through a hydrophobic region on the HiPIP surface. Although it is at present difficult to distinguish between these and other alternative mechanisms, these reactions probably follow an outer-shere electron-transfer mechanism since there is no evidence that the prosthetic group of either the cytochromes *c* or HiPIP's change their inner sphere of coordination during the reactions.

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Biosynthesis of the Macrolide Antibiotic Chlorothricin†

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ABSTRACT: Feeding experiments with ^{13}C-labeled precursors followed by ^{13}C NMR analysis of the antibiotic and its aglycon have established a polyketide mode of biosynthesis for chlorothricin, a metabolite of *Streptomyces antibioticus*. The acyl moiety is a substituted 6-methylsalicylic acid derived from four acetate units and a methyl group of methionine. The aglycon is comprised of ten acetate and two propionate units, leaving three carbon atoms, C-22, -23, and -24, unaccounted for. The two 2-deoxy-D-rhamnose moieties are derived from glucose with retention of the hydrogens at C-1, C-2, and C-6 and loss of H-3 and H-5. The hydrogen at C-4 of glucose is transferred intramolecularly to C-6 of the hexose, replacing the hydroxyl group at C-6 in an inversion mode, a result which implicates the thymidine 5′-diphosphate-glucose oxidoreductase reaction in this transformation.

In a previous paper (Holzbach et al., 1978), we presented results pointing to some of the basic building blocks of chlorothricin (I), a novel macrolide antibiotic produced by *Streptomyces antibioticus* strain Tü 99 (Keller-Schierlein et al., 1969; Muntwyler & Keller-Schierlein, 1972; Brufani et al., 1972). These studies had indicated that acetate, propionate, glucose, and methionine were good precursors of the antibiotic and that the latter provides only the *O*-methyl group but none of the *C*-methyl groups of I. In addition, the amino acids tyrosine, phenylalanine, and leucine were fairly well incorporated but not shikimic or mevalonic acid. We now report further results on the mode of incorporation of these precursors into chlorothricin.

Materials and Methods

Radioactive precursors were purchased from Amersham-Searle or from New England Nuclear Corp., except for (6*R*)- and (6*S*)-D-[4-^2H,6-^3H]glucose which had been synthesized

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Table I: ^{13}C Chemical Shifts and ^{13}C Distribution of the Acyl Portion of I Obtained Biosynthetically from [^{13}C]Acetate

carbon no.	chemical shift[a]	relative ^{13}C abundance in I derived from [1-^{13}C]-acetate	[2-^{13}C]-acetate
C-8′	16.8	1.2	2.1
C-6′	134.0	2.0	1.2
C-5′	126.8	1.3	2.4
C-4′	130.7	2.4	1.3
C-3′	109.9	1.4	3.1
C-2′	154.4	1.8	1.2
C-1′	122.8	0.9	1.8
C-7′	166.7	2.6	1.0
C-9′	56.2	1.1	1.1

[a] In ppm downfield from Me_4Si.

during earlier work in this laboratory (Snipes et al., 1977, 1979). Stable isotope-labeled precursors were obtained from Stohler Isotopes, Inc.; the ^{13}C-labeled compounds were generally of 90–95 atom % enrichment.

