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^{13}C NUCLEAR MAGNETIC RESONANCE SPECTRA OF AFLATOXIN B_1 DERIVED FROM ACETATE

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Abstract—The labeling distribution of the fungal metabolite, aflatoxin B_1, produced from [1- or 2- ^{13}C] acetate was determined by ^{13}C FT NMR. The results support the polyketide hypothesis for aflatoxin biogenesis.

INTRODUCTION

RECENT advancement in the ^{13}C magnetic resonance (CMR) technique has greatly facilitated studies of the biosynthesis of microbial and plant metabolites.[1] The technique allows determination of the labeling pattern of a product derived from a specifically ^{13}C- labeled precursor without the need for extensive chemical degradation required with ^{14}C labeling. Some examples of successful application include biosynthetic studies on sterigmatocystin, ochratoxin A, shanorellin, asperentin, rifamycin, mollisin[2-7] and the number of reports is increasing rapidly.

We report herein the determination of the labeling distribution in the toxic fungal metabolite aflatoxin B_1, synthesized from [1- or 2- ^{13}C] acetate by the fungus *Aspergillus parasiticus*. Our results support those obtained by the chemical degradation studies using ^{14}C-labeled acetate[8] which constituted important experimental evidence for the polyketide hypothesis for aflatoxin biosynthesis. In addition, it allows for assignment of label sources for skeletal carbon atoms of aflatoxin B_1 (C-8, 10 and 12) not included in the assignments of Biollaz *et al.*[8]

EXPERIMENTAL

[1- ^{13}C]- and [2- ^{13}C] acetate 50–60% enriched in ^{13}C were purchased from ICN, Cleveland, Ohio. The natural abundance spectra were of aflatoxin B_1 purchased from Calbiochem, La Jolla, Calif. and recrystallized in benzene.

^{13}C-Aflatoxin B_1. Incorporation of ^{13}C-acetate into aflatoxin B_1 was accomplished by using the resting cell culture of *Aspergillus parasiticus* ATCC15517 according to the method of Hsieh and Mateles.[9] The ^{13}C-labeled aflatoxins were purified by column chromatography on acid-washed aluminum oxide.[10] A gradient solvent system of benzene and chloroform was used, beginning with 100% benzene and continuing to 100% chloroform in 5% increments. The aflatoxins were eluted when the mixture reached a 50:50 ratio.

The ^{13}C- precursors were appropriately spiked with measured amounts of [1-^{14}C] or [2-^{14}C] acetate to monitor the incorporation efficiency using scintillation counting (Packard Tri Carb Model 2405). The radioactivity in the labeled aflatoxin B_1 revealed that the relative isotope content[11] of the products were 5·5 for the [1-^{13}C] acetate-derived and 3·0 for the [2-^{13}C] acetate-derived aflatoxin B_1. Using these values, the theoretical maximum for each product (9·0 for [1-^{13}C] acetate-derived and 7·0 for [2-^{13}C] acetate-derived aflatoxin B_1[8]) and the ^{13}C enrichment level of the acetate precursor (50–60%), the average percent of ^{13}C atoms present was computed to be 31–37% in the former and 21–26% in the latter product.

^{13}C magnetic resonance measurements. A JEOL PS-100 Fourier Transform NMR, operating at 25·2 MHz in the ^{13}C mode, with a Digilab NMR-3 data system was used. It was operated with the probe at an ambient temp of ~24° with $CDCl_3$, the solvent, used as the deuterium lock source. Concentrations are noted in Figs 1–4. All chemical shifts are reported as δ(ppm) downfield from TMS.

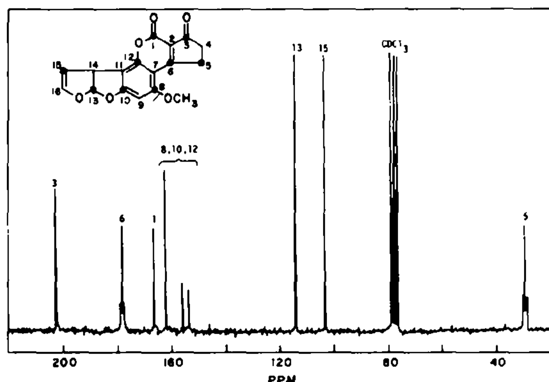

Fig. 1. CMR spectrum of aflatoxin B_1 derived from [1- ^{13}C] acetate. Concentration 0·016 M in $CDCl_3$, 16 K data points, 10KHz BDW, 10KHz filter, 20,030 pulses, 0·82 sec. pulse spacing, 15–20° pulse angle.

