THE SYNTHESIS AND BIOSYNTHESIS OF

CHLORINE CONTAINING METABOLITES

by

GRAHAM SHIRRAN MACAULAY

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Department of Chemistry

University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DEDICATION

This thesis is dedicated to my parents Jack and Myra.

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SUMMARY

The biosynthesis of cryptosporiopsinol was investigated, in particular the nature of the contraction of the 6-membered ring to the 5-membered ring. The feeding of different labelled acetates to Periconia macrospinosa has been studied. The feeding result of $CH_3{}^{13}C^{18}O_2Na$ suggests that the ring contraction previously proposed cannot be correct and that the ring contraction must go via an alternative mechanism. The isocoumarin isolated from feeding of $CD_3{}^{13}CO_2Na$ to Periconia macrospinosa showed in its ^{13}C n.m.r. spectra two different β-shifts at C-4a arising from the axial and equatorial deuteriums at C-4. A number of deuterium labelled 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarins were synthesised. The results obtained confirmed that β-shifts are additive and it was also shown that the equatorial deuterium exerted a greater β-shift than the axial deuterium.

The biosynthesis of microline and dechloromicroline was studied. 3,4-Dihydro-6,8-dihydroxy-3-methylisocoumarin was synthesised with a ^{14}C at C-3. This isocoumarin was also synthesised with deuterium atoms at C-4 and the methyl group. These compounds were fed to Gilmaniella humicola and shown to be incorporated into dechloromicroline intact.

A synthetic route was developed which gave the dimethyl ether of perimacol a metabolite of Periconia macrospinosa. It was shown that a trans arrangement exists between the methyl group and methoxyl group at C-3 and C-4 respectively. Previously it was thought that a cis arrangement existed between the substituents at C-3 and C-4.

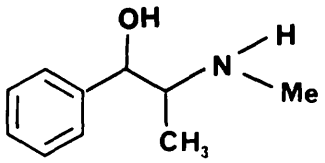
1.1 Introduction

Man has used natural products, albeit as crude plant extracts, since the dawn of time and these 'recipes' are still possessed today[1]. The term 'natural product' or secondary metabolite is commonly reserved for those organic compounds of natural origin that are unique to one organism, or common to a number of closely related organisms. Secondary metabolites are distinguished more precisely from primary metabolites by the following criteria[2,3,4]: they have a restricted distribution being found mostly in plants and micro-organisms, and are often characteristic of individual genera, species or strains; they are formed along specialized pathways from primary metabolites, and appear to serve no useful purpose to the organism that produces them. By contrast primary metabolites e.g. amino acids, carbohydrates etc. have a broad distribution in all living things and are intimately involved in essential life processes.

Primitive man found these natural extracts efficient as medicines for the relief of pain or alleviation of the symptoms of disease, as poisons for use in warfare and hunting, as effective agents for euthanasia and capital punishment, and as narcotics, hallucinogens, or

stimulants to relieve the tedium, or alleviate the fatigue and hunger in his life. Man may also have used the more odiferous and spicy compounds to obscure the odour of unwashed humanity, and to disguise the putrid or bland flavour of his food.

Many of these natural products are still used today, and usually for the same general purpose. Ephedrine (1), the basis of an ancient Chinese remedy for respiratory ailments is now used in the treatment of asthma and hay fever. Caffeine (2) was and still is, the active principle of many beverages. Crude extracts of bark, leaves and seeds produce concoctions of considerable potency. In many ways our modern coffee, cocoa, tea and cola are but poor imitations of these native brews.



