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Branched-Chain Fatty Acids Produced by Mutants of *Streptomyces fradiae*, Putative Precursors of the Lactone Ring of Tylosin

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Three branched-chain fatty acids (7-hydroxy-4,6-dimethylnona-2,4-dienoic acid [compound 1], its 7-epimer [compound 2], and 7-keto-4,6,-dimethylnona-2,4-dienoic acid [compound 3]) and a ketone (9-hydroxy-6,8-dimethylundeca-4,6-dien-3-one [compound 4]) were isolated from the culture broth of mutants of *Streptomyces fradiae* which were blocked in the biosynthesis of the macrolide antibiotic tylosin. Two phenotypic classes of mutants of this organism which were blocked in the addition of mycaminose to tylactone (compound 6) accumulated these compounds. These compounds were not produced by mutants which were blocked in lactone synthesis, in steps beyond mycaminose addition, or by the wild-type strain. Synthesis of these compounds, like synthesis of tylosin, was inhibited by the addition of cerulenin. Compounds 1, 2, and 3 were partially interconvertible by these mutants; but they were not produced from the degradation of tylactone and they were not directly incorporated into tylosin by intact cells. The structures of compounds 1 and 2 were equivalent to that of a predicted intermediate (S. Yue, J. S. Duncan, Y. Yamamoto, and C. R. Hutchinson, J. Am. Chem. Soc. 109:1253–1255, 1987) in the biosynthesis of tylactone. The ketone (compound 4) reported previously (N. D. Jones, M. O. Chaney, H. A. Kirst, G. M. Wild, R. H. Baltz, R. L. Hamill, and J. W. Paschal, J. Antibiot. 35:420–425, 1982) appears to be the decarboxylation product of the intermediate following that represented by compound 1. This represents the first report of the isolation of putative precursors of tylactone from tylosin-producing organisms.

Tylosin, a 16-membered ring macrolide antibiotic that is produced commercially from the fermentation of *Streptomyces fradiae*, is used as a supplement to swine feed for growth promotion, as a therapeutic agent for treatment of infections in swine caused by gram-positive bacteria and *Mycoplasma* species, and for treatment of respiratory infections in poultry. It is a member of the class of macrocyclic lactones which includes compounds containing 12-, 14-, and 16-membered rings. Well-known members of this class are methymycin, erythromycin, carbomycin, cirramycin, mycinamycin, and spiramycin. The biosynthetic steps leading from lactone to the final product have been delineated for many of these antibiotics (1, 3, 4, 15, 22) through the use of blocked mutants and bioconversion of intermediates. Information concerning assembly of the lactone ring, which has been the first isolable biosynthetic intermediate in macrolide synthesis, was obtained by analyzing incorporation of labeled acetate, propionate, and butyrate. Although these studies established the building blocks for the ring systems of tylosin (17, 18), erythromycin (11), pikromycin (20), and by analogy, other macrocyclic lactones, they provided no insight into the mechanisms of synthesis of the complex branched acyclic fatty acids. The notion that formation of the lactone ring precursor could be mechanistically analogous to fatty acid synthesis was suggested by the observation that cerulenin, an inhibitor of fatty acid biosynthesis (13), also inhibited macrolide synthesis (16, 19). Recently, it was shown that the *N*-acetylcysteamine ester of 2-methyl-3-hydroxypentanoic acid was incorporated intact into tylactone (the lactone of tylosin) (27) and erythromycin (6). Those experiments provided the first direct evidence for a mechanism of chain elongation, with stepwise modification, in the synthesis of the fatty acids which ultimately cyclize to form the lactones of macrolide antibiotics and, possibly, other macrocylic lactones. Biosynthetic precursors (branched-chain fatty acids) of the lactone have, however, not been demonstrated in producing organisms or blocked mutants until recently with the isolation of two branched-chain fatty acids and a branched-chain ketone from a mutant of *Micromonospora griseorubida*, a mycinamycin producer (12). We isolated and identified a similar group of branched-chain fatty acids from mutants of *S. fradiae* which are likely candidates as intermediates in the biosynthesis of tylactone.

MATERIALS AND METHODS

Bacterial strains. All mutant strains of *S. fradiae* were derived from tylosin-producing strains by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis and have been described previously (1, 4, 8). The position of mutational blocks in the tylosin pathway are shown in Fig. 1. *Micrococcus luteus* ATCC 9341 was obtained from the American Type Culture Collection (Rockville, Md.). All bacterial strains were stored in liquid nitrogen suspended in a solution containing glycerol (10%) and lactose (5%).

