Carbon-13 Nuclear Magnetic Resonance in Biosynthetic Studies

By T. J. Simpson
RESEARCH SCHOOL OF CHEMISTRY, AUSTRALIAN NATIONAL UNIVERSITY, P.O. BOX 4, CANBERRA, ACT 2600, AUSTRALIA

1 Introduction

Recent years have seen ^{13}C n.m.r. grow from a relatively obscure technique to one rivalling ^{1}H n.m.r. in utility and scope. The theory of ^{13}C n.m.r.,[1,2] biological[3] and biochemical[4] applications, and early biosynthetic work[5,6] have been reviewed, two general texts[7,8] and a compilation of spectra[9] have appeared, and the ^{13}C n.m.r. spectra of a wide range of compounds have been assigned.[10]

Although radio-isotopes, particularly ^{14}C, have proved extremely useful in biosynthetic studies, they have one severe disadvantage in the necessity of carrying out extensive degradations to locate the incorporated isotope in a metabolite. This is often very difficult owing to formidable structural complexities or the presence of carbon atoms in an unreactive aromatic framework so that only a partial analysis is obtained. Furthermore, since in many cases chemical degradations are no longer necessary as structure proofs for natural products, their use for establishing ^{14}C-labelling patterns becomes a tiresome exercise. However, since ^{13}C n.m.r. is itself an integral component of structure elucidation, its use in biosynthetic studies is very attractive and allows the establishment of labelling patterns without recourse to extensive chemical degradations.

The aim of this review is to discuss the methodology of ^{13}C n.m.r. biosynthetic studies, both as an aid to evaluating the fast-expanding literature and to the undertaking of these studies, with emphasis on recent developments and applications.

[1] J. B. Stothers, *Quart. Rev.*, 1965, **19**, 144.
[2] F. A. L. Anet and G. C. Levy, *Science*, 1973, **180**, 141.
[3] J. B. Grutzner, *Lloydia*, 1972, **35**, 375.
[4] G. A. Gray, *C.R.C. Critical Reviews in Biochemistry*, 1973, 247.
[5] G. Lukacs, *Bull. Soc. chim. France*, 1972, 351.
[6] M. Tanabe, 'Biosynthesis', ed. T. A. Geissman, Specialist Periodical Reports, The Chemical Society, London, 1973, Volume 2, p. 241.
[7] G. C. Levy and G. L. Nelson, 'Carbon-13 Nuclear Magnetic Resonance for Organic Chemists', Wiley-Interscience, New York, 1972.
[8] J. B. Stothers, 'Carbon-13 N.M.R. Spectroscopy', Academic Press, New York, 1972.
[9] L. F. Johnson and W. C. Jankowski, 'Carbon-13 Nuclear Magnetic Resonance Spectroscopy. A collection of Assigned, Coded and Indexed Spectra', Wiley-Interscience, New York, 1972.
[10] See for example: E. Wenkert, J. S. Bindra, C.-J. Chang, D. W. Cochran, and F. M. Schell, *Accounts Chem. Res.*, 1974, **7**, 46.

2 Proton Satellite Method

Before discussing ^{13}C n.m.r., brief mention must be made of the proton-satellite method. Spin–spin coupling between a ^{13}C nucleus and directly bound protons results in satellite bands appearing on either side of the main proton signal in the ^{1}H n.m.r. spectrum, so providing an indirect probe for monitoring the ^{13}C abundance at a given position in a molecule. Any incorporation of a ^{13}C-enriched precursor can be detected by the increase in intensity of these satellites, Sepedonin,[11] griseofulvin,[12] fusaric acid,[13] piercidin A,[14] mollisin,[15] and variotin[16] have all been studied by this method. However, despite its advantage of only requiring readily available instrumentation, the method has severe limitations as only carbons with attached protons can be studied, and often complex ^{1}H spectra, traces of impurities, and spinning side-bands obscure the satellites. These limitations are overcome in the direct ^{13}C n.m.r. method.

3 Carbon-13 Nuclear Magnetic Resonance

The theory of ^{13}C n.m.r. has been thoroughly reviewed[7,8] so a brief introduction only is given here, a working knowledge of ^{1}H n.m.r. being sufficient to understand ^{13}C n.m.r. Table 1 compares the relevant properties of ^{13}C and ^{1}H and it

Table 1 *Comparison of nuclear properties of* ^{1}H *and* ^{13}C

	^{1}H	^{13}C
Nuclear spin	$\frac{1}{2}$	$\frac{1}{2}$
Resonance frequency at 23.5 kG	100 MHz	25.2 MHz
Natural abundance (%)	99.99	1.11
Relative sensitivity	1.00	0.016
Normal chemical shift range for organic molecules	10 p.p.m.	200 p.p.m.

may be seen that ^{13}C, a stable isotope, natural abundance 1.1% has a nuclear spin 1/2 and so is n.m.r. active and will show the same general splitting patterns as ^{1}H. As may be deduced from Table 1, the main difficulties in obtaining a ^{13}C n.m.r. spectrum are its low abundance and low nuclear sensitivity, so that for a given sample ^{13}C is 6000 times less sensitive than ^{1}H. It is the development of techniques to overcome this that has led to the huge growth in ^{13}C studies. These include

(i) the use of large samples, which became possible with the development of high-stability spectrometers taking up to 15 mm diameter n.m.r. tubes;

(ii) multiscan techniques, initially multiple accumulation in the continuous-

[11] J. Wright, D. G. Smith, A. G. McInnes, L. C. Vining, and D. W. S. Westlake, *Canad. J. Biochem.*, 1969, **47**, 945.
[12] M. Tanabe and G. Detre, *J. Amer. Chem. Soc.*, 1966, **88**, 4515.
[13] D. Desaty, A. G. McInnes, D. G. Smith, and L. C. Vining, *Canad. J. Biochem.*, 1968, **46**, 1293.
[14] M. Tanabe and H. Seto, *J. Org. Chem.*, 1970, **35**, 2087.
[15] M. Tanabe and H. Seto, *Biochemistry*, 1970, 9, 4851.
[16] M. Tanabe and H. Seto, *Biochim. Biophys. Acta*, 1970, **208**, 151.

wave mode but subsequently pulsed Fourier transform (FT) n.m.r.[17] which enables much faster spectral determination than conventional methods;

(iii) proton-noise-decoupling, providing two gains in sensitivity. By applying a wideband proton-decoupling frequency the diffuse multiplets arising from ^{13}C–^{1}H spin–spin coupling are collapsed to a single sharp line; this also induces a nuclear overhauser effect (NOE) leading to an intensity enhancement up to three-fold, due to disturbance of the ^{13}C energy-level populations for carbon atoms with attached protons.

Owing to the wide range of chemical shifts observed for ^{13}C nuclei, even complex molecules generally give ^{13}C n.m.r. spectra in which every carbon has a discrete resonance. Much ^{13}C n.m.r. assignment data has been accumulated; nevertheless complete assignment of resonances to a new molecule requires great care, and often considerable effort, especially in biosynthetic studies where the conclusions can only be as good as the original spectral assignments. Several aids to assignment are available:

(*a*) *Known chemical shifts and substituent chemical shift effects.* The chemical shifts of different functional types fall into well-defined ranges;[7,8] carbonyl carbons resonate at low field (*ca.* 200 p.p.m.), aromatic and olefinic carbons at 160—100 p.p.m., aliphatic carbons with electronegative substituents at 50—80 p.p.m., and simple aliphatics at highest field 10—30 p.p.m. Substituent effects are generally found to be additive and rules for predicting chemical shifts in hydrocarbons[18] and benzenoid aromatics[7] are available.

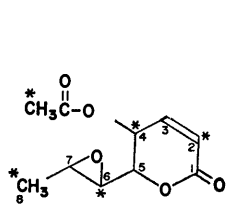
(*b*) *Off-resonance and specific proton decoupling.* In the proton noise-decoupled spectrum all ^{13}C–^{1}H coupling information is lost. In the off-resonance decoupling experiment, the ^{1}H irradiation is kept at high power levels but the centre frequency is moved *ca.* 500 Hz away from the protons being irradiated so that one-bond ^{13}C–^{1}H coupling patterns return, and the non-protonated, methine, methylene, and methyl carbons are observed as singlets, doublets, triplets, and quartets respectively. The observed or *residual* couplings, J_R, are smaller than the actual one-bond coupling and are a function of the actual coupling, J, the decoupling power, H, and the decoupler offset $\Delta\nu$:[19]

$$J_R = J\Delta\nu/H$$

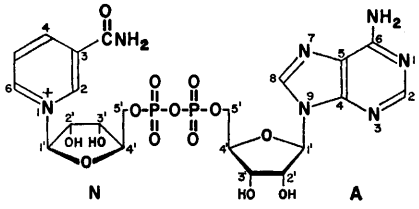
If J is known the residual coupling can be an aid to assignment. In the study of asperlin (1), a metabolite of *Aspergillus nidulans*, C-4, C-5, C-6, and C-7 all have one attached proton and are oxygen-bearing and so appear in the range 55—80 p.p.m. and give doublets in the off-resonance spectrum. However, comparison of the observed and calculated residual couplings allowed an unambiguous assignment to be made and confirmed the incorporation pattern of sodium [2-^{13}C]acetate shown.[20]

[17] E. Breitmaier, G. Jung, and W. Voelter, *Angew. Chem. Internat. Edn.*, 1971, **10**, 673.
[18] D. M. Grant and E. G. Paul, *J. Amer. Chem. Soc.*, 1964, **86**, 2984.
[19] R. R. Ernst, *J. Chem. Phys.*, 1966, **45**, 3845.
[20] M. Tanabe, T. Hamasaki, D. Thomas, and L. Johnson, *J. Amer. Chem. Soc.*, 1971, **93**, 273.