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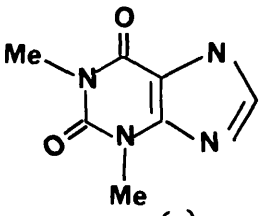

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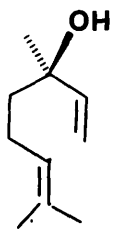

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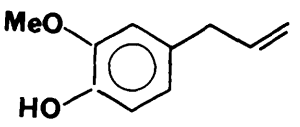

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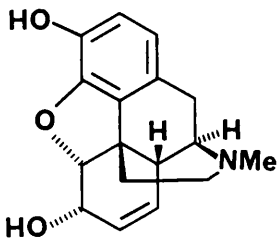
Examples of the odiferous and spicy compounds that have been used through the ages are the naturally occurring compounds linalol (3) from rose oil and eugenol (4). Faced with a plethora of natural brews and remedies, the chemists of the early nineteenth century were highly motivated to isolate and characterise these natural products. Between 1815 and 1860 more than twenty of these active principles were isolated, including caffeine (2), morphine (5), quinine (6), codeine (7) and cocaine (8). However accurate analyses were not possible before 1835 and even then it was rarely possible to do more than present molecular formulae and describe the characteristic reactions of the compounds. Many of these reactions were novel, and new ideas of molecular structure and reactivity followed. Thus it was only a matter of time before total syntheses of these compounds were then attempted, not only as a final confirmation of the structures but because their structural complexity represented a considerable challenge to the synthetic chemist.

As the structures of an increasing number of natural products became known, attempts were made to classify them in terms of structural type. This led to speculation concerning their assembly or biogenesis. In some areas it was not difficult to spot



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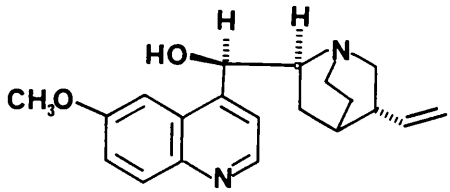

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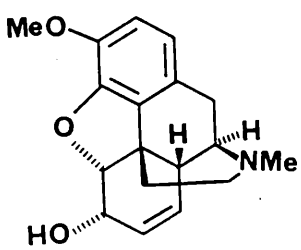

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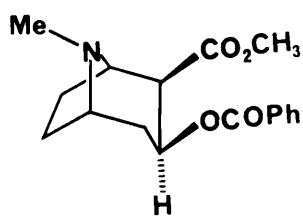
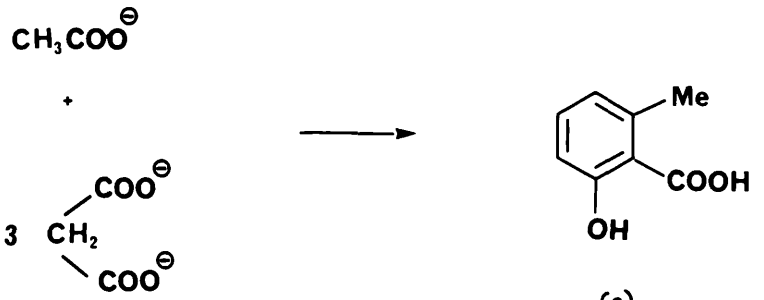


(8)

characteristic structural features which suggested a particular progenitor or precursor. For example many alkaloids incorporate the skeletons of simple amino acids, while terpenes and steroids usually contain an integral number of five-carbon units, originally thought to derive from isoprene (2-methylbuta-1,3-diene).

The most important biosynthetic role in the secondary metabolism in fungi is the acylpolymalonate or polyketide pathway. The idea that naturally occurring, highly oxygenated compounds might arise by condensation of acetate units was suggested as long ago as 1893 by J.N. Collie.[5] This inspired speculation was largely ignored for over half a century until Birch restated the hypothesis and provided experimental evidence in the form of ^{14}C-labelled acetate incorporations into 6-methylsalicylic acid (9) a metabolite of Penicillium griseofulvum and other fungi.[6,7] 6-Methylsalicylic acid (9) can be regarded as a typical example of a polyketide derived aromatic metabolite. Bu'Lock showed that 6-methylsalicylic acid (9) was formed from one acetate and three malonate units.[8]

