STRUCTURE-ACTIVITY STUDIES AMONG 16-MEMBERED MACROLIDE ANTIBIOTICS RELATED TO TYLOSIN

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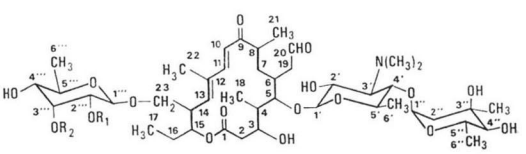
Although a substantial number of 16-membered macrolides related to tylosin have now been isolated and evaluated as antibiotics, none appeared to be superior to tylosin in treating bacterial or mycoplasmal infections caused by sensitive organisms. Nevertheless, this comparison of the antibiotic activity of 16-membered macrolides clearly indicates that novel antibiotics with potentially useful activity can be obtained from mutant strains which have been blocked at various steps in their biosynthesis of antimicrobial agents. The novel compounds thus produced may also be used as starting materials for additional chemical and microbiological modification. Furthermore, the mutant strains which produced these novel compounds should be useful recipients for interspecific genetic recombination by protoplast fusion or gene cloning to yield hybrid antibiotics.[1,2] Even greater exploitation of these methods will be required in the continuing search for new antibiotics and improved methods for producing them.

Tylosin is a 16-membered macrolide antibiotic produced commercially by strains of *Streptomyces fradiae*.[3,4] The structure of tylosin is illustrated in Fig. 1 ($R_1=R_2=CH_3$) and consists of a substituted 16-membered lactone (tylonolide), an amino sugar (mycaminose) and two neutral sugars (mycinose, mycarose).[5,6]

Recently a series of mutants of *S. fradiae* blocked in specific steps in the biosynthesis of tylosin has been described.[6] Analysis of these mutant strains in fermentation, cofermentation, *in vitro* enzymatic and *in vivo* bioconversion studies has indicated that tylactone may be converted to tylosin through a preferred series of biosynthetic steps.[6~9] Since the substrate specificity of the enzymes varied somewhat for different steps, certain of the biosynthetic steps were readily bypassed in particular mutants. Consequently, several of the mutants produced high yields of biosynthetic intermediates to tylosin while others produced shunt metabolites.[6] Many of these intermediates and shunt products had not previously been identified and consequently provided novel compounds for further chemical and microbiological modifications directed toward the discovery of more useful macrolide antibiotics.[10]

In this paper we describe the antimicrobial activity of the new 16-membered macrolides which were derived from the biosynthetically-blocked mutants described above. We have also compared the antimicrobial activity of these new compounds with that of previously reported macrolides. Such comparisons offer new insights into which functional groups of tylosin are important for antimicrobial activity and provide a basis for directed modification by chemical and microbiological methods.

Fig. 1. Structure of tylosin.



Tylosin $R_1=R_2=CH_3$

Table 1. Structures of 16-membered macrolide antibiotics related to tylosin.

Compound[a]	Oxidation level		Mycaminose	Mycarose	R_1	R_2
	C-20	C-23				
Tylosin	CHO	CH_2OR[b]	+	+	CH_3	CH_3
Macrocin	CHO	CH_2OR	+	+	CH_3	H
DOMM	CHO	CH_2OR	+	+	H	H
DMT	CHO	CH_2OH	+	+		
DMOT	CHO	CH_3	+	+		
Desmycosin	CHO	CH_2OR	+	−	CH_3	CH_3
Lactenocin	CHO	CH_2OR	+	−	CH_3	H
DOML	CHO	CH_2OR	+	−	H	H
OMT	CHO	CH_2OH	+	−		
DOMT	CHO	CH_3	+	−		
Relomycin	CH_2OH	CH_2OR	+	+	CH_3	CH_3
DODMT	CH_3	CH_2OH	+	+		
DOOMT	CH_3	CH_2OH	+	−		
5-*O*-Mycarosyl tylactone	CH_3	CH_3	−	+		
Tylactone	CH_3	CH_3	−	−		

[a] Abbreviations: DOMM : *O*-Demethylmacrocin
DMT : 23-*O*-Demycinosyltylosin
DMOT : 23-(Demycinosyloxy)tylosin
DOML : *O*-Demethyllactenocin
OMT : 5-*O*-Mycaminosyltylonolide
DOMT : 23-Deoxy-5-*O*-mycaminosyltylonolide
DODMT: 20-Deoxo-23-*O*-demycinosyltylosin
DOOMT: 20-Deoxo-5-*O*-mycaminosyltylonolide

[b] R: 6-Deoxyallose.