If the ^1H n.m.r. spectrum has been fully or partially assigned, the ^1H and ^{13}C resonances can be interrelated by single-frequency decoupling with the decoupler



(1)



N (2) A

set exactly on a specific ^1H frequency. The attached carbon appears as a singlet in the ^{13}C spectrum whereas the remaining carbons show off-resonance patterns. This process becomes tedious if several resonances require studying but can be overcome by plotting the line frequencies in the ^{13}C spectrum as the ^1H irradiating frequency is stepped through the ^1H n.m.r. spectrum. Where the lines cross gives the point where J_R is zero, and hence the ^1H and ^{13}C frequencies can be correlated.[21] Figure 1 illustrates the results obtained for NAD^+ (2).

(*c*) *Lanthanide-induced shift studies.* These are not as useful in ^{13}C studies as in ^1H n.m.r. because the actual shifts are of the same absolute value in both and so are relatively small in ^{13}C n.m.r., as are solvent and anisotropy effects. However, they can be useful for separating overlapping resonances. In complicatic acid (3), the resonances due to C-3 and C-10 both occur at 46 p.p.m. However, addition of [Eu(fod)$_3$] separated these resonances and showed that C-3 but not C-10 was enriched from [1-^{13}C]acetate-enriched cultures of *Stereum complicatum*.[22]

Another important use of shift reagents is to resolve the ^1H n.m.r. spectrum prior to specific proton-decoupling studies on the ^{13}C n.m.r. spectrum.[23]

(*d*) *Model and derivative studies.* Model compounds whose chemical shifts are known can be helpful in assigning the spectrum of a new compound, though they must be used with care. Quaternary carbons present the greatest difficulties in assignment, and studying the variation of chemical shifts in a series of closely related compounds may be the only method of reaching an unambiguous assignment. The ^{13}C n.m.r. spectrum of tajixanthone (5), a prenylated xanthone metabolite of *Aspergillus variecolor*, was fully assigned by a study of eleven derivatives.[24] Subsequent incorporation of [1-^{13}C]- and [2-^{13}C]-acetates indicated its biogenesis by prenylation and cleavage of an anthrone precursor *via* the known co-metabolite arugosin (4).

(*e*) *Synthesis* of a compound enriched at a known site with ^{13}C has been used for

[21] B. Birdsall, N. J. M. Birdsall, and J. Feeney, *J.C.S. Chem. Comm.*, 1973, 316.
[22] T. C. Feline, G. Mellows, R. B. Jones, and L. Phillips, *J.C.S. Chem. Comm.*, 1974, 63.
[23] B. Birdsall, J. Feeney, J. A. Glasel, R. J. P. Williams, and A. V. Xavier, *Chem. Comm.*, 1971, 1473.
[24] J. S. E. Holker, R. D. Lapper, and T. J. Simpson, *J.C.S. Perkin I*, 1974, 2135.

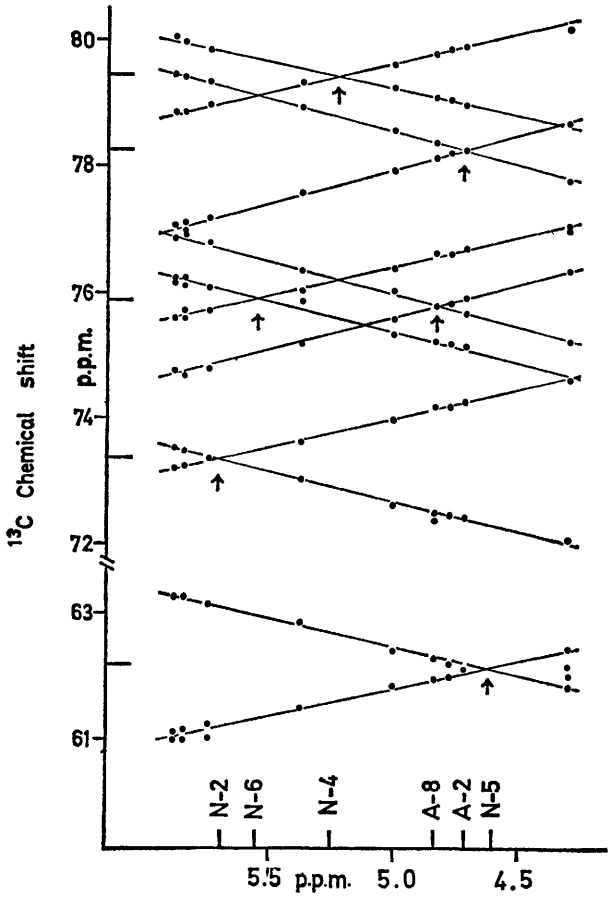
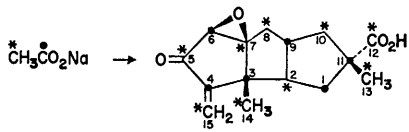
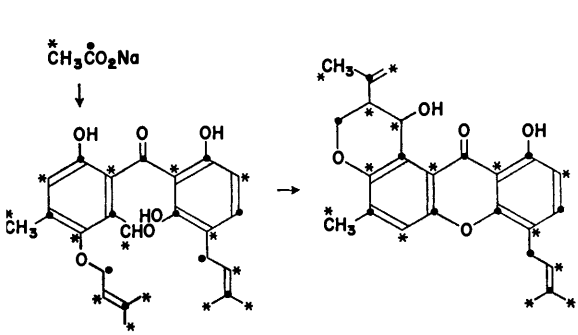


Figure 1 *Plot of peak frequencies in the* ^{1}H *off-resonance selectively decoupled* ^{13}C *spectra of* NAD^{+} *as a function of the position of irradiation in the* ^{1}H *spectrum, expressed in p.p.m. to high frequency of internal dioxan. The position of the peaks in the* ^{1}H *noise-decoupled* ^{13}C *spectrum are shown by lines on the ordinate and the position of the proton peaks by lines of the abscissa. The arrows* ↑ *indicate the point of collapse of the* ^{13}C *doublet and the connection between a given* ^{13}C *peak and the assigned proton peak. Small doublet splittings are observed on some of the signals from long-range* (C–H) *spin couplings.*



(3)

assignment. An exact assignment of the meso-carbons of protoporphyrin IX (6) was required prior to biosynthetic studies. This was accomplished by synthesis of protoporphyrins enriched specifically at the β-, γ-, and δ-meso positions respectively.[25]



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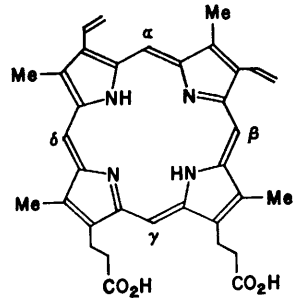


(6)

(*f*) *Partially relaxed Fourier transform* (*PRFT*) *n.m.r.* The T_1 relaxation times for ^{13}C atoms generally increase in the sequence methylene, methine, methyl, and quaternary carbon, so as the pulse internal time, τ, is increased the negative peaks obtained in PRFT for short values invert in the sequence above. This can be of great value in assigning resonances, especially in congested spectra where off-resonance may be of limited value.[26]

(*g*) *Incorporation studies.* ^{15}N is an isotope of spin $\frac{1}{2}$ and so is n.m.r. active and will couple with ^{13}C nuclei. Thus a metabolite grown in the presence of, say, $K^{15}NO_3$ will exhibit ^{15}N–^{13}C couplings for any carbons bonded to nitrogen. Similarly, any compound with adjacent ^{13}C nuclei will exhibit a ^{13}C–^{13}C coupling between these nuclei. Both these points are illustrated below.

4 Biosynthetic Methodology

The availability of ^{13}C-enriched compounds has increased rapidly and a wide range, similar to that for ^{14}C, is now available at enrichments of up to 95% and many others may be readily synthesized by standard methods from simple precursors such as $^{13}CO_2$, $^{13}CH_3I$, and $K^{13}CN$.

A. Precursor Incorporation.—Precursor efficiency may be assessed in several ways[27] but for ^{13}C studies the important criterion is *dilution* of added label. For ^{14}C, dilution per labelled site is given by:

[25] A. R. Battersby, G. L. Hodgson, M. Ihara, E. McDonald, and J. Saunders, *J.C.S. Chem. Comm.*, 1973, 441; *J.C.S. Perkin I*, 1973, 2923.
[26] K. Nakanishi, R. Crouch, I. Miura, X. Dominguez, A. Zamudio, and R. Villarreal, *J. Amer. Chem. Soc.*, 1974, **96**, 609.
[27] S. A. Brown, in 'Biosynthesis', ed. T. A. Geissman, Specialist Periodical Reports, The Chemical Society, London, 1972, Vol. 1, p. 9.

$$\frac{\text{specific activity of precursor}}{\text{specific activity of product}} \times \text{no. of labelled sites}$$

To obtain unequivocal results in ^{13}C studies using 90% enriched precursors, dilutions per labelled site of *ca.* 100 or less are required. This is due to inherent errors in ^{13}C n.m.r. resonance intensities (see below), requiring a two-fold increase in ^{13}C abundance to be certain that enrichment has occurred. Relatively large amounts of precursor, typically 1—20 mmol l^{-1}, have to be used to obtain this, especially for low precursor efficiencies. This introduces problems of expense and interference with normal metabolism; in contrast to ^{14}C studies, non-tracer amounts are now being used. A lowering of metabolite yields is common and cases of toxicity have been reported for elevated concentrations of acetate (0.2[15] and 1.6 g l^{-1});[28] propionate[28] (0.2 g l^{-1}), and mevalonate[29] (0.1 g l^{-1}). However, in other cases, higher concentrations have been used successfully, *e.g.* 2 g l^{-1} of acetate,[24] and the problem can often be overcome by pulsed feedings of precursor.[15]

Preliminary experiments with ^{14}C-labelled precursors are generally carried out to ascertain the feasibility of ^{13}C studies and to optimize conditions. Three main parameters require studying: time of precursor addition, incubation time, and amount of precursor. Maximum precursor incorporation usually occurs with addition of precursor at the start of maximum metabolite production, *i.e.* the start of the *idiophase*[30] in microbial fermentations, necessitating the determination of growth and production curves. Incorporation may be very sensitive to time of addition. Figure 2 illustrates the marked variation of incorporation with day of addition of mevalonolactone into the sequiterpenoid trichothecins.[29]

The period of growth after addition of precursor may also be critical. The variation in dilution of [^{14}C]acetate on incorporation into sepedonin (7)[12] is shown in Figure 3, which illustrates that neither maximum yield of metabolite nor even maximum total incorporation of label is the important factor, the prime consideration being *minimum dilution* of label given a *sufficient yield* of metabolite for ^{13}C spectral determination.

