Sci. U.S.A. 72, 2626-2630.

Van Holde, K. E., Sahasrabuddhe, C. G., and Shaw, B. R. (1974), *Nucleic Acids Res. 1,* 1579-1586.

Weintraub, H., and Groudine, M. (1976), *Science 193,* 848-856.

Weintraub, H., Palter, K., and Van Lente, F. (1975), *Cell 6,* 85-110.

Weintraub, H., and Van Lente, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4249-4253.

Weintraub, H., Worcel, A., and Alberts, B. (1976), *Cell 9,* 409-417.

Woodcock, C. L. F. (1973), *J. Cell Biol. 59,* 368a.

Wooley, J. C., Pardon, J. F., Richards, B. M., and Worcester, D. L. (1977), in preparation.

Studies on the Biosynthesis of 16-Membered Macrolide Antibiotics Using Carbon-13 Nuclear Magnetic Resonance Spectroscopy[†]

Satoshi Ōmura,* Hideo Takeshima, Akira Nakagawa, Jun Miyazawa, Francois Piriou,[‡] and Gabor Lukacs[‡]

ABSTRACT: The origin of the skeletal carbons in the lactone ring of 16-membered macrolide antibiotics has been studied. ^{13}C-labeled antibiotics, leucomycin and tylosin, have been obtained from the culture broth of *Streptomyces kitasatoensis* 66-14-3 and *Streptomyces fradiae* C-373, respectively, in the presence of appropriate ^{13}C-labeled precursors, and ^{13}C NMR spectra of the antibiotics thus obtained have been measured. It was shown that the aglycone of leucomycin A₃ is derived from five acetates, one propionate, one butyrate, and an un-known precursor corresponding to two carbons. The formyl carbon which is characteristic of the basic 16-membered macrolides originates from C-4 of butyrate. On the other hand, the aglycone of tylosin is formed from two acetates, five propionates and one butyrate. Butyric acid and ethylmalonic acid are metabolized to propionyl-CoA or methylmalonyl-CoA through a pathway involving methylmalonyl-CoA mutase, and subsequently incorporated into the lactone ring of tylosin.

Much attention has been paid to the biosynthesis of macrocyclic lactones of 12- or 14-membered macrolides such as methymycin and erythromycin, and the origin of these skeletal carbons has been fully explored (Bentley, 1962; Birch et al., 1964; Grisebach et al., 1961; Vaněk et al., 1961; Kaneda et al., 1962; Friedman et al., 1964).

With regard to the biosynthesis of the lactone ring of 16-membered macrolides, magnamycin A has been intensely studied as a representative unit and several reports have appeared. These investigations suggested that carbon atoms 9-16, 20, and 21 (the numbers refer to those given for leucomycin A₃ which is the dihydro product of magnamycin B as shown in Figure 1A) are derived from acetate and carbon atoms 7, 8, and 19 arise directly from a propionate residue, but that precursors other than acetate, which are readily formed from glucose or succinate, are involved in the formation of the portion consisting of carbon atoms 1-6, 17, and 18 as established by using ^{14}C-labeled precursors (Grisebach et al., 1961; Grisebach and Achenbach, 1962a,b; Achenbach and Grisebach, 1964). In another study, Srinivasan and Srinivasan (1967) concluded that the lactone moiety is synthesized from one propionate residue (carbons 7, 8, and 19) and eight acetate units (all other carbons).

Among known macrolide antibiotics, the basic 16-membered macrolides are characterized by the presence of a formyl group on the lactone ring. Considerable interest has been focused on the discussion as to the origin of the formyl group.

Grisebach and Achenbach (1962a) had originally suggested that it arises from succinate, but this possibility was eliminated later (Grisebach and Weber-Schilling, 1968). Srinivasan and Srinivasan (1967) proposed that its origin is acetate.

As seen in the biosynthetic studies of macrolides and ansamycins such as rifamycin S (White et al., 1973) and geldanamycin (Rinehart, personal communication), *propionate* is directly incorporated into the lactone ring. (A substrate written in italics indicates the chemical entity which actually participates in the biological reaction.) Analogous evidence was obtained in the formation of branched-chain fatty acids by biological systems (Gastambide-Odier et al., 1963). Branching methyl groups in these antibiotics and in the branched-chain fatty acids represent C-3 of a propionate residue incorporated as an intact unit.

On the basis of these observations, it seems more reasonable to assume that the formyl group would arise from C-4 of *butyrate* formed from acetate during the biosynthetic process rather than from acetate directly as suggested by Srinivasan and Srinivasan (1967).

In this connection, we have investigated the biosynthetic origin of the lactone ring of leucomycin and tylosin as representative 16-membered macrolides by using ^{13}C-labeled compounds and ^{13}C NMR[1] spectroscopy.

The present work strongly supports the conclusions mentioned above and provides further evidence as to the metabolism of fatty acids related to the macrolide biosynthesis by *Streptomyces* species. Preliminary reports of this work have been published (Ōmura et al., 1975a,c, 1976).

[1] Abbreviations used are: NMR, nuclear magnetic resonance; CoA, coenzyme A.