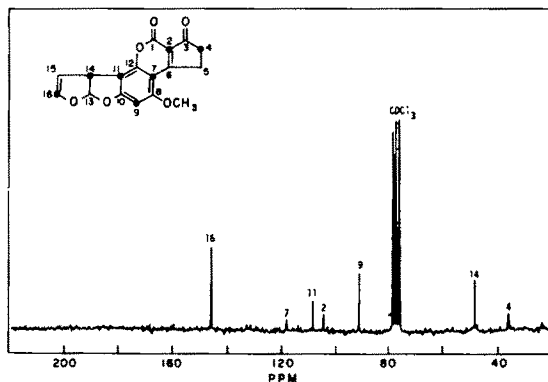

Fig. 2. CMR spectrum of aflatoxin B_1 derived from [2-^{13}C] acetate. Concentration 0·011 M in $CDCl_3$, 8 K data points, 10KHz BDW, 10KHz filter, 1,000 pulses, 0·41 sec. pulse spacing, 35° pulse angle.

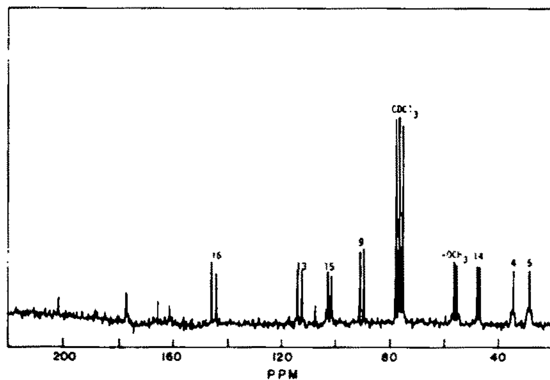

Fig. 4. CMR spectrum of natural abundance aflatoxin B_1 off resonance decoupled. Concentration 0·072 M in $CDCl_3$, 8 K data points, 10KHz BDW, 10KHz filter, 35,914 pulses, 0·41 sec. pulse spacing, 35–40° pulse angle.

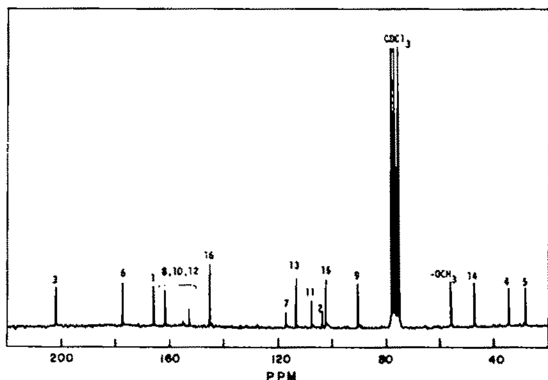

Fig. 3. CMR spectrum of natural abundance aflatoxin B_1. Concentration 0·072 M in $CDCl_3$, 8 K data points, 10KHz BDW, 10 KHz filter, 5,599 pulses, 0·41 sec. pulse spacing, 45° pulse angle.

RESULTS

The [1-^{13}C] acetate-derived aflatoxin B_1 spectrum (Fig. 1) shows nine enriched carbons. Similarly, the [2-^{13}C] acetate-derived aflatoxin B_1 spectrum (Fig. 2) shows seven enriched carbons. Assignment of the resonances in Figs 1 and 2 was aided by a natural abundance ^{13}C spectrum (Fig. 3) and an off resonance decoupled natural abundance spectrum (Fig. 4). The multiplet patterns in Fig. 4 indicate the presence of the following classes of protonated carbons; a methyl carbon at 56·5 ppm, two methylene carbons at 29·0 and 35·0 ppm, and five methine carbons at 47·9, 90·8, 102·6, 113·5 and 145·2 ppm. The remaining singlets indicate quarternary carbons.

The resonances for C-8 through C-16 were assigned on the basis of previous work on sterigmatocystin[2] (Table 1). The chemical shifts are in close agreement with those expected in most cases; however, we found it necessary to reverse the assignments for C-13 and C-15 (C-14 and C-16 in sterigmatocystin) because of the observed coupling constants for these peaks in a 400 Hz sweep width expansion of the spectrum of [1-^{13}C] acetate-derived aflatoxin B_1. The signal at 102·6 ppm does not show the shoulders (approximately 6 Hz) found on the signal at 113·5 ppm. Since C-15 can have only one geminal coupling and the signal at 113·5 ppm clearly shows an additional coupling not present in the signal at 102·6 ppm, the downfield signal (113·5 ppm) must be C-13 and the additional coupling is with C-10. Further assignments were based on other model compounds. C-3, C-4 and C-5 have chemical shifts very close to those of carbons 1, 2 and 3, respectively, of 1-indanone.[12] The signal at 103·9 ppm in the [2-^{13}C] acetate-derived aflatoxin B_1 spectrum was attributed to C-2 which compares well with C-2 of radicinin.[13]

