BIOSYNTHESIS OF THE ANTIBIOTIC CHLOROTHRICIN: ASSIGNMENT OF THE CARBON-13 MAGNETIC RESONANCE SPECTRUM

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ABSTRACT.—As part of a biosynthesis study using ^{13}C-labeled precursors, the ^{13}C-nmr spectrum of the macrolide antibiotic chlorothricin has been analyzed. The signal assignments were made based on methanolysis of the antibiotic and spectral analysis of the fragments obtained, using chemical shift and multiplicity analysis, comparison with several model compounds and derivatives and specific deuterium exchange.

Chlorothricin (**I**) is a novel macrolide antibiotic isolated from *Streptomyces antibioticus*, strain Tü 99 (1–3). It co-occurs in a ratio of about 4:1 with the corresponding deschloro analog (**II**) from which it has so far not been separated (1). In a preceding communication (4), we have reported results of experiments with radioactive precursors which indicate some of the possible building blocks of this antibiotic. In order to further probe whether these precursors are incorporated as intact units and to establish their mode of incorporation, we intended to turn to the use of ^{13}C-labeled precursors followed by analysis of the product by cmr spectroscopy. This paper reports the assignment of the cmr spectrum of chlorothricin and its hydrolysis products as a prerequisite for the analysis of the labeling patterns of chlorothricin biosynthesized from various ^{13}C-labeled precursors.

EXPERIMENTAL[1]

METHANOLYSIS OF CHLOROTHRICIN (MODIFIED PROCEDURE) (1, 4).—A solution of 1 g (1.05 mmole) of chlorothricin in 150 ml of methanol was cooled to 0° and saturated with dry HCl gas. Protected from moisture by a drying tube, the mixture was heated to reflux for about one hour; the course of the reaction was followed by *tlc* [silica gel F-254, chloroform-methanol (95:5)]. When the reaction was complete, the solution was concentrated *in vacuo* to about 30 ml and 30 ml of water were then added. The precipitated chlorothricolide methyl ester was collected by filtration, washed with water-methanol, and recrystallized from aqueous methanol. Yield 503 mg (91%), mp. 215–216°, lit. 227–228° (1).

The aqueous phase was extracted 3 times with 50 ml portions of ether; the extract was washed with water, dried and evaporated to give 322 mg of an oil consisting of the two methyl benzoyl deoxyrhamnosides (**IV** and **V**) and traces of methyl chlorothricolide. The aqueous solution was then neutralized with sodium hydroxide and extracted with ethyl acetate to give 40.5 mg α-methyl-2-deoxyrhamnoside (**VI**) as an oil.

Four hundred mg of the mixture of the two benzoylrhamnosides from two degradations were dissolved in 3 ml of chloroform and streaked on four 2-mm-thick layer plates (20 x 20 cm, silica gel F-254, Merck, precoated), which were developed twice with benzene-acetonitrile (2:1). The two major bands were scraped off, eluted with chloroform-methanol (9:1) and evaporated to dryness. The residues were taken up in 2 ml of chloroform, filtered to remove silica gel and evaporated again. The band R_f 0.83 (analytical plate) gave 208 mg of an oil identified as α-methyl-2-deoxy-3-*O*-(2'-methoxy-6'-methyl-5'-chlorobenzoyl)-D-rhamnoside (**IV**) (1). The band of R_f 0.79, yielded 138 mg α-methyl-2-deoxy-4-*O*-(2'-methoxy-6'-methylbenzoyl)-D-rhamnoside (**V**). Compounds **III**, **IV** and **VI** were identified by comparison with authentic samples (1), **V** by the proton nmr spectrum.

[1]The cmr spectra were recorded at ambient temperature on $CDCl_3$ solutions in 10-mm spinning tubes on a JEOL PFT-100 spectrometer operating at 23 Kgauss, interfaced to a JEOL EC-100 Fourier-transform computer with 20 k memory. The typical pulse-width was 25.5 μsec (90° pulse), and the repetition time between pulses was 5 sec. All proton resonances were decoupled by a broad band irradiation (2.5 kHz) from an incoherent 99.99 MHz source for proton noise-decoupled spectra. The gated decoupling technique (5, 6) was employed to measure proton coupled spectra. Chemical shift values are given relative to TMS and were derived by the relationship: $\delta(TMS) = \delta(CDCl_3) + 76.9$ ppm.