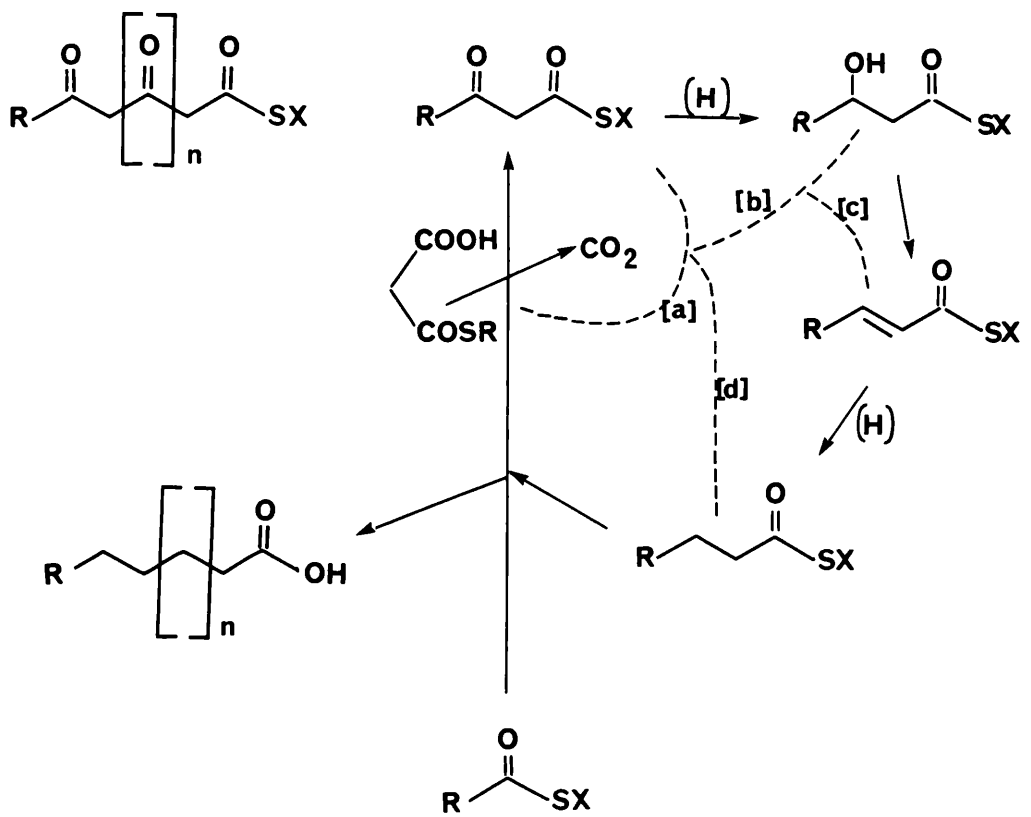


(9)

This led to many investigations of polyketide biosynthesis over the years. From these results a picture of polyketide biosynthesis has been built up in which a 'polyketide synthase', structurally related to fatty acid synthetase, assembles the enzyme-bound intermediates as shown in Scheme 1.1. Fully aromatic metabolites which retain the oxidation level of a classical poly-β-ketide are built up by a cyclic process (path a) analoguous to fatty acid biosynthesis[9] 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 the choice of which paths a-d is utilised before the next condensation reaction occurs.

SCHEME 1.1



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.

In the study of secondary metabolism the ongoing problem is two fold:

1) To identify the source(s) in primary metabolism from which a secondary metabolite has its genesis.

2) To identify by what mechanisms and manner of intermediates it is thus fashioned.

The wealth of intelligent speculation about the biosynthesis of secondary metabolites has provided a firm base from which to mount wide-ranging experimental forays into secondary metabolism in search of origins and mechanisms of formation. The dominant technique involves the administration of likely precursors to an organism and examination of the secondary metabolite produced to determine if the compounds 'fed' were used in the formation of the metabolite. To track the likely precursor through to the metabolite the precursor must be labelled, or marked in some way. Two different types of label are generally used:

1) Radioactive isotopes, e.g. ^{14}C, ^{3}H, ^{32}P.

2) Stable isotopes, e.g. ^{13}C, ^{2}H, ^{18}O, and ^{15}N.

