Tritium Nuclear Magnetic Resonance Spectroscopy. Part 7.[1] New Information from the Tritium Distribution in Biosynthetically Labelled Penicillic Acid[2]

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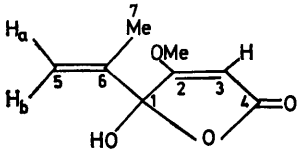
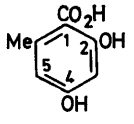
^{3}H N.m.r. spectroscopy is used to determine directly the regio- and stereo-specificity of the labelling in penicillic acid, biosynthesised by *Penicillium cyclopium* from [^{3}H]acetate, from [2-^{3}H]malonate, and from [3,5-^{3}H]orsellinic acid. In particular, the method reveals the specific labelling of the 5-methylene group—largely *trans* to *C*-methyl—and the partial loss of label from the 5-positions relative to the 3-position and from the 3-position relative to the *C*-methyl group. It thus provides new information, which needs to be incorporated in any detailed proposals concerning the biosynthetic pathway and the enzyme stereochemistry. Such detail was not available from previous studies involving the use of ^{14}C and ^{13}C as tracers.

TRITIUM n.m.r. spectroscopy[1,3] is here applied for the first time to the analysis of the labelling in a metabolite biosynthesised from tritiated precursors. Important advantages over the usual methods of tritium analysis are apparent, and indeed, in the present study, over the use of carbon isotopes.

[^{3}H]Acetate was fed to replacement cultures of *Penicillium cyclopium* under conditions established as avoiding exchange and a 7% incorporation into the metabolite, penicillic acid (1), was achieved (Table 1). Tritiated malonate and orsellinic acid (2) were also fed. Examination of the resulting penicillic acid by ^{3}H n.m.r. then gave the positions, extent, and stereochemistry of the incorporated label (Table 2). More information



(1)



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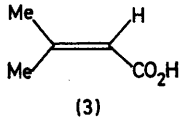

(3)

emerged directly from this rapid and non-destructive procedure than from previous studies by ^{14}C labelling,[4-7] or indeed than could be obtained from ^{13}C labelling and n.m.r. analysis.[8] This is because use of isotopic hydrogen will distinguish between non-equivalent hydrogen atoms at sp^2 (vinylic) methylene groups or at prochiral sp^3 methylene sites. Tritium (rather than deuterium) was chosen because its nuclear properties are ideal for n.m.r. analysis,[3] especially where coupling information is required.[1,9] Moreover, the radioactivity greatly facilitates location of labelled products, without posing

TABLE 1

Incorporation results with seven-day replacement cultures of *P. cyclopium*

Precursor (mCi, mCi mmol^{-1})	Incubation period (h)	Penicillic acid: Yield (mg)	Penicillic acid: Specific activity (mCi mg^{-1}, mCi mmol^{-1})	Penicillic acid: Incorporation of precursor (%)
Sodium [^{3}H]acetate				
227.3, 2 400	24	65.5	0.25, 42.5	7.2
458.7, 2 400	24	67.4	0.58, 98.6	8.5
Diethyl [2-^{3}H]malonate				
207.8, 277	8	52.9	0.020, 3.4	0.5
467.6, 1 680	24	80.3	0.117, 19.9	2.0
[3,5-^{3}H]Orsellinic acid				
97.5, 815	24	71.5	0.116, 19.5	8.4

TABLE 2

^{3}H N.m.r. incorporation data for penicillic acid

(a) Biosynthesised from [^{3}H]acetate

δ(^{3}H)	Multiplicity and J(T,H)/Hz	Assignment	Signal intensity (%)	Relative incorporation (%) per site
1.77	t, 15.5	7	69.2	43
5.27	s	3	18.5	34
5.19	m	5b	10.1	19
5.49	m	5a	2.2	4

(b) Biosynthesised from [2-^{3}H]malonate

δ(^{3}H) 5.27 (58%), 5.19 (30%), 5.49 (12%)

(c) Biosynthesised from [3,5-^{3}H]orsellinic acid

δ(^{3}H) 5.27 (57%), 5.19 (31%), 5.49 (12%)

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[3] J. P. Bloxsidge, J. A. Elvidge, J. R. Jones, and E. A. Evans, *Org. Magnetic Resonance*, 1971, **3**, 127.