Materials and Methods

Antibiotic susceptibility data given in Tables 2 and 3 were obtained by agar dilution procedures with $\log_2$ dilutions of the compounds added to Mueller-Hinton agar containing 1 percent Supplement C (Difco Laboratories). Cultures were grown overnight in brain-heart infusion broth (Difco Labs.) and diluted 1: 1,000 with sterile saline. *Streptococcus pyogenes* and *Streptococcus pneumoniae* cultures were grown in broth containing 5 percent rabbit blood. Antibiotic susceptibility to *Mycoplasma* species was determined by microtiter procedures.

Mouse protection experiments were conducted using random-sexed I.C.R. albino mice weighing 19~21 g. The infecting organism, *S. pyogenes* C203, was grown in Bacto-Beef Peptone broth (Difco Laboratories) supplemented with 5 percent defibrinated rabbit blood. Mice were infected intraperitoneally with 0.5 ml of a suspension containing approximately 50~100 LD_{50}'s of bacteria per 0.5 ml. Infected mice were treated 1 and 5 hours post-infection by either subcutaneous or oral administration of 0.25 ml of a solution of the antibiotic, prepared by dissolving the antibiotic in ethanol (10 percent of final solution volume) and diluting to final volume with sterile water. Food and water were available *ad libitum*. ED_{50} values were calculated by standard procedures.[11]

Mycoplasmal infections were induced by injection of 0.2 ml of a broth culture of *M. gallisepticum* into the abdominal air sac of 1~3 day-old male leghorn chicks. Antibiotic treatment was initiated on the day of infection. Twenty-one days after the day of infection, the chickens were weighed, a blood sample was taken, the chickens were sacrificed and the presence or absence of air sac lesions was recorded.

Table 2. *In vitro* activity of macrolide antibiotics against Gram-positive bacteria.

Compound[b,c]	MIC values (μg/ml)									
	S. aureus[a]				*S. epi.*		*S. py.*	*S. pn.*	Group D *Strep.*	
	Xl. 1	V41	X400	S13E	EPI1	EPI2	C203	Park I	X66	9960
Tylosin	0.5	1	2	1	1	1	0.25	0.5	2	1
Macrocin	0.5	1	2	2	1	2	0.25	1	2	2
DOMM	8	16	16	16	4	16	0.5	4	16	8
DMT	1	1	2	1	1	1	0.25	0.5	1	1
DMOT	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.12	0.5	0.5
Desmycosin	1	2	2	1	1	1	0.25	2	2	2
Lactenocin	2	2	4	4	2	4	0.5	0.5	4	4
DOML	4	8	16	16	4	8	2	16	16	16
OMT	1	2	2	1	1	2	0.5	4	1	1
DOMT	0.25	0.25	0.5	0.25	0.25	0.25	0.25	0.25	0.5	0.5
Relomycin	4	4	8	8	4	4	2	32	64	32
DH-Macrocin	16	32	64	64	4	64	4	32	NA[d]	NA
DH-DOMM	64	64	NA	64	16	64	32	16	NA	NA
DH-DMT	NA	NA	NA	NA	NA	NA	64	64	NA	NA
DH-DMOT	64	32	64	32	32	32	32	64	64	64
DH-Desmycosin	4	8	4	4	2	2	1	32	32	32
DH-Lactenocin	8	8	32	8	4	8	2	16	NA	NA
DH-DOML	32	64	NA	64	16	64	8	32	NA	NA
DH-OMT	64	64	NA	64	32	NA	16	64	64	64
DH-DOMT	4	4	16	4	16	16	16	16	32	32
DODMT	NA	NA	NA	64	NA	64	NA	NA	NA	NA
DOOMT	4	4	8	4	8	8	8	8	16	16
Tylactone	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5-*O*-Mycarosyl-tylactone	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Josamycin	0.5	0.25	0.5	0.5	0.5	0.5	0.12	0.25	0.5	1
Carbomycin A	0.5	0.5	0.5	0.5	0.5	0.5	0.25	NT	0.5	1
Demycarosyl carbomycin A	4	8	8	4	4	8	0.5	0.06	2	2
Rosaramicin	0.25	0.25	0.25	0.25	0.25	0.25	0.12	0.25	0.5	1
Erythromycin	0.25	NA	NA	0.25	NA	32	0.06	0.03	0.12	2

