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Terminal Stages in the Biosynthesis of Tylosin

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Tylosin, a macrolide antibiotic, was co-produced with four structurally similar antibiotics in fermentation cultures of *Streptomyces fradiae*. Macrocin, desmycosin, lactenocin, and relomycin were found to be components of a common pathway that functions in tylosin biosynthesis. Data obtained by the addition of the purified ^{14}C-labeled antibiotics to cultures of *S. fradiae* revealed that macrocin and desmycosin were direct precursors of tylosin, whereas lactenocin was an immediate precursor of both macrocin and desmycosin. Incubation of these cultures with [^{14}C]tylosin resulted in an equivalent distribution of radioactive label between relomycin and an unidentified component. The kinetics of incorporation of label into the two species were similar, suggesting that both were derived directly from tylosin. A system that supported that methylation of macrocin to tylosin by cell-free extracts of *S. fradiae* was developed. A proposed scheme defining the terminal stages of tylosin biosynthesis is presented.

Tylosin, a macrolide antibiotic synthesized by *Streptomyces fradiae*, was first described by McGuire et al. in 1961 (10). Subsequently, macrocin, a related antibiotic, was detected in cultures of this organism (5). Relomycin, a similar compound, was reported as a fermentation product of *Streptomyces hygroscopicus* (14), and it has also been observed in cultures of *S. fradiae*. It was further demonstrated that relomycin could be obtained by either microbiological (2) or chemical (14) reduction of tylosin. Other investigations have revealed the occurrence of desmycosin and lactenocin in tylosin-producing fermentations (E. T. Seno, R. L. Pieper, and F. M. Huber, unpublished observations). These antibiotics were originally described by Hamill and co-workers (4, 5) as hydrolysis products of tylosin and macrocin, respectively. The structures of tylosin and these related compounds are presented in Fig. 1 (11). This report describes the terminal stages of tylosin biosynthesis with specific regard to the interactions occurring among tylosin-like compounds.

MATERIALS AND METHODS

Culture conditions. *S. fradiae* was propagated on agar slants containing: cerelose, 1%; phytone, 1%; Meer agar, 2.5%; biotin, 0.0001%; and sodium thiosulfate, 0.1%. Slant cultures were incubated at 28°C for 10 days and then stored at 4°C. Seed cultures were obtained by the addition of a *S. fradiae* spore suspension to 600 ml of liquid medium containing: cerelose, 1.5%; cornsteep liquor, 1.0%; yeast extract, 0.625%; and calcium carbonate, 0.38%. After incubation for 48 h, this culture was used to inoculate Erlenmeyer flasks containing a previously described medium (P. G. Caltrider and H. B. Hayes, U.S. Patent 3,433,711, 1969). A 10.0% (vol/vol) concentration of inoculum was used for this stage. Fermentations were carried out either in flasks (50 ml) with 10 or 15 ml of culture or in flasks (500 ml) with 90 ml of culture. All cultures were incubated at 28°C on a 2-in [ca. 5.08-cm] throw gyratory shaker at 250 rpm.

Quantitative methodology. The concentration of tylosin-like compounds in a filtered fermentation broth was determined as previously described (12). When these substances were present in nonaqueous solvents, their concentration was estimated spectrophotometrically by measuring their absorbance at 290 nm and comparing them to known concentrations of standard materials. The relative amount of each tylosin-like substance in filtered fermentation broth was estimated by quantitative thin-layer chromatography. Microliter samples of broth were spotted on silica gel plates (20 by 20 cm) and developed in a system containing ethyl acetate and diethylamine (95:5). When it was desirable to resolve components having no mobility in this system, a second developing solvent consisting of ethyl acetate, diethylamine, and methanol (79:5:20) was employed. The plates were then analyzed on a Schoeffel thin-layer plate scanner for material absorbing at 283 nm.

Radioactivity determinations. The radioactivity associated with individual tylosin components was estimated by scraping the appropriate zone from chromatography plates into vials. The macrolide compounds were then eluted with 1 ml of methanol, after which a 10-ml quantity of Toluscint was added to each vial. Counting was done in a Nuclear-Chicago liquid scintillation counter. The location of radioactive materials on thin-layer plates was determined by radioautography. All plates were exposed to Kodak BB-54 medical X-ray film for 2 to 4 weeks prior to film development.

