# BIOSYNTHESIS OF THE MACROLIDE ANTIBIOTIC TYLOSIN

## A PREFERRED PATHWAY FROM TYLACTONE TO TYLOSIN

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The efficiencies of bioconversion of twenty-three potential intermediates in the biosynthesis of tylosin were determined with a mutant strain blocked only in tylactone biosynthesis. The results indicated that tylactone, the first intermediate excreted by *Streptomyces fradiae*, is converted to tylosin by a preferred sequence of reactions which include: (1) addition of mycaminose to the C-5 hydroxyl position of the lactone; (2) hydroxylation of the C-20 methyl group to a hydroxymethyl group; (3) dehydrogenation of the C-20 hydroxymethyl group to a formyl group; (4) hydroxylation of the C-23 methyl group to a hydroxymethyl group; (5) addition of 6-deoxy-D-allose to the C-23 hydroxymethyl group; (6) addition of mycarose to the 4′-hydroxyl group of mycaminose; (7) addition of a methyl group to the 2‴-hydroxyl position of demethylmacrocin, and (8) addition of a methyl group to the 3‴-hydroxyl position of macrocin to produce tylosin. The intermediates which lacked both neutral sugars (mycarose and 6-deoxy-D-allose) were biologically unstable, and substantial quantities of these compounds were degraded during standard bioconversion experiments. However, the amount of one such intermediate (*O*-mycaminosyltylonolide) degraded was substantially reduced when low concentrations of the compound were used for bioconversion, and under these conditions, much higher efficiencies of bioconversion to tylosin were obtained. We have shown that a mutant blocked in hydroxylation of the C-20 methyl group is also blocked in the further dehydrogenation of the C-20 hydroxymethyl group to a formyl group, and have confirmed in *in vitro* studies that the 2‴-*O*-methylation of demethylmacrocin must proceed the 3‴-*O*-methylation of macrocin to produce tylosin.

Tylosin (Fig. 1) is a 16-membered macrolide antibiotic produced by strains of *Streptomyces fradiae*[1,2,3], *Streptomyces rimosus*[4], and *Streptomyces hygroscopicus*[5]. Tylosin is composed of a branched lactone (tylonolide) and three sugars, mycarose, mycaminose, and mycinose[6~9]. The first intermediate excreted by *S. fradiae*, tylactone[1] or protylonolide[10], contains methyl groups at the C-20 and C-23 positions and lacks all three sugars. The structure of this key intermediate is consistent with predictions from $^{13}C$ precursor labeling patterns previously reported[11,12]. Cosynthesis studies with mutants blocked in different steps of tylosin biosynthesis[1] indicated that addition of mycaminose (or possibly a precursor of mycaminose) to tylactone was the essential first step in the conversion of tylactone to tylosin. Cosynthesis studies also suggested that the tylosin sugars are derived from a common intermediate[1], presumably thymidine diphospho-4-keto-6-deoxy-D-glucose[4] and that the oxidations of the C-20 position normally preceed the attachment of mycarose to the 4′-hydroxyl position of mycaminose. Further, the *O*-methylations of 6-deoxy-D-allose to produce mycinose occur in a precise order after 6-deoxy-D-allose is attached to the lactone at the C-23 position[1,13]. However, these studies were not sufficient to fully characterize the biosynthetic pathway to tylosin and additional studies were required to determine if the oxidations, neutral sugar additions and *O*-methylations normally occur in a precise order, or if

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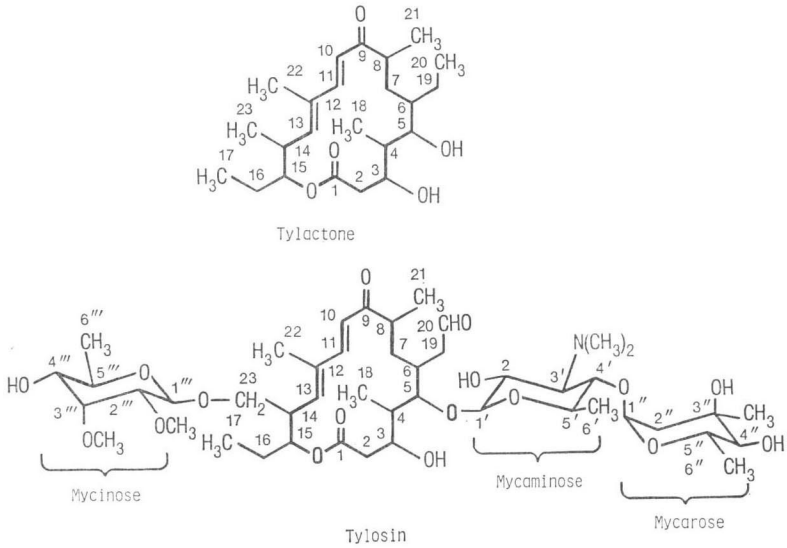
Fig. 1. The structures of tylactone and tylosin.




