chloride concentration and rate of nitrosation of proline at different pH levels. Strong activation of the nitrosation of proline was seen at high acidities (pH 0.5) and inhibiting effects were observed at lower acidities. In recent publications (Boyland, 1971; Sander, 1973) the point has been made that halide ions are catalytic agents in nitrosation reactions. As shown by our work, one must consider the pH of the system to determine whether a catalytic or inhibiting effect from chloride ions would be predicted. Whereas chloride might activate nitrosations in strongly acidic media, the same ion would be expected to produce a moderately inhibiting effect on nitrosation in mildly acidic or neutral food systems.

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Biosynthesis of Averufin from Acetate by *Aspergillus parasiticus*

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Carbon-13 nuclear magnetic resonance analysis shows that ^{13}C-labeled averufin synthesized from [1-^{13}C]acetate by *Aspergillus parasiticus* was labeled at alternating positions in the molecule. This pattern indicates the polyketide origin of the compound and its biogenetic relationship to the bisfuranoids, sterigmatocystin and aflatoxin B_1.

The hypothesis that averufin is a biosynthetic intermediate of aflatoxin B_1 (Thomas, 1965; Moss, 1972) has been experimentally supported by: (1) the accumulation of averufin in the mycelium of a nitrosoguanidine-induced deficient mutant of *Aspergillus parasiticus* ATCC15517, an aflatoxin producing fungus (Donkersloot et al., 1972), and (2) the in vivo conversion of averufin into aflatoxin by the wild-type strain (Lin et al., 1973; Yao and Hsieh, 1974). Although evidence now indicates that aflatoxin is derived from acetyl CoA via the polyketide pathway (Donkersloot et al., 1968; Biollaz et al., 1970; Hsieh and Mateles, 1970, 1971), the precursor-product relationship between acetate and averufin has not yet been demonstrated.

In this report the distribution of ^{13}C labels in averufin derived from [1-^{13}C]acetate as determined by ^{13}C nuclear magnetic resonance is presented to substantiate the proposed synthesis of averufin from acetate via a polyketide chain and biogenetic relationship between averufin and aflatoxin as well as other related metabolites.

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EXPERIMENTAL SECTION

Materials. 1-^{13}C-Labeled acetate 68% enriched in ^{13}C was synthesized via a Grignard reaction of methylmagnesium iodide and $^{13}CO_2$ generated from $Ba^{13}CO_3$ which was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. (Fitzell et al., 1975; Murray, 1958). Unlabeled averufin was produced from the culture of *Aspergillus parasiticus* ATCC24551 maintained in a medium containing sucrose, asparagine, and ammonium sulfate as described by Yao and Hsieh (1974). ^{13}C-Labeled averufin was prepared in a similar manner with [1-^{13}C]acetate added to the cultures.

Carbon-13 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 temperature of ~24° with Me_2SO-d_6, the solvent, used as the deuterium lock source and as an internal reference. All chemical shifts are reported as δ (ppm) downfield from Me_4Si, based on Me_2SO (δ 40.3). Concentrations are noted in Figures 1 and 2.

RESULTS

Assignment of the resonances for the [1-^{13}C]acetate-derived averufin spectrum (Figure 1) was aided by a natural abundance ^{13}C spectrum (Figure 2) and an off-resonance