The resonance at 177·2 ppm was initially assigned to C-1 on the basis of the chemical shifts found for model compounds. It was, however, later assigned to C-6 after analysis of observable coupling constants; $J_{5,6}$ = 34·0 Hz and $J_{3,6}$ = 11·4 Hz. There also appeared to be a coupling of about 10·5 Hz between C-6 and C-1.

The use of coupling constants for positive assignment of peaks was only possible with C-13, C-15 and C-6 in the [1-^{13}C] compound. Enhancement of resolution and homonuclear spin decoupling capability should make possible complete determination of coupling constants in the near future. No coupling constants were available from the [2-^{13}C] acetate-derived aflatoxin B_1 due to the limited quantity of the sample; it is important to note, however, that this spectrum was complementary to the spectrum of the [1-^{13}C]-derived sample.

This work indicates clearly the inherent difficulties in assigning peaks solely on the basis of comparison of chemical shifts of model compounds. Ideally, the combination of chemical shift and coupling constants should be used for absolute identification.

It was possible to estimate the enrichment of C-13 and C-15 from the [1-^{13}C] acetate-derived aflatoxin B_1 proton spectrum. By comparing the relative areas of the ^{13}C satellites to the base peak, an enrichment of approximately 37% was established, in good agreement with the average enrichment (31–37%) calculated by ^{14}C spiking. It

Table 1. ^{13}C Chemical shifts for aflatoxin B_1 and model compounds

Aflatoxin B_1			Model Compound			
Carbon	Source	δ	Compound	Carbon	δ	Reference
1	$^{13}C(1)$	165.6	COUMARIN	2	160.4	12
2	$^{13}C(2)$	103.9	RADICININ	3	98	13
3	$^{13}C(1)$	201.4	1-INDANONE	1	206.2	12
4	$^{13}C(2)$	35.0	1-INDANONE	2	36.0	12
5	$^{13}C(1)$	29.0	1-INDANONE	3	25.6	12
6	$^{13}C(1)$	177.2	COUPLING CONSTANTS*	--	--	--
7	$^{13}C(2)$	117.4	COUMARIN	10	118.7	12
8, 10, 12	$^{13}C(1)$	153-162	STERIGMATOCYSTIN	8, 10, 12	154-164	2
9	$^{13}C(2)$	90.8	STERIGMATOCYSTIN	11	91	2
11	$^{13}C(2)$	107.7	STERIGMATOCYSTIN	9	104-112	2
13	$^{13}C(1)$	113.6	COUPLING CONSTANTS*	--	--	--
14	$^{13}C(2)$	47.9	STERIGMATOCYSTIN	15	49	2
15	$^{13}C(1)$	102.6	COUPLING CONSTANTS*	--	--	--
16	$^{13}C(2)$	145.2	STERIGMATOCYSTIN	17	146	2

*see text for explanation

should be noted that this value is only approximate due to the inherent errors of integration. The overlaping of proton signals with ^{13}C satellites prevented determination of enrichment at other positions. Because pulse spacings were used which are short relative to the T_1's, and because of the differential relaxation rates of a major peak and its satellites, percent determination of the ^{13}C enrichment by integration of the ^{13}C spectra was not possible.

DISCUSSION

The labeling pattern of aflatoxin B_1 derived from the labeled acetates (Figs 1 and 2) is consonant with that determined by specific chemical degradations reported by Biollaz *et al.*[8] In the latter study, however, only 13 of the 16 skeletal carbons in the aflatoxin B_1 molecule were defined. The result of our study, therefore, offers a complete confirmation of the precursor-product relationship of acetate and aflatoxin B_1.

Establishment of the CMR spectrum of aflatoxin B_1 should facilitate a similar analysis of ^{13}C- labeled aflatoxin G_1, another carcinogenic mycotoxin produced by *Aspergillus parasiticus.* It should also allow for determination of the labeling pattern of aflatoxins derived from ^{13}C-malonate. Comparison of ^{13}C- acetate and ^{13}C- malonate incorporation into aflatoxin B_1 will yield useful information concerning the number of polyacetate chains involved in aflatoxin biosynthesis and the mechanism of assembly of the polyacetate chain. Both studies are in progress.

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