*O*-mycaminosyltylactone might be converted to tylosin through a matrix of reactions of only partially defined or undefined order. The information available from studies on tylosin blocked mutants does not rule out the latter possibilities since certain reactions can be readily bypassed[1].

The experimental approach described in this communication to further define the biosynthetic pathway to tylosin was based upon the following argument. Since it has been well documented that macrolide compounds varying widely in physical and biological properties are readily taken up by macrolide-producing *Streptomyces*[1,8,14], one might expect that normal precursors in the tylosin pathway should be taken up by *S. fradiae* and converted to tylosin. For instance, it has been shown that tylactone, a hydrophobic intermediate, and macrocin, a more hydrophilic macrolide antibiotic, are readily taken up and bioconverted to tylosin by certain *tylG* mutants of *S. fradiae*[1]. On the other hand, if a compound is not a normal precursor, it might be taken up but not be bioconverted, be taken up and converted to a shunt metabolite other than tylosin, or not be taken up. The *tylG22* mutant (*S. fradiae* GS22)[1] is particularly useful for this type of bioconversion analysis since: (1) it is blocked in formation of tylactone and therefore produces no macrolide compounds, (2) it cosynthesizes tylosin with all other classes of tylosin blocked mutants, thus indicating that it possesses many tylosin specific enzymes, (3) it produces normal levels of macrocin *O*-methyltransferase, the enzyme which converts macrocin to tylosin, and (4) it bioconverts tylactone to tylosin very efficiently, thus indicating that it possesses tylosin specific enzymes required for the biosynthesis or addition of the tylosin sugars, for the oxidations of the C-20 and C-23 methyl groups, and for the *O*-methylations of the 6-deoxy-D-allose moiety of a precursor(s) to tylosin. Since *S. fradiae* GS22 (*tylG22*) produces no macrolide compounds, the fate of non-labeled compounds added to this strain under antibiotic fermentation conditions can be readily monitored by simple chromatographic procedures.

In this communication, we present the results of a detailed analysis of bioconversion efficiencies of many potential intermediates to tylosin. We also present additional information on the oxidation reactions at the C-20 position and on the sequence of *O*-methylations. The data indicate that tylactone is converted to tylosin by a preferred sequence of reactions, but that certain reactions might proceed out

of order to produce tylosin less efficiently. Preliminary reports of part of this work have been presented elsewhere[14,15,16].

## Materials and Methods

### Bacterial Strains

*S. fradiae* C4 is a mutant strain which produces high levels of the macrocin *O*-methyltransferase enzyme and tylosin[13,17]. *S. fradiae* GS22 (*tylG22*) is a mutant of C4 blocked in formation of tylactone[1]. *S. fradiae* GS15 (*tylF15*) is a mutant which lacks macrocin *O*-methyltransferase activity and produces macrocin[1]. *S. fradiae* GS77 (*tylI77*, *tylD48*) is a mutant blocked in oxidation(s) of the C-20 methyl group of tylosin and in formation or addition of 6-deoxy-D-allose[1].

### Media, Fermentation Conditions, and Bioconversion Conditions

The media and fermentation conditions were as described[1]. Unless stated otherwise, bioconversions were carried out by adding macrolide compounds (1 mM or 2 mM) to cultures of *S. fradiae* GS22 (*tylG22*) in complex fermentation media at 48 hours, incubating the cultures for an additional 72 hours as described[1], and analyzing the bioconversion products by thin-layer chromatography and ultraviolet absorption[1].

### *In Vitro O-M*ethylations

*O*-Methyltransferase reactions were carried out with *S*-adenosyl-L-[methyl-$^{14}$C]methionine (SAM) as a methyl donor as described[13] except that undiluted cell extracts were used. Enzyme extracts were prepared from *S. fradiae* C4 and *S. fradiae* GS15 after 89 hours of fermentation, a time at which macrocin *O*-methyltransferase apparent specific activities are normally high[13,17]. Demethylmacrocin was added as substrate at the same concentration (14 $\mu$M) as that normally used with macrocin as substrate[13]. The products of the *O*-methyltransferase assay were extracted and separated by thin-layer chromatography, and radioactivity was counted as described[13].

### Preparation of Macrolide Compounds

Some of the macrolide compounds used in the bioconversion experiments were purified from fermentation broths of tylosin blocked mutants[1] or from tylosin-producing strains. Others were prepared by mild acid hydrolysis of various compounds to remove mycarose or by reduction of the C-20 formyl group to a hydroxymethyl group by treatment with sodium borohydride. The procedures for preparation and purification of these compounds will be presented elsewhere.

