NOTES

A NEW TOLYPOMYCIN-Y DERIVATIVE: *IN VITRO* AND *IN VIVO* ANTIMICROBIAL ACTIVITY

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Tolypomycin-Y is a natural antibiotic produced by *Streptomyces tolypophorus,* discovered and studied by Japanese investigators[1~3]. Chemical and physico-chemical studies have demonstrated that tolypomycin-Y has an ansamacrolide structure[4,5] similar to that of rifamycins[6,7] and streptovaricins[8,9]. Furthermore, recent studies have shown that tolypomycin-Y exerts its antibacterial action by complexing with bacterial DNA-dependent RNA polymerase in the same way as the above-mentioned antibiotics[10,11].

The clinical use of this antibiotic is limited in practice by its instability in both acid and alkaline media, and by its action spectrum which is restricted to the Gram-positive bacteria[12].

In one of our publications, we have shown that by starting from the degradation product of tolypomycin-Y, tolypomycinone, itself microbiologically inactive, one can obtain a wide range of derivatives with different antibacterial properties[13].

From the point of view of antibacterial activity and stability, the most interesting derivatives were those obtained by the addition of unbranched primary amines to the carbon α to the nitrogen atom, in position 3 of tolypomycinone.

A microbiological study has been carried out on the most interesting representative of this series of derivatives, 3-[β-(N-morpholyl) ethylamino] tolypomycinone (referred to as M/045). In this study we consistently used tolypomycin-Y as the comparison standard, this being the original starting material; we also often included rifamycin SV (RSV), and rifampicin (RMP) because

Table 1. Sensitivity of microorganisms *in vitro* to tolypomycin-Y, M/045, rifamycin SV, rifampicin: Gram-positive organisms.

Microorganism	Culture media	M. I. C. (mcg/ml)			
		Tol. Y	M/045	RSV	RMP
S. aureus FDA 209P	BHI	0.025	0.003	0.025	0.025
S. aureus ATCC 14154	〃	0.06	0.002	0.005	0.003
S. aureus ATCC 6538	〃	0.06	0.002	—	0.003
S. aureus clinical isolate	〃	0.06	0.004	—	0.003
S. aureus clinical isolate	〃	0.025	0.003	0.05	0.003
S. aureus clinical isolate	〃	0.1	0.002	0.05	0.003
S. aureus clinical isolate	〃	0.05	0.002	0.005	0.005
Strept. pyogenes ATCC 12380	BHI+serum	0.025	0.003	0.004	0.025
Strept. pyogenes clinical isolate	〃	1.0	0.005	—	1.0
Strept. pyogenes clinical isolate	〃	0.1	0.002	—	0.05
Dipl. pneumoniae NCTN 7465	〃	0.003	0.01	—	0.09
Dipl. pneumoniae clinical isolate	〃	0.003	0.01	—	0.09
B. cereus ATCC 9634	BHI	0.05	0.005	0.025	0.005
C. diphteriae v. *gravis* NCTC 3984	〃	0.003	0.0003	—	0.09
C. diphteriae v. *gravis* clinical isolate	〃	0.001	0.0002	—	0.09

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Table 2. Sensitivity of microorganisms *in vitro* to tolypomycin-Y, M/045, rifamycin SV, rifampicin: Gram-negative organisms, Mycobacterium.