Finally, mass *versus* incorporation studies will determine the minimum amount of precursor that must be added to obtain a satisfactory enrichment. Table 2 shows the variation of dilution with amount of added [^{14}C]acetate during biosynthetic studies on shanorellin (8) in *Shanorella spirotrichi.* Incorporation of [^{13}C]acetate and [^{13}C]methionine indicated its origin from a tetraketide with the methyls derived from the C_1-pool.[31]

B. Interpretation and Presentation of Results.—Having obtained a ^{13}C-enriched metabolite as above, the results from the ^{13}C n.m.r. spectrum must be evaluated.

[28] R. J. White, E. Martinelli, G. G. Gallo, G. Lancini, and P. Benyon, *Nature*, 1973, **243**, 273.
[29] J. R. Hanson, T. Marten, and M. Siverns, *J.C.S. Perkin I*, 1974, 1033.
[30] J. D. Bu'Lock in 'Essays in Biosynthesis and Microbial Development', Wiley, London, 1967.
[31] C.-K. Wat, A. G. McInnes, D. G. Smith, and L. C. Vining, *Canad. J. Biochem.*, 1972, **50**, 620.

Table 2 *Incorporation of radioactivity from* [1-^{14}C]*acetate into shanorellin*

		Shanorellin		
mmol*Acetate*	μCi mmol^{-1}	mg	μCi mmol^{-1}	*Dilution*
0.5	210	49	8.05	26
1.0	105	63	8.85	12
2.0	52.6	44	7.90	6.7
5.0	21.1	27	8.16	2.6

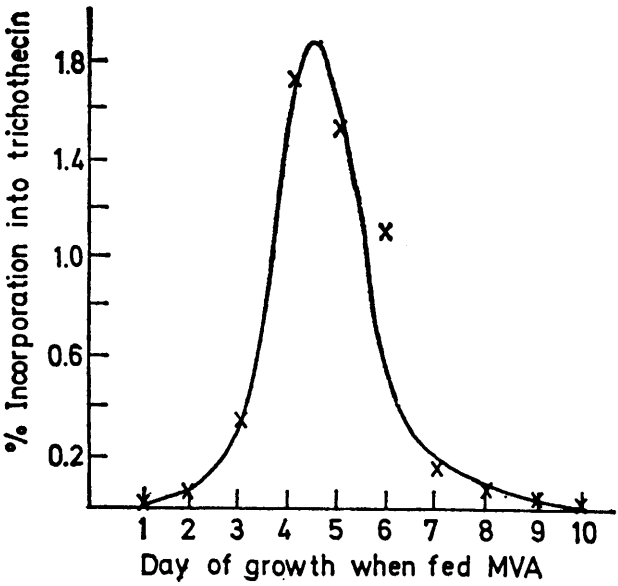
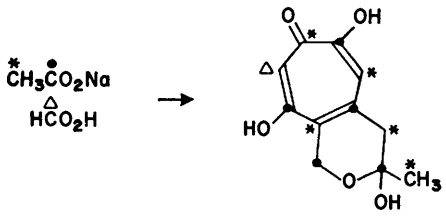


Figure 2 *Variation of incorporation of* [^{14}C]*mevalonic acid (MVA) into trichothecin with day of addition*
(Reproduced from *J.C.S. Perkin I*, 1974, 1033)



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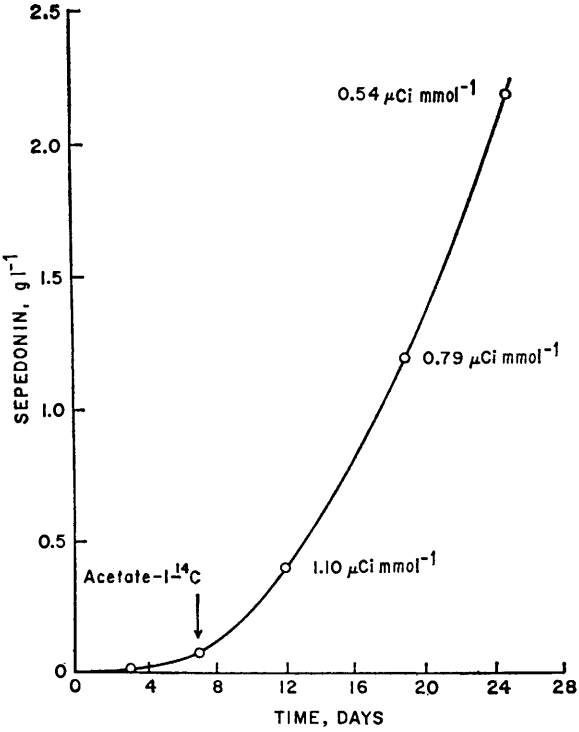
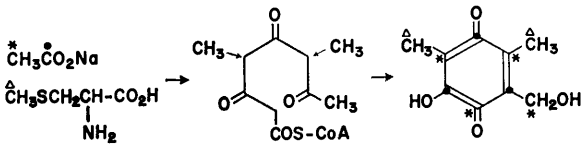
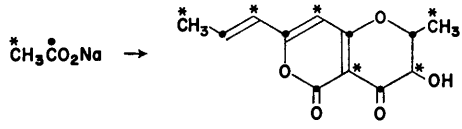


Figure 3 *Yield and specific activity of sepedonin produced by cultures of* S. chrysospermum *administered* [^{14}C]*acetate* (9 mmol l^{-1}) *on the 7th day after innoculation* (Reproduced by permission from *Canad. J. Biochem.*, 1969, **47**, 945)



(8)

If high enrichments are obtained the labelled sites will be readily apparent by visual inspection of the spectra. In the case of radicinin (9), the enrichment was so high that only the labelled peaks were visible[32] (Figure 4).



(9)

[32] M. Tanabe, H. Seto, and L. Johnson, *J. Amer. Chem. Soc.*, 1970, **92**, 2157.

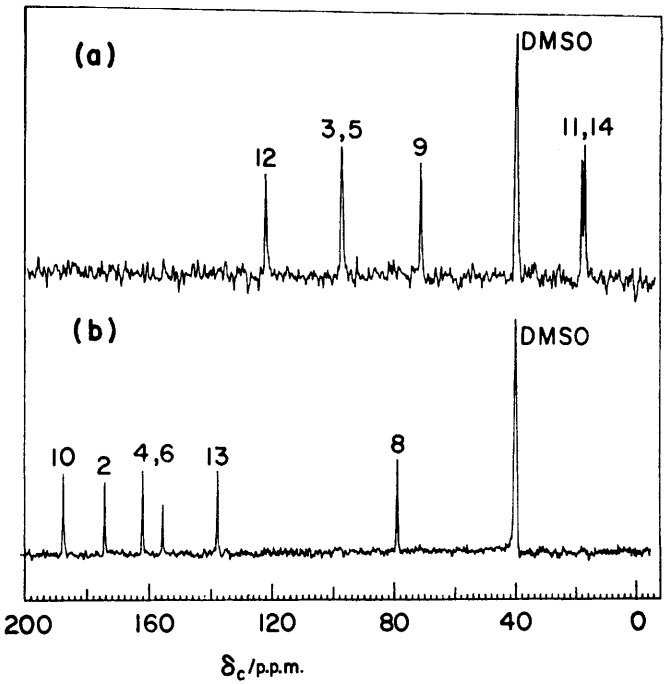


Figure 4 ^{13}C *N.m.r. spectra of radicinin from* (a) $^{13}CH_3CO_2Na$ *and* (b) $CH_3{}^{13}CO_2Na$ (Reproduced by permission from *J. Amer. Chem. Soc.*, 1970, **92**, 2157)

Incorporations are commonly given as percentage enrichments:

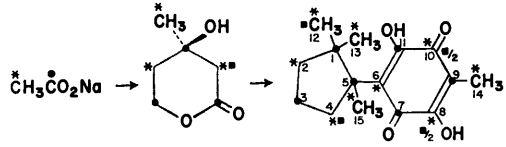
$$\text{Percentage enrichment} = \left(\frac{\text{observed } ^{13}\text{C abundance}}{\text{natural } ^{13}\text{C abundance}}\right) - 1.1$$

Very high enrichments, 5—60%, have been recorded but lower values, 0.5—5%, are more typical and can require more care in assessing, particularly at the lower values, because of the 'intensity problem'.[3]

In ^{13}C n.m.r. line intensities are non-integral owing to the variable NOE and widely varying relaxation times. With FT n.m.r. the interval between scanning pulses may be shorter than the relaxation times of individual ^{13}C nuclei, resulting in differential amounts of saturation occurring and thus variable line intensities, particularly for quaternary carbons. Addition of a free radical[33] or a paramagnetic species,[34] *e.g.* chromium trisacetoacetonate, [Cr(acac)$_3$], can partly overcome the problem. This complex quenches the NOE and shortens the relaxation times to give more uniform line intensities and was used to advantage in

[33] G. N. La Mar, *J. Amer. Chem. Soc.*, 1971, 93, 1040.
[34] R. Freeman, K. G. R. Pachler, and G. N. La Mar, *J. Chem. Phys*, 1971, **55**, 4586.

