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BIOSYNTHESIS OF TYLOSIN: OXIDATIONS OF 5-O-MYCAMINOSYLPROTYLONOLIDE AT C-20 AND C-23 WITH A CELL-FREE EXTRACT FROM STREPTOMYCES FRADIAE*

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The enzymatic conversion of various tylosin precursors was carried out using a cell-free system of the tylosin producer Streptomyces fradiae to determine the order and intermediates of oxidations of the 16-membered branched lactone ring at C-20 and C-23 in the biosynthesis of tylosin. It was found that the order of the oxidation of the lactone is: (1) hydroxylation of 5-O-mycaminosylprotylonolide at C-20, (2) oxidation of C-20 hydroxymethyl to formyl, (3) hydroxylation at C-23 to give 5-O-mycaminosyltylonolide. The formation of 23-hydroxy-5-O-mycaminosylprotylonolide from 5-O-mycaminosylprotylonolide was not observed.

INTRODUCTION

Tylosin (Fig. 1) is a commercially important, 16-membered macrolide antibiotic which is produced by strains of Streptomyces fradiae (1), S. rimosus (2), and S. hygroscopicus (3). Previous ^{13}C nuclear magnetic resonance studies (4,5) indicated that the lactone (protylonolide) is derived from two acetates, five propionates and one butyrate. Biotransformation of tylosin-related compounds by S. fradiae grown in the presence of cerulenin, an inhibitor of de novo synthesis of macrolide antibiotics (6), suggested that the addition of mycaminose to the C-5 hydroxy position of protylonolide precedes the oxidations of C-20 and C-23 positions, and subsequently the oxidations and the additions of mycinose and mycarose occur to give tylosin (7). Cofermentation and biotransformation studies by Baltz and Seno (8) with mutants of S. fradiae blocked in tylosin biosynthesis gave results similar to those described above with regard to the addition of mycaminose and oxida-

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Abbreviations: PTL, protylonolide; MPTL, 5-O-mycaminosylprotylonolide; MTL, 5-O-mycaminosyltylonolide.

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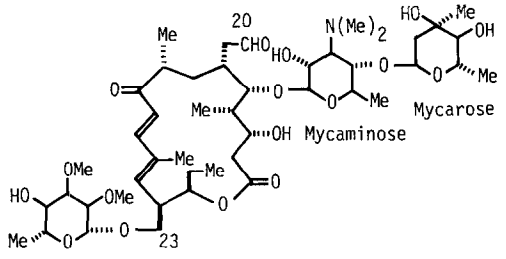

Tylosin

	R_1	R_2
5-*O*-Mycaminosylprotylonolide (MPTL)	CH_3	CH_3
23-Hydroxy-MPTL (23-OH-MPTL)	CH_3	CH_2OH
20-Hydroxy-MPTL (20-OH-MPTL)	CH_2OH	CH_3
20,23-Dihydroxy-MPTL (20,23-diOH-MPTL)	CH_2OH	CH_2OH
20-Oxo-MPTL	CHO	CH_3
5-*O*-Mycaminosyltylonolide (MTL)	CHO	CH_2OH

Fig. 1. Structures of tylosin and its related compounds.

tions of the lactone, while they indicated that 6-deoxy-D-allose instead of mycinose is added to the C-23 hydroxyl position of the lactone and subsequently O-methylated at 2" and 3" positions. However, the order and intermediates of oxidations of the lactone remain to be determined. Therefore, we attempted oxidations of the lactone with a cell-free extract from S. fradiae to clarify the problem.

In the present communications, we describe the enzymatic formation of 5-O-mycaminosyltylonolide (MTL) from 5-O-mycaminosylprotylonolide (MPTL) via 20-hydroxy-5-O-mycaminosylprotylonolide (20-OH-MPTL) and 20-oxo-5-O-mycaminosylprotylonolide (20-oxo-MPTL).

