Biosynthesis of the Aflatoxin Precursor Sterigmatocystin by *Aspergillus versicolor*; Spin-echo Resolution of ^{18}O Isotope Shifts in ^{13}C-N.M.R. Spectroscopy

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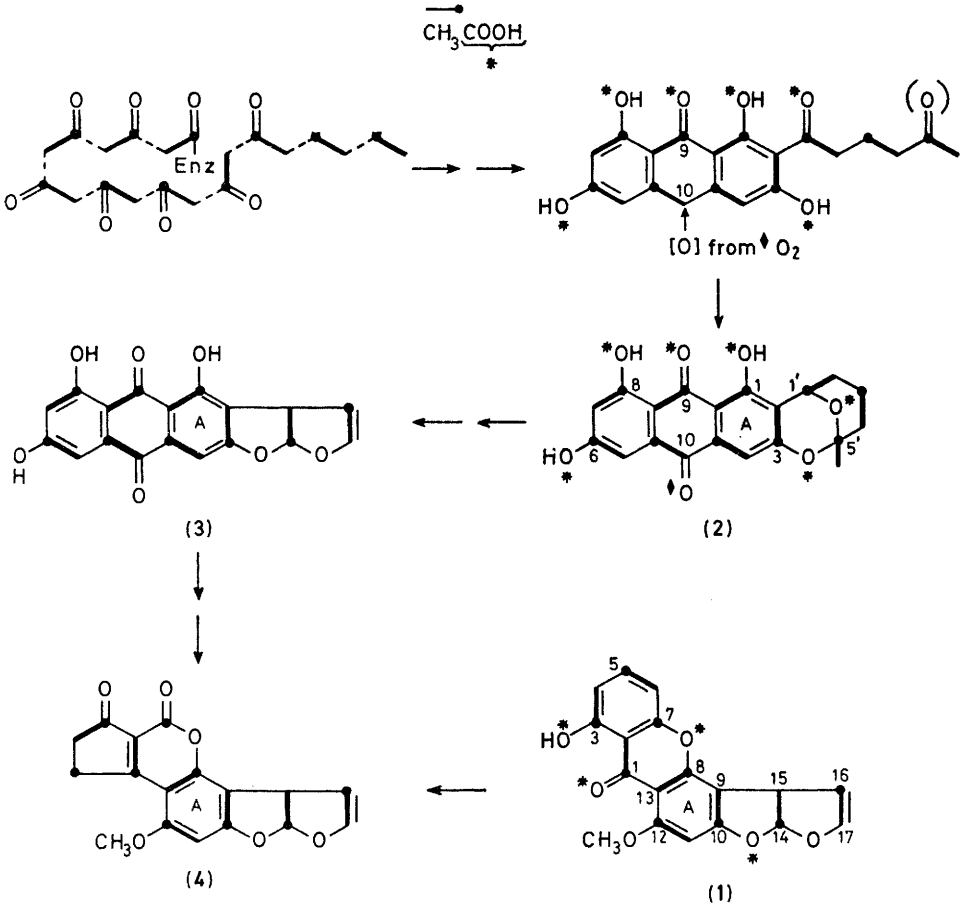
Locations of oxygen-18 label in an aflatoxin precursor, sterigmatocystin, derived from sodium [1-^{13}C,$^{18}O_2$]-acetate were determined using a spin-echo pulse sequence [90°-τ-180°-τ-acquisition, where $\tau = 1/(2J_{C-C})$] in ^{13}C-n.m.r. spectroscopy to resolve isotope shifts from interfering carbon–carbon couplings (J_{C-C}).