Microorganism	Culture media	M. I. C. (mcg/ml)			
		Tol. Y	M/045	RSV	RMP
Neiss. gonorrhoeae NCTC 10277	BHI+serum	0.2	0.05	—	0.1
Neiss. gonorrhoeae clinical isolate	〃	0.1	0.03	—	0.2
E. coli ATCC 10586	BHI	100.0	12.5	100.0	12.5
E. coli NCTC 8164	〃	25.0	3.0	—	0.7
E. coli clinical isolate	〃	50.0	12.5	75.0	6.2
E. coli clinical isolate	〃	25.0	3.0	—	3.0
Salm. typhi NCTC 8383	〃	75.0	12.5	—	4.5
Salm. typhi clinical isolate	〃	75.0	12.5	—	4.5
Salm. paratyphi clinical isolate	〃	100.0	12.5	100.0	12.5
Kleb. pneumoniae ATCC 10031	〃	100.0	50.0	50.0	25.0
Kleb. pneumoniae clinical isolate	〃	50.0	40.0	—	12.5
Kleb. pneumoniae clinical isolate	〃	100.0	50.0	100.0	25.0
Pseud. aeruginosa NCTC 10332	〃	100.0	50.0	—	50.0
Pseud. aeruginosa clinical isolate	〃	100.0	50.0	—	25.0
Shig. sonnei NCTC 9774	〃	50.0	12.5	—	25.0
Shig. sonnei ATCC 9290	〃	100.0	25.0	100.0	12.5
Shig. sonnei clinical isolate	〃	50.0	12.5	—	12.5
Shig. flexneri NCTC 9950	〃	50.0	12.5	—	12.5
Shig. flexneri clinical isolate	〃	50.0	12.5	—	12.5
Shig. flexneri clinical isolate	〃	100.0	50.0	50.0	25.0
Bruc. abortus NCTC 10093	BHI+serum	6.2	1.25	—	0.6
Bruc. abortus clinical isolate	〃	6.2	1.25	—	0.3
Bruc. abortus clinical isolate	〃	12.5	0.8	12.5	0.8
Bruc. melitensis NCTC 10094	〃	6.2	1.25	—	0.6
Bruc. melitensis clinical isolate	〃	6.2	1.25	—	0.3
Bruc. melitensis clinical isolate	〃	12.5	0.8	12.5	0.8
Proteus vulgaris NCTC 4636	BHI	25.0	12.5	—	6.2
Proteus vulgaris ATCC 7829	〃	25.0	12.5	—	6.2
Proteus vulgaris clinical isolate	〃	50.0	12.5	—	6.2
Past. pestis MRE 2482	〃	100.0	6.2	—	6.2
Past. pseudotuberculosis MRE 2444	〃	100.0	6.2	—	6.2
M. tuberculosis hominis NCTC 7016	DUBOS	25.0	12.5	—	6.2
M. tuberculosis hominis clinical isolate	〃	25.0	12.5	—	3.1

of their before-mentioned similarities in structure and activity.

The M.I.C.'s (minimal inhibitory concentrations, determined by the test-tube serial dilution method) show that M/045 is always more active than tolypomycin-Y against all the Gram-positive strains examined except for the two *Diplococci* studied. As regards the comparison with RMP, often the two antibiotics had the same activity and in some cases (*Streptococci, Diplococci, Corynebacteria*) the activity of M/045 was superior.

Against the Gram-negative bacteria studied the activity of M/045 is much greater than that of tolypomycin-Y.

Regarding the comparison with RMP, it often emerged that the action spectra of the two antibiotics were superimposable (*Pseudomonas, Shigella, Brucella*); in contrast, against *E. coli,*

Table 3. Effect of the pH of culture medium on the activity of tolypomycin-Y, M/045, rifampicin on *Staphylococcus aureus*.

pH of culture medium	M. I. C. (mcg/ml)								
	Tolypomycin-Y			M/045			Rifampicin		
	I	II	III	I	II	III	I	II	III
5.5	0.025	0.025	0.1	0.003	0.003	0.002	0.025	0.003	0.003
6	0.05	0.012	0.2	0.005	0.003	0.004	0.025	0.003	0.005
7	0.05	0.05	0.2	0.005	0.003	0.004	0.025	0.003	0.003
8	0.1	0.2	0.8	0.009	0.003	0.004	0.05	0.005	0.005

I=*S. aureus* FDA 209 P, II & III=*S. aureus* clinical isolates

Table 4. Effect of the inoculum size on the activity of tolypomycin-Y, M/045, rifampicin on *Staphylococcus aureus*.

Cells/ml	M. I. C. (mcg/ml)								
	Tolypomycin-Y			M/045			Rifampicin		
	I	II	III	I	II	III	I	II	III
10^1	0.025	0.025	0.1	0.003	0.003	0.002	0.025	0.003	0.003
10^2	0.050	0.025	0.2	0.003	0.005	0.004	0.025	0.003	0.005
10^3	0.050	0.050	0.5	0.003	0.005	0.005	0.025	0.003	0.005
10^4	0.050	0.050	0.5	0.005	0.005	0.005	0.050	0.003	0.005
10^5	0.5	0.8	0.5	0.005	0.090	0.005	0.10	0.005	0.090
10^6	2.12	1.5	4.0	0.008	0.080	0.005	0.030	0.005	0.090

I=*S. aureus* FDA 209P, II & III=*S. aureus* clinical isolates

Salmonella, Klebsiella and *Proteus* rifampicin always had a higher activity than M/045. Against *Mycobacterium* too rifampicin was always more active (Tables 1, 2).