MATERIALS AND METHODS

Chemicals: 23-OH-MPTL, 20-oxo-MPTL, 20,23-diOH-MPTL and MTL are generous gifts from Eli Lilly & Co., U.S.A. MPTL was prepared as described previously (9). 20-OH-MPTL was prepared by reduction of 20-oxo-MPTL with $NaBH_4$ according to the method described for reduction of tylosin to relomycin (10). Other chemicals were commercially obtained.

Preparation of a Cell-Free Extract: Wet mycelia (50 g) of the tylosin producer (S. fradiae KA-427) grown with aeration for 50 h at 27°C in a liquid medium (pH 7.4), containing 1.0% glucose, 2.0% starch, 0.5% peptone, 0.5% yeast extract, 0.3% L-asparagine and 0.4% $CaCO_3$, were added to 100 ml of 50 mM phosphate buffer (pH 7.2), homogenized with a Ultra-Turrax homogenizer, and disrupted with a Kubota 200 M sonicator. The sonicated suspension was centrifuged at 12,000 x g for 10 min. The supernatant fluid was used as an enzyme preparation.

Enzyme Reaction: A reaction mixture (2.0 ml), containing 100 μM substrate (a tylosin-related compound), 1.0 mM cofactor [$NAD(P)^+$ or NAD(P)H], 50 mM phosphate buffer (pH 7.2) and 1.0 ml of enzyme solution (13 mg as pro-

Table I. Conversion of tylosin-related compounds with a cell-free extract from S. fradiae KA-427.

Substrate	Cofactor	Compound formed (μM)					
		MPTL	20-OH-MPTL	23-OH-MPTL	20,23-diOH-MPTL	20-Oxo-MPTL	MTL
PTL	NADH	0	0	0	0	0	0
MPTL	NADH	(65)	25	0	1	0	0
23-OH-MPTL	NADH	0	0	(90)	6	0	0
20-OH-MPTL	NADH	0	(80)	0	5	5	0
20-Oxo-MPTL	NADH	0	0	0	0	(48)	24
20-OH-MPTL	NAD^+	0	(82)	0	2	11	2

tein) was incubated with aeration at 27°C for 20 min, unless otherwise stated. After the reaction was stopped by adding 0.5 ml of 5N NH_4OH, the product(s) was extracted twice from the reaction mixture with an equal volume of benzene. The benzene layer was concentrated to dryness and subjected to silica gel thin-layer chromatography (TLC) using $CHCl_3$-MeOH-7% NH_4OH (20:6:5, v/v, bottom layer). The product(s) was analyzed by scanning the chromatogram with a Shimadzu CS-920 chromatogram scanner at 282 nm. The Rf values of tylosin-related compounds were: PTL, 0.72; MPTL, 0.62; 20-oxo-MPTL, 0.43; 23-OH-MPTL, 0.40; 20-OH-MPTL, 0.38; MTL, 0.25; 20,23-diOH-MPTL, 0.22.

Identification of Reaction Products: A reaction mixture (200 ml) containing 50 μM substrate, 0.5 mM cofactor, 50 mM phosphate buffer (pH 7.2) and 50 ml of enzyme solution (650 mg as protein), was incubated with aeration at 27°C for 60 min, and was then extracted twice with benzene (200 ml) at pH 10. The extract was concentrated and subjected to preparative TLC. The products isolated were identified by means of TLC and mass spectrometry.

RESULTS AND DISCUSSION

In order to determine the intermediates and order of oxidations of the lactone moiety in tylosin biosynthesis in S. fradiae KA-427, we attempted the reactions with a cell-free extract from the organism. Tylosin-related compounds indicated in Fig. 1 were incubated with the cell-free extract in the presence of various cofactors, and the products were analyzed by TLC and mass spectrometry. Fig. 1 shows typical TLC profiles, and Table I summarizes the conversion of tylosin-related compounds.

