Studies on the Biosynthesis of Magnamycin*

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ABSTRACT: The biosynthesis of Magnamycin, a macrolide antibiotic, produced by *Streptomyces halstedii* was investigated with the aid of specifically labeled acetates-^{14}C and propionates-^{14}C. The Magnamycin was isolated from the supernataut of the culture medium and was chemically degraded to carimbose; the latter was further degraded to C_{12} and C_8 acids. A comparison of molar activities of propionate-labeled Magnamycins and their degradation products indicated that one intact propionate unit was incorporated into the C_{12} portion of the lactone backbone only, and that the branching methyl group stems from C-3 of propionate. Radioactivity determinations of the cellular fatty acids isolated from each of the above experiments also revealed the presence of a system capable of incorporating intact propionate units.

Studies of the Magnamycin labeled by acetate-1-^{14}C and by acetate-2-^{14}C showed that the lactone backbone is synthesized from eight acetate residues in addition to the one propionate residue.

The macrolide antibiotics comprise a group of substances which are bacteriostatic, acting in low concentrations primarily on Gram-positive pathogenic organisms and on some large viruses such as *Rickettsia*. These antibiotics have all been isolated from the fermentation broths of various species of *Streptomyces* and possess a number of structural features in common. They are all characterized by a many-membered lactone ring containing one or more branching methyl groups. In addition, the lactone ring is linked to one or two unusual deoxyhexoses.

The elucidation of the structure of Magnamycin,[1] a macrolide antibiotic (Woodward, 1957; Wagner *et al.*, 1953; Woodward *et al.*, 1965; Kuehne and Benson, 1965), brought out additional interesting features: a branching methyl group in the middle of the lactone structure, a branching acetaldehyde group, and an epoxide ring. These characteristics taken together present challenging biogenetic problems, some of which are explored in this paper.

Woodward (1957) had proposed that the branching methyl groups in the macrolide antibiotics may represent C-3 of a propionate residue incorporated as an intact unit. It has been shown that the lactone of erythromycin, another macrolide antibiotic, is formed entirely from propionate residues (Corcoran *et al.*, 1960; Corcoran, 1966; Grisebach *et al.*, 1960). The results presented in this paper on the biogenesis of the Magnamycin lactone also support this hypothesis and are in agreement with the work of Grisebach *et al.* (1961, 1962). They demonstrated that the methyl group of $^{14}CH_3$–methionine is not incorporated into the lactone residue of Magnamycin and that the label from propionate-2-^{14}C as precursor appears in the two-carbon fragment containing the branching methyl group. Our results and conclusions with regard to the biosynthesis of other portions of the lactone ring differ somewhat from those of Achenbach and Grisebach (1964).

A preliminary note on some of these findings has appeared (Gilner and Srinivasan, 1962).

Experimental Section

Streptomyces halstedii (NRRL 2331) was maintained on Asheshov's agar.[2] Magnamycin was provided by Dr. F. A. Hochstein of Chas. Pfizer Laboratories. Sodium acetate-^{14}C and sodium propionate-^{14}C were obtained from New England Nuclear Corp. and Isotopes Specialties Co.

Production and Estimation of Magnamycin. The entire growth from an Asheshov's agar slant was scraped into 10 ml of distilled H_2O. This cellular suspension (2 ml) was introduced into 200 ml of indicated medium in a 2-l. erlenmeyer flask and agitated on a New Brunswick gyrotory shaker (Model G25, approximately 250 rpm) at 30° for 60 hr. The cells from this first phase (phase I) were collected by centrifugation at 10° under sterile conditions and were resuspended in fresh medium containing a few glass beads for better dispersal of the cells. The flasks were again placed on the shaker (phase II). In the experiments with radioactive precursors, the latter were added 10 hr after commencement of phase II. Twenty-four hours

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[1] Magnamycin is the trademark of Chas. Pfizer & Co., Inc., for the antibiotic carbomycin.

