ELUCIDATION OF STRUCTURE OF NOVEL MACROLIDE ANTIBIOTICS PRODUCED BY MUTANT STRAINS OF *STREPTOMYCES FRADIAE*

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The physicochemical characterization and proof of structure are reported for several new macrolide antibiotics related to tylosin which have been obtained by fermentation of mutant strains of *Streptomyces fradiae*.

Tylosin is a 16-membered macrolide antibiotic which is produced commercially by strains of *Streptomyces fradiae*[1,2] and is composed of a substituted lactone (tylonolide), an amino sugar (mycaminose) and two neutral sugars (mycinose, mycarose)[3]. Recently, a series of mutants of *S. fradiae* which are blocked in specific steps in the biosynthesis of tylosin has been described[4]. From an analysis of these mutant strains in fermentation, cofermentation, *in vitro* enzymatic and *in vivo* bioconversion studies, it was proposed that tylosin is assembled *via* a preferred series of biosynthetic steps[4~8]. This proposal is further supported by *in vitro* studies recently reported by ŌMURA and coworkers[9]. Since the substrate specificities of certain enzymes involved in tylosin biosynthesis are not particularly stringent, blocked biosynthetic steps involving those enzymes were readily bypassed in particular mutant strains of *S. fradiae*[4,7,8]. Consequently, some of the mutants produced biosynthetic intermediates to tylosin in high yield while others produced shunt metabolites, many of which possessed antimicrobial activity[10]. Since many of these biosynthetic intermediates and shunt metabolites had not been previously reported, they were isolated and identified by physicochemical methods. After our work had been completed, a communication from another group was published describing a series of compounds which had been obtained from an independently-derived set of mutants which were blocked in various steps of tylosin biosynthesis[11]. In this paper, we report our work on the isolation and elucidation of structure of the 16-membered macrolides which have been obtained from the biosynthetically-blocked mutants of *S. fradiae* previously described[4].

Results and Discussion

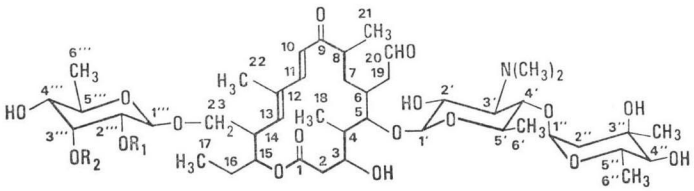
Production of Novel Macrolide Antibiotics

Table 1 summarizes the strains which were used for large scale production of several of the macrolide antibiotics characterized in this study. Representative fermentation conditions are described in the experimental section of this paper. The remaining compounds characterized in this study were obtained from the fermentation products by removal of mycarose under mildly acidic conditions, yielding the corresponding demycarosyl derivatives.

Table 1. *S. fradiae* strains used to produce macrolide antibiotics.

Strain	Macrolide(s) produced
C4	Tylosin and macrocin
GS48 (*tylD48*)	Demycinosyltylosin
GS16 (*tylE16*)	*O*-Demethylmacrocin
GS76 (*tylH76, tylD48*)	23-Deoxydemycinosyltylosin
GS77 (*tylI77, tylD48*)	20-Deoxy-20-dihydrodemycinosyltylosin; 20,23-Dideoxy-20-dihydrodemycinosyltylosin; 20-Deoxy-20-dihydro-5-*O*-mycaminosyltylonolide and 5-*O*-mycaminosyltylactone

The detailed properties of these mutants have been described elsewhere[4,5].



Tylosin (R_1=R_2=CH_3)

Table 2. Structures of 16-membered macrolide antibiotics related to tylosin.

