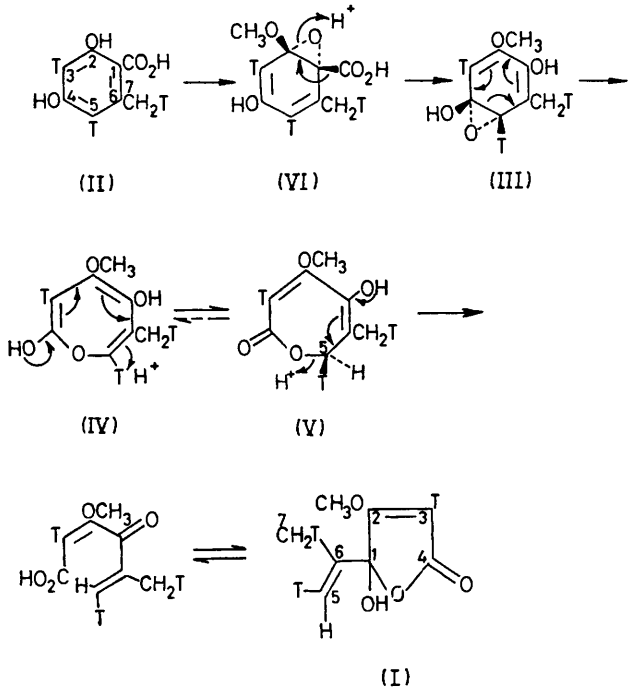
Use of Tritium Nuclear Magnetic Resonance for the Direct Location of ^3H in Biosynthetically-labelled Penicillic Acid

By JASIM M. A. AL-RAWI, JOHN A. ELVIDGE, DEVENDRA K. JAISWAL, JOHN R. JONES, and ROBERT THOMAS*
(*Department of Chemistry, University of Surrey, Guildford, Surrey* GU2 5XH)

Summary In a first application of ^3H n.m.r. spectroscopy to a biosynthetic investigation, namely the incorporation of [^3H]acetate by *Penicillium cyclopium* into penicillic acid (I), the stereospecificity of labelling of the vinyl methylene group is directly demonstrated and a biosynthetic pathway thereby proposed.

TRITIUM n.m.r. studies can be carried out safely and routinely even with simple equipment.[1] With a more sophisticated spectrometer, *e.g.* Bruker WH 90 operating by the pulse, Fourier-transform method, the increased sensitivity gives a satisfactory signal-to-noise ratio with samples containing as little as 1 mCi of tritium. It is now possible, therefore, to apply ^3H n.m.r. techniques to the study of bio-organic problems, without there being any significant radiological hazard. Here we report the first direct determination of the positions of the tritium label in biosynthetically derived penicillic acid (I) by ^3H n.m.r. spectroscopy.



(i) Numbering in (I) corresponds to (II) for clarity. (ii) Stereochemistry in (III), (V), and (VI) is only relative. (iii) Relative positions of 5-H and 5-T in (I) are yet to be determined.

The polyketide origin of penicillic acid has been established through incorporation studies with [^{14}C]-acetate[2,3] and -malonate.[4] In the present work, tritium supplied as sodium [^3H]acetate (460 mCi) of high specific activity (2·4 Ci mmol^{-1}) was incorporated with *ca.* 7% efficiency into penicillic acid by *P. cyclopium*. The acetate was dissolved in sugar-free Raulin–Thom medium (350 ml) at pH 3·0, which was incubated for 24 h with a refloated pad of mycelium, previously grown in surface culture for 7 days at 24° on a similar volume of Raulin–Thom medium containing 5% glucose. The resulting purified penicillic acid (35 mg. *ca.* 98·3 mCi mmol^{-1}) was dissolved in 100 μl of [$^2H_6$]acetone and transferred to a cylindrical micro-cell (Wilmad), which in turn was placed in a 5 mm n.m.r. tube.