[2] F. A. Hochstein (1959), Chas. Pfizer & Co., personal communication, New York, N. Y.

later the contents of the flasks were pooled, cooled in ice, acidified to pH 3 with concentrated HCl, and the aqueous solution was freed of solids by centrifugation. The supernatant solution was immediately neutralized with 10% NaOH, and in the experiments with ^{14}C precursors, 7 g of nonradioactive precursor/l. of medium was added. Magnamycin was isolated from this supernatant by previously published methods (Dutcher *et al.*, 1953; Wagner *et al.*, 1953; Tanner *et al.*, 1957) and was crystallized from 85% isopropyl alcohol-H_2O and dried at 100° *in vacuo* over P_2O_5, mp 216–220° dec (lit. (Breslow, 1955; Dutcher *et al.*, 1953; Wagner *et al.*, 1953) mp 204–218° dec). The ultraviolet and infrared spectra were identical with those published (Breslow, 1955; Tanner *et al.*, 1957; Wagner *et al.*, 1953).

The accumulation of Magnamycin was studied in the following media. Medium II (Tanner *et al.*, 1957) consisted of the following components: soluble starch (20 g), Casamino Acids (10 g), soybean meal (15 g), $CaCO_3$ (1 g), and H_2O to 1 l. Medium III contained the following ingredients: soluble starch (10 g), Casamino Acids (10 g), $CaCO_3$ (1 g), yeast extract (2 g), and H_2O to 1 l. Medium III_D was similar to medium III except that starch was replaced by glucose (5 g/l.).

The estimation of Magnamycin in solution was carried out by the disc diffusion method against *Sarcina lutea* as described by Grove and Randall (1955).

Extraction of Fatty Acids. The cells, which were obtained by centrifugation of the acidified shaking medium, were washed once with a small amount of H_2O, twice with saline, and were then lyophilized. The fatty acids were isolated as lithium salts after saponification of the lyophilized cells with alcoholic KOH.

Degradation of Magnamycin to Carimbose and C_{12} Acid. The degradation of Magnamycin to carimbose was carried out as described by Breslow (1955). The further conversion of carimbose to C_{12} acid was accomplished according to the procedure outlined by Agosta (1957) up to the obtention of the crude material. This was purified on a silicic acid column, prepared according to Bulen *et al.* (1952) before recrystallization from peroxide-free *n*-butyl ether, yield 43%, mp 138–140.5° (lit. (Agosta, 1957) mp 139–141°). The infrared spectrum was in agreement with the published one (Agosta, 1957).

Iodoform from C_{12} Acid. C_{12} acid (68 mg) was refluxed for 2 hr with 3 ml of 10% NaOH. To degrade the resulting methyl ketone the solution was cooled, 3 ml of 1 N HCl was added, followed by a slight excess of I_2 in KI, and the solution was warmed in a water bath at 60° for 10 min with addition of more KI–I_2 to maintain a visible excess. After cooling, the excess iodine was destroyed with 10% NaOH. The iodoform was collected by centrifugation and recrystallized from methanol–H_2O, yield 40%.

Degradation of C_{12} to C_8 Acid and Synthesis of Pentane-2,4,5-tricarboxylic Acid. C_8 acid was obtained by HNO_3 oxidation of C_{12} acid (Agosta, 1957) and the crude product was purified on a silicic acid column and recrystallized from nitromethane. The yield was doubled by reducing the refluxing time to 2 hr, yield 45%, mp 100–102° (lit. (Agosta, 1957) mp 99–100°). *Anal.* Calcd for $C_8H_{12}O_6$: C, 47.12; H, 5.89. Found: C, 45.96; H, 6.01.

Pentane-2,4,5-tricarboxylic acid (synthetic C_8 acid) was synthesized by the route described by Hope and Perkin (1911). The crude product was purified on a silicic acid column and the fractions under the 25% butanol (in $CHCl_3$) peak were pooled, acidified, extracted with ether, and crystallized from nitromethane. The infrared spectrum of this material was identical with the published spectrum of C_8 acid obtained by a different synthetic route (Agosta, 1957), mp 138° (lit. (Hope and Perkin, 1911) mp 158°). *Anal.* Calcd for $C_8H_{12}O_5$; C, 47.12; H, 5.89. Found: C, 47.03; H, 5.98.

The difference between the reported melting point and that obtained here is undoubtedly due to the separation of diastereoisomers on the silicic acid column. That the synthetic product is a different diastereoisomer from the C_8 acid derived from the degradation of Magnamycin is also indicated by its elution by 15% butanol while the major portion of the synthetic C_8 acid is eluted by 25% butanol.

Esterification of C_8 Acid, Dieckman Condensation, and Hydrolysis Accompanied by Decarboxylation. These procedures were based on the original method described by Hope and Perkin (1911) except that the methods had to be adapted for work on a microscale.