## Results

### Bioconversion of Potential Tylosin Intermediates by *S. fradiae* GS22

Table 1 summarizes the results of bioconversion experiments with twenty-three potential intermediates in the biosynthesis of tylosin. Tylactone (**1**), the product of *tylA* and *tylB* mutants[1], was bioconverted to tylosin very efficiently (84%), as demonstrated previously[1]. *O*-Mycaminosyltylactone (**2**) was also converted to tylosin efficiently (56%), thus supporting the interpretation that addition of mycaminose to tylactone is the first step in the conversion of tylactone to tylosin[1]. 23-Deoxy-*O*-mycaminosyltylonolide (**6**) and *O*-mycaminosyltylonolide (**7**) were also converted to tylosin efficiently (>70%) when calculations were made only on the distributions of tylosin-like products. However, about 50% of these and other potential intermediates which contained mycaminose but lacked neutral sugars (*i.e.*, **3**~**5**) was degraded to biologically inactive polar compound(s) which remained at the origin in the standard TLC system. If we disregard the degradation products of these compounds (see below), it appears that 23-deoxy-*O*-mycaminosyltylonolide (**6**) and *O*-mycaminosyltylonolide (**7**) may be normal precursors. The other potential tylosin intermediates containing mycaminose, but lacking neutral sugar(s) (**3**, **4**, and **5**) were bioconverted to tylosin less efficiently (about 40%), and were also converted to demycinosyl

Table 1. Structures and bioconversion patterns of tylosin-like compounds.

| | Compound | Oxidation level | | Sugars present | | | *O*-Methyl groups present | | Bioconversion to tylosin (%)[b] | Other major products (%)[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-20 | C-23[a] | Mycaminose | Mycarose | 6-Deoxy-D-allose | 2''' | 3''' | | |
| **1** | Tylactone | $CH_3$ | $CH_3$ | − | − | − | − | − | 83[e] | — |
| **2** | *O*-Mycaminosyltylactone | $CH_3$ | $CH_3$ | + | − | − | − | − | 56 | **12** (31), **23** (13) |
| **3** | 20-Deoxy-20-dihydro-*O*-mycaminosyltylonolide | $CH_3$ | $CH_2OH$ | + | − | − | − | − | 45[d] | **11**, **12**, **23** (55) |
| **4** | 20-Dihydro-23-deoxy-*O*-mycaminosyltylonolide | $CH_2OH$ | $CH_3$ | + | − | − | − | − | 41[d,e] | **12** (27) |
| **5** | 20-Dihydro-*O*-mycaminosyltylonolide | $CH_2OH$ | $CH_2OH$ | + | − | − | − | − | 43[d] | **11**, **12**, **23** (50) |
| **6** | 23-Deoxy-*O*-mycaminosyltylonolide | CHO | $CH_3$ | + | − | − | − | − | 74[d] | **12** (22) |
| **7** | *O*-Mycaminosyltylonolide | CHO | $CH_2OH$ | + | − | − | − | − | 73[d] | **12** (16) |
| **8** | 20-Dihydro-23-deoxy-demycinosyltylosin | $CH_2OH$ | $CH_3$ | + | + | − | − | − | 0 | **11** (40), **8** (28) |
| **9** | 23-Deoxy-demycinosyltylosin | CHO | $CH_3$ | + | + | − | − | − | 0 | **12** (45) |
| **10** | 20-Deoxy-20-dihydro-demycinosyltylosin | $CH_3$ | $CH_2OH$ | + | + | − | − | − | 0 | **10** (83) |
| **11** | 20-Dihydro-demycinosyltylosin | $CH_2OH$ | $CH_2OH$ | + | + | − | − | − | 0 | **11** (70), **23** (30) |
| **12** | Demycinosyltylosin | CHO | $CH_2OH$ | + | + | − | − | − | 13[e] | **12** (70) |
| **13** | 20-Dihydro-demethyllactenocin | $CH_2OH$ | $CH_2OR$ | + | − | + | − | − | 0 | **13** (53), **15** (29), **23** (14) |
| **14** | Demethyllactenocin | CHO | $CH_2OR$ | + | − | + | − | − | 39 | **14** (45) |
| **15** | 20-Dihydro-demethylmacrocin | $CH_2OH$ | $CH_2OR$ | + | + | + | − | − | 0 | **23** (72) |
| **16** | Demethylmacrocin | CHO | $CH_2OR$ | + | + | + | − | − | 85[e] | — |
| **17** | 20-Dihydro-lactenocin | $CH_2OH$ | $CH_2OR$ | + | − | + | + | − | (0)[f] | **23** (53), **17** (42) |
| **18** | Lactenocin | CHO | $CH_2OR$ | + | − | + | + | − | 31 | **22** (69) |
| **19** | 20-Dihydro-macrocin | $CH_2OH$ | $CH_2OR$ | + | + | + | + | − | 0 | **23** (92) |
| **20** | Macrocin | CHO | $CH_2OR$ | + | + | + | + | − | 88 | — |
| **21** | 20-Dihydro-desmycosin | $CH_2OH$ | $CH_2OR$ | + | − | + | + | + | 2[e] | **21** (62), **23** (30) |
| **22** | Desmycosin | CHO | $CH_2OR$ | + | − | + | + | + | 27[e] | **22** (55) |
| **23** | Relomycin | $CH_2OH$ | $CH_2OR$ | + | + | + | + | + | 0 | **23** (97) |
| **24** | Tylosin | CHO | $CH_2OR$ | + | + | + | + | + | − | **24** (93) |

a) R = 6-Deoxy-D-allose or the *O*-methylated derivatives, demethylmycinose or mycinose.

