NOVEL FERMENTATION PRODUCTS FROM *STREPTOMYCES FRADIAE*: X-RAY CRYSTAL STRUCTURE OF 5-*O*-MYCAROSYLTYLACTONE AND PROOF OF THE ABSOLUTE CONFIGURATION OF TYLOSIN

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5-*O*-Mycarosyltylactone has been isolated as a predominant factor from fermentation broths of a *Streptomyces fradiae* mutant. The relative configurations of mycarose and tylactone (protylonolide) have been determined by X-ray crystal structure analysis. Hydrolysis of 5-*O*-mycarosyltylactone yielded (−)-tylactone and L-(−)-mycarose. Taken together, these two experiments establish the absolute configuration of (−)-tylactone. Bioconversion of (−)-tylactone to tylosin by *tyl G* mutants of *S. fradiae* proves the absolute configuration of tylosin. Physicochemical data for tylactone and a unique component piece of tylactone are also reported.

Two minor products, which lack antibacterial activity, were initially isolated from the fermentation broths of tylosin-producing strains of *Streptomyces fradiae*. More recently, these same two products have been obtained in much higher yield from fermentations of a *tyl B* mutant strain of *S. fradiae*.[1] This mutant was blocked only in formation or addition of the amino sugar, mycaminose, to the 5-hydroxyl group of the lactone ring and accumulated tylactone (or protylonolide[2]), (**1**), and a shunt product, 5-*O*-mycarosyltylactone (**2**). Recently these same two products have been found by ŌMURA and coworkers[2,3].

The structures of **1** and **2** have been deduced from a detailed analysis of physicochemical data (Table 1), including proton NMR spectra (Table 2). Both yield single crystals suitable for X-ray analysis. Although the X-ray crystal structure of **1** was recently reported[4], the absolute configuration of the 16-membered ring was established by spectral arguments relating tylosin to leucomycin[5].

Table 1. Physicochemical properties of tylactone (**1**), 5-*O*-mycarosyltylactone (**2**), 9-hydroxy-6,8-dimethyl-undeca-4,6-diene-3-one (**4**) and its *O*-benzoyl derivative (**5**).

Compound	**1**	**2**	**4**	**5**
Formula	$C_{23}H_{38}O_5$	$C_{30}H_{50}O_6$	$C_{13}H_{22}O_2$	$C_{20}H_{26}O_3$
Parent *m/z*	394 (FDMS)	538 (FDMS)	210 (FIMS)	314 (FDMS)
M.P. (°C)	163～164.5	182～184	oil	wax
$[\alpha]_D^{25}$ (MeOH)	−56.0° (*c* 6.0)	−46.4° (*c* 4.4)	—[a]	—[a]
UV $\lambda_{max}^{95\%\ EtOH}$ nm(ε)	282 (21,600)	282 (21,300)	279 (14,100)	276 (14,800) 229 (17,800)
IR ν_{CO} cm^{-1} ($CHCl_3$)	1713, 1678	1715, 1678	1710	1707
Rf	0.22[b]	0.15[b]	0.35[c]	0.62[c]

[a] Insufficient material available.
[b] Silica gel plates (E. Merck), developed in chloroform - ethyl acetate, 3: 1.
[c] Silica gel plates (E. Merck), developed in toluene - acetone, 5: 1.

Table 2. The 360 MHz ^{1}H NMR (in $CDCl_3$) data for tylactone (**1**), 5-*O*-mycarosyltylactone (**2**), 9-hydroxy-6,8-dimethylundeca-4,6-diene-3-one (**4**) and its *O*-benzoyl derivative (**5**).

For comparison purposes, the atoms in all four compounds are numbered below as in Fig. 1.