All melting points were determined on a Fisher-Johns melting point apparatus.

Radioactivity Measurements. All compounds were recrystallized to constant melting point and to constant radioactivity.

Radioactivity measurements were performed with two types of detectors: (1) a sealed Geiger tube having a mica window of 1.4 mg/cm² in a Tracerlab shielded manual sample changer, with a background of 15–20 cpm and (2) a Nuclear-Chicago low-background gas-flow counter with a micromil window (<150 μg/cm²), having a background of 2 cpm. All samples were counted at infinite thickness (20–25 mg) on stainless-steel planchets with a plating area of 0.96 cm².

In all cases the compound in question was compared to its parent compound at the same dilution and in the same counter. All samples were counted so that the statistical error was <5%. The observed counts per minute are reported.

The data are presented as molar activities. Molar activity = counts per minute × molecular weight. Molar incorporation = molar activity of magnamycin/molar activity of precursor.

Results

The initial experiments were directed toward finding the conditions for obtaining the maximum yield of Magnamycin in a soluble and simple medium. Simultaneously, a search was made for substances which

TABLE I: Effect of Various Substances on the Production of Magnamycin.

Medium of Phases I and II	Additions in Phase II	Magnamycin Produced (mg/l.)
Medium II	None	40
Medium II	1% methanol	10
Medium II	5% methanol	2
Medium II	1% ethanol	8
Medium II	5% ethanol	None (sometimes no growth)
Medium II	1% propanol	Trace
Medium II	5% propanol	No growth
Medium II	1% NH_4Cl and omit soybean meal	No growth
Medium III	None	60
Medium III	1% NH_4Cl	Trace
Medium III_D	None	30
Medium III_D	Potassium phosphate buffer (0.25 M, pH 7)	None

might markedly increase the production of the antibiotic and thereby present a clue as to its possible origin. The results are presented in Table I and the most significant conclusions may be summarized as follows. Soybean meal can be replaced by yeast extract. Unlike the effect of propanol on enhancing the yield of erythromycin produced by *Streptomyces erytherus* (Upjohn Co., 1956), alcohols had an inhibitory effect on the yield of Magnamycin. Ammonium chloride and phosphate significantly depressed the yield of

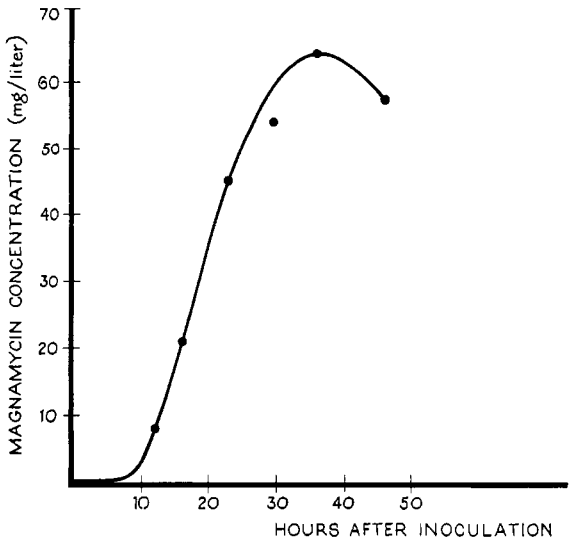

FIGURE 1: Rate of Magnamycin production.

TABLE II: Incorporation of Acetate and Propionate into Magnamycin.

Expt No.	Acetate-2-^{14}C 3	Propionate-2-^{14}C 4	Propionate-1-^{14}C 5	Acetate-2-^{14}C 6
Precursor				
Mmoles per liter	24	24	24	2.4
Counts per minute	3,180	2,150	3,900	13,150
Carrier dilution	13.7	14.7	12.7	24.5
Molar activity $\times 10^{-6}$	3.57	3.05	4.74	26.5
Magnamycin				
Counts per minute	388	380	715	454
Carrier dilution	9.7	4.7	4.4	27
Molar activity $\times 10^{-6}$	3.17	1.49	2.65	10.3
Molar incorporation	0.89	0.50	0.56	0.39

Magnamycin. The sodium salts of formic, acetic, and propionic acids did not affect the formation of Magnamycin. Since no one substance was found to stimulate Magnamycin production, the biosynthesis was explored with the aid of specifically labeled acetate and propionate in medium III. The rate of Magnamycin production in the replacement medium is depicted in Figure 1. At the end of 60 hr in phase I very little Magnamycin was produced. It begins to appear in the culture medium 10 hr after the start of phase II and reaches a maximum concentration at 35 hr. Therefore, the labeled compounds were introduced after 10 hr in the replacement medium and the isolation of Magnamycin from the culture medium was carried out 24 hr later.