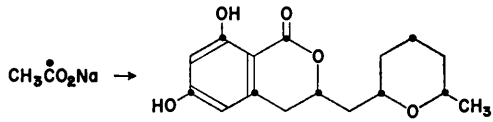
studying the biosynthesis of helicobasidin (10) in *Helicobasidium mompa*.[35] [2-^{13}C]Mevalonate was expected to label C-4 and C-12, with the remaining label distributed equally between C-8 and C-10 owing to tautomerism of the



(10)

dihydroxy–quinone system. However, in the resultant spectrum, the large variations in intensity made the labelling of C-8 and C-10 uncertain, but from the spectrum in the presence of 0.1 mol l^{-1} [Cr(acac)$_3$] the equal labelling of C-8 and C-10 was apparent (Figure 5).

A second method uses gated decoupling. In this, the NOE is eliminated by switching off the ^{1}H noise decoupling frequency during the interval between scanning pulses. This method was used to study the incorporation of [2-^{13}C] acetate into asperentin (11) by *Aspergillus flavus*.[36] The saturation problem can



(11)

be eliminated by a sufficiently long delay between the scanning pulses, but as some ^{13}C relaxation times are very long a compromise has to be made in practice to maintain reasonable spectral acquisition times.

Ultimately the only reliable method is a direct comparison of the respective line intensities in the natural-abundance and enriched spectra; both sets of intensities are subject to identical NOEs and relaxation considerations which should cancel out provided both spectra are standardized. This can be achieved by using identical concentrations and instrument parameters[15] but a more convenient technique is to normalize both spectra to a reliable standard. If the material is derivatized before spectral acquisition, as with asperentin (11) as the dimethyl ether or neomycin (see below) as the hexa-acetate, all the remaining line intensities can be normalized to the average value of the intensities of the introduced methyl groups in each spectrum. Correction for the difference of these averages in the respective spectra then allows direct comparison of indi-

[35] M. Tanabe, K. Suzuki, and W. C. Jankowski, *Tetrahedron Letters*, 1973, 4723.
[36] L. Cattel, J. F. Grove, and D. Shaw, *J.C.S. Perkin I*, 1973, 2626.

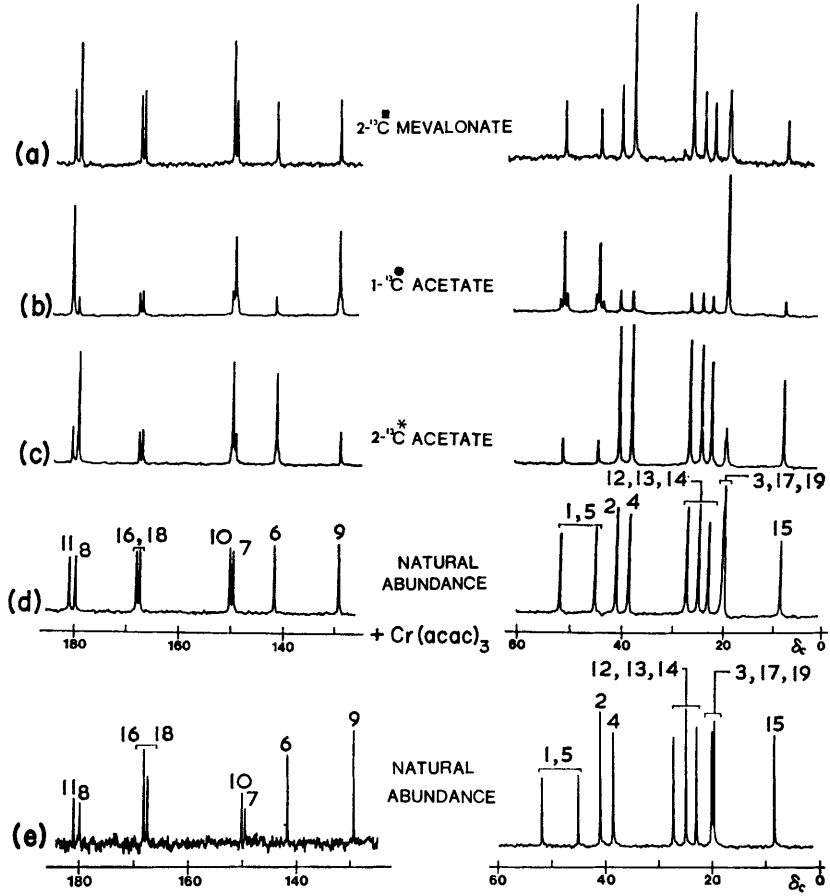
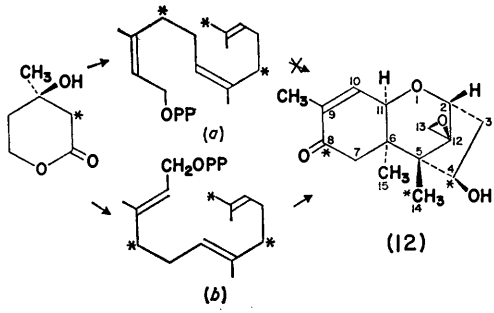


Figure 5 ^{13}C *N.m.r. spectra of helicobasidin from* (a) [2-^{13}C]*mevalonate*, (b) [2-^{13}C]-*acetate*, (c) [1-^{13}C] *acetate, and* (d) *at natural abundance, all in the presence of* 0.1 M-[Cr(acac)$_3$], *and* (e) *at natural abundance alone*
(Reproduced by permission from *Tetrahedron Letters*, 1973, 4723)

vidual intensities. However, derivatization is not always feasible. Incorporation of [2-^{13}C]mevalonate was used to distinguish between the two possible foldings of the farnesyl pyrophosphate precursor of trichothecalone (12) (Scheme 1) as ^{14}C studies provided conflicting evidence.[29] Incorporation of label at C-4 and C-8 rather than at C-10 indicated biosynthesis *via* path (*b*). The natural-abundance and enriched spectra were normalized to the line intensity of C-12 which was assumed to be unlabelled. This was not an ideal choice as, being one of the least intense peaks in the spectrum, it is most sensitive to errors. A more general method using all the unlabelled peaks in the spectrum for normalization has been proposed.[24]



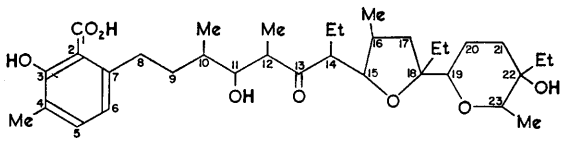
Scheme 1

However, despite these operations, there remains for FT n.m.r. spectra an uncertainty in line intensities *due* to the digitization of data during spectral accumulation and transformation[37] so that a series of experiments run on the same sample under apparently identical spectrometer parameters can give intensities that vary by $\pm 20\%$. This means that enrichments of less than 0.5% must be regarded with caution in the absence of supporting data such as multiple spectral determinations, proton-satellite enrichments, or ^{13}C–^{13}C coupling. The problem is alleviated by the use of larger data-storage facilities[3] but these are expensive and not always readily available.

5 Further Biosynthetic Studies

A large variety of metabolite types have been studied, several having been mentioned above. Further examples are discussed below.

Lasocolic acid (13) contains three unique *C*-ethyl groups. ^{14}C Studies failed



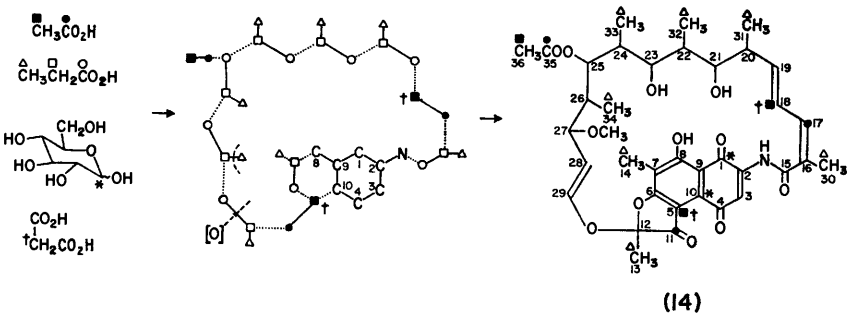
(13)

to establish the origin of these groups but addition of sodium [1-^{13}C]butyrate to the culture established their butyrate origin.[38] Incorporation of [1-^{13}C] propionate confirmed the origin of the C-4, C-10, C-12, and C-16 methyls and [1-^{13}C]acetate that of the C-23 methyl.[39]

Considerable attention has been given to the origin of the ANSA chain in the

[37] H. M. Pickett and H. L. Strass, *Analyt. Chem.*, 1972, **44**, 265.
[38] J. W. Westley, D. L. Preuss, and R. G. Pitcher, *J.C.S. Chem. Comm.*, 1972, 161.
[39] J. W. Westley, R. H. Evans, G. Harvey, R. G. Pitcher, D. L. Preuss, A. Stempel, and J. Berger, *J. Antibiotics*, 1974, **27**, 288.

rifamycins and related antibiotics. Incorporation of [1-^{13}C]-, [2-^{13}C]-, and [3-^{13}C]-propionates and of [1-^{13}C]- and [2-^{13}C]-acetates into rifamycin S (14) by *Nocadia mediterranei* confirmed the origin of the ANSA chain from eight propionate and two acetate units, linked in a clockwise manner[28] (Scheme 2),



(14)

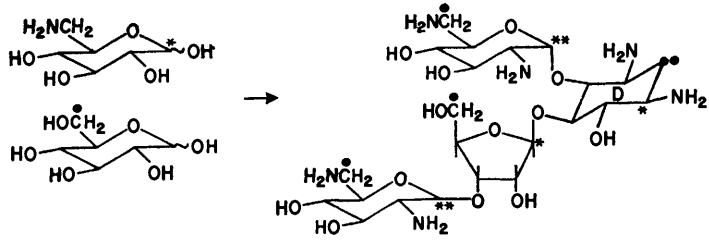
Scheme 2

since [1-^{13}C]acetate enriches C-17 rather than C-19. The exact assignment of the C-17 and C-19 ^{13}C resonances was crucial here and was achieved by specific proton decoupling.[40] Similar findings are reported for the related streptovaricins.[41] The origin of the C_7N moiety, C-1 to C-4 and C-8 to C-10, is obscure, but the enrichment of C-1 and C-10 of rifamycin S on incorporation of [1-^{13}C] glucose has been reported,[42] providing the first direct evidence for the origin of this moiety, also found in the mitomycins, validamycins, and kinamycins. In this study the first use of [2-^{13}C]malonate is reported; C-5 and C-18 are enriched but, interestingly, not the C-25 acetoxy-group.