There are several reviews [10,11,12,13,14] which cover the use of stable isotopes in biosynthesis. The use of radioactive isotopes in biosynthetic studies predominated during the period between 1946 and 1970 even though ^{2}H was the isotope first used to study metabolic processes.[15,16] The main reason for their preferential use over stable isotopes is sensitivity. It is possible to detect and quantify accurately compounds labelled with radioactive isotopes after mixing up to 10^9 times with their non-labelled forms. Since dilutions of 10^6 fold are commonly encountered when studying the biosynthesis of secondary metabolites, it is often necessary to use isotopes that can be detected with high sensitivity. On the other hand, the use of radioactive isotopes has several disadvantages, e.g. the tedious method of degradation of the final metabolite to confirm that the label is in the postulated position in the metabolite; the inability to analyze intramolecular events during bond making and breaking processes during biosynthesis. Such drawbacks have hindered the progress of research in the field.

Developments during the 1970's have resulted in a marked increase in the use of stable isotopes to study biochemical processes governing the formation of secondary natural products. Firstly, the problem of low sensitivity for the detection and quantification of stable isotopes characteristic of the period before 1970 has been overcome by the commercial availability of mass and nuclear magnetic resonance (n.m.r.) spectrometers. These instruments have high resolution and sensitivity adequate for the accurate measurement of stable isotopes at low levels.

Secondly, the study of biosynthetic pathways received a major new impetus in the 1970's with the advent of pulsed Fourier transform n.m.r. spectrometers. This has allowed the development of ^{13}C n.m.r. spectroscopy to provide a non-destructive detection system capable of determining the location and relative concentration of ^{13}C at each chemically non-equivalent carbon in a molecule. It also enables the presence of ^{2}H, ^{18}O, and ^{15}N to be demonstrated from a study of the isotope induced shifts in ^{13}C n.m.r. resonance frequencies or by spin-spin coupling to ^{13}C. Since the ^{13}C nucleus has a nuclear spin ($I=\frac{1}{2}$) nuclear magnetic resonance signals may be observed. With the development of sensitive instruments with the means to accumulate (and thus enhance) weak signals, the amount of data

available on ^{13}C chemical shifts and spin-spin coupling constants[17] to ^{13}C, ^{1}H and ^{15}N is increasing rapidly. The undecoupled natural abundance ^{13}C n.m.r. spectrum shows coupling to both directly bonded hydrogens and longer range couplings to non-bonded hydrogens. Analysis of these couplings is often of great utility in both structural elucidation and spectral assignments. The spectra are often proton-noise decoupled so that a single line is obtained for each carbon in the molecule. Thus, although the natural abundance of ^{13}C is only 1.1% it is now relatively easy to obtain ^{13}C n.m.r. spectra for any compound given a minimum amount of material. One great advantage of ^{13}C n.m.r. is the considerable spread of shifts commonly observed: about 200 p.p.m. compared with 10 p.p.m. for ^{1}H shifts.

1.2 N.M.R. Studies Using Stable Isotopes

The complete assignment of ^{1}H and ^{13}C n.m.r.'s of a natural product is usually a prerequisite for ensuing biosynthetic experiments in which stable isotopes are used.[14] Although the ever-increasing variety of new pulse sequences may dismay the uninitiated, the use of a relatively small number of these can simplify routine spectral assignment. The exact choice depends on the type of information sought. Generally after the normal ^{1}H n.m.r. and broad-band proton decoupled ^{13}C nmr.

spectra have been obtained the most widely useful sequence would be:

i) determination of the complete proton-proton coupling scheme by correlation of ^{1}H homonuclear chemical shifts (e.g. COSY, correlated spectroscopy [18,19])

ii) determination of the number of hydrogen atoms that are attached to each carbon by spin-echo Fourier transform[20] or polarisation transfer experiments[18] (e.g. DEPT, distortionless enhanced polarisation transfer)[21]

iii) determination of which protons are connected to particular carbon nuclei (e.g. correlation of ^{1}H and ^{13}C heteroscalar chemical shifts)[18,22,23]

iv) determination of the complete carbon connectivity pattern by ^{13}C homonuclear chemical shift correlation (e.g. 2D-inadequate)[18,24]

1.3 Use of ^{13}-C in Biosynthetic Studies

The early studies using singly ^{13}C labelled precursors did not provide any information which could not have been 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. This 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. Bond fragmentation and rearrangement processes could be detected by the loss of ^{13}C-^{13}C couplings. This in fact represented the real advance offered by ^{13}C labelling techniques. This type of information could not be obtained, even in principle by classical radioisotope methods.

