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Biosynthesis of the Macrolide Antibiotic Chlorothricin: Basic Building Blocks†

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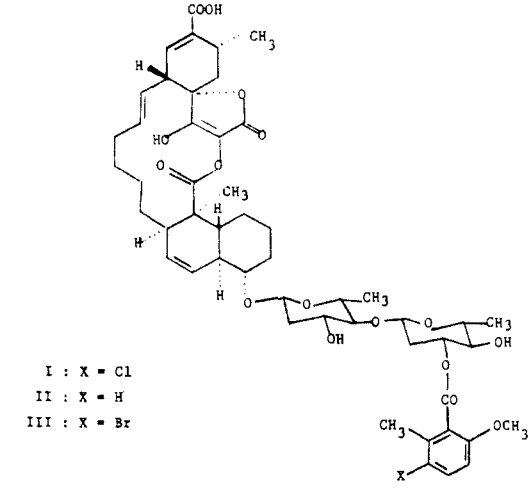
ABSTRACT: The biosynthesis of chlorothricin (I), a macrolide antibiotic isolated from *Streptomyces antibioticus* Tü 99, has been studied by feeding experiments with ^{14}C- and ^{3}H-labeled precursors. Acetate and propionate, but not methionine and mevalonate, were incorporated into the macrocyclic aglycone of the antibiotic. Glucose and the various carbon atoms of tyrosine, except the carboxyl carbon, also contributed label to the aglycone. Glucose also seems to be a specific precursor of the 2-deoxyrhamnose moiety, probably via a process involving a hydrogen shift from C-4 to C-6 of the hexose. The substituted 6-methylsalicylic acid moiety seems to be derived from acetate and one *O*-methyl group provided by methionine; shikimic acid is not incorporated.

Chlorothricin (I) is a novel macrolide antibiotic which was first isolated from *Streptomyces antibioticus* strain Tü 99 (Keller-Schierlein et al., 1969a). Its antibiotic activity against gram-positive bacteria on synthetic, but not on complex media, is due to an inhibition of the anaplerotic CO_2 fixation catalyzed by pyruvate carboxylase (Schindler & Zähner, 1972). Further mode of action studies have also shown an interaction between chlorothricin and the membrane phospholipids of *Bacillus subtilis* (Pache & Chapman, 1972). In its binding to pyruvate carboxylase from *B. stearothermophilus,* chlorothricin antagonizes the activating effect of acetyl-coenzyme A on this enzyme (Schindler & Zähner, 1973). Different inhibition



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characteristics were observed for pyruvate carboxylases from different organisms (Schindler & Scrutton, 1975), and, in an observed inhibition of porcine heart malate dehydrogenase, the antibiotic discriminated between the mitochondrial and cytoplasmic isoenzyme (Schindler, 1975). The mechanism of action of chlorothricin is clearly quite different from that of other macrolide antibiotics.

The structure elucidation (Muntwyler & Keller-Schierlein, 1972), which rests heavily on a crystal structure analysis of the aglycone, chlorothricolide methyl ester (Brufani et al., 1972b), showed that chlorothricin also represents a novel structural type of a macrolide antibiotic. The macrocyclic ring of the aglycone of chlorothricin incorporates a *trans*-decalin system and a tetronic acid moiety spiro-linked to a cyclohexene ring. The sugar component consists of two molecules of 2-deoxy-D-rhamnose, a dideoxyhexose which is only occasionally encountered in antibiotics, e.g., in the venturicidins (Brufani et al., 1972b), avilamycin (Buzzetti et al., 1968), curamycin (Gros et al., 1968), olivomycin (Berlin et al., 1964), and the chromomycins (Miyamoto et al., 1964). Esterified to one of the sugars is a substituted benzoic acid which by spectroscopic methods and by synthesis (Muntwyler et al., 1970) was identified as 5-chloro-6-methylsalicylic acid methyl ether. Chlorothricin co-occurs with the corresponding dechloro compound (II), from which it could so far not be separated (Keller-Schierlein et al., 1969a), and by adding 0.5% potassium bromide to the fermentation medium the corresponding bromo analog, bromothricin (III), can be obtained (Keller-Schierlein et al., 1969b). Some of the chemistry of chlorothricin has been described (Keller-Schierlein et al., 1969a; Muntwyler & Keller-Schierlein, 1972), particularly an unexpected rearrangement reaction of the aglycone leading to a dihydronaphthalene derivative (Gerhard et al., 1975).