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[6] R. Bentley and J. G. Keil, *J. Biol. Chem.*, 1962, **237**, 867.

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[8] M. Seto, L. W. Cary, and M. Tanabe, *J. Antibiotics*, 1974, **27**, 558.

[9] J. M. A. Al-Rawi, J. A. Elvidge, J. R. Jones, and E. A. Evans, *J.C.S. Perkin II*, 1975, 449.

undue safety and health hazards.[10a] Our present n.m.r. equipment is adequate for the detection of at least 1 mCi of tritium per labelled site in a molecule (Table 3). Because the specific activity of a fully

TABLE 3

^{3}H N.m.r. detection sensitivity, measured by using [7α,7β-^{3}H]dehydroepiandrosterone [1] in $(CD_3)_2SO$

Radioactivity in 100 μl n.m.r. cell from 7β-^{3}H (mCi)	No. of transients	Time (h)	Observed signal-to-noise ratio for 7β-^{3}H signal [a]
12	3.6×10^4	16	47
1.2	4.5×10^4	20	4
0.12	3×10^5	133	1.6

[a] Signal height/(extreme noise excursion/2.5)

monotritiated compound is 29.1 Ci mmol^{-1},[10b] the preceding level of radioactivity corresponds to 3.4×10^{-3}% of tritium. Such detection sensitivity is not yet comparable to the extreme sensitivity of counting methods,[10c] but there are the enormous advantages of dispensing with tedious chemical degradations and their inherent uncertainties.[10c] Moreover, improvement in the sensitivity of the n.m.r. technique is feasible, as superconducting spectrometers, larger probes, quadrature detection, crystal filters, *etc.*, become available.

EXPERIMENTAL

U.v. and i.r. spectra were measured with Unicam SP 800B and SP 200 spectrophotometers, respectively. Proton and tritium n.m.r. spectra were obtained as previously described [1] by using a Bruker WH 90 pulse spectrometer operating at 90 and 96 MHz (nominal), respectively, with tetramethylsilane as internal standard and perdeuterioacetone as solvent (unless otherwise stated). Tritiated samples were counted with a Beckman LS 100 liquid scintillation counter by using NE-250 liquid scintillator (Nuclear Enterprises Ltd.). Simultaneous assay of ^{3}H and ^{14}C was performed by counting in two channels and the use of an external standard ratio.[11]

Tritiated Precursors.—Sodium [^{3}H]acetate (2.4 Ci mmol^{-1}) was from The Radiochemical Centre Ltd. Aqueous sodium [^{3}H,2-^{14}C]acetate (48.67 μCi ml^{-1} of ^{3}H and 7.45 μCi ml^{-1} of ^{14}C) was prepared by mixing the two labelled acetates in water. Diethyl malonate (0.5 ml) in dioxan (0.1 ml) was treated with tritiated water (65 μl; 50 Ci ml^{-1}) and sodium carbonate (10 mg) for 7 days. Sodium sulphate was added and the organic liquid fractionated to give diethyl [2-^{3}H]malonate (1.68 Ci mmol^{-1}), b.p. 42° at 0.5 mmHg, δ 3.25 (d, 2-^{3}H), J(H,T) 16.7 Hz. Treatment of orsellinic acid [12] (0.3 mmol) with tritiated water (20 μl; 50 Ci ml^{-1}) and sodium hydroxide (1 pellet) for 4 days, neutralisation (HCl), extraction into ether, and evaporation of the extract gave [3,5-^{3}H]orsellinic acid (0.82 Ci mmol^{-1}), δ 6.26 (d,3-^{3}H) and 6.34 (d,5-^{3}H), with J(H,T) 5.3 Hz in each case.