[a] Abbreviations: *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae,* Group D *Streptococcus.*

[b] Compound abbreviations from Table 1.

[c] DH means 20-dihydro.

[d] NA means ≥128 μg/ml.

Results and Discussion

The *in vitro* activity of the 16-membered macrolide antibiotics related to tylosin is shown in Tables 2~4. Erythromycin, josamycin and rosaramicin have been included for comparison. Several trends in structure-activity relationships can be found in the data from these *in vitro* evaluations. As previously recognized, the neutral sugars mycarose and mycinose are unnecessary for good antibacterial activity; the presence or absence of either one or both of these sugars had very little effect on *in vitro* activity

Table 3. *In vitro* activity of macrolide antibiotics against Gram-negative bacteria.

Compound[b]	MIC values (μg/ml)							
	E. coli[a] EC14	*S. son.* N9	*E. clo.* EB5	*K. pneum.* KAE	*S. typh.* 1335	*P. rett.* PR7	*S. mar.* SE3	*P. aer.* PS18
Tylosin	>128	>128	>128	>128	>128	>128	>128	>128
DMT	128	64	>128	>128	128	>128	>128	>128
DMOT	>128	128	>128	>128	>128	>128	>128	>128
Desmycosin	>128	>128	>128	>128	>128	>128	>128	>128
OMT	128	128	>128	>128	128	128	>128	64
DOMT	32	32	64	64	16	32	64	32
DOOMT	>128	>128	>128	>128	>128	>128	>128	>128
Josamycin	>128	>128	>128	>128	>128	>128	>128	>128
Demycarosyl carbomycin A	>128	>128	>128	>128	>128	>128	>128	>128
Rosaramicin	16	16	32	64	16	32	32	32
Erythromycin	64	64	>128	>128	128	>128	128	64

[a] Abbreviations: *Escherichia coli, Shigella sonnei, Enterobacter cloacae, Klebsiella pneumoniae, Salmonella typhi, Proteus rettgeri, Serratia marcescens, Pseudomonas aeruginosa.*

[b] Compound abbreviations from Table 1.

Table 4. *In vitro* activity of macrolide antibiotics against *Mycoplasma* species.

Compound[a]	MIC values (μg/ml)			Compound[a]	MIC values (μg/ml)		
	M. gallisepticum 38502	*M. synoviae* 46995	*M. hyorhinis* S4155		*M. gallisepticum* 38502	*M. synoviae* 46995	*M. hyorhinis* S4155
Tylosin	0.4	0.1	1.6	DH-DMT	25	25	>50
Macrocin	0.4	0.2	3.1	DH-DMOT	25	25	>50
DOMM	0.8	1.6	0.8	DH-Desmycosin	3.1	6.2	50
DMT	0.4	0.8	0.2	DH-Lactenocin	12.5	>50	50
DMOT	0.1	1.6	0.4	DH-DOML	25	50	>50
Desmycosin	0.4	0.2	3.1	DH-OMT	12.5	25	50
Lactenocin	1.6	0.8	3.1	DH-DOMT	6.2	12.5	50
DOML	3.1	1.6	12.5	DODMT	25	25	>50
OMT	0.4	0.8	1.6	DOOMT	12.5	12.5	>50
DOMT	0.2	0.4	0.4	Josamycin	0.2	0.8	0.8
Relomycin	1.6	3.1	50	Rosaramicin	0.1	0.4	0.8
DH-Macrocin	12.5	>50	50	Erythromycin	0.8	>50	>50
DH-DOMM	50	50	>50				