The presence of several unusual structural features in chlorothricin raises the question of how this antibiotic is formed in nature and caused us to initiate a study of its biosynthesis. The present paper reports some of the results of this investigation.

Materials and Methods

Radioactive substrates were purchased commercially from the following suppliers: Amersham/Searle, [1-^{14}C]- and [2-^{14}C]acetic acid sodium salt, [1-^{14}C]- and [3-^{14}C]propionic acid sodium salt, D-[U-^{14}C]-, -[1-^{14}C]-, -[6-^{14}C,4-^{3}H]-, and -[6-^{3}H]glucose, L-[*methyl*-^{14}C]methionine, D,L-[2-^{14}C]mevalonate, L-[U-^{14}C]- and [1-^{14}C]leucine, L-[U-^{14}C]- and -[*side chain*-1-^{14}C]tyrosine, D,L-[*side chain*-2-^{14}C]tyrosine, and L-[U-^{14}C]phenylalanine; New England Nuclear Corp., [1-^{14}C]-, [2-^{14}C]-, and [3-^{14}C]propionic acid sodium salt, [G-^{14}C]shikimic acid, and D,L-[3-^{14}C]tyrosine. Double-labeled precursors were prepared by mixing the appropriate single-labeled species. The radioactivity of samples in solution was determined by liquid scintillation counting in a Beckman LS-250 or a Nuclear Chicago Mark I instrument. The scintillator solutions used were Unisolve I (obtained from Zinser, Frankfurt) for aqueous samples, 2,5-diphenyloxazole in toluene or 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in a mixture of toluene and ethanol for samples dissolved in organic solvents. Counting efficiences for individual samples were determined by external standardization, using the channel ratio method, or by internal standardization with [^{14}C]- and [^{3}H]toluene. The latter method was used for all double-labeled samples. Radioactivity on chromatograms was located with the aid of a Packard Model 7200 radiochromatogram scanner.

Preparative layer chromatography on 20 × 20 cm plates coated with a 1-mm thick layer of silica gel HR or silica gel F-245 (Merck, Darmstadt) was employed for both the purification of chlorothricin and for the separation of the methanolysis products. The following solvent systems were used: (a) ethyl acetate:chloroform, 1:1 (v:v), developed twice; (b) ethyl acetate:petroleum ether:methanol, 10:5:1; (c) petroleum ether:chloroform:methanol:benzene, 60:30:25:10. An alternative method for the purification of chlorothricin was chromatography on a column of silica gel Merck (0.05–0.2 mm), suspended in chloroform, with petroleum ether:chloroform:benzene (6:3:1) containing increasing volumes of methanol (1 to 3 parts) as the developing solvent. The quantitation of chlorothricin in crude extracts made use of the specific reaction of 2,6-dideoxy sugars with 60% sulfuric acid (Pape & Grisebach, 1965). Ten microliters of the crude chlorothricin extract were diluted with 50 μL of methanol and warmed with 200 μL of 60% H_2SO_4 for 10 min at 60 °C. After cooling to room temperature, the resulting yellow color was measured at 472 nm against a blank. A calibration curve was established with pure chlorothricin. The same quantitation was used for those methanolysis products containing the dideoxyhexose moiety, whereas the aglycone, chlorothricolide methyl ester, was determined on the basis of its UV maximum at 258 nm in alkaline solution (Keller-Schierlein et al., 1969a).