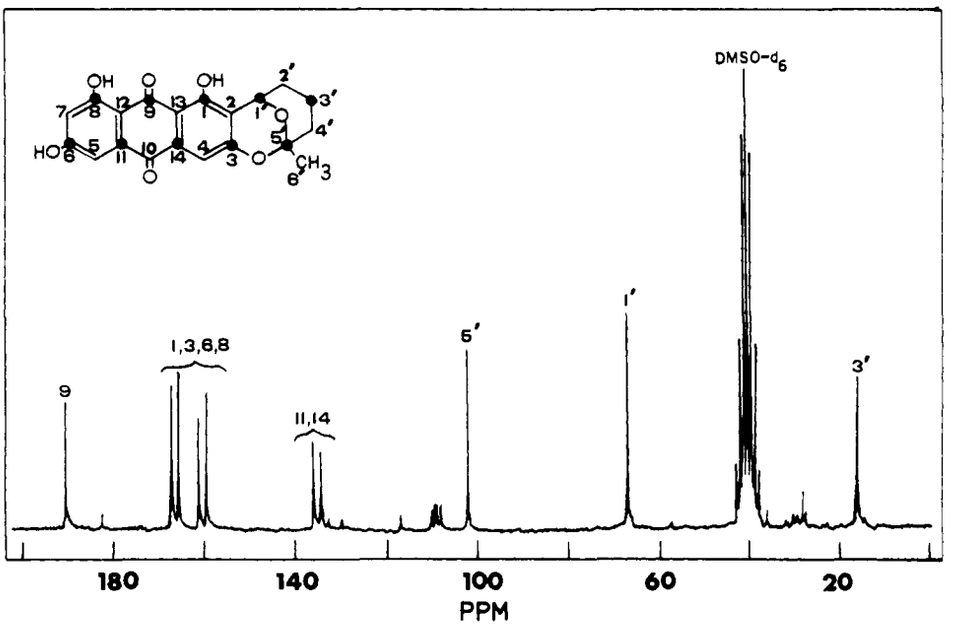

Figure 1. ^{13}C NMR spectrum of averufin from [1-^{13}C]acetate; concentration 0.65 *M* in Me_2SO-d_6; 8K data points; 10 kHz BDW; 10 kHz filter; 15,000 pulses; 0.41-sec pulse spacing; ~20° pulse angle.

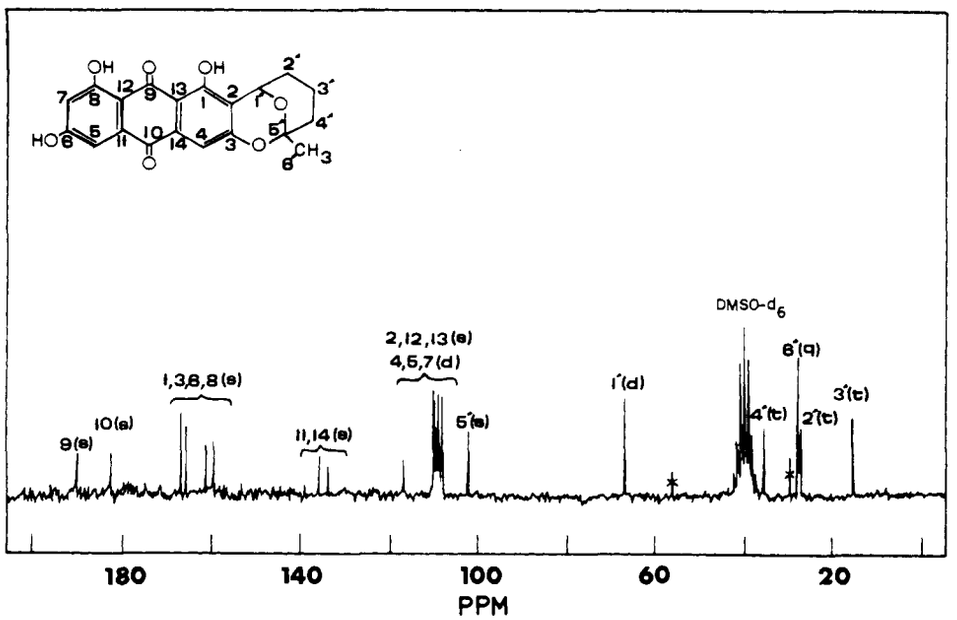

Figure 2. ^{13}C NMR spectrum of natural abundance averufin; concentration 0.70 *M* in Me_2SO-d_6; 8K data points; 10 kHz BDW; 10 kHz filter; 23,474 pulses; 0.41-sec pulse spacing; ~20° pulse angle; (s) singlet; (d) doublet; (t) triplet; (q) quartet.

decoupled natural abundance spectrum. Figure 1 shows ten enriched carbon atoms present and the off-resonance decoupled spectrum indicates the following multiplet patterns for those signals: δ 16.3 (t), 67.1 (d), 102.1 (s), 133.9 (s), 135.7 (s), 158.9 (s), 160.7 (s), 165.0 (s), 166.1 (s), and 189.7 (s).

