Chem. Soc. Rev., 1987, **16**, 123—160

Applications of Multinuclear NMR to Structural and Biosynthetic Studies of Polyketide Microbial Metabolites

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1 Introduction

The study of biosynthetic pathways received a major new impetus in the early 1970s with the advent of pulsed Fourier-transform n.m.r. spectrometers which greatly facilitated the routine determination of ^{13}C n.m.r. spectra of realistically available amounts of natural products. In addition, precursors enriched with ^{13}C and other stable isotopes were becoming more available. These were timely developments because structures had been determined increasingly by spectroscopic and other physical methods with little or no recourse to degradative chemistry with the result that classical biosynthetic studies, using radioisotopes and necessitating extensive degradative schemes to locate the position of incorporation of isotopic label, were becoming increasingly difficult. In addition, the complexity or limited available amounts of molecules that were the targets for study meant that, at best, only a partial labelling pattern might be determined. These problems were to be largely overcome by ^{13}C-labelling methods which again provided a biosynthetic technique complementary to the methods used for structure elucidation.

It should be noted that the early studies [1] using *singly* ^{13}C-labelled precursors did not provide any information which in principle at least, could not be obtained by classical radioisotope methods: they merely (!) facilitated the determination of information such as complete labelling patterns by observation of enhancements of individual ^{13}C resonances. However these studies soon led to the use of precursors *doubly* labelled with ^{13}C which *inter alia* enabled the mode of incorporation of intact biosynthetic units, and the integrity of particular carbon–carbon bonds to be established by observation of ^{13}C–^{13}C spin–spin couplings; and bond fragmentation and rearrangement processes to be detected by the loss of ^{13}C–^{13}C couplings. This in fact represented the real advance offered by ^{13}C-labelling techniques as this type of information could not be obtained, even in principle, by classical radioisotope methods.

Subsequent and equally important developments have involved the use of precursors doubly labelled with ^{13}C together with ^{18}O or ^{2}H or ^{15}N which enable the biosynthetic origins of hydrogen,[2,3] oxygen,[4] and nitrogen [2] to be determined

[1] T. J. Simpson, *Chem. Soc. Rev.*, 1975, **4**, 497.
[2] T. J. Simpson in 'Modern Methods of Plant Analysis', ed., H. F. Linskens and J. F. Jackson, Springer-Verlag, 1986, vol. 2, p. 1.
[3] C. Abell in 'Modern Methods of Plant Analysis', ed. H. F. Linskens and J. F. Jackson, Springer-Verlag, 1986, Vol. 2, 60; M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, **8**, 539.
[4] J. C. Vederas, *Can. J. Chem.*, 1982, **60**, 1637.

by observation of isotope-induced shifts (or spin coupling) in ^{13}C n.m.r. spectra; and by direct observation of the incorporation of label from ^{2}H- or ^{15}N-enriched precursors by direct ^{2}H or ^{15}N n.m.r.[5] Some limited success has also been achieved in the use of ^{17}O n.m.r. and ^{17}O-enriched precursors.[6] Besides these essentially biosynthetic techniques, n.m.r. methodology has developed to permit the rigorous assignment of spectra and of structures,[7] both of which are essential prerequisites of biosynthetic studies.

In this article the growth and development of biosynthetic studies using stable-isotope labelling methodologies over the past decade will be illustrated by a personal account of a research programme aimed at elucidating details of the biosynthesis of polyketide-derived metabolites. For this reason the work is presented in a more or less chronological order which means that certain molecules will be revisited as developments in methodology are discussed which permitted further information on their biosynthesis to be elucidated. The compounds studied are all metabolites of the lower fungi in which the requirements of high and reliable precursor incorporation rates are more easily (but not it must be emphasized always) achieved, and because the polyketide pathway is particularly characteristic of these and other microorganisms.

2 The Polyketide Biosynthetic Pathway

The polyketide pathway is one of the major routes in nature for the formation of aromatic compounds but it also produces many non-aromatic compounds many of which display biological activity as antibiotics, antitumour agents, or mycotoxins. It was first described by Birch and Donovan[8] and in common with the other pathways of secondary metabolism it can be considered in terms of a primary or assembly phase followed by a secondary or modification phase. The primary phase produces a relatively small number of compounds and is responsible for the basic unity of the pathway. However, many of these primary products can be subjected to a wide variety of modifying reactions and it is this secondary phase which is ultimately responsible for the amazing diversity of structures produced by this and indeed other pathways. A simplified picture of this which will suffice for present discussions is given in Scheme 1. In essence, varying numbers of acetate units, activated as their coenzyme A thioesters, are condensed* to form enzyme-bound 'polyketide' intermediates which then undergo stabilizing reactions, typically cyclization and aromatization, before being released from the enzyme. Thus four acetates give a tetraketide intermediate which can, for example, cyclize to produce orsellinic acid (1) which in turn can be extensively modified to produce *inter alia* the mycotoxin botryodiplodin (2) in *Penicillium roquefortii*.[9] As will be demonstrated,

* The condensation occurs *via* malonyl coenzyme A. See Scheme 21 (p. 153) for further details.

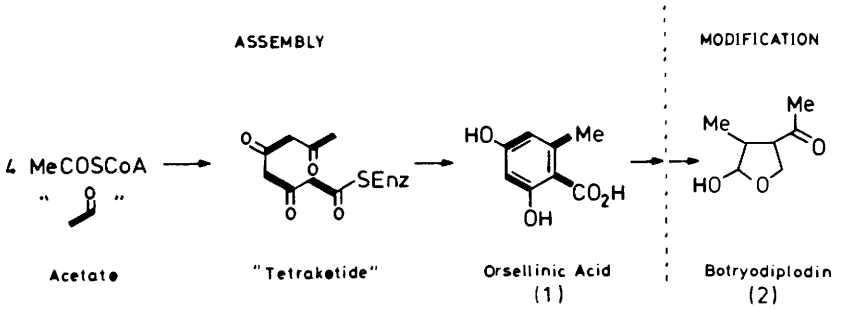
[5] K. Nanamori and J. D. Roberts, *Acc. Chem. Res.*, 1983, **16**, 35; R. L. Baxter and S. L. Greenwood, *J. Chem. Soc., Chem. Commun.*, 1986, 175.

[6] R. M. Adlington, R. T. Alpin, J. E. Baldwin, L. D. Field, E. M. M. John, E. P. Abraham, and R. L. White, *J. Chem. Soc., Chem. Commun.*, 1982, 137.

[7] R. Benn and H. Gunther, *Angew. Chem., Int. Ed. Engl.*, 1983, **22**, 350.

[8] A. J. Birch and F. W. Donovan, *Aust. J. Chem.*, 1953, **6**, 360.

[9] R. Renauld, S. Moreau, and A. Lablanche-Combier, *Tetrahedron*, 1984, **40**, 1823.



The POLYKETIDE Biosynthetic Pathway

Scheme 1

stable isotope labelling studies have enabled much invaluable information on both the assembly and modification process to be elucidated.[10]

3 ^{13}C Enrichment Studies

The labelling pattern resulting from incorporation of a ^{13}C-enriched precursor is determined by obtaining the p.n.d. ^{13}C n.m.r. spectrum of the labelled metabolite and comparing it with the spectrum of the unlabelled compound. This is illustrated in Figure 1 which illustrates the results of incorporating either [1-^{13}C]-, [2-^{13}C]-, or [1,2-$^{13}C_2$]acetate into contiguous carbons of a polyketide-derived metabolite. Normally these carbons contain only natural abundance ^{13}C (1.1%) as indicated in Figure 1a. Incorporation of a singly ^{13}C-labelled precursor will result in an increase of the ^{13}C content at a particular carbon and this manifests itself as an increase in the intensity of the signal due to that carbon in the ^{13}C n.m.r. spectrum of the enriched metabolite (Figure 1b and c). As may be seen, a severe limitation to this type of experiment is the existing ^{13}C natural abundance which as a rule necessitates precursor incorporation efficiencies which will produce a doubling of the ^{13}C content if enrichment is to be reliably observed. This means that the maximum *dilution* of label from precursor into product is *ca.* 100. These dilutions are more easily obtained in micro-organisms than in plants and it is for this reason that many of the early studies were with micro-organisms. An integral, essential, and often the most difficult part of this type of study is the rigorous assignment of the ^{13}C n.m.r. spectrum, although this aspect has tended to be underemphasized in many biosynthetic papers.

Incorporation of a precursor in which adjacent carbons are enriched with ^{13}C will lead to the observation of a ^{13}C–^{13}C spin coupling being observed in the ^{13}C n.m.r. spectrum of the enriched metabolite if the bond so labelled remains intact

[10] For regular reviews see, T. J. Simpson, *Nat. Prod. Rep.*, 1985, **2**, 321, and previous reviews in this series.

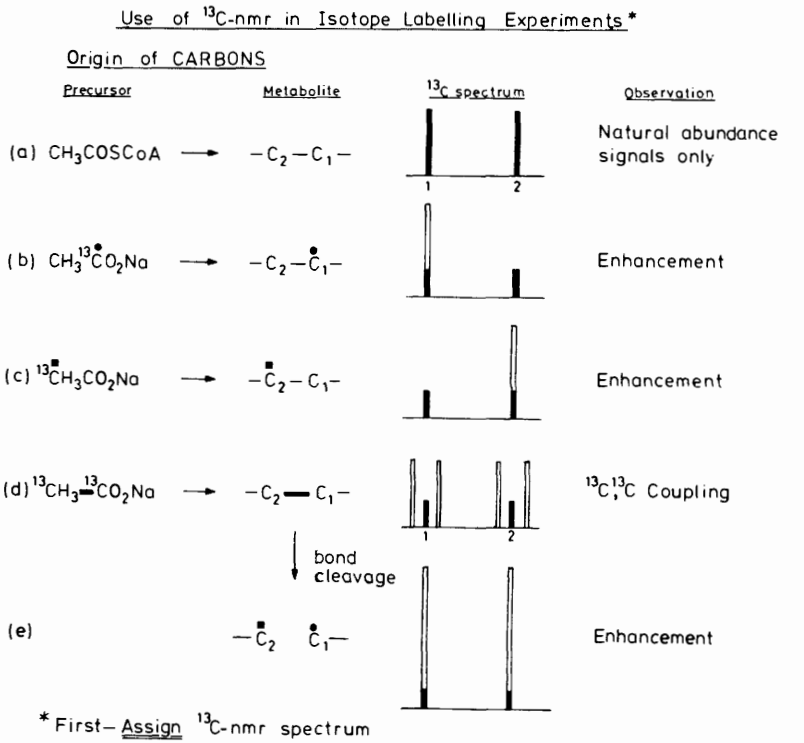


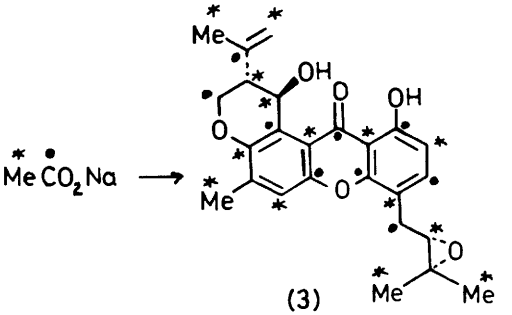
Figure 1 *Simulated p.n.d.* ^{13}C *n.m.r. spectra of a polyketide-derived moiety* (a) *at natural abundance,* (b) *enriched from* [1-^{13}C]*acetate,* (c) *enriched from* [2-^{13}C]*acetate,* (d) *enriched from* [1,2-$^{13}C_2$]*acetate, and* (e) *enriched from* [1,2-$^{13}C_2$]*acetate after bond cleavage*

throughout the biosynthetic pathway (Figure 1d). If, however, the bond is broken then the derived carbons will give rise to enhanced singlets in the final spectrum. This ability to test the integrity of carbon–carbon bonds during biosynthesis represented the first major advance offered by ^{13}C-labelling studies.[1]

4 Applications of Single and Double ^{13}C-Labelling

Aspergillus variecolor produces a group of xanthone metabolites, the major one being tajixanthone (3). This provided our first experience of the use of ^{13}C n.m.r. to identify the types of carbon present in a molecule.[11] The ^{13}C n.m.r. spectrum of tajixanthone was fully assigned on the basis of simple chemical shift and multiplicity considerations and by shift comparison studies amongst a number of

[11] K. K. Chexal, C. Fouweather, J. S. E. Holker, T. J. Simpson, and K. Young, *J. Chem. Soc., Perkin Trans. 1*, 1974, 1584.



(3)

closely related derivatives. After much thought and experimentation to work out the feeding protocols and the other requirements necessary to obtain significant ^{13}C enrichment values, incorporation of [1-^{13}C]- and [2-^{13}C]acetate into tajixanthone was achieved and from the enrichments observed in the ^{13}C n.m.r. spectra of the enriched metabolite, the origins of all the carbons in tajixanthone were deduced as shown. This enabled a biosynthetic pathway (see Scheme 9 below), *via* oxidative ring-cleavage of an anthraquinone to produce an intermediate benzophenone, to be proposed.[12] Further support for this pathway was provided by the isolation of the variecoxanthones, arugosins, and other related co-metabolites from a number of strains of *A. variecolor*.[13,14]

The spectra which result from incorporation of [1-^{13}C]- and [2-^{13}C]acetates into deoxyherqueinone (4) in *Penicillium herquei*[15] are shown in Figure 2 for the derived diacetate(11). If high enrichments are obtained, as in this case, the labelled sites are readily apparent from visual inspection of the spectrum. More often, enrichments are lower and identification of enriched sites with certainty may be more difficult.[1,2]

^{13}C N.m.r. was also used in the structural elucidation of multicolic acid (5) and related tetronic acid metabolites of *Penicillium multicolor*.[16] Their biosynthesis was studied by incorporations of singly and doubly ^{13}C-labelled acetate to provide one of the earliest applications of this then recently described technique.[17] The observations of ^{13}C–^{13}C couplings and more significantly their absence on the ^{13}C resonances of certain carbons led to the proposal that the tetronic acids were biosynthesized *via* oxidative cleavage of an intermediate containing a benzenoid ring (Scheme 2). These proposals were subsequently confirmed by the incorporation of 6-pentyl-resorcylic acid (6),[18] and from ^{18}O-labelling studies.[19]

[12] J. S. E. Holker, R. D. Lapper, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2135.
[13] J. S. E. Holker, K. Young, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1975, 543.
[14] K. K. Chexal, J. S. E. Holker, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1975, 549.
[15] T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1975, 258.
[16] J. A. Gudgeon, J. S. E. Holker, and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1974, 636.
[17] H. Seto, L. Cary, and M. Tanabe, *J. Chem. Soc., Chem. Commun.*, 1973, 867; H. Seto, T. Sato, and M. Tanabe, *J. Am. Chem. Soc.*, 1973, **95**, 8461.
[18] J. A. Gudgeon, J. S. E. Holker, T. J. Simpson, and K. Young, *Bioorg. Chem.*, 1979, **8**, 311.
[19] J. S. E. Holker, E. O'Brien, R. N. Moore, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1983, 192.