Incorporations of both ^{13}C-labelled acetate and mevalonate into terpenes have been reported. Studies on helicobasidin, complicatic acid, and trichothecalone have been discussed above; incorporation of [^{13}C]acetate into the virescenosides,[43] fusidic acid,[44] and *Acrostolagmus*[45] lactone have also been reported.

The neomycins [*e.g.* (15)] are antibiotic metabolites of *Streptomyces fradiae*. The lack of crystalline derivatives and satisfactory degradations hampered ^{14}C biosynthetic studies but incorporation of [1-^{13}C]glucosamine and [6-^{13}C]glucose led to the labelling pattern shown in Scheme 3.[46] The preferential incorporation

[40] E. Martinelli, R. J. White, G. G. Gallo, and P. J. Benyon, *Tetrahedron Letters*, 1974, 1367.
[41] B. Milavetz, K. Kakinuma, K. L. Rinehart, J. P. Rolls, and W. J. Haak, *J. Amer. Chem. Soc.*, 1973, **95**, 5793.
[42] A. Karlsson, G. Sartori, and R. J. White, *European J. Biochem.*, 1974, **47**, 251.
[43] J. Polonsky, Z. Baskevitch, N. Cognoli-Bellavita, P. Cecchivelli, B. L. Buckwalter, and E. Wenkert, *J. Amer. Chem. Soc.*, 1972, **94**, 4369.
[44] T. Riisom, H. J. Jakobsen, N. Rastrup-Andersen, and H. Lorck, *Tetrahedron Letters*, 1974, 2247.
[45] H. Kakisawa, M. Sato, T.-I. Ruo, and T. Hayashi, *J.C.S. Chem. Comm.*, 1973, 802.
[46] K. L. Rinehart, J. M. Malik, R. S. Nystrom, R. M. Stroshane, S. T. Truitt, M. Taniguchi, J. P. Rolls, W. J. Haak, and B. A. Roff, *J. Amer. Chem. Soc.*, 1974, **96**, 2263.

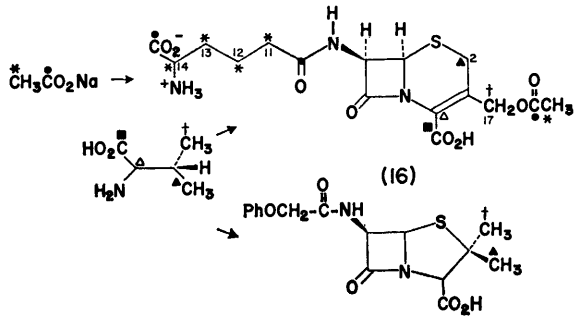


(15)

Scheme 3

of glucose rather than glucosamine into the deoxystreptamine residue (D) was unexpected and necessitated the proposal of a new pathway for deoxystreptamine biosynthesis. It and related amino-cyclitols are important constituents of several antibiotics.[47]

Incorporations of [^{13}C]-labelled acetates and valines into cephalosporin C (16) are as expected from ^{14}C studies. The higher degree of labelling with [2-^{13}C] acetate of C-14 compared with C-11, C-12, and C-13, which were similar, suggested the formation of the α-aminoadipyl side-chain from α-ketoglutaric acid and acetyl coenzyme A.[48] The stereoselective incorporation of ^{13}C in (2*RS*,3*R*)-[4-^{13}C]valine into C-2[49] and of (2*RS*,3*S*)-[4-^{13}C]valine into C-17 of (16)[50] has been demonstrated. The (3*R*)-isomer specifically labels the C-2 β-methyl group of penicillin V (17) in *Penicillium chrysogenum*[51] and the (3*S*)-isomer labels the α-methyl group.[52]



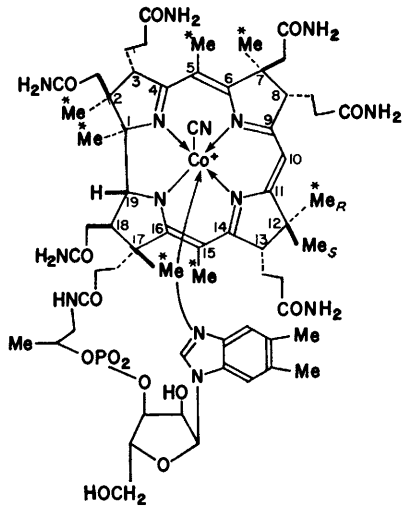
(16)

(17)

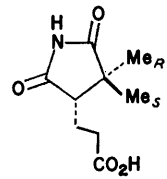
Scheme 4

[47] W. T. Dobranzki, *Chem. in Britain*, 1974, **10**, 386.
[48] N. Neuss, C. H. Nash, P. A. Lemke, and J. B. Grutzner, *J. Amer. Chem. Soc.* 1971, **93**, 2337; *Proc. Roy. Soc.*, 1971, **B179**, 335.
[49] N. Neuss, C. H. Nash, J. E. Baldwin, P. A. Lemke, and J. B. Grutzner, *J. Amer. Chem. Soc.*, 1973, **95**, 3797.
[50] H. Kleunder, C. H. Bradley, C. J. Sih, P. Fawcett, and E. P. Abraham, *J. Amer. Chem. Soc.*, 1973, **95**, 6149.
[51] P. A. Lemke, C. H. Nash, and S. N. Pieper, *J. Gen. Microbiol.*, in the press.
[52] D. J. Aberhart and L. J. Lin, *J.C.S. Perkin I*, 1974, 2320.

^{13}C N.m.r. has been used extensively in biosynthetic studies of porphyrins and vitamin B_{12} (18). Early work on the incorporation of ^{13}C-labelled porphobilinogen, δ-amino-laevulinic acids, and Urogens I—IV has been reviewed.[6] More recently, three groups have independently shown that seven of the eight methyl groups of vitamin B_{12} are enriched by feeding [^{13}C]methionine to *Propionibacterium shermanii*.[53] The methyl group not enriched is one of those at C-12. Battersby has suggested this to be the 12β; degradation of vitamin B_{12} gives the imide (19) in which the methyl ^1H n.m.r. resonances have been assigned.



(18)



(19)

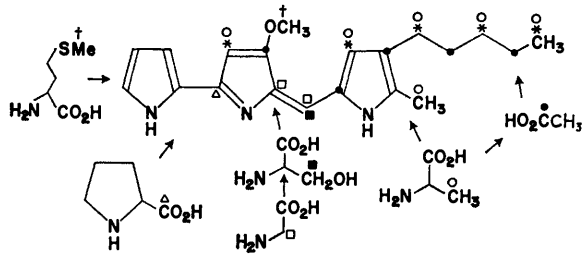
Only the signal due to the 12α-methyl showed enhanced ^{13}C satellites, Scott is in agreement with this, arguing that the C-2, C-7, C-12α, and C-17 methyls should have similar chemical shifts owing to the γ-effect[54] of the *syn*-propionate side-chain whereas that at C-12β, lacking this, should be shifted downfield. Good support was obtained for this analysis from the ^{13}C n.m.r. spectrum of the enriched vitamin B_{12} after epimerization at C-13 when one of the enriched signals, presumably the C-12α, moved downfield by *ca.* 12 p.p.m. Shemin and Katz have reached the opposite conclusion using specific proton decoupling to correlate the enriched ^{13}C resonances with the ^1H resonances. However, doubt has been cast on the crucial ^1H assignments in this case.[55]

[53] (*a*) A. R. Battersby, M. Ihara, E. McDonald, J. R. Stevenson, and B. T. Golding, *J.C.S. Chem. Comm.*, 1973, 404; *ibid.*, 1974, 458; (*b*) A. I. Scott, C. A. Townsend, and R. J. Cushley, *J. Amer. Chem. Soc.*, 1973, **95**, 5759; (*c*) C. E. Brown, D. Shemin, and J. J. Katz, *J. Biol. Chem.*, 1973, **248**, 8015.

[54] D. K. Daling and D. M. Grant, *J. Amer. Chem. Soc.*, 1972, **94**, 9318.

[55] E. McDonald, *Ann. Reports* (*B*), 1974, **70**, 597.

