Acknowledgments

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Biosynthetic Studies with Carbon-13: Mollisin*

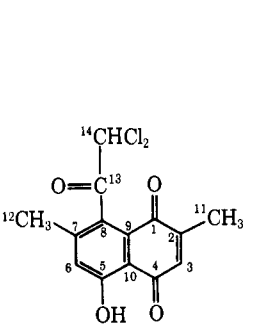
Masato Tanabe† and Haruo Seto‡

ABSTRACT: The biosynthesis of mollisin has been studied using nuclear magnetic resonance spectroscopy techniques. Isotope-enriched mollisin was produced from [2-^{13}C]sodium acetate. Measurement of the position and intensity of the ^{13}C-proton satellite bands and J^{13}C-H in the proton nuclear magnetic resonance spectrum of the labeled mollisin was accomplished with a time-averaging computer. These spectral data show that carbons-3, -6, -11, -12, and -14 of mollisin are derived from the methyl group of acetate. These positions all incorporate acetate at approximately equal intensities, indicating that the biosynthetic pathway of mollisin is from two simultaneously formed polyketide chains which cyclize to mollisin. Confirmation of the level of isotope enrichment in labeled mollisin was achieved by mass spectrometry. The results reported here are in agreement with previous studies of the biosynthesis of mollisin using ^{14}C techniques.

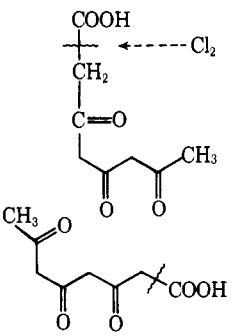
Mollisin (I) is a yellow napthoquinone pigment produced by the mold *Mollisia caesia* grown on solid malt agar medium. Bentley and Gatenbeck (1965) have studied the biosynthesis of mollisin using ^{14}C-labeled substrates. Since radioactive methionine was not incorporated into the pigment, the C_{11} and C_{12} methyl groups are not biosynthetically introduced by methylation of a naphthoquinone unit. The observed incorporation of ^{14}C-labeled acetate and malonate into mollisin can be explained by a biogenetic scheme involving two separate polyketide units.

Bentley and Gatenbeck (1965) obtained evidence for the acetate origin of the C_{11} and C_{12} methyl groups by Kuhn–Roth oxidation of the pigment, which yielded radioactive acetic acid along with nearly the expected level of labeled carbon dioxide from the remaining carbon atoms.

The two hypothetical tetraacetate chains involved in mollisin biosynthesis can be oriented in two ways. Bentley and Gatenbeck prefer the arrangement shown in II since it afforded a rational biochemical route for the introduction of the unique dichloracetyl side chain present in mollisin.



I



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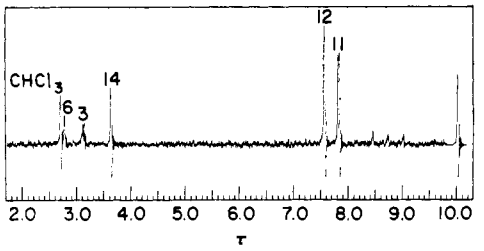

FIGURE 1: Nuclear magnetic resonance spectrum (60 MHz) of mollisin (I), 18 mg in 0.5 ml of deuteriochloroform. The numbers refer to the proton-bearing carbons (see structure I).

Two separate polyketide chains are also involved in the biosynthesis of the antibiotics citromycetin (Gatenbeck and Mosbach, 1963) and rotiorin (Holker *et al.*, 1964). Studies with ^{14}C showed that the two polyketide chains involved in the biosynthesis of citromycetin were nearly equally labeled, whereas the level of radioactivity was higher in the acetoacetate unit of the β-keto lactone in rotiorin than in the main chain.

