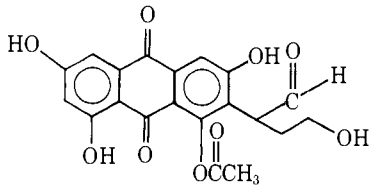
Communications to the Editor

Carbon-13 Nuclear Magnetic Resonance Studies of the Structure and Biosynthesis of Versiconal Acetate

Sir:

Previous reports have shown that aflatoxin B_1 production by *Aspergillus flavus* and *A. parasiticus* was greatly reduced in the presence of the insecticide dichlorvos.[1-3] The reduction in aflatoxin production was accompanied by the concomitant appearance of an orange pigment which was tentatively identified as versiconal acetate (**1**).[3] We wish to report a revised structure for versiconal acetate based on ^{13}C NMR studies and a ^{13}C NMR study of the biosynthesis of versiconal acetate using ^{13}C labeled acetate. The results suggest that versiconal acetate is an intermediate in the biosynthesis of aflatoxin B_1 and the versicolorins.



I

The proton decoupled, natural abundance ^{13}C NMR spectrum of versiconal acetate[4] is given in Figure 1A.[5] Careful examination of this spectrum reveals several aspects which are not consistent with the spectrum expected from the previously reported structure (I) of versiconal acetate. First, there is no absorption in the aldehyde carbonyl region of the spectrum. Second, there are more peaks in the spectrum than would be expected. Repeated recrystallization of versiconal acetate yielded material which gave spectra identical with that in Figure 1A. Furthermore, this material appeared pure by TLC (silica gel).

Examination of the ^{13}C spectra of several anthraquinones[6] and other spectral data[3] indicate that the basic anthraquinone structure suggested for versiconal acetate is correct. However, calculated chemical shifts for the side chain carbons using known substituent effects[7] and the previously reported ^1H NMR data[3] indicate that the acetate group is located on the side chain primary alcohol and not on the anthraquinone ring. The lack of an aldehyde carbonyl peak in Figure 1A and the appearance of a peak at 113.0 ppm characteristic of a five-membered hemiacetal carbon indicated that versiconal acetate exists in the form of a five-membered ring hemiacetal involving the phenolic hydroxy group on C-3 and/or C-1 of the anthraquinone ring. The previous report of an aldehyde absorption in the ^1H NMR spectrum of the methylated derivate of I probably resulted from opening of the hemiacetal ring during methylation.

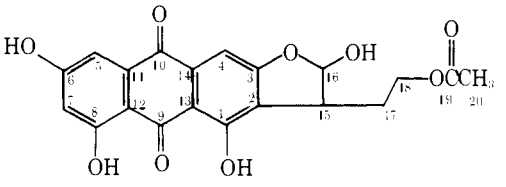
The quinone carbonyl region of Figure 1A (180–190 ppm) provides some valuable information. The four "extra peaks" in the ^{13}C spectrum at 180.9, 181.7, 184.9, and 189.0 ppm can be explained as follows. Examination of the ^{13}C spectra of several related hydroxy anthraquinones[6,8] shows that hydrogen bonding between one hydroxy group and the carbonyl group produces a downfield shift of ~5 ppm for the ^{13}C chemical shift of the carbonyl carbon whereas hydrogen bonding with two hydroxy groups produces a downfield shift of ~10 ppm. Based on these data, the four peaks in the quinone carbonyl region of Figure 1A indicate the presence of two carbonyl carbons not involved in hydrogen bonding, one carbonyl carbon hydrogen bonded to one hydroxyl group, and one carbonyl carbon hydrogen bonding with two hydroxyl groups.

Table I. Carbon-13 Chemical Shifts[a] of Versiconal Acetate and Versicolorin C[5]

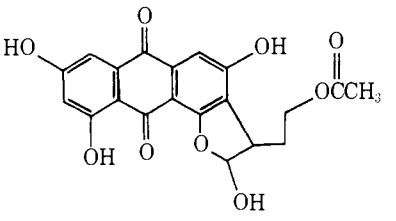
Carbon/compound	IIa	III
1	159.1	158.6
2	120.5	119.7
3	164.2	165.1
4	103.3	100.9
5	108.8	108.6
6	165.1	164.8
7	107.	107.6
8	164.2	163.8
9	188.9	188.4
10	180.9	180.1
11	134.7	134.8
12	107.9	107.9
13	110.0	110.4
14	134.7	134.2
15	43.6	43.3
16	113.0	112.9
17	28.9	30.0
18	61.7	66.9
CH_3	20.5	
C=O	170.1	

[a] In ppm downfield from Me_4Si.

