THE USE OF SODIUM [2-$^2H_3$,1,2-$^{13}C_2$]ACETATE IN DETERMINING THE BIOSYNTHETIC ORIGINS OF HYDROGEN ATOMS IN FUNGAL METABOLITES: THE BIOSYNTHESIS OF CITRININ BY *PENICILLIUM CITRINUM*

Sir:

Various methods have been developed in order to determine the origins of the hydrogen atoms in fungal metabolites such as the polyketides.[1] The α-deuterium shift in the ^{13}C NMR spectrum is used to detect intact incorporation of ^{13}C-D from acetate.[2] More recently the smaller β-deuterium shift has been used very successfully to detect intact incorporation of ^{13}C-C-D from acetate.[3] Another approach, which allowed the separate detection of hydrogen atoms from acetate, water and nicotinamide coenzymes, involved growing the fungus on a medium based on D_2O in the presence of $^{13}CH_3{}^{13}COOH$ and natural abundance glucose.[4] All these techniques are powerful but all have associated problems. The α-shift is difficult to detect because the signal intensity is reduced (relative to the corresponding C-H signal) by C-D coupling and by loss of the nuclear Overhauser effects. The β-shift is small (variable but typically 0.04 ppm), the signal is broadened by long range C-D coupling, and a large ^{13}C-CH signal can thus swamp the β-shifted signal. The third method avoids these problems by using protium as a tracer; however, many fungi fail to produce metabolite when grown on D_2O. In this paper we describe a new method for determining the origins of hydrogen atoms in fungal metabolites which combines some of the important features of these methods and yields some new results.

In this experiment the fungus is treated with $^{13}CD_3{}^{13}COOH$ and the resulting metabolite analysed by ^{13}C and ^2H NMR. Thus the presence of deuterium may be monitored by analysis of α and β shifts in the ^{13}C NMR spectrum, with the advantage (described previously) that ^{13}C-^{13}C coupling enables the acetate derived carbon atoms to be distinguished from those arising from natural abundance ^{13}C from other carbon sources.

The biosynthesis of citrinin (**1**) by *Penicillium citrinum* was chosen as a case study. Citrinin is biosynthesised from $5C_2$ and $3C_1$ units as shown in Scheme 1.[5] In this experiment *P. citrinum* was grown from spores, sodium [2-$^2H_3$,1,2-$^{13}C_2$]acetate mixed with 2 equivalents of unlabelled sodium acetate administered, and citrinin harvested as previously described.[4] The ^{13}C NMR spectrum of this sample was recorded at 63 MHz and the features of particular interest are shown in Fig. 1.

The region of the spectrum around δ 16 shows a natural abundance singlet due to $C(9)H_3$ flanked by a very small doublet (J=37.8 Hz)[4] due to ^{13}C-$^{13}CH_3$, indicating complete exchange of deuterium at C(9) by a few molecules. This would not have been discernible using a single ^{13}C label. Fortuitously, the ^{13}C-D coupling

Scheme 1. The biosynthesis of citrinin by *Penicillium citrinum*.