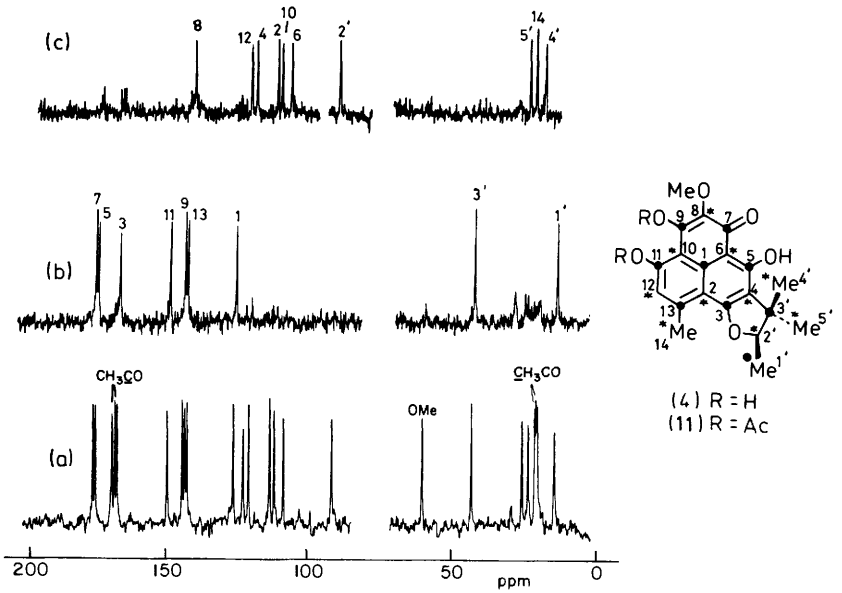
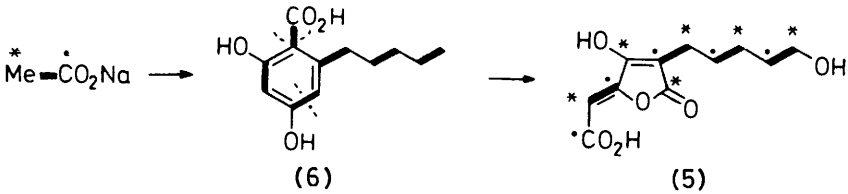


Figure 2 15.04 MHz *p.n.d.* ^{13}C *n.m.r. spectra of deoxyherqueinone diacetate* (a) *at natural abundance, and enriched from* (b) [1-^{13}C]acetate (●) (c) [2-^{13}C]*acetate* (✱)

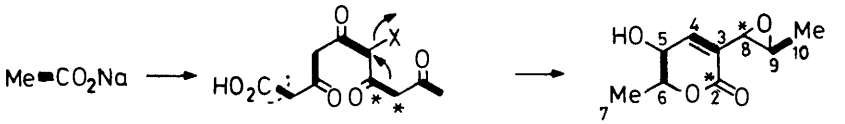


Scheme 2

Incorporation of [$^{13}C_2$]acetate was next applied to the biosynthesis of aspyrone (7) a metabolite of *Aspergillus melleus*. The resultant labelling pattern (Scheme 3) suggested that its biosynthesis was either *via* a ring cleavage pathway or by rearrangement.[20] The rearrangement pathway shown was supported by the observation of a two-bond ^{13}C–^{13}C coupling of 6.2 Hz between C-2 and C-8 in the ^{13}C n.m.r. spectrum (Figure 3) of [$^{13}C_2$]acetate-enriched aspyrone.[21] This was the first observation of such a coupling in biosynthetic studies.

At about this time the idea was conceived that the involvement of a symmetrical

[20] T. J. Simpson, *Tetrahedron Lett.*, 1975, 175.
[21] T. J. Simpson and J. S. E. Holker, *Tetrahedron Lett.*, 1975, 4693; *J. Chem. Soc., Perkin Trans. 1*, 1981, 1397.



(7)

Scheme 3

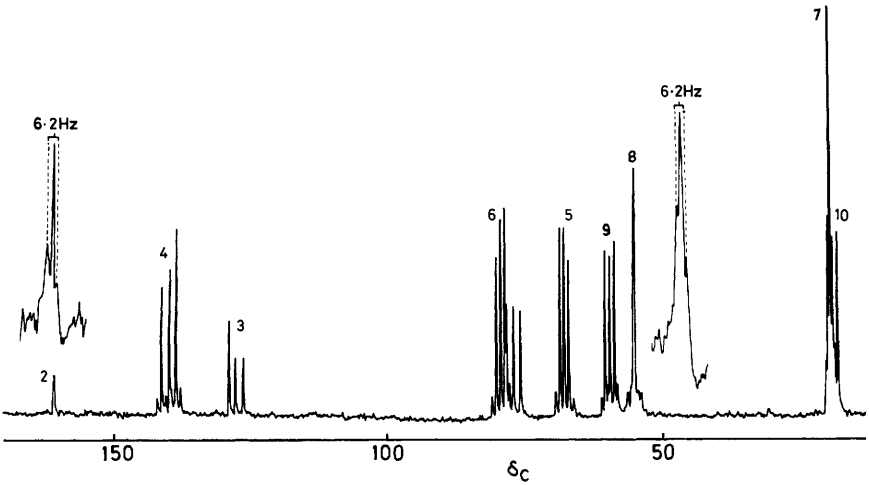
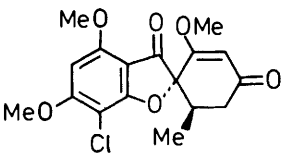
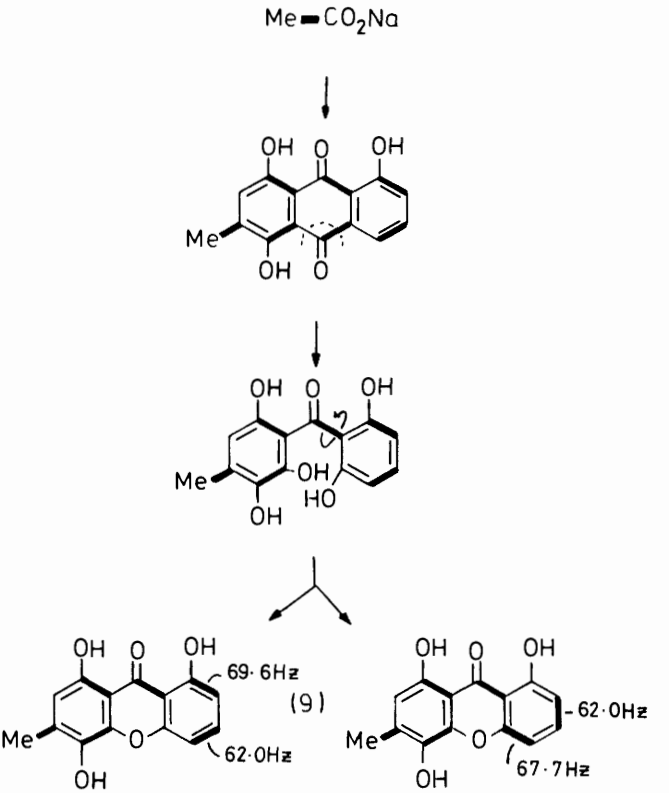


Figure 3 25.2 MHz *p.n.d.* ^{13}C *n.m.r. spectrum of aspyrone* (7) *enriched from* [1,2-$^{13}C_2$]*acetate*



(8)

intermediate at any stage in a biosynthetic pathway would result in a randomization of labelling, *e.g.* of ^{13}C–^{13}C spin couplings if a double ^{13}C-labelled precursor were used. Griseofulvin (8) was chosen as a model to test this hypothesis and [$^{13}C_2$]acetate was incorporated using a high-yielding commercial strain of *Penicillium patulum*. However the results obtained in this study turned out to be very complicated. Rapid metabolic turnover of exogenous acetate resulted in



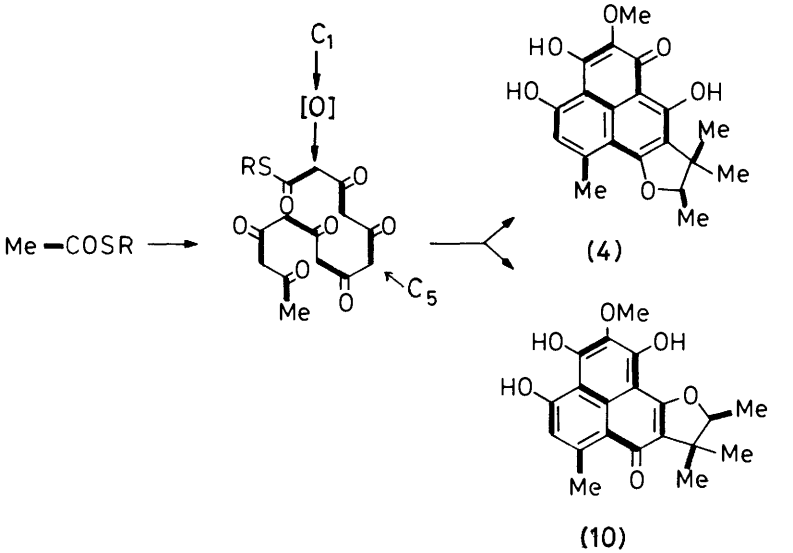
Scheme 4

multiple labelling of individual molecules and therefore the observation of extensive *inter*-acetate and long range ^{13}C–^{13}C couplings in addition to the desired *intra*-acetate couplings. In fact the most efficient route for incorporation of label from acetate was *via* the C_1-pool into the methoxyl carbons.[22]

However the hypothesis was soon proved to be correct when a study on the xanthone metabolite ravenelin (9) was carried out. Incorporation of [$^{13}C_2$]acetate into ravenelin in cultures of *Helminthosporium ravenelii* resulted in the predicted randomization of ^{13}C–^{13}C couplings in ring-C consistent with the intermediacy of a symmetrical benzophenone intermediate (Scheme 4), itself derived from cleavage of an anthraquinone.[23] This type of information has subsequently found extensive use in biosynthetic studies.

In this study the ^{13}C n.m.r. spectrum of ravenelin was partially assigned by

[22] T. J. Simpson and J. S. E. Holker, *Phytochemistry*, 1977, **16**, 229.
[23] A. J. Birch, T. J. Simpson, and P. W. Westerman, *Tetrahedron Lett.*, 1975, 4173; A. J. Birch, J. Baldas, J. R. Hlubucek, T. J. Simpson, and P. W. Westerman, *J. Chem. Soc., Perkin Trans. 1*, 1976, 898.



Scheme 5

analysis of long-range ^{1}H–^{13}C couplings in the fully ^{1}H-coupled ^{13}C n.m.r. spectrum, a technique which was to form the basis of many future studies. It remains one of the best but under-utilized methods for both structural elucidation and spectral assignment studies (see below). This was exemplified by a study of deoxyherqueinone (4) and herqueichrysin (10), phenalenone metabolites of *Penicillium herquei*.[24] The aromatic ring system of these metabolites could be derived *a priori* by any one of three foldings of a heptaketide precursor or by numerous possible multi-chain condensations. Deoxyherqueinone was isolated and purified as its diacetate derivative. Due to the tautomeric possibilities in these hydroxyphenalenone structures the actual structure of this diacetate was uncertain. However, analysis of the long range ^{1}H–^{13}C couplings by selective ^{1}H irradiation and D_2O exchange experiments simultaneously defined the precise structure (11), (Figure 4) and simultaneously produced an unambiguous spectral assignment. Similar studies defined the structure of herqueichrysin. Subsequent incorporation experiments with singly and doubly ^{13}C-labelled acetates and malonate and analysis of the resultant enrichments and ^{13}C–^{13}C couplings indicated formation of the phenalenone ring system as shown in Scheme 5 by a specific folding of a single heptaketide precursor.

Another study finally established the structure of phomazarin (12), an aza-anthraquinone, produced by the plant pathogen *Phoma terrestris*, for which numerous structures had been proposed since its original isolation in 1940. The exact structures of phomazarin and its co-metabolite isophomazarin (13), including

[24] T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 1233.