Protons (s) on atom	Shift (δ)			
	1	**2**	**4**	**5**
C 2	2.48 / 1.93	2.47 / 1.93		
C 3	3.71	3.68		
C 4	1.47	~1.58		
C 5	3.75	3.76		
C 6	—[a]	~1.58		
C 7	1.55 / —[a]	~1.58 / ~1.58		
C 8	2.69	2.75	2.61	2.62
C 10	6.31	6.26	6.12	6.13
C 11	7.30	7.32	7.22	7.12
C 13	5.64	5.64	5.90	5.93
C 14	2.77	2.71	2.69	2.98
C 15	4.70	4.70	3.44	5.08
C 16	1.85 / ~1.61	1.84 / ~1.58	1.53 / 1.42	1.71 / 1.66
C 17	0.93	0.93	0.96	0.94
C 18	0.99	0.93		
C 19	~1.61	~1.58		
C 20	0.91	~0.93		
C 21	1.22	1.21	1.12	1.13
C 22	1.82	1.79	1.82	1.85
C 23	1.08	1.07	1.05	1.08
C 1′		4.85		
C 2′		2.15 / 1.80		
C 4′		2.96		
C 5′		3.73		
C 6′		1.29		
C 7′		1.25		
O 4′		2.14		
Hydroxyl	3.36[a]	3.14[a]		
		3.99[a]		
Benzoyl				7.50 (3H)
				8.13 (2H)

[a] Not assigned

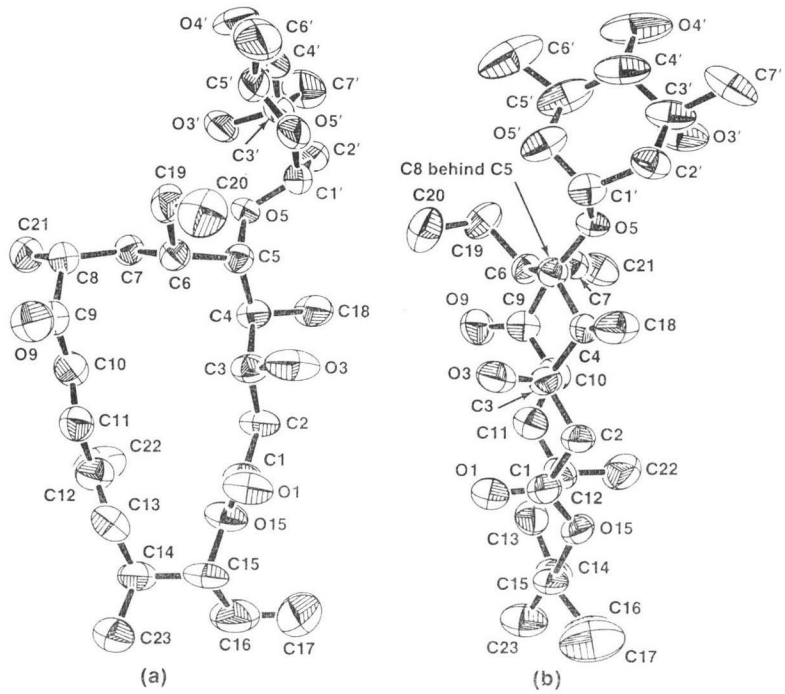
We now report the X-ray crystal structure of 5-*O*-mycarosyltylactone, which unequivocally proves the absolute configuration of the lactone ring, since the absolute configuration of L-(−)-mycarose has been previously determined[6]. Fig. 1 (a) is an ORTEP drawing of the structure in the crystal, showing that the lactone ring has the same absolute configuration and a very similar conformation to that found in protylonolide (tylactone)[4]. In Fig. 1 (b), the molecule has been rotated 90° about the vertical axis of the drawing, showing the three pseudo-axial oxygen atoms which protrude on the same face of the rather planar, but staggered, lactone ring.