The chlorothricin fermentation was carried out using *Streptomyces antibioticus* strain Tü 99, which was obtained from Professor H. Zähner, Tübingen, and maintained on slants of Emerson agar or yeast extract agar (yeast extract 0.4%, malt extract 0.4%, Bacto-agar 1.5%, glucose 2.4%, pH 7.3). Fermentations and feeding experiments were carried out independently at the University of Tübingen and at Purdue University under similar, but not completely identical conditions. The fermentation medium was a complex medium consisting of 2% soybean meal and 2% mannitol in distilled water with (Tübingen) or without (Purdue) an additive of 0.2% $CaCO_3$. Cultures were grown in 500-mL indented Erlenmeyer flasks containing 100 mL of medium, which were incubated at 27 °C on a rotary shaker at 120 rpm (Tübingen) or 350 rpm (Purdue). Seed cultures were prepared by inoculating a flask from a slant and incubating for 48–72 h. About 24 h after the appearance of the brown color, 5 mL (Tübingen) or 10 mL (Purdue) of these suspensions was used to inoculate the production flasks. Radioactive precursors were added to these at 24 h (Purdue) or 48 h (Tübingen) after inoculation as aqueous solutions sterilized by passage through a Millipore filter, and the cultures were harvested 24 h later.

For the isolation of chlorothricin, the mycelium was separated from the culture medium by either of two methods. One involved centrifugation of the cultures at 1000*g*, decanting the supernatant, washing the mycelium with water (50 mL per flask), and lyophilization of the mycelium. Alternatively, Celite (2 g per flask) was added to the cultures followed by filtration through three layers of cheesecloth. The mycelium was washed with water and compressed to force out most of the water. The mycelium obtained by either method was then extracted three times with methanol, the extract was concentrated to a small volume, and after the addition of an equal volume of water the pH was adjusted to 5. This mixture was then extracted three times with ethyl acetate; the extract was dried with sodium sulfate and evaporated to dryness in a rotary evaporator. In one procedure (Tübingen), the oily residue was dissolved in methanol and defatted by extraction with 2 volumes of petroleum ether. The amount of chlorothricin in this crude extract was determined colorimetrically and the chlorothricin was then purified by column chromatography or preparative layer chromatography (see above). Constant specific radio-

TABLE I: Incorporation of Radioactively Labeled Precursors into Chlorothricin by *Streptomyces antibioticus* strain Tü 99.