Media and growth conditions. Mutant and wild-type cultures of *S. fradiae* were grown by a two-stage scheme as described previously (1). Fatty acids were added to fermentation-stage cultures in methanol solution; the final methanol concentration was 1%. Cerulenin (20 μg/ml) was added to fermentation-stage cultures at the time of inoculation and at 24-h intervals thereafter. [^{14}C]tylactone addition (final concentration, 0.5 mg/ml) to *S. fradiae* GS62 was made 24 h after inoculation of the fermentation medium.

Isolation of compounds. Analytical high-pressure liquid chromatography (HPLC) was conducted on a μBondapak C-18 reversed-phase column (3.9 by 300 mm; Waters Associates, Inc., Milford, Mass.). The compounds were eluted by using a linear gradient from 50 to 80% methanol in 0.3% (wt/vol) ammonium formate (pH 4.0) at a flow rate of 1.0 ml/min. The effluent was monitored at 268 or 282 nm.

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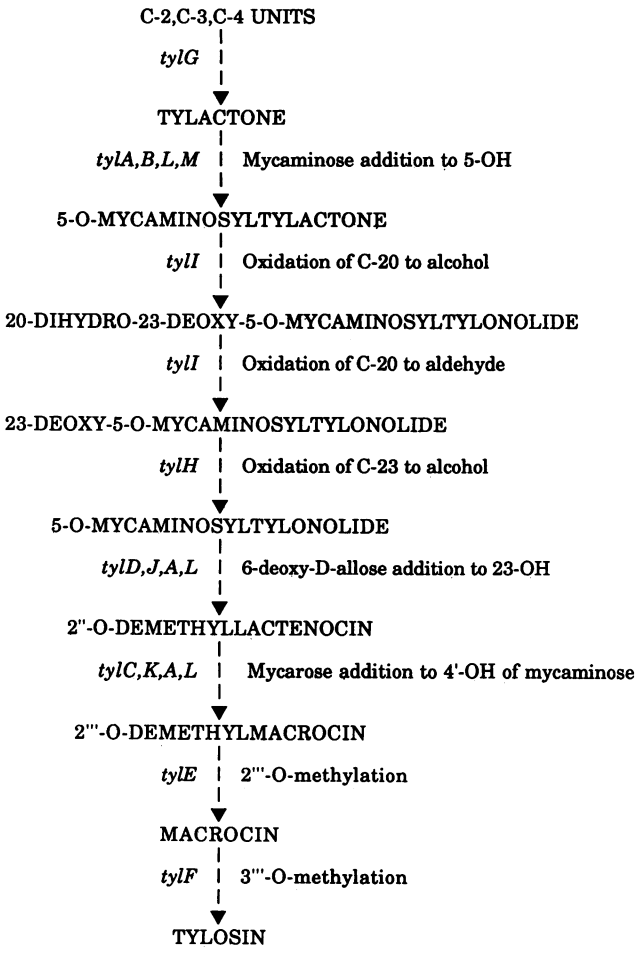

FIG. 1. Mutant blocks in the tylosin biosynthetic pathway. The mutant designations are to the left, the intermediates are indicated in the center, and the modification at each step is indicated to the right (2).

Tylosin and precursors of tylosin were isolated from culture broths by ethyl acetate extraction at pH 8.5 (tylactone and compound 5; see Fig. 2) or pH 3.0 (fatty acids). Samples from growing cultures were prepared for HPLC analysis by the addition of 2 volumes of methanol to a portion of the culture followed by centrifugation to remove cells and particulate material. For large-scale preparation, 7-hydroxy-4,6-dimethylnona-2,4-dienoic acid (compound 1), its 7-epimer (compound 2), and 7-keto-4,6-dimethylnona-2,4-dienoic acid (compound 3) were recovered from the fermentation broth by extraction with ethyl acetate at pH 3.0. After removal of the ethyl acetate, the residue was dissolved in a minimal amount of acetonitrile-acetic acid-water (96:2:2) and adsorbed onto silica gel. The silica gel was poured into a column (2 by 50 cm), which was then eluted with acetonitrile-acetic acid-water (98:1:1). Compounds 1, 2, and 3 were eluted together in the first 2 column volumes of solvent. Final purification was accomplished by preparative HPLC by using a Rainin Dynamax C-18 column (21.4 by 300 mm) in an isocratic system by elution with acetonitrile–0.2% formic acid (1:1) at a flow rate of 34 ml/min. Antibiotic activity was assayed by standard agar diffusion methods by using *M. luteus* as the assay organism.

Identification of compounds. Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured in deuteriochloroform solution on an ^{1}H NMR spectrometer (QE-300; General Electric Co., Schenectady, N.Y.); chemical shifts were measured from $CHCl_3$, which was the internal standard (δ = 7.25). Field desorption mass spectra (FD-MS) were obtained on a mass spectrometer (MAT 731; Varian Instrument, Inc.) by using carbon dendrite emitters. Optical rotations were measured on a polarimeter (model 241; The Perkin-Elmer Corp., Norwalk, Conn.).