Culture.—*P. cyclopium* IMI 89372 (Commonwealth Mycological Institute, Kew) was grown under aseptic conditions on potato–dextrose–agar [13] slopes. After 6—7 days, spores were transferred by suspension in sterile water to Raulin–Thom medium [14] (350 ml portions) in 1 l flasks and grown in surface culture at 24 °C in the dark. The metabolism solution was concentrated (to 1/10th volume) at 45—50 °C and 10 mmHg and extracted with chloroform (2 × 90 ml), and the extract was dried (Na_2SO_4) and evaporated under reduced pressure to give penicillic acid hydrate [from water (charcoal)], m.p. 59—63°.[15] Dehydration (over $CaCl_2$) gave penicillic acid (1) [from chloroform–cyclohexane (1 : 3)], m.p. 83—84° [15] (Found: C, 56.8; H, 5.9. Calc. for $C_8H_{10}O_4$: C, 56.5; H, 5.9%), $\lambda_{max.}$ (H_2O) 227 nm (log ε 4.06),[16] $\nu_{max.}$ (Nujol) 3 270s, 1 741s, 1 639s, 1 278, 1 014, 906, and 806 cm^{-1}, δ 1.77 (CMe), 3.98 (OMe), 5.27 (3-H), 5.18 (5-H_b), 5.44 (5-H_a), and 6.70 (OH), and, by selective decoupling, J(Me,H_b) 1.4 ± 0.3, $|J(H_a,H_b)|$ 0.88 ± 0.3, and J(H_a,Me) 0 Hz. Paper chromatography [6] of the metabolism solution showed that orsellinic acid (2) was also present.

Feeding Experiments.—The metabolism solution was cautiously removed by suction from seven-day-old cultures and replaced under the mycelium with sterile sugar-free Raulin–Thom medium at pH 3.0 (HCl) containing the chosen tritiated precursor. The resulting labelled penicillic acid was harvested (as before) after 8 or 24 h at 24 °C in the dark. Observations and results are given in Tables 1 and 2. By feeding [^{3}H,2-^{14}C]acetate (^{3}H : ^{14}C 7.04 : 1), labelled penicillic acid resulted with ^{3}H : ^{14}C 2.51 : 1.

Safety Considerations.—Standard precautions [10a] were observed in operations with [^{3}H]compounds, including measurement of ^{3}H n.m.r. spectra.[3]

During the feeding experiments, vials separately containing NE-250 liquid scintillator and silica gel were kept in the incubator and subsequently counted, the silica first being soaked in water (5 ml) and an aliquot portion of the water (0.5 ml) then being counted in NE-250 liquid. In the highest level [^{3}H]acetate feeding experiment (Table 1) observations were: scintillator, 3.77×10^{-2} μCi; silica gel, 3.64×10^{-2} μCi. This level of radioactivity released in 24 h represents 8×10^{-7}% of the total activity of the [^{3}H]acetate. Even if this radioactivity release had occurred at one instant into the laboratory air (3.3×10^8 cm^3), the contamination would have been far below the permissible maximum of 5×10^{-6} μCi cm^{-3} as tritiated water vapour in air.[17]

RESULTS AND DISCUSSION

Optimal conditions for the growth and feeding of *P. cyclopium* cultures were obtained from experiments on still, shake, and replacement culture, and involving

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[14] G. Smith and H. Raistrick, 'An Introduction to Industrial Mycology,' Arnold, London, 4th edn., 1954, p. 238.
[15] *Cf.* J. H. Birkinshaw, A. E. Oxford, and H. Raistrick, *Biochem. J.*, 1936, **30**, 394.
[16] *Cf.* J. H. Ford, A. R. Johnson, and J. W. Hinman, *J. Amer. Chem. Soc.*, 1950, **72**, 4529.
[17] Internat. Comm. Radiological Protection Publ. 2, ' Report of Committee II on Permissible Dose for Internal Radiation,' Pergamon, Oxford, 1959.

addition of 'cold' and of low-level radioactive precursors.

Possible trivial loss of tritium label was shown not to pose problems. Sodium [^{3}H]acetate showed no detectable detritiation in water or aqueous acid, or even in 0.2M-sodium hydroxide. Bonhoeffer [18] obtained a velocity constant of 1.6×10^{-8} s^{-1} at 25 °C for protium–deuterium exchange by extrapolation of results at 100 °C for acetate in 1M-potassium deuterioxide in deuterium oxide. Exchange detritiation of [^{3}H]acetate during biological feeding experiments (even under physiologically 'alkaline' conditions) could therefore be discounted. On the other hand, diethyl [2-^{3}H]-malonate was quite rapidly detritiated [19] in either aqueous acid or alkali and so might only be used for comparatively short times (*e.g.* <2 days): our measured rate of detritiation in Raulin–Thom medium at pH 4.0 and 25 °C was 1.24×10^{-5} s^{-1}. Hence the [^{3}H]malonate feeding experiments were appropriately brief. Exchange detritiation of the (biosynthesised) [^{3}H]penicillic acid was also examined but no significant loss of label was found in water or acetate buffer (pH 4.6) during 10 days. A possibility that tritium in precursors or product might be labilised during the biosynthesis of penicillic acid from [^{3}H]acetate was examined by feeding a mixture of [^{3}H]- and [2-^{14}C]-acetate. A loss of 0.73 atom of tritium per molecule of penicillic acid, during biosynthesis from the acetate, was found in comparison with the uptake of ^{14}C. It was hardly feasible to assess isotope effects because of the unknown number of steps in the biosynthesis.