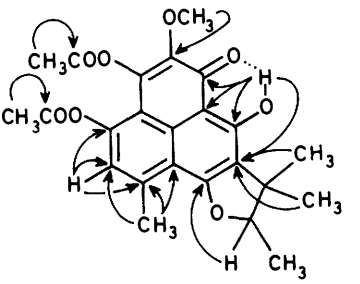
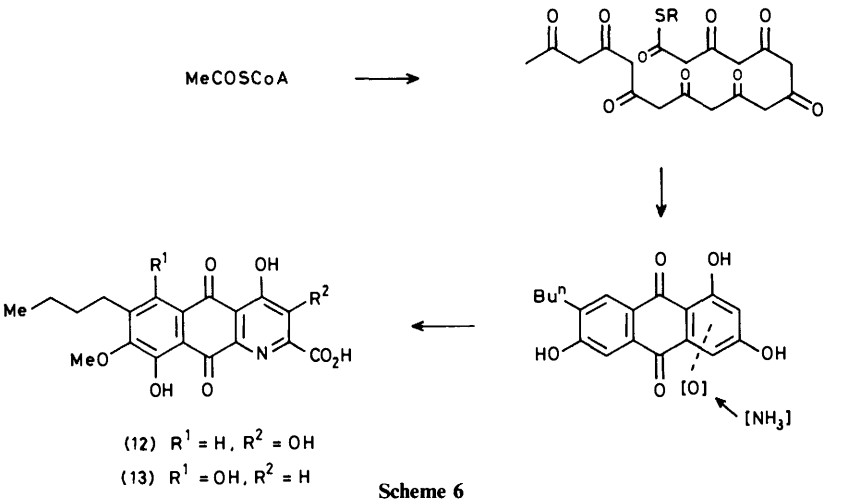


Figure 4 *Long range* ^{1}H–^{13}C *spin–spin couplings observed in the fully* ^{1}H *coupled* 15.04 MHz ^{13}C *n.m.r. spectrum of deoxyherqueinone diacetate* (11)



(12) $R^1 = H$, $R^2 = OH$

(13) $R^1 = OH$, $R^2 = H$

Scheme 6

the tautomeric form of the 4-hydroxypyridine ring were established using ^{1}H–^{13}C couplings and in particular ^{15}N chemical shifts (by INDOR), ^{1}H–^{15}N, and ^{15}N–^{13}C couplings in derivatives of biosynthetically ^{15}N-enriched phomazarin.[25,26] Subsequent labelling studies with ^{13}C-labelled acetates and malonate showed that phomazarin was biosynthesized *via* oxidative ring fission of an anthraquinonoid intermediate itself formed by a specific folding and condensation of a single nonaketide precursor rather than the two-chain pathways that had been previously proposed (Scheme 6).[27] This study helped to confirm a suspicion that whenever a two-chain pathway is proposed in polyketide biosynthesis the

[25] A. J. Birch, D. N. Butler, R. E. Effenberger, R. W. Rickards, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 807.

[26] R. E. Effenberger and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 823.

[27] A. J. Birch and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 816.

possibility, however remote, of formation *via* a single-chain modification should not be excluded. Studies on multicolic acid, aspyrone, ravenelin, and tajixanthone all exemplify this.

5 Monitoring of ^{2}H and ^{18}O by n.m.r.

The studies described above mainly concerned determining the origins of the carbon skeletons of metabolites. However, in studying the nature of the intermediates on a biosynthetic pathway and in particular elucidating the detailed mechanisms of their interconversions, it is essential to determine the biosynthetic origins and fate of the hydrogen and oxygen atoms. Studies in recent years have been more concerned with these aspects. Deuterium incorporation can be monitored directly by ^{2}H n.m.r.[3] or indirectly from isotope-induced shifts in ^{13}C n.m.r.[2,3] (see below). These methods are essentially complementary.

^{2}H n.m.r. despite several inherent disadvantages has been the nucleus of choice in many biosynthetic studies. Its major limitations are mainly as a consequence of the low magnetogyric ratio, and the relaxation behaviour of the ^{2}H nucleus. Because it is a quadrupole nucleus (spin 1) and thus very efficiently relaxed, the spectral lines are rather broad and this, coupled with the low magnetogyric constant and the small chemical shift range for hydrogen nuclei, often results in poorly resolved spectra. However, the rapid relaxation and lack of any n.O.e. mean that accurate integration of ^{2}H n.m.r. spectra is possible so that the relative enrichment at different sites in a metabolite can be accurately assessed. Another major advantage is that as a consequence of its low natural abundance (0.012%) much greater dilutions are tolerable than in the case of ^{13}C-labelling: a 100% ^{2}H-labelled precursor may be diluted 6 000 fold and still result in a doubling of intensity over the corresponding natural abundance signal. This makes ^{2}H-labelling particularly suitable for studying the incorporation of advanced intermediates on a biosynthetic pathway.

The inherent lack of resolution in ^{2}H n.m.r. can be overcome by the use of isotope-induced shifts in ^{13}C n.m.r. The use of ^{13}C as a 'reporter' nucleus for both hydrogen and oxygen represents the great recent advance in biosynthetic studies with stable isotopes and makes use of the observation that substitution of a proton *alpha* or *beta* to a ^{13}C by deuterium causes a change (usually upfield) in the ^{13}C chemical shift. Similarly the presence of ^{18}O alpha to a ^{13}C atom can be detected by an upfield shift in the ^{13}C n.m.r. spectrum. These effects are summarized in Figure 5. When the deuterium label is directly attached to a ^{13}C nucleus in the precursor molecule, the p.n.d. ^{13}C n.m.r. spectrum of the enriched metabolite shows, for carbons which have retained deuterium label, a series of signals upfield of the normal resonance.[3] The presence of each deuterium shifts the centre of the resonance by 0.3—0.6 p.p.m. and spin–spin coupling ($^{1}J_{CD}$) produces a characteristic multiplet, hence CD appears (Figure 5a) as a triplet whereas CD_2 and CD_3 would give respectively a quintet and septet. Shifted signals arising from carbons which bear no hydrogen suffer reduced signal-to-noise ratio caused by poor relaxation and lack of n.O.e. enhancement, a disadvantage of the method

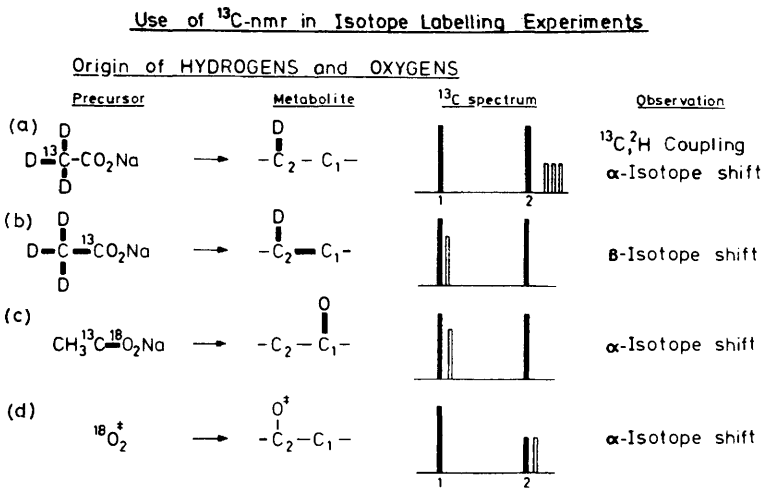


Figure 5 *Simulated p.n.d.* ^{13}C *n.m.r. spectra of a polyketide-derived moiety enriched from* (a) [2-^{13}C,$^{2}H_3$]*acetate*, (b) [1-^{13}C,$^{2}H_3$]*acetate*, (c) [1-^{13}C,$^{18}O_2$]*acetate, and* (d) $^{18}O_2$ *gas*

which is compounded by the multiplicities due to coupling. Deuterium decoupling[28] can assist in this by removing the ^{13}C–^{2}H coupling. However, information not obtainable by direct ^{2}H n.m.r. spectroscopy, such as the distribution of label as CH_2D, CHD_2, and CD_3 and the integrity of carbon–hydrogen bonds during biosynthesis, may be gained.

More recently a pulse sequence has been described which allows the selective observation of deuterated ^{13}C signals by selective suppression of signals from protonated carbons.[29] This makes the technique more sensitive but like the simultaneous proton and deuterium decoupling method requires instrumentation and expertise which are not widely available.

Many of the problems associated with directly attached deuterium are avoided by placing the deuterium label two bonds away from the ^{13}C reporter nucleus.[30] The isotope shift, although reduced, is still observable, and as β-hydrogens only contribute markedly to the relaxation of non-protonated ^{13}C nuclei, the shifted signals otherwise retain any n.O.e. also experienced by the unshifted signals on proton decoupling. As geminal carbon–proton coupling constants are generally small anyway,[31] and carbon–deuteron couplings are over six times smaller again, the shifted signals are effectively singlets (Figure 5b), even without deuterium

[28] C. R. Hutchinson, I. Kurobane, C. T. Mabuni, R. W. Kumola, I. G. McInnes, and J. A. Walter, *J. Am. Chem. Soc.*, 1981, **103**, 2474.
[29] D. M. Doddrell, J. Staunton, and E. D. Laue, *J. Chem. Soc., Chem. Commun.*, 1983, 602.
[30] C. Abell and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 856.
[31] J. L. Marshall, 'Carbon–Carbon and Carbon–Proton NMR Couplings', Verlag Chemie International, Deerfield Beach, 1983.

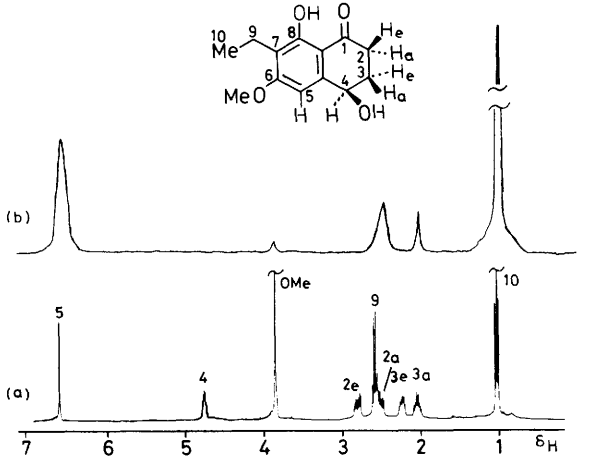


Figure 6 (a) 360.13 MHz ^{1}H *n.m.r. spectrum of* O-*methylasparvenone* (14), (b) 55.28 MHz ^{2}H *n.m.r. spectrum of* (14) *enriched from* [$^2H_3$]*acetate*

decoupling, and this gives a further increase in the signal-to-noise ratio compared with the corresponding α-shift experiment.

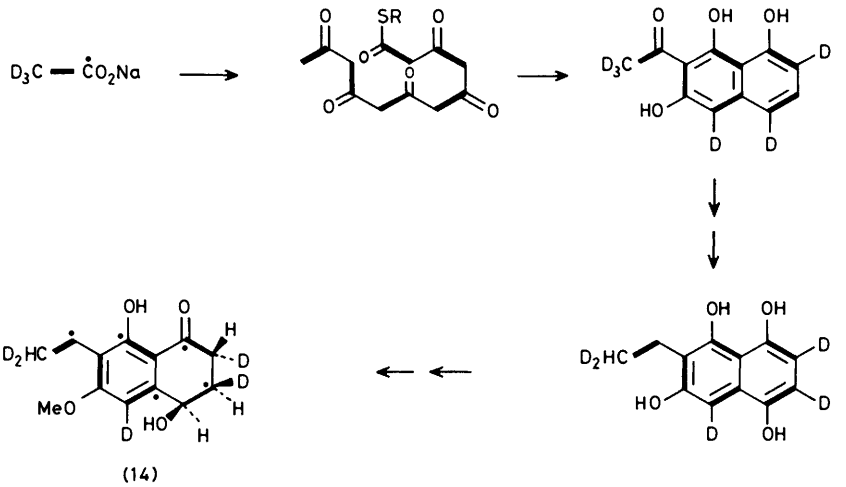
However, neither of these methods provides reliable information on the stereospecificity of deuterium labelling. Although ^{2}H n.m.r. spectra are disadvantaged by their inherently low dispersion and broad lines, they do have the advantage of providing information on the stereospecificity as well as regiospecificity of labelling. ^{2}H N.m.r. however does not prove the number of deuteriums incorporated.

At about the same time, the first biosynthetic application of ^{18}O isotope-induced shifts in ^{13}C n.m.r. was reported,[32] as shown in Figure 5; the ^{18}O may be conveniently introduced *via* a doubly labelled precursor or by growth in an $^{18}O_2$ atmosphere. The resulting shifts are generally not much larger than 0.05 p.p.m. These are very small effects and are the same general size as β-^{2}H isotope shifts and are only readily observed with high field spectrometers. These techniques for elucidating the origins of hydrogen and oxygen provided the basis for much of the work described below.

6 Applications of ^{2}H and ^{18}O Labelling

Incorporations of singly and doubly ^{13}C-labelled acetates confirmed the formation of *O*-methylasparvenone (14) from specific folding and condensation of a hexaketide precursor in *Aspergillus parvulus*. ^{2}H N.m.r. analysis (Figure 6) of the [$^2H_3$]acetate-enriched metabolite showed that ^{2}H label was incorporated specifically into the 10-methyl, 5-, 2-axial, and 3-axial hydrogens with none at C-4. This indicated that oxygen is introduced at C-4 by an aromatic hydroxylation

[32] J. C. Vederas and T. T. Nakashima, *J. Chem. Soc., Chem. Commun.*, 1980, 183.



Scheme 7

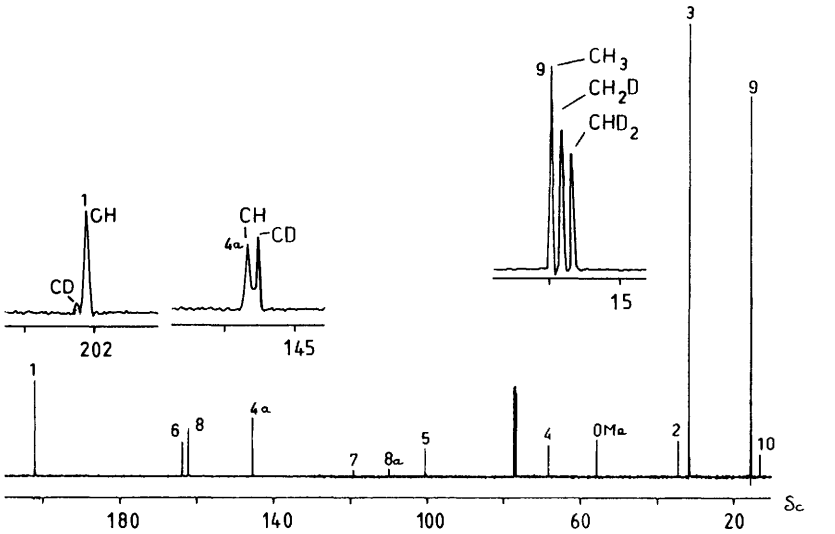


Figure 7 90.56 MHz *p.n.d.* ^{13}C *n.m.r. spectrum of* O-*methylasparvenone enriched from* [1-^{13}C,$^{2}H_3$]*acetate*

process with an accompanying NIH shift of hydrogen from C-4 to C-3 and that reduction from the naphthalene to the dihydroaromatic level occurs with stereospecific *trans* introduction of hydrogen at C-2 and C-3 (Scheme 7).[33] In a

[33] T. J. Simpson and D. J. Stenzel, *J. Chem. Soc., Chem. Commun.*, 1981, 239.