Thin-layer chromatography (TLC) was conducted by using commercially available plates (E. Merck AG, Darmstadt, Federal Republic of Germany) of silica gel 60 with a fluorescence indicator (F-254); visualization was effected by UV light. Preparative chromatographic separations were performed on silica gel by using either a Chromatotron (model 7924; Harrison Research, Inc., Palo Alto, Calif.) or flash chromatography techniques (23).

Chemical synthesis: reduction of ketone (compound 3) to alcohol compounds 1 and 2. A solution of ketone (compound 3) (15.8 mg, 0.08 mmol) in 2-propanol (5 ml) was treated with sodium borohydride (3 mg) at room temperature; after 1 h, the starting material was still predominant, so another portion of sodium borohydride was added. The solution was allowed to stand overnight, at which point the starting material was almost completely consumed. The reaction was quenched with 1 N sulfuric acid (10 ml) and then partitioned between ethyl acetate (50 ml) and water (25 ml); additional 1 N sulfuric acid (10 ml) was added to the aqueous layer, and product was further extracted with ethyl acetate (25 ml). The combined organic extracts were dried (sodium sulfate), filtered, and evaporated; and the residue was separated by reversed-phase semipreparative HPLC (Rainin Dynamax C-18 column, 21.4 by 300 mm; 20-mg load in 1 ml of acetonitrile-water [2:3]; eluted with acetonitrile–water–0.1% formic acid [45:35:20]; flow rate, 15 ml/min; UV detection at 268 nm). Fractions containing compound 2 and those containing compound 3 were individually acidified with 1 N sulfuric acid (10 ml), diluted with water (25 ml), and extracted with ethyl acetate (two times, 25 ml each time); the organic extracts were dried, filtered, and evaporated to give oils which solidified upon standing, yielding 5.5 mg of compound 2 (first eluted product) and 7.2 mg of compound 1 (second eluted product).

Synthesis of methyl ester of compound 1. A solution of the hydroxyacid (compound 1; 210 mg) in acetonitrile (50 ml) was treated successively with iodomethane (0.32 ml) and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.30 ml) at room temperature for 3 h. Volatile material was evaporated under reduced pressure, and the residue was dissolved in methanol (1.5 ml) and separated by flash chromatography on silica gel by elution with ethyl acetate. Fractions containing the desired product were located by TLC (developed with ethyl acetate), combined, and evaporated to dryness under reduced pressure to yield 200 mg of oily product: ^{1}H NMR ($CDCl_3$) δ 0.97 (3H, t, J = 7.5 Hz, H_9), 1.04 (3H, d, J = 9 Hz, 6-CH_3), 1.36–1.59 (2H, m, H_8), 1.82 (3H, s, 4-CH_3), 2.61–2.68 (1H, m, H_6), 3.43 (1H, m, H_7), 3.76 (3H, s, OCH_3), 5.83 (1H, d, J = 16 Hz, H_2), 5.85 (1H, d, J = 10 Hz, H_5), 7.35 (1H, d, J = 16 Hz, H_3).

Synthesis of *N*-acetylcysteaminyl derivative of compound 1. To a solution of *N*-acetylcysteamine (110 mg, 0.92 mmol) in *N*,*N*-dimethylformamide (1.2 ml) were added hydroxyacid (compound 1) (120 mg, 0.61 mmol) and diphenylphosphoryl azide (0.13 ml, 0.61 mmol) (26). After the yellow solution was stirred at 0°C for 15 min, triethylamine (0.085 ml, 0.61 mmol) was added. After stirring at 0°C for another 30 min and then at room temperature overnight, unreacted hydroxy-

acid was still present (TLC), so additional *N*-acetylcysteamine (73 mg), diphenylphosphoryl azide (0.067 ml), and triethylamine (0.04 ml) were added as described above. After stirring at 0°C for 30 min, the mixture was diluted with toluene (10 ml) and extracted with saturated sodium bicarbonate solution. The organic layer was dried (sodium sulfate), filtered, and evaporated to give a pale yellow syrup, which was dissolved in toluene and extracted four times with 5% citric acid solution. The combined aqueous solution was carefully neutralized with sodium bicarbonate and then extracted with toluene. The organic layer was dried, filtered, and evaporated to provide 48 mg of a colorless syrup: $[\alpha]_D^{25}$ −33.4° (c 5.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.96 (3H, t, J = 7.5 Hz, H_9), 1.05 (3H, d, J = 8 Hz, 6-CH_3), 1.33–1.6 (2H, m, H_8), 1.81 (3H, s, 4-CH_3), 1.96 (3H, s, *N*-acetyl), 2.66 (1H, m, H_6), 3.13 (2H, t, J = 6 Hz, CH_2S), 3.48 (3H, m, CH_2N and H_7), 5.93 (1H, broad s, NH), 5.97 (1H, d, J = 10 Hz, H_5), 6.14 (1H, d, J = 15 Hz, H_2), 7.30 (1H, d, J = 17 Hz, H_3); FD-MS, m/z 300 (MH^+).