b) Compound **2** was added at 0.5 mM; compounds **3**, **4**, **5**, **8** and **17** were added at 1.0 mM; and the remaining compounds were added at 2.0 mM. Several of the compounds were tested at both 1 mM and 2 mM and the bioconversion efficiencies to tylosin were essentially the same when efficiencies wer edetermined on recovered tylosin-like compounds; some minor differences were seen in the distribution of non-tylosin bioconversion products.

c) The compound numbers are followed by percents in parentheses. Combination of more than one compound number before the percent indicates that roughly equivalent amounts of each were included in the total percent.

d) These percentages were based upon recovered tylosin-like compounds. About one-half of the added compounds was degraded to biologically inactive polar compounds.

e) Average of two experiments.

f) The 20-dihydro-lactenocin used was only 83% pure and contained 17% lactenocin. Some tylosin was produced in this bioconversion, but it was assumed to be derived from lactenocin.

tylosin (**12**), 20-dihydro-demycinosyltylosin (**11**) or relomycin (**23**) in substantial quantities (Table 1).

The compounds which contained methyl or hydroxymethyl groups at the C-20 position and either mycarose or 6-deoxy-D-allose (or its *O*-methylated derivatives) (**8**, **10**, **11**, **13**, **15**, **17**, **19**, **21** and **23**, see Table 1) were not further oxidized at the C-20 position by *S. fradiae* GS22. Therefore, both oxidation reactions at the C-20 position must occur before the addition of either neutral sugar. The C-23 methyl group of 23-deoxy-demycinosyltylosin (**9**) was oxidized to produce demycinosyltylosin, indicating that this reaction can take place after the addition of mycarose; however, none of the demycinosyltylosin was further bioconverted to tylosin.

When demycinosyltylosin was added directly to GS22, only 13% was converted to tylosin, while the majority remained unreacted. This suggests that 6-deoxy-D-allose is normally added before mycarose. This possibility was further supported by the finding that demethyllactenocin (**14**), which contains 6-deoxy-D-allose but lacks mycarose (Table 1), was bioconverted to tylosin more efficiently (39%) than demycinosyl tylosin. A major portion of demethyllactenocin, however, remained unreacted.

Demethylmacrocin (**16**) and macrocin (**20**), which contain both neutral sugars, were bioconverted to tylosin very efficiently (>80%), suggesting that both are intermediates to tylosin. On the other hand, lactenocin and desmycosin, which lack mycarose but which contain one or two *O*-methyl groups, respectively, were bioconverted to tylosin much less efficiently than demethylmacrocin and macrocin (Table 1). Therefore it appears that mycarose is normally added to demethyllactenocin prior to both *O*-methylations.

### Enzyme Deficiencies in *S. fradiae* GS77 (*tylI77*, *tylD48*)

It was shown previously[1] that *S. fradiae* GS77, which contained *tylD* and *tylI* mutations, produced 20-deoxy-20-dihydro-*O*-mycaminosyltylonolide (**3**) and 20-deoxy-20-dihydro-demycinosyltylosin (**10**). Since both of these compounds contained C-20 methyl groups, and since the conversion of C-20 methyl to formyl probably proceeds by a hydroxylation of the methyl group to a hydroxymethyl group followed by a dehydrogenation to produce a formyl group, it was of interest to know if both reactions were blocked by the *tylI* mutation. To test this we carried out a bioconversion with *S. fradiae* GS77 of 20-dihydro-*O*-mycaminosyltylonolide (**5**) which contained a hydroxymethyl group at C-20. Thirty-two percent of compound **5** was converted to 20-dihydro-demycinosyltylosin (**11**), none was further oxidized to any compound containing C-20 formyl, and the rest was degraded to inactive polar compounds (not shown). Therefore, both oxidations at the C-20 position were blocked in *tylI77*.

### Effects of Precursor Concentration on the Efficiency of Bioconversion of *O*-Mycaminosyltylonolide to Tylosin

Since about 50% of all compounds containing mycaminose but lacking both neutral sugars was degraded to biologically inactive polar compound(s), it seemed possible that none of these compounds was a normal intermediate. However, since the high degree of degradation might have been due to the relatively high levels of the compounds added to *S. fradiae* GS22, we carried out bioconversions of *O*-mycaminosyltylonolide at different substrate concentrations. Fig. 2 shows that bioconversion of *O*-mycaminosyltylonide to tylosin was very efficient at low substrate concentrations: it appears therefore that degradation reaction(s) may compete with the biosynthetic reactions more efficiently at high substrate concentrations.

Fig. 2. The effect of *O*-mycaminosyltylonolide concentration on the efficiency of bioconversion to tylosin.