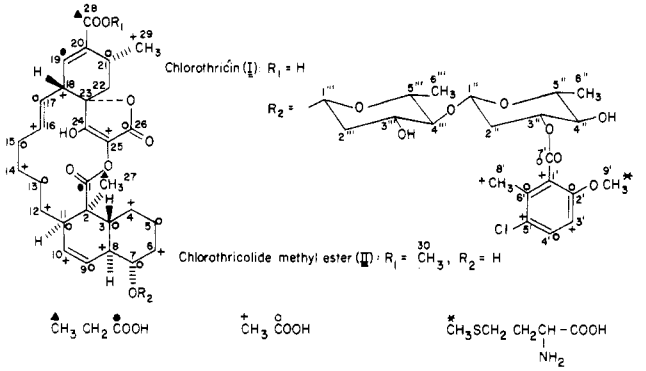
General methods for the maintenance and cultivation of *S. antibioticus*, the conditions of the feeding experiments, and the procedures for the isolation and purification of I and its degradation by methanolysis and Kuhn–Roth oxidation were as detailed in our previous publications (Holzbach et al., 1978; Mascaretti et al., 1979), as were the general methods for radioactivity analysis. In all the experiments with ^{13}C-labeled precursors, a small amount of the ^{14}C-labeled compound was fed along with the stable isotope-labeled sample in order to allow determination of the approximate average enrichment from the specific radioactivity of the product. The concentrations of precursor added in the ^{13}C experiments and the amounts of the product obtained were as follows: [1-^{13}C]-acetate, 9.9 mmol/L, 283 mg of I from 20 100-mL cultures; [2-^{13}C]acetate, 9.9 mmol/L, 300 mg of I from 10 cultures; [1,2-$^{13}C_2$]acetate, 9.2 mmol/L, 420 mg of I from 13 cultures; [1-^{13}C]propionate, 7.8 mmol/L, 146 mg of I from 20 cultures; [3-^{13}C]propionate, 4.6 mmol/L, 205 mg of I from 10 cultures; [2-^{13}C]tyrosine, 2.9 mmol/L, 63 mg of I from 10 cultures; [1,4-$^{13}C_2$]succinate, 9.63 mmol/L, 348 mg of I from 9 cultures.

The chirality analysis of acetate samples was carried out by the general procedure of Eggerer as modified in our laboratory (Floss & Tsai, 1979).

Results

Since our preliminary studies (Holzbach et al., 1978) had shown good incorporation of radioactive acetate and propionate into both the acyl and the aglycon moieties of chlorothricin, we carried out experiments with ^{13}C-labeled acetate and propionate to determine the mode of their incorporation. *S. antibioticus* Tü 99 was used, and optimum times for precursor addition and harvesting were those established earlier, i.e., addition 24 h and harvest 48 h after inoculation. The optimum concentration of precursor was determined in a series of experiments with ^{14}C-labeled material, and on the basis of these results we fed acetate at a concentration of 10 nmol/L and propionate at 5–8 mmol/L. Chlorotricin was isolated as described (Holzbach et al., 1978) with the one modification that the product was recrystallized alternately from acetone and from methanol. In all the experiments, the ^{13}C NMR spectrum of the purified chlorothricin was recorded; the antibiotic was then degraded by methanolysis (Keller-Schierlein et al., 1969; Mascaretti et al., 1979), and the spectrum of the purified chlorothricolide methyl ester (II) was then recorded. A complete ^{13}C NMR spectral analysis of chlorothricin and

Scheme I: Labeling Pattern of the Acyl and Aglycon Moieties of Chlorothricin



methyl chlorothricolide has been carried out (Mascaretti et al., 1979), which has provided the signal assignments essential for this study.

Table I shows the enrichment values for the carbon atoms of the acyl moiety taken from the ^{13}C NMR spectra of the enriched chlorothricin from [1-^{13}C]acetate and [2-^{13}C]acetate. The data clearly indicate that the acyl moiety is derived from four acetate units and shows the alternate labeling pattern from C-1 and C-2 of acetate predicted for a polyketide-derived product. An experiment with [1,2-$^{13}C_2$]acetate showed that all the labeled carbon atoms gave doublets due to one-bond ^{13}C–^{13}C coupling (results not shown), indicating that all four acetate molecules are incorporated as intact units. The methoxy carbon was not enriched, in agreement with its origin from the methyl group of methionine as demonstrated by ^{14}C labeling (Holzbach et al., 1978). The demonstrated biosynthetic origin and lebeling pattern of the acyl moiety are shown in Scheme I.