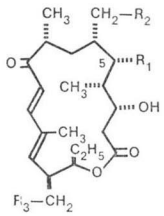
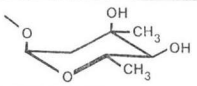
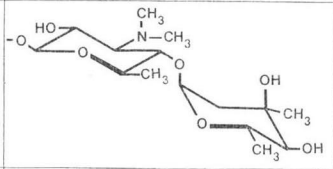
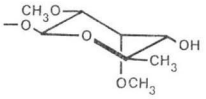
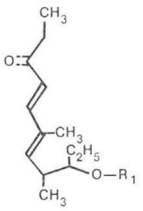
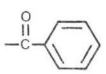
Acidic hydrolysis of **2** yielded L-(−)-mycarose[7], identical in all respects to a sample of mycarose obtained from acidic hydrolysis of tylosin. The other hydrolysis product was (−)-tylactone, identical in all respects to a sample which was efficiently bioconverted to tylosin by *tyl G* mutants of *S. fradiae*[1,8]. Thus, the absolute configuration of both tylactone and tylosin (**3**) can be rigorously defined on the basis of the crystal structure of **2**.

During the course of our work on the production of 5-*O*-mycarosyltylactone, one fermentation became contaminated by an unidentified bacterium. Upon workup of the broth from this fermentation, a new product was observed in addition to **1** and **2** (TLC analysis, visualization by ultraviolet light).

Fig. 1. ORTEP plots of 5-*O*-mycarosyltylactone (a) with lactone ring oriented as in ŌMURA *et al.*[4], (b) rotated 90° around vertical axis of drawing.

The thermal ellipsoids are drawn at the 50% probability level.



		R_1	R_2	R_3
	1	—OH	$-CH_3$	—H
	2		$-CH_3$	—H
	3		—CHO	
	4	—H		
	5			

This new compound was less polar than either **1** or **2** and was readily separated from them by chromatography on silica gel. It was identified as 9-hydroxy-6,8-dimethylundeca-4,6-dien-3-one (**4**) on the basis of physicochemical data for it and its O-benzoyl derivative (**5**) (Tables 1 and 2). This new compound was clearly a fragment of tylactone, corresponding to carbon atoms 8 to 15 (with substituents) of tylactone. Since **4** was not observed in an uncontaminated fermentation, it most likely resulted from microbial degradation of tylactone*. Microbial degradation products of macrolide rings have not been previously identified[11], but microorganisms which can specifically degrade macrolide rings should be obtainable by routine screening procedures.

Experimental

Production and Isolation of Tylactone (**1**) and 5-*O*-Mycarosyltylactone (**2**)

A lyophilized pellet of *Streptomyces fradiae* GS-50[1] dispersed in 1~2 ml water was inoculated into 150 ml of complex vegetative medium[1]. Six ml portions of the vegetative culture were transferred to several 86 ml volumes of complex fermentation media[1] in 500-ml Erlenmeyer flasks and incubated in a closed box shaker at 29°C for 6 days at 300 rpm.

The pooled fermentation broth (~900 ml) obtained above was extracted with 900 ml of petroleum ether and the extract was concentrated to an oil. The oil was dissolved in 15 ml ethyl acetate, 15~20 ml heptane was added, and the ethyl acetate was allowed to slowly evaporate to permit crystallization. The crystals were filtered and dried to yield 450 mg of a mixture of 5-*O*-mycarosyltylactone and tylactone. The crystals (400 mg) were dissolved in benzene and applied to a silica gel (Woelm) column packed in benzene. The elution was monitored by silica gel TLC using a benzene - ethyl acetate (3: 2) system and conc. sulfuric acid spray for detection. The column was eluted sequentially with 1 liter benzene, 1 liter benzene - ethyl acetate (9: 1), 1.4 liter benzene - ethyl acetate (6: 1), and 0.9 liter benzene - ethyl acetate (3: 1) collecting 150 ml fractions. Tylactone was eluted in fractions 14~19 and then 5-*O*-mycarosyltylactone in fractions 22~26. The fractions containing each were combined, evaporated *in vacuo* and crystallized from heptane. Analysis of tylactone: C 69.82%, H 9.75%, calcd. C 70.02%, H 9.71%. Analysis of 5-*O*-mycarosyltylactone: C 67.06%, H 9.60%, calcd. C 66.88%, H 9.36%.