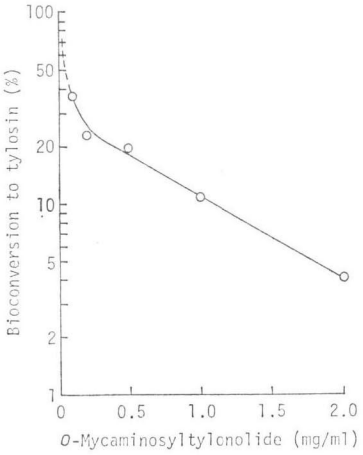
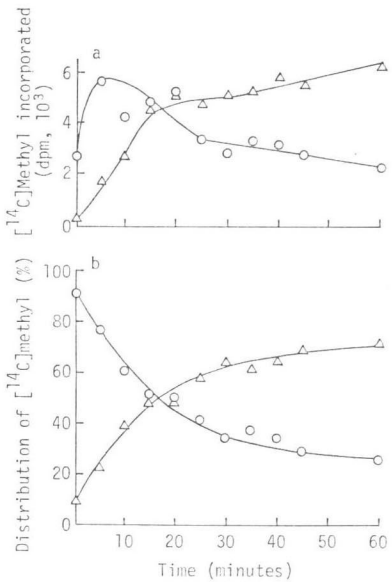



Fig. 3. *In vitro* *O*-methylation of demethylmacrocin to macrocin and macrocin to tylosin.

The macrocin *O*-methyltransferase assay was carried out with demethylmacrocin substituted for macrocin as substrate. At 10 minutes, a 20-fold excess of non-radioactive SAM was added to the assay mixture. At the times indicated 10 $\mu$l samples of the assay mixture were removed, spotted on silica gel TLC plates and immediately dried. The TLC plates were developed, and zones containing macrocin or tylosin were scraped into scintillation vials and the radioactivity was counted.

(a) Incorporation of methyl-$^{14}$C from [methyl-$^{14}$C]SAM into macrocin (○) and tylosin (△).

(b) Percent of radioactivity in macrocin (○) and tylosin (△).




### The Sequence of *O*-Methylations

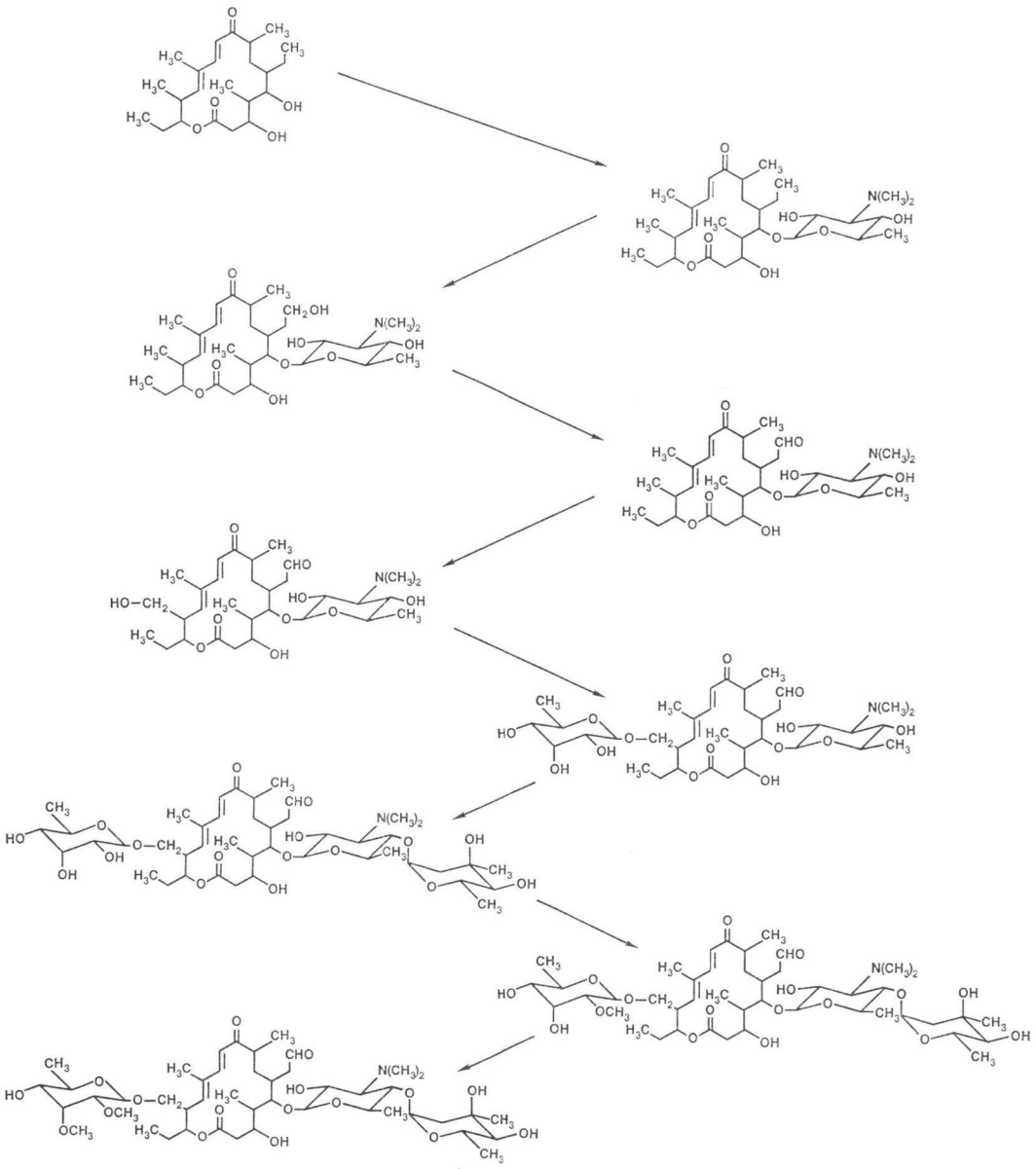
Previous studies with mutants blocked in specific *O*-methylations of demethylmacrocin or macrocin indicated that the 2‴-*O*-methylation preceded the 3‴-*O*-methylation[1,18]. We present here additional evidence to support these conclusions. When demethylmacrocin was used as a substrate in the macrocin *O*-methyltransferase assay with an extract from a *S. fradiae* strain which produces both *O*-methyltansferase activities, demethylmacrocin was rapidly converted to macrocin, and macrocin was subsequently converted to tylosin (Fig. 3). When a similar experiment was carried out with an extract of *S. fradiae* GS15, a mutant strain deficient in macrocin *O*-methyltransferase[1], demethylmacrocin was converted to macrocin. Acid hydrolysis of the product to liberate the tylosin sugars yielded one radioactive sugar which comigrated with 3-*O*-demethylmycinose (data not shown) confirming that the initial methylation measured in Fig. 3 was in fact an *O*-methylation of the 2‴ position of 6-deoxy-D-allose, and not a *C*-methylation of a precursor of mycarose or an *N*-methylation of a precursor of mycaminose (Fig. 4).