All of the above data and that reported previously[3] suggest that versiconal acetate exists as a ~60:40 mixture of the two hemiacetal forms (IIa and IIb). Individual peaks are observed



IIa



IIb

for many of the carbons of IIa and IIb. It is interesting that the 100-MHz ^1H NMR spectrum of versiconal acetate gives no indication of a mixture of isomers. Chemical studies also support the basic carbon skeleton suggested for versiconal acetate (II). Both acid and base hydrolysis of versiconal acetate result in a compound which is identical in all respects with versicolorin C[9] (III, Figure 1C, Table I). Apparently, under the hydrolysis reaction conditions, IIa and IIb are in equilibrium such that only one of the two possible bisfuran ring compounds is formed. The yield from the hydrolysis reaction

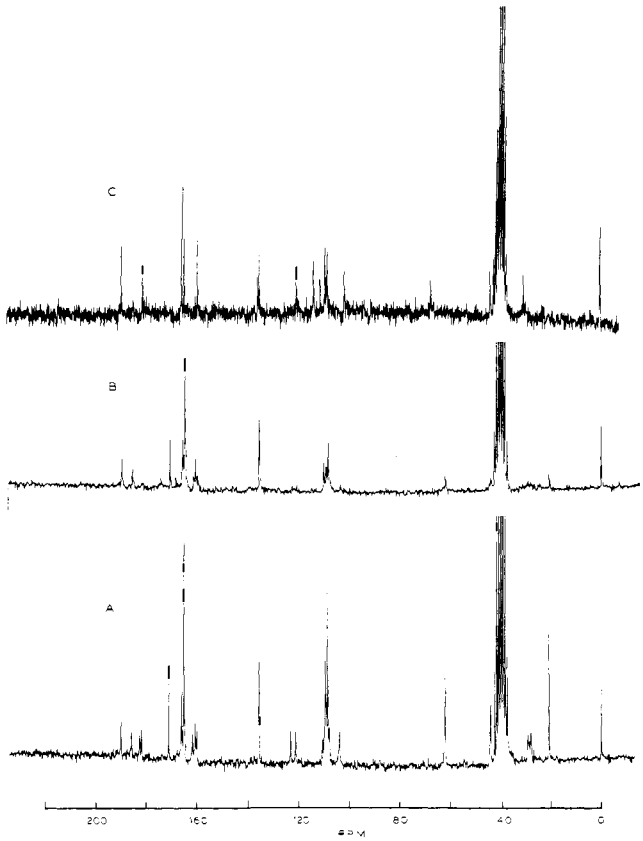
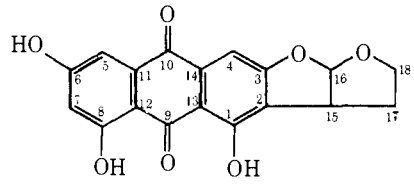


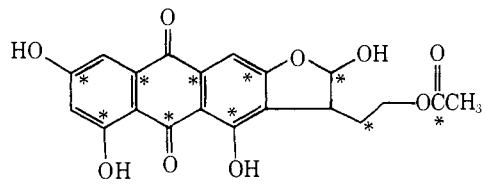
Figure 1. (A) The ^{13}C NMR spectrum of versiconal acetate in Me_2SO. (B) The ^{13}C NMR spectrum of versiconal acetate (Me_2SO) produced when the culture was supplemented with $^{13}C_1$ acetate. (C) The ^{13}C NMR spectrum of versicolorin C in Me_2SO.

clearly indicated that both isomers (IIa and IIb) are giving the same product (III).

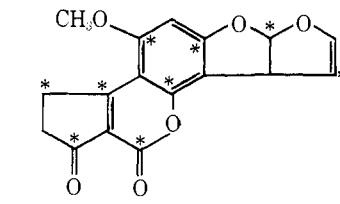


III

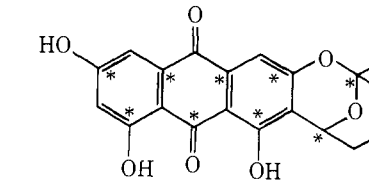
Previous studies[10-12] have shown that aflatoxin B_1 is formed from a polyketide precurser. In an attempt to confirm the possible intermediacy of versiconal acetate in aflatoxin biosynthesis, versiconal acetate was produced by cultures supplemented with 90% ^{13}C-1 enriched acetate. The ^{13}C NMR spectrum of this material is given in Figure 1B. Only two of the quinone carbonyl carbons at 184.9 and 189.0 ppm appear to be labeled. This gives further support for the existence of versiconal acetate as a mixture of two hemiacetal forms (IIa and IIb). Comparison of Figures 1A and 1B indicates an alternating labeling pattern for IIa and IIb as indicated in IV. The correct carbons are labeled, assuming versiconal acetate to be an intermediate in the biosynthesis of aflatoxin B_1 (V).