Experimental Procedure

Preparation of ^{13}C-Labeled Leucomycin A_3. The culture employed for preparation of ^{13}C-labeled leucomycin was *Streptomyces kitasatoensis* 66-14-3 which had been kept on a Bact-oatmeal agar (Difco Laboratories). The slant cultures were used to inoculate flasks for the preparation of vegetative inoculum. The vegetative culture was incubated in Sakaguchi flasks for 48 h at 27 °C using a medium containing the following components (g/L): glucose, 20.0; peptone, 5.0; meat extract, 5.0; dried yeast, 3.0; NaCl, 5.0; and CaCO$_3$, 3.0. The resulting vegetative inoculum was then transferred (inoculum size, 2-3%, v/v) into a production medium (glucose, 7.0 g; peptone, 5.0 g; meat extract, 5.0 g; yeast extract, 1.0 g; NaCl, 5.0 g; and CaCO$_3$, 3.0 g, in 1000 mL of tap water) and its pH adjusted to 7.0 before sterilization at 120 °C for 15 min in an autoclave. All fermentations were conducted in 500-mL Sakaguchi flasks containing 100 mL of the medium on a reciprocal type shaker (about 250 rpm) at 27 °C. After 8-h cultivation, an appropriate ^{13}C-labeled precursor was added to the culture, and the cultivation was continued for an additional 40 h. The concentrations of these precursors added were as follows: [1-^{13}C]acetate, [2-^{13}C]acetate, [1,2-^{13}C]acetate, [1-^{13}C]glycine, diethyl [1-^{13}C]oxalate, [2-^{13}C]malonic acid, and diethyl [1,4-^{13}C]succinate were 0.2% (w/v), and [1-^{13}C]propionate, [1-^{13}C]butyrate, and [1'-^{13}C]ethylmalonic acid were 0.1% (w/v). The fermentation broth was filtered, and the filtrate was extracted three times with a half volume of chloroform at pH 8.0. The combined extracts were dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed on a silica gel thin-layer plate using benzene-acetone (2:1) as a developer to isolate pure ^{13}C-labeled leucomycin A_3. From 1 L of culture broth supplemented with each ^{13}C-labeled precursor, 10-30 mg of purified ^{13}C-labeled leucomycin A_3 was obtained.

Preparation of ^{13}C-Labeled Tylosin. The culture employed for preparation of ^{13}C-labeled tylosin was *Streptomyces fradiae* C-373 which was kindly supplied by Eli Lilly and Co. and maintained on a Bact-oatmeal agar (Difco Laboratories). Spores from these slants were used as the inoculum for a seed medium of the following composition: starch, 15 g; corn steep liquor, 10 g; yeast extract, 5 g; CaCO$_3$, 4 g; and tap water to 1000 mL. The inoculated medium was incubated at 27 °C on a reciprocating shaker for 72 h. The fermentation medium used consisted of the following components: starch, 20 g; corn steep liquor, 10 g; sugar cane molasses, 10 g; soybean meal, 15 g; soybean oil, 10 g; CaCO$_3$, 2 g; and tap water to 1000 mL; pH being adjusted to 7.5 before sterilization at 120 °C for 15 min in an autoclave. This medium was inoculated with 2% (v/v) of the seed culture and incubated at 27 °C on a reciprocal type shaker (about 250 rpm). Fermentation was conducted in 500-mL Sakaguchi flasks, containing 80 mL of the medium. After cultivating for 72 h, each of the ^{13}C-labeled precursors, [1-^{13}C]acetate, [1-^{13}C]propionate, [1-^{13}C]butyrate, [1'-^{13}C]ethylmalonic acid, [1,3,1'-^{13}C]ethylmalonic acid, and diethyl [1,4-^{13}C]succinate, was added at a concentration of 0.1% (w/v) to the culture, and the fermentation was continued for an additional 96 h. The culture was filtered by suction, the filtrate was extracted three times with a half volume of chloroform at pH 8.0, and the combined extracts were dried over anhydrous sodium sulfate and evaporated. The residue was purified by thin-layer chromatography on a silica gel plate eluting with chloroform-methanol (10:1). From 1 L of culture broth, 70-120 mg of pure ^{13}C-labeled tylosin was obtained.

Chemicals. Sodium [1-^{13}C]acetate (90 atom% C-13), so-dium [2-^{13}C]acetate (90 atom% C-13), sodium [1,2-^{13}C]-acetate (90 atom% C-13), sodium [1-^{13}C]propionate (90 atom% C-13), sodium [1,4-^{13}C]succinate (90 atom% C-13), [1-^{13}C]glycine (90 atom% C-13), diethyl [1-^{13}C]oxalate (90 atom% C-13), [1-^{13}C]ethyl iodide (90 atom% C-13), [2-^{13}C]malonic acid (90 atom% C-13), diethyl [1,3-^{13}C]malonate (90 atom% C-13), and [^{13}C]carbon dioxide [90 atom% C-13] were purchased from Merck, Sharp, and Dohme (Japan) Co., Ltd.

Preparation of [1-^{13}C]butyric acid was performed according to a modified procedure of Gilman and Kirby (1964). Bromopropane (0.25 mL, 3 mmol) was added to a stirred mixture of 1.04 g (40 mmol) of magnesium powder and 4.0 mL of anhydrous ether under nitrogen gas and gently refluxed for about 20 min at room temperature. After adding 4.0 mL of anhydrous ether to the reaction mixture, a solution of 3.45 mL (37 mmol) of bromopropane and 30 mL of anhydrous ether was added dropwise and refluxed for about 1 h. The reaction mixture was cooled to −12 °C in ice-salt bath. After 10 mL of anhydrous ether had been added, excess ^{13}CO$_2$ (about 1000 mL) was introduced into the closed reaction vessel, followed by dropwise addition of 46 mL of 18 N sulfuric acid. After stirring for 30 min, the reaction mixture was extracted three times with 50 mL of ether. The ether extract was dried over anhydrous sodium sulfate and concentrated to yield 2.33 g (60%) of viscous colorless oil. ^{13}C NMR (D$_2$O): δ 12.9 (*C*H$_3$CH$_2$CH$_2$COOH), δ 18.2 (CH$_3$*C*H$_2$CH$_2$COOH), δ 36.0 (CH$_3$CH$_2$*C*H$_2$COOH), and δ 179.7 ppm (CH$_3$-CH$_2$CH$_2$*C*OOH). The enrichment of [1-^{13}C]butyric acid thus obtained was found to be 86.3%, as determined by the intensity in the ^{13}C NMR spectrum.