Compound*	Oxidation level		Mycaminose	Mycarose	R_1	R_2
	C-20	C-23				
Tylosin	CHO	CH_2OR**	+	+	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	+	−		
DODMOT	CH_3	CH_3	+	+		
DODOMT	CH_3	CH_3	+	−		
5-*O*-Mycarosyl-tylactone	CH_3	CH_3	−	+		
Tylactone	CH_3	CH_3	−	−		

* Abbreviations: DOMM: *O*-Demethylmacrocin
DMT: Demycinosyltylosin
DMOT: 23-Deoxydemycinosyltylosin
DOML: *O*-Demethyllactenocin
OMT: 5-*O*-Mycaminosyltylonolide
DOMT: 23-Deoxy-5-*O*-mycaminosyltylonolide
DODMT: 20-Deoxy-20-dihydrodemycinosyltylosin
DOOMT: 20-Deoxy-20-dihydro-5-*O*-mycaminosyltylonolide
DODMOT: 20,23-Dideoxy-20-dihydrodemycinosyltylosin
DODOMT: 5-*O*-Mycaminosyltylactone

** R: 6-Deoxyallose.

Purification

All of the compounds in Table 2 except the last six were isolated by methods suitable for organic solvent-extractable basic compounds. The fermentation broths were filtered, extracted using amyl acetate or ethyl acetate at pH 9.0~9.5, and then back-extracted into water at about pH 4.0 to separate the products from organic solvent-soluble neutral compounds. Since mycarose is readily removed by hydrolysis under acidic conditions, the pH of the aqueous phase was carefully controlled to avoid hydrolysis of mycarose from these products. The products were then either crystallized directly from the aqueous solution, or extracted into a volatile solvent such as methylene chloride or ethyl acetate at pH 9.0~9.5; evaporation of the solvent yielded a dry product which could be used directly or further purified by crystallization.

A complex of compounds having a methyl group at C-20, *i.e.*, DODMT, DOOMT, DODMOT, and DODOMT (Table 2), were produced together by *S. fradiae* GS77. To separate them, one or more six-stage countercurrent extraction separations were used after the extraction procedure described above. DODMOT and DODOMT also required chromatography on silica gel for purification. The countercurrent distribution systems used were 0.5 M phosphate buffer ranging from pH 4.0 to 6.2 as the aqueous phase and ethyl acetate or a mixture of heptane - ethyl acetate, 2: 1 as the organic phase. The silica gel columns were eluted with mixtures of heptane, ethyl acetate and diethylamine or with methanol.

The isolation of tylactone and mycarosyltylactone has been previously described[12].

Structure Elucidation

The structures of the new 16-membered macrolides were determined primarily from their proton NMR spectra and their field-desorption mass spectra. Comparison of the proton NMR spectrum of each new compound with the spectra of previously-identified, tylosin-related macrolides readily indicated the nature of the structural difference(s) between the compounds. The presence or absence of the three sugars and the *O*-methyl groups, as well as the oxidation states of carbon atoms 20 and 23, were readily ascertained from the proton NMR spectra. The fully assigned spectra of tylosin and the new macrolides are recorded in Table 3. The proton NMR spectra of two other related compounds, tylactone and 5-*O*-mycarosyltylactone, have been reported in an earlier publication[12].

Further confirmation of the structures proposed for the new macrolide compounds was obtained from the physicochemical data recorded in Table 4. Field desorption mass spectrometry showed the parent molecular ion at the *m/z* value expected for the proposed structure, thereby confirming the molecular weight of each compound. The UV spectrum of each compound established the presence of the dienone as the chromophore in each case. This combination of physicochemical data conclusively established the structure of each new macrolide.

Two of the macrolide antibiotics described in this paper, 20-deoxy-20-dihydro-5-*O*-mycaminosyltylonolide (DOOMT) and 5-*O*-mycaminosyltylactone (DODOMT), have also been obtained by partial chemical synthesis[13]. Since this partial synthesis requires three chemical steps, the potential economic advantage of producing DOOMT and DODOMT by fermentation of the mutant strain GS77 is readily apparent. Three other macrolide antibiotics described in this paper, demycinosyltylosin (DMT), 23-deoxydemycinosyltylosin (DMOT) and *O*-demethylmacrocin (DOMM) appear to be the same as YO-9010, YT-3927 and YO-7625, which were recently obtained from an independently-derived set of mutant strains of *S. fradiae*[11]. However, these workers have not reported any mutant strains which lack the ability to oxidize the C-20 methyl group of tylactone, as we have described for the mutant strain GS77.

