Some New N.M.R. Methods for Tracing the Fate of Hydrogen in Biosynthesis

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1 Introduction

The experimental study of biosynthesis has developed largely through the application of tracer studies using compounds istopically labelled with ^{14}C or ^{3}H. More recently, the introduction of sensitive Fourier Transform nuclear magnetic resonance techniques[1] has led to the widespread adoption of ^{13}C as an isotopic label. This rapid, non-destructive technique for detecting specifically the sites of enrichment in a molecule has already contributed enormously to our knowledge of biosynthesis,[2] particularly in the polyketide, terpene, and porphyrin fields.

An important advantage in the use of ^{13}C is that studies with doubly-labelled precursors such as [1,2-$^{13}C_2$]acetate enable a detailed picture of the bonds broken or formed during biosynthetic processes to be built up. Hydrogen isotopes may also be used with ^{13}C isotopes in multiple label studies, providing evidence concerning the integrity of carbon–hydrogen bonds. This is the basis of one of a number of interesting n.m.r. methods using hydrogen isotopes by which the fine details of biological reactions may be probed. This review sets out to evaluate these new techniques in relation to more standard approaches.

A. **Choice of Isotope.**—The choice of isotope to be used in a biosynthetic experiment is determined by several factors: the first but not normally decisive consideration is the method of synthesis of the labelled precursor; secondly, the method of detection of the isotope as determined by its physical and magnetic properties (See Tables 1 and 2). A third consideration are the kinetic isotope effects[3] (stemming from differences in thermodynamic bond energies) which can profoundly alter the reactivity of isotopically-substituted molecules, thus exerting a dramatic influence upon experiments with labelled compounds in biological

[1] T. D. Farrer and E. D. Becker, 'Pulse and Fourier Transform NMR, Introduction to Theory and Methods', Academic Press, New York, 1971; J. B. Stothers, 'Carbon-13 N.M.R. Spectroscopy', Academic Press, New York, 1972; G. C. Levy and G. L. Nelson, 'Carbon-13 Nuclear Magnetic Resonance for Organic Chemists', Wiley-Interscience, New York, 1972.

[2] J. B. Grutzner, *Lloydia*, 1972, **35**, 375; M. Tanabe, 'Biosynthesis', ed. T. A. Geissman (Specialist Periodical Reports), The Chemical Society, London, 1973, Vol. 2, 241; *ibid.*, 1974, Vol. 3, 247; *ibid.*, 1975, Vol. 4, 204; T. J. Simpson, *Chem. Soc. Rev.*, 1975, **4**, 497—523; A. G. McInnes and J. L. C. Wright, *Accounts Chem. Res.*, 1975, **8**, 313—320; A. G. McInnes, I. A. Walter, J. L. C. Wright, and L. C. Vining, 'Topics in Carbon-13 NMR Spectroscopy', ed. G. C. Levy, Vol. II, Ch. 3, Wiley-Interscience, New York, 1976.

[3] R. P. Bell, *Chem. Soc. Rev.*, 1974, **3**, 513—544.

Table 1 *The three isotopes of hydrogen*

Isotope	*Mass*	*Natural abundance*	*Mean energy of bond to carbon* (kcal per mole)
Protium(^{1}H)	1.00797	99.985	4.15
Deuterium(^{2}H)	2.01410	0.0160	3.00
Tritium(^{3}H)	3.01605	1 in 10^{17}	—

Table 2 *The magnetic properties of hydrogen isotopes*

	^{1}H	^{2}H	^{3}H
Nuclear Moment	2.79	0.86	2.96
Nuclear Spin	½	1	½
Quadrople Moment	—	0.0027	—
Sensitivity	1.00	0.00965	1.21
Relative gyromagnetic ratio	1.000	0.154	1.067
Resonant Frequency (10 kG)	42.577	6.536	42.414

systems. Careful design and interpretation of experiments combined with a full awareness of the limitations of any techniques used are essential to the success of all incorporation studies with labelled precursors.

B. Tritium.[4,5]—Tritium has negligible natural abundance. It is a weak β-emitter with a half-life of 12.26 years and is detected routinely by liquid scintillation counting. The advantages of tritium in tracer work may be summarised as the sensitivity of detection and the zero natural abundance which allows its use at low isotopic enrichment. However, the need to ensure radiochemical purity by extensive recrystallisation of a suitable derivative and the chemical degradation required to establish unambiguously the sites of labelling both require skill in manipulating small amounts of compound and can be time-consuming.

C. Deuterium.—In contrast, deuterium is a stable isotope, readily available at a high level of enrichment, which has up to now been detected routinely by mass spectrometry,[6] sometimes in conjunction with proton n.m.r. The method is tatistical and of low sensitivity, but evidence for sites of labelling can be obtained

[4] E. A. Evans, 'Tritium and its Compounds', Butterworth, London, 2nd Edn., 1974.
[5] E. Buncel and G. C. Lee, 'Isotopes in Organic Chemistry', Vol. 4, Elsevier, Amsterdam, 1978.
[6] D. H. Williams and I. Howe, 'Principles of Organic Mass Spectrometry', pp. 170—174, McGraw-Hill, London, 1972.

without recourse to degradative experiments. Additionally, deuterium is a more suitable substitute for protium than is tritium since the properties of deuterium-labelled compounds mirror more closely those of the protio-compound.

D. Protium.—Although protium, hereafter called hydrogen, cannot be used as a tracer under normal circumstances, proton n.m.r.[7] has played a central role in biosynthetic study. Its value will be further emphasised in this review because some of the methods discussed depend upon ^{1}H n.m.r. for their success.

2 Direct N.M.R. Methods for Detecting Hydrogen Isotopes

A. Tritium N.M.R.[8]—The nuclear properties of tritium are ideal for detection by n.m.r. spectroscopic methods (See Table 2). The magnetogyric ratio is the highest known for any isotope and the n.m.r. resonant frequency is 106.7 MHz at 23 kG (compare ^{1}H at 100 MHz). The nuclear spin of $\frac{1}{2}$ results in proton-like spectra with narrow line-widths being obtained. Chemical-shift values are very similar to those of the equivalent proton; hence signals can be readily assigned by reference to the ^{1}H n.m.r. spectrum. Coupling constants are also easily evaluated as the ratio of coupling constants $J(^3\text{H}–^1\text{H})/J(^1\text{H}–^1\text{H})$ is proportional to the ratio of magnetogyric constants (1.067). The Nuclear Overhauser Effect observed with tritium is small and negative, the theoretical maximum being 47% compared with 199 % for ^{13}C with ^{1}H decoupling.[9] Tritium n.m.r. spectra may therefore be integrated to give a measure of isotopic content at each site which has been found to agree reasonably well (± 10%) with tritium analysis by conventional counting methods.

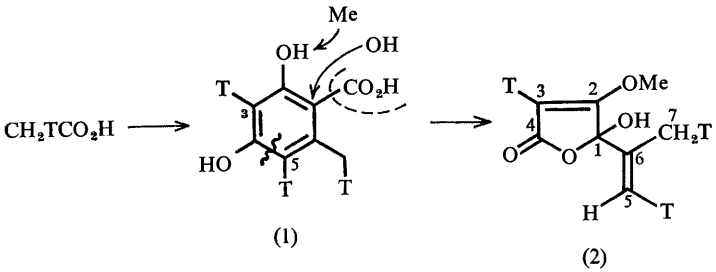
A consequence of the high magnetogyric ratio is that tritium n.m.r. is the most sensitive n.m.r. method available. This, combined with the negligible level of tritium at natural abundance, enables spectra to be run on samples containing as little as 1 mCi of radioactivity (0.0034 atom % tritium), a level which presents only slight radiological hazard. Special screening precautions are not therefore necessary, although it is advisable for this type of work to be segregated from normal radioactive tracer work because of the high risk of cross-contamination. The method is less sensitive than liquid scintillation counting for tritium analysis, but obviates the need for extensive chemical degradation to locate the sites of enrichment.

