The Use of ^2H N.m.r. Spectroscopy and β-Isotopic Shifts in the ^{13}C N.m.r. Spectrum to Measure Deuterium Retention in the Biosynthesis of the Polyketide 6-Methylsalicylic Acid

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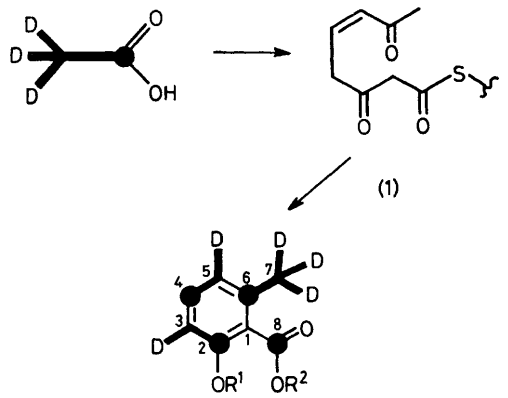
Summary A comparison is made between the use of ^2H n.m.r. spectroscopy and β-isotopic shifts in the ^{13}C n.m.r. spectrum for measurement of deuterium incorporation from [2-$^2H_3$]acetate into the polyketide 6-methylsalicylic acid (**2**); a high degree of retention is observed for the chain-building sites but the distribution of deuterium is not uniform.

THE fate of deuterium in biosynthesis can be monitored by ^2H n.m.r. spectroscopy or indirectly by ^{13}C n.m.r. spectroscopy.[1] The indirect method has been widely used with the deuterium directly attached to a ^{13}C nucleus, the resonance of which is shifted upfield (the α-shift) and shows deuterium coupling. For quantitative measurements of deuterium enrichment however this technique has the disadvantage that the signal to noise ratio of the shifted resonance is reduced by poor relaxation, signal multiplicity, and loss of nuclear Overhauser effect (n.O.e.).

We have therefore explored a variation of the indirect technique in which the reporter ^{13}C nucleus is placed β to the deuterium. The presence of the deuterium is again detected by an upfield shift in the resonance of the ^{13}C nucleus (the β-shift)[2] but the ^2H–^{13}C coupling over two bonds is negligible (< 1 Hz) so that the shifted signal is effectively a singlet in the proton noise decoupled (p.n.d.) ^{13}C n.m.r. spectrum. It is more easily detected and the level of enrichment can be more reliably determined because the problems with relaxation and n.O.e. are avoided.



SCHEME

The β-shift technique is ideal for a quantitative study of deuterium exchange in the biosynthesis of the polyketide 6-methylsalicylic acid from [2-$^2H_3$,1-^{13}C]acetate. The Scheme shows how the molecule is formed from four C_2-units *via* a linear polyketide such as (**1**)[3] with connectivities to be investigated indicated by heavy lines.

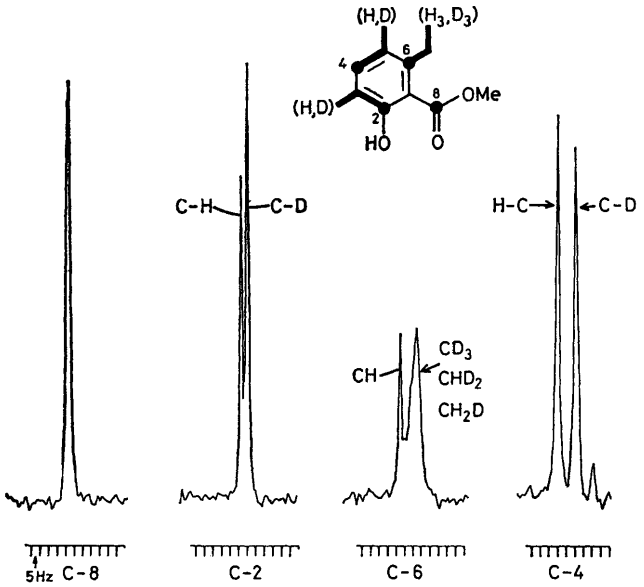


FIGURE 1. Signals from the p.n.d. ^{13}C n.m.r. spectrum of methyl 6-methylsalicylate derived from [2-$^2H_3$,1-^{13}C]acetate.