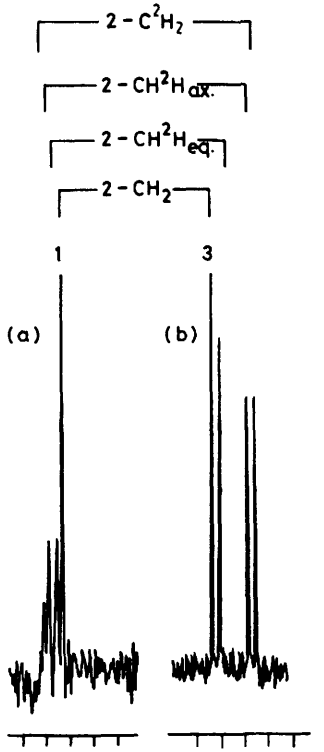
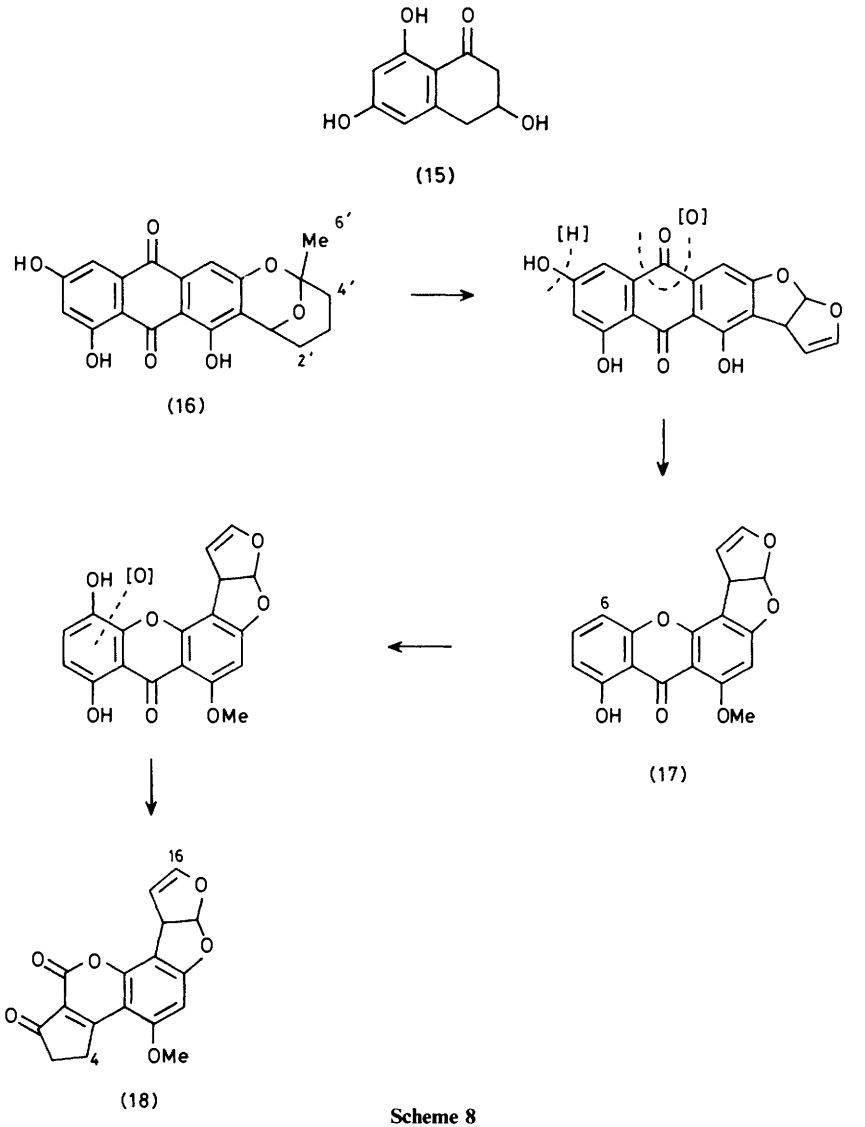


Figure 8 *Signals from the* 90.56 MHz *p.n.d.* ^{13}C *n.m.r. spectrum of* O-*methylasparvenone* (14) *partially deuteriated at* C-2. *Resonances are for* (a) C-1 *and* (b) C-3

further study,[34] incorporation of [1-^{13}C,$^{2}H_3$]acetate and analysis of the ^{2}H β-isotope shifts in the resultant ^{13}C n.m.r. spectrum (Figure 7) showed that one hydrogen was lost from the C-10 methyl to indicate formation of the ethyl moiety by a reduction–elimination–reduction sequence on the corresponding acetyl group. The magnitude and direction of the β-isotope shifts were observed to depend markedly on the functionality of the reporter ^{13}C nucleus and surprisingly on the stereospecificity of ^{2}H incorporation. This was confirmed by an *in vitro* experiment when the C-2 methylene hydrogens were exchanged in equimolar MeOH and MeO^{2}H to give the spectrum shown in Figure 8. For carbonyl groups the observed shift could be *downfield* or even *zero* in contrast to the usually observed *upfield* shifts, thus indicating the necessity for caution in the interpretation of results when carbonyl groups are involved. The incorporation of ^{2}H label from [$^{2}H_3$]acetate into the similar dihydronaphthalene scytalone (15) in *Phialaphora lagerbergii* was

[34] T. J. Simpson and D. J. Stenzel, *J. Chem. Soc., Chem. Commun.*, 1982, 1074.



Scheme 8

also studied by ^2H n.m.r. In contrast to *O*-methylasparvenone, reduction of the aromatic ring was not stereospecific.[35]

^2H-Labelling has also been applied to good effect in studies on the biosynthesis of aflatoxin B_1. Although averufin (16) was generally held to be an early

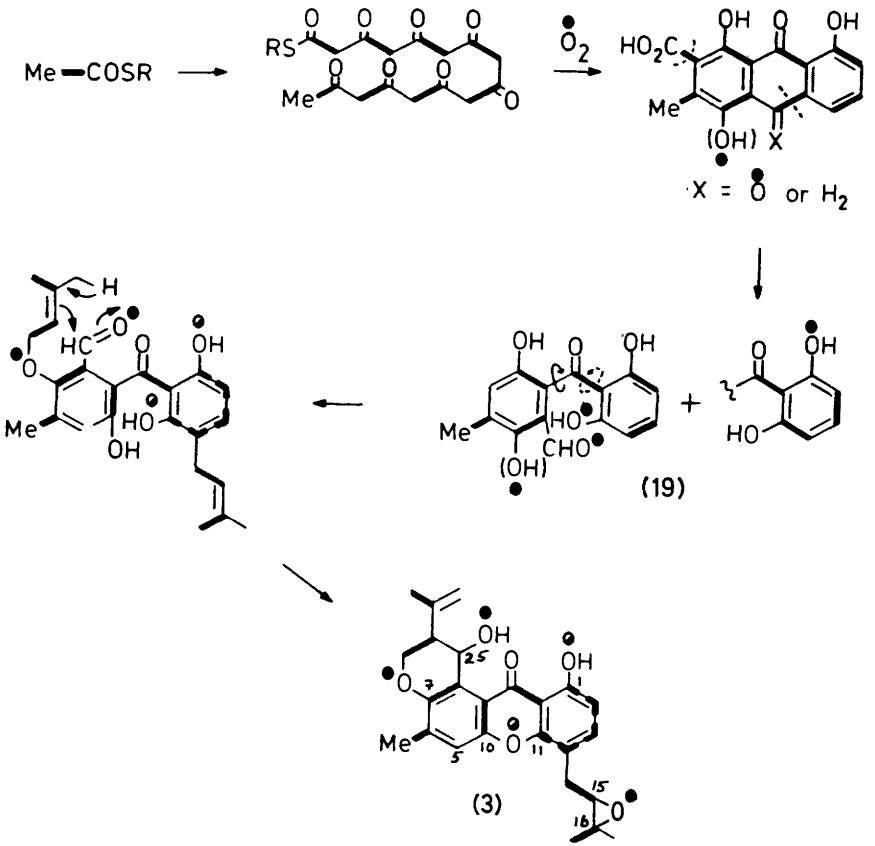
[35] E. Bardshiri and T. J. Simpson, *Tetrahedron*, 1983, **39**, 3539.

intermediate on the biosynthetic pathway to aflatoxin B_1 (18), this had never been rigorously established. Accordingly, [4′-$^2H_2$]averufin was prepared and incorporated into aflatoxin B_1 by cultures of *Aspergillus flavus*. ^2H N.m.r. analysis showed that ^2H label was incorporated specifically at C-16 of aflatoxin B_1.[36] The incorporation of ^2H from [1-^{13}C,$^2H_3$]acetate into averufin, sterigmatocystin (17) and aflatoxin B_1 was studied both by direct ^2H n.m.r. analysis and by observation of β-isotope shifts. The results showed that one ^2H was incorporated stereospecifically into the C-2′ and C-4′ positions of the side chain of averufin[37] and that these are retained on conversion of the C_6 side chain into the C_4-bisfuranoid side chain of the aflatoxins.[38] Other important observations included the retention of ^2H label at C-6 of sterigmatocystin, so ruling out previously proposed mechanisms for xanthone ring formation requiring the introduction of a phenolic hydroxyl group on this carbon.[39] However the appearance of ^2H at C-4 of aflatoxin B_1 shows that such a hydroxylation with accompanying NIH shift does occur in the biosynthesis of aflatoxin B_1. These results are in accord with the biosynthetic sequence shown in Scheme 8.

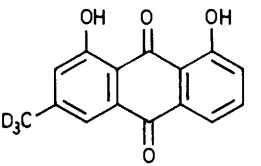
Further studies were carried out on tajixanthone. Incorporations of [$^{13}C_2$]-acetate and [$^2H_3$]acetate gave the results summarized in Scheme 9. The absence of ^2H label on C-25 and C-5 indicated that cleavage of an anthraquinone rather than an anthrone intermediate occurred and that decarboxylation of the octaketide precursor occurs after cyclization and aromatization. The observed scrambling of ^{13}C–^{13}C couplings in ring C implies the involvement of a symmetrical benzophenone intermediate (19) which in turn means that ring cleavage of the anthraquinone precursor must precede introduction of the *C*-prenyl residue, *cf.* ravenelin. The stereospecificity of labelling in the dihydropyran ring, however, suggests its formation from an *O*-prenylaldehyde intermediate by a *concerted* 'ene' reaction.[40]

In order to obtain information on the mechanism of xanthone ring-closure tajixanthone was isolated from *A. variecolor* growing under an atmosphere containing $^{18}O_2$ using the closed system shown in Figure 9 which allows the oxygen pressure to be kept constant and the oxygen uptake to be monitored. The ^{18}O isotope shifts observed in the ^{13}C n.m.r. spectrum are shown in Figure 10. The intensities of the isotopically shifted signals for carbons 1, 10, and 11 are half of those for the other shifted signals and so are consistent with the intermediacy of a symmetrical benzophenone and a ring-closure mechanism *via* a Michael addition–elimination process in which a ring C hydroxyl attacks ring A.[41] Mass spectral analysis of tajixanthone produced in an $^{18}O_2$, $^{16}O_2$ mixture also showed that each aerobically derived oxygen atom was derived separately by mono-oxygenation so that previously proposed mechanisms *via* dioxygenases can be ruled out. In a more recent study, [*methyl*-H_3]chrysophanol (20) has been shown to be a specific

[36] T. J. Simpson, A. E. de Jesus, P. S. Steyn, and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1982, 631.
[37] T. J. Simpson, A. E. de Jesus, P. S. Steyn, and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1982, 632.
[38] T. J. Simpson, A. E. de Jesus, P. S. Steyn, and R. Vleggaar, *J. Chem. Soc., Chem. Commun.*, 1983, 338.
[39] T. J. Simpson and D. J. Stenzel, *J. Chem. Soc., Chem. Commun.*, 1982, 890.
[40] E. Bardshiri and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1981, 195.
[41] E. Bardshiri, C. R. McIntyre, T. J. Simpson, R. N. Moore, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1984, 1404.



Scheme 9



(20)

precursor for tajixanthone. ^{2}H N.m.r. analysis (Figure 11) showed that specific incorporation of label into the aromatic methyl of (3) had occurred.[42]

[42] S. A. Ahmed, E. Bardshiri, and T. J. Simpson, unpublished work.