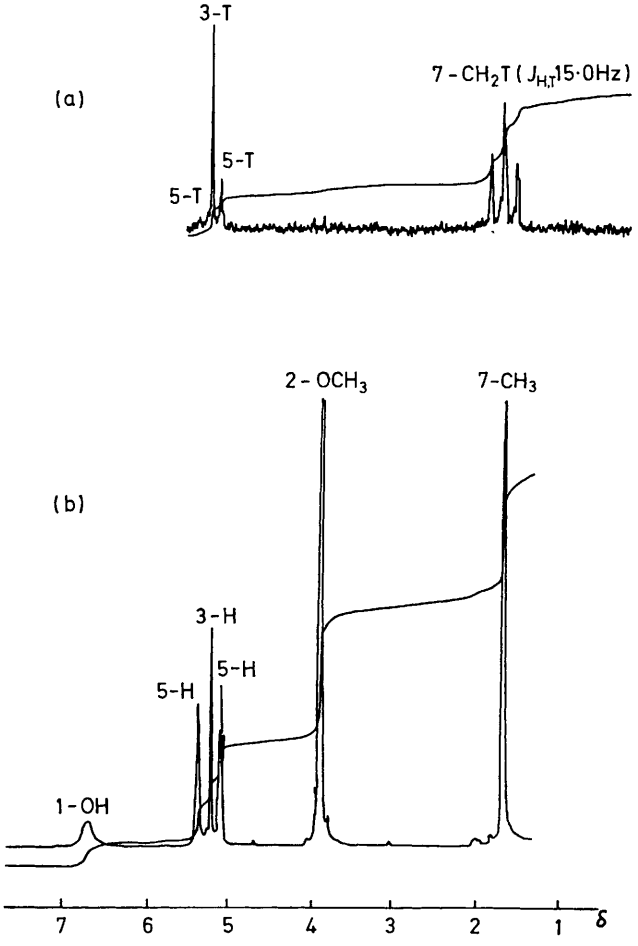


FIGURE. Fourier-transform n.m.r. spectra of penicillic acid (I) in [$^2H_6$]acetone at 25°; (a) ^3H spectrum, 1·12 × 10^4 pulses at 1·7 s intervals, (b) ^1H spectrum (Me_4Si).

The ^3H n.m.r. spectrum (96·02 MHz) is shown in the Figure together with the ^1H spectrum (90·02 MHz). A satisfactory signal-to noise ratio for the ^3H signals was obtained even with penicillic acid of lower specific activity (42·5 mCi mmol^{-1}), which had been obtained in another feeding

experiment using 230 mCi of [^{3}H]acetate. The tritium spectra clearly showed the expected labelling pattern, *i.e.* there was a triplet from the 7-CH_2T group and a singlet from the 3-T, with relative intensities of 3:1, and also a signal, weaker than the last, from a CHT:C< group, corresponding to the higher field one of the two terminal vinylic protons at C-5. The assignment of the signals from these two protons is ambiguous[5] and currently under investigation. A very weak tritium signal corresponding to the other 5-H was also present.

This direct evidence for the specific incorporation of ^{3}H at C-5 illustrates the value of the technique as a mechanistic probe. The observations require that the known intermediate (II) undergoes 4,5-cleavage. This agrees with Mosbach's demonstration,[3] based on chemical degradations of (I) derived metabolically from specifically labelled [^{14}C]-orsellinic acid (II). The alternative 1,2-cleavage of the aromatic ring would not result in the incorporation of tritium from [^{3}H]acetate into the vinylic methylene group.

The apparent specific partial loss of tritium at C-5 is probably a consequence of the ease of tritium exchange in one of the biosynthetic intermediates formed subsequent to the ring scission of orsellinic acid (II). This aromatic C–C cleavage, and also that leading to patulin, are both unusual in that they require a lower degree of oxidation than the majority of known ring scissions.[6]

A possible scheme is indicated, involving cleavage through an electrocyclic conversion of the hypothetical epoxide (III) to an oxepin (IV), which could account mechanistically for the formation of (I) (and, by analogy, for the formation of patulin *via* an appropriate epoxide of gentisic aldehyde). The scheme would also be consistent with the apparently selective partial exchange of tritium at the chiral centre (C-5) of (V). This could result from comparatively rapid enzyme-catalysed stereospecific isomerisation of (IV) to (V), together with a slower spontaneous reversible interconversion of these intermediates, which in one direction would effect some loss of tritium, and in the reverse direction would yield a racemate of (V). The epoxide (VI) could intermediate the required oxidative decarboxylation (II) → (III).

The present study demonstrates that tritium n.m.r. spectroscopy is a convenient and direct probe of ^{3}H-incorporation at moderate levels of radioactivity. Useful aspects of this new technique relative to ^{13}C n.m.r. spectroscopy include the absence of detectable isotope at natural abundance, the radioactivity as a monitor of isotopic content the closely predictable chemical shifts and coupling constants (from the ^{1}H n.m.r. spectra), and the direct indication of stereospecific hydrogen labelling, as in non-equivalent olefinic and prochiral methylene groups.

We thank the Radiochemical Centre and S.R.C. for support, and the Association of Commonwealth Universities for a Scholarship (to D.K.J., on leave from the Research and Development Organisation, New Delhi).

(*Received*, 29*th January* 1974; *Com.* 132.)

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