Preparation of [1'-^{13}C]ethylmalonic acid was carried out according to a modified procedure of Adams and Kamm (1964). To 10 mL of absolute ethanol, 290 mg of sodium (12.5 g-atom) was added and stirred for 30 min at room temperature. To the resulting alcoholate, 2.0 g of diethyl malonate (12.5 mmol) was added dropwise with stirring at room temperature. After 20 min, 2.0 g (12.7 mmol) of [1-^{13}C]ethyl iodide was added dropwise and the reaction mixture was refluxed for an additional 90 min in an oil bath. The solvent was then evaporated and the residue was extracted three times with 30 mL of ethyl acetate. The combined extracts were concentrated under reduced pressure, and the oily residue was refluxed in an oil bath with 4.0 mL of 10 N potassium hydroxide for 6 h. The reaction mixture was adjusted to pH 2.0 and extracted four times with 30 mL of ethyl acetate. Combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield 1.5 g (60%) of [1'-^{13}C]ethyl-malonic acid. ^{13}C NMR (D$_2$O): δ 11.8 (-CH$_2$*C*H$_3$), δ 22.8 (-*C*H$_2$CH$_3$), δ 54.1 (>*C*HCH$_2$CH$_3$), and δ 174.1 ppm (-*C*OOH).

[1,3,1'-^{13}C$_3$]Ethylmalonic acid was prepared by the same procedure used for [1'-^{13}C]ethylmalonic acid. From 648 mg (4.0 mmol) of diethyl [1,3-^{13}C]malonate and 750 mg (4.0 mmol) of [1-^{13}C]ethyl iodide, 285 mg (52%) of [1,3,1'-^{13}C$_3$]ethylmalonic acid was obtained. ^{13}C NMR (D$_2$O): δ 11.8 (-CH$_2$*C*H$_3$), δ 22.8 (-*C*H$_2$CH$_3$), δ 54.1 (>*C*HCH$_2$CH$_3$), and δ 174.1 ppm (-*C*OOH).

Diethyl [1,4-^{13}C]succinate was prepared by esterification of [1,4-^{13}C]succinic acid as follows. To 5 mL of absolute ethanol containing 500 mg of [1,4-^{13}C]succinic acid (4.0 mmol), concentrated sulfuric acid was added dropwise, and the reaction mixture was slowly heated. Water and ethanol were removed by distillation. This procedure was repeated three to four times to obtain a high yield. The mixture was neutralized
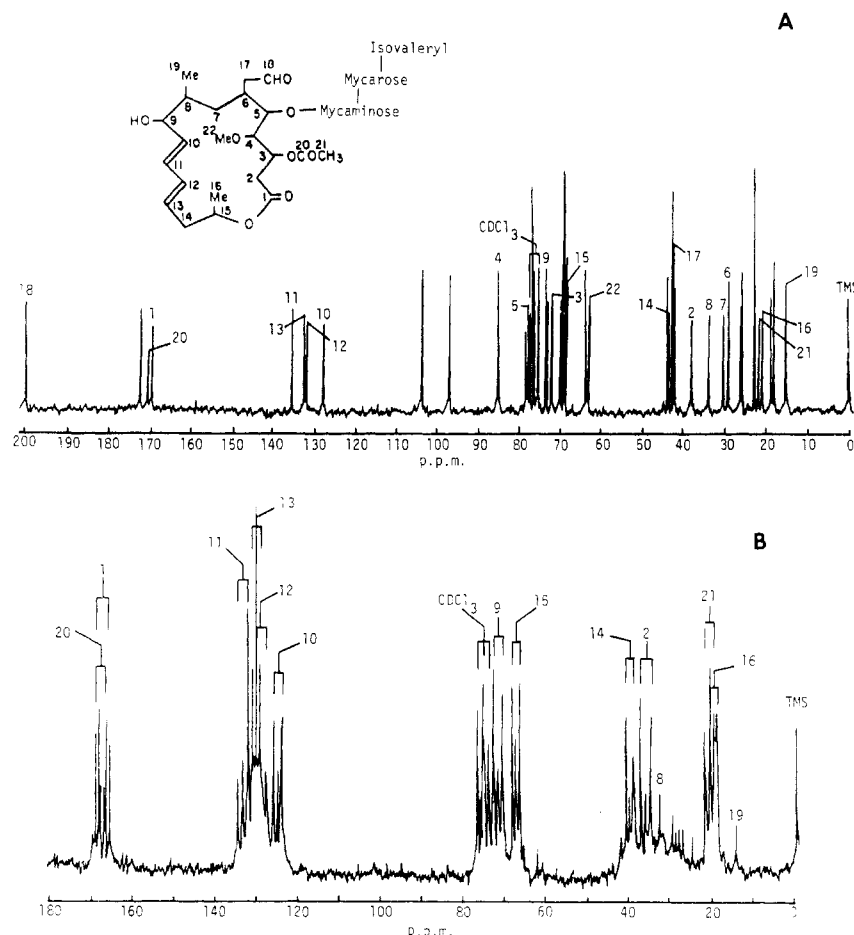
FIGURE 1: Proton noise-decoupled Fourier transformed carbon nuclear magnetic resonance spectra of leucomycin A₃. Spectra were registered at 22.63 MHz on a Bruker HX FT-NMR spectrometer equipped with Fourier transform accessory and deuterium lock. The tubes measured 8 mm and the sample concentration was ~0.4 M in CDCl₃. The number of scans was 100 000 with a repetition time of 1.1 s. Chemical shifts are reported in ppm downfield from tetramethylsilane (TMS). (A) Natural abundance; (B) biosynthetically enriched with [1,2-¹³C]acetate (90%).