The variations in the pH did not affect the M.I.C. values for M/045 and for rifampicin, as has already been reported in the literature[9]; however, the M.I.C. values for tolypomycin-Y were 4~10 times lower in pH 5.5 medium than those observed in pH 8 medium (Table 3).

Furthermore the antibacterial activities of M/045 and rifampicin were independent of the inoculum concentration, whereas the M.I.C. values for tolypomycin-Y were clearly affected (Table 4).

Different concentrations of bovine and equine sera did not influence the *in vitro* activity of the antibiotics studied.

A high degree of resistance to tolypomycin-Y, M/045 and rifampicin (studied by the transfers methods) by *S. aureus* FDA 209 P develops after 4~5 transfers.

Table 5. Development of resistance to tolypomycin-Y, M/045, rifampicin by *Staphylococcus aureus*.

Transfer	MIC (mcg/ml)		
	Tolypomycin-Y	M/045	Rifampicin
1	0.2	0.2	0.2
2	1.0	1.0	1.5
3	8.3	8.0	8.0
4	97.0	55.0	81.0
5	590.0	240.0	600.0

The speed of development of the resistance to the three antibiotics is practically the same, even if in absolute terms the values for M/045 are about half those obtained with tolypomycin-Y and rifampicin (Table 5).

The bactericidal activity of M/045 compared

Fig. 1. Bactericidal activity of tolypomycin Y, M/045, rifamycin RSV and rifampicin on *Staphylococcus aureus*.

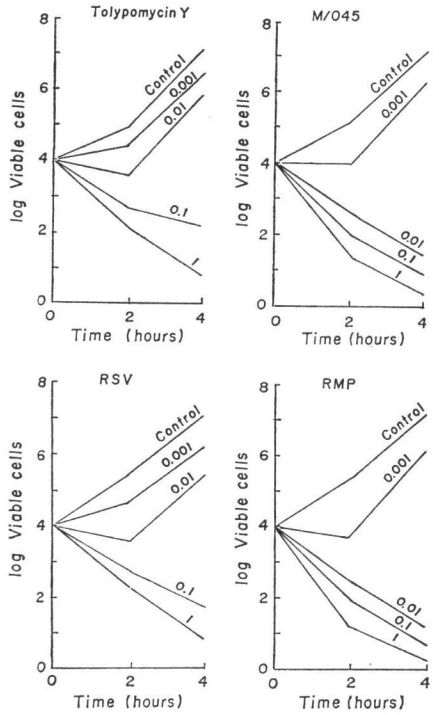
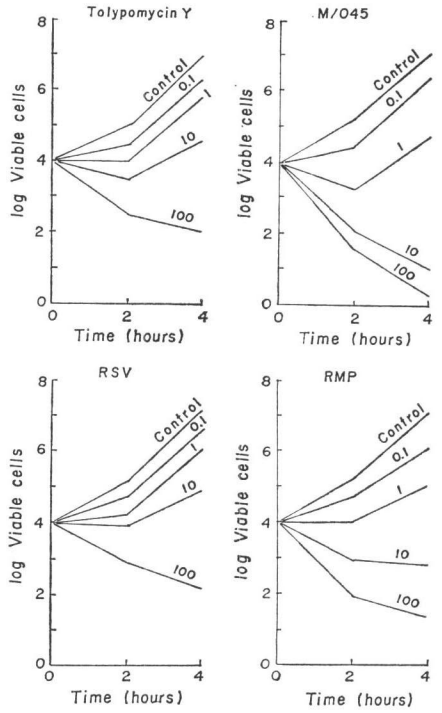


Fig. 2. Bactericidal activity of tolypomycin Y, M/045, rifamycin RSV and rifampicin on *E. coli*.



