

T-2636 C in the concentrations of 1.56 to 50 mcg/ml was effective against clinically isolated staphylococci, in which 86 of 90 strains showed growth inhibition at the minimum concentrations from 1.56 to 12.5 mcg/ml, whereas, the standard laboratory staphylococci showed minimum inhibitory concentration at 6.25 mcg/ml. This inhibitory pattern of T-2636 C shows a sharp contrast to macrolide antibiotics, which present wide range of minimum inhibitory concentration against clinically isolated staphylocci. As shown in Table 9 and Figs. 4~7, T-2636 C exerted its full activity against clinically isolated staphylococci resistant to macrolide antibiotics.

Fig. 3. Distribution of sensitivity of clinically isolated *Staph. aureus* against T-2636 C and macrolide antibiotics.



Bacteriocidal activity: The viability of *S. aureus* FDA 209 P cultivated in TSB containing various concentrations of T-2636 C was determined by plate count, as illustrated in Fig. 8.

The logarithm of the viable count was plotted against exposure time to the antibiotic. When the antibiotic was added simultaneously weak bacteriocidal action was demonstrated at concentrations of 10 mcg/ml and 100 mcg/ml of T-2636 C. At the concentration of 1 mcg/ml, the viable cell count did not vary through a period of 8 hours. The

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

increase of viable cell count at the concentration of 0.1 mcg/ml was only slightly less than that in control. These experimental results reveal that T-2636 C is rather bacteriostatic than bacteriocidal.

Stability of T-2636 C measured by antibacterial activity: T-2636 C solution diluted in pH 2, 4, 7 or 9 buffer was kept at 37°C and the growth inhibitory activity against *S. aureus* FDA 209 P was observed 1, 2, 3, 4, 5, 6 and 7 hours thereafter. As shown in Table 10, T-2636 C was stable in pH 4 solution, whereas relatively unstable in other pH solutions.

Mode of action of antimicrobial activity: The effect of T-2636 C on the growth of *S. aureus* FDA 209 P is shown in Fig. 9. At the concentrations of 10 mcg /ml and 100 mcg/ml, partial growth inhibition was observed after addition of the drug. Synthesis of cellular protein was inhibited completely and immediately after addition of 10 mcg/ml and 100 mcg/ml, but synthesis of DNA and RNA were not significantly

Table 9. Effect of T-2636 C against resistant organisms to macrolide antibiotics in clinically isolated *Staph. aureus*

Strain No.	M. I. C in mcg/ml				
	T-2636 C	Erythromycin	Triacetyl-oleandomycin	Spiramycin	Leucomycin
T-86	1.56	>100	>100	>100	>100
T-11	3.125	>100	>100	>100	>100
T-42	3.125	>100	>100	>100	>100
T-44	3.125	>100	>100	>100	>100
T-47	3.125	>100	>100	>100	>100
T-76	3.125	>100	>100	>100	>100
T-3	6.25	>100	>100	>100	>100
T-24	6.25	>100	>100	>100	>100
T-26	6.25	>100	>100	>100	>100
T-31	6.25	>100	>100	>100	>100
T-48	6.25	>100	>100	>100	>100
T-71	6.25	>100	>100	>100	>100
T-73	6.25	>100	>100	>100	>100
T-7	12.5	>100	>100	>100	>100
T-18	25	>100	>100	>100	>100
T-33	50	>100	>100	>100	>100
T-36	3.125	>100	>100	>100	3.125
T-20	6.25	25	>100	25	25
T-35	6.25	3.125	>100	>100	>100
T-4	6.25	>100	>100	6.25	6.25
T-49	3.125	>100	50	6.25	3.125
T-67	25	>100	50	12.5	3.125
T-40	3.125	>100	25	12.5	1.56
T-41	3.125	>100	25	6.25	1.56
T-42	3.125	>100	25	12.5	1.56
T-77	6.25	>100	25	12.5	3.125
T-70	12.5	>100	25	12.5	1.56
T-59	3.125	>100	12.5	12.5	3.125
T-97	3.125	>100	12.5	12.5	3.125
T-52	6.25	>100	12.5	12.5	1.56
T-91	6.25	100	12.5	12.5	3.125
T-13	6.25	25	3.125	12.5	3.125
T-28	6.25	1.56	50	12.5	6.25
T-27	12.5	0.39	12.5	100	1.56

Fig. 4. Correlation of the sensitivity of clinically isolated *Staph. aureus* against T-2636 C and erythromycin.