Table 3. Proton NMR assignments for tylosin and related macrolides.[a]

Position of proton	Tylosin	DOMM[b]	DMT	DMOT	DOML	DODMT	DOOMT	DODMOT	DODOMT
2	2.49	2.49	2.52	2.49	2.53	2.49	2.50	2.48	2.48
	1.95	1.96	2.00	1.93	1.96	2.03	2.00	1.96	1.97
3	3.83	3.84	3.86	3.82	3.84	3.75	3.80	3.75	3.75
4	1.62	1.63	1.54	1.65	1.64	1.61	1.64	1.5	1.5
5	3.72	3.72	3.77	3.72	3.73	3.77	3.81	3.78	3.78
6	2.18	2.16	2.12	2.16	2.20	NA[c]	NA	NA	NA
7	1.6	1.63	1.7	1.6	1.64	1.6	NA	1.5	1.5
	1.5	1.48			1.48	1.44			
8	2.61	2.62	2.75	2.60	2.63	2.78	2.80	2.74	2.70
10	6.28	6.28	6.34	6.28	6.27	6.28	6.27	6.24	6.30
11	7.32	7.31	7.35	7.29	7.30	7.27	7.27	7.24	7.30
13	5.93	5.92	5.90	5.66	5.92	5.78	5.78	5.62	5.61
14	2.97	2.92	2.92	2.73	2.96	2.90	2.91	2.74	2.70
15	5.00	4.94	4.96	4.72	4.96	4.95	4.96	4.73	4.72
16	1.89	1.83	1.9	1.84	1.84	1.86	NA	1.83	1.82
	1.65	1.63	1.7	1.54	1.64	1.61	1.64	1.6	1.6
17	0.95	0.95	1.01	0.93	0.95	0.95	0.96	0.94	0.94
18	1.02	1.00	1.03	1.01	1.00	1.02	1.05	1.03	1.03
19	2.90	2.90	2.92	2.88	2.96	1.61	1.49	1.5	1.5
	2.47	2.49	2.47	2.49	2.47				
20	9.68	9.68	9.69	9.68	9.69	0.85	0.89	0.86	0.87
21	1.23	1.22	1.28	1.22	1.22	1.18	1.18	1.19	1.19
22	1.81	1.80	1.88	1.79	1.80	1.83	1.82	1.79	1.76
23	4.00	4.04	3.77	1.07	4.03	3.74	3.76	1.09	1.08
	3.54	3.54			3.55				
1′	4.22	4.22	4.24	4.22	4.26	4.26	4.31	4.26	4.28
2′	3.55	3.54	3.56	3.54	3.50	3.57	3.55	3.57	3.51
3′	2.48	2.49	2.51	2.49	2.41	2.49	2.40	2.50	2.41
4′	3.28	3.27	3.30	3.27	3.07	3.30	3.08	3.30	3.06
5′	3.28	3.27	3.30	3.27	3.28	3.30	3.33	3.30	3.29
6′	1.25	1.25	1.30	1.25	1.27	1.28	1.31	1.29	1.31
NMe_2	2.48	2.49	2.52	2.49	2.53	2.49	2.51	2.50	2.50
1″	5.09	5.07	5.08	5.07		5.09		5.10	
2″	2.04	2.03	2.08	2.03		2.03		2.04	
	1.77	1.76	1.81	1.76		1.76		1.76	
4″	2.94	2.94	2.96	2.94		2.94		2.95	
5″	4.07	4.06	4.08	4.06		4.07		4.07	
6″	1.31	1.29	1.35	1.29		1.29		1.27	
7″	1.24	1.23	1.29	1.23		1.24		1.24	
1‴	4.56	4.54			4.55				
2‴	3.04	3.45			3.45				
3‴	3.76	4.21			4.20				
4‴	3.18	3.27			3.28				
5‴	3.52	3.68			3.68				
6‴	1.27	1.31			1.31				
2‴-OMe	3.49								
3‴-OMe	3.62								

[a] Chemical shifts in δ values (ppm downfield from internal TMS). Deuteriochloroform was used as solvent.
[b] Compound abbreviations from Table 2.
[c] Not assigned.