Since one bond carbon-carbon coupling constants are much larger than those of two or three bond couplings, ^{13}C n.m.r. spectroscopy is an ideal method to detect adjacent carbon nuclei in a secondary metabolite. There are several ways in which such coupling may arise - see Figure 1.1.[14]

i) two singly labelled precursor molecules may form a new bond in the product (path a)

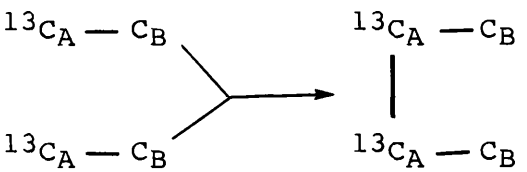
ii) a singly labelled precursor may be metabolised by a branch pathway to a doubly labelled form (path b).

iii) a doubly labelled precursor in which there are isolated carbon-13 atoms, may form a bond between them (path c).

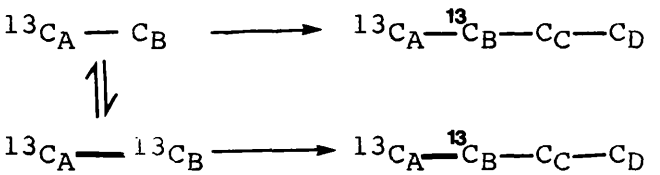
iv) a doubly labelled precursor in which the carbon-13 atoms are adjacent, may be incorporated intact.