The ^{13}C distributions in chlorothricolide methyl ester biosynthesized from [1-^{13}C]- and [2-^{13}C]acetate and from [1-^{13}C]- and [3-^{13}C]propionate are shown in Table II. Both samples of propionate each label two atoms specifically, indicating that two molecules of propionate are incorporated into II.[1] One of these gives rise to carbons 19, 20, and 28, the methyl group having been oxidized to a carboxyl function, and the other provides the carboxyl end of the carbon chain, i.e., carbons 1, 2, and 27. The labeling patterns from [^{13}C]acetate are not as clear-cut; the considerable scrambling seen reflects extensive metabolism of the acetate, for example, to propionate. Nevertheless, it is evident that carbon atoms 3–18 of II are labeled by C-1 and C-2 of acetate in the alternating pattern typical of a polyketide. Furthermore, carbon atoms 29 and 21 are derived from C-2 and C-1, respectively, of acetate. These two carbons must thus represent the starter unit of a polyketide chain extending from C-29 and C-21 via C-20 to C-1 in the direction of decreasing numbers of the atoms with C-28 and C-27 as one-carbon branches derived from the methyl group of propionate. Again an experiment with [1,2-$^{13}C_2$]acetate clearly showed that all the directly acetate derived carbons

[1] Our previous study (Holzbach et al., 1978) had indicated some incorporation of propionate into the acyl moiety of I and some of the initial spectra of I derived from [^{13}C]propionate indicated the presence of a third enriched carbon atom. However, the spectra of highly purified I and those of II show only two carbon atoms to be enriched. As a possible explanation, we suggest that the addition of high levels of propionate to the fermentation may prime the aberrant synthesis of small amounts of an analogue of I containing the highly enriched *C*-ethyl homologue of the normal acyl moiety, which is only removed upon rigorous purification of I. No attempts have been made to verify the correctness of this explanation.

Table II: ^{13}C NMR Data of Chlorothricolide Methyl Ester Obtained Biosynthetically from Various ^{13}C-Labeled Precursors

carbon no.	chemical shift[a]	$^1J_{C-C}$ in II from [1,2-$^{13}C_2$]acetate (Hz)	relative ^{13}C abundance in II derived from [1-^{13}C]acetate	[2-^{13}C]acetate	[1-^{13}C]propionate	[3-^{13}C]propionate	[1,4-$^{13}C_2$]succinate
C-1	177.5		1.20	2.20	4.25		3.94
C-2	47.4		1.63	1.83			
C-3	37.6	39.5	4.40	1.47			
C-4	26.6	39.5	1.20	2.20			
C-5	24.1	33.9	4.00	1.47			
C-6	36.2	33.9	1.41	2.63			
C-7	73.3	38.5	5.01	1.89			
C-8	45.4[c]	38.5[d]	1.10	2.83			
C-9	123.2	*d*	5.54	1.28			
C-10	129.9	*d*	1.46	2.44			
C-11	46.8	30.5[d]	4.71	1.90			std: 1.10
C-12	33.0	30.5[d]	1.83	2.70			
C-13	28.0[b]	30.4[d]	4.52	1.70			
C-14	28.4[b]	30.4[d]	1.34	2.31			
C-15	32.3	39.6	4.64	1.44			
C-16	138.0	39.6	2.82	3.04			
C-17	124.4	48.4	5.43	1.10			
C-18	45.9[c]	48.4[d]	1.10	1.82			
C-19	136.5		5.07	2.11	5.86		3.38
C-20	134.0		1.10	2.08			
C-21	27.1	35.4	4.11	1.30			
C-22	35.0		1.98	1.70			
C-23	80.4		0.90	1.15			
C-24	159.4		1.10	1.10			1.10
C-25	115.5	91.5	1.10	1.83			
C-26	164.9	91.5	2.75	2.30			
C-27	16.6		1.51	2.79		5.6	
C-28	166.5		1.00	2.00		4.4	
C-29	20.7	35.4	1.63	3.47			
C-30 ($\underline{COOCH_3}$)	51.6		std: 1.10	1.10	1.10	1.10	

[a] In ppm downfield from Me_4Si. [b,c] These values may be reversed. The assignments listed are the ones compatible with biosynthetic theory. [d] Not clearly observed.