Hydroxylation of the Lactone Moiety at C-20 When MPTL as substrate and NADH as cofactor were incubated with the cell-free extract from the tylosin producer at 27°C for 20 min, a product having the Rf value of 0.38 was detected on the chromatogram, as shown in Fig. 2-(1). The product was identified as 20-OH-MPTL by TLC and mass spectrometry [m/z: 583 (M^+), 409 (aglycone), and 174 (mycaminose)]. On the other hand, 23-OH-MPTL (Rf, 0.40)

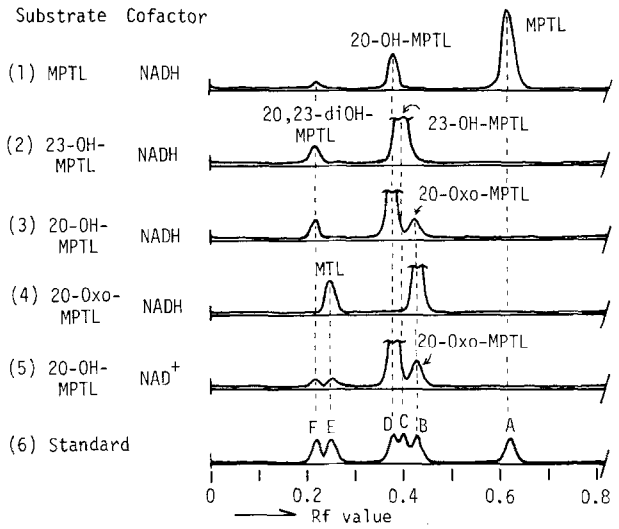

Fig. 2. Thin-layer chromatogram of the products from tylosin-related compounds with a cell-free extract from S. fradiae KA-427. Standard: A, MPTL; B, 20-oxo-MPTL; C, 23-OH-MPTL; D, 20-OH-MPTL; E, MTL; F, 20,23-diOH-MPTL.

could not be detected in the extract from the reaction mixture incubated as described above. This indicates that the enzyme catalyzes the hydroxylation of MPTL at C-20 but not at C-23. As shown in Table II, the enzyme reaction required NADH or NADPH and aeration, suggesting that the enzyme is the monoxygenase-type. The enzyme activity was lost when the enzyme preparation was dialyzed against 50 mM phosphate buffer (pH 7.2), indicating that the enzyme requires another cofactor(s). When PTL instead of MPTL was used as substrate, no products were detected. When 23-OH-MPTL was used, however, 20,23-diOH-MPTL (Rf, 0.22) was formed as shown in Fig. 2-(2) and Table I.

From these results, it is concluded that the hydroxylation of the lactone in tylosin biosynthesis first occurs at C-20 but not at C-23 after the addition of mycaminose at C-5. Because the formation of 23-OH-MPTL from MPTL was not observed, it is speculated that 23-OH-MPTL is not a real intermediate although the hydroxylation of 23-OH-MPTL at C-20 occurred as described above and its microbial transformation into tylosin was previously reported (7).

Oxidation of C-20 Hydroxymethyl to Formyl When 20-OH-MPTL as substrate and NAD^+ as cofactor were incubated with the cell-free extract,

Table II. Requirements for the hydroxylation of MPTL at C-20.

Reaction mixture	20-Hydroxy-MPTL formed (μM)
Under aerobic conditions	
Complete	11.0
- NADH	3.5
- NADH + NADPH	6.5
- Enzyme + heated enzyme	0
- Enzyme + dialyzed enzyme	0.1
Under anaerobic conditions*	
Complete	0

A reaction mixture (2.0 ml) containing 50 μM MPTL, 500 μM NADH, 50 mM phosphate buffer (pH 7.2) and 0.5 ml of enzyme solution (6.5 mg protein) was incubated with aeration at 27°C for 20 min. After the reaction was stopped by adding 0.5 ml of 5N NH_4OH, 20-hydroxy-MPTL was extracted from the reaction mixture with benzene and then analyzed as described in the text.

*The tube containing the reaction mixture was evacuated, the air being substituted in N_2 gas.

20-oxo-MPTL (11 μM), 20,23-diOH-MPTL (2 μM) and MTL (2 μM) were formed as shown in Fig. 2-(5) and Table I. The formation of 20-oxo-MPTL from 20-OH-MPTL required $NAD(P)^+$ (data not shown). When 20,23-diOH-MPTL was used as substrate, substantial portion of the substrate was degraded to the substance(s) showing no UV absorptions and no appreciable products were detected (data not shown). These data indicate that oxidation of C-20 hydroxymethyl to formyl preceeds probably the hydroxylation of C-23.