Sterigmatocystin (**1**) is a toxic hepatocarcinogen produced by *Aspergillus* and other fungi which can contaminate food and pose a serious health hazard.[1–3] It also acts as a biosynthetic precursor to the even more potent aflatoxin B_1 (**4**), although recent pulse labelling studies suggest that (**1**) may be an intermediate in only one of two possible pathways.[4] Co-occurrence of (**1**) with small amounts of 5,6-dimethoxysterigmatocystin in *Aspergillus multicolor* may be biogenetically significant in this regard.[5] Extensive carbon-13 labelling work has established the arrangement of acetate-derived carbons in sterigmatocystin (**1**)[6] and its ostensible precursors, averufin (**2**)[7] and versicolorin A (**3**)[8] (Scheme 1). Since detection of oxygen-18 by upfield isotope shifts induced in ^{13}C n.m.r. spectra[9,10] has opened a new avenue for exploration of biosynthetic mechanism,[11–16] these earlier studies make sterigmatocystin (**1**) a prime candidate for oxygen-labelling experiments. Interestingly, a frequent problem is excessive incorporation of [^{13}C,^{18}O]-precursor which leads to multiple ^{13}C labels within a particular molecule, thereby causing long range carbon–carbon couplings which obscure the small ^{18}O isotope shifts.[15,16] In the present work we apply a spin-echo sequence in ^{13}C-n.m.r. spectroscopy to overcome this difficulty and determine the ^{18}O-labelling pattern in sterigmatocystin (**1**) derived from [1-^{13}C,$^{18}O_2$]-acetate.

In the incorporation experiment ten standing cultures of *Aspergillus versicolor* NRRL 5219 (100 ml low salt medium[17]/500 ml flask) were grown in the dark at 29 °C for 120 h prior to the first administration of precursor. An aqueous solution (pH 10) of doubly labelled sodium [1-^{13}C,$^{18}O_2$]acetate[15] (isotopic purity 90% ^{13}C, 18% $^{18}O_1$, 81% $^{18}O_2$) was injected below the surface of each culture (50 mg/injection) every 24 h until five injections had been made. After an additional three days, pure [^{18}O,^{13}C]sterigmatocystin (79 mg) was isolated by literature procedures.[17]

Examination of the normal proton-decoupled 100.6 MHz ^{13}C-n.m.r. spectrum of (**1**) showed that the signals were complex patterns of long range couplings arising from multiple intramolecular precursor incorporation. However spin-echo



Scheme 1

Fourier Transform[18,19] (SEFT) ^{13}C-n.m.r. allowed separation of the uncoupled ^{13}C–^{16}O and ^{13}C–^{18}O singlets from signals (doublets) coupled to nearby ^{13}C atoms. The necessary pulse sequence is $(90°$-τ-$180°$-τ-aquisition-$T)_N$ where τ is set to $1/(2\ J_{CC})$, T is the delay between acquisition and the next pulse sequence, and N is the number of accumulations. With this sequence the coupled resonances (doublets) are phase-modulated as a function of J_{CC} (the long range coupling constant) such that they are inverted relative to the normal ^{13}C-n.m.r. spectrum at $\tau = 0, 90°$ out of phase at $\tau = 1/(4\ J_{CC})$, and in phase (positive) at $\tau = 1/(2\ J_{CC})$. Regardless of what τ value is employed, the uncoupled singlets always remain inverted.

This technique resolved even the smallest expected isotope shift[9,10] at C-3 and sharpened resonances for carbons with no isotopically shifted peaks (such as C-7) to single lines (Figure 1). The presence of [^{18}O]acetate-derived oxygens could be detected in sterigmatocystin (**1**) at C-1, C-3, C-8, and C-10 (Table 1). Except for C-3, all of the carbons showing isotope shifts gave base line resolution of the ^{13}C–^{16}O and ^{13}C–^{18}O inverted peaks in the spin-echo experiments. Improved signal to noise ratio and resolution may be partly due to necessary changes in acquisition parameters and partial suppression of broad resonances with T_2 values shorter than the τ value used. Although quantitative estimates of isotope ratios (Table 1) must be viewed with extreme caution because the exact influence of SEFT on signal intensities is uncertain, the technique does permit the qualitative identification of ^{18}O attached to carbon. Our results support a proposed[1,4,6] biosynthetic scheme [(**2**) → (**3**) → (**1**)] in light of previous work on the source of oxygens in averufin (**2**),[11] although the absence of an oxygen substituent at C-5 of (**1**) remains puzzling.