Table 4. Physicochemical data for tylosin and related macrolides.

	Tylosin	DOMM[a]	DMT	DMOT	DOML	DODMT	DOOMT	DODMOT	DODOMT
Formula	$C_{46}H_{77}NO_{17}$	$C_{44}H_{73}NO_{17}$	$C_{38}H_{63}NO_{13}$	$C_{38}H_{63}NO_{12}$	$C_{37}H_{61}NO_{14}$	$C_{38}H_{65}NO_{12}$	$C_{31}H_{53}NO_{9}$	$C_{38}H_{65}NO_{11}$	$C_{31}H_{53}NO_{8}$
Parent m/z	915	887	741	725	743	727	583	771	567
MP (°C)[b]	126~130	~160	~150	165~167	134~140	198~200	214~217	140~145	107~111
$[\alpha]_D^{25}$ (MeOH)	−44° (*c* 10)	−39.7° (*c* 1)	−53.5° (*c* 1)	−62.8° (*c* 1)	−8.4° (*c* 10)	−46.3° (*c* 3.3)	−7.4° (*c* 6)	−55.5° (*c* 5)	−6.8° (*c* 5)
UV λ_{max} nm (ε) (95% EtOH)	282 (24,500)	283 (22,550)	283 (22,300)	283 (21,500)	283 (21,300)	283 (21,800)	282 (22,400)	282 (21,300)	282 (21,000)
IR ν_{CO} cm^{-1} ($CHCl_3$)	1720, 1675	1723, 1678	1710, 1676	1709, 1669	1720, 1678	1715, 1676	1714, 1677	1717, 1685	1707, 1670
TLC Rf									
Solvent 1[c]	0.78	0.22	0.68	0.84	0.18	0.70	0.61	0.84	0.76
Solvent 2[d]	0.24	0.01	0.15	0.45	0.00	0.17	0.14	0.48	0.31

[a] Compound abbreviations from Table 2.

[b] All of the compounds gradually softened at temperatures well below their melting point.

[c] Ethyl acetate - diethylamine - methanol (95: 5: 5).

[d] Ethyl acetate - heptane - diethylamine (50: 50: 5).

Experimental

Strains

The *S. fradiae* mutants used in this study are listed in Table 1. The isolation and properties of these strains have been described elsewhere[4,5].

Physicochemical Determinations

Proton NMR spectra were obtained on a Bruker WH-360 NMR spectrometer, using 16K of memory and 5 KHz sweep width. Field desorption mass spectra were obtained on a Varian-MAT 731 spectrometer using carbon dendrite emitters. Ultraviolet spectra were obtained on either a Cary 219 or Cary 118 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. IR spectra were obtained on a Nicolet MX-1 FT-IR spectrometer. Melting points were measured on a Mel-temp apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed using E. Merck plates of Silica gel 60 with a fluorescent indicator (F-254); visualization was effected by either ultraviolet light or anisaldehyde spray reagent.