^{14}C Studies clearly indicate that the mechanism of pyrrole-ring formation in prodigiosin (20) is unrelated to that operative in the porphyrins and so is of special interest. A complete assignment of the ^{13}C spectrum of prodigiosin has been made,[56] enabling ^{13}C biosynthetic studies to be carried out. ^{13}C-Labelled acetates, alanine, proline, glycine, and serine have been incorporated by cultures of *Serratia marcescens* (Scheme 5), allowing a biosynthetic path to be proposed.[57]



(20)

Scheme 5

Recently metacycloprodigiosin and undecylprodigiosin have been isolated, and ^{13}C studies indicate a similar biosynthesis.[58]

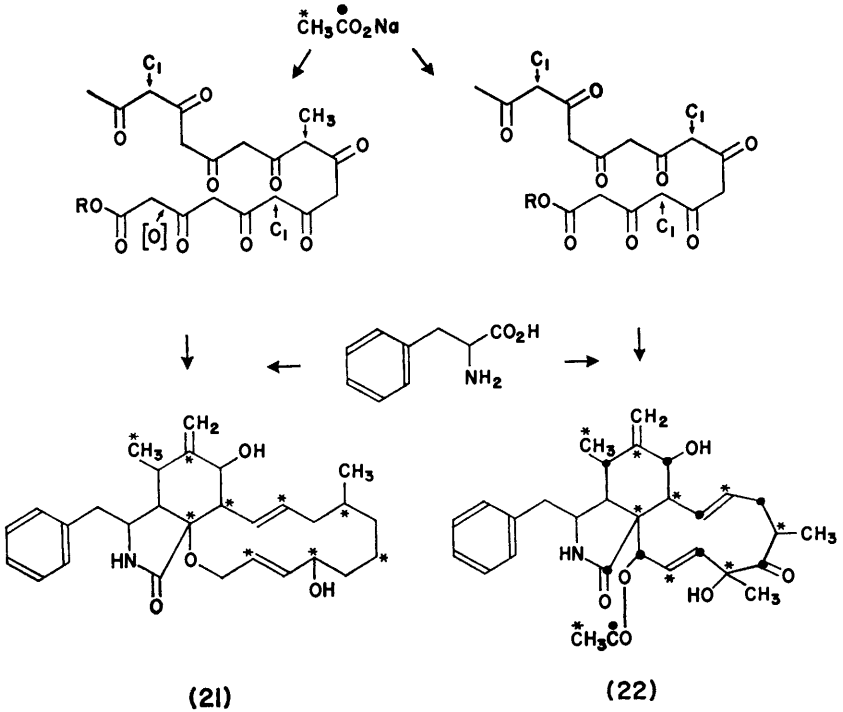
Specific proton decoupling and PRFT methods have been used to assign the ^{13}C n.m.r. spectra of cytochalasin B (21) and cytochalasin D (22). Incorporation of sodium [1-^{13}C]- and [2-^{13}C]-acetate[59] confirms previous proposals of biosynthesis of the cytochalasins from phenylalanine, methionine, and a C_{18} or C_{16} polyketide (Scheme 6).

6 ^{13}C–^{13}C Spin–Spin Coupling

In natural-abundance ^{13}C n.m.r. spectra ^{13}C–^{13}C spin–spin coupling is not observed as the probability of ^{13}C nuclei being adjacent is equal to the square of the natural abundance, giving satellites of 0.55% of the intensity of the main signal. However, in enriched material the probability is much higher and the detection of a ^{13}C–^{13}C coupling can provide conclusive evidence that two labels have been incorporated into adjacent positions in a molecule.

A. Singly Labelled Precursors.—A ^{13}C–^{13}C coupling can arise from administration of singly labelled precursors in a variety of ways.

[56] R. J. Cushley, D. R. Andersen, S. R. Lipsky, R. J. Sykes, and H. H. Wasserman, *J. Amer. Chem. Soc.*, 1971, **93**, 6284.
[57] H. H. Wasserman, R. J. Sykes, P. Peverada, C. K. Shaw, R. J. Cushley, and S. R. Lipsky, *J. Amer. Chem. Soc.*, 1973, **95**, 6874.
[58] H. H. Wasserman, R. J. Sykes, C. K. Shaw, and R. J. Cushley, *Tetrahedron Letters*, 1974, 2787.
[59] W. Graf, J.-L. Robert, J. C. Vederas, C. Tamm, P. H. Solomon, I. Miura, and K. Nakanishi, *Helv. Chim. Acta*, 1974, **57**, 1801.



Scheme 6

Molecular rearrangement of a biosynthetic intermediate may give rise to a ^{13}C–^{13}C coupling. The pyrone (23), a metabolite of *Aspergillus quercinus*, when enriched from [2-^{13}C]acetate shows a coupling of 61 Hz between C-2 and C-7 (Figure 6b). This coupling probably arises from an intramolecular rearrangement of the precursor polyketide chain[60] (Scheme 7). A similar coupling from head-to-head linkage of acetate units is observed on incorporation of [2-^{13}C]acetate into sterigmatocystin (24).[61]

Folding of a pentaketide chain to give a five-membered ring results in a ^{13}C–^{13}C coupling between C-5 and C-4a of dihydrolatumicidin (25) enriched from [2-^{13}C]acetate.[62] Folding of a terpenoid chain enriched from [1-^{13}C]acetate gives rise to a coupling between C-1 and C-5 in helicobasidin, (Figure 5), and between C-8 and C-14 in fusidic acid (26). The head-to-head linkage of farnesyl units, *via* squalene, gives rise to the coupling observed between C-11 and C-12.[44]

Conversion of [2-^{13}C]acetate into succinate in the Krebs cycle results in a

[60] T. J. Simpson, *Tetrahedron Letters*, 1975, 175.
[61] M. Tanabe, T. Hamasaki, H. Seto, and L. Johnson, *Chem. Comm.*, 1970, 1539.
[62] H. Seto, T. Sato, H. Yonehara, and W. C. Jankowski, *J. Antibiotics*, 1973, **26**, 609.

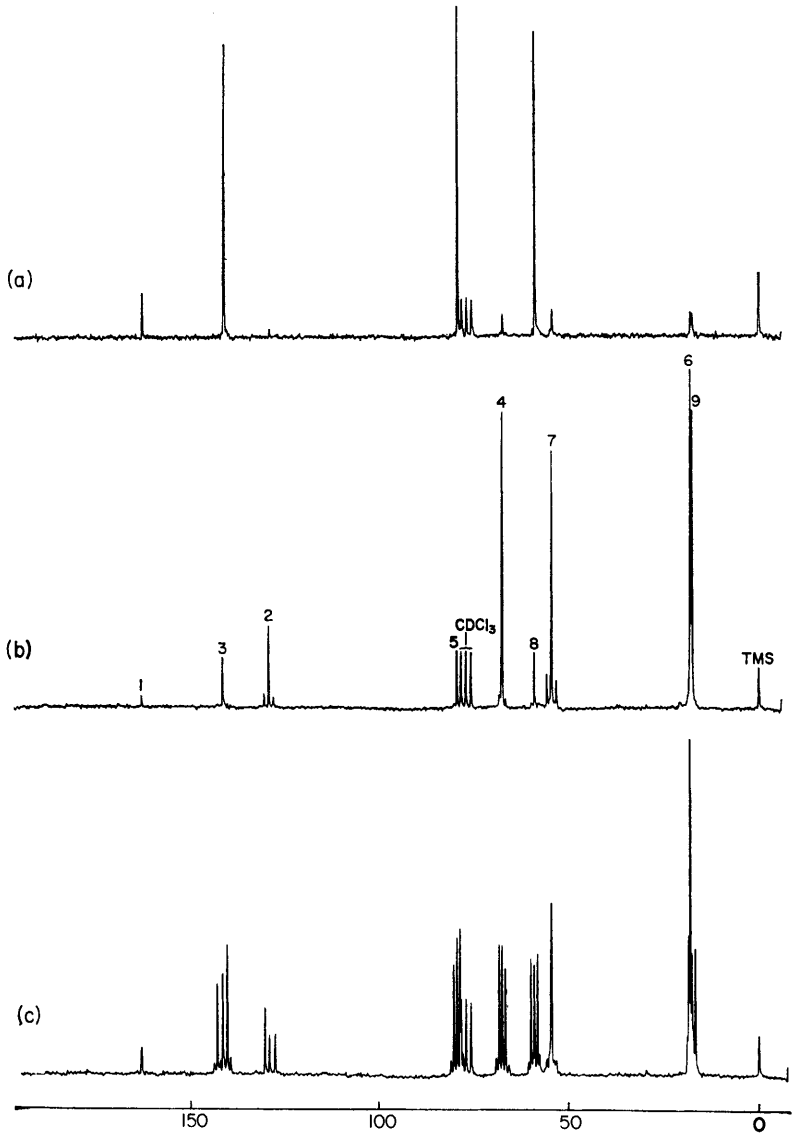
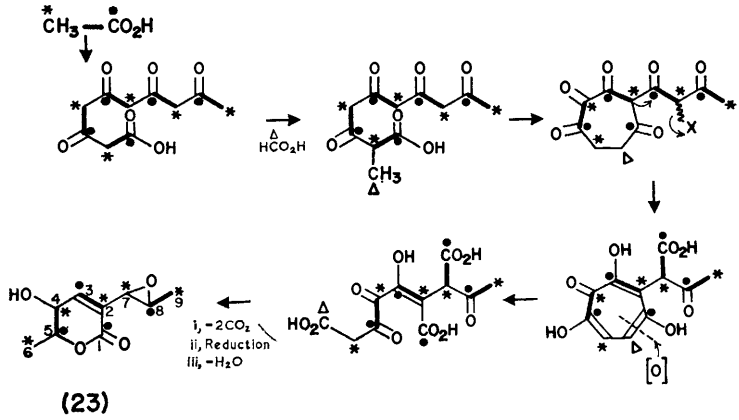


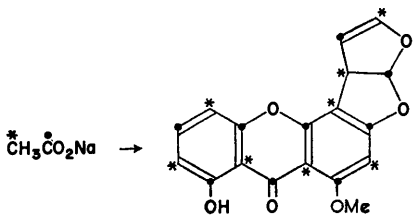
Figure 6 ^{1}H *Noise-decoupled* ^{13}C *n.m.r. spectra of pyrone* (23) *from* (a) $CH_3{}^{13}CO_2Na$, (b) $^{13}CH_3CO_2Na$, *and* (c) $^{13}CH_3{}^{13}CO_2Na$

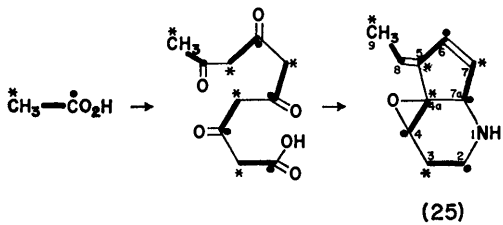
^{13}C–^{13}C coupling between C-11 and C-15 in avenaciolide (27).[63] Similar metabolic transformations give rise to ^{13}C–^{13}C couplings when [2-^{13}C]glycine is

[63] M. Tanabe, T. Hamasaki, Y. Suzuki, and L. F. Johnson, *J.C.S. Chem. Comm.*, 1973, 212.



