Biosynthesis of Averufin by *Aspergillus parasiticus*; Detection of ^{18}O-label by ^{13}C-N.M.R. Isotope Shifts

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Summary The incorporation of oxygen-18 gas and doubly-labelled sodium [1-^{13}C, $^{18}O_2$]acetate into averufin (**1**) by an aflatoxin deficient mutant of *Aspergillus parasiticus* was detected by ^{18}O-induced isotope shifts in ^{13}C-n m r spectra

AVERUFIN (**1**) is implicated as an intermediate in the biosyntheses of carcinogenic fungal food contaminants such as sterigmatocystin and aflatoxin B_1 (**2**) [1,2] An *Aspergillus parasiticus* mutant (ATCC 24551) blocked in aflatoxin production accumulates averufin (**1**), and has been used to determine the carbon labelling patterns

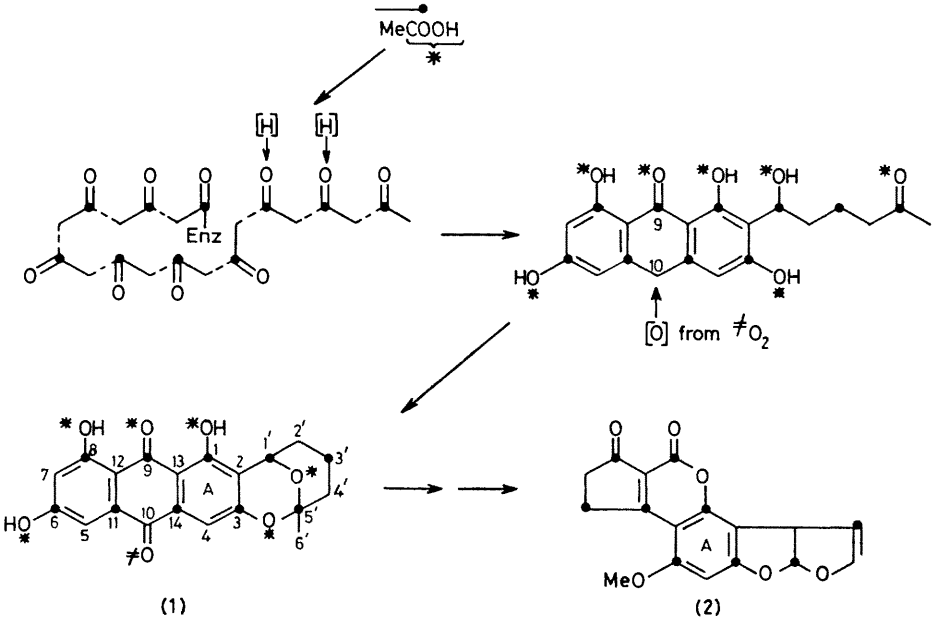

FIGURE 1 Incorporation of acetate and oxygen gas into averufin (**1**)

(Figure 1) after incorporation of [^{13}C]acetates.[3] However, the origin of the oxygen atoms in (**1**), and indeed in most natural products, remained unknown because of difficulty in ascertaining the position of the label(s) by mass spectrometry. We now report the first biological application of ^{18}O-induced isotope shifts in ^{13}C-n.m.r. spectra[4] resulting in elucidation of the source of all of the oxygen atoms in averufin (**1**).

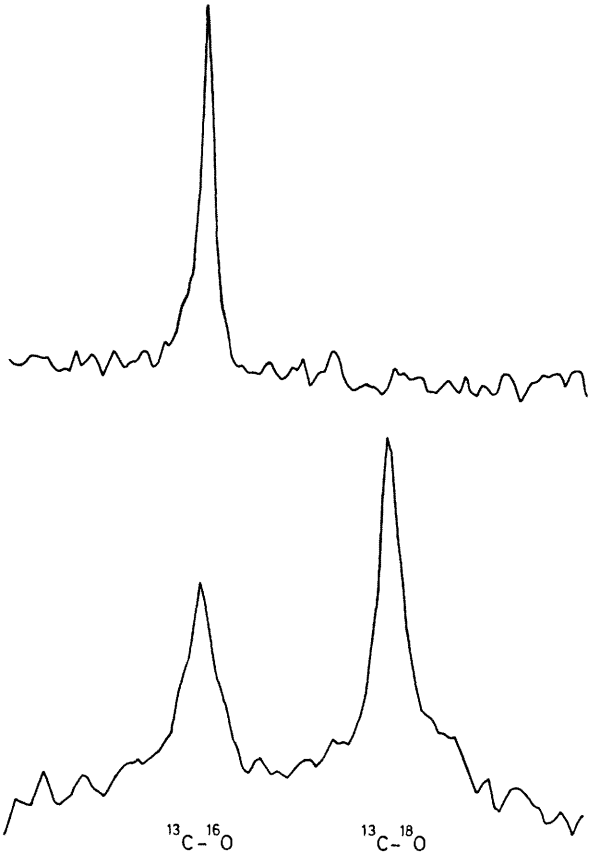

FIGURE 2. 100·6 MHz ^{13}C-n.m.r. spectra of C(10) of averufin (**1**) after incorporation of sodium [1-^{13}C, $^{18}O_2$]acetate (above) and [^{18}O]oxygen gas (below).