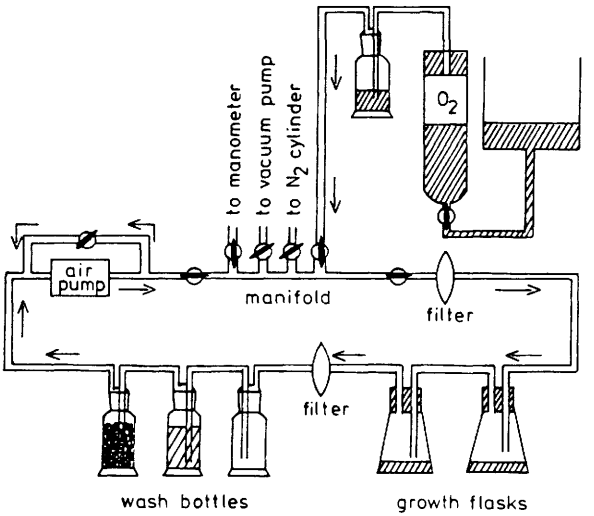


Figure 9 *Apparatus for growth of fungal cultures in an* $^{18}O_2$ *atmosphere. The wash bottles are arranged so that the first acts as a suck-back trap, the second contains* 5 M KOH *to absorb* CO_2 *produced by the cultures and the third contains cotton wool to remove any alkaline spray*

The work described above on *O*-methylasparvenone was initiated due to an interest in the biosynthesis of polyketide-derived molecules containing an ethyl side chain. This also promoted an investigation of LL-D253α, a chromanone first isolated from *Phoma pigmentivora* and subsequently from several other plant pathogens. In the course of ^{13}C assignment studies it became apparent that the previously assigned structure (21) was incorrect and analysis of the fully ^{1}H coupled ^{13}C n.m.r. spectrum (Figure 12) of the diacetate (23) identified the long range ^{1}H–^{13}C couplings indicated in Figure 13. This effectively defined the structure (22) for LL-D253α which was subsequently confirmed by unambiguous synthesis.[43] Its biosynthesis has been studied by incorporation of ^{13}C-, ^{2}H-, and ^{18}O-labelled acetates and the resulting labelling patterns are summarized in Scheme 10.[43] A particularly interesting feature was the *partial* randomization of label from singly ^{13}C-labelled acetates between C-10 and C-11 in the hydroxyethyl side chain. On incorporation of [1-^{13}C,$^{2}H_3$]acetate two ^{2}H atoms were incorporated at both C-10 and C-11 and only one at C-3. Taken along with the ^{18}O labelling (Scheme 10) this indicated that the chromanone ring was formed by conjugate addition of a phenolic hydroxyl to the corresponding αβ-unsaturated ketone. As LL-D253α is optically active the ring-closure is stereospecific with respect to C-2, but ^{2}H n.m.r. analysis showed that both hydrogens at C-3 were

[43] C. R. McIntyre and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1984, 704; C. R. McIntyre, T. J. Simpson, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1984, 706.

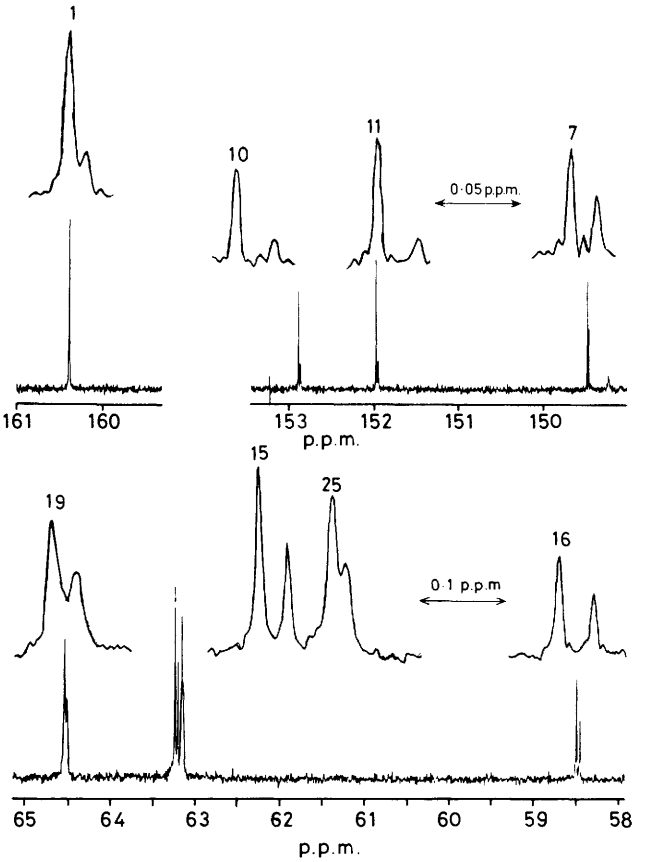
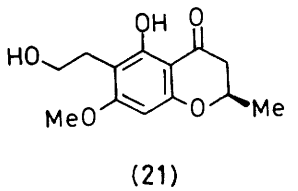
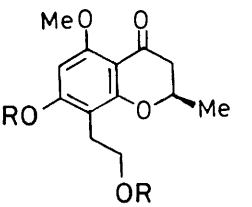


Figure 10 *Sections of the* 90.56 MHz *p.n.d.* ^{13}C *n.m.r. spectrum of tajixanthone* (3) *labelled by* $^{18}O_2$ *gas*



(21)



(22) R = H

(23) R = Ac

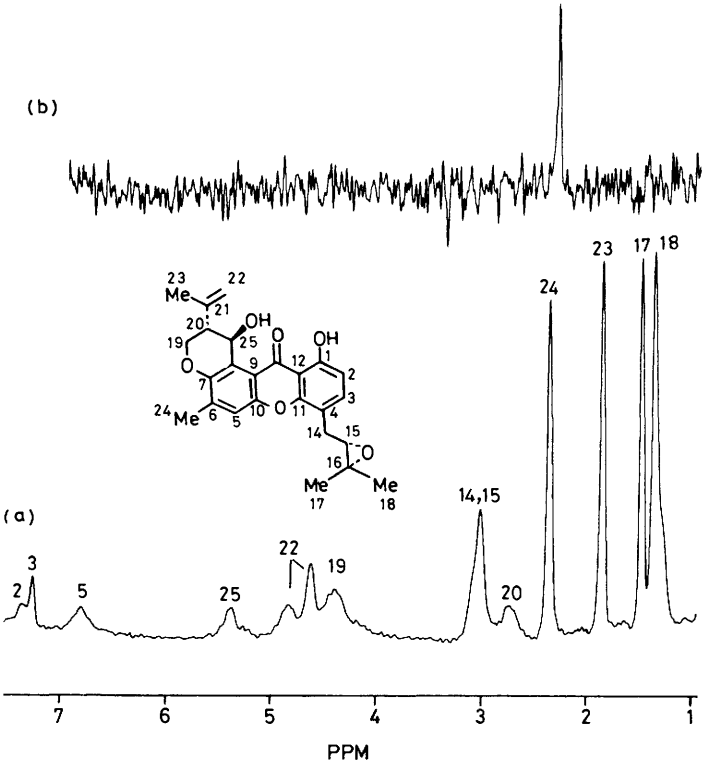


Figure 11 55.28 MHz ^2H *n.m.r. spectra of tajixanthone* (3) (a) *produced in a culture medium supplemented with* 5% ^2H_2O, (b) *labelled from feeding* [**m**ethyl-$^2H_3$]*chrysophanol* (20)

labelled to an equal extent so that protonation of the intermediate enolate must occur with equal facility from both faces as indicated in Scheme 11. This contrasts with the corresponding chalcone to flavanone ring-closure which is known to be stereospecific with respect to both positions. LL-D253α must be biosynthesized *via* two preformed polyketide chains. One possibility is shown in Scheme 12. The observed randomization of labelling in 80% of the molecules is accounted for by formation of a symmetrical cyclopropyl intermediate (24) as shown. This intermediate can undergo hydrolytic ring-opening at either the α or β carbon. According to this scheme the 20% of the molecules *not* undergoing randomization should have the 11-hydroxyl derived from the atmosphere and in accord with this, fermentation in an $^{18}O_2$ atmosphere resulted in an ^{18}O isotope shift being observed on the resonance due to C-11 in the ^{13}C n.m.r. spectrum, the intensity of the shifted peak being *ca.* 20% that of the unshifted peak. It is not clear whether the randomization is an *in vivo* or an *in vitro* process.

The success of these methods in revealing subtle biosynthetic information

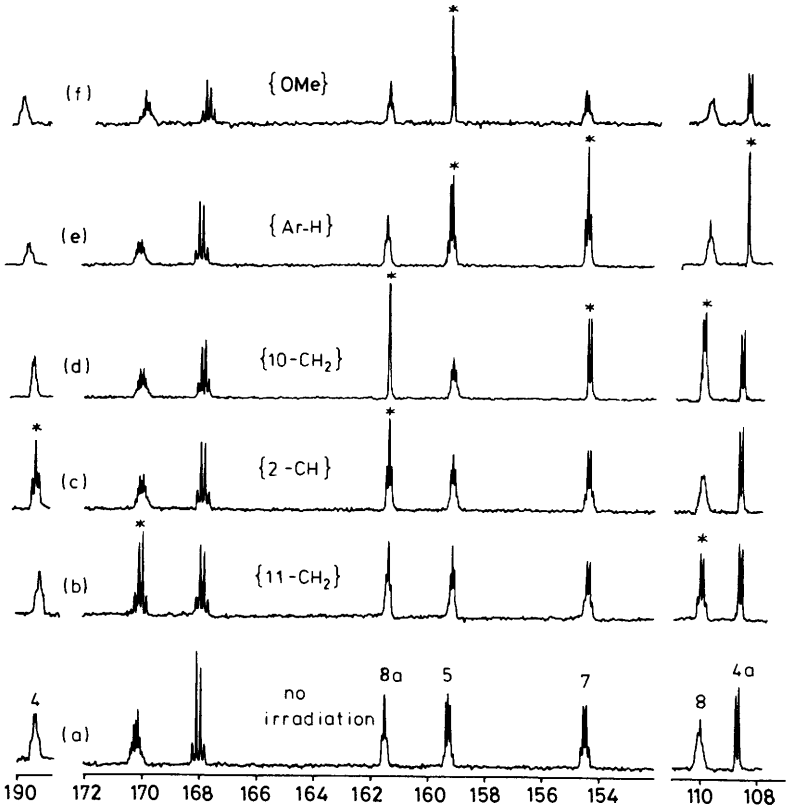


Figure 12 *The high frequency region of the fully* ^{1}H*-coupled* 200 MHz ^{13}C *n.m.r. spectrum of* LL-D253α *diacetate* (23), *and results of selective low-power* ^{1}H *decoupling experiments. Positions where decoupling is observed are indicated* (✱)

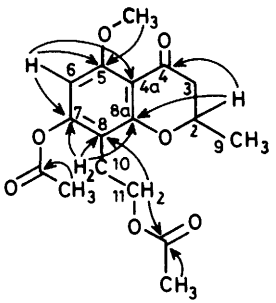
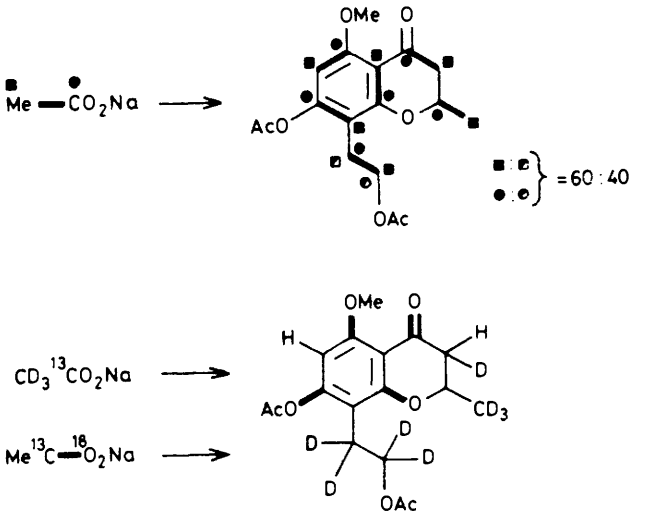
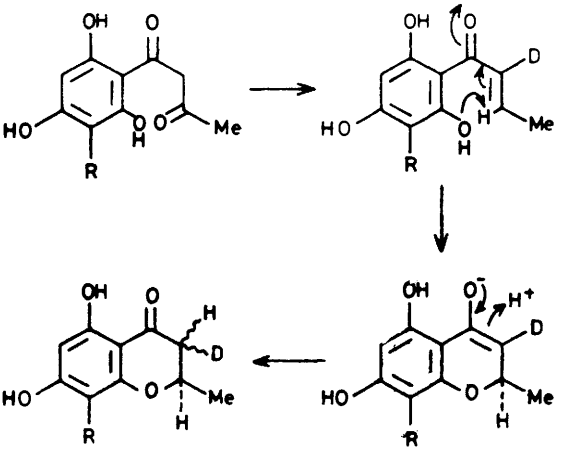


Figure 13 *Long-range* ^{1}H–^{13}C *couplings in* LL-D253α *diacetate* (23) *selectively removed by low-power* ^{1}H *decoupling experiments shown in Figure* 12



Scheme 10

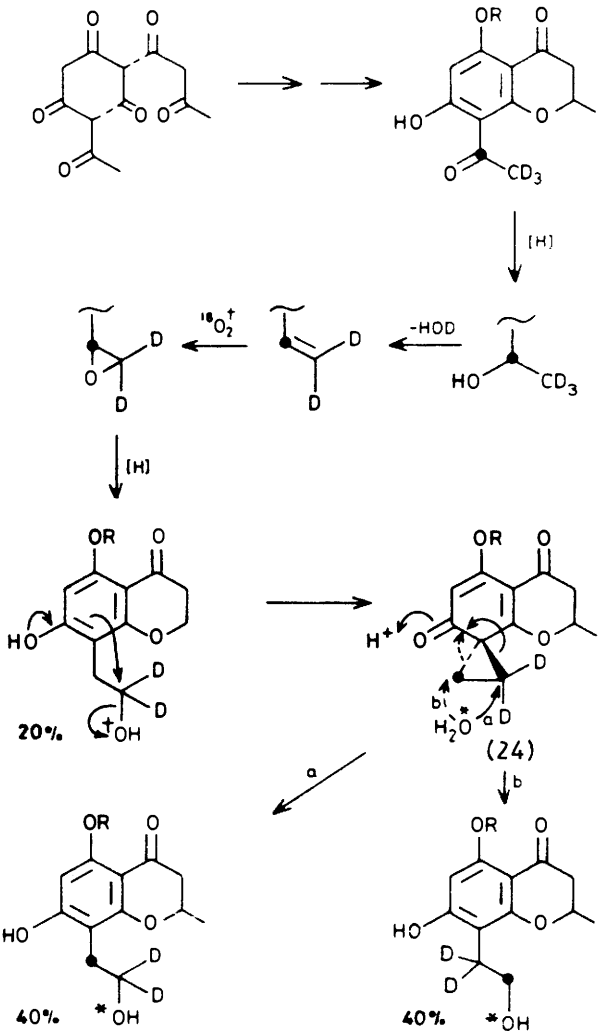


Scheme 11

encouraged a re-examination of the formation of aspyrone (7). Experiments with [1-^{13}C,$^{18}O_2$]acetate and $^{18}O_2$ revealed the surprising result that *none* of the oxygens were derived from acetate, three being derived from the atmosphere and one from the medium.[44] As indicated in Figure 14, the lactone carbonyl carbon C-2 showed shifts due to the presence of an aerobically derived ^{18}O oxygen in either the

[44] S. A. Ahmed, T. J. Simpson, J. Staunton, A. C. Sutkowski, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1985, 1685.