## Discussion

The rationale for the experimental approach described in this report was that if tylosin is synthesized by a preferred sequence of reactions, then only the normal intermediates should be *efficiently* converted to tylosin by a strain capable of carrying out all of the appropriate enzymatic reactions. The success of this approach depended on two factors: that intermediates and shunt metabolites be readily taken up by the bioconverting strain; and that the fate of individual compounds be readily monitored. The results of this study indicated that all compounds tested were in fact readily taken up by *S. fradiae* GS22

Fig. 4. The proposed pathway from tylactone to tylosin.

The compounds which appear in this sequence are tylactone (**1**) → *O*-mycaminosyltylactone (**2**) → 20-dihydro-23-deoxy-*O*-mycaminosyltylonolide (**4**) → 23-deoxy-*O*-mycaminosyltylonolide (**6**) → *O*-mycaminosyltylonolide (**7**) → demethyllactenocin (**14**) → demethylmacrocin (**16**) → macrocin (**20**) → tylosin (**24**). (See Table 1).



(see below). In addition, the use of GS22 permitted us to quantify all bioconversions of exogenously added compounds without having to account for any endogenously-produced macrolide components, since GS22 produces no macrolide components, but produces all of the tylosin biosynthetic enzymes required to efficiently convert tylactone to tylosin[1].

Tylactone (**1**) is the least modified lactone excreted by *S. fradiae*[1]. It is produced by *tylA* and *tylB* mutants[1,10], and was predicted to be an intermediate to tylosin from $^{13}C$ nuclear magnetic resonance studies[11,12]. Since *tylB* mutants are blocked only in formation or addition of mycaminose (or a pre-

cursor to mycaminose)[1], addition of mycaminose (or its precursor) to tylactone must be the first step in conversion of tylactone to tylosin[1]. The efficient bioconversions of both tylactone and *O*-mycaminosyltylactone to tylosin by GS22 further support this conclusion.

*O*-Mycaminosyltylactone (**2**) was not a major product of *S. fradiae* mutants blocked in the oxidations of either the C-20 or the C-23 methyl groups[1]. Thus these reactions need not proceed in a particular order since either can be bypassed. We have shown that 23-deoxy-*O*-mycaminosyltylonolide (**6**) was bioconverted very efficiently to tylosin, suggesting that it is a normal precursor. However, 20-deoxy-20-dihydro-*O*-mycaminosyltylonolide (**3**) and 20-dihydro-*O*-mycaminosyltylonolide (**5**), both of which contained the C-23 hydroxymethyl group, but which were incompletely oxidized at the C-20 position, were bioconverted to tylosin less efficiently than **6**. Therefore, it appears that the oxidation reaction(s) at the C-20 position normally occur before the oxidation of the C-23 methyl group. On the other hand, 20-dihydro-23-deoxy-*O*-mycaminosyltylonolide (**4**) was converted to tylosin less efficiently than **6** and **7**, even though it should be immediate precursor to **6**. This might be expected if both oxidation reactions at the C-20 position are normally carried out in succession by a single enzyme or by an enzyme complex containing both enzyme activities. The partially oxidized intermediate (**4**) may have a weaker binding affinity for the enzyme or enzyme complex than the fully reduced intermediate. The finding that a *tylI* mutant was incapable of carrying out either of the oxidations of the C-20 position argues in favor of at least a close coupling of these reactions. However, we have not ruled out the possibility that the *tylI* mutant contains separate mutational blocks in both activities.