Incorporation of radioactive substrates. A 25-μCi

	R_1	R_2	R_3
TYLOSIN	$-CHO$	$-CH_3$	MYCAROSYL
DESMYCOSIN	$-CHO$	$-CH_3$	$-H$
MACROCIN	$-CHO$	$-H$	MYCAROSYL
RELOMYCIN	$-CH_2OH$	$-CH_3$	MYCAROSYL
LACTENOCIN	$-CHO$	$-H$	$-H$

FIG. 1. *Chemical structures of the tylosin fermentation components.*

portion of L-[*methyl*-^{14}C]methionine in sterile aqueous solution was added to 100 ml of culture at the indicated times after inoculation. Samples were mixed with acetone and filtered through Whatman no. 1 filter paper. The filtrates were evaporated to dryness, and the residues were dissolved in a minimal volume of methanol. The total concentration of tylosin and tylosin-like materials and the distribution of radioactive label among the tylosin components were determined as described in the preceding sections.

Preparation of radioactive antibiotics. Labeled tylosin-like antibiotics were prepared by the addition of 12.5 μCi each of D-[U-^{14}C]glucose, L-[*methyl*-^{14}C]methionine, and [2-^{14}C]sodium propionate to each of four 90-ml cultures at 90 h postinoculation. After incubation for 29 h at 25°C on a gyratory shaker, the broth was harvested by the addition of 3 volumes of acetone. The whole broth-acetone mixture was filtered through Whatman no. 42 paper. The filter cake was washed twice with 70% aqueous acetone, and the wash was combined with the filtrate. The acetone was removed in vacuo, the pH of the remaining solution was adjusted to 9.3, and the tylosin was extracted into 3 volumes of chloroform. The extract was evaporated to dryness, and the residue was dissolved in 100 ml of amyl acetate. The labeled macrolide antibiotics were extracted from the amyl acetate into 0.5 M phosphate buffer at pH 4.5. The pH was adjusted to 8.5, and the macrolide components were reextracted into chloroform. The chloroform solution was applied to a silica gel (Merck 7734) column prepared in chloroform and developed with a 1:1 mixture of ethyl acetate-acetone. Tylosin was eluted from the column in the early fractions and was followed by a partially resolved mixture of macrocin and relomycin. The fractions containing the [^{14}C]tylosin were pooled and evaporated to dryness, and the residue was dissolved in methanol.

Fractions containing [^{14}C]macrocin and [^{14}C]relomycin were pooled and dried in vacuo, and the residue was dissolved in a small volume of ethyl acetate. [^{14}C]macrocin was separated from [^{14}C]relomycin and residual [^{14}C]tylosin by chromatography on a column of activated aluminum oxide (Woelm, activity grade I) prepared in ethyl acetate. The column was developed with an 80:20 mixture of ethyl acetate-ethanol. Fractions containing the [^{14}C]macrocin were pooled and evaporated to dryness, and the residue was dissolved in methanol.

[^{14}C]lactenocin was prepared by mild acid hydrolysis of [^{14}C]macrocin. The labeled macrocin was dissolved in 20 ml of distilled water, and the pH was adjusted to 2.0 with dilute sulfuric acid. Hydrolysis was complete in 19 h at 28°C, yielding a component with the chromatographic mobility of lactenocin. Treatment of the developed thin-layer plate with xanthydrol reagent indicated the presence of free mycarose, thus confirming the hydrolysis reaction. The pH of the hydrolysate was adjusted to 8.5, and the [^{14}C]lactenocin was extracted into chloroform. The chloroform was removed in vacuo, and the residue was dissolved in methanol.

[^{14}C]desmycosin was derived from [^{14}C]tylosin by the same method used in the preparation of [^{14}C]lactenocin from [^{14}C]macrocin.