Figure 14 ^{18}O *Isotope-induced shifts observed in the* 100.6 MHz *p.n.d.* ^{13}C *n.m.r. spectrum of aspyrone* (7) *labelled by* $^{18}O_2$ *gas*



Scheme 12

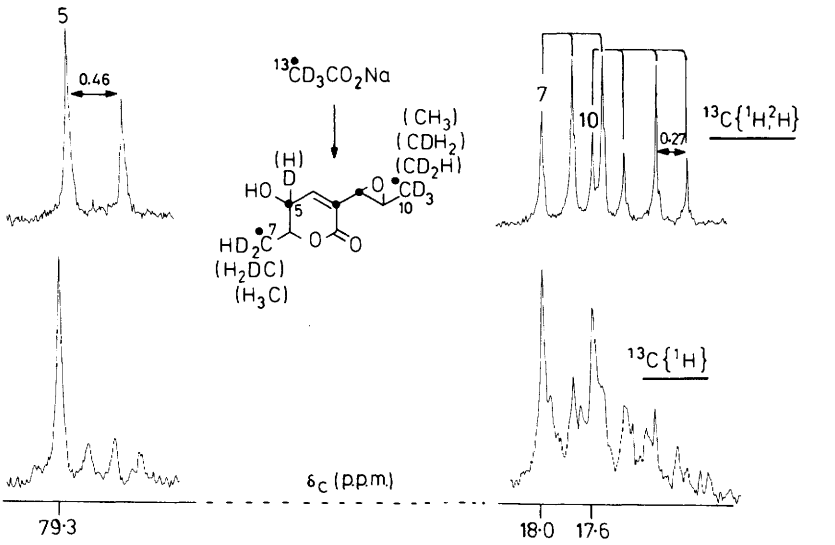


Figure 15 *α* ^{2}H *Isotope-induced shifts observed in the* 100.6 MHz ^{1}H *and* ^{2}H *noise decoupled* ^{13}C *n.m.r. spectrum of aspyrone* (7) *labelled from* [2-^{13}C,^{2}H$_3$]*acetate*

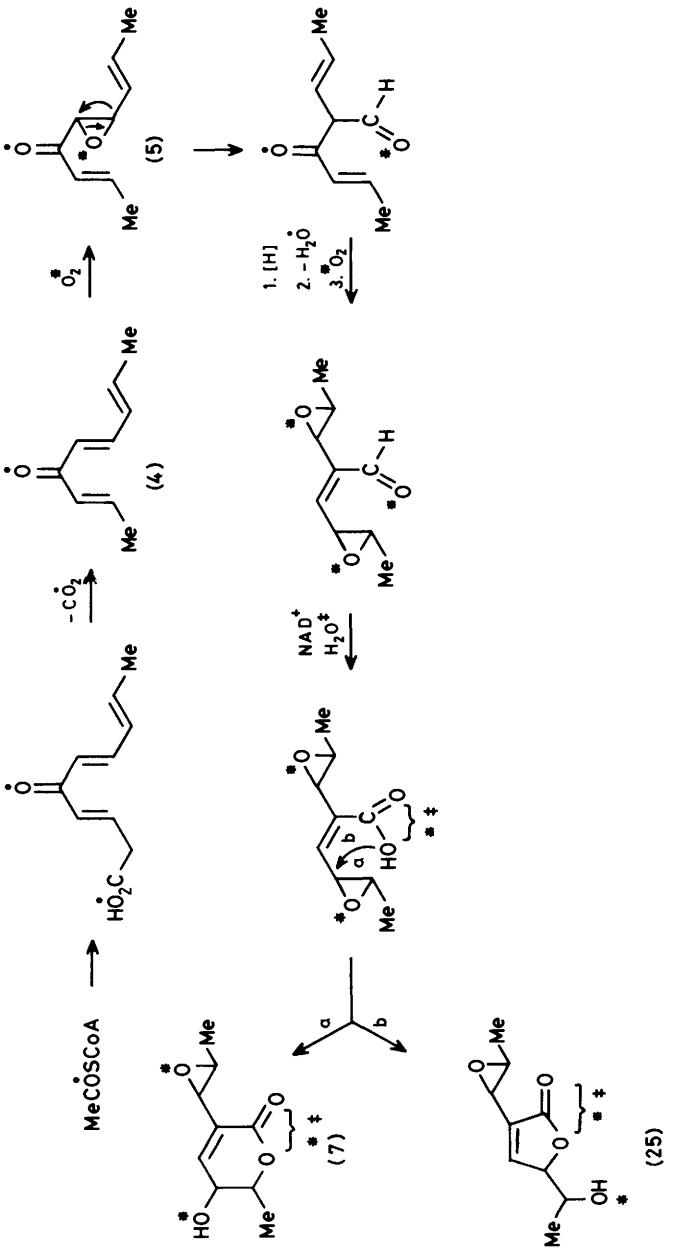
doubly or singly bonded oxygen. Incorporation of [2-^{13}C,^{2}H$_3$]acetate gave the spectra shown in Figure 15. As may be seen, deuterium noise decoupling leads to a great simplification of the otherwise uninterpretable results for C-7, which complements previous results[45] for the related co-metabolite asperlactone (25), and makes it unlikely that C-7 was ever part of a double bond. To accommodate these results, a pathway involving epoxide-mediated rearrangement and ring-closure reactions, Scheme 13, was proposed, thus providing a relatively simple model for similar processes which appear to be involved in the formation of the much more complex polyether and ionophore antibiotics.[46]

7 Polyketide Assembly Processes

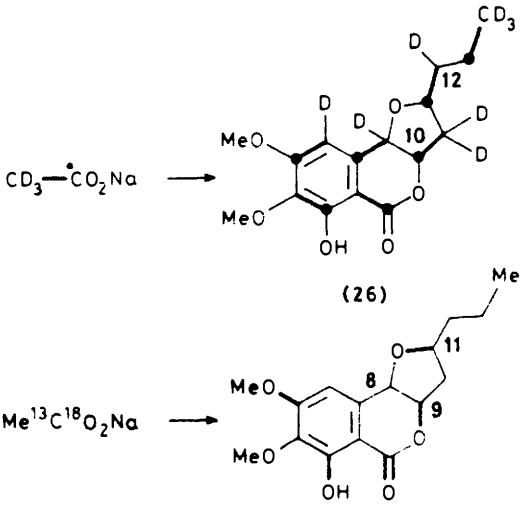
A longstanding problem is the exact relationship between polyketide biosynthesis and the corresponding pathway in primary metabolism *viz.* fatty acid biosynthesis. Labelling studies with ^{18}O and ^{2}H on appropriate polyketide-derived molecules permit indirect information on the processes which must be occurring on the polyketide synthetase enzymes to be obtained and compared with the much better understood processes catalysed by fatty acid synthetases. These studies required molecules with intermediate oxidation levels between the highly oxygenated fully aromatic polyketides and fatty acids to be examined.

Incorporation of ^{13}C-, ^{2}H-, and ^{18}O-labelled acetates into monocerin (26) by cultures of *Dreschlera ravenelii* and analysis by ^{13}C and ^{2}H n.m.r. gave the labelling

[45] R. G. Brereton, M. J. Garson, and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1027.
[46] D. E. Cane, W. D. Colmer, and J. W. Westley, *J. Am. Chem. Soc.*, 1983, **105**, 4110.



Scheme 13

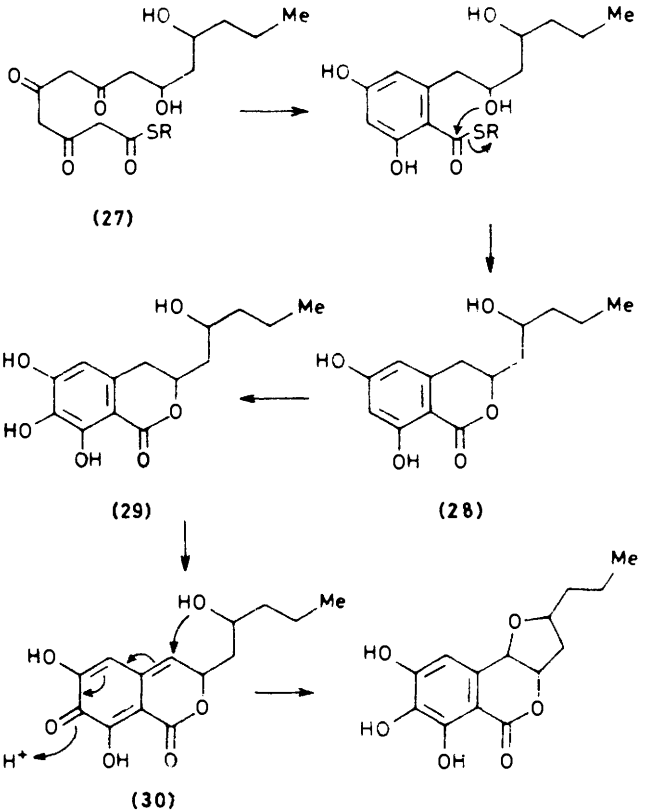


Scheme 14

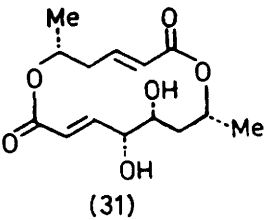
patterns shown in Scheme 14.[47] A particularly interesting feature is the retention of two acetate-derived hydrogens at C-10 which suggests that reduction of the β-ketoacyl intermediate to the corresponding β-hydroxyacyl intermediates takes place during chain assembly. ^{2}H N.m.r. showed that only one of the diasterotopic hydrogens at C-12 is labelled. Thus the dihydroxy moiety (27) rather than a classical heptaketide can be proposed as the likely enzyme-bound precursor as shown in Scheme 15. The ^{18}O-labelling pattern means that the benzopyrone ring must be formed by nucleophilic attack at the terminal carboxy moiety by a hydroxy group at C-9. It is likely that the cyclization takes place on the enzyme-bound thioester to give (28) as the first enzyme-free intermediate. The retention of the acetate carbon–oxygen bond at C-11 indicates that the tetrahydrofuran ring is formed by nucleophilic attack of a C-11 hydroxy function on C-8. A mechanism for this would be nucleophilic addition onto a quinonemethide intermediate (30) formed by oxidation of (29), the hydroxylated derivative of (28).

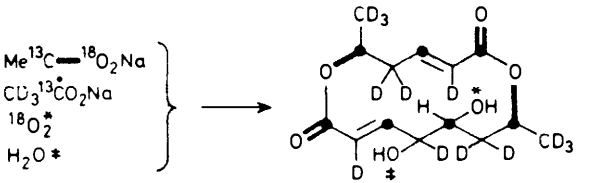
Colletodiol (31) is a macrocyclic dilactonic metabolite originally isolated from the plant pathogenic fungus *Colletotrichum capsici*. The origins of all the oxygen and hydrogen atoms have been elucidated by incorporation of label from [1-^{13}C,$^{18}O_2$]- and [1-^{13}C,$^{2}H_3$]acetates and $^{18}O_2$ gas in cultures of a *Cytospora* sp. and analysis of the resultant ^{2}H and ^{18}O isotope-induced shifts in the p.n.d. ^{13}C n.m.r. spectra of the enriched metabolites and by ^{2}H n.m.r. analysis.[48] The results are summarized in Scheme 16. From these results it can be concluded that the lactone-ring formation occurs by an acyl substitution mechanism as shown in Scheme 17; and that the 1,2-diol formation most likely occurs by epoxidation of a (*Z*)-alkene from the α-face, followed by hydrolytic S_N2 ring-opening of the epoxide by attack of

[47] F. E. Scott, T. J. Simpson, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1984, 756.
[48] T. J. Simpson and G. I. Stevenson, *J. Chem. Soc., Chem. Commun.*, 1985, 1822.