[a] Compound abbreviations from Tables 1 and 2.

against Gram-positive bacteria and *Mycoplasma* species; however, some activity against Gram-negative bacteria was observed when both neutral sugars were absent (*e.g.*, OMT). In contrast, the basic sugar mycaminose (or desosamine, its counterpart in some related macrolides) appears to be important for antibiotic activity in this series since it could not be interchanged with the neutral sugar mycarose without loss of activity (see reference 12 for additional examples). However, an absolute requirement for an amino sugar on the 5-hydroxyl group of the lactone in order to have antimicrobial activity cannot be stated because other macrolide antibiotics such as chalcomycin are known which do not conform to this

pattern.

The precise nature of the substituent at the C-23 position appears to be very important for antimicrobial activity. MIC values were lowest for those compounds with lipophilic substituents on C-23 and increased as the hydrophilicity of the substituent at the C-23 position increased. Similar trends have been observed with derivatives of these macrolides (HAK, unpublished results; also see reference 13). DMOT and DOMT, both of which have a C-23 methyl group, were the most active macrolides in this series whereas DOMM and DOML, both of which contain 23-*O*-(6-deoxyallose), were the least active of the 23-modified macrolides. As illustrated in Table 3, only DOMT showed any significant *in vitro* activity against Gram-negative bacteria, having activity comparable to that of rosaramicin, to which it is closely related in structure.[14] The smaller structure of these molecules may allow better penetration through the outer membrane of Gram-negative bacteria, but other factors must be involved since demycarosylcarbomycin A, a close analog, was relatively inactive against Gram-negative bacteria.

Reduction of the aldehyde group to a primary alcohol decreased the antimicrobial activity of the parent macrolide in all cases. However, the presence of a methyl group instead of an

Table 5. *In vivo* activity of macrolide antibiotics against experimental infections induced by *S. pyogenes* C203 in mice.

Compound[a]	ED_{50} values (mg/kg×2)	
	Subcutaneous	Oral
Tylosin	0.7	33
Macrocin	1.7	71
DOMM	1.1	>100
DMT	1.9	82
DMOT	5.7	>50
Desmycosin	0.8	80
Lactenocin	1.8	81
DOML	14	>100
OMT	2.6	97
DOMT	20	62
DOOMT	>30	75
Rosaramicin	5.7	83
Josamycin	7.1	19
Erythromycin	0.9	10

[a] Compound abbreviations from Table 1.

Table 6. Parenteral efficacy of macrolide antibiotics against experimental infections induced by *M. gallisepticum* in chicks.

Compound[a]	Dose (mg/kg)	Number of deaths per number treated	Number with air sac lesions per number treated	Number with antibodies to MG per number tested
Tylosin	12.5	0/29	0/29	0/29
Macrocin	〃	0/30	14/30	20/30
Desmycosin	〃	11/28	19/28	11/17
Untreated	—	6/29	29/29	23/23
DMT	15	10/30	24/30	20/20
DMOT	〃	3/24	21/24	20/21
OMT	〃	2/ 9	9/ 9	7/ 7
DOMT	〃	2/24	21/24	20/22
DOMM	30	8/24	24/24	16/16
DMT	〃	10/30	27/30	16/20
DMOT	〃	3/24	15/24	17/21
OMT	〃	0/10	9/10	9/10
DOMT	〃	5/24	6/24	7/19
DMT	60×4	0/10	0/10	0/10
OMT	〃	0/10	0/10	1/10

[a] Compound abbreviations from Table 1.

aldehyde group gave mixed results for two compounds which have been isolated from fermentation of a blocked mutant of *S. fradiae*. The antibiotic activity of 20-deoxo-OMT was reduced somewhat when compared to that of OMT whereas the activity of 20-deoxo-23-demycinosyltylosin was more substantially reduced when compared to that of DMT. It appears that the degree of antibiotic activity which is lost upon reduction of the aldehyde group varies greatly, depending upon the particular macrolide being examined.

