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A Carbon-13 Nuclear Magnetic Resonance Study of Mollisin and Its Biosynthesis

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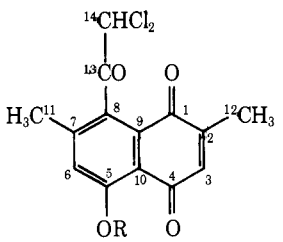
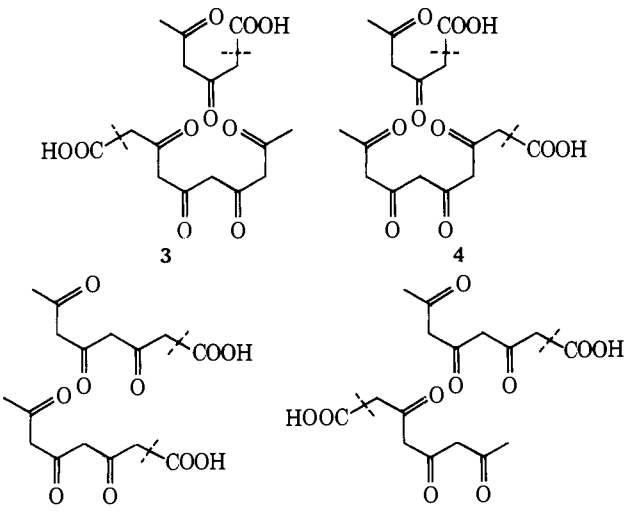
Abstract: The biosynthesis of mollisin (**1**) has been studied, utilizing both sodium [1-^{13}C]- and [2-^{13}C]acetate and sodium [1,2-^{13}C]acetate. The results confirm the biosynthetic scheme proposed by Tanabe and Seto. High levels of acetate incorporation and conversion of mollisin to the heretofore unknown mollisin acetate (**2**) made possible assignment of all ^{13}C resonance frequencies and ^{13}C–^{13}C coupling constants.

The work reported herein was undertaken with the aim of elucidating the acetate connectivity pattern in the biosynthesis of mollisin (**1**). During the course of our work a similar study of this molecule appeared[1] wherein it was concluded that the biosynthesis of mollisin had an acetate connectivity pattern consistent with biosynthesis **3** rather than alternatives such as **4**, **5**, or **6**.[2] As the carbon NMR spectra reported[1] suffered from low signal-noise (S-N) ratios, a number of important resonances and couplings germane to the problem were not observed. Since we were able to surmount the S-N difficulties (traceable to a combination of factors including low acetate incorporation, low isolated