with a saturated solution of sodium bicarbonate and then extracted four times with 5 mL of ether. The extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield 635 mg (86%) of a colorless liquid. ¹³C NMR (CDCl₃): δ 14.2 (-OCH₂CH₃), δ 29.2 (-CH₂CH₂-), δ 60.6 (-OCH₂CH₃), and δ 172.2 ppm (-CO-).

Results and Discussion

Biosynthesis of Leucomycin A₃. The ¹³C NMR spectra of labeled leucomycin A₃ obtained by feeding appropriate ¹³C-enriched precursors were measured. The natural abundance ¹³C NMR spectrum of leucomycin A₃ is illustrated in Figure 1A. The carbon signal assignments were based on our recent report (Ōmura et al., 1975b). Assignments of peaks due to sugar carbons were deliberately omitted for the sake of simplicity in the present discussion.

Although precise quantitative analysis of the data of the proton-decoupled ¹³C NMR spectra is precluded by a nuclear Overhauser effect (Jones et al., 1969), relative intensities of these signals can be estimated from the peak heights of ¹³C-enriched spectra. Evaluations of the degree of enrichment in the samples from the three precursors, [1-¹³C]acetate, [2-¹³C]acetate, and [1-¹³C]butyrate, are shown in Table I, in which peak heights are reported relative to the highest peak (100%). Relative peak heights of the spectra of the samples labeled with the [¹³C]acetate indicate that carbons 1, 5, 9, 11, 13, 15, 17, and 20 originate from C-1 of acetate and that carbons 2, 6, 7, 8, 10, 12, 14, 16, 18, 19, and 21 are derived from

C-2 of acetate (Table I). These observations are further corroborated by the ¹³C-enriched leucoymcin A₃ labeled with [1,2-¹³C]acetate. The proton-noise decoupled ¹³C NMR spectrum of leucomycin A₃ thus obtained is shown in Figure 1B, which reveals that carbons 1, 2, 9–16, 20, and 21 were enriched and that each of these signals was accompanied by a doublet arising from vicinal ¹³C–¹³C coupling ($J_{^{13}C_1-^{13}C_2}$ = 63.2 Hz, $J_{^{13}C_9-^{13}C_{10}}$ = 51.0 Hz, $J_{^{13}C_{11}-^{13}C_{12}}$ = 59.8 Hz, $J_{^{13}C_{13}-^{13}C_{14}}$ = 45.3 Hz, $J_{^{13}C_{15}-^{13}C_{16}}$ = 38.7 Hz and $J_{^{13}C_{20}-^{13}C_{21}}$ = 64.1 Hz). The experiment using the doubly labeled acetate provided the spectrum which shows singlets for the propionate-derived carbons 8 and 19. The weak enrichment on these sites is due to indirect incorporation, indicating that carbons 1, 2, 9–16, 20, and 21 are directly derived from the intact form of acetate. In the sample derived from [1-¹³C]propionate, only carbon 7 is specifically enriched and no enrichment on other carbons was observed. This suggests that carbons 7, 8, and 19 arise directly from a propionate unit in agreement with the results of Grisebach and Achenbach (1962b) and Srinivasan and Srinivasan (1967). Table 1 shows that carbons 5, 6, 17, and 18 are negligibly enriched by [¹³C]acetates compared to other carbons, suggesting that these four carbons are indirectly derived from acetate. We therefore assumed *butyrate* to be the direct origin of these carbons in analogy with the incorporation of *propionate* as described earlier. In order to confirm this hypothesis, two ¹³C-labeled compounds, [1-¹³C]butyric acid and [1'-¹³C]ethylmalonic acid, were prepared, and ¹³C-labeled leucomycin A₃ was obtained by feeding each compound. The

TABLE I: Incorporation of [13C]-Labeled Precursors into Leucomycin A₃.

Carbon atom	Chemical shift δ (ppm) from (CH₃)₄Si	Relative intensity (%)[a,b]		
		[1-13C]-Acetate	[2-13C]-Acetate	[1-13C]-Butyrate
C-1	169.9	11.6		2.7
C-2	37.0		81.7	
C-3	71.6			
C-4[c]	84.9			
C-5[c]	77.5	19.9		100
C-6	28.8		9.0	
C-7	30.4		<5.0	
C-8	33.5		8.2	
C-9	73.1	100		4.1
C-10	127.6		100	
C-11	135.7	61.2		6.5
C-12	132.1		74.3	
C-13	132.6	60.3		4.9
C-14	40.9		81.5	
C-15	68.8	71.1		4.6
C-16	20.3		92.1	
C-17	42.4	4.8		
C-18	201.2		8.0	
C-19	14.7		3.1	
C-20	170.8	21.6		7.2
C-21	21.3		64.9	

[a] Relative intensity (%); peak heights are reported in percent relative to the highest peak in the spectrum. [b] Under these spectral conditions natural abundance peaks were not observed. [c] Biosynthetic evidence indicates that the previously assigned signals (Ōmura et al., 1975b) due to carbons 4 and 5 should be reversed.