(i.e., C-3 through C-18, C-21, C-29) gave doublets due to ^{13}C–^{13}C coupling, indicating that all these carbons are derived from intact acetate units.

These data account for all but five of the carbon atoms of the aglycon moiety of I. Our previous work (Holzbach et al., 1978) had shown significant incorporation of ^{14}C from [2-^{14}C]-, [3-^{14}C]-, and [U-^{14}C]tyrosine, but not from [1-^{14}C]-tyrosine, into I, with a majority of the label (72%) residing in the aglycon moiety. This caused us to explore the possibility that the five carbon atoms of the tetronic acid moiety might arise by a novel ring cleavage from C-2 and C-3 of the side chain and three carbon atoms of the ring of tyrosine. A feeding experiment with DL-[2-^{13}C]tyrosine gave chlorothricin containing 5.9 atom % excess ^{13}C. However, the ^{13}C NMR spectrum of this material showed no selective enrichment of any one carbon, and it was therefore concluded that label from tyrosine is only incorporated in an indirect fashion, presumably following metabolic breakdown to acetate. A clue to the origin of the missing carbon atoms was obtained upon careful examination of the spectrum of II obtained from [1,2-$^{13}C_2$]-acetate. The data on the incorporation of single-labeled [^{13}C]acetate already indicate some labeling of C-25 and C-26 by acetate; however, it was not clear whether this represents a direct conversion of acetate into these two carbons or some indirect incorporation of the label. The spectrum of II from the double-labeled acetate clearly shows doublets for both C-25 and C-26 (Figure 1), and the unusually large coupling constant (90.3 Hz) typical for carbon atoms in a tetronic acid ring (Jacobsen et al., 1977) unequivocally indicates that these two carbons are coupled to each other and are thus both derived from one intact acetate unit. Thus, the problem is reduced

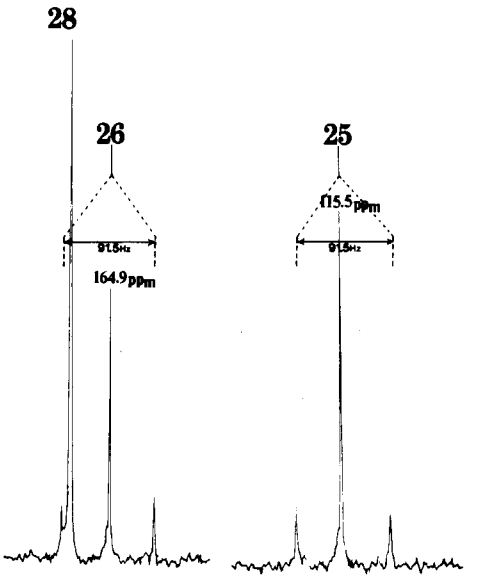


FIGURE 1: Section of the ^{13}C NMR spectrum of chlorothricolide derived from a feeding experiment with [1,2-$^{13}C_2$]acetate, showing the one-bond coupling between C-25 and C-26.

to accounting for the origin of three remaining carbon atoms, carbons 22, 23, and 24. The biosynthetic origin and labeling pattern of the aglycon are shown in Scheme I.