Dimerization of hydroxyacid (compound 1). Hydroxyacid (compound 1; 50 mg, 0.25 mmol) was dissolved in tetrahydrofuran (1 ml) and treated sequentially with triethylamine (0.04 ml, 0.25 mmol) and diethyl chlorophosphate (0.04 ml, 0.25 mmol) (10, 25). After the mixture was stirred for 0.5 h, the white precipitate was removed by filtration and the filtrate was diluted with toluene (40 ml). This solution was added dropwise over a period of 4 h to a solution of 4-(dimethylamino)pyridine (92 mg, 0.75 mmol) in toluene (60 ml) at 80°C. After it was stirred and heated for 18 h, the solution was cooled to room temperature, extracted twice with saturated sodium bicarbonate solution, dried (sodium sulfate), and evaporated under reduced pressure. The residue was dissolved in ethyl acetate and purified by chromatography (Chromatotron [1-mm layer of silica gel 60 F_{254}], elution with a stepwise gradient of ethyl acetate containing increasing concentrations of methanol [200 ml, 0%; 100 ml, 5%; 100 ml, 10%; 150 ml, 20% methanol]). Fractions containing the desired product were located by TLC (developed with dichloromethane-methanol-concentrated ammonium hydroxide [90:10:2]) and evaporated to yield 18 mg (40%) of a pale yellow amorphous solid: ^1H NMR ($CDCl_3$) δ 0.98 (6H, t, J = 7.5 Hz, H_9), 1.06 (6H, d, J = 7.5 Hz, 6-CH_3), 1.55 and 1.83 (2H each, m, H_8), 1.71 (6H, s, 4-CH_3), 2.67 (2H, m, H_6), 4.73 (2H, t of d, J_t = 10 Hz, J_d = 4 Hz, H_7), 4.47 (2H, d, J = 9 Hz, H_5), 5.54 (2H, d, J = 16 Hz, H_2), 7.04 (2H, d, J = 17 Hz, H_3); FD-MS m/z 361 (MH^+) and 360 (M^+).

Materials. Cerulenin was obtained from Sigma Chemical Co. (St. Louis, Mo.). Tylactone was isolated from the culture broth of a tylosin-blocked mutant as described previously (9) and was kindly supplied by G. M. Wild (Eli Lilly & Co.). [^{14}C]tylactone was isolated from a fermentation stage culture of *S. fradiae* GS14 supplemented with [1-^{14}C]propionate.

RESULTS

During examination of the kinetics of tylactone synthesis in *tylA*, *tylB*, *tylL*, and *tylM* mutants of *S. fradiae*, compounds that cochromatographed with the penultimate intermediate in tylosin biosynthesis (macrocin) were discovered. Figure 2 shows the structures of these compounds as determined after chemical isolation and purification.

The HPLC profile of a sample from a 168-h culture broth of *S. fradiae* GS62 (*tylM*, blocked in the addition or synthesis of mycaminose) is shown in Fig. 3. The peaks at 20.97 and 23.62 min were identified as tylactone and 5-*O*-mycarosyltylactone (9), respectively, based on their chromatographic behaviors relative to reference standards. The peak at 16.12 min showed a UV maximum at 282 nm, whereas the peaks at 9.0, 9.88, and 11.45 min showed a UV maximum at 268 nm; tylosin and its precursors exhibited a characteristic UV maximum at 282 nm. The compounds (Fig. 2) corresponding to these elution times were purified from the culture broth as described in Materials and Methods. Mass spectroscopy and ^1H NMR analysis (Table 1) established the structure of the 11.45-min peak as compound 1, the 9.88 min peak as compound 2, the 9.00 min peak as compound 3, and the 16.12 min peak as compound 4 (9-hydroxy-6,8-dimethylundeca-4,6-dien-3-one). Hydroxyacids 1 and 2 were