The first reported biosynthetic application of ^{3}H n.m.r. was a study of the incorporation of [^{3}H]acetate into penicillic acid (2),[10] a metabolite of *Penicillium*

[7] J. W. Emsley, J. Feeney, and L. H. Sutcliffe, 'High Resolution Nuclear Magnetic Resonance Spectroscopy', Pergamon Press, New York, 1965.
[8] J. A. Elvidge, J. R. Jones, V. M. A. Chambers, and E. A. Evans, Ch. 1 in ref. 6, pp. 1—49.
[9] J. P. Bloxsidge, J. A. Elvidge, D. K. Jaiswal, J. R. Jones, and R. Thomas, *J. Chem. Res.* (*S*), 1977, 258.
[10] J. M. A. Al-Rawi, J. A. Elvidge, D. K. Jaiswal, J. R. Jones, and R. Thomas, *J.C.S. Chem. Comm.*, 1974, 220; J. A. Elvidge, D. K. Jaiswal, J. R. Jones, and R. Thomas, *J.C.S. Perkin I*, 1977, 1080.

cyclopium which has been studied extensively with carbon isotopes.[11] In preliminary experiments, no loss of tritium label was detected from either [^{3}H]acetate or the tritium-enriched metabolite under physiological conditions, but some loss of tritium relative to ^{14}C was observed during the biosynthesis.

The tritium n.m.r. spectrum (Figure 1a) of (2) derived from [^{3}H]acetate was assigned by reference to the corresponding proton n.m.r. spectrum (Figure 1b). Tritium was found to be present at the 3-, 5-, and 7-positions consistent with the overall mode of biosynthesis shown in Scheme 1. The 7-position showed less



Scheme 1

exchange of tritium label than the 3- and 5-positions since it is derived from a chain starter methyl group rather than an activated chain-building methylene position.

Incorporation of [$^3H_2$]malonate caused only the 3- and 5-positions to be labelled. The C-5 was selectively labelled; this, together with the very high exchange at this position compared with C-3, although both are derived from chain-extending units, provided clues to the nature of some of the complex steps in the biosynthesis which could not have been obtained from studies with carbon isotopes. Further, [3,5-$^3H_2$]orsellinic acid (1) was established as an advanced precursor by incorporation of tritium into (2) with the same pattern of distribution of label between the 5α- and 5β-positions as the established precursors. ^{3}H n.m.r. analysis may thus be of use in establishing the nature of advanced precursors.

The same group have also used ^{3}H n.m.r. to study the steric course of the biotransformation of testosterone to androsta-1,4-diene-3,17-dione[12] in the bacterium *Cyclindrocarpon radicicola*.

In another pioneering study,[13] tritium n.m.r. has been used as an alternative to the classic method (involving enzymatic degradation and radioactive counting)

[11] A. J. Birch, G. E. Blance, and A. H. Smith, *J. Chem. Soc.*, 1958, 4582; K. Mosbach, *Acta Chem. Scand.*, 1960, **14**, 457.
[12] J. M. A. Al-Rawi, J. A. Elvidge, R. Thomas, and B. J. Wright, *J.C.S. Chem. Comm*, 1974, 1031
[13] L J Altman, G. Y. Han, A. Bertolino, G. Handy, D. Laungani, W. A. Muller, S. Schwartz, D. Shanker, W. H. de Wolf, and F. Yang, *J. Amer. Chem. Soc.*, 1978, **100**, 3235.

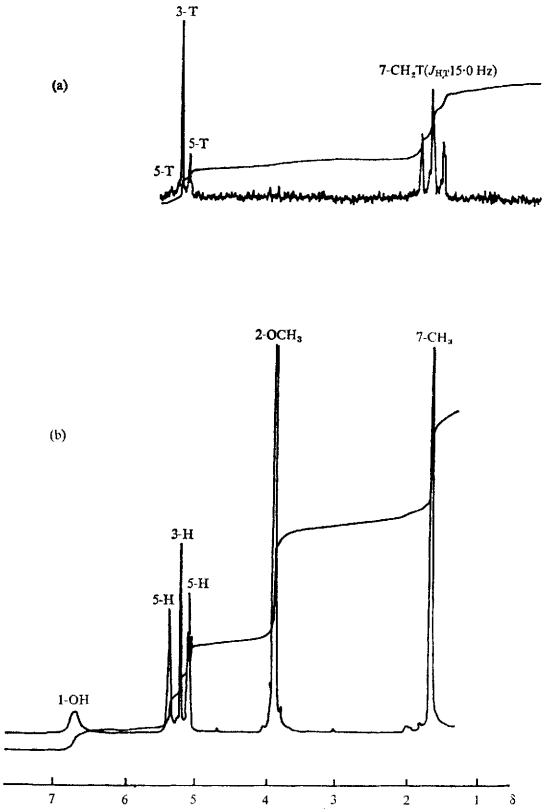
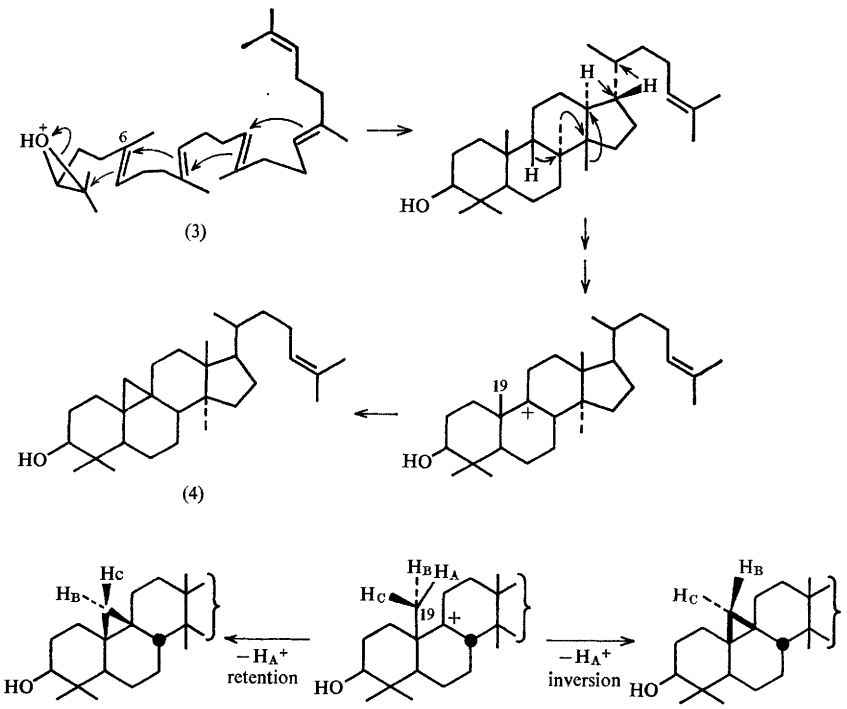


Figure 1 *Fourier Transform n.m.r. spectra of penicillic acid* (2) *in* [$^2H_6$]*acetone at* 25 °C; (a) ^3H *n.m.r. spectrum,* 1.12 × 10^4 *pulses at* 1.75 s. *intervals.* (b) ^1H *n.m.r. spectrum,* (Me_4Si)
(Reproduced from *J.C.S. Chem. Comm.*, 1974, 220)

devised by Cornforth, Arigoni *et al.*[14] for determining the configuration of isotopic labelling in methyl groups. The cyclisation of 2,3-oxidosqualene (3) to cycloartenol (4) requires a 1,3-proton loss from the C-19 methyl group to form the cyclopropane ring, a process which may involve either inversion or retention of configuration at this centre (Scheme 2). The two cyclopropyl resonances in the proton n.m.r. of (4) were distinguishable since the lower field signal showed a long-range coupling to $H_{1\alpha}$, which enabled it to be assigned to the *endo* proton. Additional support for this assignment was obtained from T_1 measurements and experiments with lanthanide shift reagents.

A sample of chiral (3) in which each molecule containing tritium in the C-6

[14] J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature*, 1969, **221**, 1212; J. Lüthey, J. Rétey, and D. Arigoni, *ibid.*, p. 1213; J. W. Cornforth, *Chem. Soc. Rev.*, 1973, **2**, 1—20.



Scheme 2

methyl group was also shown to contain one deuterium and one hydrogen at this position was prepared from D-malic acid and converted into (4) by a cell-free microsomal preparation from *Ochromonas malhamensis*. In the NOE-suppressed, proton-decoupled tritium n.m.r. spectrum of the product, it was possible to assign the most intense signal at δ 0.168 p.p.m. to molecules containing *exo*-tritium and *endo*-deuterium. A signal at δ 0.456 p.p.m. corresponded to molecules with *endo*-tritium and *exo*-hydrogen. Thus, the overall process occurs with retention of configuration.

These few experiments demonstrate convincingly the potential of tritium n.m.r. Its present use is limited by the attendant radioactive properties. However, with instrumentation becoming available which will increase the sensitivity of n.m.r. detection, it is likely that the technique will be more fully exploited in the future.