The results presented in Table II (expt 3–5) clearly show that both acetate and propionate were incorporated into Magnamycin. The molar incorporation of propionate-2-^{14}C and propionate-1-^{14}C was similar indicating that propionate was incorporated as an intact unit rather than *via* the methylmalonyl-CoA[3] → acetyl-CoA or *via* the acrylyl-CoA → acetyl-CoA pathways. In either of these latter cases there would have been no direct incorporation of ^{14}C from propionate-1-^{14}C. These thoughts are further substantiated by examining the relative extent of incorporation of the respective precursors into fatty acids (Table III). If the molar activities of the precursors are all corrected to the same value in each of the experiments, namely 357×10^4, then the corrected activities recovered in the fatty acids are 4060, 1030, and 194 cpm, respectively. These results follow the pattern expected on the basis of our general understanding of the biosynthesis of

[3] Abbreviation used: CoA, coenzyme A.

TABLE III: Incorporation of Acetate and Propionate into the Cellular Fatty Acids.

Expt No.	Acetate-2-^{14}C 3	Propionate-2-^{14}C 4	Propionate-1-^{14}C 5
Precursor			
Molar activity ($\times 10^4$)	357	305	474
Fatty acids			
Counts per minute	4060	878	258

fatty acids. The activity found in the fatty acids derived from propionate-2-^{14}C probably represents incorporation of intact propionate units (into fatty acids containing an odd number of carbon atoms) as well as the incorporation of the label *via* the methylmalonyl-CoA → acetyl-CoA pathway. The former possibility was substantiated by the low yet significant incorporation of propionate-1-^{14}C into the fatty acids.

The next experiment was performed with a view to increasing the molar activity of the Magnamycin produced in the presence of the radioactive precursor. This was achieved by increasing the molar activity of the acetate-2-^{14}C and by reducing the concentration of acetate added to the culture medium (expt 6, Table II). Although the molar incorporation was significantly lower than in expt 3 (Table II), the molar activity of the Magnamycin produced was much higher. Based on this, a further experiment was performed using 3.0 mc of sodium acetate-1-^{14}C. The production of the Magnamycin and its extraction were carried out as described earlier except that 510 mg of carrier Magnamycin was added prior to its isolation from the culture medium.

The Magnamycin obtained from expt 3–7 was degraded to carimbose (Figure 2). The activities of the carimbose and the parent Magnamycin are presented in Table IV. Essentially all the activity of the Magnamycin was found in the carimbose in every experiment. This is not surprising in view of the fact that the shaking medium was rich in starch and in Casamino Acids and would thus serve as a good carbon source for mycarose and isovaleric acid, respectively, and also for mycaminose. Further comparisons of activities were therefore made with respect to carimbose.

The carimbose from each expt 3–7 was further degraded (Figures 2 and 3). The molar activities of the various compounds and the per cent activity of each compound with respect to the parent carimbose are shown in Table V.

It is evident that C_{12} acids obtained from propionate-1-^{14}C and propionate-2-^{14}C contain almost all of the activity of the carimbose. The results also indicate that the C_{12} and the C_8 acids from both acetate-1-^{14}C and acetate-2-^{14}C incorporated acetate residues but that considerable activity was lost in the degradation of carimbose to C_{12} acid.

Carimbose contains an *O*-acetyl unit which may have a higher molar activity than the other acetate units incorporated into the lactone residue of carimbose. It is conceivable that in the course of the many reactions involved in the incorporation of acetate residues into the lactone backbone, the intermediates may be considerably more diluted than the acetate which is incorporated into the *O*-acetyl. It is therefore very difficult to estimate the per cent molar activity which should be assigned to each acetate residue simply on the basis of the degradation of carimbose to the C_{12} acid. However, the C_{12} acid and its degradation to the C_8 acid (Figure 3) do shed more light on this question (Table V) and this is examined in the discussion.