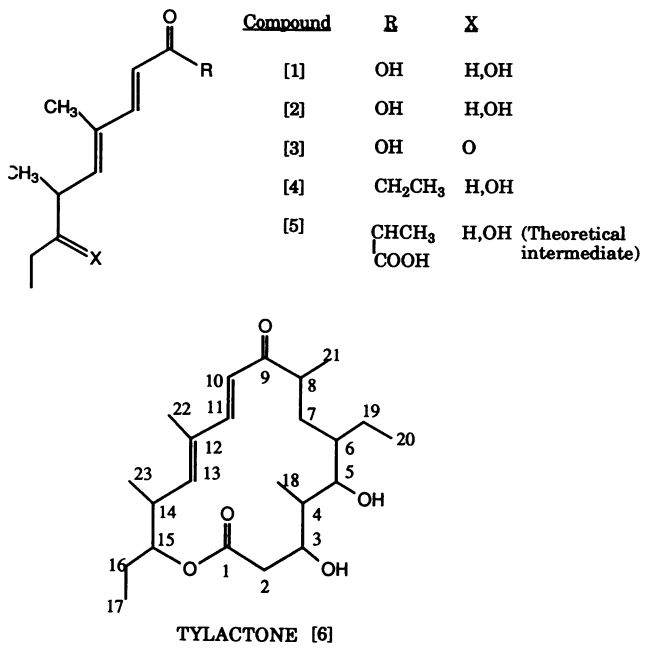

FIG. 2. Structures of putative precursors of tylactone compounds 1, 2, 3, and 4; a theoretical intermediate compound (compound 5); and tylactone (compound 6).

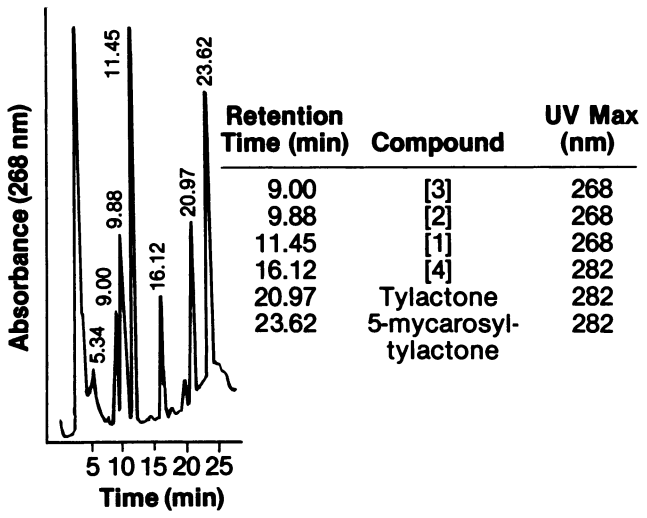

FIG. 3. Analytical HPLC chromatography of a methanol extract of a culture of *S. fradiae* GS62 (*tylM*) (see text).

TABLE 1. Physical characterization of putative tylosin precursors

Position	^{1}H NMR of compound[a]:		
	1	2	3
2	5.83	5.81	5.87
3	7.42	7.39	7.39
5	5.90	5.81	5.86
6	2.67	2.62	3.57
7	3.44	3.39	
8	1.56/1.42	1.54/1.34	2.45
9	0.95	0.95	1.03
4-CH_3	1.83	1.82	1.87
6-CH_3	1.06	1.06	1.21
FD-MS	198 (M^+)	198 (M^+)	197 (MH^+)
UV maximum	268	268	268
Optical rotation	−36.8	−52.37	+57.5

[a] Spectra were recorded in $CDCl_3$ solution. Chemical shifts were measured from $CHCl_3$ which was the internal standard (δ = 7.25).

TABLE 2. Accumulation of compounds 1 and 4 by mutants of *S. fradiae* blocked in tylosin synthesis

Strain	Concn (μg/ml)	
	Compound 1	Compound 4
GS62 (*tylM*)	68	49
GS50 (*tylB*)	6	15
GS51 (*tylB*)	17	20
GS14 (*tylA*)	15	22
GS33 (*tylL*)	28	34
GS52 (*tylC*)	ND[a]	ND
GS48 (*tylD*)	ND	ND
GS16 (*tylE*)	ND	ND
GS15, GS28 (*tylF*)	ND	ND
GS76 (*tylH tylD*)	ND	ND
GS77 (*tylI tylD*)	ND	ND
GS18 (*tylG*)[b]	ND	ND
C373.10 (wild type)	ND	ND

[a] ND, Not detected.

[b] Identical results were found with the tylactone responder (1) *tylG* mutants GS5, GS13, GS20, GS22, GS53, and GS73 and the tylactone nonresponder *tylG* mutants GS40 and GS41 (GS18 belongs to this group).

epimeric at C-7, and the exact stereochemistry at C-7 is now being determined. These structural assignments were further verified by the chemical reduction of hydroxyketone (compound 3) into the pair of hydroxyacids (compounds 1 and 2) as described in Materials and Methods.