Since all of the potential intermediates between *O*-mycaminosyltylactone and *O*-mycaminosyltylonolide are bioconverted to tylosin relatively efficiently, we cannot rule out the possibility that all of the possible sequences of oxidations are physiologically significant. However, it should be noted that none of the compounds which are postulated to be direct precursors to tylosin in the proposed linear pathway (Figs. 4 and 5) were converted to 20-dihydro shunt metabolites such as 20-dihydro-demycinosyltylosin (**11**) or relomycin (**23**) in substantial quantities, whereas **3** and **5** were. On the other hand, the proposed precursors **2**, **4**, **6**, and **7** were converted in fairly substantial quantities to demycinosyltylosin (**12**). These observations can be explained in the following manner. If substantial quantities of *O*-mycaminosyltylonolide (**7**) or 20-dihydro-*O*-mycaminosyltylonolide (**5**) accumulate intracellularly they may become substrates for mycarose addition. In the former case, this would lead to formation of the shunt metabolite demycinosyltylosin (**12**). In the latter case, 20-dihydro-demycinosyltylosin (**11**) would be produced since addition of mycarose would preclude further oxidation at the C-20 position. Thus accumulation of **11** would indicate that the second oxidation reaction at the C-20 position is proceeding relatively slowly. Since this was observed only with compounds not oxidized or partially oxidized at the C-20 position and oxidized at the C-23 position (**3** and **5**), it appears that the second oxidation reaction at C-20 can proceed rapidly enough to avoid being shunted to **11** only if it preceeds the C-23 oxidation. The relatively low amount of **4** (41%) converted to tylosin may be somewhat misleading, since it may have been converted very efficiently to *O*-mycaminosyltylonolide and efficiently shunted (27%) to demycinosyltylosin (**12**) in addition to being converted to tylosin. Thus our data suggest that both oxidations of the C-20 group normally precede the oxidation of the C-23 group. Recent *in vitro* bioconversion experiments also support this interpretation[18].

Both oxidation reactions at the C-20 positions must precede the addition of 6-deoxy-D-allose or mycarose, since none of the compounds tested which contained either or both of these sugars, or methylated derivatives of 6-deoxy-D-allose, plus a hydroxymethyl group at the C-20 position (compounds **8**, **11**, **13**, **15**, **17**, **19**, **21**, **23**) were further oxidized at the C-20 position in bioconversion experiments. In addition, oxidation of the C-23 methyl group normally precedes addition of mycarose to the 4′-hydroxyl group of mycaminose since 23-deoxy-demycinosyltylosin (**9**) was not appreciably converted to tylosin. Therefore, since oxidation of the C-23 position must precede addition of 6-deoxy-D-allose at the C-23 position, all three oxidation reactions at the C-20 and C-23 positions normally occur before either neutral sugar (6-deoxy-D-allose or mycarose, see below) is added to the lactone ring.

From the foregoing analysis, *O*-mycaminosyltylonolide, which contains a formyl group at the C-20 position and a hydroxymethyl group at the C-23 position, should be a central precursor to tylosin. This possibility was supported by previous bioconversion experiments in which *O*-mycaminosyltylonoli-

de was efficiently converted to tylosin by a tylosin-producing strain of *S. fradiae* which was inhibited in tylactone biosynthesis by addition of the antibiotic cerulenin[19]. In our initial experiments, *O*-mycaminosyltylonolide was converted to tylosin only very poorly by *S. fradiae* GS22 (*tylG22*), while the majority of the compound was degraded to polar material(s) which lacked biological activity. However, *O*-mycaminosyltylonolide was converted to tylosin very efficiently at low substrate concentrations. Therefore, it appears that at high substrate concentrations, the biosynthetic reactions compete unfavorably with a degradative enzymatic reaction(s) which perhaps has a higher $K_m$. The degradative reaction(s) was also observed with other potential intermediates to tylosin which contained mycaminose but lacked the neutral sugars. None of the compounds which contained one or both of the two neutral sugars was degraded appreciably to the inactive polar compound(s). Thus the addition of a neutral sugar(s) appears to increase the biological stability of tylosin-like compounds. Since a substantial amount of macrolide-like material(s) produced in normal fermentations remains at the origin in the TLC system used in this study (E. T. SENO, G. M. WILD & R. H. BALTZ, unpublished), we suspect that the degradation reaction discussed above is biologically significant, and that addition of *O*-mycaminosyltylonolide to *S. fradiae* GS22 at low concentrations more closely mimics normal *in vivo* conditions. Since tylactone is relatively insoluble, it may be taken up more slowly, and converted to tylosin more efficiently than *O*-mycaminosyltylonolide by maintaining lower concentrations of the biologically unstable intermediates.