Scheme 7

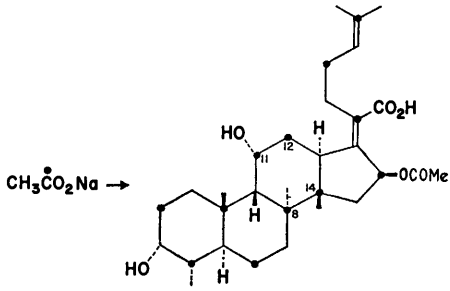




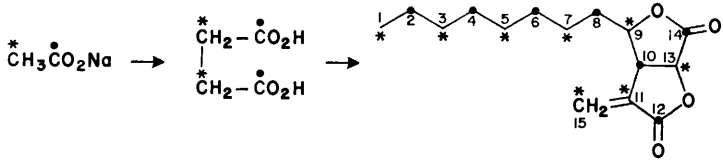
incorporated *via* serine into prodigiosin and [5-^{13}C]-δ-aminolaevulinic acid is incorporated into vitamin B_{12} *via* porphobilinogen.[64]

B. Doubly Labelled Precursors.—This has been without doubt one of the most important developments in biosynthetic studies in recent years. Most studies have used [1,2-^{13}C]acetate in which both the carboxyl and methyl carbons are highly enriched. This means that with this precursor all acetate-derived atoms are

[64] C. E. Brown, J. J. Katz, and D. Shemin, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 2585.



(26)



(27)

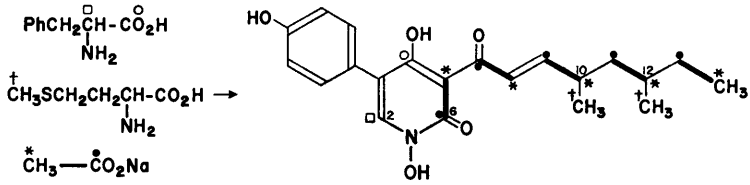
labelled and adjacent atoms derived from incorporation of an intact acetate unit will exhibit a ^{13}C–^{13}C coupling. Generally, no coupling will be observed between *adjacent* units owing to the low probability of more than one added precursor unit being incorporated into any one metabolite molecule. This coupling can be of great use in structural and spectral assignment studies in addition to providing biosynthetic information.

The first application of this technique was to dihydrolatumicidin (25). On incorporation of [1,2-^{13}C]acetate all 10 carbons exhibited ^{13}C–^{13}C couplings, confirming its origin from five acetate units.[65] Feeding of a 50:50 mixture of [1-^{13}C]acetate and [2-^{13}C]acetate gave rise to couplings for the carbon–carbon bonds between adjacent acetate units. Although four combinations between these singly labelled acetates are possible, only one ($\cdots CH_3{}^{13}CO\cdots{}^{13}CH_3CO\cdots$) gives the desired coupling. This latter technique requires high incorporations and so is of limited use. The size of the ^{13}C–^{13}C coupling, generally 30—90 Hz, is related to the hybridization of the atoms involved,[8] increasing with increased '*s*' character, and so in conjunction with chemical shift data is an important source of structural information.

Tenellin (28), a metabolite of *Beauvaria* sp., has been studied using [1,2-^{13}C]-acetate to provide both structural and biosynthetic information. An interesting feature of this study was growth of the organism using $K^{15}NO_3$ as the sole nitrogen source, resulting in ^{13}C–^{15}N couplings on C-6 and C-2. [^{13}C]Methionine indicated the origin of the C-10 and C-12 methyl groups and feeding of [1-^{13}C]-

[65] H. Seto, T. Sato, and H. Yonehara, *J. Amer. Chem. Soc.*, 1973, **95**, 8461.

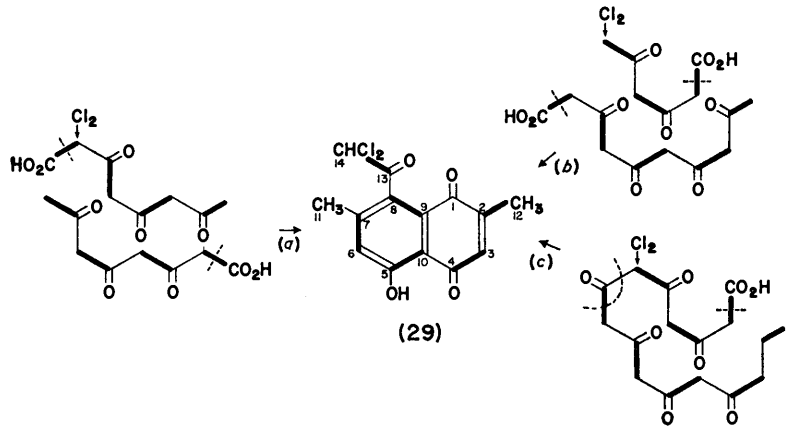
and [2-^{13}C]-phenylalanine proved the origin of the remainder of the molecule and indicated that a carboxy-carbon migration must take place during biosynthesis.[66]



(28)

A major advantage of doubly labelled precursors is in the elucidation of anomalous biosynthetic pathways. If the biosynthesis involves cleavage of an original acetate unit, the ^{13}C–^{13}C coupling is lost and the respective carbons now appear merely as enhanced singlets. When [1,2-^{13}C]acetate is incorporated into pyrone (23), three of the nine carbons appear as singlets; the remaining six all exhibit ^{13}C–^{13}C coupling (Figure 6c), suggesting biosynthesis *via* cleavage of a pre-formed carbocyclic ring as in Scheme 7.

The lack of couplings on C-1 and C-11 of mollisin (29) enriched with [1,2-^{13}C] acetate was interpreted in favour of a two-chain derivation[67] as in path (*b*) of Scheme 8; path (*a*) had been suggested on the basis of proton satellite studies



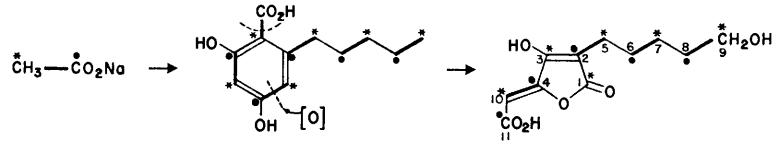
(29)

Scheme 8

[66] A. G. McInnes, D. G. Smith, C.-K. Wat, L. C. Vining, and J. L. C. Wright, *J.C.S. Chem. Comm.*, 1974, 281; *ibid.*, p. 283.
[67] H. Seto, L. W. Cary, and M. Tanabe, *J.C.S. Chem. Comm.*, 1973, 867.

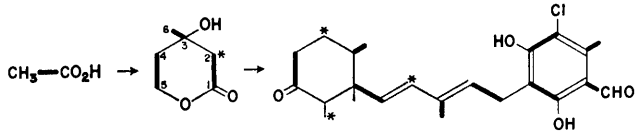
with singly labelled acetate. However, cleavage of a single polyketide chain, path (*c*), would give similar results and cannot be excluded.

^{13}C–^{13}C Couplings from incorporation of [1,2-^{13}C]acetate aided in the placing of the substituents on the tetronic acid ring of multicolic acid (30), a metabolite of *Penicillium multicolor*.[68] The labelling pattern from [1-^{13}C]- and [2-^{13}C] acetate was not as expected from the normal pathway of fungal tetronic acid biosynthesis and suggested formation *via* ring cleavage of n-pentylresorcylic acid (31). The absence of couplings on C-1, C-3, and C-11 on feeding [1,2-^{13}C] acetate confirmed this hypothesis.



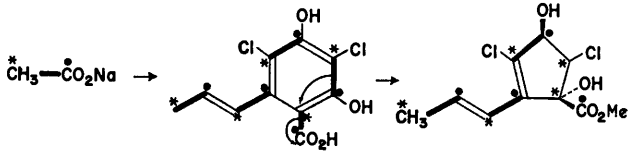
(31) (30)

Incorporation of [1,2-^{13}C]acetate into ascochlorin (32) in *Nectria coccinea* resulted in only five ^{13}C–^{13}C couplings in the triprenyl side-chain, showing that the methyl from C-6 rather than from C-2 of mevalonate migrates during biosynthesis.[69]



(32)

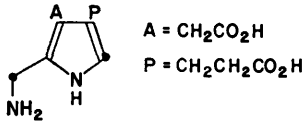
It has been suggested that the cyclopentenol (33), a metabolite of *Periconia macrospinosa*, and related fungal cyclopentenones are formed by ring contraction of a benzenoid precursor.[70] The incorporation of singly and doubly labelled [^{13}C]acetate shows that this is the case, but the labelling pattern obtained indicates a different mechanism from that proposed.[71]



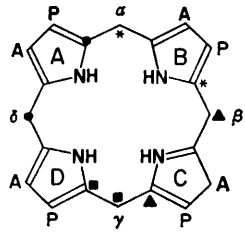
(33)

[68] J. A. Gudgeon, J. S. E. Holker, and T. J. Simpson, *J.C.S. Chem. Comm.*, 1974, 636.
[69] M. Tanabe and K. T. Suzuki, *J.C.S. Chem. Comm.*, 1974, 445.
[70] W. B. Turner, 'Fungal Metabolites', Academic Press, London, 1971, p. 126.
[71] J. S. E. Holker and K. Young, *J.C.S. Chem. Comm.*, 1975, 525.