Figure 4 shows the kinetics of accumulation of tylactone and compounds 1, 2, 3, and 4 during growth of mutant strain GS14 (*tylA*). The concentration of these putative lactone precursors peaked at the same time (72 h) that tylactone concentrations peaked in this culture. Tylactone reached a level of about 1.1 μmol/ml, while the total amount of precursors (the majority of which is represented by compounds 1 and 4) reached levels of about 0.7 μmol/ml. The kinetics of appearance of these compounds in a culture of mutant GS62 (*tylM*) was slightly different, in that compounds 1, 2, 3, and 4 accumulated prior to the accumulation of significant quantities of tylactone (data not shown). A primary difference between these two mutants was the kinetics of tylactone accumulation and not the kinetics of accumulation of compounds 1, 2, 3, and 4 (J. R. Turner, unpublished data).

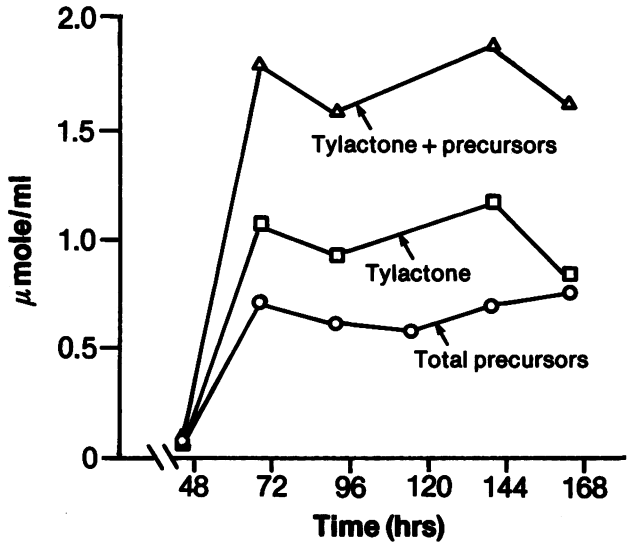

FIG. 4. Accumulation of tylactone and precursors by *S. fradiae* GS14 (*tylG*). Culture samples (1 ml) were taken at 24-h intervals and analyzed by HPLC for the various fatty acids, tylosin, and tylosin intermediates (see text). Total precursors represent the sum of the quantities of compounds 1, 2, 3, and 4 determined at each time point.

Other mutants of *S. fradiae* were examined for synthesis of intermediates 1 through 4. Compounds 2 and 3 were minor products found in culture broths of all mutants that produced compound 1. Table 2 lists the mutants that were examined and the amounts of compounds that were found in the culture broth after 168 h of growth. Accumulation of these putative intermediates was found in only two phenotypic classes of mutants (*tylB* and *tylM*, *tylA* and *tylL*), both of which accumulated tylactone. These classes differed in that the *tylB* and *tylM* mutants were blocked in the addition or synthesis of mycaminose, whereas the *tylA* and *tylL* mutants were apparently blocked in synthesis of mycaminose, mycinose, and mycarose (1, 8). Mutant strain GS62 clearly accumulated more of these products than the other mutants did and was used to isolate material for identification. Neither the wild-type nor the mutants that were blocked prior to tylactone (*tylG* type) or beyond 5-*O*-mycaminosyltylactone (*tylC*, *tylD*, *tylF*, *tylH*, and *tylI* types) synthesis accumulated these fatty acids.

Compounds 1, 2, 3, and 4 were excreted into the culture broth (Table 3), as were tylosin and its intermediates. Assay of the culture broth and combined cell washes showed that 95 to 100% of the putative precursors were found outside the cells. Supplementation of a culture of a *tylG* mutant GS5 with compounds 1, 2, or 3, in an attempt to establish tylosin synthesis in this lactone-blocked mutant, did not result in antibiotic synthesis but showed some interconversion of the three compounds (Table 4). About 12% of the total recovered material was present as compound 2 and as compound 3 after the addition of compound 1. Compounds 2 and 3 were interconvertible to the extent of 13 to 14%; however, neither was converted to compound 1. No change in any of these compounds was seen when they were added to uninoculated medium and incubated for a similiar time period. The fact that this interconversion occurred indicates that the compounds are transported into the cells but are not extensively metabolized, as has been observed previously with 2-methyl-3-hydroxypentanoic acid (6, 27). The fact that these compounds accumulated in cultures of mutants that were

TABLE 3. Distribution of tylactone precursors in a 96-h culture of GS62 (*tylM*)[a]

Culture fraction	Concn (mg/ml [% of total]) of compound(s):		
	2 + 3	1	4
Culture broth	30.8 (65.9)	126.0 (62.6)	83.6 (56.6)
Cell washes	15.9 (34.0)	72.6 (36.0)	57.0 (38.4)
Broth + cell washes	46.7 (100)	198.6 (98.6)	140.6 (95)
Cell pellet	0.0	2.8 (1.4)	7.2 (4.9)
Total	46.7	201.4	147.8