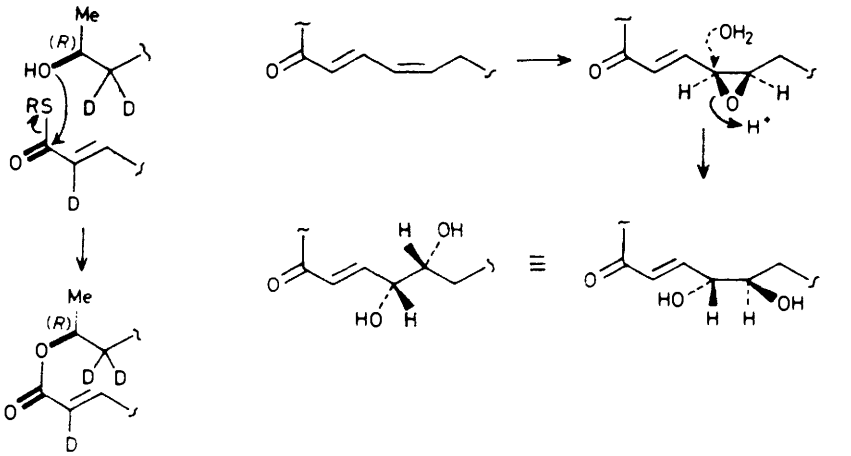


Scheme 15

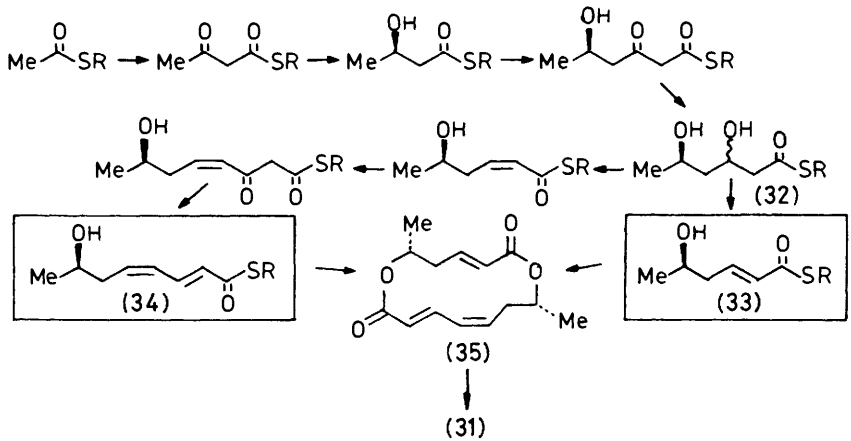




Scheme 16



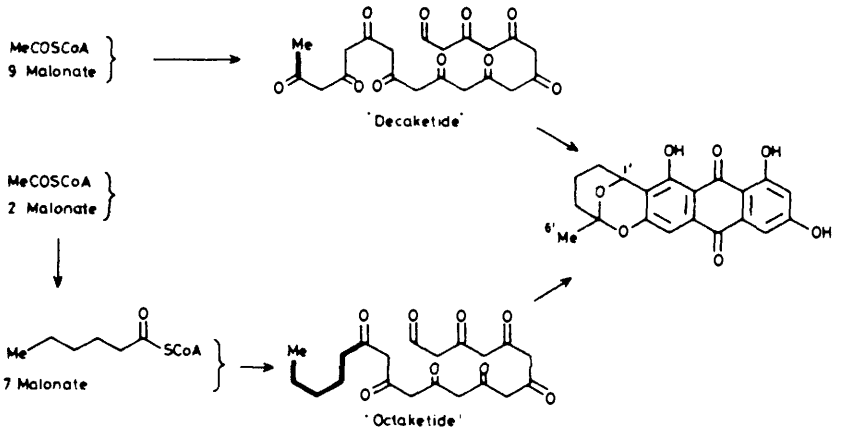
Scheme 17



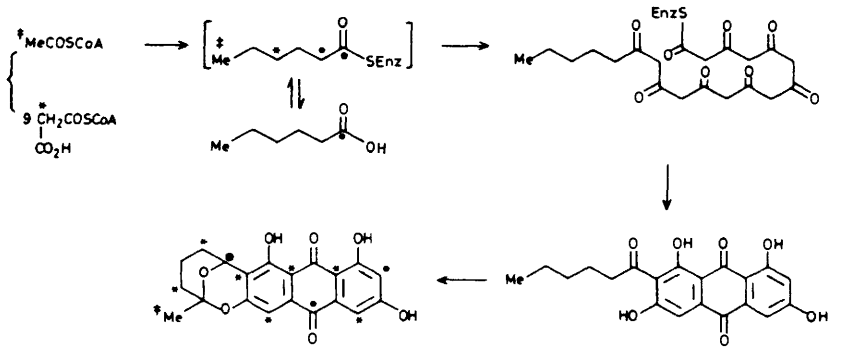
Scheme 18

water from the α-face at C-4, Scheme 17. On the basis of these results the thioesters (33) and (34), Scheme 18, were proposed as the actual enzyme-bound precursors and their formation *via* the sequence shown in Scheme 18 where the diol (32) in which the C-3 stereochemistry is uncertain was proposed as a common intermediate, *trans*-elimination of water giving rise to (33) directly whereas *cis*-elimination followed by elaboration of a further C_2 unit produces (34). The relative timing of the diol formation is uncertain but macrocycle formation to give (35) followed by epoxidation is an attractive possibility.

It was recently reported that ^{13}C-labelled hexanoate is incorporated intact into averufin and as a result it was suggested that averufin is not a decaketide but is an



Scheme 19

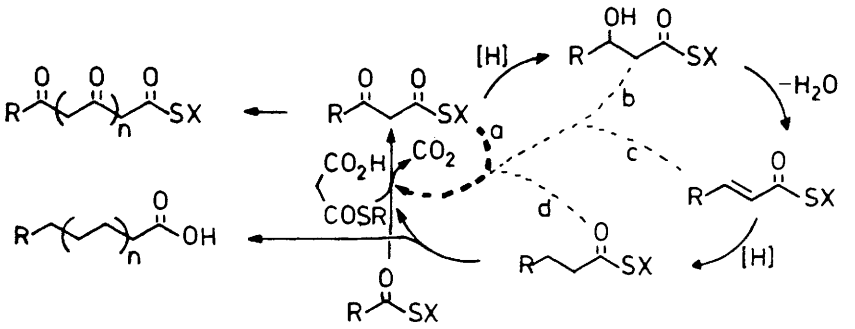


Scheme 20

octaketide formed as shown in Scheme 19 from a hexanoate 'starter' (from fatty acid metabolism) and 7 malonate chain-extending units.[49] However incorporation of [2-^{13}C]malonate into averufin and analysis of enrichments in the ^{13}C n.m.r. spectrum shows high and equal incorporation at nine positions (Scheme 20) to indicate a clear acetate 'starter' effect.[50] Thus averufin is a decaketide, but the significance of the original observation is that exogenous hexanoate can equilibrate with the enzyme-bound intermediate and so be incorporated without prior degradation.

These results, and others, are contributing to an overall picture of polyketide biosynthesis in which a 'polyketide synthase' structurally related to fatty acid synthetase assembles the enzyme-bound intermediates as shown in Scheme 21.

[49] C. A. Townsend and S. B. Christensen, *Tetrahedron*, 1983, **39**, 3575.
[50] I. M. Chandler and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1987, 17.



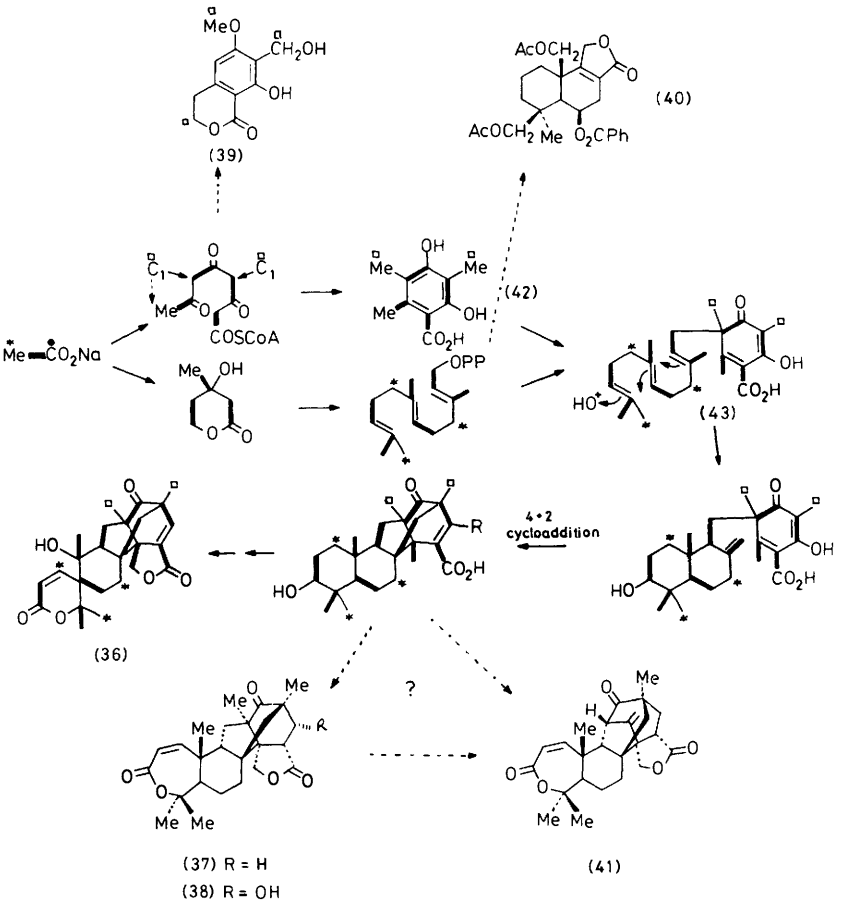
Scheme 21

Fully aromatic metabolites which retain the oxidation level of a classical poly-β-ketide are built up by a cyclic process (path a) analagous to fatty acid biosynthesis[51] but omitting the reduction–elimination–reduction sequence responsible for the loss of acetate-derived oxygen. The majority of metabolites however, show varying degrees of reduction and/or deoxygenation. It is envisaged that after each malonate condensation step the synthase has a choice of which of paths a—d is utilized before the next condensation reaction occurs. In this way 'polyketide' precursors which show varying degrees of reduction and deoxygenation can be assembled in a stepwise manner on the synthase before being released from the enzyme by a stabilizing ring condensation or some other such process. Substitution of acyl CoA starter units, other acetate and methylmalonate, ethylmalonate, *etc.* as chain-extending units accounts for the other polyketide-derived structural types formed in nature.

8 Meroterpenoids

A major interest has involved a group of compounds of mixed polyketide–terpenoid origins—the so-called meroterpenoids.[52] Our interest in these compounds began with the reported isolation of andibenin (36) from *A. varie-colour*.[53] Its structure which was elucidated by *X*-ray crystallography strongly suggested a sesterterpenoid origin. However the results of incorporation experiments with ^{13}C-labelled acetates and methionine showed this hypothesis to be incorrect.[54] The labelling pattern which resulted was consistent with a biosynthetic pathway in which the key step was alkylation of a bis-*C*-methylated tetraketide-derived phenolic precursor (42) by farnesyl pyrophosphate to give (43) followed by further cyclization and oxidative modifications as shown in Scheme 22. A number of closely related co-metabolites *e.g.* andilesin C (37) and A (38) were isolated in the

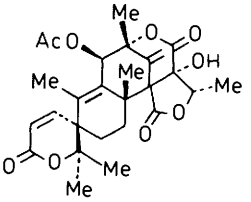
[51] B. Sedgwick and C. Morris, *J. Chem. Soc., Chem. Commun.*, 1980, 96; F. Lynen, *Eur. J. Biochem.*, 1980, **112**, 431.
[52] J. W. Cornforth, *Chem. Br.*, 1968, **4**, 102.
[53] A. W. Dunn, R. A. W. Johnstone, B. Sklarz, and T. J. King, *J. Chem. Soc., Chem. Commun.*, 1976, 270.
[54] J. S. E. Holker and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1978, 626.



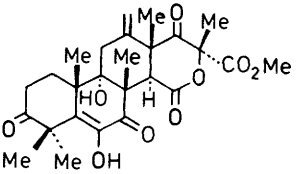
Scheme 22

course of these studies and their structures were assigned by ^{1}H and ^{13}C spectral comparisons and chemical correlations.[55] Two further, structurally unrelated but biosynthetically relevant, metabolites were isolated from mutant strains of *A. variecolor*. These were the bis-*C*-methylated tetraketide metabolite stellatin (39) whose structure was defined almost entirely from analysis of the fully ^{1}H-coupled ^{13}C n.m.r. spectrum,[56] and the sesquiterpenoid astellolide A (40) whose structure was deduced from ^{1}H and ^{13}C n.m.r. studies and confirmed by *X*-ray crystallography.[57]

[55] T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, 2118; A. W. Dunn, R. A. W. Johnstone, B. Sklarz, L. Lersinger, and T. J. King, *ibid.*, 2113.
[56] T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1978, 627.
[57] R. O. Gould, T. J. Simpson, and M. D. Walkinshaw, *Tetrahedron Lett.*, 1981, 1047.



(44)

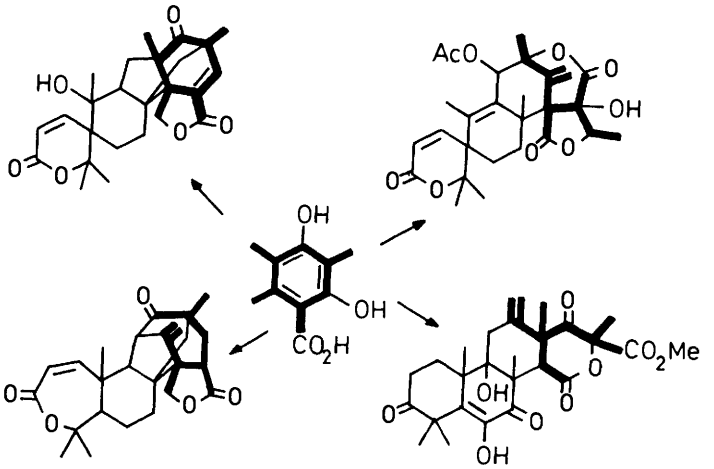


(45)

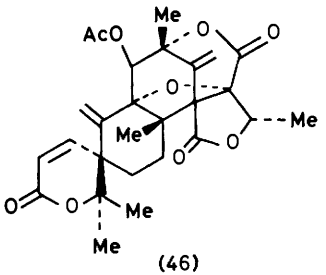
A further significant metabolite was isolated along with andilesin C from another strain of *A. variecolor*. This was anditomin (41) whose structure was deduced by spectroscopic methods and confirmed by *X*-ray analysis[58] and whose biosynthesis was confirmed by ^{13}C-labelling experiments.[59] It represented an important modification of the meroterpenoid pathway as it was the first metabolite in which the carbocyclic ring of the tetraketide-derived moiety had been fragmented. While this work was in progress, attention was drawn to two further metabolites whose structures could be rationalized by extensions, albeit drastic ones, of the meroterpenoid pathway. These metabolites were austin (44) and terretonin (45) which had been isolated as toxic metabolites of *Aspergillus ustus*[60] and *Aspergillus terreus*[61] respectively. Modified terpenoid origins had been suggested for both metabolites. However, incorporation of ^{13}C-labelled acetates and methionine gave results which supported the hypothesis that these were further metabolites of the meroterpenoid pathway formed *via* (43).