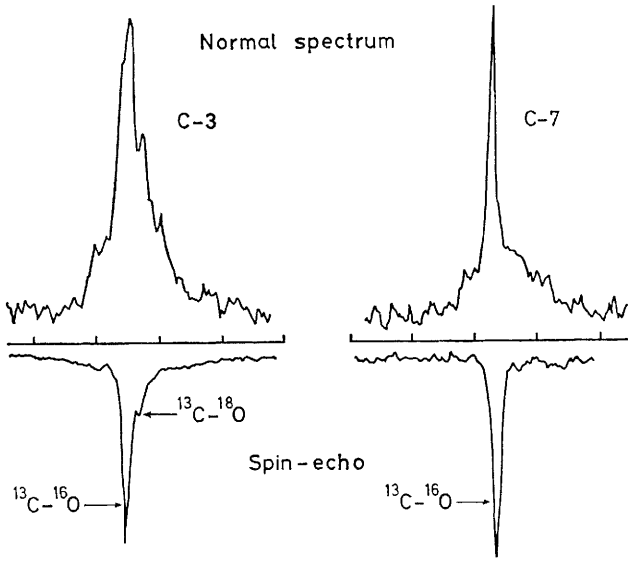


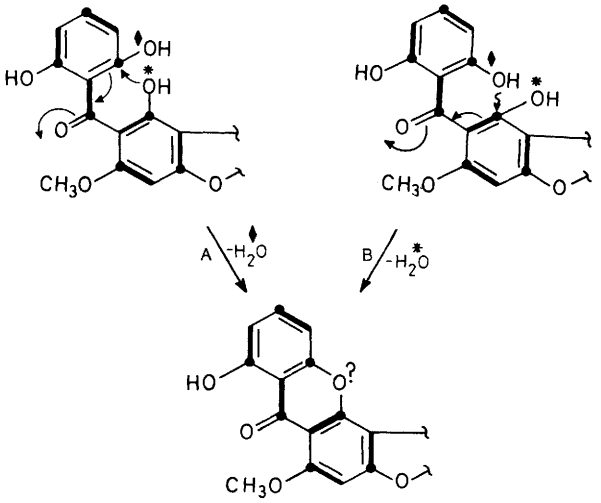
Figure 1. ^{1}H-Decoupled 100.6 MHz ^{13}C-n.m.r. spectra expansions (0.05 p.p.m./division) of C-3 and C-7 of sterigmatocystin (**1**) derived from sodium [1-^{13}C,$^{18}O_2$]acetate.

The nature of the intermediate between versicolorin A (**3**) and sterigmatocystin (**1**) is unknown, but the unscrambled carbon labelling pattern[1,6] shows that xanthone ring closure occurs through an unsymmetrical species. Given the ease of xanthone formation by addition-elimination reactions,[20] oxidative cleavage of (**3**) to an enzyme-bound hydroxylated

Table 1. 100.6 MHz ^{13}C-n.m.r. of sterigmatocystin (**1**) after incorporation of sodium [1-^{13}C,$^{18}O_2$]acetate.[a]

	δ/p.p.m.	Δδ[b]/ p.p.m. × 100	Isotope ratio[c]
C-1	181.3	2.9	62:38
C-3	162.5	1.0	76:24
C-8	154.1	2.1	81:19
C-10	164.6	1.5	80:20

[a] Proton noise decoupled spectra of *ca.* 0.1 M (**1**) in $CDCl_3$ on Bruker WH 400; signals were first expanded using 32 K data block/2000 Hz, 500—3500 scans, 16.4 s acquisition, 45° pulse angle; subsequent spin-echo expansions employ 6.5 s acquisition, τ = *ca.* 0.07 s. [b] ^{18}O Upfield isotope shifts are ±0.1 (p.p.m. × 100). [c] Ratio of ^{16}O to ^{18}O. Relative peak heights of ^{13}C–^{16}O and ^{13}C–^{18}O inverted signals in the spin-echo experiment; the relative intensities are ±5% of estimates from normal spectra.



Scheme 2. Possible modes of xanthone ring formation.

benzophenone (Scheme 2) seems likely. In principle the ring closure can proceed in two ways; either an acetate-derived oxygen (*) adds in Michael fashion to eliminate an oxygen (◆) derived from the atmosphere (or medium) as water (path A) or *vice versa* (path B). Doubly labelled [^{13}C,^{18}O]-precursors allow detection of intact carbon–oxygen bonds because of the low probability of an adjacent carbon being ^{13}C in a particular product molecule. Hence the appearance of an isotope shift at C-8 of (**1**) (but not at C-7) shows that the hydroxy-group in ring A of versicolorin A (**3**) (or a closely related intermediate) becomes the xanthone ring oxygen of sterigmatocystin (**1**), thereby supporting path A in Scheme 2.

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