1, R = H
2, R = $COCH_3$



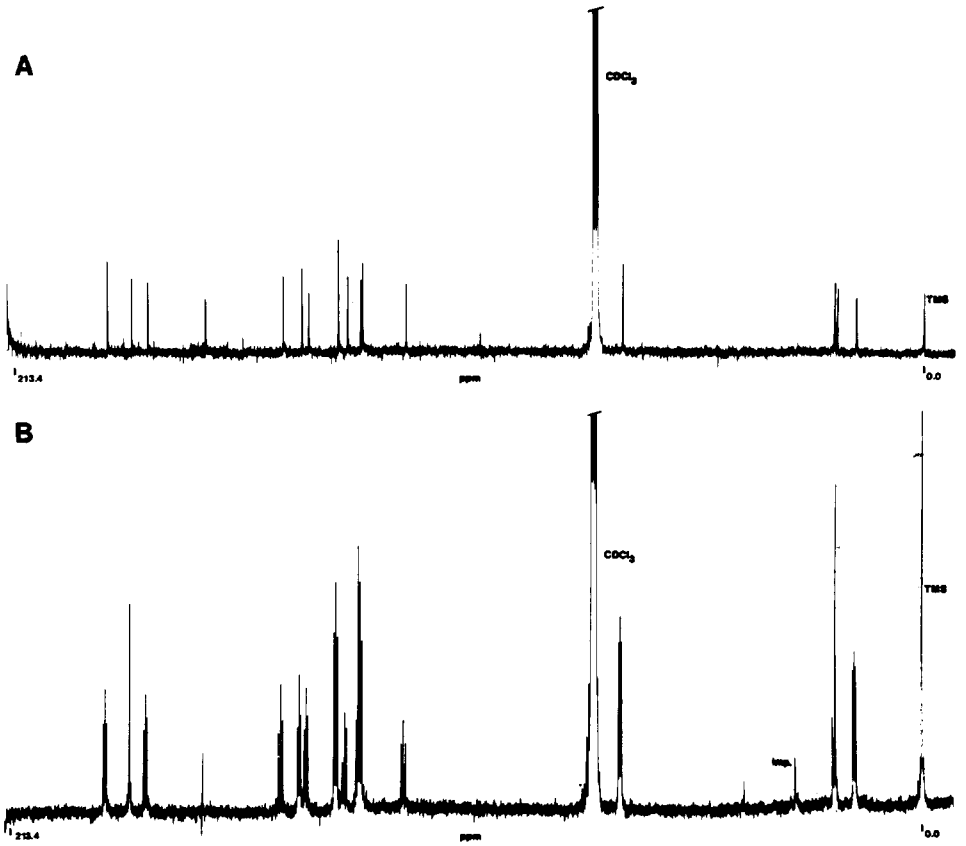


Figure 1. Proton noise-decoupled Fourier transform ^{13}C NMR spectrum of mollisin acetate at 67.92 MHz: A, from ^{13}C-natural abundance [160 mg + 45 mg of $Cr(acac)_3$], 10K transients, 1.1-s pulse delay; B, from $^{13}CH_3{}^{13}CO_2Na$ enrichment [85 mg + 18 mg of $Cr(acac)_3$], 23K transients, 3.0-s pulse delay.

yields of mollisin, the poor solubility of mollisin in NMR solvents, and long relaxation times of non-hydrogen-substituted carbons) we have pursued this problem to its conclusion and report here our results that support the original proposal by Tanabe and co-workers.[1]

Method. The basic approach used by ourselves and Tanabe and co-workers[1] is that one employing acetate-*1,2*-$^{13}C_2$ of high isotopic purity. Consider the biosynthetic incorporation of doubly labeled acetate into an acetogenin C-C(O)-C-C(O). If the level of incorporation (on a per acetate unit) is comparable to the natural abundance of ^{13}C one will observe in the ^{13}C NMR of the acetogenin a superposition of three types of peaks. (1) Singlets due to endogenously synthesized acetate units; at the low level of incorporation envisioned (1-2%) satellites arising from ^{13}C-^{13}C coupling of this carbon with either endogenous acetate or exogenous acetate-*1,2*-^{13}C will be too small to distinguish from baseline noise. (2) Doublets arising from exogenous acetate-*1,2*-^{13}C; if no ^{13}C chemical shift isotope effect is observed and $\Delta\nu \gg J$ holds for the two carbons derived from the labeled acetate, one expects a symmetrical doublet centered at the frequency of the corresponding peak in the unlabeled material. (3) Singlets arising from exogenous acetate-*1,2*-^{13}C wherein the biosynthesis of the acetogenin proceeds with either rearrangement of the carbon skeleton or cleavage and loss of one of the added acetate's carbons.

The ability to trace out acetate incorporation patterns on a two-carbon basis and detect skeletal rearrangements and cleavages is a unique property of doubly labeled acetate. It is moreover a technique that does not require accurate integration of carbon spectra.