IV



V



VI

Previously, averufin (VI), another anthraquinone intermediate in the biosynthesis of V has been isolated. Labeling studies with enriched ^{13}C-1 acetate give the alternating labeling pattern shown.[13] Further studies have shown that VI is converted into aflatoxin B_1 (V).[14] The labeling pattern found for versiconal acetate is identical with that found for VI in the anthraquinone ring. Therefore, this as well as the accumulation of II concomitant with inhibition of aflatoxin biosynthesis suggests that versiconal acetate is an intermediate in the biosynthesis of aflatoxin B_1 and the versicolorins. To further confirm the intermediacy of versiconal acetate in the biosynthesis of aflatoxin B_1, the ^{13}C-labeled versiconal acetate was converted into aflatoxin B_1 by *A. flavus*.[15] The labeling pattern of the labeled aflatoxin B_1 produced was identical with that previously reported[11,12] for aflatoxin B_1 produced with enriched ^{13}C-1 acetate. These data indicate that versiconal acetate is an intermediate in the biosynthesis of aflatoxin B_1.

Kingston et al.[16] and Thomas[17] have proposed a scheme consistent with the labeling pattern in which VI is converted into the versicolorins. Although versiconal acetate does not appear in their scheme, only a slight modification is required to accommodate versiconal acetate. One question concerning the biosynthesis of aflatoxin B_1 which was not clear in the previous studies is whether the carbons in the furan rings are derived from the same polyketide chain as the carbons in the anthraquinone ring.[14] The intensities of the peaks observed in Figure 1B would indicate that all carbons are derived from the same polyketide chain.

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References and Notes

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(3) H. W. Schroeder, R. J. Cole, R. D. Crigsby, and H. Hein, Jr., *Appl. Microbiol.*, **27**, 374 (1974).
(4) Versiconal acetate was produced in solid-state rice shake cultures supplemented with 25 ppm dichlorvos by *Aspergilius parasiticus* (NRRL 2999). Cultures were incubated at room temperature for 6 days. The pigment was extracted with chloroform and allowed to precipitate from chloroform solution at 5 °C. The precipitate was collected and reprecipitated three times from chloroform solution to yield purified versiconal acetate.
(5) Carbon-13 NMR spectra were obtained on a JEOL P FT-100 spectrometer equipped with the EC-100 data system. Chemical shifts were assigned on the basis of single frequency off-response decoupling experiments, known substituent effects, and comparison with the spectra of related anthraquinones such as versicolorin C, averufin, versicolorin A, and norsolorinic acid.
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(8) The quinone carbonyl chemical shifts for some anthraquinones are as follows: 1,8-dihydroxy-9,10-anthraquinone, 181.2 and 191.9 ppm; 1,2,5,8-tetrahydroxy-9,10-anthraquinone, 185.0 and 190.7 ppm; and 1,3,8-trihydroxy-6-methyl-9,10-anthraquinone, 180.9 and 189.4 ppm.[6]

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(15) Labeled aflatoxin B_1 was produced by coating 300 g of rice with 60 mg of ^{13}C-labeled versiconal acetate. This mixture was placed into a 2800-mL Fernbach flask, sterilized, inoculated with an aflatoxin B_1 producing strain of *A. flavus*, and incubated at room temperature for 6 days via solid-state shake culture. The aflatoxin B_1 produced was isolated as reported previously.[3]
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(17) R. Thomas, personal communication to M. O. Moss, "Aflatoxin and Related Mycotoxins, Phytochemical Ecology", J. B. Harborne, Ed., Academic Press, London, 1972, p 140.

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Characterization of the Acetyl-chymotrypsin Intermediate by ^{13}C Nuclear Magnetic Resonance Spectroscopy

Sir:

Carbon-13 NMR is a valuable tool in studies of proteins in solutions at natural abundance of ^{13}C.[1] However, problems of sensitivity and assignment arise in the observation of single carbon atom resonances in proteins. To overcome these problems several applications have been carried out in which ^{13}C enriched chemically modifying groups have been attached to proteins as ^{13}C NMR reporter groups.[2,3] We wish to report the first observation of the resonance of a ^{13}C enriched enzyme-substrate intermediate.

The mechanistic pathway in chymotrypsin catalysis is thought to involve an acyl-enzyme intermediate.[4] The evidence for such intermediates is based upon enzyme activity studies,[5-7] and upon spectrophotometric observations.[8,9] Labeled ^{14}C acetate was used to show the position of acetylation on the active serine 195.[10] In order to study the properties of the active site environment of the enzyme in solution, a ^{13}C NMR study of ^{13}C labeled acyl-chymotrypsin intermediates has been undertaken. We report the characterization of the first member of this series [1-^{13}C]acetyl-α-chymotrypsin ($CH_3{}^{13}CO$-E).