The signal at 16.3 ppm was assigned to C-3′. It is the farthest removed methylene carbon from the deshielding oxygen atoms, indicating an upfield position relative to the two other methylene carbons [δ 28.3 (t) and 40.4 (t)]. The signal at δ 67.1 was found to be C-1′. It is the only aliphatic methine carbon in the molecule and its chemical shift is far upfield from that of aromatic carbons (Levy and Nelson, 1972). The signals at δ 158.9, 160.7, 165.0, and 166.1 were collectively assigned to C-1, -3, -6, and -8 because of their downfield shift characteristic of aromatic carbons bonded directly to oxygen (Tanabe et al., 1970; Lauterbur, 1961). Similarly the signals at δ 133.9 and 135.7 are characteristic of disubstitution of oxygen functions only in the meta positions, therefore attributed jointly to C-11 and -14. The strong deshielding effect of two adjacent oxygen atoms caused C-5′ to resonate far downfield at 102.1 ppm.

While it would not be possible to conclusively assign δ 189.7 to C-9 rather than C-10 solely by comparison of chemical shifts of model compounds, that assignment can be made on the basis of coupling constants (Hsieh et al., 1975). If C-10 were the labeled carbonyl there would be a splitting of ~70 Hz (sp^2–sp^2) (Stothers, 1972) with C-11 and -14 which would be easily observable. The absence of any one bond coupling in Figure 1 indicates δ 189.7 is C-9 and confirms the alternating labeling pattern.

Of the remaining signals, none of which are from [1-^{13}C]acetate, only limited assignments can be made. C-10

is assigned to δ 181.5 once C-9 has been determined. The sole methyl group (C-6′) was found to be the lower field signal at δ ~28.3. Although an impurity slightly downfield from these two signals changed the relative intensities of the multiplet patterns in the off-resonance spectrum due to overlap, the lower field signal could be identified as a quartet and the upper field signal as a triplet. The triplet at δ 40.4 would appear to be C-4′ because of its proximity to the two oxygen atoms, although no proof is available at this time. It remains for C-2′ to be assigned to the upfield signal at δ ~28.3 (t) through elimination and similar analysis with respect to its position relative to the oxygen atoms.

An expansion of the region of δ 108–110 indicated there were five signals present, due to three methine and two quarternary carbons. Because of the overlap of the multiplet patterns in the off-resonance spectrum, it was not possible to positively assign a splitting pattern to each signal from this single spectrum. Thus, the remaining signals at δ 108–117 were collectively assigned to C-2, -4, -5, -7, -12, and -13. All of these signals have upfield shifts indicative of carbons ortho and/or para to oxygen functions (Lauterbur, 1961).

DISCUSSION

The alternating labeling pattern of averufin derived from [1-^{13}C]acetate indicates the polyketide origin of the molecule and that it is synthesized by a head-to-tail assembly of ten acetate units. This labeling pattern agrees with the prediction of Donkersloot et al. (1972).

In the study of the fungal polyketide pathway, it is generally accepted that the following steps prevail: anthraquinones → xanthones → coumarins. This general biosynthetic scheme is supported by our experimental findings that (1) averufin can be converted in vivo into sterigmatocystin by *A. versicolor* (unpublished data), (2) averufin can be converted into aflatoxins by *A. parasiticus* (Lin et al., 1973), and (3) sterigmatocystin can also be converted into aflatoxins by *A. parasiticus* (Hsieh et al., 1973).

An important question in this pathway is how the C_4 bisfuran ring system of sterigmatocystin and aflatoxins is formed. Thomas proposed a mechanism for the conversion of the C_6 side chain of averufin into the bisfuran moiety involving an acetyl furan intermediate (Moss, 1972), although no experimental evidence is yet available. Alternately, sterigmatocystin and aflatoxin B_1 were proposed to be synthesized from a fusion of two preformed polyketide units (Holker and Mulheirn, 1968; Heathcote et al., 1973), implying that the C_6 side chain of averufin was replaced by an acetoacetate-like C_4 unit in the formation of the bisfuran ring system.

We have recently found that in the incorporation of labels from [^{14}C]acetoacetate into aflatoxin, averufin did not serve as a carrier to enhance the incorporation efficiency of [^{14}C]acetoacetate despite the fact that averufin is readily convertible into aflatoxin (unpublished data). The experimental findings so far seem to better support the derivation of sterigmatocystin and aflatoxin from a single polyketide intermediate; however, the mechanism of formation of the bisfuran ring system as well as absolute confirmation of the carbon source are still areas for future research.

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