Results and Discussion

Satisfactory carbon NMR spectra of mollisin are difficult to obtain for two reasons. (1) The compound is relatively insoluble in organic solvents. Its solubility in chloroform-*d*, the best solvent we have found, is only about 40 mg/2-3 ml. (2) The nonproton bearing carbons have as expected long relaxation times and appear as very weak signals relative to those bearing protons. Employing pulse delays on the order of 10 s did not improve the situation. These difficulties are evident in the spectra published by earlier workers.[1]

Table I. Dependence of Yield of Mollisin and Percent of Acetate Incorporation on Pulse Concentration of Acetate[a]

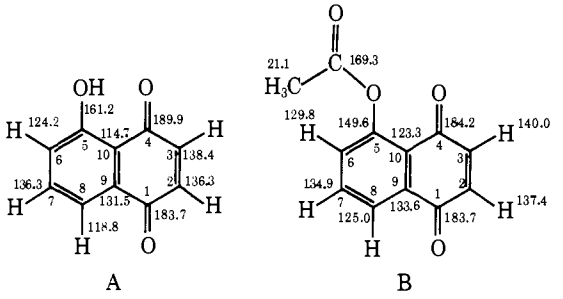
Acetate (mmol) added per 6 tubes	Mollisin produced (mmol) per 6 tubes	% incorpn of added acetate[b]
0.622	0.100	2.17
0.730	0.0831	4.62
0.876	0.0732	5.13
1.021	0.0633	6.28
1.165	0.0591	6.55

[a] Culture conditions were as described in the Experimental Section. [b] Calculated on a per acetate of mollisin basis, assuming eight acetate units per mollisin and even distribution of the exogenous acetates in the mollisin molecule.

Contrary to literature reports,[3] mollisin can be acetylated under carefully controlled conditions to afford its acetate **2** in high yield. Unlike mollisin, **2** is quite soluble in chloroform-*d* and is compatible with tris(acetylacetonato)chromium(III) [$Cr(acac)_3$].[4] Good spectra of **2** in chloroform-*d* containing 25 mol % of $Cr(acac)_3$ could be obtained with only minimal pulse delays (2-3 s). Under these conditions all of the carbon peaks of **2** are distinct and of comparable size (Figure 1, A), a necessary requisite for use of monolabeled acetate.

Culture and Isolation. Considerable effort was expended to maximize the yield and level of acetate incorporation of mollisin. It was found (Table I) that mollisin production continued well past the 2-week period reported[5] and that

Chart I. Chemical Shift Assignments for Juglone (5-Hydroxy-1,4-naphthoquinone) (A) and Juglone Acetate (B)[a]



A B

[a] The assignments of C-1 and C-4, and of C-2 and C-3, could be reversed (although the corresponding assignments in mollisin acetate are firm, see text).

mollisin production still occurred at acetate concentrations as high as 1.6 mg/0.5 ml. While a decrease in mollisin production occurred this was compensated for by increased levels of incorporation. Sufficient production and incorporation occurred at pulsing concentrations of 1.6 mg/0.5 ml that only 24 culture tubes were needed per labeling experiment.

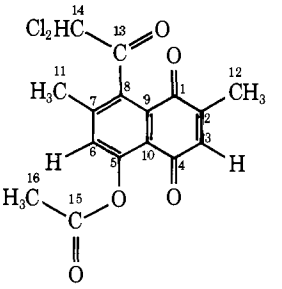
Chemical Shift Assignment. Accurate assignment of the chemical shift of each carbon of mollisin is essential to establishing which pairs of carbons originate from the same molecule of acetate and thus establishing the biosynthetic pathway. The chemical shifts of the carbons in the model compound juglone acetate (5-acetoxy-1,4-naphthoquinone) given in Chart I, B, were used as the basis for predicting the carbon shifts for mollisin acetate.[6] Parameters for ^{13}C substituent effects[8,9] were added to the chemical shift of the appropriate carbon in juglone acetate in order to obtain predicted chemical shifts for carbons 1-10 of mollisin acetate. As shown in Table II, the calculated and observed shifts agree within 2.5 ppm or less. The chemical shifts of the methyl carbons (C-11 and C-12) were assigned by single-frequency irradiation,[10] while model compound studies confirmed the assignment of carbons 13-16 (as discussed in the footnotes to Table II).