Incorporation of [^{14}C]tylosin fermentation components. Biosynthetic studies employing labeled materials were carried out in shake flasks (50 ml) with culture volumes of 10 to 15 ml. [^{14}C]desmycosin was added to the cultures as an aqueous solution, whereas all other labeled substances employed in these studies were dissolved in methanol. The quantity of methanol introduced into the culture did not exceed 2.5% by volume. Sample collection and preparation were as described under "Incorporation of radioactive substrates."

Methylation of macrocin to tylosin by cell-free extracts of *S. fradiae*. Cells of *S. fradiae* were obtained from tylosin-producing cultures by centrifugation at 17,000 × *g* for 15 min in a Sorvall RC-2 centrifuge. The cells were washed twice in 0.02 M potassium phosphate buffer (pH 6.5) containing 0.85% NaCl and 0.02% Tween 80. The pellet derived from centrifugation was resuspended in an equal volume of the same buffer, and the cells were broken in a French pressure cell at 20,000 lb/in^2. The soluble fraction was obtained as the supernatant after centrifugation at 12,000 × *g* for 10 min. The cells were maintained at 4°C throughout the preparation of the extract.

The methylation reaction mixture contained in a volume of 200 μl: 100 μg of $MgCl_2 \cdot 6H_2O$; 50 μl of 3.3 mM tris(hydroxymethyl)aminomethane buffer (pH 7.0); 64 μg of macrocin in 20 μl of ethanol; 1.4 μg of *S*-[*methyl*-^{14}C]adenosylmethionine or 2.6 μg of L-[*methyl*-^{14}C]methionine and 17.8 μg of dipotassium adenosine 5′-triphosphate; 110 μl of cell extract. The mixture was incubated at 22°C, and the reaction was stopped by the addition of 1 ml of amyl acetate and 20 μl of 6 N NaOH. Solvent-extractable radioactivity and radioactivity associated with tylosin were

determined as previously described. Protein was estimated by the method of Lowry et al. (7).

Radioactive isotopes. All radioactive chemicals employed in this study were purchased from the New England Nuclear Corp.

RESULTS

Course of antibiotic synthesis. The time course of antibiotic synthesis in a liquid culture at 28°C is illustrated in Fig. 2. Tylosin was detectable at approximately 30 h post-inoculation and its concentration increased linearly until 120 h. At this point, the synthetic rate significantly decreased. Macrocin and relomycin were first detected between 70 and 80 h. Relomycin continued to increase through the remainder of the fermentation, whereas macrocin attained peak concentration at 140 h.

Incorporation of L-[*methyl*-^{14}C]methionine into tylosin-related substances. Previous reports have indicated that the methyl groups on the sugar moieties of erythromycin were derived from methionine (1, 8, 13). These findings were later extended to include methylation of mycarose, a sugar present in the tylosin molecule (3). Therefore, it seemed likely that the methyl group of methionine could be incorporated into tylosin and its related factors.

Figure 3 illustrates the pattern of incorporation of radioactive label into tylosin, macrocin, and relomycin when L-[*methyl*-^{14}C]methionine was introduced into the fermentation at 68 h. Macrocin rapidly accumulated the label and was the most highly radioactive component at 15 min. Its activity peaked at 1 h and steadily declined thereafter. Tylosin also exhibited a high initial rate of incorporation, but it continued to accumulate radioactive label until 50 h after the addition of the isotope. The appearance of label in relomycin was initially slow, but its rate of incorporation was enhanced at about the time that tylosin attained its maximal activity.

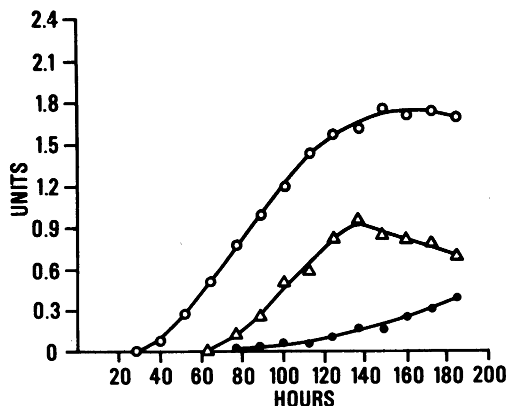

FIG. 2. *Time course of synthesis of tylosin (○), macrocin (△), and relomycin (●) in submerged cultures of S. fradiae.*