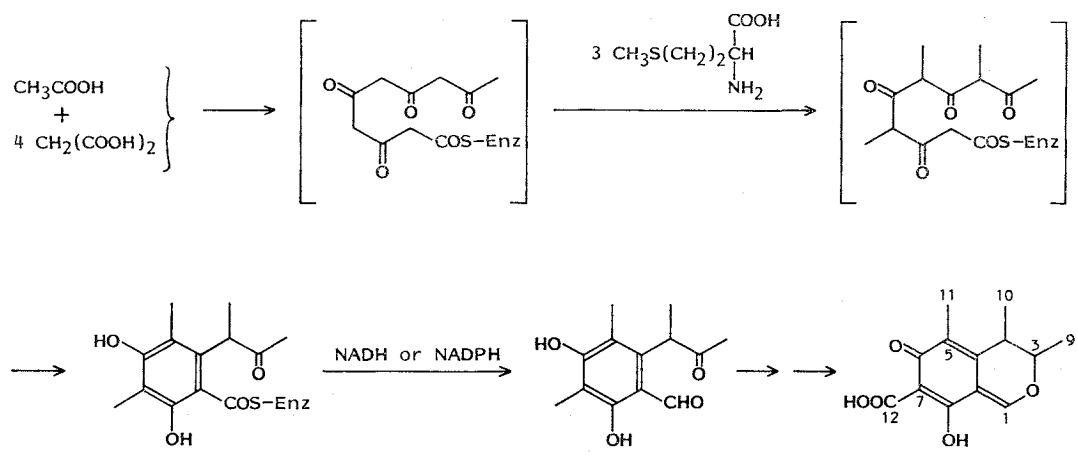

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Fig. 1. ^{13}C NMR spectrum of citrinin from [2-$^{2}H_3$,1,2-$^{13}C_2$]acetate: Selected signals.

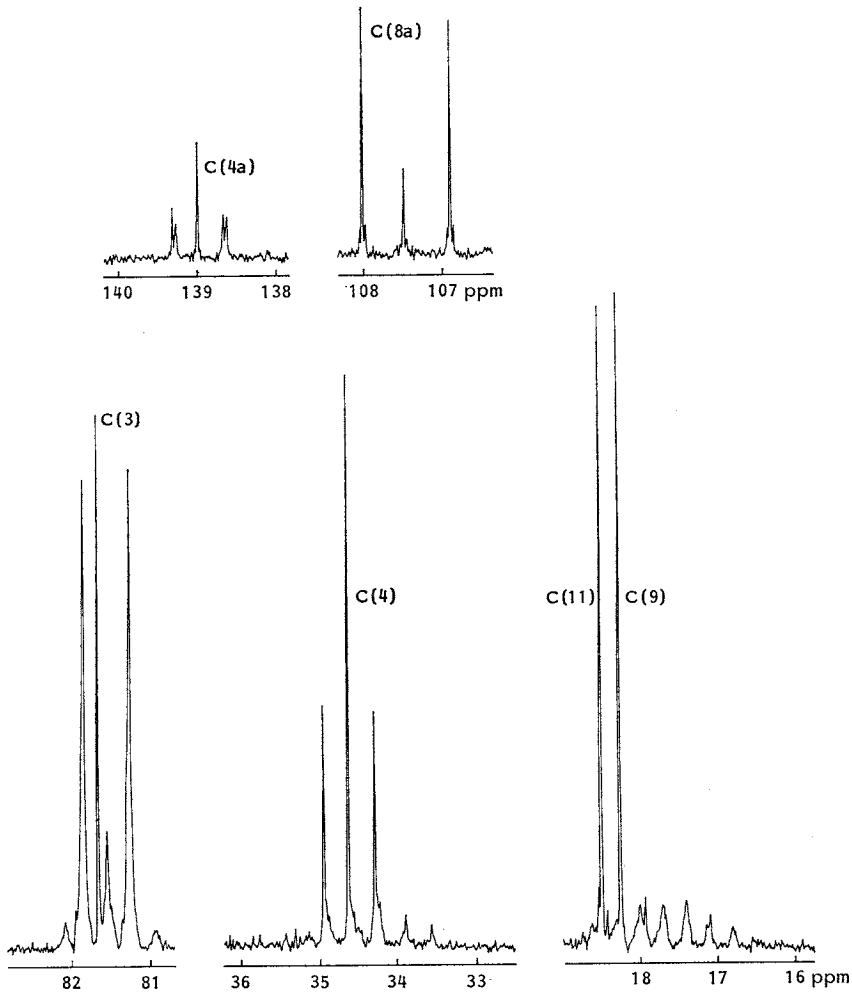


Table 1.

C(9) Signal at 63 MHz[a]									
ppm	18.6	18.3	18.0	17.7	17.4	17.1	16.8	16.5	16.2
CH_2D (low field signal)	1	1	1						
CH_2D (high field signal)			1	1	1				
CHD_2 (low field signal)	1	2	3	2	1				
CHD_2 (high field signal)			1	2	3	2	1		
CD_3 (low field signal)	1	3	6	7	6	3	1		
CD_3 (high field signal)			1	3	6	7	6	3	1

[a] The coincidence of the ^{13}C-D coupling constant (38 Hz) with the upfield shift (0.3 ppm) simplifies the spectrum and might influence the choice of instrument for this type of experiment.