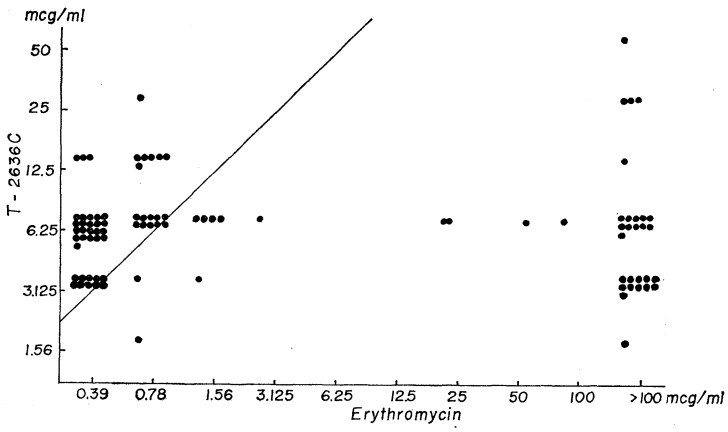


Fig. 5. Correlation of the sensitivity of clinically isolated *Staph. aureus* against T-2636 C and leucomycin.

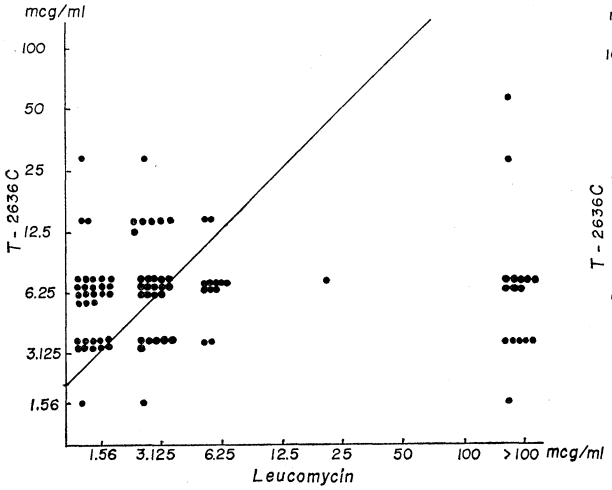


Fig. 6. Correlation of the sensitivity of clinically isolated *Staph. aureus* against T-2636 C and spiramycin.

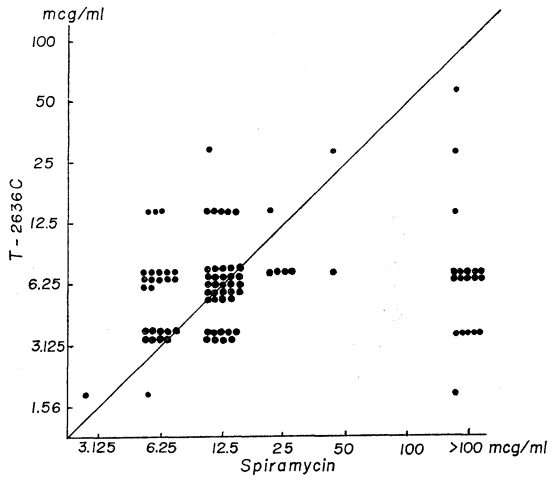


Fig. 7. Correlation of the sensitivity of clinically isolated *Staph. aureus* against T-2636 C and triacetyl-oleandomycin.

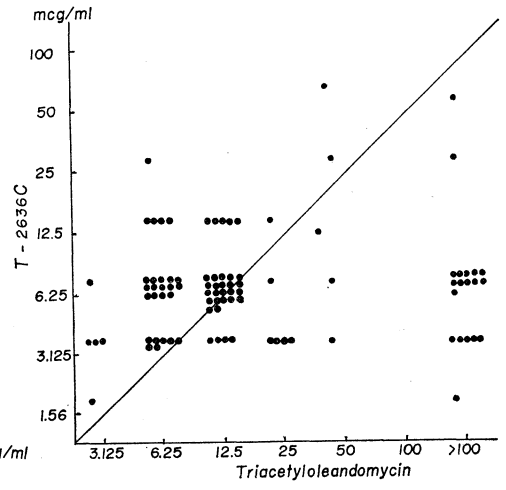


Fig. 8. Bactericidal activity of T-2636 C on *Staph. aureus* FDA 209 P.