Most of these 16-membered macrolide antibiotics demonstrated good efficacy, when administered parenterally, against infections in mice induced by Gram-positive bacteria (see Table 5). Many showed parenteral activity comparable to that shown by tylosin, but none surpassed it. Unexpectedly, some of the compounds which had been most active *in vitro* (DMOT, DOMT, rosaramicin, josamycin) were significantly less active parenterally in this mouse infection model than were other macrolides which had comparable or higher MIC values against the infecting organism. Improved parenteral antibiotic activity did not appear to parallel improved *in vitro* activity. When administered orally, tylosin was also more effective than related macrolides in treating the *S. pyogenes* infection in mice. Of the tylosin-

Table 7. Oral efficacy of macrolide antibiotics against experimental infections induced by *M. gallisepticum* in chicks.

Compound	Dose[a]	Number of deaths per number treated	Number with air sac lesions per number treated	Number with antibodies to MG per number tested	Average weight per chick (g)
DMT base	2 g/gal	9/30	26/30	21/21	397
DMT tartrate	〃	0/30	13/30	17/30	458
Tylosin tartrate	〃	0/30	0/30	0/30	474
DMT base	1 g/gal	13/30	28/30	15/17	392
DMT tartrate	〃	2/30	22/29	27/28	354
Tylosin tartrate	〃	4/30	6/27	5/26	534
Untreated	—	15/30	30/30	15/15	231

[a] Dose given as g of antibiotic/gallon of drinking water.

Table 8. Oral efficacy of tylosin factors against experimental infections induced by *M. gallisepticum* in chicks.

Compound	Dose*	Number of deaths per number treated	Number with air sac lesions per number treated	Number with antibodies to MG per number tested	Average weight per chick (g)
Tylosin	1,000 g/T	0/27[a]	5/27[a]	5/27[a]	482[a]
Macrocin	〃	14/27[b]	27/27[c]	13/13[c]	289[d]
Desmycosin	〃	14/27[b]	27/27[c]	13/13[c]	304[abcd]
Relomycin	〃	14/27[b]	27/27[c]	13/13[c]	308[abcd]
Tylosin	600 g/T	2/27[a]	14/27[b]	15/25[b]	458[ab]
Macrocin	〃	22/27[c]	27/27[c]	5/ 5[c]	242[d]
Desmycosin	〃	19/27[bc]	27/27[c]	8/ 8[c]	367[abcd]
Relomycin	〃	13/27[b]	27/27[c]	14/14[c]	285[bcd]
Untreated	—	29/54[b]	53/54[c]	25/25[c]	281[cd]
Uninfected	—	0/30[a]	0/30[a]	0/30[a]	440[abc]

* Dose given as g of antibiotic/ton of feed, 5 day feeding period. Values with different superscripts are significantly different from one another, $P \leq 0.05$.

related macrolides, DOMT showed the best efficacy as an oral antibiotic, comparable to rosaramicin in this model infection. Only erythromycin and josamycin showed oral activity greater than that obtained with tylosin. A correlation was not observed between ED_{50} values obtained from subcutaneous and oral administration of macrolides; obviously factors other than antibiotic potency, such as degree of oral absorption, metabolism and tissue distribution are important for *in vivo* efficacy.

Against experimentally-induced infections by *M. gallisepticum* in chicks, tylosin was more effective than any of the macrolides which were tested (see Tables 6~8). Most of the macrolides did treat the *M. gallisepticum* infection parenterally if a sufficiently large dose and/or multiple injections were given. However, tylosin was the most effective antibiotic at the lower doses given parenterally and was also the most effective macrolide after oral administration, whether incorporated in feed or in drinking water.

Note Added in Proof

After submission of this manuscript, a publication appeared from another group independently describing the isolation and characterization of several of the new macrolide antibiotics described in this paper.[15] It appears that YT-3927, YO-9010 and YO-7625 are the same as our compounds DMOT, DMT and DOMM, respectively.

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