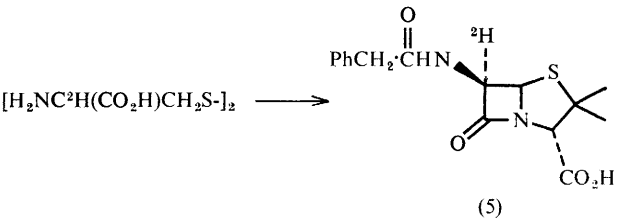
B. Deuterium N.M.R.[15]—The potential of deuterium n.m.r. was first demonstrated[16] in 1964, but it is only in the last five years that the technique has been widely adopted by organic chemists.

Deuterium is a quadrupole nucleus with spin 1. As a consequence of the lower magnetogyric ratio (Table 2), deuterium resonates at 15 MHz in a magnetic field of 23 kG compared with 100 MHz for ^1H. The chemical shift values obtained in deuterium n.m.r. spectra are closely similar to those of the equivalent protons, although the scale in Hz is only 15% that of the proton n.m.r. Coupling constants $J(^2H–^1H)$ also approximate to one-sixth the value of $J(^1H–^1H)$. The relaxation behaviour of deuterium is dominated by a quadrupole exchange mechanism and this results in extensive line-broadening. The two major limitations of deuterium n.m.r. are spectral crowding and poor resolution, but there is one major advantage over other n.m.r. techniques. The short relaxation times, combined with the absence of an NOE effect, minimise the possibility of partial saturation. The extent of enrichment in a partially deuteriated molecule may therefore be determined accurately by integration.

Other advantages of using ^2H n.m.r. in biosynthetic study are, firstly, that it is an inexpensive tracer which, unlike tritium, does not require special handling. Secondly, compared with ^{13}C (natural abundance 1.1%), the low natural abundance (0.016%) enables the incorporation of deuterium-labelled precursors to be positively identified (by a doubling in peak height over natural abundance) even after 6600 fold dilution of the precursor in the metabolic pool. The incorporation of molecules singly labelled with ^{13}C can only be detected with confidence at dilution levels of less than 100 fold. Thus ^2H n.m.r. is effectively sixty times more sensitive than ^{13}C n.m.r. when applied to biosynthetic study. This extra sensitivity may be particularly useful in studies on non-microbial systems where incorporations are generally lower.

In the earliest study using ^2H n.m.r., Bycroft *et al.* fed L-[1-^2H]cystine to *P. chrysogenum* and were able to show that all of the isotopic label was present at C-6 of the derived penicillin G (5).[17] An $\alpha\beta$-cysteinyl residue could thus be discounted as an intermediate in the biosynthetic transformations (Scheme 3) which lead to (5).

The biosynthesis of the fungal metabolite griseofulvin (8) in *Penicillium*



(5)

Scheme 3

[15] P. Diehl, 'Nuclear Magnetic Resonance Spectroscopy of Nuclei other than Protons', ed. T. Axenrod and G. A. Webb, Wiley-Interscience, New York, 1974, 257.
[16] P. Diehl and T. Leipert, *Helv. Chim. Acta*, 1964, **47**, 545.
[17] B. W. Bycroft, C. M. Wels, K. Corbett, and D. A. Lowe, *J.C.S. Chem. Comm.*, 1975, 123.

urticae has been extensively studied with deuteriated precursors.[18] The ^2H n.m.r. spectrum of (8) derived from [$^2H_3$]acetate was assigned by comparison with the ^2H n.m.r. spectra of specifically deuteriated analogues (see Figure 2). Label was

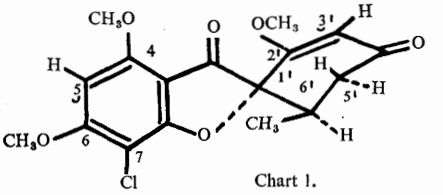


Chart 1.

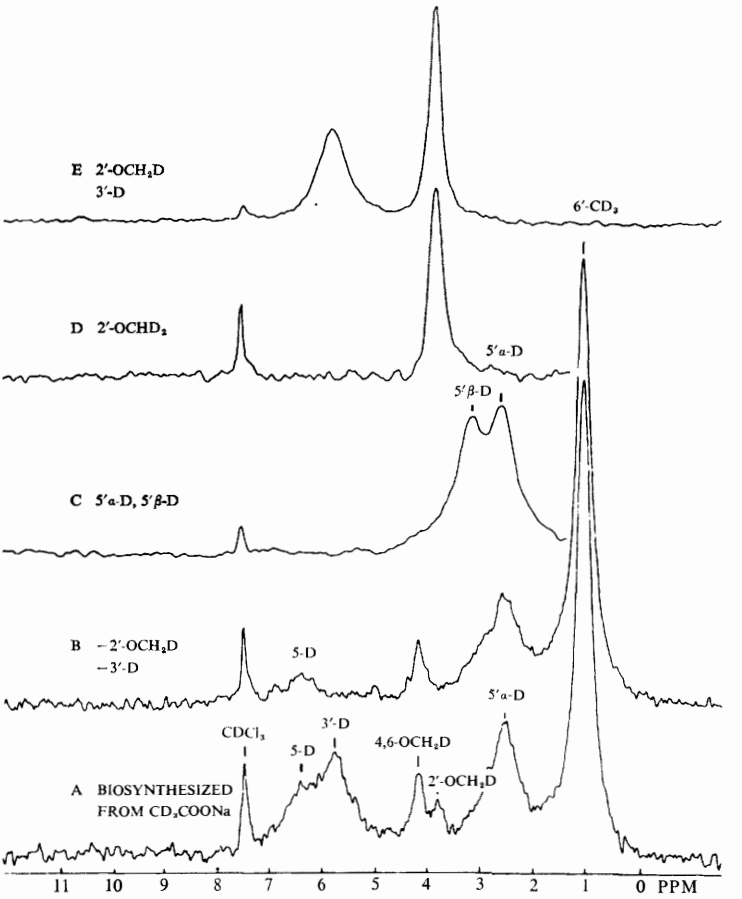
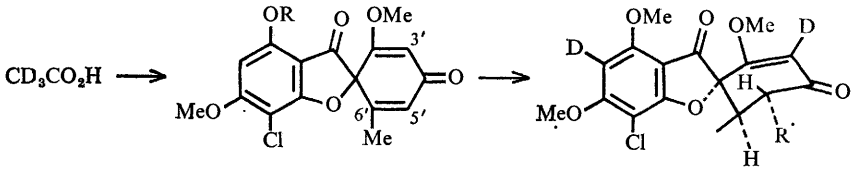


Figure 2 ^2H *n.m.r. spectra of griseofulvin* (8) *samples in* $CHCl_3$ *solutions. (A) Biosynthetically deuteriated; (B) Removed the deuteriums at 2' and 3' positions from A; (C)* 5'α-D, 5'β-D_2; *(D)* 2'-$OCHD_2$; *(E)* 2'-OCH_2D,3'-D

(Reproduced by permission from *Tetrahedron Letters*, 1976, 2695)

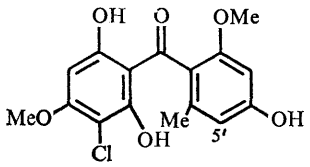
[18] Y. Sato, T. Oda, and H. Saito, *Tetrahedron Letters*, 1976, 2695.

incorporated into the methoxy-group as well as the expected sites along the carbon chain. The label at C-5′ was shown to be present exclusively in the α-position. Since the intensity of the signal for the 6′-methyl group was only twice that of the 5′α-D and furthermore sharpened on proton-decoupling, the authors concluded that the 6′-methyl group was $-CHD_2$ rather than $-CD_3$. It should be emphasised, however, that the use of ^2H n.m.r. does not allow firm conclusions to be drawn concerning the labelling of individual molecules; thus it can only be stated that the average isotopic distribution (^2H:^1H) at this position is 2:1.