All of the tabulated chemical shifts could be made with reasonable certainty. Although C-2 and C-3 were not distinguishable in juglone acetate, in mollisin acetate, the presence of the additional methyl group (C-12) made these carbons clearly distinguishable (by chemical shift differences and by single-frequency irradiation of C-3). C-1 and C-4 also could not be definitely distinguished in juglone acetate. However, in mollisin acetate, C-1 and C-4 were distinguished by comparison of chemical shifts for the acetylated and unacetylated compound. In mollisin itself, C-1 and C-4 can be distinguished by irradiation of the phenolic proton which causes enhancement of the carbon signal for C-4 (the carbonyl whose oxygen is hydrogen bonded to the irradiated phenol). Upon acetylation of mollisin and loss of the hydrogen bond between the phenolic proton and the C-4 carbonyl oxygen, the signal for C-4 moves upfield (from 189.4 to 182.4 ppm) as expected,[11] while the signal for C-1 remains at the same chemical shift as it is in mollisin (186.1 ppm in mollisin, 186.2 ppm in mollisin acetate). A similar upfield shift of ~7 ppm was observed when juglone was acetylated and the chemical shifts of C-4 in the acetylated and unacetylated material were compared (see Chart I).

Incorporation of Singly Labeled Acetates. *Mollisia caesia* was cultured separately in the presence of sodium [1-^{13}C]- and [2-^{13}C]acetate. In each case, the resulting mollisin was chemically acetylated for NMR analysis. Because ^{13}C NMR conditions had been established under which all of the carbon signals of the unlabeled mollisin acetate were of approximately the same peak height, the spectra of the mollisin acetate labeled with [1-^{13}C]acetate and [2-^{13}C]acetate were easily analyzed. The peak height of carbon signals which contained incorporated ^{13}C were approximately 2.5-5 times the height of the other carbon signals. The results of [1-^{13}C]acetate and [2-^{13}C]acetate labeling experiments are summarized in Table III. The results clearly show that the labeling pattern in the mollisin acetate is as shown in Chart II. Six carbons originate from the C-1 of acetate and eight from C-2 of acetate (i.e., two decarboxylations are involved in the biosynthesis).

Since the extent of labeled acetate incorporation is presumably the same for all carbons, the ratio of peak heights for the [1-^{13}C]acetate-derived and [2-^{13}C]acetate-derived mollisin acetate (column 4, Table III) should theoretically be 1.00 for no incorporation, ~4.0 for [2-^{13}C]acetate incorporation, and ~0.4 for [1-^{13}C]acetate incorporation. The variation in ratio recorded in Table III is partly due to the insufficient resolution of the computer on the XL-100 spectrometer which was used to obtain these spectra (4096 words for the real part of the spectrum). At the required spectral width of 5500 Hz, the resolution is 1.47 Hz/point and since most of the carbon peaks were observed to be 2-3 Hz wide, highly accurate peak height/peak area definition could not be expected. Experimentally the variation observed between spectra in the peak height of a given signal

Table II. Chemical Shift Assignments for Carbons of Mollisin Acetate (Tabulated by the Numbering System Shown Below)



Carbon (chemical shift)[a]	Predicted/calcd chemical shift[b]	Carbon (chemical shift)[a]	Predicted/calcd chemical shift[b]
C-1 (186.2)	~184[c]	C-9 (132.3)	133.6[e]
C-2 (146.2)	148.9-146.3	C-10 (121.7)	120.4[e]
C-3 (137.5)	140.0-137.4[d]	C-11 (20.4)	20.6[f]
C-4 (182.4)	~184[c]	C-12 (16.0)	16.3[f]
C-5 (150.4)	149.6	C-13 (191.9)	~185-190[g]
C-6 (132.0)	130.5[d]	C-14 (70.8)	~68[g]
C-7 (144.6)	143.8	C-15 (169.0)	169.3[h]
C-8 (135.4)	~134	C-16 (21.0)	21.1[h]