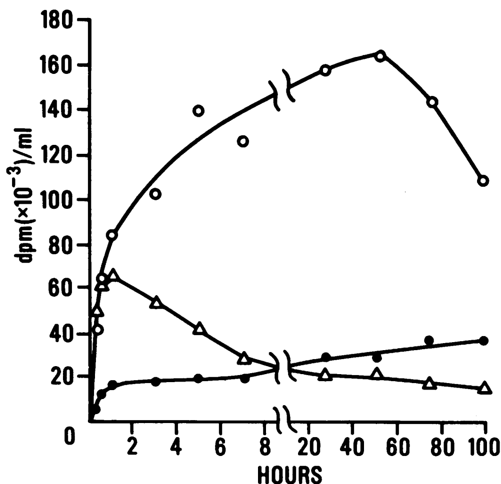

FIG. 3. *Incorporation of radioactive label into tylosin (○), macrocin (△), and relomycin (●) after addition of L-[methyl-^{14}C]methionine.*

Incorporation of [^{14}C]tylosin fermentation components. Desmycosin has been infrequently observed in tylosin fermentation. In those instances where it did occur, it appeared very late in the cycle and in relatively small quantity. Table 1 illustrates the results obtained when [^{14}C]desmycosin was introduced into a tylosin fermentation at 116 h. After incubation at 30°C for 22 h, virtually all of the radioactivity originally present in the [^{14}C]desmycosin was associated with tylosin. The label present in tylosin components other than desmycosin and tylosin remained essentially unchanged over the period of incubation.

Macrocin differs from tylosin in the absence of a methyl group at the 3'-hydroxyl position of the mycinose residue. When [^{14}C]macrocin was introduced into fermentation cultures at 42 h, its radioactivity steadily decreased, with a concomitant increase in labeled tylosin (Fig. 4). After 95 h at 28°C, essentially all of the radioactivity was recovered in tylosin and relomycin, the latter having incorporated about one-fifth of the total label. The total radioactivity in tylosin, macrocin, and relomycin remained virtually constant throughout the period of incubation.

Relomycin is an inevitable component of tylosin fermentation. Early data obtained from the examination of antibiotic concentration changes during the fermentation suggested that relomycin was produced at the expense of

TABLE 1. *Distribution of radioactive label among tylosin fermentation components after addition of [^{14}C]desmycosin*

Time after addition (h)	Radioactivity (dpm/ml)				
	Desmycosin	Tylosin	Macrocin	Relomycin	Total
0	13,721	2,016	2,334	1,292	19,363
22	2,275	14,286	2,687	1,305	20,553

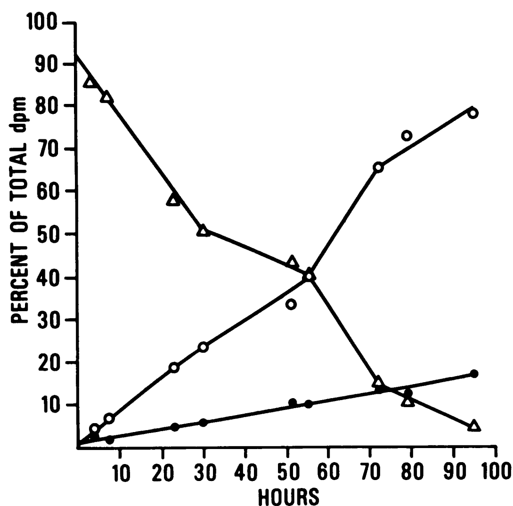

FIG. 4. *Incorporation of radioactive label into tylosin (○) and relomycin (●) after addition of [^{14}C]macrocin (△).*

tylosin. This hypothesis was confirmed by observing the fate of [^{14}C]tylosin in liquid cultures of *S. fradiae*. Labeled tylosin, upon extended incubation at 32°C, lost about 46% of its radioactivity (Fig. 5). Approximately one-half of this label was recovered in relomycin; the other half was associated with material remaining at the origin after thin-layer chromatography. The rates of incorporation of radioactive label into both species were nearly equal.