Crystal Structure of 5-*O*-Mycarosyltylactone (**2**)

5-*O*-Mycarosyltylactone was recrystallized from heptane containing a small amount of ethyl acetate to obtain crystals suitable for X-ray crystal structure analysis. The compound forms yellowish prisms in the space group $P2_1$, with two molecules in a unit cell having the dimensions $a=14{,}986\pm0.006$ Å, $b=15.797\pm0.004$, $c=6.820\pm0.001$ and $\beta=102.20\pm0.02°$. The density determined by the flotation method was 1.12 g/cm^3, while that calculated for $C_{30}H_{50}O_8$ (mol. wt. 538.7) is 1.13 g/cm^3. The intensities of 2262 independent reflections were measured on a four-circle, computer automated diffractometer, using monochromatic copper Kα-radiation. The structure was solved using the direct methods program MULTAN-78 and was refined by the least-squares method to an R index of 0.069. In the final refinement cycle, all carbon and oxygen atoms had anisotropic temperature factors and all hydrogen atoms, except the three on oxygen, were included with isotropic temperature factors at assumed positions. The final non-hydrogen atom coordinates and their standard deviations are given in Table 3. The atoms are numbered as in Fig. 1.

Hydrolysis of 5-*O*-Mycarosyltylactone (**2**)

5-*O*-Mycarosyltylactone (0.8 g) was dissolved in methanol (50 ml) and the solution was treated with 1 N HCl (1 ml) and heated at 60°C for one hour. The solution was cooled to room temperature and adjusted to pH 11 with 1 N NaOH. The solvent was evaporated under reduced pressure and the residue was partitioned between water and ethyl acetate. The aqueous layer was lyophilized and the organic

* An alternative explanation for **4** is microbially induced release and subsequent decarboxylation of a partially assembled polyketide chain from the enzyme complex of the *S. fradiae* mutant which normally produces tylactone.

Table 3. Atomic coordinates (standard deviations) $\times 10^4$ for 5-*O*-mycarosyltylactone.

Atom	x	y	z	Atom	x	y	z
C 1	3075 (6)	−2132 (6)	−3679 (14)	C 20	−15 (10)	891 (9)	−3499 (18)
C 2	2331 (6)	−2295 (6)	−2621 (14)	C 21	2515 (8)	689 (8)	4374 (14)
C 3	1669 (6)	−1545 (6)	−2705 (12)	C 22	4558 (10)	−1494 (11)	1359 (16)
C 4	1042 (6)	−1630 (5)	−1246 (12)	C 23	6096 (7)	−1664 (10)	−3285 (19)
C 5	433 (5)	−866 (5)	−1209 (11)	O 1	2980 (4)	−1708 (6)	−5230 (10)
C 6	930 (5)	−12 (5)	−660 (11)	O 3	1121 (5)	−1448 (6)	−4684 (10)
C 7	1407 (5)	8 (5)	1585 (11)	O 5	−156 (3)	−1011 (4)	185 (7)
C 8	2068 (6)	754 (6)	2180 (13)	O 9	2892 (5)	1363 (5)	−92 (11)
C 9	2809 (6)	762 (6)	914 (12)	O 15	3810 (4)	−2541 (4)	−2898 (9)
C 10	3370 (6)	8 (7)	982 (12)	C 1′	−1060 (5)	−1250 (5)	−686 (13)
C 11	3912 (6)	−144 (7)	−347 (13)	C 2′	−1423 (6)	−1818 (6)	822 (14)
C 12	4467 (6)	−896 (8)	−341 (12)	C 3′	−1674 (6)	−1392 (8)	2537 (14)
C 13	4809 (6)	−1050 (7)	−1930 (13)	C 4′	−2256 (6)	−635 (9)	1884 (17)
C 14	5299 (6)	−1842 (7)	−2357 (13)	C 5′	−1841 (7)	−78 (7)	557 (19)
C 15	4632 (6)	−2438 (7)	−3777 (16)	C 6′	−2456 (12)	630 (10)	−441 (45)
C 16	5010 (10)	−3341 (10)	−3774 (27)	C 7′	−2158 (8)	−2015 (11)	3716 (18)
C 17	4381 (11)	−3948 (13)	−5004 (25)	O 3′	−873 (4)	−1072 (6)	3952 (10)
C 18	484 (7)	−2462 (6)	−1624 (19)	O 4′	−2424 (6)	−141 (8)	3472 (16)
C 19	290 (7)	747 (6)	−1198 (15)	O 5′	−1635 (4)	−539 (5)	−1240 (11)