(6) R = Me
(7) R = H

(8) R = D
(9) R = OH



(10)

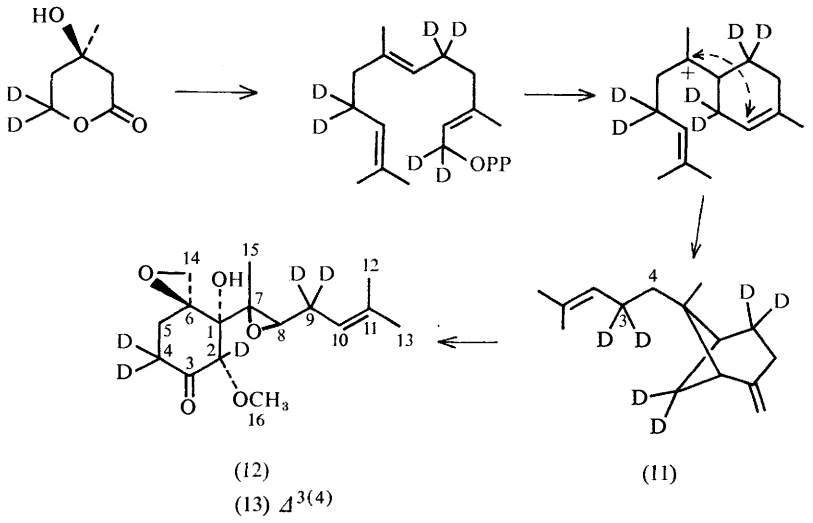
Scheme 4

In subsequent work,[19,20] the microbial hydrogenation of dehydrogriseofulvin (6) to griseofulvin (8) in *Streptomyces cinereocrocatus* was shown to proceed with *trans* diaxial reduction at the 5′- and 6′-positions (Scheme 4). Additionally, hydroxylation of (8) to 5′-hydroxygriseofulvin (9) proceeded with direct replacement of the 5′α-hydrogen by a hydroxy-group.[19] Incorporation studies with [5′-^{2}H]griseophenone, [5′-^{2}H]-(10), and 4-demethyl-[5′-^{2}H]dehydrogriseofulvin, [5′-^{2}H]-(7), have also been carried out, confirming results obtained by standard ^{14}C methods.

Many aspects of terpene biosynthesis are amenable to investigation using hydrogen isotopes and the application of deuterium n.m.r. has produced some exciting results in this area. Ovalicin (12), an antibiotic produced by cultures of *Pseudeurotium ovalis*, has been examined by Cane's group.[21] The isolation of β-*trans*-bergamotene (11) from this fungus supported the suggestion that it is an

[19] Y. Sato, T. Oda, and H. Saito, *J.C.S. Chem. Comm.*, 1977, 415.
[20] Y. Sato, T. Oda, and H. Saito, *J.C.S. Chem. Comm.*, 1978, 135.
[21] D. E. Cane and S. E. Buchwald, *J. Amer. Chem. Soc.*, 1977, **99**, **6132**.

intermediate in the biosynthesis of (12) which is known to involve 1,3-migration of the eight-carbon side-chain of a bisabolyl cation formed by cyclisation of farnesyl pyrophosphate (Scheme 5).[22] The proton n.m.r. spectrum of ovalicin



(12)
(13) $\Delta^{3(4)}$

(11)

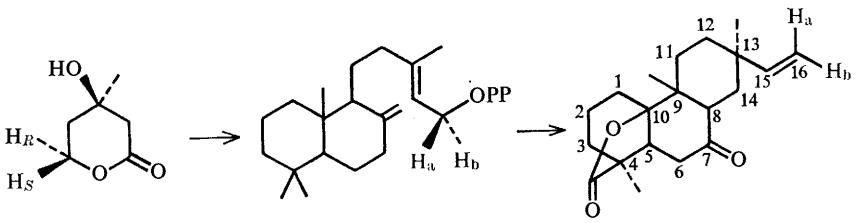
Scheme 5

was assigned unambiguously with the aid of specific proton–proton decoupling and chemical deuteriation experiments. [5,5-$^2H_2$]Mevalonate, mixed with (3*RS*, 5*RS*)-[2-^{14}C, 5-$^3H_2$]mevalonate (as internal monitor) was supplied to the organism; a total incorporation of 4.5% was obtained corresponding to an enrichment of 7.5% at each labelled site, while the change in $^{14}C/^3H$ ratio indicated that five out of six mevalonoid hydrogen labels had been retained. The sites of labelling were established as C-2 (1D), C-4 (2D), and C-9 (2D) from the ^2H n.m.r. of the biosynthetically derived (12). Retention of two labels at C-9 rules out the intervention of a dehydrobergamotene (13) whilst oxidative cleavage of the methylene bridge of (11) is excluded because of the presence of label at the corresponding position in (12).

The formation of ring C of the fungal diterpene rosenonolactone (16) from the bicyclic precursor labda-8(17),13-dien-15-yl-pyrophosphate (15) has similarly been shown to proceed by means of an allylic (S_N2') displacement with antarafacial stereochemistry.[23] Thus a 5-*pro-R* mevalonoid deuterium uniquely labelled the 16*Z* hydrogen of (16) in *Tricothecium roseum* (Scheme 6).

The ^2H n.m.r. of the biosynthetically deuteriated triterpene tetrahymanol (17) was compared with those of the model compounds 3α- or 3β-[$^2H_1$]-5α-cholestane

[22] D. E. Cane and R. E. Levin, *J. Amer. Chem. Soc.*, 1976, **98**, 1183.
[23] D. E. Cane and P. P. N. Murthy, *J. Amer. Chem. Soc.*, 1977, **99**, 8327.



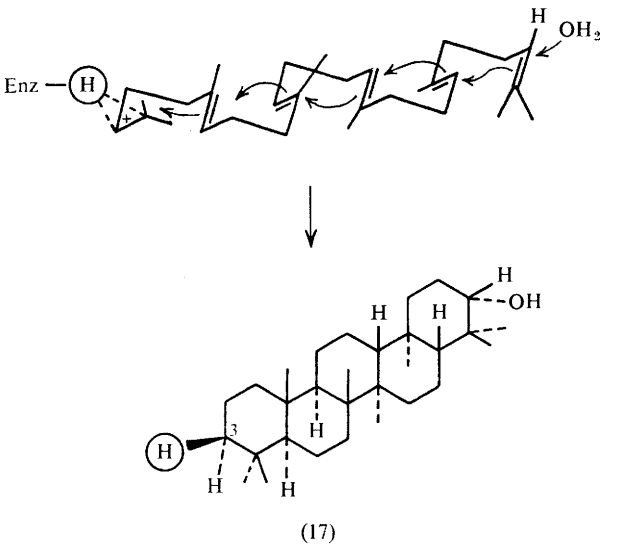
(15) (16)

H_a = 16*Z* = 5-*pro-R*
H_b = 16*E* = 5-*pro-S*.

Scheme 6

and 3α- or 3β-[$^2H_1$]-4,4-dimethyl-5α-cholestane. Aberhart and Caspi[24] were thus able to conclude that the non-oxidative cyclisation of squalene in D_2O by *Tetrahymena pyriformis* proceeds with the introduction of a 3β-deuterium atom as shown in Scheme 7. Useful additional support for these results was obtained from a comparison of the carbon–deuterium stretching frequencies in the infrared spectra of the various compounds.

Two examples of the application of ^2H n.m.r. to biosynthetic processes in

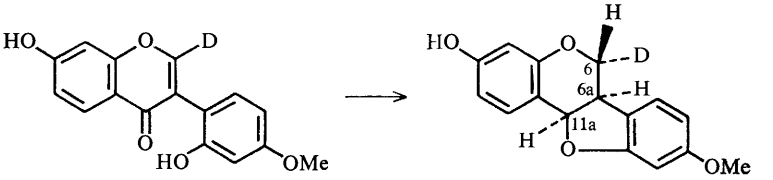


(17)

Scheme 7

[24] D. J. Aberhart and E. Caspi, *J. Amer. Chem. Soc.*, 1979, **101**, 1013.

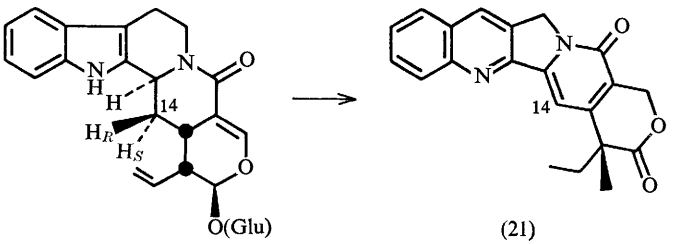
higher plants have now been reported.[25,26] One of these[25] studied pterocarpan biosynthesis in fenugreek seedlings. The intermediate to be tested, 2′,7-dihydroxy-4′-methoxy[2-^{2}H]isoflavone (18), was first converted into a mixture of the two isomeric (6a*R*,11a*R*)-demethylhomopterocarpans (19) by reduction with $NaBH_4$ in order to assign the 6-*pro-R* and 6-*pro-S* resonances in the ^{2}H n.m.r. spectrum. When (18) was converted biosynthetically into (19), the deuterium label was shown to be present exclusively at the 6-*pro-R* position. Because of the high (56%) incorporation which was obtained, the corresponding signal in the proton n.m.r. was substantially reduced in intensity relative to the other signals. Overall *trans*-addition of hydrogen across the 6,11-double bond has occurred (Scheme 8).