[a] Chemical shifts were measured in $CDCl_3$ in parts per million relative to internal Me_4Si. [b] The chemical shifts observed in juglone acetate (Chart I, B) formed the basis for the predicted/calculated chemical shifts of carbons 1-10 of mollisin acetate. As appropriate, substituent effect parameters were added to the basic juglone acetate shifts to obtain the predicted chemical shifts. All substituent effect parameters (except that for methyl) were taken from Stothers, ref 9. The methyl parameter was taken from Levy and Nelson, ref 10. Since a parameter for $-COCHCl_2$ has not been determined, either the parameter for $-COCH_3$ was substituted or, in the case of potentially small effects (meta and para substituent effects), the effect of $COCHCl_2$ was ignored. [c] Carbons 1 and 4 were distinguished by comparison of the ^{13}C NMR spectrum of acetylated and unacetylated mollisin and juglone (see text). [d] Signal appears as a doublet in off-resonance decoupling experiments. [e] Signal appears as a sharp singlet in off-resonance decoupling experiments. [f] Assignment based on single-frequency decoupling of published proton resonances.[4] [g] Model compounds used for assignment were $PhCOCHCl_2$ (carbonyl carbon at 185.3 ppm, dichloromethyl carbon at 68.2 ppm) and $PhCOCH_3$ (carbonyl carbon at 196.0 ppm). See Stothers, ref 6, pp 151, 280, 288. [h] Chemical shifts identical with those observed for acetate carbons of juglone acetate (Chart I, B).

Chart II

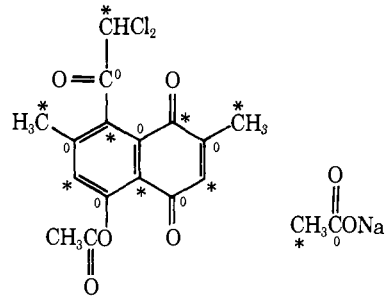


Table III. Observed Labeling Pattern in Mollisin Grown from [1-^{13}C] Acetate and from [2-^{13}C] Acetate

Carbon	Rel height of signal in mollisin grown from [1-^{13}C]-acetate	Rel height of signal in mollisin grown from [2-^{13}C]-acetate	Ratio of peak heights[a]	Derivation of C atom[b]
1	8	31	3.9	*
2	77	24	0.31	0
3	18	93	5.2	*
4	46	12	0.26	0
5	65	24	0.37	0
6	18	87	3.8	*
7	61	28	0.46	0
8	8	33	4.1	*
9	52	18	0.35	0
10	9	39	4.3	*
11	15	56	3.7	*
12	18	77	4.3	*
13	48	19	0.40	0
14	24	100	4.2	*
15	13	12	0.92	
16	20	20	1.00	

[a] The relative peak height of the signal in mollisin grown from [2-^{13}C] acetate divided by the peak height of the signal in mollisin grown from [1-^{13}C] acetate. A ratio of ~1 was established for the unlabeled acetate carbons (C-15 and C-16). The variation in the ratio from one [1-^{13}C] acetate derived (or [2-^{13}C] acetate derived) carbon to another is caused by insufficient instrumental resolution which precludes obtaining highly accurate peak heights or areas. [b] Carbons which the results indicate are derived from C-1 of acetate are labeled 0, while those derived from C-2 are labeled *

could be traced in part to where the computer points fell on a given peak.

A further obvious cause of the peak height variation is intrinsic differences in carbon relaxation times from one sample to another. These differences could be caused by different sample concentrations, different Cr(acac)$_3$ concentrations (although the same mole ratio of mollisin to Cr(acac)$_3$ was used throughout), and/or instrumental variation in spite of standard settings, etc.