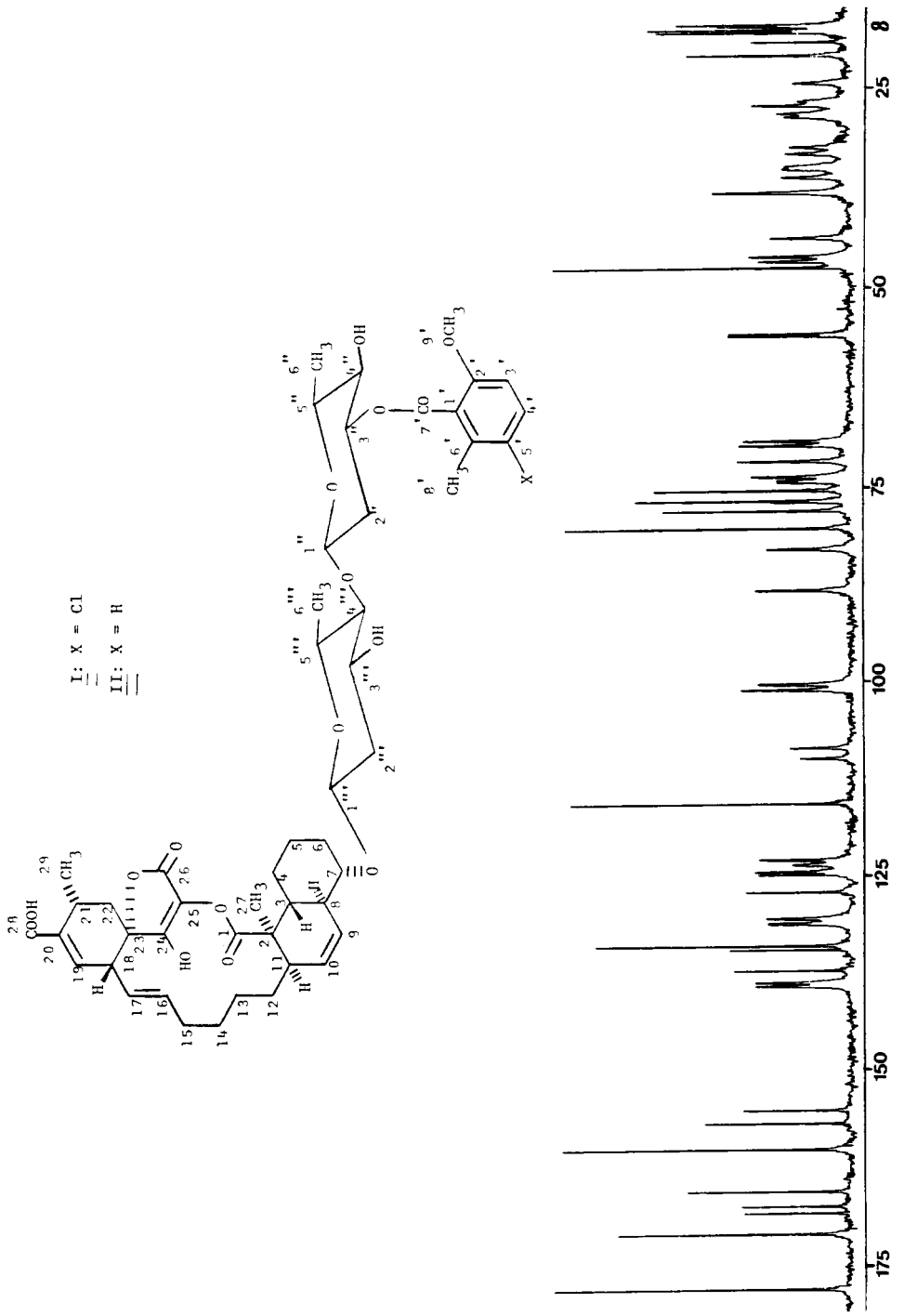