(18) (19)

Scheme 8

More recently, Hutchinson *et al.*[26] have used ^{2}H n.m.r. in a study of camptothecin (21) biosynthesis. Previous experiments with [14-^{3}H, 5-^{14}C]strictosamide lactam (20) had revealed only a 5–9% loss of tritium from C-14 although a hydrogen must be lost from this position during the biosynthesis of (21). [14-^{3}H,



(20) (21)

Scheme 9

14-^{2}H]-(20) was shown by ^{2}H n.m.r. and by ^{13}C n.m.r. spectroscopy to contain deuterium only at C-14. From the reduction in intensity of the relevant signals in the proton n.m.r., it was clear that both diastereotopic hydrogens were equally

[25] P. M. Dewick and D. Ward, *J.C.S. Chem. Comm.*, 1977, 338.
[26] C. R. Hutchinson, A. H. Heckendorf, J. L. Straughn, P. E. Daddona, and D. E. Cane, *J. Amer. Chem. Soc.*, 1979, **101**, 3358.

labelled by deuterium, and therefore also by tritium. Furthermore, when [14-^{3}H, 14-^{2}H]-(20) was converted into (21), deuterium label was found by ^{2}H n.m.r. to be present only at C-14. These experiments, together with others, indicated that the removal of hydrogen from C-14 of (20) was not subject to enzymic control.

It is clear that deuterium n.m.r. has already made a useful contribution to biosynthetic study. The problems associated with signal resolution are likely to diminish as n.m.r. equipment becomes more sophisticated; it seems probable that deuterium n.m.r. will become a very attractive alternative to ^{13}C n.m.r. spectroscopy in the near future.

3 Indirect Approaches to Monitoring Hydrogen Isotopes

Tracing the fate of hydrogen is an important aspect of biosynthesis which allows detailed information to be gained concerning the pathway under investigation. Both of the direct n.m.r. methods discussed above allow the average degree of enrichment at each labelled site to be measured; they therefore yield evidence for the retention or transfer of hydrogen, from which it is possible to deduce information about transformations in the carbon skeleton. An attractive alternative is to monitor the presence of isotopic hydrogen indirectly through its interaction with the adjacent carbon nucleus. A precursor is chosen so that the isotopically labelled site is also enriched with ^{13}C; the presence of hydrogen isotope in the biosynthetic product is thus detected by its coupling to ^{13}C in the ^{13}C n.m.r. spectrum. To date, this approach has been successfully applied to deuterium. In addition to the detection of deuterium, the integrity of carbon–hydrogen bonds throughout a biosynthetic pathway can be probed and may yield information about the nature of intermediates which is not available by conventional methods. An important advantage is that the labelling pattern of individual molecules can be deduced from the multiplicity and chemical shift values of the n.m.r. signals.

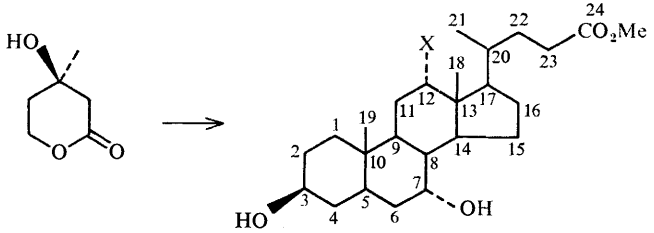
In a proton-decoupled ^{13}C n.m.r. spectrum, a carbon with one directly attached deuterium appears as a triplet[27] whose lines are of equal intensity, because the nuclear spin of deuterium is one. The $J(^{13}C–^{2}H)$ values are one sixth those of the equivalent $J(^{13}C–^{1}H)$ and the signal is centred 0.3—0.6 p.p.m. upfield of the normal protonated signal. The presence of each additional deuterium shifts the signal a further 0.3—0.6 p.p.m. upfield and increases the multiplicity; thus, $–CD_2–$ appears as a quintet with signals of intensity 1:2:3:2:1 and a $–CD_3$ as a septet (1:3:6:7:6:3:1). The different signals for a methyl group overlap to a certain extent and are also superimposed upon the normal signal from protonated nuclei. A partially deuteriated methyl thus gives rise to a complex signal comprising lines from $–CD_3$, $–CD_2H$, $–CDH_2$ as well as $–CH_3$ labelled species. Fortunately, the spectral analysis can be simplified by re-running the spectrum with deuterium decoupling instead of proton decoupling; interference from protonated nuclei is greatly reduced in this mode of operation because of the loss of NOE. It is also possible to carry out simul-

[27] 'Topics in C-13 NMR Spectroscopy', ed. G. C. Levy, Wiley-Interscience, New York, 1974, Vol. I, pp. 234—238.

taneous proton and deuterium decoupling although it is more useful for only one decoupling mode to be used at a time.

^{13}C–^{2}H n.m.r. is limited by the relative weakness of the ^{13}C–^{2}H n.m.r. signal.[28] This is the result of three factors: firstly, ^{13}C–^{2}H relaxation is much less efficient than ^{13}C–^{1}H relaxation, since the rate of relaxation is proportional to the square of the magnetogyric ratio; secondly, the replacement of hydrogen by deuterium is accompanied by loss of NOE enhancement and, thirdly, increased multiplicity causes loss of signal strength in individual lines.

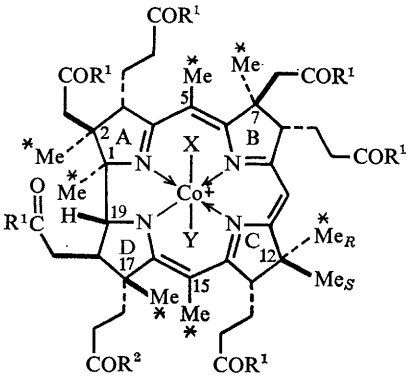
The feasibility of indirect deuterium monitoring was first shown in 1974 when a study of the metabolism of [1-^{13}C, 1-$^{2}H_2$]- or [2-$^{2}H_3$]-ethanol in rats was reported.[29] The isotopic labels were incorporated into the bile-acid esters methyl cholate (22) or chenodioxycholate (23) from either [1-^{13}C]- or [2-$^{2}H_3$]-acetate and also *via* the reduced pyridine coenzymes labelled with deuterium at the 4-position; hence, at no stage was the integrity of any ^{13}C–^{2}H bond maintained. The amount of deuterium label at each site was estimated from the reduction in intensity of the peaks in the natural-abundance ^{13}C n.m.r. spectrum (simultaneous proton and deuterium decoupling) or by analysis of isotopically-shifted peaks.



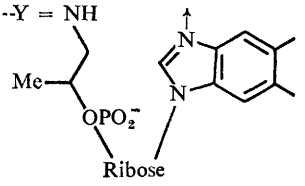
(22) X = OH
(23) X = H

Three groups[30–32] have studied the incorporation of [$^{13}CD_3$]methionine into Vitamin B_{12} (24) in order to study the transfer of the seven methyl groups (starred). Earlier investigation using mass-spectroscopic methods had given inconclusive results. Dicyanocobalamine (25) or cobester (26) obtained after feeding [*methyl*-$^{13}CD_3$]methionine to *Propionibacterium shermanii* gave a deuterium decoupled n.m.r. spectrum in which the seven signals corresponding to the methyl groups were all shifted upfield as a consequence of isotopic substitution; more importantly, none of these signals showed the large 120 Hz coupling

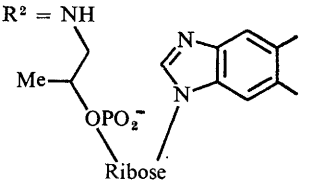
[28] G. C. Levy, *Accounts Chem. Res*, 1973, **6**, 161.
[29] D M. Wilson, A. M. Burlinghame, T. Cronholm, and J. Sjovall, *Biochem. Biophys. Res. Comm.*, 1974, **56**, 828.
[30] M. Imfeld, C. A. Townsend, and D. Arigoni, *J.C.S. Chem. Comm.*, 1976, 541.
[31] A. R. Battersby, R. Hollenstein, E. McDonald, and D. C. Williams, *J.C.S. Chem. Comm.*, 1976, 543.
[32] A. I. Scott, M. Kajiwara, T. Takahashi, I. M. Armitage, P. Demou, and D. Petrocine, *J.C.S. Chem. Comm.*, 1976, 544.