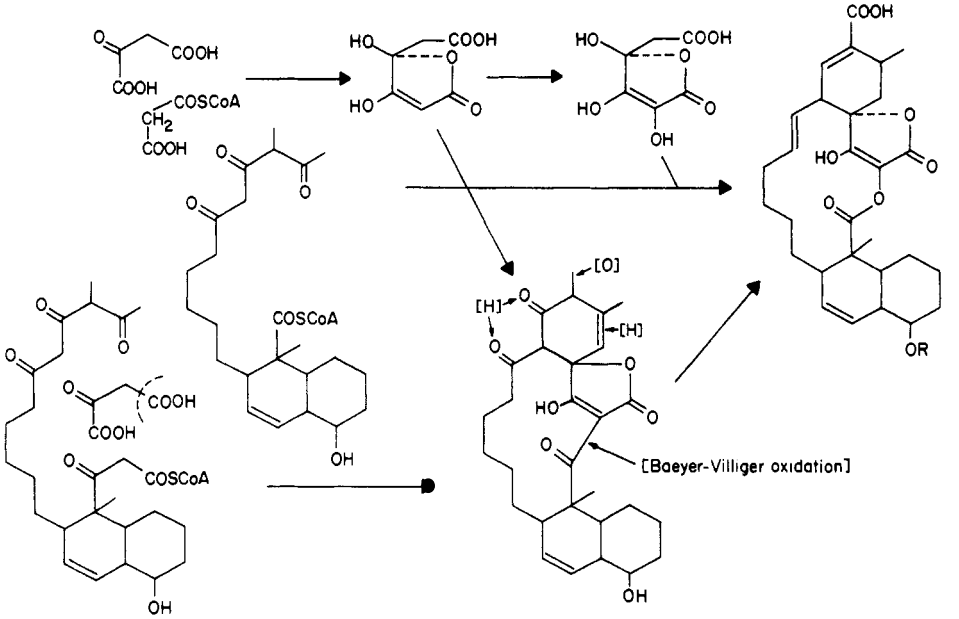
At this point, we carried out a number of additional feeding experiments with ^{14}C-labeled precursors in order to identify likely proximate precursors of the three-carbon unit in the aglycon moiety. As shown in Table III, of the compounds tested, the C_4 dicarboxylic acids, malate and succinic acid,

Table III: Incorporation of Radioactively Labeled Precursors into Chlorothricin by *S. antibioticus* Strain Tü 99

precursor fed				chlorothricin formed				
name	sp radioact (μCi/mmol)	amount added (mmol/L)	total radioact added (μCi/L)	yield (mmol/L)	sp radioact (μCi/mmol)	total radioact (μCi/L)	incorp rate (%)	dilution factor
[1-^{14}C]ribose	*a*		10	0.12	0.45	0.054	0.54	
sodium [1-^{14}C]pyruvate	3.3	7.9	26	0.055	0.034	0.002		97
α-keto-[U-^{14}C]glutaric acid	*a*		10	0.055	0.144	0.008	0.08	
L-[U-^{14}C]lysine	18.3	0.68	12.5	0.15	0.13	0.19	0.15	
L-[1-^{14}C]ascorbic acid	*a*		14.0	0.34	0.011	0.0037	0.03	
L-[U-^{14}C]malic acid	*b*		28.3	0.47	0.55	0.26	0.90	
L-[U-^{14}C]malic acid	2.83	10	28.3	0.16	0.41	0.064	0.23	7
[1-^{14}C]glyoxylic acid	*b*		10.2	0.21	0.025	0.0053	0.05	
[2,3-$^{14}C_2$]succinic acid	1.73	10	17.3	0.56	0.43	0.24	13.8	4
[1,4-$^{14}C_2$]succinic acid	*b*		7.76	0.46	0.048	0.022	0.28	

[a] Specific radioactivity, >1 mCi/mmol, cold acetate added. [b] Specific radioactivity, >1 mCi/mmol, no carrier added.