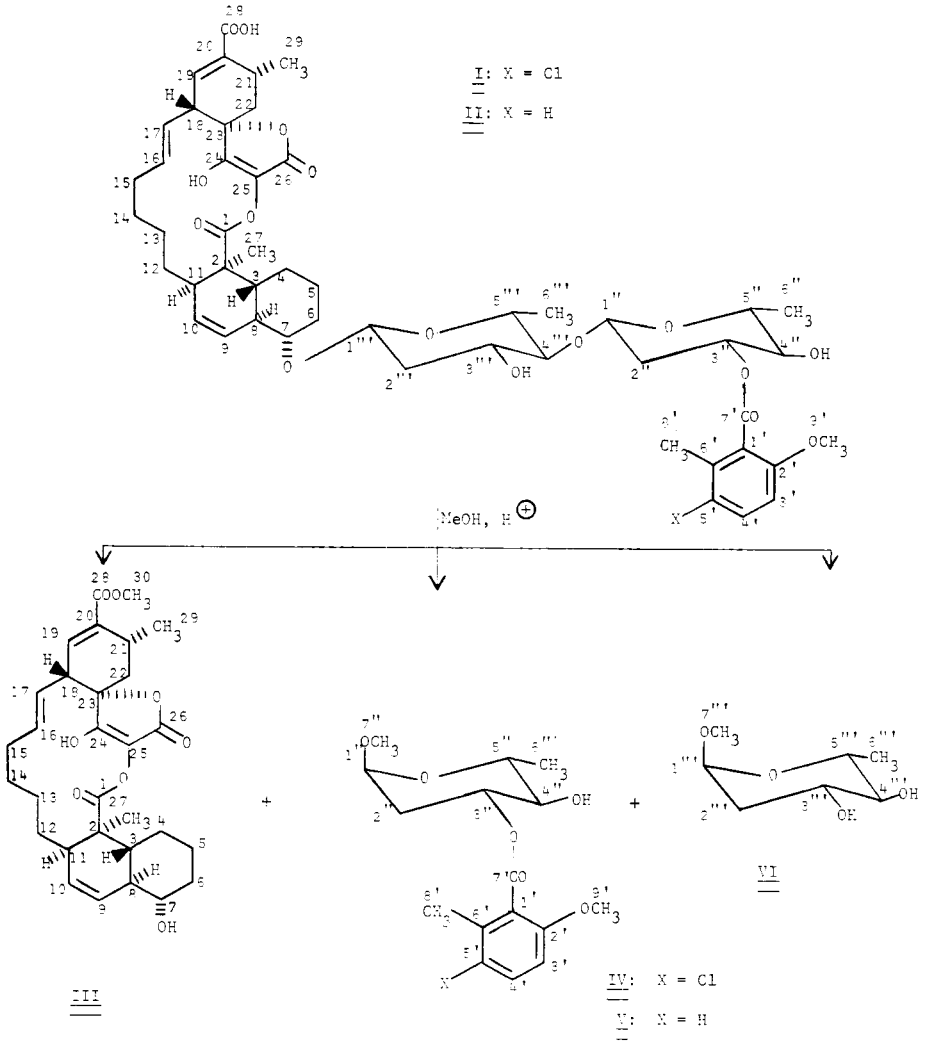