(24) $R^1 = NH_2$, X = CN



(25) $R^1 = NH_2$, X = Y = CN

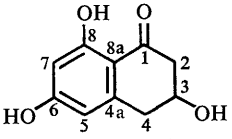


(26) $R^1 = R^2 = OMe$, X = Y = CN

expected if some hydrogen were present, although long-range coupling broadened some of the resonances. The seven methyl groups were thus incorporated intact without significant hydrogen exchange; the retention of all three hydrogen atoms is particularly important since this discounts mechanisms for the formation of the corrin ring in which C-1 becomes a methylene group (barring the unlikely possibility that the label removed in such a step is replaced without exchange with the medium). Scott's group[32] reached similar conclusions using proton Fourier-transform difference spectroscopy to detect the lack of hydrogen (^1H) attached to ^{13}C.

Polyketide biosynthesis is an area which has been studied in great detail with carbon isotopes but where hydrogen isotopes have found little direct application; however, their use in conjunction with ^{13}C has already proved fruitful. Although much of the hydrogen label is lost by exchange from the activated methylenes of the polyketone chain, the possibility remains that more than one isotopic label may be retained at positions derived from the methyl group of acetate. The number of deuteriums detected at a given site can be used to identify the chain starter unit or to distinguish between possible biosynthetic intermediates, thereby yielding information about the mode of biosynthesis. The extent of deuterium retention may be correlated to the amount of hydrogen exchange during biosynthesis and may thus provide clues to the complex mechanisms involved in the building up on the carbon skeleton.

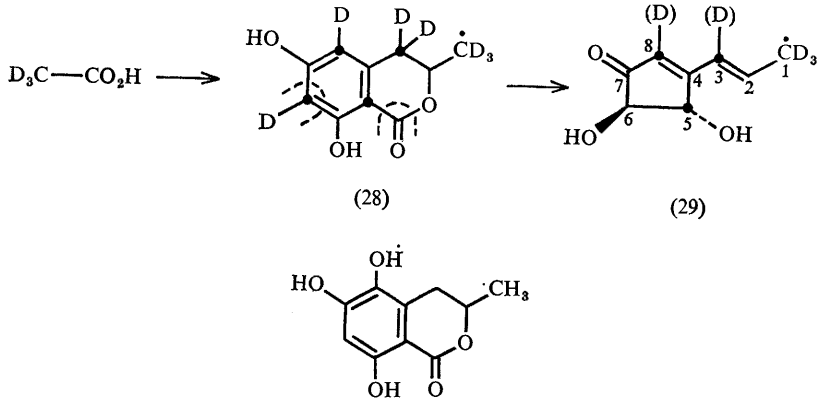
In 1977, a brief report of the incorporation of [2-^{13}C, 2-$^{2}H_3$]acetate into the fungal metabolite skytalone (27) was published.[33] The enriched (27) showed enhancements for C-2, C-4, C-5, C-7, and C-8a but the signal intensities for C-4 and C-5 were lower than expected, indicating the presence of deuterium. A triplet (J = 20 Hz) was observed 0.3 p.p.m. upfield of the normal ^{13}C signal for C-4



(27)

but no corresponding deuteriated signal was visible for C-5. When the spectrum was re-run with deuterium decoupling,[34] the signal for C-4 appeared as a double (J = 127 Hz) which established the labelling pattern as –CHD– while a singlet at 108.7 p.p.m. was assigned to molecules carrying deuterium at C-5. Although several other sites in the molecule could carry deuterium, no evidence as to their labelling was obtained. ^{2}H n.m.r. might be of assistance in this connection.

A more searching study of the application of this technique to polyketide biosynthesis was completed in 1977 with two reports on terrein biosynthesis.[35,36] Terrein (29), a metabolite of *Aspergillus terreus*, has been extensively studied with carbon isotopes;[37] the biosynthesis is known to proceed through the condensation of five acetate units to give, after aromatisation, the dihydroisocoumarin (28) which then undergoes an interesting ring contraction as shown in Scheme 10.



(28) (29)

(30)

Scheme 10

[33] U. Sankawa, H. Shimada, T. Sato, T. Kinoshita, and K. Yamasaki, *Tetrahedron Letters*, 1977, 483.
[34] U. Sankawa, H. Shimada, and K. Yamasaki, *Tetrahedron Letters*, 1978, 3375.
[35] M. J. Garson, R. A. Hill, and J. Staunton, *J.C.S. Chem. Comm.*, 1977, 624.
[36] M. J. Garson, R. A. Hill, and J. Staunton, *J.C.S. Chem. Comm.*, 1977, 921.
[37] R. A. Hill, R. H. Carter, and J. Staunton, *J.C.S. Chem. Comm.*, 1975, 380.

Hydroxylated intermediates such as (30) are potential intermediates although these are not easy to synthesise in labelled form. Preliminary experiments showed that the deuteriated acetate was as good a precursor as normal acetate and that the presence of deuterium did not distort the uptake of precursor into different acetate-derived units.

In the proton-decoupled ^{13}C n.m.r. spectrum (Figure 3) of terrein enriched with [2-^{13}C, 2-$^{2}H_3$]acetate, the signal for C-5 was enriched five-fold relative to that for C-6 which was unlabelled. Using C-5 as internal standard (since the proton at this position is derived from the medium) the signals for C-3, C-8, and C-1 were less intense than expected, indicating the presence of deuterium. The signal for C-1 was a broad envelope of peaks from which signals at 18.4 and 18.7 p.p.m. indicated, respectively, molecules containing $-CH_3$ and $-CH_2D$ at this position. A more detailed analysis was undertaken after re-running the spectrum with deuterium decoupling (Figure 4a); the signal at 17.95 p.p.m., 0.8 p.p.m. upfield of the normal signal for protonated molecules, related to molecules triply substituted with deuterium, while a doublet (J = 123 Hz) centred at 18.2 p.p.m. arose from molecules labelled as $-CHD_2$. The detection of some molecules with three deuteriums at C-1 confirmed that this methyl is derived intact from C-2 of acetate and is therefore part of a chain starter unit. This technique thus provides a useful alternative to the standard procedure of using either ^{14}C- or ^{13}C-labelled malonate to pick out the chain starter unit.

Adjacent to the ^{13}C–^{1}H signals for C-3 and C-8 (which have similar chemical shift values) were visible two of the three lines of a ^{13}C–^{2}H signal (J = 27 Hz), which could be assigned to C-8; however, both C-3 and C-8 carried deuterium since, on re-running the spectrum with deuterium decoupling (Figure 4b), two sharp singlets at 123.5 (C-8) and 124.8 p.p.m. (C-3) were seen, with that for C-8 the more intense. The retention of some deuterium at C-8 rules out the involvement of a 5-hydroxy-derivative (30) in terrein biosynthesis.

Other important contributions in this area have been made in Japan. Apart from the work on skytalone discussed above, a recent paper reports the preparation of rugulosin (31) from [2-^{13}C, 2-$^{2}H_3$]acetate.[34] The sites of deuteriation were established by comparison with the spectrum of (31) derived solely from [2-^{13}C]-acetate. The analysis of the methyl group was aided by deuterium decoupling; the presence of signals for molecules labelled as $-CD_3$, $-CD_2H$, and (from the proton decoupled spectrum) $-CH_3$ indicated, as in the case of terrein, extensive loss of label from this position. Brief reports on the incorporation of [2-^{13}C, 2-$^{2}H_3$]acetate into 2-hexyl-5-propylresorcinol (32)[34] and sterigmatocystin (33)[38] have also appeared.

McInnes *et al.*[39] have studied the incorporation of [2-^{13}C, 2-$^{2}H_3$]acetate into methyl palmitate (34). The ^{13}C n.m.r. spectrum, run under conditions of simultaneous proton and deuterium decoupling, contained a single isotopically-shifted peak for every methyl-derived carbon, indicating that each bore not more than

[38] H. Shimada, T. Sato, T. Kinoshita, E. Ebizuka, T. Akiyama, and H. Noguchi, 21st Symposium on the Chemistry of Natural Products, Sapporo, Japan, 1978.
[39] A. G. McInnes, J. A. Walters, and J. L. C. Wright, *Tetrahedron Letters*, 1979, 3245.