	Precursor fed				Chlorothricin formed				
Expt no.[a]	Name	Spec radioact. [mCi/mmol]	Amount added [mmol/L]	Total radioact. added [mCi/L]	Yield [mmol/L]	Spec radioact. of purified antibiotic [mCi/mmol $\times 10^{-3}$]	Total radioact. [mCi/L $\times 10^{-3}$]	Incorp rate [%]	Dilution factor
1 (T)	[1-^{14}C]Acetate[c]	8.7	0.023	0.2	0.226	9.0	2.03	1.02	967
2 (P)	[1-^{14}C]Acetate[c]	0.0029	1.19	0.0035	0.120	2.91	0.349	9.98	1.0
3 (P)	[2-^{14}C]Acetate[c]	0.0096	1.13	0.011	0.219	1.76	0.385	3.5	5.5
4 (T)	[1-^{14}C]Propionate[c]	5.9	0.034	0.2	0.120	8.0	0.96	0.48	738
5 (P)	[1-^{14}C]Propionate[c]	0.0134	1.12	0.015	0.331	0.07	0.023	0.15	191
6 (P)	[2-^{14}C]Propionate[c]	0.010	1.0	0.010	0.228	0.81	0.185	1.8	12.3
7 (T)	[3-^{14}C]Propionate[c]	3.2	0.063	0.2	0.243	3.5	0.85	0.43	914
8 (P)	[3-^{14}C]Propionate[c]	0.0075	1.14	0.0086	0.233	1.30	0.30	3.5	5.8
9 (T)	[U-^{14}C]-D-Glc[d]	3.0	0.125	0.375	0.314	11.9	3.73	0.99	252
10 (P)	[1-^{14}C]-D-Glc[d]	>1	<0.01	0.0088	0.137	0.46	0.063	0.72	nd[b]
11 (T)	[6-^{14}C]-D-Glc[d]	2.9	0.172	0.5	0.299	20	5.98	1.20	145
12 (P)	[6-^{14}C,6-^{3}H]-D-Glc[d]	^{14}C: >2.5	<0.004	^{14}C: 0.0097	0.210	^{14}C: 0.295	^{14}C: 0.062	^{14}C: 0.64	nd[b]
		^{3}H: >5		^{3}H: 0.0182		^{3}H: 0.157	^{3}H: 0.033	^{3}H: 0.18	nd[b]
13 (P)	[6-^{14}C,4-^{3}H]-D-Glc[d]	^{14}C: >2.5	<0.004	^{14}C: 0.0097	0.172	^{14}C: 0.481	^{14}C: 0.083	^{14}C: 0.86	nd[b]
		^{3}H: >5		^{3}H: 0.0222		^{3}H: 0.0605	^{3}H: 0.0104	^{3}H: 0.045	nd[b]
14 (T)	[*methyl*-^{14}C]-L-Met	5.4	0.037	0.2	0.210	116	24.36	12.2	46.6
15 (P)	[*methyl*-^{14}C]-L-Met	0.0112	1.09	0.0122	0.146	3.2	0.467	3.8	3.5
16 (T)	[2-^{14}C]Mevalonate	6.3	0.032	0.204	0.151	0	0	0	
17 (P)	[U-^{14}C]Shikimic acid	>5	>0.001	0.0054	0.156	0.0006	0.0001	0.002	nd[b]
18 (P)	[U-^{14}C]-L-Tyrosine	0.0128	0.866	0.0111	0.286	0.308	0.088	0.79	41.6
19 (P)	[3-^{14}C]-D,L-Tyrosine	0.0144	0.795	0.0114	0.073	0.395	0.029	0.25	36.5
20 (P)	[2-^{14}C]-D,L-Tyrosine	0.0075	0.831	0.0062	0.114	0.35	0.040	0.64	21.4
21 (P)	[1-^{14}C]-L-Tyrosine	0.0147	0.818	0.012	0.197	0.013	0.0026	0.02	1131
22 (P)	[U-^{14}C]-L-Phe	0.0094	1.01	0.0095	0.167	0.345	0.058	0.61	27.2
23 (P)	[U-^{14}C]-L-Leucine	0.009	0.98	0.0088	0.189	0.779	0.147	1.67	11.6
24 (P)	[1-^{14}C]-L-Leucine	0.006	1.02	0.0059	0.193	0.100	0.019	0.32	60

[a] Experiments marked (T) were carried out at the University of Tübingen, those marked (P) at Purdue University. [b] nd = not determined. [c] Fed as the sodium salt. [d] Glc, glucose.

TABLE II: Degradation of Labeled Chlorothricin by Methanolysis.

Expt[a]	Precursor	Chlorothricin (I) (dpm/μmol)	Chlorothricolide methyl ester (dpm/μmol)[c]	α-Methyl-3-acyl-2-deoxyrhamnoside (dpm/μmol)[c]	α-Methyl-2-deoxy-rhamnoside (dpm/μmol)[c]
1	[1-^{14}C]Acetate	2 070	1095 (53)	538 (26)	0 (0)
4	[1-^{14}C]Propionate	15 650	3700 (24)	1 760 (11)	0 (0)
7	[3-^{14}C]Propionate	4 150	2410 (58)	1 380 (33)	0 (0)
9	[U-^{14}C]-D-Glucose	5 290	850 (16)	1 950 (37)	912 (17)
11	[6-^{14}C]-D-Glucose	8 550	2720 (32)	2 540 (30)	1280 (15)
14	[*methyl*-^{14}C]-L-Methionine	33 690	1780 (5)	24 700 (73)	0 (0)
18	[U-^{14}C]-L-Tyrosine	290	209 (72)	nd[b]	nd[b]
22	[U-^{14}C]-L-Phenylalanine	361	264 (73)	nd[b]	nd[b]

[a] Experiment numbers refer to Table I. [b] nd = not determined. [c] % of I given in parentheses.

activity was assured by repeated rechromatography on preparative layers. In the alternative procedure (Purdue), the crude chlorothricin was dissolved in ether and precipitated by the gradual addition of hexane. The antibiotic was then recrystallized to constant specific radioactivity from methanol/water and ether/hexane. Yields of chlorothricin ranged from 120 to 160 mg of purified material per L of culture; the crystalline material had mp 201–209 °C.