An interesting feature of mollisin biosynthesis that remains undetermined is the extent of labeling of both polyacetate units. This problem can be conveniently investigated by the use of [2-^{13}C]sodium acetate as the substrate. The position and intensity of the ^{13}C-proton satellite bands in the nuclear magnetic resonance spectra of isotopically enriched mollisin yield information on the position of isotope incorporation and the extent of labeling. This nuclear magnetic resonance method has been successfully applied to studies of the biosynthesis of griseofulvin (Tanabe and Detre, 1966), variotin (Tanabe and Seto, 1970a), piercidin (Tanabe and Seto, 1970b), fusaric acid (Desaty *et al.*, 1968), and sepedonin (McInnes *et al.*, 1969).

Materials and Methods

[2-^{13}C]Sodium acetate (56%) was obtained from Merck, Sharp and Dohme (Canada). The mollisin-producing organism *Mollisia caesia* was obtained from Professor Ronald Bentley and maintained at 24° on slopes of agar (Krainsky). Higher yields of mollisin were obtained on this Krainsky medium supplemented with 0.25 g/l. of sodium chloride than on the malt Oxoid medium previously employed.

For inoculations, pieces of mycelium and agar about 2 mm^2 were transferred to culture test tubes with screw caps (1.5 × 15 cm), each containing slants of 10 ml of Krainsky–NaCl medium. After inoculation (6 or 7 days), an aqueous solution of [2-^{13}C]sodium acetate (0.8 mg/0.5 ml) was added to each culture tube. On the next day the added sodium acetate solutions were removed with a sterile micropipet from the slants and replaced by fresh solutions. This procedure was repeated for 6 days.

The labeled mollisin was extracted from the slants with chloroform and purified by crystallization from hot methanol: mp 201–202°, lit. mp 202–203° (Van de Kerk and Overeem, 1957). From a total of 50 culture tubes, 19 mg of purified mollisin, homogeneous by thin-layer chromatography in several solvent systems, was obtained. We observed that the addition of sodium acetate solutions of concentrations higher than 0.8 mg/0.5 ml to *M. caesia* not only stopped production of mollisin, but also inhibited growth of the organism. This limitation prevented us from obtaining high incorporation yields of ^{13}C into mollisin.

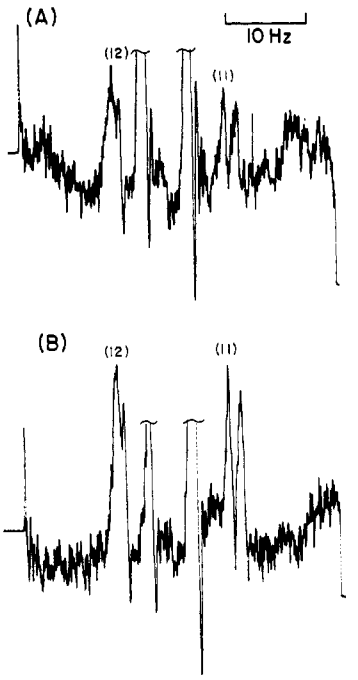

FIGURE 2: C-1024 readout showing ^{13}C–H satellite resonances of carbons-11 and -12 of mollisin; 30 mg/0.5 ml of CD_2Cl_2. (A) Unlabeled mollisin; (B) [^{13}C]CH_3COONa-labeled mollisin. Each readout represents 500 scans at 50 sec/scan, sweep width 40 Hz, sweep offset 100 Hz, readout 2^{12} at 100 sec.

Measurement of Nuclear Magnetic Resonance Spectra. The proton nuclear magnetic resonance spectra of mollisin were obtained on a Varian A-60A spectrometer. For observation of the satellite peaks, the spectrometer was equipped with a Varian C-1024 time-averaging computer for accumulation of the spectral data. Peak areas were determined by means of a planimeter.

Mass Spectrometry. Mass spectrum measurements were obtained on a CEC Model 21-110B spectrometer at an ionization potential of 70 eV with the samples introduced by a direct insertion probe. Peak intensity measurements were taken from slow oscillograph scans of the relevant peaks.