[13C] NMR spectra of the antibiotics thus obtained revealed that carbons 5 (Table I) and 17 are enriched by [1-13C]butyric acid and [1'-13C]ethylmalonic acid, respectively. These results can be regarded as supporting evidence for these carbons (5, 6, 17, and 18) being derived from butyrate. Secondary metabolites containing *acetate* and *propionate* units are probably synthesized *via* the same process as fatty acids; that is, acetyl-CoA and propionyl-CoA function as initiators, while malonyl-CoA and methylmalonyl-CoA participate in chain extension (Friedman et al., 1964). In the same manner, ethylmalonic acid should be incorporated in the form of ethylmalonyl-CoA as an actual participant of *butyrate* unit in chain extension. Supporting evidence for our conclusion that *butyrate* should be incorporated into carbons 5, 6, 17, and 18 has been reported by Furumai et al. (1975) that the aglycone containing a methyl group instead of the formyl group was afforded by a mutant of the 16-membered macrolide platenomycin-producing strain (Figure 2). Namely, the methyl group on the lactone ring was oxidized into the formyl group at a later stage.

In the samples obtained by feeding [1-13C]butyrate, only carbon 5 was highly enriched and negligible incorporation was observed for all of the six carbons (1, 9, 11, 13, 15, and 20) which should originate from C-1 of acetate (Table I). This result may be explained by the conversion of butyrate into acetate by β oxidation in analogy with the biosynthetic incorporation of butyrate into the antibiotic X-537A (Westley et al., 1972). The enrichment of carbon 7 will be discussed in a later section of this paper.

It is now clear that no enrichment on carbons 3 and 4 was observed by any [13C]-labeled compounds employed in the present study. In addition [1-13C]glycine, diethyl [1-13C]-oxalate, [2-13C]malonic acid, and diethyl [1,4-13C]succinate gave the same results. These results indicate that these two
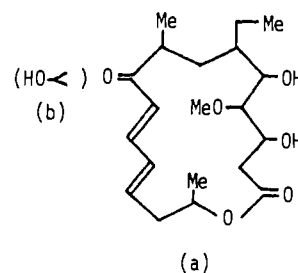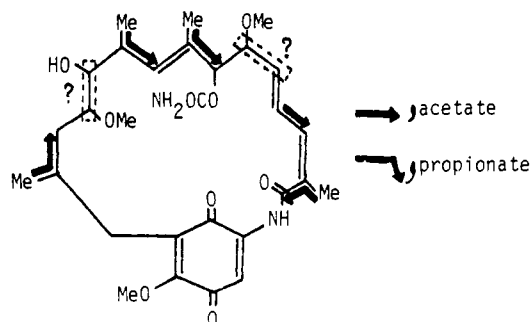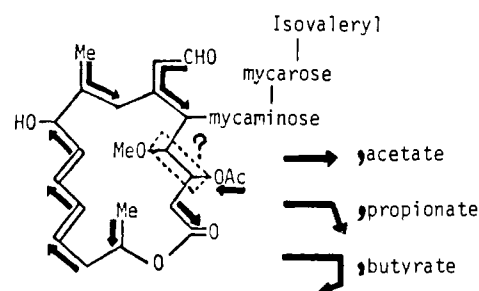

FIGURE 2: Structure of platenolide I(a) and II(b).

FIGURE 3: Biosynthetic origin of geldanamycin.

FIGURE 4: Biosynthetic origin of leucomycin A₃.

carbon atoms may be derived by a different mechanism or from a different precursor. Achenbach and Grisebach (1964) previously reported that with [3,4-14C]glucose as a precursor 88% of the total activity in the lactone ring of magnamycin A is localized in carbon atoms 1–4. Similar evidence for our conclusion can be found in the result of Rinehart (personal communication) that unknown precursors other than acetate and propionate are involved in geldanamycin biosynthesis. It should be noted that the segment consisting of the two carbons in each antibiotic has a rather similar structure, as shown in Figure 3. There still remains, however, an intriguing problem with regard to the biosynthesis of antibiotics.

From these results, it is concluded that the carbon skeleton of the aglycone of leucomycin A₃ is formed from five *acetates*, one *propionate*, one *butyrate*, and an unknown precursor corresponding to two carbons, as shown in Figure 4. This conclusion is somewhat inconsistent with the results obtained by Srinivasan and Srinivasan (1967), who concluded from their study of magnamycin A biosynthesis that carbons 3 and 4 arise from acetate. Achenbach and Grisebach (1964) suggested that in the formation of carbons 1–6, 17, and 18 other precursors besides *acetate* are involved which are readily formed from glucose or succinate, although they could not identify these precursors.

Biosynthesis of Tylosin. The basic 16-membered macrolide antibiotics, among which more than 40 compounds have been known, can be divided into two major groups according to the
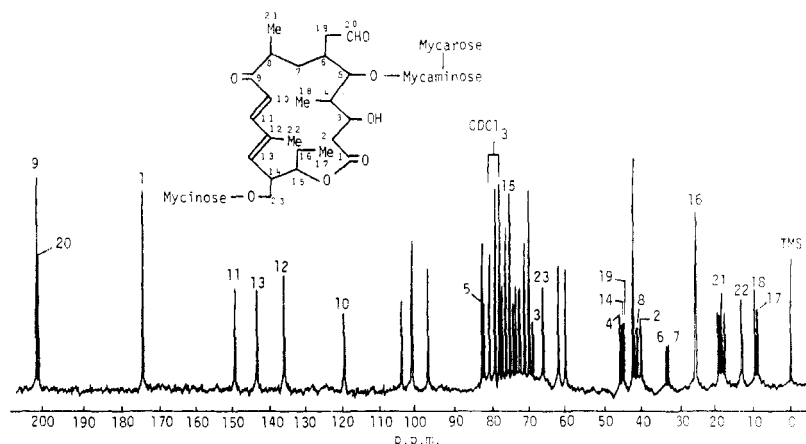
FIGURE 5: Proton noise-decoupled Fourier transformed carbon nuclear magnetic resonance spectrum of natural tylosin. Identical spectral conditions were employed as described in Figure 1.
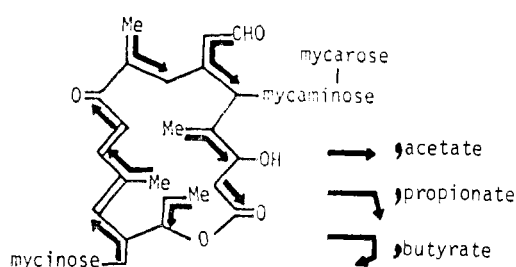