Methanolysis of chlorothricin (Keller-Schierlein et al., 1969a). The chlorothricin (120 mg) was dissolved in 10 mL of anhydrous methanol and heated with 5–10 drops of concentrated H_2SO_4 for 3 h on a steam bath. The solution was then poured on ice and the mixture was extracted three times with ethyl acetate. The extract, which contained the aglycone, chlorothricolide, as the methyl ester and the α-methyl 2-deoxy-3-*O*-acyl-D-rhamnoside, was dried and then evaporated to dryness in a vacuum. The two products were separated by preparative layer chromatography. The aqueous phase contained the third degradation product, α-methyl 2-deoxy-D-rhamnoside. The two glycosides were quantitated colorimetrically for the determination of their specific radioactivity. In an abbreviated version of this degradation (Purdue), only the chlorothricolide methyl ester was isolated.

The Kuhn–Roth oxidation of chlorothricin and chlorothricolide methyl ester followed standard literature procedures (Simon & Floss, 1967) and the Zeisel degradation of chlorothricin was carried out as described for ricinine (Dubeck & Kirkwood, 1952). The methyl iodide from the *O*-methyl group was isolated as tetramethylammonium iodide.

Incorporation rates were calculated as specific molar ra-

dioactivity of the purified chlorothricin (μCi/μmol) times amount of chlorothricin in the extract (μmol), as determined by colorimetry (Tübingen) or weight (Purdue), divided by total radioactivity of the added precursor. In cases where the amount of precursor added exceeds by a large margin the amount of product formed, the dilution factor (specific molar radioactivity of product) is also given, because it reflects more realistically the proximity of the precursor:product relationship in those cases. Tritium retention is defined as

$$\frac{[T]/[^{14}C] \text{ of product}}{[T]/[^{14}C] \text{ of precursor}} \times 100\%$$

Results

Experiments to establish the basic building blocks of the antibiotic chlorothricin were initiated independently at the University of Tübingen and at Purdue University. Both laboratories used the same organism, strain Tü 99 of *Streptomyces antibioticus*, and established optimum conditions for radiotracer feeding experiments using [1-^{14}C]acetate.