The ^{13}C enriched (90%) *p*-nitrophenyl acetate was prepared by the general method of Okawa and Hase.[11] The compound was recrystallized from hot ethanol, and exhibited a single carbon signal at 175.0 ppm downfield from Me_4Si. The methyl resonance of the acetyl group was split into a doublet (J = 7 Hz) instead of a singlet, as in the unenriched sample.

The purified acetyl-α-chymotrypsin intermediate was prepared by the method of Bender et al.,[12] with an enzyme (0.2 mM) to substrate ratio of 1:10. Phosphate buffer (pH 5.1) and a reaction time of 4 h were employed. Membrane ultrafiltration (Diaflo ultrafilter PM10) was used to purify the intermediate instead of column chromatography. Enzyme activity was determined with the substrate, carbobenzyloxy-L-alanine *p*-nitrophenyl ester, at pH 5.1.[13] In this pilot run approximately 95% of the protein was recovered with a residual enzyme activity of 4-5% of the control. On stirring the protein solution at pH 8.0, the enzyme activity was fully recovered within 30 min.

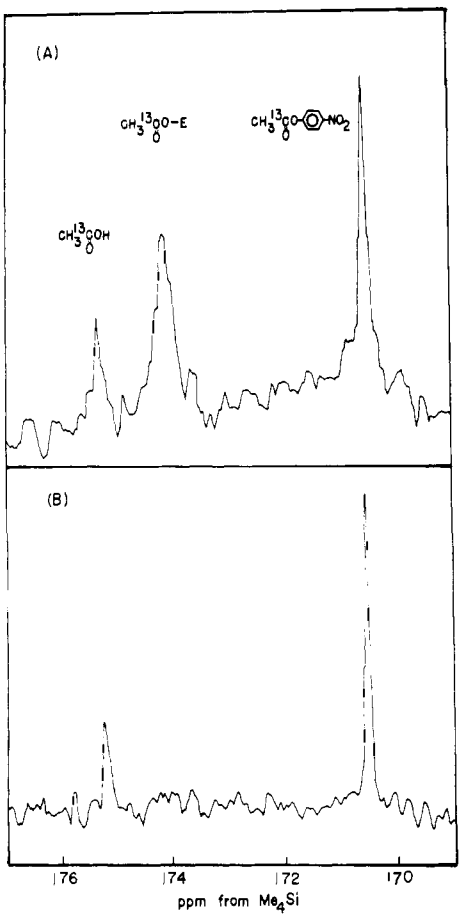

Figure 1. Proton-decoupled 68-MHz ^{13}C NMR spectra: (A) α-chymotrypsin (2.0 mM) plus *p*-nitrophenyl [1-^{13}C]acetate (3.0 mM) at room temperature in 0.2 M phosphate buffer (pH 5.1). (After 2 h, the pH of the solution was adjusted to 3.2.); (B) 3.7 mM *p*-nitrophenyl [1-^{13}C]acetate under the same conditions after 3 days.

With the ^{13}C enriched substrate at a molar ratio of 1:1 with enzyme, the acyl-α-chymotrypsin intermediate was prepared as above, without ultrafiltration. Upon ^{13}C NMR examination of the reaction mixture three major signals were observed (170.4, 174.0, 175.1 ppm downfield from Me_4Si) (Figure 1A). The signals at 170.4 and 175.1 ppm were readily attributed to substrate and acetic acid, respectively (Figure 1B). After 4 h the carbon signal of the substrate had disappeared (Figure 2A). Increasing the pH of a reaction mixture to 8.0 at room temperature eliminated the signal at 174.0 ppm, while the intensity of the signal due to acetic acid was correspondingly increased (Figure 2B). These results were consistent with the enzyme activity studies. In another experiment, in which the purified ^{13}C enriched intermediate was prepared in larger quantity (4 mM) using ultrafiltration, 90% of the protein was recovered with a residual enzyme activity of 15%. Nonetheless, only one major carbon signal at 174.0 ppm was observed. Incubation of this solution at 37 °C and pH 4.5 for 30 min resulted in a dramatic increase in the intensity of the acetic acid carboxyl carbon signal with a concomitant reduction in the signal at 174.0 ppm. Upon reassay of this solution it was found that 80% of the original activity had been recovered.

The results of these preliminary experiments are consistent with the proposal that the signal at 174.0 ppm arises from the [1-^{13}C]acetyl-chymotrypsin intermediate. This provides further direct evidence for the formation of acyl-chymotrypsin intermediates during the hydrolysis of esters by chymotrypsin.