FIG. 1. Proton-Decoupled Carbon-13 Natural Abundance Spectrum of Chlorothricin (**I**) and Deschlorothricin (**II**) (4:1) in Deuterio-chloroform Solution.

Nmr spectrum (60 MHz, $CDCl_3$), . (ppm), multiplicity, no. of hydrogens, J, assignment: 1.31, d, 3H, 6 Hz, 6"-CH_3; 1.70, td, 1H, 13.0 Hz and 4.0 Hz, 2"-H_a; 2.18, m, 1H, 2"-H_e; 2.32, s, 3H, 8'-CH_3; 3.10, s, 1H, OH; 3.36 s, 3H, 7"-OCH_3; 3.78, s, 3H, 9'-OCH_3; 3.0–3.7, m, 4"-CH and 5"-CH; 4.78, dd, 1H, 3.5 Hz and 1.5 Hz, 1"-CH; 5.40, m, 1H, 3"-CH; 6.62–6.90, m, 2H, 3' and 5' CH; 7.22, t, 1H, 8 Hz, 4'-CH.

OXIDATION OF CHLOROTHRICOLIDE METHYL ESTER.—A modification of the procedure of Djerassi *et al.* (7) was used to oxidize chlorothricolide methyl ester. An aliquot of an oxidizing mixture (13.4 g CrO_3 in 25 ml of water and 12 ml of conc. sulfuric acid) was added dropwise to a stirred solution of 730 mg (1.38 mmole) **III** in 20 ml of acetone under nitrogen maintained at −10 to −15° by cooling with acetone-dry ice until the orange color of the reagent persisted for 5 min. Tlc [silica gel, chloroform-methanol (95:5)] showed that at that time the reaction was complete. Excess reagent was then destroyed by addition of 0.5 ml of isopropanol; the reaction mixture was neutralized with solid sodium bicarbonate and filtered through a layer of silica gel, which was washed with 15 ml acetone. The combined filtrate was evaporated, and the residue was recrystallized from aqueous methanol. Yield 600 mg (83%), mp 156–157°. Molecular wt found 524.237, calc. for $C_{30}H_{36}O_8$, 524.240. Ir (KBr), ν(cm^{-1}): 1780 (C=O), 1715 (C=O), 1680 (C=C), 1645 (C=C). Ms (E.I.), m/e (% rel. intensity): 525 (1.9) (M−1), 524 (2.5), 492 (3.7), 478 (3.1), 337 (8.5), 161 (100), 105 (16.2), 91 (12.5).



ISOMERIZATION OF 7-OXOCHLOROTHRICOLIDE METHYL ESTER (**VII**).—A solution of 200 mg of **VII** in 30 ml methanol and 1 ml of con. HCl was stirred under nitrogen for 10 minutes at room temperature at which time tlc [silica gel F-254, chloroform-ethanol (96:4)] indicated complete conversion. After evaporation *in vacuo* to a volume of about 5 ml an equal volume of water was added, and the precipitated product **VIIIa** was collected by filtration and washed with aqueous methanol. Yield 179 mg=90%, mp 148–149 . Molecular wt found 524.243, calc. for $C_{30}H_{36}O_8$, 524.240. Ir (KBr), ν(cm^{-1}): 1780 (C=O), 1715 (C=O), 1690 (C=O), 1645 (C=C), 1620 (C=C). Ms (E.I.), *m/e* (% rel. intensity): 525 (2.8) (M+1), 524 (3.7), 492 (3.7), 478 (2.3), 344 (8.4), 337 (6.5), 199 (12.2), 183 (100), 161 (85.4), 149 (38.5), 113 (23.5), 105 (11.2), 91 (12.2). The deuterated rearrangement product **VIIIb** was obtained in the same fashion with CH_3OD and DCl with a reaction time of 16 hours. Mass spectrometry indicated the presence of 19.4% tetradeuterated, 36.7% trideuterated, 25.1% dideuterated and 4.31 monodeuterated molecules. The deuteration reaction was not optimized because even incompletely deuterated carbon atoms give strongly altered signals in the ^{13}C nmr.