Incorporation of Doubly Labeled Acetate. Analysis of the ^{13}C NMR spectrum of the mollisin acetate grown from ^{13}C doubly labeled acetate (and chemically acetylated) was more complicated than analysis of the singly labeled material (see Figure 1, B). Twelve of the 16 carbons appear as triplets showing that six molecules of doubly labelled acetate have been incorporated. (Coupling occurs only when adjacent carbons come from a single molecule of acetate.) The amount of acetate incorporation was 2.5-3% so that the ^{13}C-^{13} satellite signals of the coupled carbons appear approximately the same size as the center natural abundance peak. Therefore, the 12 coupled carbons appear to be triplets, although all of the triplets could be distinguished only in the 67-MHz ^{13}C NMR spectrum. (In the 25-MHz spectrum the triplets for the coupled pair C-8 and C-9 were not apparent because the difference in chemical shift between the carbons is close enough in magnitude to the coupling constant at this field (chemical shift Δ = 80 Hz, J = 41 Hz) that a first-order pattern was not obtained.)

Table IV. ^{13}C-^{13}C Coupling Constants for [1,2-^{13}C] Acetate Enriched Mollisin Acetate

Coupled C	J, Hz	Distance (Hz) between natural abundance center peak and each half of ^{13}C-^{13}C doublet[a]
C(2)-C(12)	44.1	+22.0, -22.1 (C-2)
C(12)-C(2)	44.1	+21.9, -22.2 (C-12)
C(3)-C(4)	55.1	+26.5, -28.6 (C-3)
C(4)-C(3)	54.7	+28.4, -26.3 (C-4)
C(5)-C(10)	71.5	+37.2, -34.3 (C-5)
C(10)-C(5)	71.0	+33.6, -37.4 (C-10)
C(6)-C(7)	58.0	+25.9, -32.1 (C-6)
C(7)-C(6)	57.9	+30.5, -27.4 (C-7)
C(8)-C(9)	~41	-20.6 (C-8)[b]
C(9)-C(8)	~41	+20.2 (C-9)[b]
C(13)-C(14)	48.3	+23.5, -24.8 (C-13)
C(14)-C(13)	48.2	+23.4, -24.8 (C-14)

[a] Positive downfield, negative upfield from center peak. These data indicate the presence of an isotope effect due to the two continuous ^{13}C molecules or an approach to an AB spin system. [b] In these cases, overlap was too extensive to measure accurately the indicated distances.

The remaining four carbons in the doubly labeled mollisin acetate appear as singlets. The singlets for the two carbons incorporated chemically from acetylation (C-15, C-16) have approximately half the intensity of the signals for the two carbons (C-1, C-11) which are incorporated biosynthetically from ^{13}C-enriched acetate with loss of a neighboring ^{13}C-labeled carbon. (Compare the intensity of carbonyl carbons C-1 and C-15 and methyl carbons C-11 and C-16 in Figure 1, B).

Using the chemical shifts given in Table II, we could establish that the following pairs of carbons are coupled and therefore come from a single molecule of doubly labeled acetate: C-2 and C-12, C-3 and C-4, C-5 and C-10, C-6 and C-7, C-8 and C-9, C-13 and C-14. Our results clearly confirm the original proposal[1] that C-1 and C-11 are uncoupled. The observed coupling constants for all the coupled carbons are tabulated in Table IV. The fact that each coupling constant could be measured and shown to be of the same magnitude for each carbon in a given pair of coupled carbons firmly establishes the biosynthetic pathway as in structure **3**.[1,12] In addition, it confirms the accuracy of the chemical shift assignments.

The wide variation in ^{13}C-^{13}C coupling constants for relatively similar sp^2-sp^2 bonds ($J_{8,9}$ = 41 Hz, $J_{5,10}$ = 71Hz) was unexpected from previous results. ^{13}C-^{13}C coupling contstants have been found to vary with substituents but not to the extent observed here.[13] Table IV, column 3, shows that the distance between the upfield ^{13}C satellite peak and the central natural abundance peak varied significantly from the corresponding distance for the downfield ^{13}C satellite peak. The observed variations are too large to be explained by a lack of instrumental resolution (which was 1.47 Hz/point in these spectra). The observed effect must be due either to an isotope effect on the ^{13}C chemical shifts or to second-order effects arising from the coupling patterns starting to assume unsymmetrical character.[14] The latter explanation is indicated by the fact that as the difference in chemical shift between coupled carbons becomes smaller, the observed assymmetry around the natural abundance center peak becomes greater.