The conclusive evidence for the meroterpenoid origins of these metabolites was provided by the synthesis of labelled 3,5-dimethylorsellinic acid (42)[62] and its specific incorporation into andibenin (36),[63,64] and austin (44) and terretonin (45).[65] This was established by ^{2}H n.m.r. analysis of the metabolites enriched from feeding experiments with (42) specifically labelled with ^{2}H in the 5-methyl group. The mode of incorporation of the carbon skeleton of 3,5-dimethylorsellinate into these metabolites is summarized in Scheme 23. Whereas the skeleton is incorporated intact into andibenin B and andilesin C, and suffers one bond cleavage only on incorporation into anditomin, it is fragmented to an unprecedented degree on incorporation into austin and terretonin. This was the subject of further studies described below. Further evidence for the biosynthetic relationship of austin and

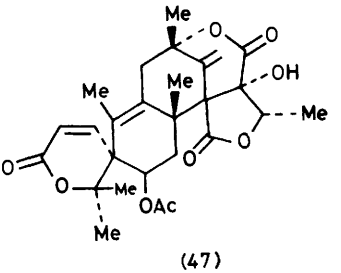
[58] T. J. Simpson and M. D. Walkinshaw, *J. Chem. Soc., Chem. Commun.*, 1981, 914.
[59] T. J. Simpson, *Tetrahedron Lett.*, 1981, 3785.
[60] K. K. Chexal, J. P. Springer, J. Clardy, R. J. Cole, T. W. Kirksey, J. W. Dorner, H. G. Cutler, and W. J. Strawter, *J. Am. Chem. Soc.*, 1976, **98**, 6748.
[61] J. P. Springer, J. W. Corner, R. J. Cole, and R. H. Cox, *J. Org. Chem.*, 1979, **44**, 4852.
[62] T. J. Simpson and D. J. Stenzel, *J. Chem. Soc., Chem. Commun.*, 1981, 1042; C. R. McIntyre and T. J. Simpson, *ibid.*, 1043.
[63] A. J. Bartlett, J. S. E. Holker, T. J. Simpson, and E. O'Brien, *J. Chem. Soc., Perkin Trans. 1*, 1983, 667.
[64] A. J. Bartlett, J. S. E. Holker, E. O'Brien, and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1981, 1198.
[65] C. R. McIntyre, T. J. Simpson, D. J. Stenzel, A. J. Bartlett, E. O'Brien, and J. S. E. Holker, *J. Chem. Soc., Chem. Commun.*, 1982, 781.



Scheme 23



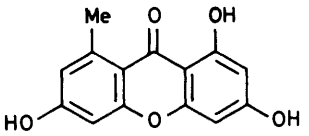
(46)



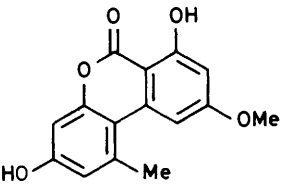
(47)

andibenin B was provided by the isolation of both metabolites from *A. variecolor*[66]. The ^{13}C n.m.r. spectral assignment of austin (44) and the structures of dehydroaustin (46) and iso-austin (47) related metabolites isolated from *A. ustus* and *Penicillium diversum* were largely established by detailed analysis of fully ^{1}H-coupled ^{13}C spectra and ^{1}H–^{13}C correlation experiments.[66] *P. diversum* produces an amazing range of metabolites. Apart from iso-austin, it produces the known polyketides lichenxanthone (48), alternariol monomethyl ether (49) and two new structural types. These are the diversolonic esters (50) whose structure were elucidated by extensive ^{1}H and ^{13}C n.m.r. studies,[67] and a novel isocoumarin (51) whose structure was confirmed by *X*-ray studies.[68] This metabolite is probably biosynthesized by a novel aromatic ring contraction from the known co-metabolite (49).

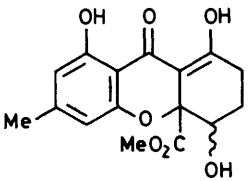
[66] T. J. Simpson, D. J. Stenzel, A. J. Bartlett, E. O'Brien, and J. S. E. Holker, *J. Chem. Soc., Perkin Trans. 1*, 1982, 2687.
[67] J. S. E. Holker, E. O'Brien, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1982, 1365.
[68] J. S. E. Holker, E. O'Brien, T. J. Simpson, and M. D. Walkinshaw, unpublished results.



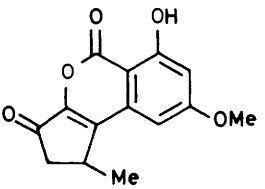
(48)



(49)



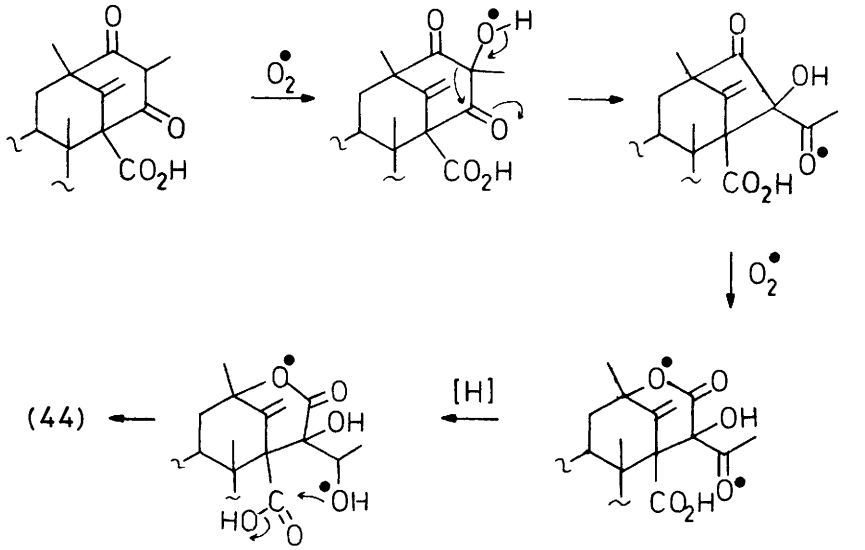
(50)



(51)

The origins of the oxygen atoms in the meroterpenoid metabolites were then studied by incorporation experiments in the presence of $^{18}O_2$ and [1-^{13}C,$^{18}O_2$]acetate, to try to elucidate information on the mechanisms by which the extensive modifications observed for the orsellinate-derived moiety in austin and terretonin occurred and also for the formation of the spiro-δ-lactone systems in andibenin B and austin. Whereas ^{18}O label from acetate was successfully incorporated into andibenin B,[69] the low level of incorporation obtained precluded the observation of the necessary isotope shifts for andilesin A and austin. Nonetheless the results from incorporation of label from $^{18}O_2$ into austin[70] were consistent with a modification scheme in which the orsellinate moiety undergoes a ring-contraction *via* an α-ketol rearrangement followed by biological Baeyer–Villiger type of oxygen insertions to form the δ-lactone moieties found in both the polyketide- and terpenoid-derived portions of the molecules as shown in Scheme 24. The problem of low incorporation of labelled acetate was overcome by synthesizing 3,5-dimethylorsellinate doubly labelled with ^{13}C and ^{18}O in both the carboxyl carbonyl and at the C-6 position. This was incorporated with high efficiency into austin (Figure 16) to confirm the $^{18}O_2$ results,[71] and also into andilesin A to rule out the possible involvement of deoxyorsellinate intermediates in the biosynthesis of the andibenins and andilesins.[72]

[69] C. R. McIntyre, T. J. Simpson, R. N. Moore, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1984, 1499.
[70] T. J. Simpson, D. J. Stenzel, R. N. Moore, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1984, 765.
[71] F. E. Scott, T. J. Simpson, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1986, 214.
[72] C. R. McIntyre, F. E. Scott, T. J. Simpson, L. A. Trimble, and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1986, 501.



Scheme 24

Interestingly, further metabolites related to austin have been isolated from *Emericella dentata*,[73] and two unrelated metabolites which are almost certainly further products of the meroterpenoid pathway, fumigatonin (52) and paraherquonin (53), have been isolated from *Aspergillus fumigatus*[74] and *Penicillium paraherquei*.[75] It is of interest to note that studies which were initiated on the mistaken assumption of a sesterterpenoid origin for andibenin B have led to the unravelling of a complex, novel, and now apparently widespread biosynthetic pathway. The meroterpenoid pathway as it stands at present is summarized in Scheme 25. This will clearly be an area in which much biosynthetic and synthetic work will be carried out in the future.

9 Conclusions

The use of stable-isotope labelling methodology has enabled otherwise unobtainable information on both the early and later stages of biosynthetic pathways to be obtained. These results, besides their own intrinsic merit, enable further work using advanced intermediates and cell-free enzyme studies to proceed on a more rational basis.

Acknowledgements. The author expresses his gratitude to the many other people who have carried out labelling studies with stable isotopes. The ideas and

[73] Y. Maebayashi, E. Okuyama, M. Yamazaki, and Y. Katsube, *Chem. Pharm. Bull.*, 1982, **30**, 1911.
[74] E. Okuyama, M. Yamasaki, and Y. Katsube, *Tetrahedron Lett.*, 1984, 3233.
[75] E. Okuyama, M. Yamasaki, K. Kobayashi, and T. Sakurai, *Tetrahedron Lett.*, 1983, 3113.

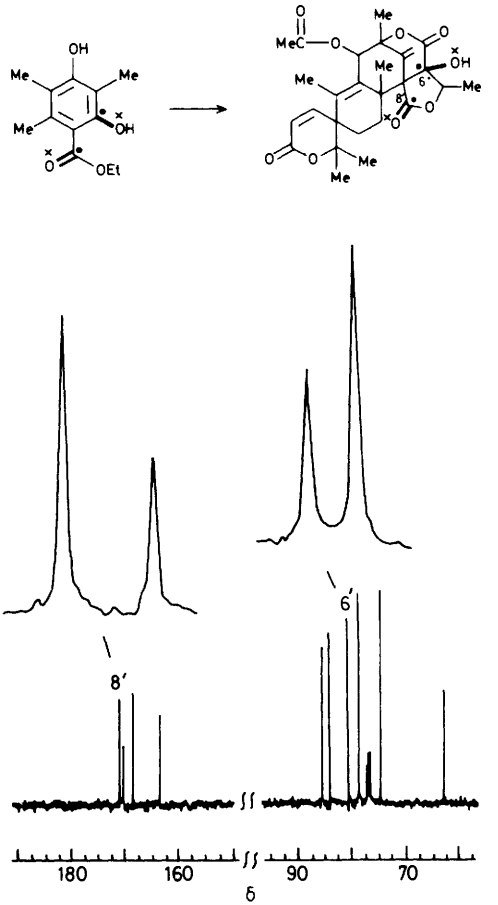
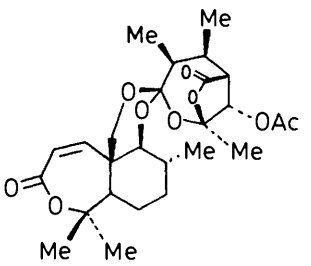
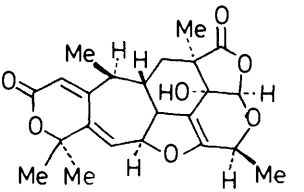


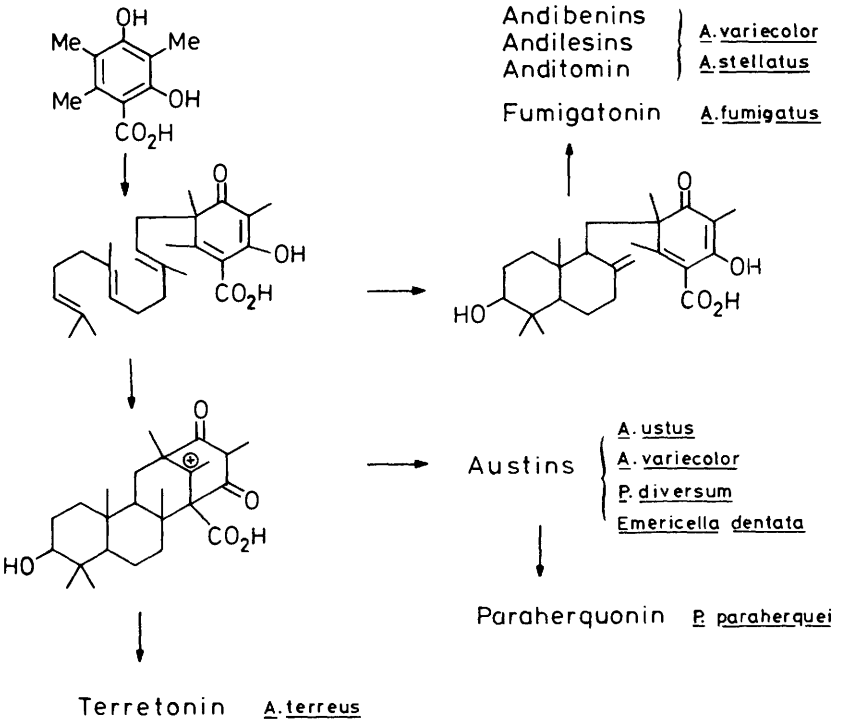
Figure 16 ^{18}O-*Isotopically shifted resonances in the* 100.6 MHz *p.n.d.* ^{13}*C n.m.r. spectrum of austin* (44) *enriched by ethyl* [^{13}C,^{18}O]-3,5-*dimethylorsellinate*



(52)



(53)



The Fungal Meroterpenoid Pathway

Scheme 25

experiments described by them have provided much of the stimulation and rationale for the work described above. Particular thanks must go to some of the collaborators who over the years have contributed to these studies—these include A. J. Birch, J. S. E. Holker, P. S. Steyn, J. C. Vederas, and J. Staunton.