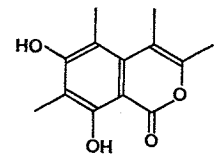
constant (18.75 Hz) is equivalent to the upfield deuterium shift (0.3 ppm, 18.9 Hz per deuterium) and to half the ^{13}C-^{13}C coupling constant so that the signals due to CH_2D, CHD_2 and CD_3 superimpose as shown in Table 1. The presence of a peak at δ 16.5 demonstrates the presence of $^{13}CD_3$. The peak at δ 18.0 is of a similar size to those at δ 17.7 and 17.4 suggesting that the species contributing most to this complex multiplet is $^{13}CHD_2$, and that this species is much

more abundant than $^{13}CH_2D$ or $^{13}CH_3$. However, since fully deuterated ^{13}C relaxes poorly, the relative amounts of $^{13}CD_3$ and $^{13}CHD_2$ cannot be deduced from this signal. Indeed in the absence of a relaxation agent and of a pulse delay it is probably only the presence of the adjacent ^{13}C that enables the $^{13}CD_3$ signal to be seen at all. ^{13}C-^{13}C dipole interactions provide an additional mode of relaxation which, though not important for protium-bearing ^{13}C, can lead to shorter T_2s for quaternary and fully deuterated ^{13}C. Thus the intensities of the two septets due to ^{13}C-$^{13}CD_3$ are high enough for the signal to be clearly visible. Similarly, it can be seen (Fig. 1) that the doublet due to $^{13}C(8a)$-^{13}C is enhanced by a factor of about 3 relative to the singlet due to $^{13}C(8a)$-^{12}C.

Complementary information about the H/D composition at C(9) is obtained from an analysis of the signal at δ 80, due to C(3). The major signal is shifted upfield 0.12 ppm, together with a small doublet upfield shifted by 0.08 ppm. These signals are due to ^{13}C-$^{13}CD_3$ and ^{13}C-$^{13}CHD_2$ respectively and, when combined with the data above, show that relative amounts of the species at C(9) are in the order $CD_3 \gg CHD_2 \gg CH_2D$, CH_3.

Analysis of the peaks at δ 34, due to C(4), and at δ 136 due to C(4a), were carried out similarly. The α-shifted triplets due to $^{13}C(4)D$ are clearly visible (Fig. 1), although we were unable to discern them using a single ^{13}C label,[4] although they have been seen in the proton-coupled spectrum.[6] The signal due to C(4a) is also clarified by the use of two ^{13}C labels. The 0.04 ppm shift appears only as a broadening when a single ^{13}C label is used, but, with the second ^{13}C, the peaks due to ^{13}CH and ^{13}CD are comparable in size and are easily distinguished. It is clear from these data that deuterium from acetate can be retained at C(4) of citrinin, providing further evidence that the isocumarin (**2**) is not an obligatory precursor in citrinin biosynthesis.[4]

The ^2H NMR spectrum of this sample shows two doublets due to $^{13}C(4)D$ and $^{13}C(9)D_n$ (n=1, 2 and 3). The doublet due to deuterium at the methine carbon C(4) is an especially clear demonstration of retention of intact D-^{13}C at this position. The doublet due to deuterium at C(9) does not allow the various deuterated species at this position to be distinguished but



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the relative integrals show that there is about six times as much deuterium at C(9) than at C(4), consistent with $^{13}CD_3$ being the dominant species at C(9), and C(4) retaining about 50% deuterium.

In conclusion, the use of $^{13}CD_3{}^{13}COOH$ as a biosynthetic precursor provides not merely a convenient method of analysing both α and β shifts but has other advantages. The second ^{13}C allows ^{13}C from labelled acetate to be distinguished from that from other sources, especially important when enrichment is low. In addition, ^{13}C not bearing a proton relaxes much more readily when an adjacent carbon is a ^{13}C facilitating detection without the need for a relaxation agent or long relaxation delays, important when samples are small. We anticipate therefore that this new method will find wide application in natural product biosynthesis.

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