Experimental Section

Carbon spectra were run on either a Varian XL-100-15 spectrometer operating at a carbon frequency of 25.16 MHz or a Bruk-

er WH-270 spectrometer at 67.92 MHz. Chemical shifts are in parts per million relative to internal tetramethylsilane. The computer associated with the Varian instrument yielded 4K data points in the real transformed spectrum and the computer associated with the Bruker instrument afforded 16K real data points. Standard techniques were used for single-frequency, broad-band noise, and off-resonance decoupling experiments.

Organism. *Mollisia caesia* Sacc. Sensu Sydow was obtained from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, and from Dr. M. Tanabe, Stanford Research Institute, Menlo Park, Calif. The latter culture originates from Dr. R. Bentley, University of Pittsburgh. No difference was detected in the strains.

Culture. Blakeslee malt extract[15] agar (plus sodium chloride) was prepared in the following manner. A mixture of dextrose (20 g), Bacto malt extract (Difco, 20 g), Bacto agar (Difco, 20 g), Bacto peptone (Difco, 1 g), and sodium chloride (0.5 g) in 1 l. of distilled water was autoclaved for 20 min and poured into 150 × 25 mm culture tubes (15–18 ml per tube). The slant was inoculated by smearing the surface with a small piece of mycelium and allowed to grow at 20–24° for up to 24 days.

Isolation of Mollisin. The contents of the culture tubes were mixed with water and blended. The resulting mixture was repeatedly extracted with chloroform until the organic layers were colorless. The combined organic layers were evaporated to dryness and the crude product (5–7 mg per tube) was sublimed (110–150°, 0.05–0.1 mm) as described earlier[16] to afford 3–5 mg per tube of yellow sublimate, identified as mollisin by comparison with an authentic sample.[17]

Preparation of Sodium Acetate Solutions. Sodium acetate-*2*-^{14}C (2.0 mCi/mmol), 2.1 mg, was dissolved in 100 ml of water and cold sodium acetate was added to bring the concentration up to that desired (1.6, 1.8, 2.0, 2.4, 2.8, 3.2 mg/ml). The solution was sterilized and the radioactivity was determined on a Packard Tri-Carb Model 3375 counter in Aquasol (New England Nuclear) solution. Sodium acetate-*2*-^{13}C, -*1*-^{13}C, and -*1,2*-^{13}C solutions were prepared analogously from 90% ^{13}C acetates (Merck) with added acetate-2-^{14}C as tracer.

Addition of Sodium Acetate. Solutions (0.5 ml) of known sodium acetate concentration were added daily to the culture starting at day 7. Excess liquid was removed but kept and added to the crude material during isolation. The yield of mollisin, and the percent incorporation of acetate-^{14}C as a function of acetate concentration, is shown in Table I.

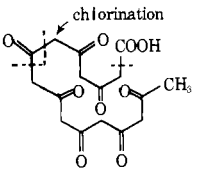
Mollisin Acetate. To a mixture of 104 mg (0.33 mmol) of mollisin and 1.0 ml (10.6 mmol) of acetic anhydride in a small test tube was added 0.8 ml (9.9 mmol) of pyridine. The test tube was shaken until solution occurred and then allowed to stand at room temperature for 5 min. During this period a precipitate of light yellow needles appeared. The mixture was poured into an excess of 2 N hydrochloric acid and worked up (chloroform) to afford 117 mg (100% yield) of mollisin acetate: mp 211–213° (methanol); NMR ($CDCl_3$, 270 MHz) δ 2.13 (3 H, d, J = 1.5 Hz), 2.44 (3 H, s), 2.48 (3 H, s), 6.38 (1 H, s), 6.74 (1 H, q, J = 1.5 Hz), 7.32 (1 H, s); ir (KBr) ν 1772, 1711, 1647, 1581, 1278, 1178, 1046, 910, 794 cm^{-1}; uv λ_{max} ($CHCl_3$) 354 mμ (2660), 272 (14 900, sh), 255 (22 250); MS *m/e* 271 ($M\cdot - CHCl_2$), 229 ($M\cdot - CHCl_2 - C_2H_2O$).