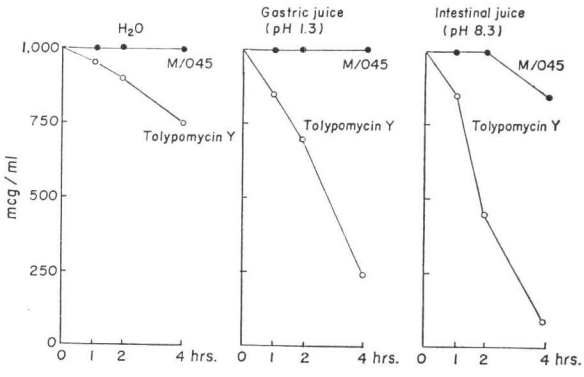
with those of tolypomycin-Y and rifampicin was studied, by the technique of counting on plates, against *S. aureus* FDA 209 P and *E. coli* K 12 in the logarithmic phase of growth.

As can be seen in Fig. 1, M/045 and rifampicin are highly bactericidal against *S. aureus* at a concentration of 0.01 mcg/ml, whereas tolypomycin-Y shows the same degree of activity at concentrations 10 times higher (0.1 mcg/ml). The bactericidal activity of M/045 and rifampicin is also considerable (10 mcg/ml) against *E. coli* K 12 (Fig. 2), whereas tolypomycin-Y shows the same degree of activity at concentrations 10 times higher (100 mcg/ml).

As can be seen from Fig. 3, M/045 is considerably more stable than the starting prototype (tolypomycin-Y) in water, artificial gastric juice and artificial intestinal juice.

The therapeutic activity of M/045, tolypo-

Fig. 3. Stability of tolypomycin Y and M/045 in water, artificial gastric juice and artificial intestinal juice.



mycin-Y and rifampicin was studied in the mouse. Table 6 shows that the ED_{50} values for our derivative *in vivo* are again considerably better than those observed for tolypomycin-Y. It should be noted that the differences between the ED_{50} values for oral and subcutaneous administration

Table 6. Comparative activity of tolypomycin-Y, M/045, rifampicin in experimental staphylococcal infections in mice.

Antibiotic	*Staphylococcus aureus* clinical isolate I			*Staphylococcus aureus* clinical isolate II		
	M. I. C. (mcg/ml)	ED_{50} (mg/kg)		M. I. C. (mcg/ml)	ED_{50} (mg/kg)	
		p. o.	s. c.		p. o.	s. c.
Tol. Y	0.05	25 (14.90~41.80)	0.91 (0.54~2.10)	0.025	16 (11.70~24.30)	1.6 (1.20~2.20)
M/045	0.003	1.85 (1.29~ 2.65)	0.41 (0.31~0.72)	0.005	0.9 (0.76~1.45)	0.31 (0.15~0.55)
RMP	0.003	0.21 (0.09~ 0.47)	0.10 (0.08~0.12)	0.005	0.17 (0.12~0.23)	0.17 (0.13~0.22)

are much more marked with tolypomycin-Y than with M/045. These results can be partly explained by the results obtained in the stability tests. From the results reported in the table, rifampicin proves to be superior in terms of therapeutic activity.

Conclusions

Study of the antibacterial spectrum *in vitro* has revealed that M/045 has a considerable activity against the greater part of Gram-positive bacteria examined; furthermore, this activity is nearly always superior to that of the original antibiotic, tolypomycin-Y.

The Gram-negative strains examined, with a low sensitivity to tolypomycin-Y, have shown a sensitivity to M/045 comparable at times to that seen with rifampicin, used in this study for comparison purposes.

The studies concerning the influence of the inoculum and of the pH of the medium have shown a clear superiority of M/045 over the original antibiotics.

The development of resistance by *S. aureus* FDA 209 P has been shown to be equally rapid for all the three antibiotics studied.

In addition, M/045 has shown a high bactericidal activity against both *S. aureus* and *E. coli*; this activity was always about 10 times higher than that of the prototype compound.

A notable therapeutic effect, greater than that of tolypomycin-Y, has been observed for M/045 in experimental infections produced with Gram-positive bacteria in the mouse; this effect was obtained with both oral and subcutaneous routes of administration.

Stability studies in aqueous solution and artificial gastric and intestinal juices have again confirmed the favorable characteristics of our derivative, thus giving encouragement for its possible clinical use.

Thus, 3-[β-(N-morpholyl)ethylamino] tolypomycinone was proved to be considerably superior to the original product (tolypomycin-Y) in terms of activity and stability, under the conditions used in the present work.

The experimental results obtained in this study therefore confirm the validity of the chemical hypothesis[13], and show M/045 to have microbiological characteristics comparable to those of other antibiotics belonging to the same family of ansamycins.

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