The precursor was added in equal portions over five days to an eleven day-old surface culture of *Penicillium griseofulvum* and the p.n.d. ^{13}C n.m.r. recorded for the methyl ester (**3**) of the metabolite under conditions designed to optimise the reliability of integration (small pulse angle, long relaxation delay). Figure 1 shows the resonances corresponding to the four sites of ^{13}C enrichment (*ca.* 7%). As expected, upfield β-shifted resonances were observed for C(2), C(4), and C(6) but not for C(8). The β-shifted resonance for C(2) (0·04 p.p.m. upfield) establishes the retention of deuterium at C(3). In the case of C(4), the shifted resonance for molecules labelled with deuterium at C(5) (0·1 p.p.m. upfield) is accompanied by a second weaker resonance (0·2 p.p.m. upfield) which is attributed to molecules containing deuterium at both C(3) and C(5).† This multiple labelling arises from incorporation of labelled C_2-units at adjacent sites in the metabolite due to inadequate dilution of the added precursor with endogenous material during the biosynthesis; due allowance is made for this in the quantitative analysis. The resonances for C(6) consist of a sharp unshifted signal arising from molecules with no deuterium at C(7) and a broad envelope of signals (up to 0·1 p.p.m. upfield) arising from molecules carrying one or more deuterium atoms at C(7). The relative areas of the shifted and unshifted peaks were measured and after allowing for the incidence of ^{13}C at natural abundance, the percentage of molecules in which the ^{13}C labels retain at least one deuterium partner was calculated for the four C_2-units (Table).

TABLE. Percentage of ^{13}C-labelled C_2-units retaining one or more deuterium atoms in 6-methylsalicylate derived from [2-$^2H_3$,1-^{13}C]acetate.

C(2) + C(3)	C(4) + C(5)	C(6) + C(7)	C(8) + C(1)
80%	70%	95%	0%

For comparison a parallel incorporation study was carried out using [2-$^2H_3$]acetate as precursor and ^{2}H n.m.r. spectroscopy to monitor deuterium retention. The spectrum of the methyl ester of the metabolite is shown in Figure 2.

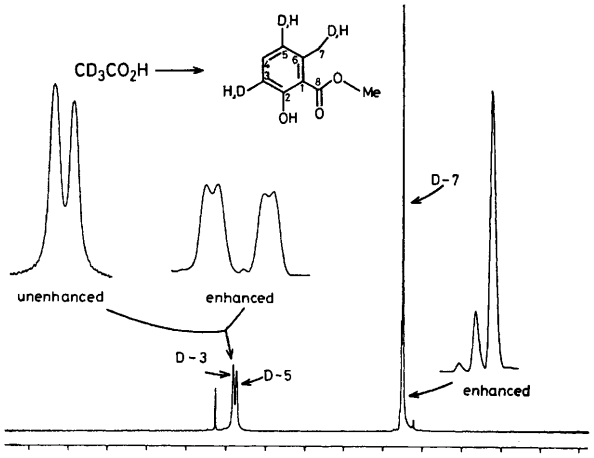


FIGURE 2. ^{2}H n.m.r. spectrum of methyl 6-methylsalicylate derived from [2-$^2H_3$]acetate.

The two downfield resonances at 6·80 and 6·72 p.p.m. correspond to deuterium attached to C(3) and C(5) respectively. For the chain starter methyl there is an intense singlet (2·51 p.p.m.) arising from molecules containing a CD_3 group and weaker additional multiplets assigned to CD_2H and CDH_2 groups. The relative intensities of the signals

† The assignments and validity of the quantitative analyses described later were confirmed by control experiments with synthetic samples of specifically deuteriated (**4**).

for D(3), D(5), and D(7) measured by peak area were 1·2 : 1 : 4·9 but uncertainty was introduced by overlap of the resonances for D(3) and D(5).

Poor resolution is frequently a limitation of ^{2}H n.m.r. spectroscopy because the peaks are inherently broad and the spectral dispersion is low; with increasing molecular size this problem is exacerbated. In contrast the β-shift technique offers superior resolution which is not lost with increasing molecular size. The technique also provides information on incorporation of intact units (^{13}C–C–D) not available from ^{2}H n.m.r. spectroscopy.

Both techniques indicate that there is significantly more deuterium retained at C(3) than at C(5), contrary to a previous suggestion.[4] This non-uniform distribution could arise from different degrees of random exchange in the process of chain assembly, or more intriguingly, it could be the consequence of different mechanisms of deuterium loss at C(3) and C(5) during the steps of cyclisation and aromatisation. Another noteworthy feature of our results is the high degree of deuterium retention at the chain building sites which is in marked contrast with results reported for certain non-aromatic polyketide metabolites.[5]

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