FIGURE 6: Biosynthetic origin of tylosin.
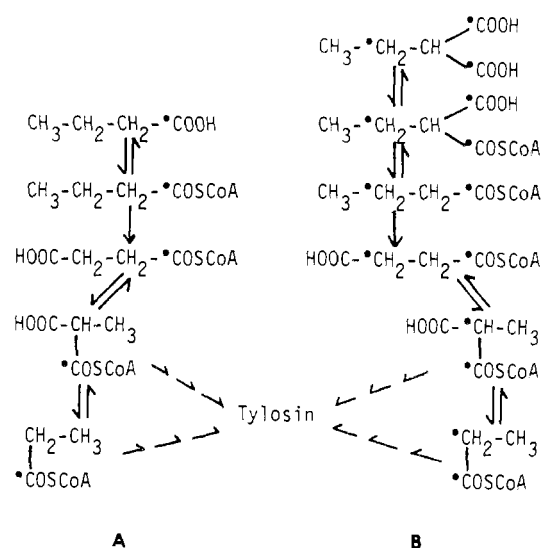

FIGURE 7: Proposed pathway of butyric acid (A) and ethylmalonic acid (B) into methylmalonyl-CoA or propionyl-CoA in a tylosin-producing organism.

carbon skeleton of the lactone ring, namely, the magnamycin–leucomycin group containing niddamycin, spiramycin, maridomycin, SF-837, platenomycin, and espinomycin, and the tylosin–cirramycin group containing rosamicin, B-58941, angolamycin, and relomycin.

On the basis of the biosynthetic studies of methymycin (Birch et al., 1964), erythromycin (Friedman et al., 1964), and leucomycin, it is suggested that the lactone ring of tylosin may be derived from two *acetate,* five *propionate* and one *butyrate* units. However, since the origin of carbons 3 and 4 of leucomycin still remains unknown, it would not be entirely reasonable to conclude that the corresponding carbons of tylosin, i.e., carbons 3, 4, and 18 (the numbers refer to those given for Figure 5), originated from *propionate.* In order to establish this point, a biosynthetic investigation of tylosin was undertaken using the same experimental techniques used for leucomycin biosynthesis.

The ^{13}C NMR spectrum of naturally occurring tylosin is shown in Figure 5. The carbon signal assignments are mostly based on our recent report (Ōmura et al., 1975b). The assignments of signals due to sugar carbons are omitted for the sake of simplicity. The data of the feeding experiments using ^{13}C-labeled precursors are collected in Table II. In the sample derived from [1-^{13}C]acetate, carbons 1 and 9 are highly enriched and incorporation was also detected in the two butyrate-derived carbons 5 and 19, as a result of indirect incorporation of *acetate* at these sites as observed in leucomycin. In the spectrum of the sample obtained from [1-^{13}C]propionate, carbons 3, 7, 11, 13, and 15 are enriched, indicating that five *propionates* are incorporated in the lactone ring. In the experiment using [1-^{13}C]butyric acid, a high degree of incorporation was observed in not only carbon 5 but also carbons 3, 7, 11, 13, and 15, which should be derived from C-1 of *propionate.* The sample from [1'-^{13}C]ethylmalonic acid reveals

enrichment in carbons 4, 8, 12, 14, and 16, which should arise from C-2 of *propionate,* as well as in carbon 19. These results indicate that the latter two compounds are metabolized into propionyl-CoA or methylmalonyl-CoA, followed by incorporation into the lactone ring. The incorporation pattern of each precursor into tylosin is summarized in Figure 6 from which one can reasonably conclude that the lactone ring of tylosin is formed from two *acetate,* five *propionate,* and one *butyrate* units.