[a] A 96-h fermentation culture of strain GS62 was centrifuged, and the cells were washed with phosphate buffer (0.05 M, pH 7.0). The culture supernatant and the supernatant from each wash were diluted with methanol and assayed by analytical HPLC. The cell pellet was extracted with an equal volume of methanol and centrifuged, and the methanol supernatant was assayed. Compounds 1, 2, and 3 were measured at 268 nm; compound 4 was measured at 282 nm.

capable of tylactone synthesis supports the notion that reentry of the free acids into the biosynthetic pathway to tylosin is not favored. An attempt to establish antibiotic biosynthesis in a *tylG* mutant of *S. fradiae* by the addition of the unlabeled *N*-acetylcysteamine ester of compound 1 (prepared as described above) was also unsuccessful. This result was not unexpected since relatively poor incorporation rates of lactone precursors are common in whole-cell experiments with macrolide-producing organisms, even when this ester is used (6, 27).

These fatty acids did not accumulate as a result of degradation of tylactone, as evidenced by the lack of detectable label in compound 1, 2, 3, or 4 when [^{14}C]tylactone was added to the cultures (Fig. 5). Figure 5A shows the HPLC trace of an extract of a culture of mutant GS62 after growth in the presence of [^{14}C]tylactone. Figure 5B shows the radioactivity associated with fractions collected from the analytical HPLC eluate. [^{14}C]tylactone was transported into cells of the mutant GS62 since radioactivity appeared in the shunt product mycarosyltylactone (retention time, 24.92 min). However, except for this product and the added tylactone (retention time, 23.15 min), radioactivity was not found in any other compound.

The kinetics of appearance of these compounds by mutant GS62 suggests that they may be precursors of tylactone rather than degradation products, since compounds 1, 2, and 3 accumulated before tylactone accumulation occurred (see above). Additionally, a probable common origin of compounds 1, 2, and 3 and tylactone was supported by the findings that all four compounds were labeled to the same

TABLE 4. Interconversion of compounds 1, 2, and 3 by *S. fradiae* GS5 (*tylG*)[a]

Compound added	Compound recovered	% of total
1	1	76
	2	12
	3	12
2	1	1
	2	85
	3	14
3	1	0
	2	13
	3	87

[a] HPLC analysis (see text) of cultures was done 9 days after the addition of compound 1, 2, or 3 at an initial concentration of 0.5 mg/ml.

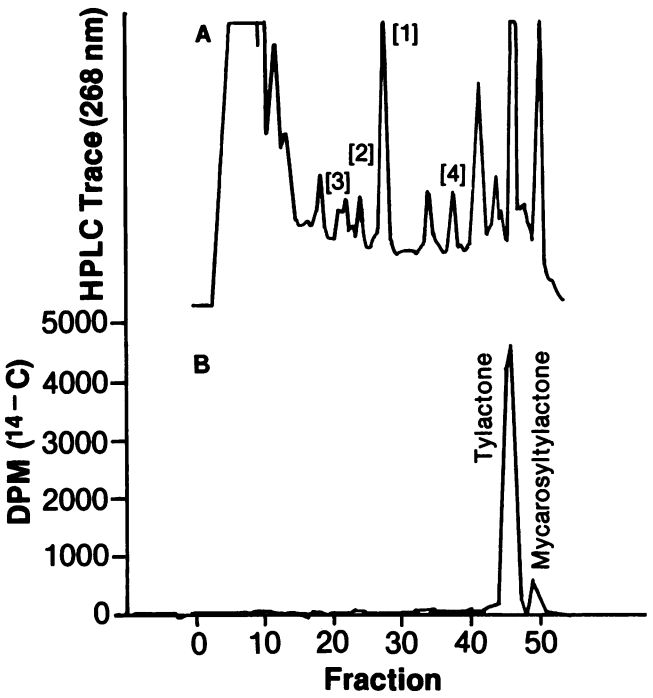

FIG. 5. [^{14}C]tylactone (specific activity, 0.039 mCi/mmol; 10^5 dpm/ml) was added at 24 h to a fermentation culture of strain GS62, and the culture was incubated for an additional 144 h. An ethyl acetate extract was made of the culture broth, which was then analyzed for tylactone and precursors by HPLC analysis and for radioactivity in fractions collected from the HPLC effluent. The analytical system was intentionally overloaded to pick up trace amounts of any degradation products that formed.

specific activity by the addition of [^{14}C]propionate to a culture of mutant GS14 (*tylB*) and that synthesis of all four compounds was inhibited by cerulenin (data not shown).