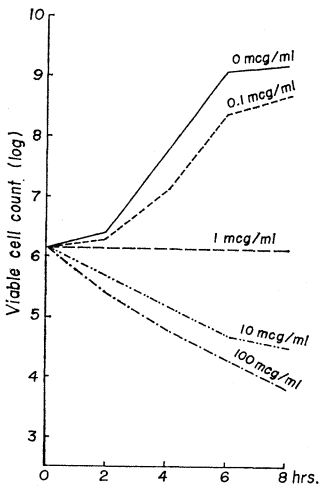
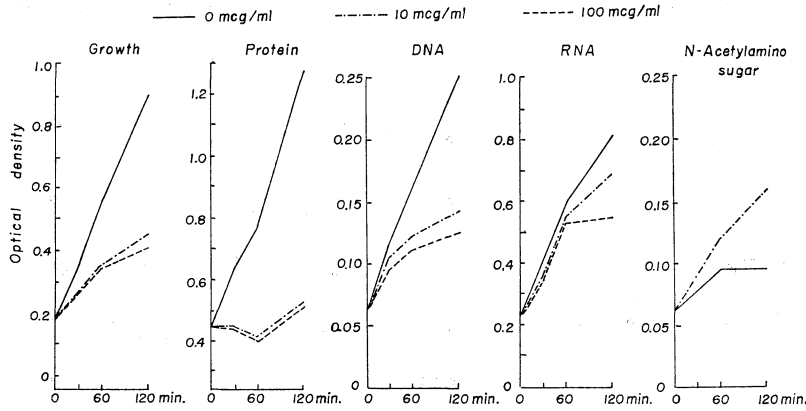


Table 10. Stability of T-2636 C in solution at various pH at 37°C

pH	M. I. C. in mcg/ml							
	0 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.
2	6.25	6.25	6.25	6.25	6.25	6.25	25	50
4	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
7	6.25	6.25	6.25	6.25	6.25	12.5	12.5	50
9	6.25	6.25	6.25	12.5	12.5	25	25	100

Fig. 9. Effect of T-2636 C on growth, protein, DNA, RNA and N-acetylamino sugar synthesis in *Staph. aureus* FDA 209 P.



inhibited at the same concentration. N-Acetylamino sugar was accumulated at the concentration of 10 mcg/ml of the antibiotic.

Antimicrobial Test *In Vivo*

Therapeutic effect of T-2636 A and C against experimental infections of mice produced by *S. aureus* 308 A-1 and *S. pyogenes* E-14 are shown Tables 11~14, and the average ED_{50} values of each strain are summarized in Table 15. Also, Table 16 shows the comparative therapeutic effects of T-2636 A, T-2636 C and macrolide antibiotics by oral administration.

1. Therapeutic Effect of T-2636 A

In experimental *S. aureus* infection, T-2636 A was most effective by intraperitoneal administration and moderately by oral administration. But the antibiotic is not effective by subcutaneous administration (Table 11).

In experimental *S. pyogenes* infection, T-2636 A was most effective by intraperitoneal administration. The therapeutic effect of the antibiotic was weaker in oral and subcutaneous administration (Table 12).

2. Therapeutic Effect of T-2636 C

In experimental *S. aureus* infection, the therapeutic effect of T-2636 C by intraperitoneal administration was two to three times more effective than that by subcutaneous one. Although therapeutic effect of the antibiotic was found by oral administra-

Table 11. Effect of T-2636 A against *Staph. aureus* infection in CF 1/H mice

Exp. No.	ED_{50} in mg/kg			Challenge dose ($\times LD_{50}$)
	Subcutaneous	Intraperitoneal	Oral	
I	640	3.84	45.9	3.16
II	—	7.69	113.5	3.16
III	640	—	40	5.62
IV	—	3.07	80	28.5
V	640	10	112.7	31.6
Average	640	6.15	78.42	—

In vitro sensitivity (MIC) : 100 mcg/ml.

Table 12. Effect of T-2636 A against *Strept. pyogenes* infection in CF 1/H mice

Exp. No.	ED_{50} in mg/kg			Challenge dose ($\times LD_{50}$)
	Subcutaneous	Intraperitoneal	Oral	
I	72	4.5	26	178
II	80	8.93	40	316
III	—	13.3	17.9	316
IV	73.4	4.2	25.9	3,160
Average	75.13	7.73	21.96	—

In vitro sensitivity (MIC) : 6.25 mcg/ml.