Scheme II: Variations of a Hypothetical Pathway for the Biosynthesis of the Aglycon Moiety of Chlorothricin



appear to be the most efficient precursors, and particularly the center carbons of succinic acid are well utilized. Based on these results, we hypothesized that the formation of the tetronic acid moiety of II may parallel that of other, simpler tetronic acids, e.g., carolic and carlosic acid, which were studied by Lybing & Reio (1958) and Bentley et al. (1962). In analogy to carolic acid biosynthesis, a precursor unit having all the necessary functionalities could be formed by condensation of oxalacetate and malonyl-CoA. This compound would have to be functionalized at C-3 of the lactone ring or, in analogy to carlosic acid formation, may be acylated in that position by the carboxy terminus of the polyketide chain, followed by a Baeyer–Villiger oxidation. Even more attractive is the possibility that the main polyketide chain does not terminate with propionate at C-1, but rather with the acetate unit which gives rise to C-25/C-26. These possibilities are outlined in Scheme II. To further test the basic premise of this scheme, we fed [1,4-$^{13}C_2$]succinic acid (9.6 mmol/L) to cultures of *S. antibioticus* Tü 99. The above hypothesis would be strongly supported if specific incorporation of ^{13}C into C-24 were observed. The isolated chlorothricin (208 mg) had a specific radioactivity, from added [1,4-$^{14}C_2$]succinate, corresponding to 4.6% specific incorporation, and II obtained by methanolysis of this sample retained 92% of the specific radioactivity of I. Thus, the incorporation of this succinate is specifically into the aglycon and not into other acetate-derived parts of the antibiotic. However, ^{13}C NMR spectral analysis of the aglycon (Table II) shows no significant enrichment of C-24. Instead, the labeling pattern closely parallels that seen with [1-^{13}C]propionate, indicating that the main pathway of utilization of added succinate in the culture is activation to succinyl-CoA, isomerization to methylmalonyl-CoA, and incorporation into the polyketide chain in the positions of the "propionate" units. The experiment thus does not provide a definite answer to the question whether carbons 22, 23, and 24 of II are derived from a C_4 acid.

The origin of the two 2-deoxy-D-rhamnose moieties in I had in earlier experiments been traced to glucose as the basic precursor (Holzbach et al., 1978). In a series of additional experiments, we further probed the mode of transformation of glucose into the deoxy sugar moiety by determining the fate of the various hydrogens of glucose in this process. In these experiments, summarized in Table IV, the tritium retention from glucose labeled with ^{14}C at C-6 and with tritium in various positions in the conversion to chlorothricin as a whole and into its dideoxyhexose moieties was determined. The

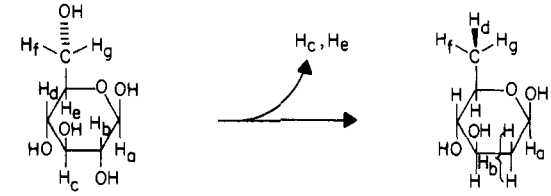
Table IV: Incorporation of Specifically Labeled Glucoses into Chlorothricin and Methyl 2-Deoxy-α-D-rhamnoside

position of label in glucose fed	$^3H/^{14}C$ of glucose	chlorothricin			methyl 2-deoxy-α-D-rhamnoside	
		^{14}C incorpd (%)	$^3H/^{14}C$	^3H ret (%)	$^3H/^{14}C$	^3H ret (%)
6-^{14}C, 1-^3H	1.45	0.7	0.21	14.4	0.98	67.5
6-^{14}C, 2-^3H	1.93	0.5	0.38	19.6	1.93	100.0
6-^{14}C, 3-^3H	1.81	1.2	0.13	7.1	0.19	10.4
6-^{14}C, 4-^3H	2.50	1.9	0.12	4.8	0.90	36.0
6-^{14}C, 5-^3H	3.97	2.3	0.04	1.0	0.13	3.2
6-^{14}C, 6-^3H	2.10	1.4	0.54	25.7	2.10	100.0