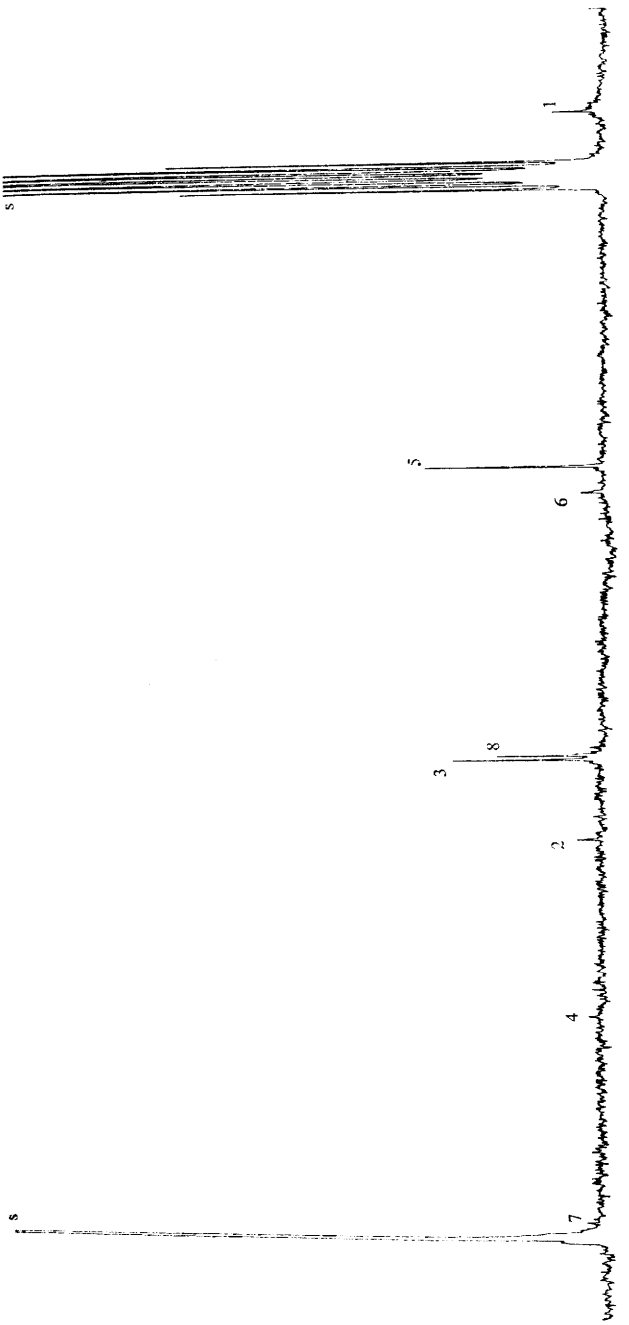


Figure 3 *Proton-decoupled* ^{13}C *n.m.r. spectrum of terrein* (29) *enriched with* [2-^{13}C,2-$^{2}H_3$]*acetate.* S = $(CD_3)_2CO$

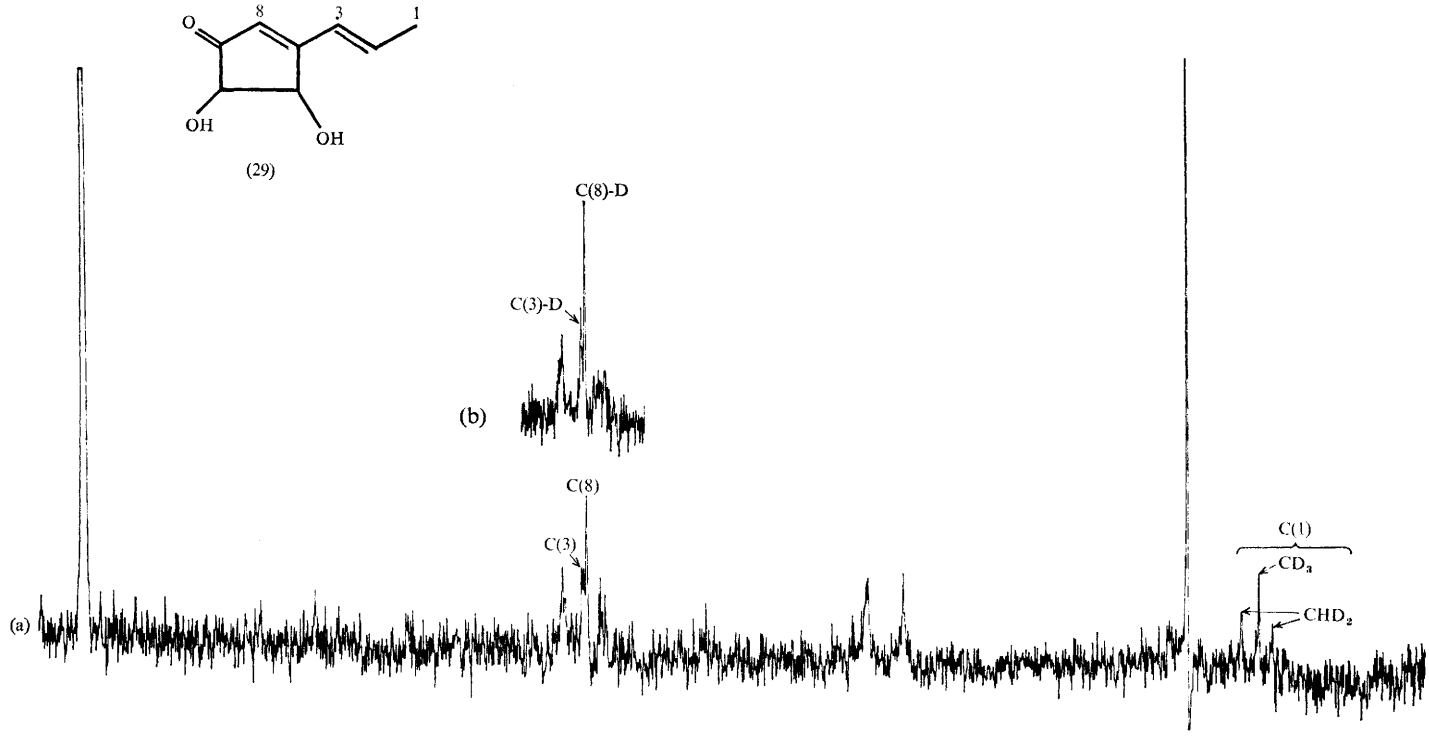
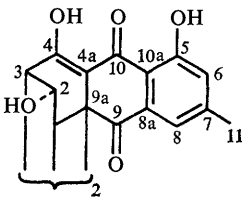
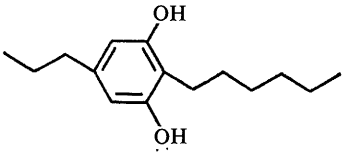


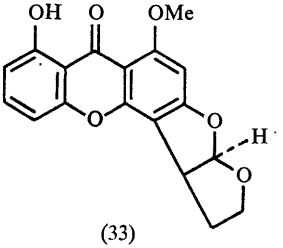
Figure 4 *Deuterium-decoupled* ^{13}C *n.m.r. spectra of terrein* (29) *enriched with* [2-^{13}C,2-$^{2}H_3$]*acetate;* (a) *with optimum conditions for decoupling at* C-1 (b) *with optimum conditions for decoupling at* C-3 *and* C-8



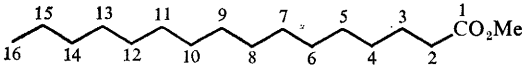
(31)



(32)



(33)



(34)