These compounds also represent a fermentation-derived source of highly functionalized chiral molecules that could be valuable starting materials for chemical synthesis. Some practical difficulties encountered in the synthesis of related hydroxyacids have been noted (6, 27). Furthermore, numerous natural products within the diolide class are known (14) and represent targets for synthesis (5, 24); dimerizations of 7-hydroxy-α,β,γ,δ-unsaturated fatty acids have been conducted as model systems for the synthesis of azalomycin B (elaiophylin) (21, 25). The potential synthetic utility of these new fermentation-derived compounds was exemplified by the dimerization of hydroxyacid 1 (see Materials and Methods) to yield a 16-membered α,β,γ,δ-unsaturated diolide which is a C-methyl analog of the ring system found in azalomycin B.

DISCUSSION

Cane and Yang (6) and Yue et al. (27) recently reported the results of experiments in which incorporation of the *N*-acetylcysteamine ester of a ^{13}C-labeled branched-chain fatty acid (2-methyl-3-hydroxypentanoic acid) into erythromycin and tylactone was demonstrated. Those results support a pathway for biosynthesis of macrocyclic lactones (27) in which oxidation states are fixed immediately after the addition of each C_2, C_3, or C_4 subunit rather than occurring as subsequent modifications after the complete carbon chain is

formed. The data reported here from *S. fradiae* and by Kinoshita et al. (12) from *M. griseorubida*, which document the accumulation of possible lactone precursors by macrolide-producing strains of *Streptomyces*, are consistent with this mechanism of synthesis.

These branched-chain fatty acids are excreted into the culture medium, as are tylosin and tylosin precursors containing intact lactone rings (1, 3), and can be transported back into the cells, but they are apparently not degraded or extensively interconverted or incorporated into tylosin. Accumulation of these fatty acids was not observed in cultures of *S. fradiae* mutants other than the two phenotypic classes that accumulated tylactone. All *S. fradiae tylA*, *tylB*, *tylL*, and *tylM* mutants that we examined accumulated the putative lactone precursors, although these mutants have genetically distinct loci (7, 8). All are thought to be involved with synthesis of mycaminose, mycarose, and mycinose or attachment of these sugars to the lactone ring and share the common trait of producing tylactone. No previous report has linked these mutations to tylactone synthesis per se. These findings suggest a common pleiotropic effect of the *tylA*, *tylB*, *tylL*, and *tylM* mutations or, possibly, a common secondary defect that causes a perturbation of lactone synthase resulting in the premature release of truncated fatty acid chains. The lesion in *M. griseorubida* may represent a specific mutation of the lactone synthase, since it reportedly (12) does not accumulate lactone as did the *S. fradiae* mutants used in this study. Examination of the *M. griseorubida* mutant by cross-feeding experiments with nonproducing strains might be warranted, since we found that although tylactone is undetectable by HPLC analysis of cultures of mutant GS62, this mutant excretes sufficient tylactone to be detected in a cross-feeding experiment with a nonproducing mutant (*S. fradiae* GS22) (8).

The fatty acids accumulated by *M. griseorubida* (12) and *S. fradiae* mutants contain the substitution and oxidation patterns expected for precursors of the lactones of mycinamycin and tylosin, respectively. The likelihood that compounds 1, 2, and 3 are precursors of tylactone (compound 6) is shown in Fig. 2 by comparison of their structures with that of tylactone. Compound 1 and its epimer, compound 2, represent the first three propionate units and the first acetate unit involved in biosynthesis (17, 18). Compound 3 could represent an incomplete reduction product preceding compounds 1 or 2 in the biosynthetic pathway (27). Compound 4 is presumably derived from compound 5 by spontaneous decarboxylation (9) and represents the next biosynthetic intermediate after compound 1 (addition of the next propionate unit) minus the terminal carboxyl group. The fact that compounds 1, 2, and 3 are interconverted to some extent by *S. fradiae* mutants and that none of these three compounds supports tylosin biosynthesis in lactone-blocked mutants complicates delineation of the role of the individual compounds as precursors of tylactone. Reduction of the ketone (compound 3) and oxidation of the alcohols (compounds 1 and 2) could conceivably be accomplished by enzymatic reactions not involved in tylosin biosynthesis.

Alternatively, accumulation of both C-7 epimers (compounds 1 and 2) and the ketone (compound 3) suggests the possibility that the stereochemistry of the substituents on C-15 (Fig. 2) of the lactone ring may not be permanently fixed in the initial steps of lactone synthesis. This could represent a departure from the mechanism for synthesis of these compounds as proposed by Yue et al. (27) and Cane and Yang (6). Definitive proof of the roles that any of these compounds play in the synthesis of tylactone or the roles that the analogous compounds play in the synthesis of mycinamycin (12) must await clear demonstration of the incorporation of labeled material into the lactone ring.

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