Anal. Calcd for $C_{16}H_{12}O_5Cl_2$: C, 54.24; H, 3.42; Cl, 19.76. Found: C, 54.18; H, 3.14; Cl, 19.98.

Counting Protocol. Radioactivity of sodium acetate-^{14}C (New England Nuclear) was determined by counting in Aquasol. Radioactive mollisin (~20 mg) was dissolved in 25 ml of chloroform. A 25-μl. aliquot was added to 5 ml of toluene-based scintillation fluid (5 g of PPO, 0.066 g of POPOP per liter of toluene) and counted. Self-quenching was corrected for by means of a concentration–count plot.

References and Notes

(1) H. Seto, L. W. Cary, and M. Tanabe, *Chem. Commun.*, 867 (1973).
(2) McInnes and Wright have independently suggested the biosynthetic connectivity pattern as in structure i, this clearly being consistent with our labeling data. Since very accurate integration methods, much higher levels of incorporation, and rate of incorporation studies would be necessary to decide between the two alternatives (these being very difficult without a cell-free system), application of Occam's razor allows this to be classified as a null hypothesis [see A. G. McInnes and J. L. C. Wright, *Acc. Chem. Res.*, **9**, 313 (1975)].
(3) G. J. M. van der Kerk and J. C. Overeem, *Recl. Trav. Chim. Pays-Bas*, **76**, 425 (1957).



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(4) O. A. Gansow, A. R. Burke, and W. D. Vernon, *J. Am. Chem. Soc.*, **94**, 2552 (1972).
(5) M. Tanabe and H. Seto, *Biochemistry*, **9**, 4851 (1970).
(6) The chemical shifts for juglone acetate were assigned by use of the model compound 1,4-naphthoquinone,[7] with addition of substituent effect parameters[8,9] to predict chemical shifts, and by use of single-frequency decoupling. The assignment of carbons 1 and 4 is based on a comparison of juglone and juglone acetate (Chart I); in the former compound, C-1 and C-4 were distinguished by irradiation of the phenolic proton and observation of the enhanced carbon (see text).
(7) L. F. Johnson and W. C. Jankowski, "Carbon-13 NMR Spectra", Wiley-Interscience, New York, N.Y., 1972, p 366.
(8) J. B. Stothers, "Carbon-13 NMR Spectroscopy", Academic Press, New York, N.Y., 1972, from which the majority of parameters were taken.
(9) G. C. Levy and G. L. Nelson, "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists", Wiley-Interscience, New York, N.Y., 1972, p 81.
(10) The ^1H NMR spectrum of mollisin has been assigned. See ref 5.
(11) For a discussion of the effect of hydrogen bonding on carbon chemical shifts, see F. A. Bovey, "Nuclear Magnetic Resonance Spectroscopy", Academic Press, New York, N.Y., 1969, p 233, and ref 6, p 495.
(12) Bentley and Gatenbeck had previously proposed other biosynthetic pathways for mollisin based on ^{14}C-tracer experiments: R. Bentley and S. Gatenbeck, *Biochemistry*, **4**, 1150 (1965).
(13) Reference 8, p 375.
(14) K. B. Wiberg and B. J. Nist, "Interpretation of NMR Spectra", W. A. Benjamin, New York, N.Y., 1962, p 3.
(15) The American Type Culture Collection Catalog of Strains, 11th ed, 1974, medium number 325.
(16) See Bentley and Gatenbeck in ref 10.
(17) We thank Professor R. Bentley for this sample.