Hydroxylation of C-23 Methyl When 20-OH-MPTL or 20-oxo-MPTL as substrate and NADH as cofactor were incubated with the cell-free extract, the formation of the corresponding compounds hydroxylated at C-23, 20,23-diOH-MPTL (Rf, 0.22) or MTL (Rf, 0.25), respectively, was observed as shown in Fig. 2-(3) and 2-(4). The conversion rate of 20-oxo-MPTL to MTL was higher than that of 20-OH-MPTL to 20,23-diOH-MPTL (Table I). On the other hand, the hydroxylation of PTL or MPTL at C-23 was not recognized as described above. These data indicate that the hydroxylation of the lactone at C-23 follows the oxidation of C-20 hydroxymethyl. The hydroxylation at C-23 required NADH and aeration as the C-20 hydroxylation did (data not shown).

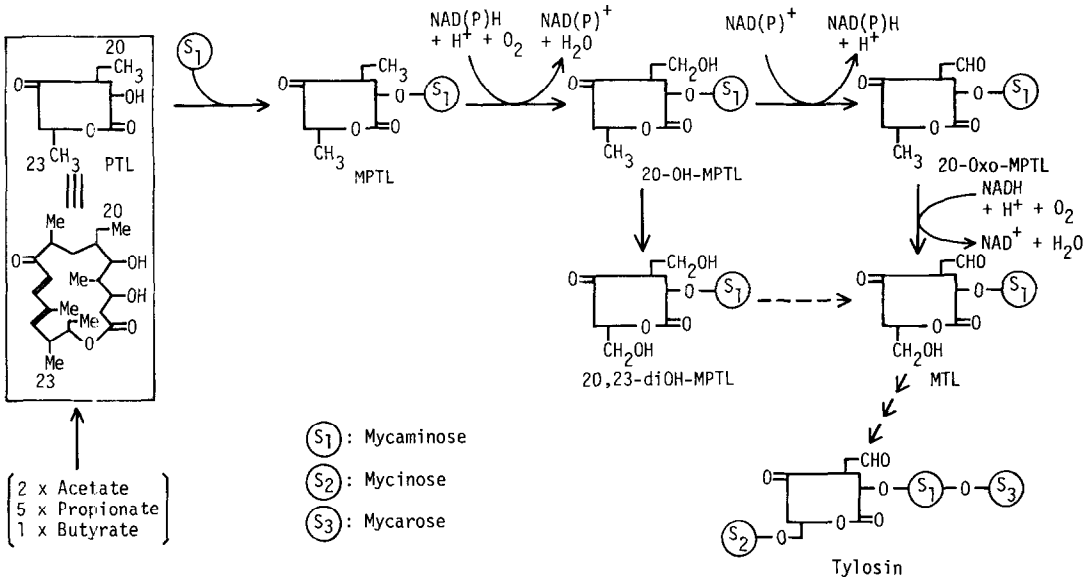

Fig. 3. Possible pathway of tylosin biosynthesis in S. fradiae.

From the above cell-free experiments using the extract from the tylosin producer, it is concluded that (1) the first oxidation of the lactone in tylosin biosynthesis is the hydroxylation of MPTL at C-20, (2) next, C-20 hydroxymethyl is oxidized to formyl, (3) and then the hydroxylation of C-23 occurs to give MTL as shown in Fig. 3. In addition, a pathway via 20,23-di-OH-MPTL may operate. However, since the formation of 23-OH-MPTL from MPTL was not recognized, 23-OH-MPTL does not seem to be a real intermediate. To ensure the results, the prufication and characterization of enzymes involved in the above reactions are in progress. The present communication is the first one concerning enzymatic oxidation of the lactone ring of a 16-membered macrolide although the hydroxylation of the 14-membered macrolide erythromycin at C-6 was recently reported (11). The oxidation of the lactones of the other 16-membered macrolides, leucomycins, spiramycins and so on, are of interest.

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