Absolute configuration is represented by the right-hand coordinate system.

layer was dried over Na_2SO_4.

The residue from lyophilization was dissolved in 2 ml of water and passed through a column of Dowex 50W-X8 (25 ml, H^+ cycle). The column was eluted with water (75 ml), the eluate was adjusted to pH 1.4 with 1 N HCl and the solution was allowed to stand at room temperature for 3 days. The solution was then concentrated to about 10 ml under reduced pressure and passed through a column of BioRad AG1-X8 (50 ml, OH^- cycle). The column was eluted with 150 ml of water and the eluate was lyophilized to yield 66 mg of mycarose. The material was crystallized from $CHCl_3$ in the manner described by WOODWARD[9], yielding crystalline mycarose identical in all respects (melting point, optical rotation, proton NMR and TLC mobility) to an authentic sample of mycarose obtained from the hydrolysis of tylosin in an analogous manner.

The dried ethyl acetate solution was filtered and the filtrate was evaporated under reduced pressure. The oily residue was dissolved in a small volume of toluene and purified by flash chromatography[10] on silica gel (E. Merck, grade 60). The column was eluted with a linear gradient of toluene (500 ml) and toluene - ethyl acetate, 4: 1 (500 ml), followed by an additional 250 ml of the latter solvent. Fractions containing tylactone were located by TLC analysis and appropriate fractions were combined and evaporated to dryness to yield 360 mg of product. Tylactone was crystallized by dissolving it in a small volume of ethyl acetate, diluting the solution with heptane and evaporating the ethyl acetate by warming on a steam bath. The sample of tylactone thus obtained was identical in all respects (melting point, optical rotation, proton NMR, TLC mobility and IR and UV spectra) to samples of tylactone isolated from wild type or *tyl A* or *tyl B* mutants of *S. fradiae* which have been bioconverted to tylosin by *tyl G* mutants of *S. fradiae*[1,8].

Isolation of 9-Hydroxy-6,8-dimethylundeca-4,6-diene-3-one (**4**) and Its *O*-Benzoyl Derivative (**5**)

A sample of crude 9-hydroxy-6,8-dimethylundeca-4,6-diene-3-one, obtained as a byproduct of workup of a fermentation broth as described above, was further purified by chromatography on silica gel, eluting with a linear gradient of toluene and toluene - acetone, 3: 1. Fractions containing the desired product were located by TLC analysis, combined and evaporated to dryness to yield 457 mg of

a yellow oil. A small sample (about 30 mg) was used to obtain physicochemical data and the remainder was dissolved in 10 ml pyridine and treated with benzoic anhydride and a catalytic amount of dimethylaminopyridine (10 mg) overnight at room temperature. After evaporation of solvent under reduced pressure, the residue was purified by flash chromatography on silica gel (E. Merck, grade 60), eluting with a linear gradient of 600 ml toluene and 600 ml of toluene - ethyl acetate, 5: 1. Fractions containing the desired product were located by TLC analysis, combined and evaporated to dryness to yield benzoate **5**.

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