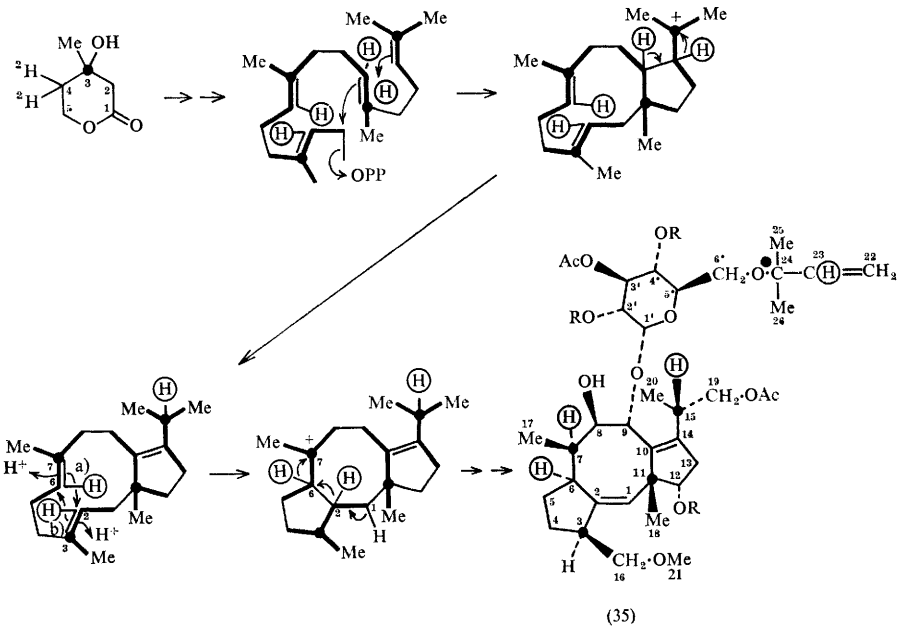
one deuterium. The methyl group, C-16, was mainly $-CD_3$ but contained successively smaller amounts of $-CD_2H$, $-CDH_2$, and $-CH_3$; this suggested that the malonate pool was small and rapidly turned-over without reconversion into acetate. The gradual reduction in hydrogen loss along the carbon chain from C-16 to C-2 was discussed in terms of the known steps of fatty-acid biosynthesis.

The biosynthesis of terpenes is an area which has been studied extensively with tritiated precursors, often in conjunction with ^{14}C, in order to prove the way in which the carbocyclic rings are built up. Two recent reports[40,42] elegantly show the contribution that ^{13}C–^{2}H n.m.r. can make to investigations of this type. [3-^{13}C, 4-$^{2}H_2$]Mevalonate was supplied to cultures of *Fusicoccum amygdali* which produce the phytotoxin fusicoccin (35);[40] this is known to be derived from geranyl geranyl pyrophosphate with retention of four out of five mevalonoid hydrogens.[41] The ^{13}C n.m.r. spectrum of enriched (35) was compared with that of unlabelled material run under identical operating conditions. The signals for C-3, C-11, and C-24 were enhanced, as would be expected, but those for C-7 and C-15 were not significantly increased although they are also derived from C-3 of

[40] A. Banerji, R. Hunter, G. Mellows, K.-Y. Sim, and D. H. R. Barton, *J.C.S. Chem. Comm.* 1978, 843.

[41] A. Banerji, R. B. Jones, G. Mellows, L. Phillips, and K.-Y. Sim, *J.C.S. Perkin I*, 1976, 2221.

[42] R. Hunter and G. Mellows, *Tetrahedron Letters*, 1978, 5051.



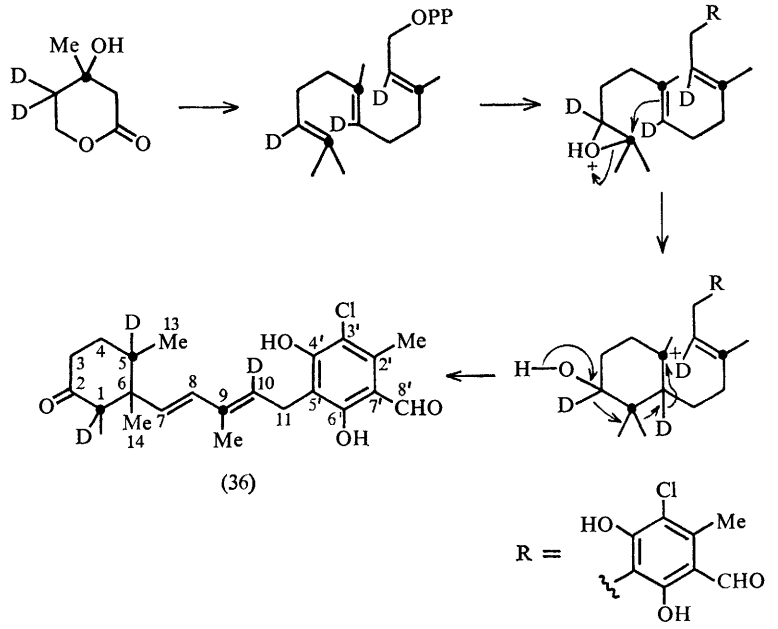
(35)

Scheme 11

mevalonate. This suggested the presence of deuterium at these two carbons; however, no ^{13}C–^{2}H multiplets were visible above the background noise level. The presence of deuterium at C-7 and C-15 requires that the biosynthesis involve a series of 1,2-hydride shifts as shown in Scheme 11. These shifts are within a discrete isoprenoid unit and therefore result in the direct transfer of deuterium to ^{13}C. A 1,3-hydride shift would involve an interisoprenoid transfer, *e.g.* from C-2 to C-7; since dilution in the metabolic pool makes it unlikely that two labelled units are adjacent, this would not result in the transfer of ^{2}H to ^{13}C.

The fungal triprenylphenol ascochlorin (36) has been studied[42] in a similar manner in order to substantiate the biosynthetic pathway (Scheme 12) suggested by Tanabe.[43] In the ^{13}C n.m.r. spectrum of (36) prepared from [3-^{13}C, 4-$^{2}H_2$] mevalonate, the signal for C-9 was enhanced relative to natural abundance and showed two lines, one for molecules with ^{13}C at C-9 and ^{2}H at C-10; and the other, more intense peak for molecules protonated at C-10. Neither C-1 nor C-5, which are also derived from C-3 of mevalonate, were significantly enhanced relative to natural abundance, consistent with some deuterium suppression of these signals. Two lines of a triplet (J = 24 Hz) centred at 40.65 p.p.m. were detected upfield of the normal signal for C-5 but no deuteriated signal was visible for C-1. Detection of deuterium at these centres provides evidence for the hydrogen transfers suggested by Tanabe.

[43] M. Tanabe and K. T. Susuki, *J.C.S. Chem. Comm.*, 1974, 445.



Scheme 12

It is clear from these examples that indirect monitoring of ^{2}H *via* the ^{13}C n.m.r. spectrum is now widely used. Compared with ^{2}H n.m.r., it has the disadvantage that the sensitivity of detection is low and that it does not provide a reliable measure of the amount of deuterium at different positions in the molecule. On the other hand, the technique does provide information concerning the numbers of deuteriums attached to particular carbons. This extra insight was decisive in some of the investigations discussed in this section. The direct-observe and indirect-observe techniques are complementary rather than competitive, the choice being determined by the nature of the biosynthetic problem.

4 Conclusions

The biosynthetic chemist might be forgiven for being bewildered by the choice of techniques for tracing hydrogen. The two direct n.m.r. techniques reviewed here became available almost simultaneously and it is therefore interesting to compare the number of applications of each to date; two for tritium n.m.r. and six for deuterium n.m.r. This presumably reflects the reluctance of many biosynthetic workers to use ^{3}H at the levels of enrichment necessary for this type of work. The balance will undoubtedly change when n.m.r. instrumentation improves to the extent that ^{3}H can be used at levels comparable with radioactive tracer work.

As explained above, the indirect method of using ^{13}C n.m.r. is complementary

It seems likely that ^{2}H rather than ^{3}H will continue to be used for this type of study in the forseeable future because the method relies upon the presence of hydrogen isotope at high enrichment. Nevertheless, ^{3}H has advantages for this type of study which may persuade future investigators to explore its potential. There is also scope for using adjacent protons rather than ^{13}C nuclei as the indirect probe. The list of techniques for tracing hydrogen is by no means complete[44] so that this will undoubtedly be an active field of exploration for several years to come.

We thank Dr. C. Jones for pointing out some textual inaccuracies during the final preparation of the manuscript. M.J.G. acknowledges financial support from the Royal Society and from New Hall, Cambridge.

[44] J. Barber and J. Staunton, *J.C.S. Chem. Comm.*, 1979, 1098; Y. Sato, T. Oda, E. Miyata, and H. Saito, *F.E.B.S. Letters*, 1979, **98**, 271.