The tylosin components reported thus far were those that occur with some measurable frequency in the tylosin fermentation. Lactenocin has been detectable only in broths of fermentations carried out at low temperature. When [^{14}C]lactenocin was added at 63 h to a tylosin-producing culture, the initial response was a sharp increase in the concentrations of desmycosin and macrocin (Table 2). Within 24 h after the addition of the labeled lactenocin, the specific radioactivities of desmycosin and macrocin attained peak values, after which the activity in each component rapidly declined (Fig. 6). The label in tylosin continued to increase for an additional 24 h and thereafter remained relatively constant.

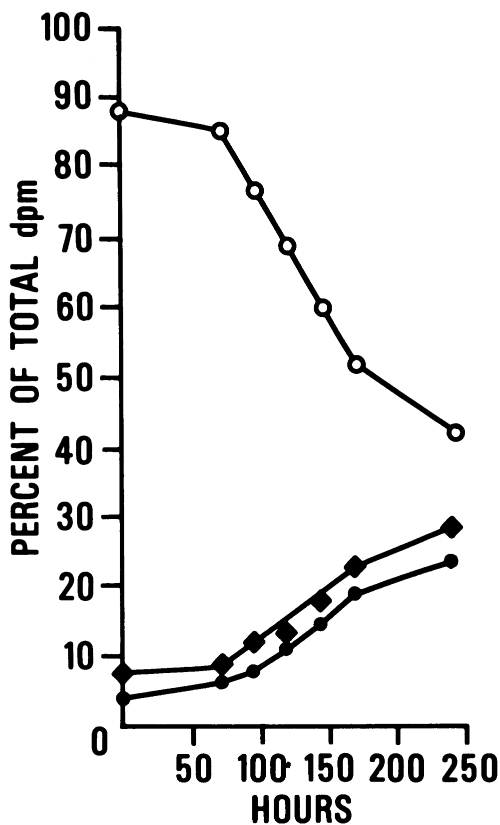

FIG. 5. *Incorporation of radioactive label into relomycin (●) and an unidentified component (◆) after addition of [^{14}C]tylosin (○).*

Methylation of macrocin in cell-free extracts of *S. fradiae*. To study the conversion of macrocin to tylosin in more detail, a cell-free system that was capable of supporting the methylation of macrocin by *S*-[*methyl*-^{14}C]adenosylmethionine or by L-[*methyl*-^{14}C]methionine and adenosine 5'-triphosphate (Table 3) was developed. The data reveal that the incorporation of the labeled methyl group into tylosin was totally dependent on the presence of macrocin. Similarly, adenosine 5'-triphosphate was required in the system employing L-methionine as the methyl donor.

TABLE 2. *Composition of tylosin fermentation broth after addition of [14C]lactenocin*

Time after addition (h)	% Total concn				
	Tylosin	Desmycosin	Relomycin	Macrocin	Lactenocin
0	36.5	0	0	0	63.5
23	55.6	20.8	0	3.8	19.9
47	53.4	12.1	0.9	12.9	20.7
72	64.1	5.0	1.4	11.9	17.6
96	65.7	6.3	2.4	12.0	13.5
169	62.6	11.0	12.2	10.1	4.2
188	64.5	9.8	18.9	4.3	2.5

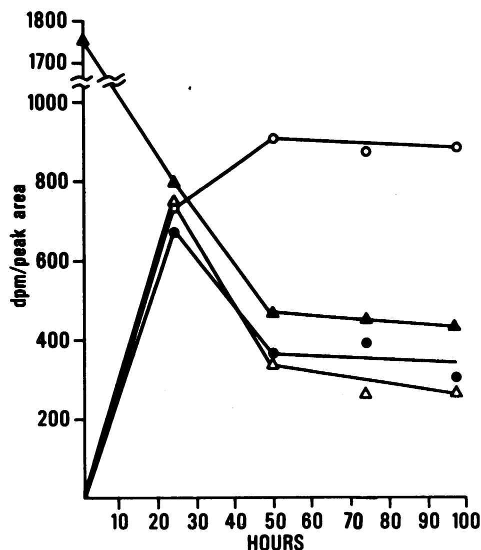

FIG. 6. *Distribution of radioactive label among tylosin fermentation components after addition of [14C]lactenocin. Values represent the ratio of disintegrations per minute to the peak area of each factor as determined from the thin-layer chromatography scan.*