RESULTS AND DISCUSSION

The ^{13}C natural abundance spectrum of chlorothricin (fig. 1) is rather complex, in part because of the contamination with deschlorothricin (**II**), which could so far not be separated from **I**. However, the antibiotic can be cleaved by methanolysis (scheme I), and we have been able to separate the methyl 2-deoxy rhamnosides of the chlorinated and the unchlorinated benzoic acids (**IV** and **V**). Spectral analysis of the various products of the methanolysis, aided by the preparation of some derivatives and by the availability of labeled samples from bio-

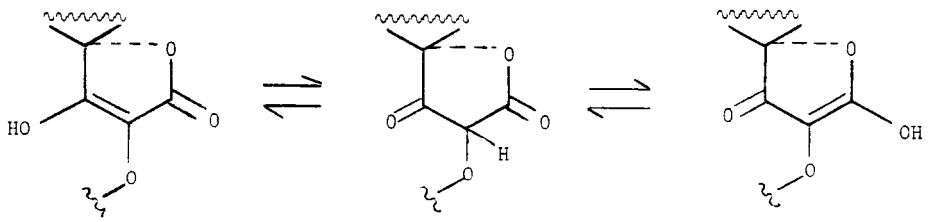
TABLE 1. ^{13}C chemical shifts[a] of the aglycone portion of chlorothricin (**I**), chlorothricolide methyl ester (**III**), 7-oxochlorothricolide methyl ester (**VII**), and the rearranged 7-oxochlorothricolide methyl ester (**VIII**) in deuteriochloroform solution.

	I	III	VII	VIII
C-1	177.3	177.5	176.4	178.6
C-2	47.5	47.4	47.4	46.4
C-3	37.9	37.6	39.6	37.4
C-4	26.5	26.6	26.2	25.0
C-5	24.3	24.1	25.5	22.2
C-6	36.1	36.2	40.7	39.7
C-7	83.0	73.3	209.0	199.4
C-8	43.7	45.4[c]	49.7	135.9
C-9	123.2	123.2	119.4	132.7
C-10	130.0	129.9	130.6	31.5
C-11	46.6	46.8	46.1	42.3
C-12	33.1	33.0	32.4[b]	33.0[b]
C-13	28.0[b]	28.0[b]	27.7[c]	27.9[c]
C-14	28.4[b]	28.4[b]	28.1[c]	28.5[c]
C-15	32.3	32.3	32.0[b]	32.5[b]
C-16	138.1[c]	138.0	137.9	137.5
C-17	124.5	124.4	124.0	124.6
C-18	46.0	45.9[c]	45.6	45.8
C-19	138.4[c]	136.5	136.3	136.5
C-20	133.6	134.0	133.8	134.0
C-21	26.9	27.1	26.9	27.1
C-22	34.9	35.0	34.7	34.9
C-23	80.4	80.4	80.2	80.4
C-24	159.4	159.4	159.3	159.5
C-25	115.5	115.5	115.1	115.5
C-26	165.0	164.9	164.7	165.0
C-27	16.7	16.6	16.2	18.2
C-28	170.1	166.5	166.2	166.4
C-29	20.7	20.7	20.5	20.7
C-30	—	51.6	51.3	51.6

[a]In ppm downfield from TMS.
[b,c,d]These values in any vertical column may be reversed.

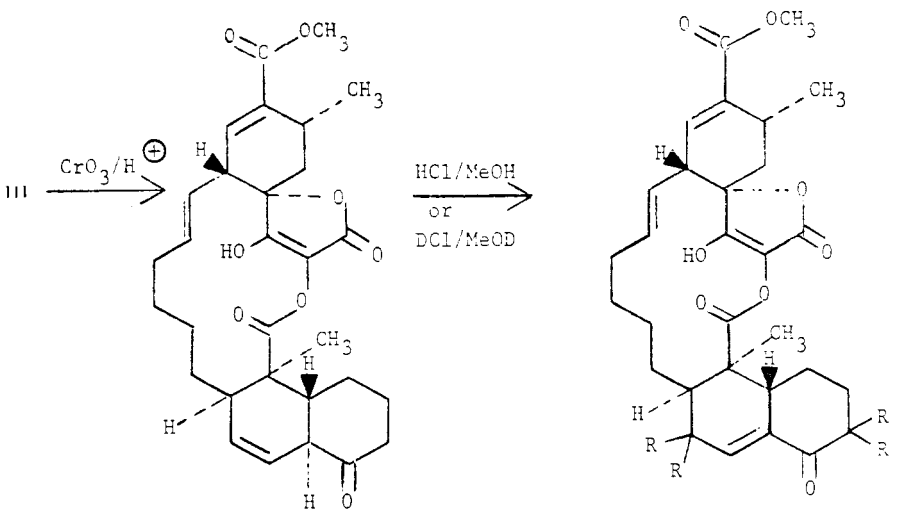
synthetic feeding experiments with $^{13}CH_3{}^{13}COONa$, has allowed the assignment of essentially all the signals in the spectrum of chlorothricin.