In order to get even more precise information on the relative activity of individual carbon atoms, the C_{12} acid was subjected to a reverse aldol condensation with aqueous sodium hydroxide, followed by treatment with NaOI as depicted by the reactions in Figure 3. The resulting iodoform should represent C-3 of the lactone. The iodoform (40% yield based on C_{12} acid) was essentially inactive from *both* C_{12} acids (*i.e.*, C_{12} acid labeled by acetate-1-^{14}C and C_{12} acid labeled by acetate-2-^{14}C) (Table V). On the basis of the experimental results obtained from the C_{12} and C_8 acids, it was expected that at least one of the C_{12} acids should yield radioactive I_3CH. This raises some doubts as to the identity of the carbon atom represented by the iodoform. Further investigations to clarify this finding are in progress.

The problem was then approached from a different direction. Synthetic C_8 acid was prepared as described earlier. The methyl ester of the synthetic C_8 acid was

TABLE IV: Activity in Magnamycin and Carimbose.

Expt No.	Precursor	Magnamycin (molar act. $\times 10^{-4}$) *a*	Carimbose (molar act. $\times 10^{-4}$) *b*	$(b/a) \times 100$
3	Acetate-2-^{14}C	4.87	4.36	90
4	Propionate-2-^{14}C	7.90	7.66	97
5	Propionate-1-^{14}C	7.56	7.18	95
6	Acetate-2-^{14}C	14.7	14.3	97
7	Acetate-1-^{14}C	124	130	105

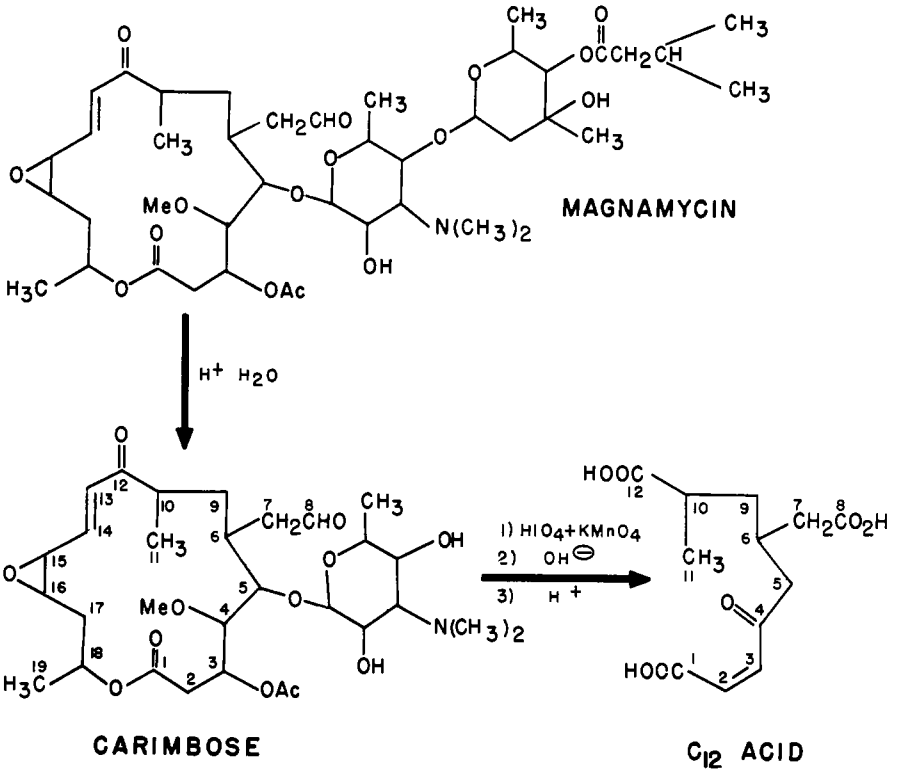

FIGURE 2: Scheme for degradation of Magnamycin to C_{12} acid.