The Metabolism of Butyric Acid and Ethylmalonic Acid into Methylmalonyl-CoA or Propionyl-CoA. A metabolic pathway of the two precursors, butyric acid and ethylmalonic acid, into tylosin can be envisaged based on the results mentioned above. As the most probable metabolic pathway which includes the conversion of the ^{13}C-enriched carbon of [1-^{13}C]butyric acid into the C-1 of *propionate* unit, one can consider a pathway involving methylmalonyl-CoA mutase (EC 5.4.99.2). In order to prove this pathway, the diethyl derivative of [1,4-^{13}C]succinate was prepared and was fed on tylosin fermentation. The ^{13}C NMR spectrum of the labeled tylosin thus obtained revealed enrichment on carbons 11 (δ 148.2), 13 (δ 142.3), and 15 (δ 75.2), which should be essentially derived from C-1 of *propionate.* Enrichment on these carbons
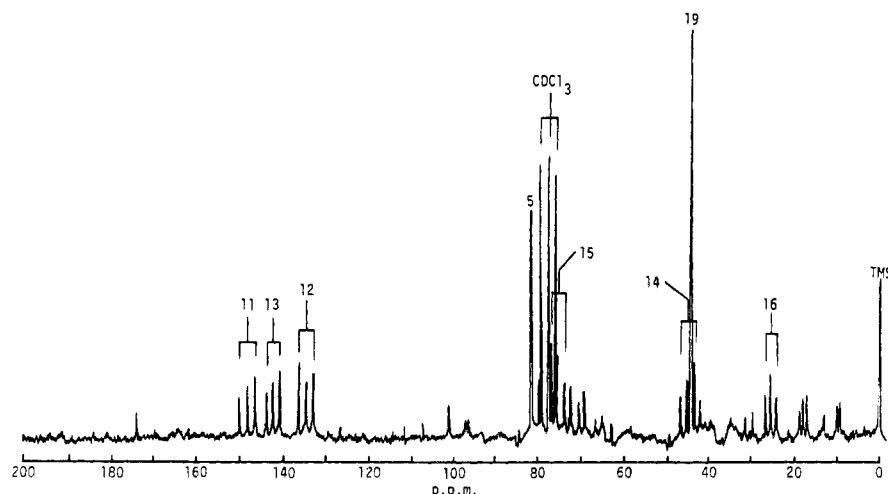
FIGURE 8: Proton noise-decoupled Fourier transformed carbon nuclear magnetic resonance spectrum of tylosin biosynthetically enriched with [1,3,1'-^{13}C]ethylmalonic acid. Identical spectral conditions were employed as described in Figure 1.

TABLE II: Incorporation of ^{13}C-Labeled Precursors into Tylosin.

Carbon atom	Chemical shift δ (ppm) from (CH$_3$)$_4$Si	Enrichment factor[a]			Relative intensity (%)[b] of [1'-^{13}C]-ethylmalonic acid
		[1-^{13}C]-Acetate	[1-^{13}C]-Propionate	[1-^{13}C]-Butyrate	
C-1	173.9	2.8	0.8	1.7	
C-2	39.4	1.5	1.8	2.0	
C-3[c]	68.2	0.1	3.8	9.8	
C-5	45.1	1.7	1.3	1.1	156
C-5	81.6	2.2	1.3	22.7	
C-6	32.2	0.1	1.9	2.5	
C-7	32.9	2.5	6.1	17.3	
C-8	40.3	1.3	1.3	0.8	133
C-9	202.8	3.9	1.6	3.2	
C-10	118.8	1.0	0.9	0.8	
C-11	148.0	2.0	6.0	17.0	
C-12	134.9	1.0	1.0	1.0	100
C-13	142.2	2.0	5.7	15.6	
C-14	44.7	1.4	1.5	0.7	233
C-15	75.3	1.2	4.0	9.7	
C-16	25.5	1.1	1.1	0.9	200
C-17	9.0	0.7	1.0	0.8	
C-18	9.6	1.1	1.0	0.6	
C-19	43.9	2.8	1.3	1.8	367
C-20	203.0	1.9	0.5	1.3	
C-21	17.4	1.1	1.3	1.3	
C-22	13.0	1.1	1.2	0.8	
C-23[c]	69.5	0.8	0.7	2.3	

[a] Peak height relative to that of natural tylosin. [b] Peak heights reported in percent relative to C-12. Under these spectral conditions, natural abundance peaks were not observed. [c] Biosynthetic evidence indicates that the previously assigned signal (Ōmura et al., 1975b) due to carbon 23 at 68.2 ppm in the spectrum of tylosin is actually carbon 3. The carbon 23 of the antibiotic appears, probably, at 69.5 ppm, although ambiguity cannot be entirely avoided.

is entirely consistent with the labeling experiment with [1-^{13}C]butyric acid. From these results, it is suggested that butyrate would be converted initially to succinate by ω-oxidation, further isomerized to methylmalonyl-CoA via succinyl-CoA, and finally incorporated into the aglycone of tylosin (Figure 7A). This scheme can also explain enrichment on carbon 7 on the aglycone of leucomycin A$_3$ by [1-^{13}C]butyric acid.

Subsequently, since the incorporation of ^{13}C-enriched carbons of [1'-^{13}C]ethylmalonic acid into C-2 of *propionate* units suggests a pathway analogous to that of the butyric acid metabolism involving methylmalonyl-CoA mutase, [1,3,1'-^{13}C]ethylmalonic acid was prepared and added to the fermentation medium. As shown in Figure 8, the ^{13}C NMR spectrum of the sample thus obtained reveals enrichment on carbons 5 and 19 as expected. In addition to these carbons, enriched signals due to carbons 11-16 were observed, and each of these signals was accompanied by a doublet arising from vicinal ^{13}C-^{13}C coupling ($J_{^{13}C_{11}-^{13}C_{12}}$ = 49.0 Hz, $J_{^{13}C_{13}-^{13}C_{14}}$ = 41.0 Hz, and $J_{^{13}C_{15}-^{13}C_{16}}$ = 36.5 Hz). These observations suggest that [1,3,1'-^{13}C]ethylmalonic acid is also metabolized via a pathway involving methylmalonyl-CoA mutase, as illustrated in Figure 7B. It is noteworthy that the enrichment of carbons 3, 4, 7, and 8 in the tylosin molecule was not ob-

served in these experiments. It seems that there is a difference in the mode of biosynthesis between these four carbons and the other carbons (carbons 11–16).

The investigation dealing with the metabolism of low-molecular-weight fatty acids using ^{13}C NMR is not yet numerous.