DISCUSSION

This investigation was undertaken to determine whether tylosin and the tylosin-like compounds observed in fermentation cultures of *S. fradiae* were components of a common biosynthetic pathway. Early studies of the effect of temperature on tylosin synthesis had indicated that the concentrations of desmycosin, macrocin, and lactenocin in fermentation broths were enhanced at lower temperatures, whereas the accumulation of relomycin was favored at higher incubation temperatures. These observations suggested that if a common pathway were involved, the former three components were likely to be precursors of tylosin whereas the latter was likely to be an end product of the pathway. The data obtained from studies on the incorporation of label from L-[*methyl*-14C]methionine into several of these factors were consistent with this hypothesis (Fig. 3). The relatively rapid turnover of radioactive label in macrocin suggests that it is a transient intermediate in the pathway. The slower sustained accumulation of radioactivity in tylosin and relomycin indicates that they are more stable species with lower rates of turnover.

That macrocin is a direct precursor to tylosin was demonstrated by the nearly complete transfer of label from [14C]macrocin to tylosin after the introduction of this radioactive component into a fermentation culture of *S. fradiae* (Fig. 4). The relationship between macrocin and tylosin is analogous to that existing between erythromycin C and erythromycin A, the former being a demethyl form of the latter. Erythromycin C has been shown to be a precursor of erythromycin A in fermentation cultures of *Streptomyces erythreus* (9).

[14C]desmycosin was converted to tylosin upon introduction into the fermentation broth at 30°C (Table 1). This reaction, involving the addition of mycarose to the mycaminose residue of desmycosin, occurs readily under these conditions. The conservation of a label characterizing this conversion suggests that desmycosin is a direct precursor to tylosin.

The production of tylosin in cultures of *S. fradiae* is always associated with the later appearance of relomycin. The tendency of relomycin to accumulate upon extended incubation had suggested that this antibiotic was formed from tylosin, a reaction involving reduction of the tylosin aldehyde group to an alcohol function (14). Jensen et al. had reported that tylosin appeared before relomycin in cultures of *S. hygroscopicus* (6). The addition of tylosin to these cultures was observed to enhance production of relomycin. Feldman and his collaborators have demonstrated that several species of *Streptomyces* and one *Nocardia* species were capable of converting tylosin to relomycin (2). [14C]tylosin was converted to two components in fermentations of *S. fradiae*, one of which was clearly relomycin (Fig. 5). The identity of the

other component is, as yet, unknown. This substance is considerably more polar than the known tylosin-like antibiotics, as judged by its lack of mobility in the thin-layer chromatography system. The kinetics of incorporation of label from [^{14}C]tylosin into relomycin and the unidentified factor were virtually identical throughout the incubation period (Fig. 5), suggesting a direct, equimolar conversion of tylosin to the two species. It is conceivable that the reduction of the tylosin aldehyde is coupled to a second reaction, perhaps one involving oxidation of the aldehyde to a carboxyl function. The test of this hypothesis awaits the identification of the unknown compound.