Table 13. Effect of T-2636 C against *Staph. aureus* infection in CF 1/H mice

Exp. No.	ED_{50} in mg/kg			Challenge dose ($\times LD_{50}$)
	Subcutaneous	Intraperitoneal	Oral	
I	7.7	—	80	3.16
II	—	—	57.9	31.6
III	8.4	2.5	99.4	31.6
IV	8.4	3.2	142.9	31.6
V	4.46	2.5	80	35.1
VI	4.46	1.77	60.6	316
Average	6.68	2.49	87.4	—

In vitro sensitivity (MIC) : 6.25 mcg/ml.

Table 14. Effect of T-2636 C against *Strept. pyogenes* infection in CF 1/H mice

Exp. No.	ED_{50} in mg/kg			Challenge dose ($\times LD_{50}$)
	Subcutaneous	Intraperitoneal	Oral	
I	5.0	—	—	46.4
II	3.8	—	24.8	316
III	6.5	—	22.5	316
IV	5.0	—	—	316
V	5.6	—	20	3,160
Average	5.18	—	22.43	—

In vitro sensitivity (MIC) : 0.39 mcg/ml.

Table 15. Correlation of the sensitivity and the therapeutic effect of T-2636 A and C against *Staph. aureus* and *Strept. pyogenes*

Organism		*Staph. aureus* 308 A-1		*Strept. pyogenes* E-14	
Antibiotic		T-2636 A	T-2636 C	T-2636 A	T-2636 C
In vitro sensitivity in mcg/ml		100	6.25	6.25	0.39
Administration route and ED_{50} in mg/kg	Subcutaneous	640	6.68	75.13	5.18
	Intraperitoneal	6.15	2.49	7.73	14.2
	Oral	78.42	87.4	21.96	22.43

Table 16. Comparison of effect of T-2636 A, T-2636 C and several macrolide antibiotics against *Staph. aureus* infection in CF 1/H mice

Antibiotic		T-2636 A	T-2636 C	Erythromycin	Leucomycin	Spiramycin	Triacetyl-oleandomycin	Challenge dose ($\times LD_{50}$)
In vitro sensitivity in mcg/ml		100	6.25	0.39	1.56	12.5	6.25	—
ED_{50} in mg/kg	Exp. No. I	38.5	76.9	—	—	—	33.5	20
	Exp. No. II	55.8	—	—	—	286	63.3	31.6
	Exp. No. III	113.5	285.7	320	446	282	—	31.6
	Exp. No. IV	99.4	92	258.1	571.4	397.5	49.7	31.6
	Average	76.8	118.2	302.55	508.7	321.8	48.8	—

tion, its activity was weaker than that by other administration routes (Table 13).

In experimental *S. pyogenes* infection, the therapeutic effect of T-2636 C by subcutaneous administration was more effective than that by oral administration (Tables 14 and 15).

3. Comparison of Therapeutic Effect of T-2636 A, T-2636 C and Macrolide Antibiotics

Table 16 demonstrates the therapeutic effect by oral administration of these antibiotics against *S. aureus* infection. The therapeutic effects of both T-2636 A and T-2636 C were found nearly equal. Although the therapeutic effects of T-2636 A and T-2636 C are inferior to that of triacetyl-oleandomycin, T-2636 antibiotics are superior to that of erythromycin, leucomycin and spiramycin.

In experimental *S. aureus* infection, the therapeutic effect of these antibiotics were found not necessarily parallel to *in vitro* activity.

Discussion

T-2636 antibiotics are chemically comparable to lankacidin or bundlins A and B. Lankacidin was reported by GÄUMANN *et al.*[13], and bundlins A and B were reported by SAKAMOTO *et al.*[14] However, the full characteristics of the antimicrobial activity of these antibiotics have not been reported.

The *in vitro* activity of T-2636 A against *S. aureus* is lower than that of T-2636 C. This observation seems to be comparable to the activity of bundlins A and B reported by SAKAMOTO *et al.*[14], but the activity seems to be different from that of lankacidin reported by GÄUMANN *et al.*[13]

The antimicrobial spectrum of T-2636 antibiotics was determined by the usual agar dilution method. T-2636 antibiotics were growth-inhibitory against many strains of Gram-positive bacteria, *N. gonorrhoeae* and *V. cholerae*. T-2636 C was most effective among T-2636 antibiotics. However, these antibiotics did not show the antibacterial activity against *B. subtilis* and many species of Gram-negative bacteria. Although the basic structure of T-2636 seems to be essential for the antibacterial activity, the acetylation at C-14 reduces the *in vitro* activity of T-2636 C and T-2636 F. Also, the hydrogenation at C-2′ decreases the *in vitro* activity of T-2636 A and T-2636 C.