Fermentation Conditions

Vegetative cultures of the various *S. fradiae* mutants were prepared as described[4]. Sixty ml of vegetative culture were used to inoculate 40 liters of a second-stage vegetative growth medium composed of corn steep liquor 1%, soybean oil meal 0.5%, yeast extract 0.5%, calcium carbonate 0.3%, soybean oil 0.5% and lecithin 0.015% in water. The pH was adjusted to 8.5 with 50% sodium hydroxide and the mixture was incubated at 29°C for about 40 hours with adequate agitation and aeration. Four liters of this culture was then used to inoculate 44 liters of sterile production medium composed of fish meal 0.875%, corn meal 1.5%, corn gluten 0.875%, calcium carbonate 0.2%, sodium chloride 0.1%, ammonium monohydrogen phosphate 0.04%, beet molasses 2%, soybean oil 3% and lecithin 0.09% in water. The pH was adjusted to 7.2 with 50% sodium hydroxide and the mixture was fermented for about four days at 28°C, while maintaining the dissolved oxygen level at 30~50% by aeration with sterile air and agitating at about 250 rpm.

Representative Isolation Procedures

A. Isolation of *O*-Demethylmacrocin (DOMM): Fermentation broth (38 liters) which was harvested from a tank fermentation to produce DOMM, as described above, was filtered using a filter aid. The mycelial cake was washed with water and the combined filtrates were adjusted to pH 9.1 with 25% sodium hydroxide. The filtrate was extracted twice with ethyl acetate (9, 5 liters) and the combined extracts were mixed with water (3.5 liters) and adjusted to pH 4.1 with 28% aqueous phosphoric acid. After separating the aqueous layer, the ethyl acetate layer was extracted again with water (3.5 liters). The combined aqueous layers were adjusted to pH 8.5 with aqueous sodium hydroxide and were then extracted twice with chloroform (3, 1.2 liters). The combined chloroform extracts were dried under reduced pressure to yield 40 g of crude *O*-demethylmacrocin. Crystallization from aqueous acetone yielded 30 g of purified DOMM.

B. Isolation of 20-Deoxy-20-dihydrodemycinosyltylosin (DODMT): Fermentation broth (38 liters) which was harvested from a tank fermentation to produce DODMT, as described under fermentation conditions above, was extracted as described in Section A to yield 32 g of crude solid material. A portion (5 g) of this material was dissolved in ethyl acetate and separated by a six-stage countercurrent distribution procedure, using ethyl acetate and 0.5 M phosphate buffer (pH 5.5) as solvents. DOOMT (2.5 g) and impure DODMT (1.4 g) were obtained. The former was crystallized from dry acetone while the latter was further purified by a second six-stage countercurrent distribution procedure, using ethyl acetate - heptane (1 : 2) and 0.5 M phosphate buffer (pH 6.2) as solvents. The purified DODMT thus produced was crystallized from aqueous methanol.

C. Isolation of 20,23-Dideoxy-20-dihydrodemycinosyltylosin (DODMOT) and 5-*O*-Mycaminosyltylactone (DODOMT): These two compounds were enriched in the first solventfraction of the pH 5.5 countercurrent distribution separation (Section B). They were further enriched by a six-stage countercurrent distribution procedure using ethyl acetate and 0.5 M phosphate buffer at pH 4.0. Material from the resulting first three solvent stages was further purified in a countercurrent distribution

system using ethyl acetate - heptane (1: 2) and 0.5 M phosphate buffer at pH 5.2. Material from the first solvent fraction of this system was then chromatographed two successive times on silica gel, using heptane - ethyl acetate - diethylamine mixtures ranging from 50: 50: 5 to 80: 20: 5. The fractions containing only the above two compounds (which tended to move together) were combined, yielding material composed of about 95% DODMOT and 5% DODOMT. These were separated essentially quantitatively by chromatography on silica gel, using methanol as eluant; approximately 1.5 g of DODMOT was obtained from 75 g of crude solid material.

Representative Hydrolytic Removal of Mycarose: Preparation of *O*-Demethyllactenocin (DOML)

O-Demethylmacrocin was dissolved in aqueous hydrochloric acid and adjusted to pH 1.8 with aqueous hydrochloric acid. After standing for 24 hours at room temperature, the pH of the solution was adjusted to 9.0 with aqueous sodium hydroxide. The product was extracted into dichloromethane and the organic extract was dried and evaporated under reduced pressure to yield *O*-demethyllactenocin.

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