Before the ^{3}H n.m.r. spectrum of labelled penicillic acid could be interpreted it was necessary to assign all the signals in the ^{1}H n.m.r. spectrum. This had not been done for the 5-methylene signals because neither the chemical shifts [20] nor the allylic coupling constants [21]

TABLE 4

Overhauser enhancements (%) for penicillic acid (purified) in out-gassed 99.95% $CDCl_3$

	Enhancement on irradiating at frequency of		
Observed signal	5-H (higher field)	5-H (lower field)	*C*-Me
5-H (higher field)		6.2	2.2
5-H (lower field)	14		−4.2
C-Me	0	1.7	

provided a sure basis. We therefore attempted their assignment through observation of nuclear Overhauser enhancements (n.O.e.s) but the n.O.e.s found (Table 4) were so much smaller than *e.g.* for dimethylacrylic acid [22] (3) that conclusions could not be drawn safely. Experiments with the shift reagent Eu(thd)$_3$[tris(2,2,6,6-tetramethylheptane-3,5-dionato)europium(III)], however, proved diagnostic. Progressive addition of this reagent to penicillic acid in deuteriochloroform produced down-field shifts of the higher-field 5-proton signal and of the signal from the *C*-methyl group which were similar in magnitude (Table 5). This implied that the respective

TABLE 5

Europium-induced shifts (Hz) and altered positions of ^{1}H n.m.r. signals (Hz from Me_4Si, at 90 MHz) of penicillic acid in $CDCl_3$

Eu(thd)$_3$ (mg)	$CHCl_3$ [a]	5-H		3-H	OMe	CMe
0	654.6	494.5	470.1	462.2	352.7	159.8
0.5	654.6	494.1	470.4	461.1	352.4	160.4
1.5	654.6	493.8	470.7	461.1	351.9	160.7
3.3	654.6	494.1	472.8	445.8	351.3	161.9
5.5 [b]	654.6	493.8	474.5	446.1	350.7	163.6
Max. shift	0.0	*ca.* 0	+4.4	−16.1	−2.0	+3.8

[a] Trace present in solvent. [b] At higher concentrations, signals were significantly broadened.

protons came within similar distances of the europium atom in the transient complex (and subtended similar acute angles to the principal axis at the Eu atom). A Dreiding model showed that as the penicillic acid side chain rotates about the C(6)–C(1) bond, 5-H$_b$ and the *C*-methyl group traverse the same region of space. The high-field 5-methylene signal was therefore assigned to H$_b$, *trans* to the *C*-methyl group.

The approximate geometry of the complex was further indicated by the upfield shifts of the methoxy and 3-proton signals. Evidently the europium atom was preferentially co-ordinated to the 1-hydroxy-group, alongside the adjacent face of the lactone ring. This would make the angles to the principal axis subtended at the europium atom by the methoxy-group (in its average position), and by the 3-proton, increasingly (in that order) greater than *ca.* 55°, at which ($3\cos^2\theta - 1$), and so the induced shift, changes sign.

The ^{3}H n.m.r. spectrum of the [^{3}H]penicillic acid biosynthesised from [^{3}H]acetate (see ref. 2 and Table 2) at once showed that the groups at the 3-, 5-, and 7-positions [see (1)] were derived from the methyl group of the acetate precursor. This pattern of labelling was consistent only with 4,5-ring scission of the presumed intermediate orsellinic acid (2), which would necessarily have been labelled with tritium at the 3- and 5-positions and in the 6-methyl group. It was also immediately evident that the 5-methylene group in penicillic acid had largely been specifically labelled at H$_b$, *trans* to the *C*-methyl group, with slight labelling at 5-H$_a$. Any detailed mechanistic proposals for penicillic acid biosynthesis have to take into account these new findings with their important implications for the stereochemistry of the enzymic processes involved.

