Biosynthesis of Aflatoxins. Incorporation of [2-$^2H_3$]Acetate and [1-^{13}C,2-$^2H_3$]Acetate into Averufin

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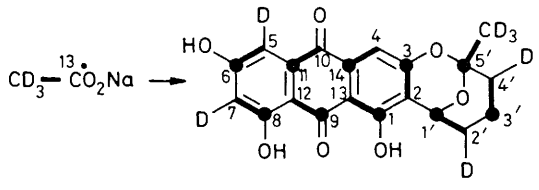
The regiospecificity of incorporation of ^2H from [1-^{13}C,2-$^2H_3$]acetate into averufin by cultures of *Aspergillus toxicarius* has been determined using the β-^2H isotope shift effect in ^{13}C n.m.r. and by ^2H n.m.r. spectroscopy; the results are discussed in relation to aflatoxin biosynthesis and polyketide biosynthesis in general.

Despite much notable work in recent years,[1] the biosynthetic pathway leading to the aflatoxins, potent carcinogenic mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, is still poorly understood in its key steps. The intermediates and mechanisms involved in the conversion of the C_6 side-chain of averufin (**1**) into the bisfuranoid moiety, the conversion of the anthraquinone system into the xanthone system, and the conversion of the xanthone system into the coumarin system are not at all clear. We are currently carrying out systematic studies to elucidate the details of these steps. This paper reports the results of incorporation of ^2H-labelled acetates into averufin. As averufin is the earliest established intermediate on the aflatoxin biosynthetic pathway,[2] it is important to determine the mode of incorporation of acetate-derived hydrogen as a basis for studies on later metabolites. In addition averufin comprises an aromatic nucleus with a relatively large, highly reduced side-chain and so the results of ^2H incorporation are of intrinsic interest in relation to recent studies on both aromatic and highly saturated polyketide metabolites such as brefeldin A[3] and the macrolide antibiotics.[4]

Both direct (^2H n.m.r.) and indirect methods (^{13}C n.m.r.) have been used to trace the incorporation of ^2H into a metabolite.[5] Staunton has recently suggested a technique in which



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^2H is attached β to ^{13}C in a precursor; the incorporation of ^2H into a metabolite can then be detected by β-^2H isotope-induced shifts in the ^{13}C n.m.r. spectrum of the enriched metabolite.[6] This has proved particularly useful in the present study.

The ^{13}C n.m.r. spectrum resulting from incorporation of [1-^{13}C,2-$^2H_3$]acetate into averufin by static cultures of a mutant of *A. toxicarius* (ATCC 24551) is shown in Figure 1. The isotopically shifted signals accompanying the resonances due to C-6 ($\Delta\delta$ −0.04), C-8(−0.04), C-1′ (−0.04), C-3′ (−0.11), and C-5′ (−0.02 p.p.m. per deuterium) indicate that ^2H is incorporated at C-5, C-7, C-2′, C-4′, and C-6′.[7] The level

Table 1. Isotopically enriched resonances in the proton noise decoupled ^{13}C n.m.r. spectrum of averufin isolated from cultures of *A. toxicarius* supplemented with sodium [1-^{13}C,2-$^2H_3$]acetate.

Carbon	δ_c/p.p.m.	$\Delta\delta_c$/p.p.m.	Relative intensity[a]
1	158.28	—	—
3	159.83	—	—
6	165.18	—	—
	165.14	−0.04	0.5
8	164.36	—	—
	164.32	−0.04	0.2
9	188.77	—	—
11	134.62[b]	—	—
14	132.94[b]	—	—
1′	66.05	—	—
	66.01	−0.04	0.5
3′	15.30	—	—
	15.19	−0.11	0.4
5′	100.81	—	—
	100.78	−0.03	0.6
	100.76	−0.02	1.6
	100.73	−0.03	2.0

[a] Intensity of isotopically shifted resonance relative to the corresponding non-isotopically shifted resonance. [b] Mutual ^{13}C–^{13}C coupling of 13.3 Hz observed.