Results and Discussion

Mollisin presents a useful application of this nuclear magnetic resonance technique for biosynthetic studies, since all of the carbons bearing hydrogen atoms that can be observed in the proton nuclear magnetic resonance spectrum are derived from the methyl group of acetate. The proton resonances are fortuitously positioned (Figure 1) for observation of most of the respective upfield and downfield ^{13}C satellite bands. The only uncertainty in the interpretation of the nuclear magnetic resonance spectrum of mollisin is the proper identification of the C_{11} and C_{12} methyl groups and the C_3 and C_6 vinyl protons. These chemical shifts in the mollisin nuclear magnetic resonance spectrum were assigned with the aid of the nuclear magnetic resonance spectrum of a model

compound, 2-methyl-1,4-naphthoquinone, in which the 2-methyl group appears at τ 7.85 as a doublet and the C_3-proton at τ 3.24 as a quartet. Comparable chemical shifts and multiplicities are apparent for C_{11} and C_3 protons in mollisin. The C_{14} proton appears as expected as a singlet at τ 3.67.

The distribution of ^{13}C in the labeled mollisin was determined by comparison of the satellite peak areas with an unlabeled sample of mollisin, since an internal unlabeled proton standard is not available in the molecule. The satellite peaks were identified by their positions relative to the parent proton peak and their band shapes, as is illustrated in Figure 2 which shows the accumulation of 500 spectral scans of the downfield satellites of the C_{11} and C_{12} methyl groups. The nuclear magnetic resonance data, presented in Table I, provide direct evidence for ^{13}C isotope incorporation into five carbon atoms of mollisin (C_3, C_6, C_{11}, C_{12}, and C_{14}) and fully corroborate that these carbon atoms are derived from the methyl group of acetate. Isotope enrichment level of the labeled mollisin was determined by mass spectrometry. The mass spectrum of mollisin showed a base peak at 229 (M − 83) which represents the loss of $CHCl_2$. Comparison of this peak of labeled and unlabeled pigment showed a difference of 5.6 or 0.8% excess at each of seven labeled carbons assuming equal distribution in the fragment ion. This corresponds to a total isotopic excess of 1.9% per carbon including natural abundance. The average value of ~2% found by nuclear magnetic resonance is in agreement with the mass spectral data.

The incorporation yield of the five carbon atoms observed indicates that both polyketide chains, the one commencing from the C_{11} methyl and the other from the C_{12} methyl, are equally labeled. These results also add further support to the polyketide origin of mollisin.

This ^{13}C biosynthetic study supplements and amplifies the data available from the ^{14}C biosynthetic study of mollisin.

TABLE I: Nuclear Magnetic Resonance Data on Carbon-13-Enriched Mollisin.

Position	Shift (τ)	$J_{^{13}C-H}$ (Hz)[a]	Atom %[b] Excess
C_{11}-3H[c]	7.85	128 downfield	2.2
C_{12}-3H[c]	7.60	130 downfield	1.9
C_3-H[d]	3.17	160 upfield[e]	2.3
		174 downfield	2.1
C_6-H[d]	2.80	160 upfield	1.8
		158 downfield	1.8
C_{14}-H[d]	3.67	174 upfield[e]	2.3
		188 downfield	2.0

[a] 500 Scans for observation of each satellite band. [b] These values were approximated by comparison of the peak satellite areas with the corresponding areas from unlabeled material. Standard deviation is ±0.4 as determined by repeated determinations of the same satellite at different times. [c] Determined in deuteriomethylene chloride (4% solution). Overlapping signals from an impurity in the labeled mollisin obscured the upfield satellite band of these methyl groups. [d] Determined in deuteriochloroform (4% solution). [e] The 14-Hz difference in upfield and downfield shifts of these bands is probably due to error in recording absolute positions of resonances from the C-1024 computer in readout.

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