References

Achenbach, H., and Grisebach, H. (1964), Z. Naturforsch. B 19, 561.

Adams, R., and Kamm, R. M. (1964), Organic Syntheses, Collect. Vol. I, 2nd ed, Blatt, A. H., Ed., New York, N.Y., Wiley, p 250.

Bentley, R. (1962), Annu. Rev. Biochem. 31, 606.

Birch, A. J., Djerassi, C., Dutcher, J. D., Majer, J., Perlman, D., Pride, E., Rickards, R. W., and Thompson, P. J. (1964), J. Chem. Soc., 5274.

Friedman, S. M., Kaneda, T., and Corcoran, J. W. (1964), J. Biol. Chem. 239, 2386.

Furumai, T., Takeda, K., and Suzuki, M. (1975), J. Antibiot. 28, 789.

Gastambide-Odier, M., Delaumeny, J. M., and Lederer, E. (1963), Biochim. Biophys. Acta 70, 670.

Gilman, H., and Kirby, R. H. (1964), Organic Syntheses, Collect. Vol. I, 2nd ed, Blatt, A. H., Ed., New York, N.Y., Wiley, p. 361.

Grisebach H., and Achenbach, H. (1962a), Z. Naturforsch. B 17, 6.

Grisebach, H., and Achenbach, H. (1962b), Tetrahedron Lett., 565.

Grisebach, H., Achenbach, H., and Hofheinz, W. (1961), Tetrahedron Lett., 234.

Grisebach, H., and Weber-Schilling, C. A. (1968), Z. Naturforsch. B 23, 655.

Jones, A. J., Grant, D. M., and Kuhlman, K. F. (1969), J. Am. Chem. Soc. 91, 5013.

Kaneda, T., Butte, J. C., Taubman, S. B., and Corcoran, J. W. (1962), J. Biol. Chem. 237, 322.

Ōmura, S., Nakagawa, A., Neszmelyi, A., Gero, S. D., Sepulchre, A. M., Piriou, F., and Lukacs, G. (1975b), J. Am. Chem. Soc. 97, 4001.

Ōmura, S., Nakagawa, A., Takeshima, H., Atsumi, K., Miyazawa, J., and Lukacs, G. (1975a), J. Am. Chem. Soc. 97, 6600.

Ōmura, S., Nakagawa, A., Takeshima, H., Miyazawa, J., Kitao, Pirious, F., and Lukacs, G. (1975c), Tetrahedron Lett. 4503.

Ōmura, S., Takeshima, H., Nakagawa, A., Kanemoto, N., and Lukacs, G. (1976), Bioorg. Chem. 5, 451.

Srinivasan, D., and Srinivasan, P. R. (1967), Biochemistry 6, 3111.

Vaněk, Z., Puža, M., and Doležilová L. (1961), Folia Microbiol. 6, 408.

Westley, J. W., Priess, D. L., and Pitcher, R. G. (1972), J. Chem. Soc., 161.

White, R. J., Martinelli, E., Gallo, G. G., Lancini, G., and Beynon, P. (1973), Nature (London) 243, 273.

DNA Polymerase γ of Human Lymphoblasts[†]

Marjorie Robert-Guroff, Anthony W. Schrecker, Bonnie J. Brinkman, and Robert C. Gallo*

ABSTRACT: DNA polymerase γ, a component of normal cells, exhibits several properties similar to those of the DNA polymerase of RNA tumor viruses (reverse transcriptase). We have purified DNA polymerase γ more than 9000-fold from a human lymphoblast cell line (NC37) and have reexamined the biochemical nature of the enzyme, its distinguishing features with regard to reverse transcriptase, and its possible relationship to the viral enzyme. The final enzyme preparation is demonstrably free of other DNA polymerase activities by immunological and biochemical criteria. Only one form of the enzyme was detected. It has a molecular weight of 120 000. The enzyme is moderately sensitive to N-ethylmaleimide, exhibits a broad pH optimum around 7.4 in imidazole buffer, and is stimulated by ammonium sulfate. Like reverse transcriptase, DNA polymerase γ prefers $(dT)_{12-18} \cdot (A)_n$ to $(dT)_{12-18} \cdot (dA)_n$ as template with either Mg^{2+} or Mn^{2+} present. However, unlike the viral enzyme its activity with $(dG)_{12-18} \cdot (C)_n$ is low, and it does not transcribe the heteropolymeric portions of natural RNA templates. It has been reported that $(dG)_{12-18} \cdot poly(2'-O-methylcytidylate)$ is a specific primer template for viral reverse transcriptase (Gerard, G. F. (1975), Biochem. Biophys. Res. Commun. 63, 706). The observation that DNA polymerase γ does not use this template was confirmed with the NC37 enzyme. However, it was shown that the reverse transcriptases of avian, murine, and primate RNA tumor viruses do not use it with very great efficiency. The same reverse transcriptases do not transcribe RNA-primed DNA templates, in contrast to DNA polymerase γ which exhibits good activity with these templates. The problems of distinguishing reverse transcriptase and DNA polymerase γ in cells are discussed with respect to these properties.

DNA polymerase γ (Weissbach et al., 1975), variously called R-DNA polymerase, DNA polymerase III, DNA polymerase A, or "synthetic" RNA-dependent DNA polymerase, has been studied in a number of cell types from several animal species. These studies have shown that the enzyme is a component of normal cells and is distinct from other cellular DNA polymerases, as well as from RNA-dependent DNA polymerase (reverse transcriptase) of RNA tumor viruses (Weissbach, 1975; Lewis et al., 1974a,b; Spadari and Weiss-