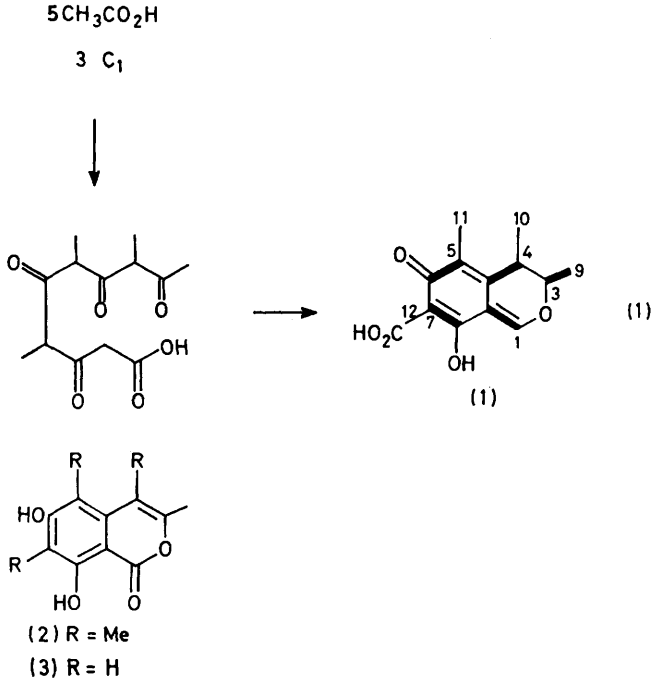
Protium as a Tracer in Polyketide Biosynthesis: Incorporation of $^{13}CH_3{}^{13}CO_2H$ into Citrinin Produced on a Medium Based on D_2O

By JILL BARBER and JAMES STAUNTON*
(*University Chemical Laboratory, Lensfield Road, Cambridge*, CB2 1EW)

Summary Evidence concerning the origin of the hydrogen atoms in the polyketide antibiotic citrinin has been obtained by a novel technique in which the metabolite is produced from protium-labelled carbon sources on a medium based on D_2O.

To date experiments to investigate the biosynthetic origin of C–H bonds in polyketide metabolites have concentrated upon the use of tritium or deuterium to label the methyl group of acetate. The sites of labelling have been detected directly by n.m.r. spectroscopy[1,2] or indirectly through spin–spin coupling to an adjacent ^{13}C nucleus.[3,4] The utility of such isotopic methods is limited, however, by low sensitivity in the case of deuterium and by radiochemical hazard in the case of tritium. We have therefore developed a simple and economical technique which avoids these limitations by using protium as the tracer in the deuteriated environment.



The biosynthesis of the antibiotic citrinin (**1**) by the fungus *Penicillium citrinum* was selected as a case study. The carbon skeleton is built up from five acetate units and three C_1 units as indicated in reaction (1). Evidence concerning the identity of advanced intermediates has been sought from mutant studies[5] and by incorporation experiments.[6] In the latter it was found that a radioactive label is incorporated specifically from the isocoumarin (**2**) but not from its relative (**3**).

After growing sluggishly for 14 days on a Czapek–Dox medium made up in D_2O, a culture of *Penicillium citrinum* was transferred to fresh medium made up in D_2O and equal doses of [1,2- $^{13}C_2$]acetate† were administered daily for 10 days. Citrinin was isolated 10 days later.

The ^{13}C n.m.r. data from a control experiment in which citrinin was produced on a medium containing H_2O are presented in the Table. They confirm that the polyketide nucleus is formed by head-to-tail linkage of five intact C_2 units. From the corresponding spectrum from citrinin produced in the presence of D_2O, some signals (mainly from carbons remote from hydrogen nuclei) are not changed significantly from the control, whereas others (all from carbons which normally bear protons) are profoundly changed in character owing to partial replacement of protons by deuterons. Two relevant regions of the spectra run in the absence of a relaxation agent so as to enhance the sensitivity of detection of protonated ^{13}C nuclei are shown in the Figure.

† The use of [1, 2- $^{13}C_2$]-acetate allowed carbon incorporated from this source to be distinguished from that derived from glucose which could therefore be of normal isotopic composition.