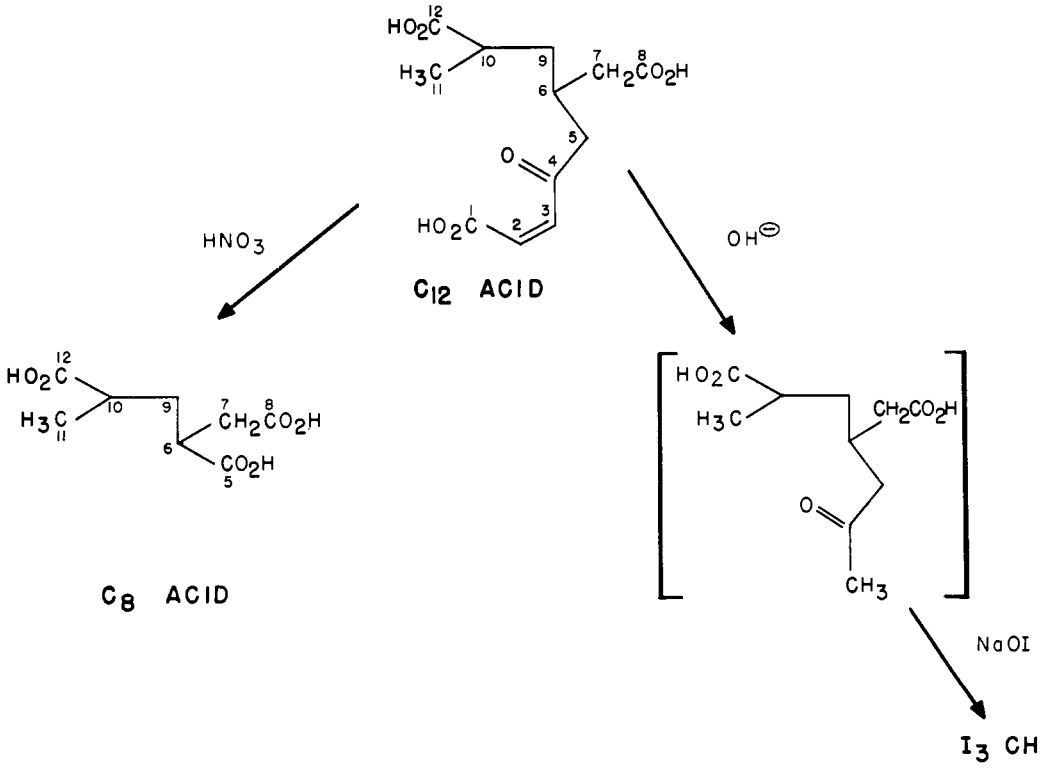

FIGURE 3: Scheme for the degradation of C_{12} acid to C_8 acid and iodoform.

TABLE V: Activity in Degradation Products of Carimbose.

Expt No.	Acetate-2-^{14}C 3	Propionate-2-^{14}C 4	Propionate-1-^{14}C 5	Acetate-2-^{14}C 6	Acetate-1-^{14}C 7
Carimbose					
Counts per minute	16	32	31	233	396
Molar activity (*a*)	99.4×10^2	196×10^2	190×10^2	1430×10^2	2430×10^2
C_{12} acid					
Counts per minute	13	59	65	174	356
Molar activity (*b*)	36.2×10^2	161×10^2	177×10^2	474×10^2	970×10^2
$(b/a) \times 100$	36	82	93	33	40
C_8 acid					
Counts per minute				50	167
Carrier dilution[a]				2.3	1.56
Molar activity (*c*)				234×10^2	530×10^2
$(c/a) \times 100$				16	22
Iodoform					
Counts per minute				(2.5)[b]	(4)[b]
Carrier dilution[a]				1.32	

[a] The C_{12} acid was diluted prior to chemical degradation. [b] This value is insignificant.

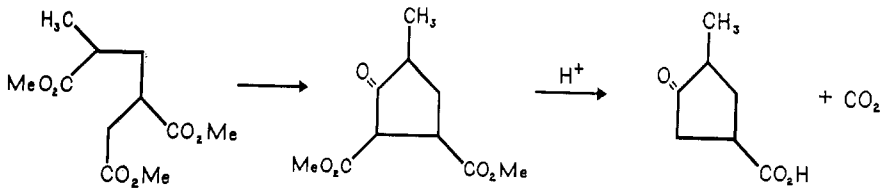

FIGURE 4: Dieckman condensation followed by acid hydrolysis of methyl ester of pentane-2,4,5-tricarboxylic acid.