In spite of macrolide-like chemical structure of T-2636, the antibacterial activity of T-2636 A and T-2636 C was activated in the acidic medium than in the basic medium. The activity of these antibiotics was decreased by addition of horse serum to the media. The most significant feature of T-2636 C was the antibacterial effectiveness not only against *S. aureus* 1840, a resistant strain, to benzylpenicillin, streptomycin, tetracycline and sulfa drug, but also against clinically isolated macrolide resistant staphylococci.

Mode of action of T-2636 C seems to be the inhibition of protein synthesis in staphylococci. However, T-2636 C did not show the cross resistance between the antibiotics inhibiting the protein synthesis. Accordingly, the inhibition of protein synthesis by T-2636 may be different in mechanism from that of other antibiotics. Furthermore, N-acetylamino sugar accumulation in the cell was observed by the treatment of T-2636 C.

Remarkable therapeutic effect of T-2636 A and C is also observed against *S. aureus* and *S. pyogenes* infection in mice. T-2636 A and T-2636 C show approximately equal effect against experimental infection of mice with both organisms when they are given intraperitonealy or orally. But the therapeutic activity of T-2636 C by subcutaneous administration shows stronger effect than that of T-2636 A. It may be related to the solubility in water or the absorption from injected site of both antibiotics. In spite of relatively weak antibacterial activity *in vitro*, T-2636 A shows still therapeutic effect *in vivo*.

References

1) HARADA, S.; T. KISHI & K. MIZUNO : Studies on T-2636 antibiotics. II. Isolation and chemical properties of T-2636 antibiotics. J. Antibiotics 24 : 13~22, 1971
2) FUGONO, T.; S. HARADA, E. HIGASHIDE & T. KISHI : Studies on T-2636 antibiotics. III. A new component, T-2636 F. J. Antibiotics 24 : 23~28, 1971
3) HIGASHIDE, E.; T. FUGONO, K. HATANO & M. SHIBATA : Studies on T-2636 antibiotics. I. Toxonomy of *Streptomyces rochei* var. *volubilis* and production of the antibiotics with an esterase. J. Antibiotics 24 : 1~12, 1971
4) HARADA, S.; E. HIGASHIDE, T. FUGONO & T. KISHI : Isolation and structures of T-2636 antibiotics. Tetrahedron Letters 1969-27 : 2239~2244, 1969
5) KAMIYA. K.; S. HARADA, Y. WADA, M. NISHIKAWA & T. KISHI : X-ray analysis of an antibiotic, T-2636 A (bundlin B). Tetrahedron Letters 1969-27 : 2245~2248, 1969

6) FUGONO, T.; E. HIGASHIDE, T. SUZUKI, H. YAMAMOTO, S. HARADA & T. KISHI : Interconversion of T-2636 antibiotics produced by *Streptomyces rochei* var. *volubilis*. Experimentia 26 : 26～27, 1970
7) PARK, J. T. & R. HANCOCK : A fractionation procedure for studies of the synthesis of cell wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. J. Gen. Microbiol. 22 : 249～258, 1960
8) LOWRY, O. H.; N. J. ROSEBROAGH, A. L. FARR & R. J. RANDAL : Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193 : 265～275, 1960
9) STROMINGER, J. L. : Microbial uridin-5′-pyrophosphate N-acetylamino sugar compounds. I. Biology of the penicillin-induced accumulation. J. Biol. Chem. 224 : 509～523, 1957
10) REISSING, J. L.; J. L. STROMINGER & L. F. LELOIR : A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. 217 : 959～966, 1955
11) SCHNEIDER, W. C. : Determination of nucleic acids in tissues by pentose analysis. Methods in Enzymology 3 : 680～684, 1957
12) REED, L. J. & H. MUENCH : A simple method of estimating fifty percent endopoints. Am. J. Hyg. 27 : 493～497, 1938
13) GÄUMANN, E.; R. HÜTTER, W. KELLER-SCHIERLEIN, L. NEIPP, V. PRELOG & H. ZÄHNER : Stoffwechselprodukte von Actinomyceten. Lankamycin und Lankacidin. Helv. Chim. Acta 43 : 601～606, 1960
14) SAKAMOTO, J. M. J.; S. KONDO, H. YUMOTO & M. ARISHIMA : Bundlins A and B, two antibiotics produced by *Streptomyces griseofuscus* nov. sp. J. Antibiotics, Ser. A 15 : 98～102, 1962