incorporation of [6-^{14}C,2-^3H]- and [6-^{14}C,6-^3H]glucose without change in the $^3H/^{14}C$ ratios leaves little doubt that the biosynthesis of 2-deoxy-D-rhamnose involves intact conversion of the carbon skeleton of glucose. The data in Table IV furthermore show that in the process the hydrogens at C-1, C-2, C-4, and C-6 of glucose are completely or partially retained whereas those at C-3 and C-5 are replaced by hydrogens from other sources. The retention of tritium from C-4 of glucose, which according to earlier results (Holzbach et al., 1978) is located at C-6 of the deoxyhexose moieties, suggests the involvement of a sugar nucleotide oxidoreductase reaction, presumably the TDP-glucose oxidoreductase reaction (Gabriel, 1973), in this transformation. Loss of H-5 of the hexose and transfer of H-4 to C-6 as a hydride are typical of the reaction catalyzed by TDP-glucose oxidoreductase (Melo et al., 1968; Gabriel & Lindquist, 1968) and support the role of this enzyme in the first step of the transformation of the glucose skeleton into that of 2-deoxy-D-rhamnose.

To further probe for the involvement of the TDP-glucose oxidoreductase reaction, we determined the steric course of the hydrogen transfer from C-4 to C-6 of the hexose moiety in order to establish whether it conforms with that deduced for this reaction in other microbial systems. (*6R*)- and (*6S*)-D-[6-^{14}C,4-^2H,6-^3H]glucose,[2] available from earlier work (Snipes et al., 1977, 1979), were fed to cultures of *S. antibioticus* Tü 99, and the resulting I was degraded to methyl 2-deoxy-α-D-rhamnoside, which was then subjected to Kuhn–Roth oxidation to give acetic acid from C-5″,-5‴ and C-6″,-6‴. The latter was analyzed for the chirality of the methyl group by the method of Arigoni, Cornforth, and coworkers [cf. Floss & Tsai (1979)], which involves activation to acetyl-CoA, condensation with glyoxylate catalyzed by malate synthase, and incubation of the resulting malate with fumarase to equilibrate the *pro-R* hydrogen at C-3 with solvent protons. Due to an isotope effect in the malate synthase reaction, either more than half of the tritium [(*R*)-acetate, *F* values of 50–79] or less than half of the tritium [(*S*)-acetate, *F* values of 50–21] will be retained in the fumarate, depending on the configuration and chiral purity of the methyl group. Analysis of the acetate samples obtained gave an *F* value of 39 for the material from the *6R* isomer of glucose; hence, the methyl group had *S* configuration, and $F = 60$, i.e., *R* configuration, for the sample derived from (*6S*)-glucose. The result shows that the replacement of the hydroxyl group at C-6 by H-4 occurs in an inversion mode. It also indicates that the transfer of the hydrogen from C-4 to C-6 must be intramolecular. This follows from the facts that the added labeled glucose has undergone considerable dilution with unlabeled endogenously synthesized glucose during the conversion and that a methyl group is only chiral if ^1H, ^2H, and ^3H are present in the same molecule. Hence, any significant intermolecular crossover of the H-4 from unlabeled into tritiated molecules would have resulted in loss of the chirality of the methyl group. The fate of the various hydrogens of glucose in the transformation to the 2-deoxy-D-rhamnose moieties of I is summarized in Scheme III.

Scheme III: Fate of the Hydrogens of Glucose during Its Conversion into the 2-Deoxy-D-rhamnose Moieties of Chlorothricin



Finally, an attempt was made to determine whether attachment of the sugar and acyl moieties to the aglycon is the terminal step in the biosynthesis of I. Chlorothricolide, the free acid corresponding to II, was prepared in radioactive form from I labeled biosynthetically by [^4C]acetate and fed to a culture of *S. antibioticus* Tü 99. The isolated I from this experiment had no significant radioactivity (data not shown), indicating nonincorporation of chlorothricolide into I.