found to be readily cyclized in a Dieckman condensation (Figure 4) and on acid hydrolysis it was decarboxylated to yield CO_2 representing C-5 of the lactone. The other product of the above reactions was isolated and identified as 1-methyl-4-carboxycyclopentan-2-one through its semicarbazone derivative. However, when this same procedure was carried out on the natural C_8 acid (obtained from C_{12} acid), the yield of $BaCO_3$ was negligible. Consequently, it became questionable as to whether this C_8 acid and the synthetic C_8 acid were structurally identical. High-resolution mass spectra of the natural and synthetic C_8 acids did not reveal any differences. Woodward *et al.* (1965) have confirmed our findings concerning the Dieckman cyclization and reexamined the structure of racemized natural C_8 acid and compared it to synthetic C_8 acid obtained by two different routes and found that these compounds were identical by means of nuclear magnetic resonance, infrared, and mass spectrometric methods and attributed the differences in reactivity in the Dieckman condensation to stereochemical differences.

Other means of obtaining identifiable carbon atoms from the C_{12} acid or the C_8 acid were also considered. The Schmidt degradation was carried out on the C_8 acid. If one could obtain a yield of at least 90% $BaCO_3$ from the three carboxyls of the C_8 acid, then the *maximum* error in estimating the activity per carboxyl would be 30%. However, it was not possible to obtain a yield higher than 60%. Thus, this was not an appropriate method for estimating the activity per carboxyl since it would be difficult to assess which of the three carboxyl groups (or what relative amount of each carboxyl) the $BaCO_3$ represented.

Discussion

The foregoing results point to a biogenetic scheme for the lactone residue of Magnamycin involving both propionate and acetate residues as precursors.

Propionate-1-^{14}C and propionate-2-^{14}C are incorporated into Magnamycin to the same extent, *i.e.*, the molar incorporation is 0.56 and 0.50, respectively, indicating that propionate is incorporated as an intact unit. This is further borne out by the fact that the C_{12} acids from

each of these experiments contain all of the activity of Magnamycin derived from propionate. It is therefore reasonable to conclude that the branching methyl group has its origin in the C-3 of propionate. The above evidence is in agreement with the work of Grisebach *et al.* (1961, 1962) who showed that $^{14}CH_3$–methionine was not incorporated into the lactone residue of Magnamycin and that the acetate obtained from a Kuhn–Roth oxidation of C_{12} acid derived from propionate-2-^{14}C contained all the activity present in the C_{12} acid.

In this connection it is interesting to point out that propionate-1-^{14}C is also incorporated to a small but significant extent (20% as much as with propionate-2-^{14}C) into the cellular fatty acids (Table III). The organism, therefore, does possess a mechanism whereby it can introduce intact propionate units into fatty acids.

The results of the degradation of Magnamycin formed in the presence of acetate-1-^{14}C and acetate-2-^{14}C give considerable insight into the origin of the rest of the carbon atoms of the lactone moiety (Table V). The C_{12} acid labeled with acetate-1-^{14}C contains 7% higher activity than the C_{12} acid from acetate-2-^{14}C. The C_8 acids also present a similar pattern, namely, the C_8 acid from the acetate-1-^{14}C experiment had 6% greater activity than the C_8 acid from acetate-2-^{14}C.

The C_8 acid contains eight carbons of which three are derived from propionate, leaving five carbon atoms derived from other sources. Since the difference between the C_{12} acids (from acetate-1-^{14}C and acetate-2-^{14}C) is 7%, this figure may well represent the activity equivalent to one acetate residue. Using this value of 7% as an approximate measure of one acetate residue, it fol ows that the C_8 acid from acetate-2-^{14}C incorporates two residues, and that the C_8 acid from acetate-1-^{14}C contains the equivalent of three residues: (for acetate-2-^{14}C and acetate-1-^{14}C, respectively) number of acetate residues in C_8 acid, 2 and 3; per cent activity of acetate residue, 7 and 7; and calculated per cent activity of C_8 acid, 14 and 21; found in C_8 acid, 16 and 22. Furthermore, the data indicate that the C_{12} acids, in each case, possess at least two additional acetate residues over their respective C_8 acids: (for acetate-2-^{14}C and acetate-1-^{14}C, respectively) per cent activity in C_8 acid, 16 and 22; two acetate residues = 14%, 14 and 14; and per cent calculated activity of C_{12} acid, 30 and 36; found in C_{12} acid, 33 and 40. This is also consistent with a loss of four carbons in the degradation of the C_1 acid to the C_8 acid. It therefore appears that the C_{12} acid from acetate-2-^{14}C has incorporated four (two plus two) acetate residues, and the C_{12} acid from acetate-1-^{14}C has incorporated five (three plus two) acetate residues. The apparent additional acetate residue incorporated into the C_{12} acid and the C_8 acid from acetate-1-^{14}C represents the incorporation of the carboxyl group of an acetate molecule into carbon atom 12. The experimental evidence presented strongly favors the view that carbon atoms 1–12 are derived from 4.5 acetate residues and 1 propionate residue.