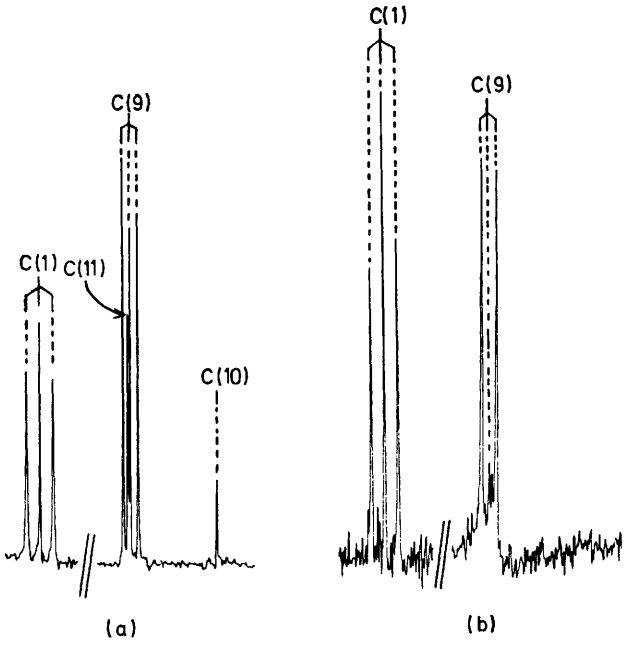


FIGURE. Proton-noise-decoupled ^{13}C n.m.r. spectrum of critinin produced on (a) H_2O; (b) D_2O [$CDCl_3$ solution; no added tris-(acetylacetonato) chromium].

Compared with the control spectrum the natural abundance singlets in spectrum (b) for the three methyl groups C(9), C(10), and C(11) are greatly diminished in intensity, their place being taken by weak multiplets, owing to ^{13}C–D coupling. At these sites, therefore, virtually all the methyl groups produced from glucose have incorporated at least one deuteron. This is confirmed in the ^1H n.m.r. spectrum in which the CH_3 singlet for C(11) is replaced by a strong 1:1:1 triplet corresponding to CH_2D with additional weaker satellites corresponding to CHD_2 (the multiplets for the other methyl groups overlap). In contrast the $^{13}CH_3$–^{13}C doublet for C(9) is still intense in spectrum (b). The great enhancement of the intensity of these satellites compared with the natural abundance singlet can be explained by incorporation of intact $^{13}CH_3$–^{13}C residues from the administered acetate, which confirms that C(9) is part of the chain starter unit.

The signals for C(4) in spectrum (b) follow the pattern set by C(9) with a strong ^{13}CH–^{13}C doublet and a relatively weak associated singlet. This evidence that the proton can be retained at this site is inconsistent with (**2**) being an intermediate because C(4) would then be expected to wholly deuteriated from the medium at a subsequent step. The possibility of an unexpected addition of a proton rather than a deuteron can be ruled out by the relative intensities of the signals which show that carbons derived from administered acetate are protonated to a greater extent than those from glucose.

TABLE. Carbon chemical shifts and ^{13}C–^{13}C coupling

Carbon	δ (^{13}C)/p.p.m.[b]	J[c]/Hz
1	161·95	69·6
3	80·91	37·8
4	33·72	40·9
4a	139·17	40·9
5	99·36	63·6
6	176·15	63·6
7	122·01	56·9
8	182·73	56·9
8a	106.44	69·6
9	17·56	37·8
10	8·72	—[a]
11	17·83	—[a]
12	173·48	—[a]

[a] Singlet. [b] Relative to Me_4Si. [c] ^{13}C–^{13}C coupling in citrinin labelled with [1,2-$^{13}C_2$]acetate.

The signal for C(1) is essentially the same shape in spectrum (b) as it is in the control, and the same applies to that from C(3). Apparently both sites are protonated to a significant extent but, in contrast to C(4) and C(9), the chances of a carbon bearing a proton are the same whether it originates from glucose or the administered acetate. This is consistent with the proton being derived from an external reducing agent, almost certainly a nicotinamide coenzyme, the proton coming originally from oxidative cleavage of C–H bonds in metabolic intermediates derived from glucose.

The superiority of protium as a tracer in the present investigation was demonstrated by a parallel experiment in which [2-$^2H_3$, 2-^{13}C]acetate was used as a precursor in the presence of H_2O. At the levels of enrichment attained, the ^{13}C–D multiplets in the proton-noise-decoupled ^{13}C n.m.r. spectrum were too weak to allow reliable identification of sites of deuteriation.

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