Lactenocin was originally described as the product of the hydrolytic cleavage of the mycarose residue of macrocin (5). Since methylation of lactenocin at the 3'-hydroxyl position of mycinose would form desmycosin and the addition of mycarose to the 4'-hydroxyl position of its mycaminose residue would yield macrocin, it was postulated that lactenocin could serve as a common precursor to both of these intermediates. These reactions have been demonstrated in the conversions of macrocin to tylosin and desmycosin to tylosin, respectively. The hypothesis was confirmed by examining the fate of [^{14}C]lactenocin in tylosin-producing cultures. The rapid accumulation of desmycosin after lactenocin addition was especially revealing, since this component is rarely detected in fermentation broths under these conditions (Table 2). The relatively high specific radioactivities of desmycosin and macrocin after addition of the labeled intermediate strongly suggests that they are formed from lactenocin (Fig. 6). Lactenocin, therefore, occupies a pivotal position in the tylosin biosynthetic pathway, since it is methylated to form desmycosin and glycosylated to produce macrocin. The rather large incorporation of label into tylosin reflects the precursor roles that were previously demonstrated for desmycosin and macrocin (Table 1; Fig. 4).

The evidence presented in this report is consistent with the scheme detailed in Fig. 7, which defines the terminal steps in tylosin biosynthesis. Two principal types of reactions occur just prior to tylosin formation: the addition of mycarose to the 4'-hydroxyl position of the mycaminoase residue and the methylation of the 3'-hydroxyl position of the mycinose residue. The sequence of the two reactions in the desmycosin branch of the pathway is the re-

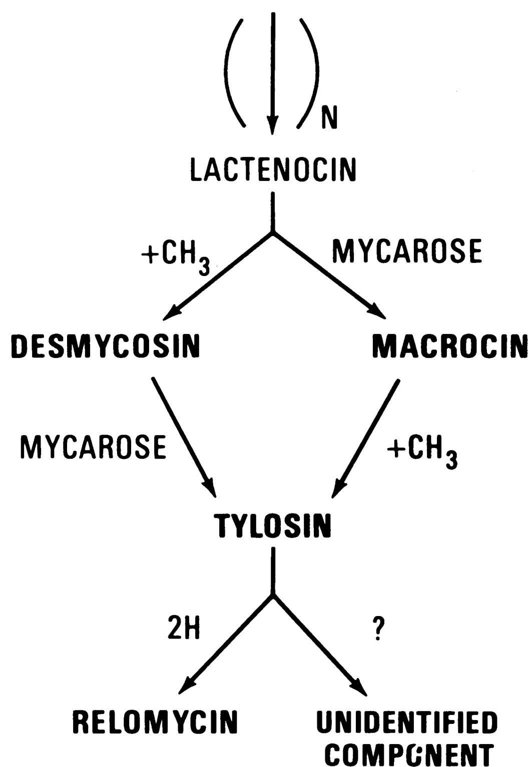

FIG. 7. *Proposed scheme for terminal steps in tylosin biosynthesis.*

TABLE 3. *Methylation of macrocin to tylosin in a cell extract of S. fradiae*[a]

System	Conditions	Dpm/ml in tylosin
S-[*methyl*-^{14}C]adenosylmethionine	Complete	49,960
S-[*methyl*-^{14}C]adenosylmethionine	Complete	50,090
S-[*methyl*-^{14}C]adenysylmethionine	Minus macrocin	350
S-[*methyl*-^{14}C]adenosylmethionine	Minus macrocin	260
L-[*methyl*-^{14}C]methionine-ATP[b]	Complete	5,430
L-[*methyl*-^{14}C]methionine-ATP[b]	Complete	6,850
L-[*methyl*-^{14}C]methionine-ATP[b]	Minus ATP	180
L-[*methyl*-^{14}C]methionine-ATP[b]	Minus ATP	110
L-[*methyl*-^{14}C]methionine-ATP[b]	Minus macrocin	240
L-[*methyl*-^{14}C]methionine-ATP[b]	Minus macrocin	220

[a] The reaction mixture was incubated at 22°C for 2.5 h.
[b] ATP, Adenosine 5'-triphosphate.

verse of that occurring via the macrocin route to tylosin.

The conversions of the radioactive tylosin pathway intermediates to their respective products were found to occur with varying rates and over extended periods of time. This was especially pronounced in the conversion of [^{14}C]tylosin to relomycin and the unidentified species. It is likely that the rate-determining step in these reactions is the uptake of the exogenous precursor. As such, the observed rates of conversion probably reflect the differential rates of uptake of the respective intermediates rather than the rates of the corresponding enzymatic reactions.

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