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[19] J. A. Elvidge, D. K. Jaiswal, J. R. Jones, and R. Thomas, *J.C.S. Perkin II*, 1976, 353.
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[22] F. A. L. Anet and A. J. R. Bourn, *J. Amer. Chem. Soc.*, 1965, **87**, 5250.

The simplicity (and power) of the ^3H n.m.r. analysis of the biosynthesised [^3H]penicillic acid compares favourably with the procedures used in previous studies involving carbon isotopes. Birch *et al.*[4] first proposed that penicillic acid was effectively a tetraketide derived from acetate, and from the results of feeding [2-^{14}C]-acetate, degradation of the penicillic acid, and counting, suggested a biosynthetic pathway *via* orsellinic acid and 1,2-ring scission thereof. Mosbach[5] subsequently pointed out that 4,5-ring scission would equally well have fitted the observations. He decided in favour of the second possibility on the basis of the relatively elaborate expedient of synthesising and feeding [1-^{14}C]-orsellinic acid. More recently, the feeding of [1,2-^{13}C]-acetate and analysis of the resulting penicillic acid by ^{13}C n.m.r.[8] have confirmed Mosbach's deduction.

Table 1 shows that the highest incorporation of tritium into penicillic acid was effected with labelled orsellinic acid, in agreement with orsellinic acid being an advanced precursor. The specific incorporation of tritium into the 5-position of penicillic acid [Table 2, (c)] is consistent only with 4,5-ring cleavage of the intermediate [3,5-^3H]orsellinic acid; the very different distribution between the 5-H_a and 5-H_b in penicillic acid [Table 2, (c)], similar to that obtained from precursor [^3H]acetate [Table 2, (a)], strongly supports the role of orsellinic acid as a true intermediate on the pathway from acetate.

Bentley and Keil[6] found that in penicillic acid derived from malonate, C-7 and C-6 are not appreciably labelled from either the methylene or the carbonyl carbon atoms of malonate. Therefore they concluded that malonate is not incorporated through decarboxylation to acetate but represents a precursor beyond acetate. The biosynthesis of penicillic acid thus proceeds from one acetate unit and three malonate units. Our feeding of [2-^3H]malonate gave penicillic acid labelled only at the 3- and 5-positions [Table 2, (b)]. The complete absence of label in the *C*-methyl group confirmed that this group was acetate-derived. Moreover the relative degrees of incorporation in the 3- and 5-sites as shown by integration of the ^3H n.m.r. signals were very similar to those in the penicillic acid derived solely from [^3H]acetate, again confirming that the same enzymic pathway is involved. The condensation of acetate with three malonate units presumably gives rise on the enzyme surface to a tetraketide which then undergoes ring closure to orsellinic acid. Exchange detritiation of the tetraketide might be significant: it would be expected to be least from the methyl group of the 'starter' acetate and most from the other methylene groups because of their possible activation. This would lead to lower incorporation of tritium into the aromatic ring relative to the methyl group in the intermediate biosynthesised orsellinic acid and show correspondingly in the penicillic acid, the *C*-methyl carrying relatively more label than *e.g.* the 3-position (Table 2). Exchange loss of tritium at a stage subsequent to the 4,5-ring scission of orsellinic acid would account for the lower incorporation at the 5- than at the 3-position in penicillic acid (Table 2). This and the observed selective labelling within the 5-methylene group could be explained by the intermediacy of an enzyme-held oxepin, as already outlined by us.[2] Further discussion of the implications of the newly found details of the biosynthesis of penicillic acid is intended (by R. T. and D. K. J.).

The present experiments demonstrate clear advantages of ^3H n.m.r. spectroscopy over conventional degradation and counting for the analysis of tritium-labelled products. The potentialities of tritium labelling for biosynthetic investigations and the precautions necessary for obtaining meaningful results have been underlined. Tritium can at least be usefully complementary to carbon isotopes: it may, as here, be more powerful.[2] Even incidental exchange-loss of tritium can provide detailed mechanistic information not accessible through the use of carbon isotopes.

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