The results of a number of feeding experiments with various precursors are listed in Table I. Experiments 1–3 and many more feeding experiments under a variety of conditions always consistently showed good incorporation of [^{14}C]acetate. It is evident that both C-1 and C-2 of acetate are utilized, in accordance with a possible involvement of the polyketide pathway in this biosynthesis. Degradation of chlorothricin by methanolysis (Keller-Schierlein et al., 1969a) showed (expt 1, Table II) that [1-^{14}C]acetate labeled not only the aglycone, but also the α-methyl 2-deoxy-3-*O*-acylrhamnoside. Since the α-methyl 2-deoxyrhamnoside from this experiment is not radioactive, the label must reside in the acyl moiety. It thus seems likely that the 2-methyl-3-chloro-6-methoxybenzoic acid portion is also polyketide derived rather than originating from the shikimate acid pathway. The nonincorporation of shikimic acid (expt 17, Table I) further supports this tentative conclusion, although such a negative result has to be interpreted with caution because on occasion one finds that shikimic acid does not enter the cells of the producing organism. With the same caveat, the nonincorporation of mevalonate would seem to rule out the participation of any isoprenoid moieties in the biosynthesis of chlorothricin. The aglycone of chlorothricin contains several C_1 units branching off the main skeleton, two of them C-methyl groups, and these could arise either from propionate or by transfer of the methyl group of methionine. Despite some biological variation it is evident from the data in Table I (expt 4–8) that all three carbon atoms of propionate are effectively incorporated, pointing to the involvement of this compound in the biosynthesis. Degradation of [1-^{14}C]- and [3-^{14}C]propionate-derived chlorothricin by methanolysis (expt 4 and 7, Table II) indicated that in both cases about two-thirds of the recovered radioactivity resides in the aglycone, whereas about one-third seems to be associated with the acyl moiety. Kuhn–Roth oxidations were carried out on the propionate-derived chlorothricin samples from experiments 5, 6, and 8. The resulting acetic acid was found to contain 29% of the total radioactivity of the antibiotic derived from [3-^{14}C]propionate, 18% in the [2-^{14}C]propionate experiment, but only 4% of the radioactivity of the chlorothricin derived from [1-^{14}C]propionate. These data suggest that C-2 and C-3 of propionate give rise to at least one C-methyl group and its attached carbon in the antibiotic. Methyl-labeled methionine, on the other hand, is also incorporated effectively into chlorothricin (expt 14 and 15, Table I). However, methanolysis of the antibiotic showed (expt 14, Table II) that in this case almost all the label resides in the acyl moiety. Further degradations of the material from expt 15 (648 dpm/μmol) showed that the acetic acid produced by a Kuhn–Roth oxidation of the antibiotic is essentially devoid of radioactivity (0.2 dpm/μmol = <0.2% of I), whereas the methyl iodide produced from the *O*-methyl group of the acyl moiety by treatment with HI has the same specific molar radioactivity as the parent antibiotic (653 dpm/μmol = 100.8% of I). Thus, the methyl group of methionine provides exclusively the *O*-methyl group of chlorothricin, but is not incorporated into any of the C-methyl groups or any other part of the molecule.

Experiments 9–11 (Table I) show that glucose is effectively incorporated into chlorothricin. Methanolysis of the antibiotic obtained from [U-^{14}C]glucose and [6-^{14}C]glucose (expt 9 and 11, Table II) indicates labeling of the 2-deoxyrhamnose moieties, as expected, but also shows that a considerable portion of the radioactivity is incorporated into the aglycone and into the acyl moiety. At least the latter must be due to metabolic breakdown of the precursor and reincorporation of the radioactive fragments, presumably via acetate. Evidence in support of a direct conversion of glucose into the 2-deoxyrhamnose moieties comes from the double-labeling experiments 12 and 13 (Table I). [6-^{14}C,6-^{3}H]Glucose ([T]/[^{14}C] 1.87) loses 72% of its tritium, relative to ^{14}C, during incorporation into chlorothricin ([T]/[^{14}C] 0.53 = 28% T retention), reflecting the fact that much of the tritium is released into water during the metabolic breakdown of glucose. However, the acetic acid obtained by Kuhn–Roth oxidation of this chlorothricin, which originates inter alia from C-5 and C-6 of the 2-deoxyrhamnose moieties, has almost the same [T]/[^{14}C] ratio ([T]/[^{14}C] 1.55 = 83% T retention) as the precursor, suggesting that most of the radioactivity in the deoxy sugar moieties is due to a direct conversion of glucose into the 2-deoxyrhamnose portion of the antibiotic. Moreover, [6-^{14}C,4-^{3}H]glucose ([T]/[^{14}C] 2.29) gave rise to chlorothricin ([T]/[^{14}C] 0.13 = 6% T retention) with an even more pronounced loss of tritium, but the acetic acid from the Kuhn–Roth oxidation clearly contained tritium ([T]/[^{14}C] 0.56 = 24% T retention) and had a substantially higher [T]/[^{14}C] ratio than the antibiotic itself. This is to be expected for formation of the 6-deoxy sugar moiety from glucose via the glucose nucleotide oxidoreductase reaction, which has been shown to involve a hydride transfer from C-4 of the substrate to C-6 of the product (Melo et al., 1968; Gabriel & Lindquist, 1968).