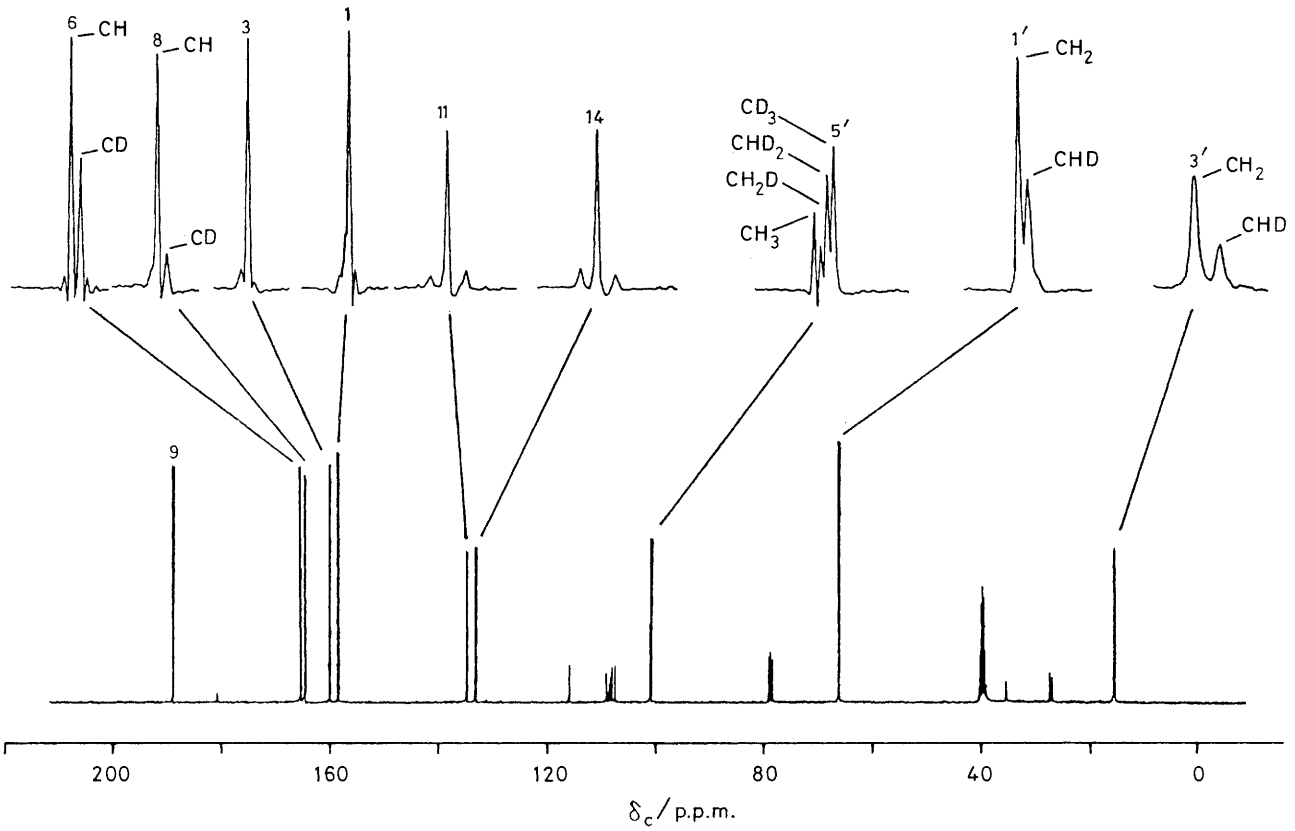

Figure 1. Resolution enhanced proton noise decoupled ^{13}C n.m.r. spectrum of [1-^{13}C,2-$^{2}H_3$]acetate-enriched averufin determined at 90.56 MHz in $CDCl_3$, [$^{2}H_6$]DMSO solution. Spectral width 20 kHz, 32 K data points, line broadening —2Hz, and gaussian multiplier 0.5.