^{13}C SPECTRAL ANALYSIS OF CHLOROTHRICOLIDE METHYL ESTER (**III**).—The chemical shifts for the aglycone portion of **I**, for **III** and two derivatives are listed in table 1. Only two downfield carbon signals of the aglycone portion change in going from **I** (170 and 138.1 or 138.4 ppm) to **III** (166.5 and 136.5 ppm) and are, therefore, suggestive of the resonance signals of C-28 and C-19, respectively. The remaining two carbonyl carbon signals can thus be assigned to C-1 (177.5 ppm) and C-26 (164.9 ppm) (conjugated carbonyl carbon). This leaves the most downfield and the most upfield quaternary aromatic signals at 159.4 and 115.5 ppm to be allocated to C-24 and C-25, respectively, because of the tautomeric equilibrium (A⇌B⇌C) in solution:



A B C

The quaternary carbon signal at 134.0 ppm can then only be assigned to C-20. The remaining four olefinic methine carbon signals cannot be directly distinguished. However, Jones oxidation of **III** at low temperature yielded the oxo derivative **VII**, which was then rearranged to **VIIIa** or **VIIIb**:



VII

VIII a: R = H
VIII b: R = D

Analyzing the corresponding spectral changes (tables 1 and 2), we can assign the 138.0 and 124.4 ppm signals to C-16 and C-17 and the 123.2 and 129.9 ppm signals to C-9 and C-10, respectively. The C-17 signal is shifted upfield relative

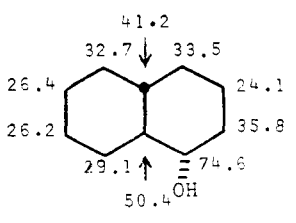
to the C–16 signal due to the γ-effect of the spiro moiety. Transforming the 7-hydroxy group of **III** into a keto function not only affects the C–7 resonance, but also influences the surrounding carbon signals (carbons 3, 4, 5, 6, 8, 9 and 10) (table 2). These carbon resonances can thus be tentatively recognized on the

TABLE 2. Differences between chemical shifts of chlorothricolide methyl ester (**III**) and 7-oxochlorothricolide methyl ester (**VII**) for the α, β and γ carbons.

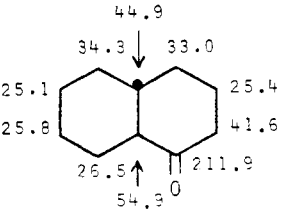
α-effect		β-effect		γ-effect
CH	CH_2	CH	CH_2	
4.3 (C–8)	4.5 (C–6)	2.0 (C–3)	1.4 (C–5) −3.8 (C–9)[a]	−0.4 (C–4)[a] 0.0 (C–2) 0.7 (C–10)

[a]Negative values denote downfield shifts relative to the parent compound.

basis of the reported (8, 9) values for the model compounds **IX** and **X**, which provide calculated values for the α-, β- and γ-effects:



IX



X

The intensity of the C–6 and C–10 signals of **VIIIb** is dramatically reduced relative to **VIIIa** due to the combined effect of quadrupolar broadening, spin-spin coupling and reduction of nuclear Overhauser enhancement resulting from deuterium substitution (10, 11). This observation verifies the peak assignments for C–6 and C–10. The deuterium labeling at C–6 and C–10 also causes intensity variations of the adjacent carbon signals (carbon 5 (52% decrease), 7, 9 (40% decrease) and 11 (25% decrease) due to modification of their relaxation times (10, 11). The rearrangement of the double bond (**VII**→**VIII**) also results in significant chemical shift changes of C–1 and C–27 relative to C–28 and C–29. The signal at 164.9 ppm can then be assigned to C–26. Based on the spectral studies of these derivatives and simple chemical shift theory (12–14), the signals at 80.4, 45.8 and 27.1 ppm can be readily assigned to C–23, C–18 and C–21 of **VIII**. These values provide the basis for the corresponding assignments of **III**. The C–22 resonance (35.0 ppm) is the methylene carbon signal showing the least variation. The C–15 resonance peak (32.3 ppm) can be distinguished from the C–12 peak (33.0 ppm) by measuring the ^{13}C–^{13}C one-bond couplings [$1J(^{13}C_{15}$–$^{13}C_{16})$: 39.6 Hz and $1J(^{13}C_{11}$–$^{13}C_{12})$: 30.6 Hz]. However, an unequivocal differentiation of C–13 and C–14 (28.0 and 28.4 ppm) cannot be achieved on the basis of previous studies on olefinic and aliphatic compounds. The assignments listed in

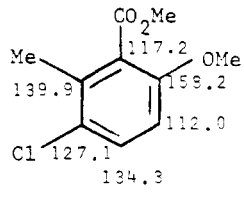
table 1 for these carbons in **I** and **II** are those supported by biosynthetic ^{13}C-labeling experiments. As predicted by biogenetic theory, C-8, C-12, C-14 and C-18 are labeled by $^{13}CHC_3OONa$; C-13 and C-15 are labeled by $CH_3{}^{13}COONa$ (15).

^{13}C SPECTRAL ANALYSIS OF THE AROMATIC AND SUGAR MOIETIES.—From the results for a large variety of substituted benzenes, the shielding values or substituent effects have become a variable. The carbon-13 resonances of substituted aromatic molecules usually have been assigned on the basis of the additivity of these substituent effects. However, this calculation often provides only approximate values, particularly for ortho-substituted compounds because of steric effects and intramolecular interactions. From the multiplicity and from the calculated values for the model compounds **XI** and **XII** (13), we can interpret the spectra of **IV** and **V** (table 3):

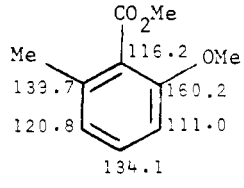
TABLE 3. ^{13}C Chemical shifts[a] of the acetyl deoxyrhamnosyl portions of chlorothricin and derivatives (**I**, **II**, **IV** and **V**).

	I	II	IV	V
C-1‴, C-1″	100.2, 101.0	100.2, 101.0	97.8	97.7
C-2‴, C-2″	34.6, 34.6	34.6, 34.6	34.9	34.9
C-3‴, C-3″	72.1, 73.8[b]	72.1, 73.8[b]	73.7[b]	73.4[b]
C-4‴, C-4″	88.2, 74.3[b]	88.2, 74.3[b]	75.2[b]	75.2[b]
C-5‴, C-5″	69.4, 70.0	69.4, 70.0	67.5	67.5
C-6‴, C-6″	17.8, 17.4	17.8, 17.4	17.6	17.6
C-7″	—	—	54.3	53.4
C-8′	16.8	19.0	16.9	19.0
C-6′	134.0	136.6	134.0	136.6
C-5′	126.8	122.6	126.8	122.4
C-4′	130.7	130.7	130.5	130.6
C-3′	109.9	108.6	109.8	108.4
C-2′	154.4	156.0	154.4	156.0
C-1′	122.8	124.1	122.6	124.0
C-7′	166.7	167.6	167.2	168.0
C-9′	56.2	56.0	56.1	55.9

[a]In ppm downfield from TMS.
[b]These values in any vertical column may be reversed.



XI



XII

The C-5′ peak (122.4 ppm) is differentiated from the C-3′ peak (108.4 ppm) by its ^{13}C–^1H three-bond coupling with H-8′. The assignments of the deoxyrhamnosyl portion are straighforward, except that C-3″ and C-4″ have not been distinguished. Since a distinction between these two closely spaced signals was not important in our ^{13}C nmr studies of chlorothricin biosynthesis, no further efforts have been made to resolve this ambiguity.

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