Discussion

The ^{13}C-labeling studies reported here demonstrate quite clearly that both the substituted benzoic acid moiety and the aglycon of I are biosynthesized by the polyketide pathway in the manner indicated in Scheme I. The acyl moiety shows the labeling pattern from acetate which is typical of 6-methylsalicylic acid, a known acetate-derived metabolite of many microorganisms (Birch et al., 1955). The aglycon is formed from, presumably, a single polyketide chain, starting at C-29 and ending either at C-1 or, possibly, at C-26. This polyketide chain thus contains either nine or ten acetate and two propionate units. In one of the propionate units, the methyl group (C-28) has been oxidized to a carboxyl group, a phenomenon also observed in the biosynthesis of lucensomycin (Manwaring et al., 1969) and in the streptovaricins (Milavetz et al., 1973). For the most part, the formation of the aglycon of I thus follows similar patterns as the biosynthesis of other macrolides (Grisebach & Hofheinz, 1964; Grisebach, 1965; Corcoan & Chick, 1966). However, the results leave the origin of three carbon atoms, C-22 to C-24, unaccounted for. These are clearly not derived from the breakdown of an aromatic amino acid, as we suspected at one point. Whether they were provided by a four-carbon Krebs cycle acid like oxalacetate, as outlined in Scheme II, remains to be determined. The negative outcome of the experiment with [1,4-$^{13}C_2$]succinic acid does not completely rule out this posibility because it is possible that the exogenously supplied succinate is subject to activation and rearrangement to methylmalonyl-CoA preferential to dehydrogenation to fumarate. Alternatively, the origin of these remaining three carbon atoms may be related to carbohydrate metabolism. As indicated in Table III, [1-^{14}C]ribose was efficiently incorporated into I,

[2] The precursor is labeled in such a way that essentially every tritiated molecule also contains deuterium.

and degradation of the antibiotic showed that the majority of the label (>90%) resides in the aglycon. The sugar moieties were virtually devoid of radioactivity, and the acyl portion had 7.5% of the label. From the latter value, one estimates that each acetate unit contains 2% of the label of I. Hence, only 28% of the radioactivity of I would be accounted for by the acetate-derived carbons, and the rest, about 70%, must be located in the propionate-derived carbons and/or carbons 22–24 of the aglycon.[3] Further experiments will be necessary to elucidate the origin of these three carbon atoms and the mode of formation of the tetronic acid moiety.

The pathway of transformation of glucose into the two 2-deoxy-D-rhamnose moieties of I parallels closely that of the formation of the 2,6-dideoxyhexose moiety in the antibiotic granaticin (Snipes et al., 1979). In both cases, the hydrogens from C-1, C-2, and C-6 of glucose are retained, and the hydrogen from C-4 is transferred to C-6. The characteristics of this latter process are the same in *S. antibioticus* as in the granaticin-producing *S. violaceoruber* and match those determined in vitro for TDP-glucose oxidoreductase purified from *E. coli* (Snipes et al., 1977); i.e., the migration of the hydrogen is strictly intramolecular, and the OH group at C-6 is replaced by H-4 in an inversion mode. Loss of H-5 from the hexose is obligatory in the oxidoreductase reaction, and the loss of H-3, also observed in granaticin formation, is probably incurred during the removal of the hydroxyl group from C-2, which presumably requires transposition of the keto group from C-4 to C-3. In the biosynthesis of granaticin, the OH group at C-2 is replaced by a hydrogen in a retention mode (Snipes et al., 1979), compatible with a mechanism involving pyridoxamine phosphate as a cofactor as in the formation of CDP-4-keto-3,6-dideoxyhexose (Rubenstein & Strominger, 1974). A similar experiment with [2-^{2}H]glucose was carried out with the chlorothricin fermentation but gave inconclusive results.

The mode of assembly of the components of the antibiotic requires further study. However, it appears that chlorothricolide itself is not a precursor of I, and, thus, attachment of the sugar and acyl moieties must occur at the stage of a precursor of II rather than the aglycon itself.

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[3] This argument would not be valid if the acetate units in II had a much higher specific activity than those in the acyl portion. However, the ^{13}C NMR spectra show no dramatic differences in the enrichments of the acetate-derived carbons in the acyl and the aglycon moieties.

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