*O*-Mycaminosyltylonolide (**7**) appears to be converted to tylosin by a specific sequence of reactions. The first is addition of 6-deoxy-D-allose to C-23 hydroxymethyl to give demethyllactenocin (**14**). Demethyllactenocin was converted to tylosin less efficiently than its immediate precursors (**6** and **7**) (*i.e.* 39 compared to >70%). However, none of the demethyllactenocin was degraded to inactive polar compound(s), and 45% remained unchanged. This suggests that lack of more efficient conversion may have been due to less efficient uptake of demethyllactenocin relative to other tylosin-like compounds, or that this conversion may be substrate inhibited.

An alternate first step in the conversion of *O*-mycaminosyltylonolide to tylosin could be addition of mycarose to the 4′-hydroxyl of mycaminose to produce demycinosyltylosin (**12**). Demycinosyltylosin, however, was converted to tylosin very poorly (13%). Poor conversion of demycinosyltylosin to tylosin was not due to inefficient uptake of the compound since 23-deoxy-demycinosyltylosin (**9**) was converted to demycinosyltylosin with 45 percent efficiency, but none of the endogenously produced demycinosyltylosin was further converted to tylosin. We therefore conclude that demethyllactenocin, and not demycinosyltylosin, is a normal intermediate in the pathway.

Demethyllactenocin might be converted to tylosin by at least three routes: **14** → **18** → **22** → **24**; **14** → **18** → **20** → **24**; or **14** → **16** → **20** → **24**. The first two routes do not appear to be normal pathways since neither lactenocin (**18**) nor desmycosin (**22**) were converted to tylosin very efficiently. Inefficient uptake of these compounds was ruled out since lactenocin was efficiently converted to desmycosin (69%), but the endogenously produced desmycosin was not further converted to tylosin.

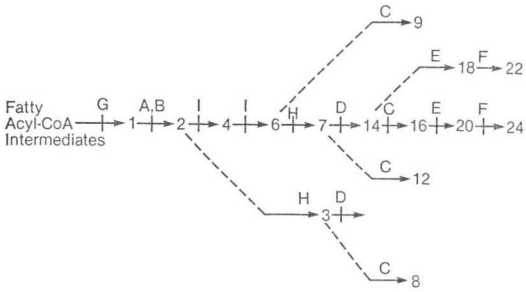
Demethylmacrocin and macrocin (**16** and **20**), however, were converted to tylosin very efficiently (>80%), and therefore appeared to be normal intermediates to tylosin. We have shown previously that a *tylE* mutant blocked specifically in the *O*-methylation of the 2‴-hydroxyl of demethylmacrocin produced demethylmacrocin[1,13]. Since this mutant cosynthesized tylosin with a *tylF* mutant blocked specifically in the 3‴-*O*-methylation, and since the *tylE* mutant produced normal levels of macrocin *O*-methyltransferase (3‴-*O*-methyltransferase), we concluded that the 2‴-*O*-methylation must precede the 3‴-*O*-methylation[1,13]. We have further demonstrated here in *in vitro* studies that demethylmacrocin was converted to macrocin by *O*-methylation of the 6-deoxy-D-allose moiety, and that macrocin was subsequently converted to tylosin.

A summary of the proposed preferred pathway from tylactone to tylosin is shown in Fig. 4. This pathway is consistent with previous information obtained from mutants blocked in tylosin biosynthesis[1], with data on the specificity and sequence of *O*-methylations[1,13] and with previous bioconversion data[19,20]. However, it does not support the suggestion that lactenocin and desmycosin may be normal precursors to tylosin[3]. The present study strongly suggests that lactenocin and desmycosin are shunt metabolites.

Also, the data presented here and elsewhere[1,3,13,14,15] do not support the biosynthetic pathway

Fig. 5. The relationship between the tylosin intermediates and shunt metabolites produced by various blocked mutants.

The letters indicate the locations of various genetic blocks [*e.g. tylA, tylB, etc.*[1]].




from *O*-mycaminosyltylonolide to tylosin proposed by ŌMURA and coworkers[20]. Their biosynthetic scheme assumes that the *O*-methylation reactions to convert 6-deoxy-D-allose to mycinose occur before mycinose is attached to the lactone. Our data clearly support the interpretation that both *O*-methylation reactions normally occur after 6-deoxy-D-allose is attached to the lactone.

Fig. 5 shows a schematic representation of the tylosin biosynthetic pathway; shows the locations of genetic blocks previously identified[1]; and shows the sequence of biosynthetic reactions which occur in various tylosin blocked mutants. Only three of the biosynthetic steps cannot be bypassed [*i. e.* addition of mycaminose and the two *O*-methylations][1]. However, certain oxidation reactions and the neutral sugar glycosyl transfer reactions can be bypassed in genetically blocked mutants. Therefore, several novel macrolide structures were identified in certain blocked mutants[1,14].

Although the pathway presented in Figs. 4 and 5 is consistant with all genetic and biochemical data currently available, we cannot rule out the possibility that the *N*-methylation reactions might occur very rapidly after a non-methylated precursor to mycaminose is attached to tylactone. Although we feel that this is unlikely, additional biochemical and genetic work may be warranted to further probe this question.

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