In our first experiment, a culture of *A. parasiticus* ATCC 24551 (1 l) was grown for 8 days on a 20% sucrose–yeast extract medium[5] in a closed system having a carbon dioxide trap containing 3 N KOH. The fermentation was begun in a normal atmosphere but depleted oxygen was replaced twice daily with [^{18}O]oxygen gas (96%). Mass spectrometric analysis of the resulting averufin (**1**) showed that 62% of the molecules contained one ^{18}O label, but did not allow exact determination of its position. Examination of the ^{1}H-decoupled 100·6 MHz ^{13}C-n.m.r. spectrum (Bruker WH400 spectrometer) in $CDCl_3$–$(CD_3)_2SO$ (1 : 1) showed that all of the signals were singlets except for the one corresponding to C-10. This carbon appeared as two signals at 181·149 and 181·108 p.p.m. (isotope shift = 4·17 Hz), the upfield peak being due to carbon bearing ^{18}O (Figure 2). Comparison of n.m.r. integrals of these signals indicated an isotopic content of 59%, in good agreement with mass spectrometric results.

In a separate experiment the culture was grown in a normal atmosphere and injected below the surface after 106 and 144 h with sodium [1-^{13}C, $^{18}O_2$]acetate (0·40 g/injection, isotopic purity 90% ^{13}C, 96% ^{18}O). The required precursor was prepared from sodium [1-^{13}C]acetate by exchange with [^{18}O] water containing HCl followed by basification with sodium hydride. As expected,[3] C(1), C(9), C(8), C(6), C(11), C(14), C(3), C(1′), C(3′), and C(5′) of averufin (**1**) were enhanced by a factor of five in the ^{1}H-decoupled ^{13}C-n.m.r. spectrum. Expansion of the individual signals for carbons directly attached to oxygen showed that all of the oxygens except the one at C(10) were labelled. Comparison of the integral ratios and correction for natural abundance ^{13}C suggested that roughly half of the oxygen label was lost at each site to the medium (Table). Similar levels of exchange during

TABLE. Results of 100·6 MHz ^{13}C-n.m.r. spectra of averufin (**1**) after incorporation of sodium [1-^{13}C, $^{18}O_2$]acetate.

Carbon	% ^{18}C retention[a]	shift/Hz[b]
1	36	1·13
3	53	1·98
6	52	1·13
8	41	1·05
9	50	2·95
10	unlabelled	—
1′	40	2·35
5′	not resolved	—

[a] Corrected for natural abundance and estimated as ±5%. [b] ^{18}O substitution shifts signals upfield (see ref. 4); values are ±0·06 Hz.

polyketide biosynthesis have been observed in several cases, and, as proposed by Hutchinson, may be due to hydration of the growing polyketide chain on the enzyme surface.[6] Although the isotope shifts at C(3) and C(1′) clearly show that both acetal oxygens are partially labelled, we were not able to resolve the signal for C(5′) and are currently investigating this problem further.

Our results support the hypothesis that the whole polyketide chain is assembled and reduced at C(1′) [and possibly C(3′)] on the enzyme surface. Intramolecular aldol condensations and release of a free anthrone are followed by aerobic oxidation at C(10). It is unknown whether this reaction is enzymatic since anthrones readily air oxidize to anthraquinones.[7]

This new non-degradative technique allows semi-quantitative localization of ^{18}O in highly oxygenated systems where extensive degradations and preliminary labelling would be necessary to determine structures of mass spectrometric fragments. We are currently applying this methodology to aflatoxins and their immediate precursors.

We thank Dr. P. S. Steyn (National Chemical Research Laboratory, South Africa), Prof. W. A. Ayer, and Dr.

S. P. Lee (University of Alberta) for generous gifts of unlabelled averufin. The [^{18}O]-gas and [^{18}O]-water were donated by the Stable Isotopes Resource of Los Alamos National Laboratory. We acknowledge financial support from Natural Sciences and Engineering Research Council of Canada (N.S.E.R.C.).

(*Received*, 26*th November* 1979; *Com.* 1228.)

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