of incorporation of deuterium varies widely at the different positions, as indicated in Table 1. By far the highest level of incorporation of deuterium occurs into the 6′-methyl group: C-5′ shows three isotopically shifted signals corresponding to the incorporation of 1—3 ^{2}H atoms into the 6′-methyl group with the signals due to $^{13}C^{1}H^{2}H_2$ and $^{13}C^{2}H_3$ species being the most intense. This confirms that the 6′-methyl is part of the 'starter' acetate unit of the polyketide chain. Only one ^{2}H is incorporated at C-4′ and one at C-2′. Studies on brefeldin A show that both hydrogens from the first malonate unit to be condensed with the acetyl coenzyme A starter unit are retained.[3] The retention of only one of these hydrogens in averufin may be due either to a rapid stereospecific exchange of either malonyl or polyketide methylene hydrogen; or to the involvement of the 4′-methylene in the loss of ketide oxygen from C-3′. This latter possibility would in turn imply that reduction of the side-chain occurs *after* ring closure and aromatisation of the polyketide precursor. In the aromatic portion of the molecule it can be seen that significantly more ^{2}H is retained at C-5 than at C-7 and that none at all is retained at C-4.‡ These differing levels of ^{2}H retention cannot be readily rationalised at this stage but it will be interesting to see if they are reflected in the subsequent metabolites on the biosynthetic pathway.

The ^{2}H n.m.r. spectrum of [2-$^{2}H_3$]acetate enriched averufin, Figure 2, is in agreement with the above results and confirms the preferential retention of ^{2}H at C-6′ and the differing levels

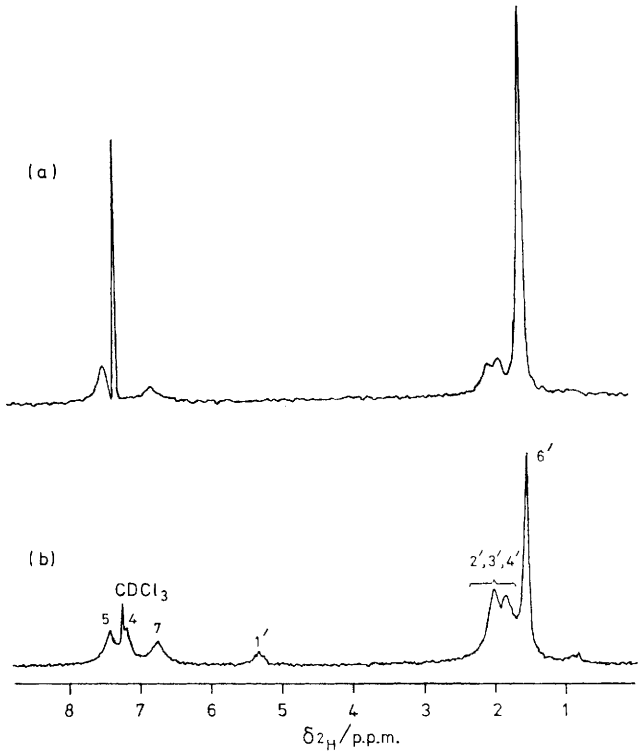

Figure 2. The 55.28 MHz ^{2}H n.m.r. spectra of 6,8-di-*O*-methylaverufin in $CHCl_3$ solution (a) enriched with sodium [2-$^{2}H_3$]-acetate and (b) uniformly labelled by addition of 10% $^{2}H_2O$ to cultures of *A. toxicarius*.

‡ C-11 and C-14 show a relatively large 2-bond ^{13}C–^{13}C coupling of 13.3 Hz *via* the C-10 carbonyl since a degree of multiple labelling had occurred.

of retention on carbons 4, 5, and 7. It was hoped that ^2H n.m.r. spectroscopy would provide information on the stereospecificity of ^2H incorporations into the side-chain but this is not possible owing to inadequate chemical shift dispersion. However specific deuteriation studies on averufin which are in progress may help to resolve this aspect of the problem.

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