The use of a doubly labelled precursor other than acetate is illustrated by the elegant work of Battersby on the biosynthesis of uroporphyrinogen III (35).[72] [2,11-^{13}C]-PBG (34) was synthesized and showed a long-range coupling of *ca.* 4 Hz. This was incorporated into (35) using a cell-free enzyme system from avian blood. Analysis of the resultant ^{13}C n.m.r. spectrum showed three doublets,



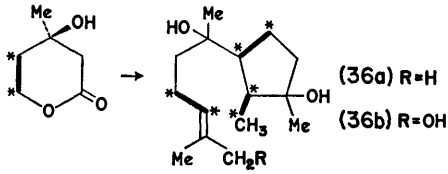
(34)



(35)

each of 5 Hz splitting, corresponding to the α-, β-, and δ-carbons, and one doublet of 72 Hz for the γ-carbon, indicating that PBG unit D must undergo an intramolecular rearrangement with respect to itself during biosynthesis.

To date, the only other doubly labelled precursor that has been employed is [4,5-^{13}C]mevalonate. This was synthesized by Hanson and incorporated into the fungal sesquiterpenes cyclonerodiol (36a) and cyclonerotriol (36b). All three sets of ^{13}C–^{13}C couplings are retained, showing that mevalonate is incorporated without rearrangement.[73]

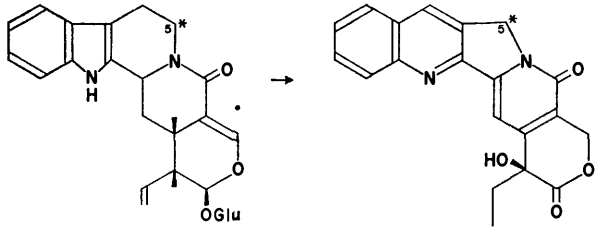


7 Higher Plant Metabolites

All the above ^{13}C studies have involved micro-organisms or partially purified enzyme systems. The main problems with higher plants are (*a*) the low incorporations generally obtained and (*b*) dilution of the labelled metabolite by unlabelled endogenous material, which can be so large that the ^{13}C content of the isolated substance does not differ significantly from natural abundance despite good incorporation. Despite these difficulties, two successful studies have been reported.

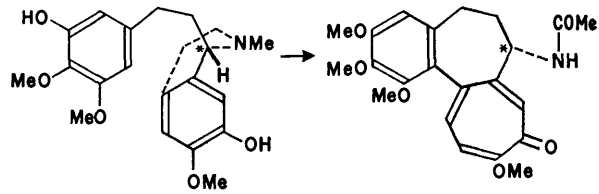
[72] A. R. Battersby, E. Hunt, and E. McDonald, *J.C.S. Chem. Comm.*, 1973, 442.
[73] J. R. Hanson, personal communication.

^{14}C-Labelling studies indicated that the lactone (37) was an intermediate in the biosynthesis of camptothecin (38) in *Camptotheca acuminata*.[74] Owing to the absence of suitable degradations, ^{13}C n.m.r. was used to prove specificity of incorporation. [1-^{13}C]Tryptamine was synthesized from K^{13}CN and thereby the [5-^{13}C]-lactone (37), 38 mg of which was wick-fed to intact plants. After two days growth, 20 mg of camptothecin was isolated which showed an enhancement of only the C-5 resonance intensity of *ca.* 55%.



(37) (38)

[1-^{13}C]Autumnaline (39) has been synthesized and injected as the hydrochloride (300 mg) into seed capsules (1 mg per capsule) of *Colchicum autumnale* (autumn crocus). After two weeks growth 1.24 g colchicine (40) was isolated. The resultant ^{13}C n.m.r. spectrum showed *ca.* 2.5-fold enrichment of the C-7 signal only.[75]



(39) (40)

However, the general extension of ^{13}C n.m.r. biosynthetic studies to higher-plant metabolites is likely to prove difficult. This may be alleviated by the use of cell-free enzyme systems in which the problems of penetration of precursors to the active site and dilution by endogenous material are eliminated. Work with ^{14}C-labelled material indicates that the dilution values and yields obtainable make ^{13}C studies feasible.[76] Similar considerations apply to studies using tissue cultures.

A second possibility is the use of ^{12}C-labelled compounds completely flushed of ^{13}C which are becoming available as a by-product of ^{13}C-enrichment and so are

[74] C. R. Hutchinson, A. H. Heckendorf, P. E. Doddona, E. Hagaman, and E. Wenkert, *J. Amer. Chem. Soc.*, 1974, **96**, 5609.
[75] A. R. Battersby, P. W. S. Sheldrake, and J. A. Milner, *Tetrahedron Letters*, 1974, 3315.
[76] D. H. Bowen, J. MacMillan, and J. Graebe, *Planta*, 1972, **102**, 261.

relatively inexpensive. Their use in mechanistic[77] and biosynthetic studies[78] has already been advocated; elimination of a peak intensity rather than enhancement would be observed. Though their direct use in biosynthetic studies is impractical owing to the high enrichments that would be required to obtain significant results,[79] they may be of use. Scott has suggested growth of a plant from seed or tissue culture in an atmosphere of $^{12}CO_2$ which should ensure a very low (0.1 to 0.2%) ^{13}C content in the various pools of organic intermediates.[80] Thus the ^{13}C 'natural abundance' is lowered by an order of magnitude, making subsequent ^{13}C-enrichment studies possible. It is perhaps noteworthy at this stage that it is only the fact that ^{13}C natural abundance is so relatively low that makes any biosynthetic studies possible at all, despite making spectra difficult to determine.

8 Conclusions

Besides the examples given many other metabolites have been studied. These studies include the incorporation of [^{13}C]acetate into ochratoxin,[81] palmiteolic acid,[82] niobomycin,[83] maleimycin,[84] showdomycin,[85] epoxydon,[86] and thermozymocidin,[87] of [1,2-^{13}C]acetate into penicillic acid,[88] ovalicin,[89] sterigmatocystin,[90] and bikaverin,[91] of [2-^{13}C]mevalonate into gibberellic acid,[73] of DL-tryptophan-[3-^{13}C]alanine into pyrrolnitrin,[92] and of [1-^{13}C]glycerate into rifamycin.[93] There can be no doubt that the use of ^{13}C methods will continue to expand rapidly, perhaps even superseding ^{14}C in biosynthetic studies of micro-organisms.

The use of doubly labelled precursors makes simultaneous determination of structure and biosynthesis possible for the first time and makes possible studies where classical ^{14}C methods could not provide unequivocal answers.

The extension of the method to higher plants and metabolic studies in man where the risk attached to the use of radio-isotopes is removed, seems imminent.

[77] J. Prestien and H. Gunther, *Angew. Chem. Internat. Edn.*, 1974, **13**, 276.
[78] S. B. W. Roeder, *J. Magn. Resonance*, 1973, **12**, 343.
[79] T. J. Simpson, *J. Magn. Resonance*, 1975, **14**, 262.
[80] A. I. Scott, *Science*, 1974, **186**, 101.
[81] Y. Maebayashi, K. Miyaki, and M. Yamazaki, *Chem. and Pharm. Bull. (Japan)*, 1972, **20**, 2172.
[82] A. L. Burlingame, B. Balogh, J. Welch, S. Lewis, and D. Wilson, *J.C.S. Chem. Comm.*, 1972, 318.
[83] N. M. J. Knoll, R. J. Huxtable, and K. L. Rinehart, *J. Amer. Chem. Soc.*, 1973, **95**, 2704.
[84] E. F. Elstner, D. M. Carnes, R. J. Suhadolnik, G. P. Krushmen, M. P. Schweizer, and R. K. Robins, *Biochemistry*, 1973, **12**, 4992.
[85] E. F. Elstner, R. J. Suhadolnik, and A. Allerhand, *J. Biol. Chem.*, 1973, 5385.
[86] K. Nabeta, A. Ichihara, and S. Sakamura, *J.C.S. Chem. Comm.*, 1973, **814**.
[87] F. Aragozzini, M. G. Beretta, G. S. Ricca, C. Scolastico, and F. W. Wehrli, *J.C.S. Chem. Comm.*, 1973, 788.
[88] H. Seto, L. W. Cary, and M. Tanabe, *J. Antibiotics*, 1974, **27**, 558.
[89] M. Tanabe and K. T. Suzuki, *Tetrahedron Letters*, 1974, 4417.
[90] H. Seto, L. W. Cary, and M. Tanabe, *Tetrahedron Letters*, 1974, 4491.
[91] A. G. McInnes, D. G. Smith, J. A. Walter, L. C. Vining, and J. L. C. Wright, *J.C.S. Chem. Comm.*, 1975, 66.
[92] L. L. Martin, C.-J. Chang, H. G. Floss, J. A. Mabe, E. W. Hageman, and E. Wenkert, *J. Amer. Chem. Soc.*, 1972, **94**, 8942.
[93] R. J. White and E. Martinelli, *F.E.B.S. Letters*, 1974, **49**, 233.