Other precursors which showed significant incorporation into chlorothricin are several amino acids, e.g., phenylalanine, tyrosine, and leucine (expt 18–24, Table I). Methanolysis of the chlorothricin samples obtained from [U-^{14}C]tyrosine and [U-^{14}C]phenylalanine (expt 18 and 22, Table II) indicated that the majority of the label in both cases resides in the aglycone. Experiments with tyrosine labeled specifically in the various side chain positions showed incorporation of C-2 and C-3 of the side chain, in addition to the aromatic ring, but not of the carboxyl carbon (expt 19–21, Table I). These data can be taken to suggest a specific participation of tyrosine in the biosynthesis of the aglycone of chlorothricin.

Discussion

An inspection of the structure of chlorothricin together with our knowledge of the biosynthesis of other macrolide antibiotics (Grisebach, 1967) suggests a variety of possible precursors. The substituted benzoic acid moiety could be derived from shikimic acid or from 4 acetate/malonate units. The latter is more likely because the substitution pattern of this acid is that of a 6-methylsalicylic acid, known to be acetate-derived in other microorganisms, e.g., *Mycobacterium phlei* (Hudson

et al., 1970). Our results, incorporation of acetate, but not shikimate, into the acyl moiety, support the assumption that this portion of the antibiotic chlorothricin is polyketide derived.

Acetate, propionate, methionine, or even mevalonate can be considered as possible precursors of the aglycone of chlorothricin. The data clearly show incorporation of acetate and of all three carbons of propionate and rule out any participation of mevalonate or methionine. The latter only provides the methoxy group in the acyl portion. The results presented here do not establish which parts of the aglycone are derived from acetate and from propionate, and how the label is distributed in the various carbon atoms of the macrolide system. However, the data from the Kuhn–Roth oxidations of chlorothricin from 1, 2, and 3-labeled propionate strongly suggest that at least one C-methyl group and its attached carbon must be derived from C-2 and C-3 of propionate. Since the structure of the acyl moiety does not easily account for this, the propionate-derived methyl group must presumably be located in the aglycone. Rather surprising is the incorporation of about one-third of the label from both [1-^{14}C]- and [3-^{14}C]propionate into the acyl-deoxyrhamnoside fragment (V), apparently into its acyl moiety, since VI is not labeled (expt 4 and 7, Table II). This result, which is not readily interpreted in terms of the structure of the acyl portion and of biogenetic theory, will be examined further in order to arrive at a plausible explanation.

The two deoxyrhamnose moieties of the antibiotic are presumably of identical origin and should be both derived from glucose. The data showing incorporation of glucose into both sugar portions support this assumption and the double-labeling experiments tentatively point to intact conversion of the carbon skeleton of glucose into that of 2-deoxyrhamnose by a route involving a 4 → 6 hydrogen shift, as in the well-known sugar nucleotide oxidoreductase reactions. In addition, considerable metabolic breakdown of glucose is also evident from the degradation data, followed by incorporation of label into both the acyl moiety and the aglycone, presumably *via* acetate. Whether in addition glucose is also incorporated more directly into the aglycone, for example, into the tetronic acid portion, remains to be determined. The feeding experiments with tyrosine labeled in various positions finally suggest that the ring and carbons 2 and 3 of the side chain of this amino might also contribute to the biosynthesis of the aglycone of chlorothricin. The nonincorporation of shikimic acid, on the other hand, argues against this possibility. However, in our experience shikimic acid sometimes does not penetrate the cell wall of microorganisms and can therefore not be excluded as a biogenetic intermediate with complete certainty. Clearly, the role of tyrosine in this biosynthesis needs to be clarified further.

In conclusion, these exploratory studies on the biogenetic precursors of the antibiotic chlorothricin indicate that the acyl moiety is derived from acetate, and possibly propionate, and a methyl group from methionine, the two sugar moieties come from glucose, and, in addition to acetate and propionate, glucose and/or tyrosine are possible precursors of the aglycone portion. The mode of incorporation of these precursors into the antibiotic is not yet established by these experiments, and this is the main objective of further studies which are in progress.

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