Achenbach and Grisebach (1964) conclude that carbons 1–8 originate from precursors which can be formed more directly from glucose or succinate than from acetate. In our experiments the labeled precursors were added to a medium relatively rich in starch and under conditions of growth (the conditions which also gave the highest yields of Magnamycin) which should optimize the use of the most immediate precursors for specific biosynthetic activities. If, a glycolytic or Krebs cycle intermediate other than acetate were a more direct precursor to carbons 1–8 of the lactone ring, then this would be reflected in expt 3 and 6 of Tables II and V. In expt 6 one-tenth the concentration of acetate was added to the medium and yet the per cent activity recovered in the C_{12} acids was the same in each experiment even though the molar incorporation into magnamycin was one-half in the latter experiment. The molar incorporation may be used as an index of the availability of the exogenous precursor to the relevant pools of the organism. The per cent activity in the C_{12} acid in expt 6 should therefore have been a maximum of one-half of 35% if carbons 1–8 preferentially originate from glycolytic or Krebs cycle intermediates other than acetate. The discrepancy between the conclusions of Achenbach and Grisebach and our findings is difficult to resolve for the present since the experimental conditions and the evaluation of the results were different.

The degradation of carimbose derived from acetate-2-^{14}C and from acetate-1-^{14}C acid results in a considerable loss of activity in contrast to the propionate experiments where all of the activity of the carimbose is retained by the C_{12} acid, suggesting that the rest of the lactone moiety of seven carbon atoms, other than the C_{12} acid, is also derived from acetate. Since the C_{12} acid represents the incorporation of four acetate residues, one propionate residue plus one carboxyl from acetate, the seven carbon atoms of the lactone ring lost during the degradation of carimbose to C_{12} acid are made up of three acetate units plus one carbon from the methyl of acetate. Therefore, the lactone backbone is probably built up of eight acetate residues plus one propionate residue. On this basis the following calculations can be made: per cent activity in C_{12} acid, 35, 33, and 40; no. of incorporated acetate residue, 4, 4, and 5; calculated per cent activity of lactone ring, $^{35}/_4 \times 8 = 70$, $^{33}/_4 \times 8 = 66$, and $^{40}/_5 \times 8 = 64$ for acetate-2-^{14}C, acetate-2-^{14}C, and acetate-1-^{14}C, respectively. The activity due to the *O*-acetyl is therefore equal to 100% − the per cent activity in the lactone ring: 30, 34, and 36%, respectively, in each of the above experiments. The *O*-acetyl seems to have consistently higher activity than the acetate units incorporated into the lactone backbone. This difference in molar activity might be expected if the acetylation of the lactone backbone occurs at a different stage of its biosynthetic sequence, or, if this acetyl comes from a different pool. Also, it may well be that the relative activity of the primer precursor residue is different than that of the residues incorporated subsequently. This has been found to be the case in the biosynthesis of erythromycin, another macrolide antibiotic (Friedman *et al.*, 1964; Wawszkiewicz and Lynen, 1964).

In addition to this, the incorporation of a propionate residue in the middle of a fatty acid like substance strongly suggests further reason for some variations in

the molar activities of individual residues. The propionate residue may represent an elongation reaction of an α,β-unsaturated fatty acid with methylmalonyl-CoA analogous to phthiocerol (Gastambide-Odier *et al.*, 1963) or mycocerosic acid (Lederer, 1964) and the resultant C_{11} fatty acid may then undergo further reactions involving a Claisen-type condensation with another C_8 dicarboxylic fatty acid or ω-OH C_8 fatty acid with an activated β-carbon.

In conclusion, the evidence presented lends support to a biogenetic scheme for the lactone backbone of Magnamycin being synthesized from eight acetate units and one propionate unit, the branching methyl group arising from C-3 of propionate.

Acknowledgments

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