Figure 1.1

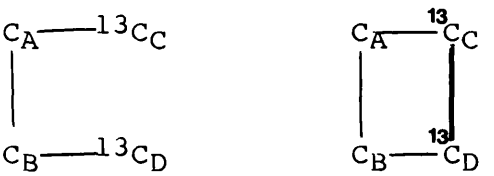
Path a



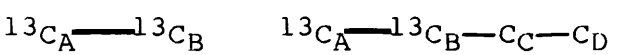
path b



path c

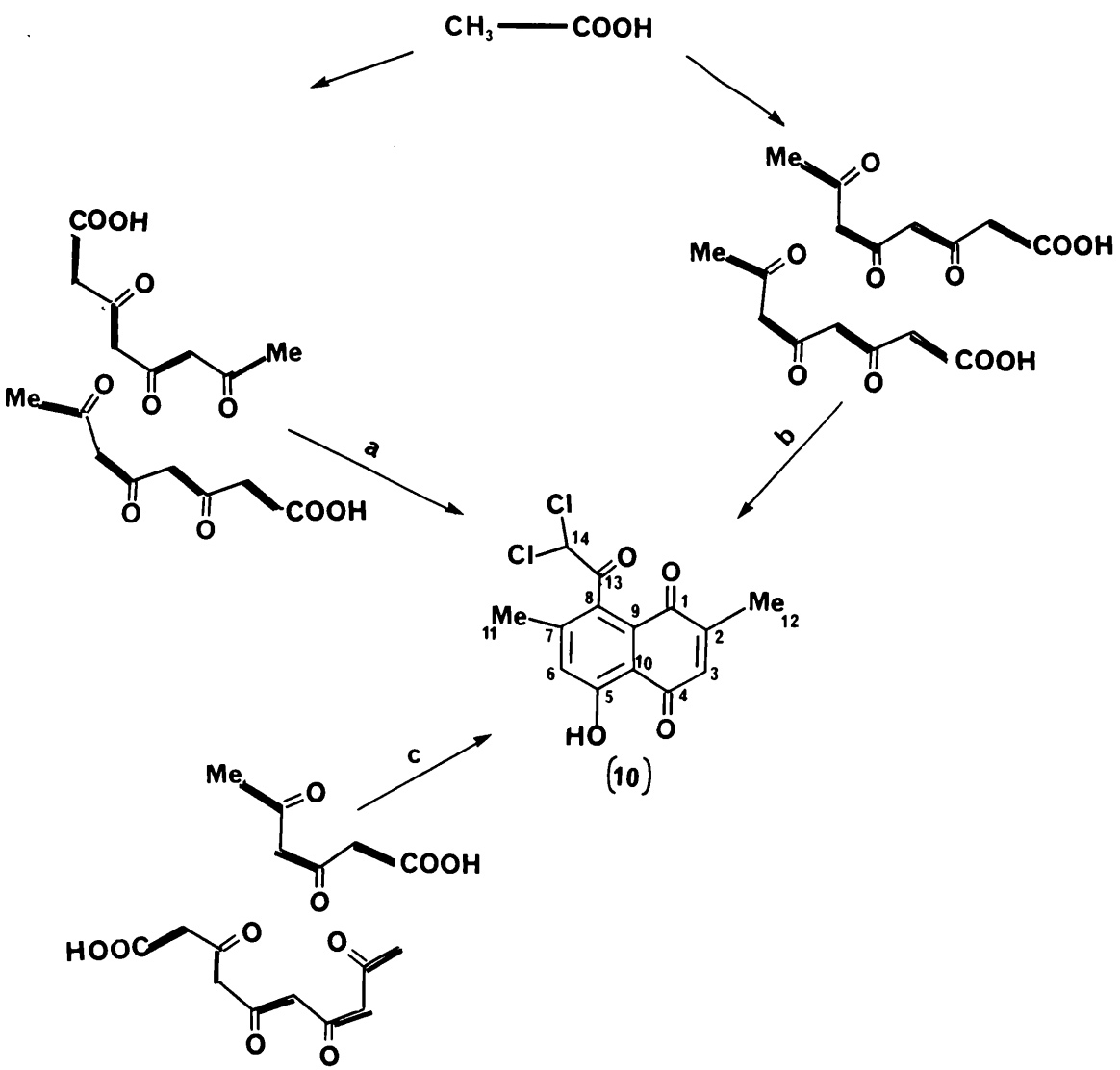


path d



In most cases, a sufficient pool of unlabelled precursor is naturally present during biogenesis, such that paths a and b invariably lead to a much lower concentration of product molecules in which adjacent carbon atoms are labelled than paths c and d. This usual dilution of ^{13}C-labelled precursor permits 'bond labelling'. Therefore, a high concentration of coupled species in the product generally indicates that

SCHEME 1 . 2



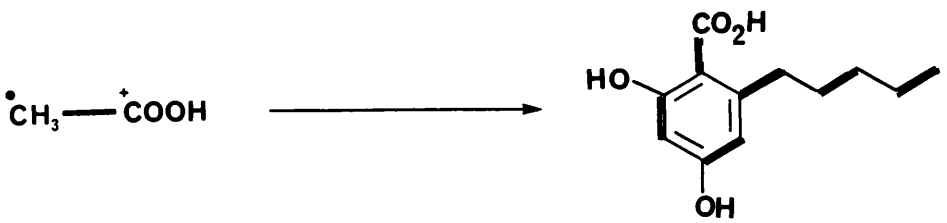
the bonds between the carbon-13 atoms in a doubly labelled precursor have not been cleaved to give separate fragments.

An early application of this technique was reported by Seto et al[25] who studied the incorporation of ^{13}C from sodium [1,2-^{13}C]acetate into the polyketide mollisin (10), a metabolite of Mollisia Caesia (see Scheme 1.2). Two biosynthetic pathways for mollisin (10) had been suggested by Bentley et al[26] based on the labelling pattern obtained by ^{14}C-tracer experiments (pathways (a) and (b)). Pathway (a) was favoured since this route uses an activated methylene for the chlorination reactions. For pathway (a) to be the correct one, ^{13}C-^{13}C couplings should be observed between C-11 and C-7, and between C-12 and C-2 but not with C-14. For pathway (b) to take place coupling between C-11 and C-7, and between C-14 and C-13 and no coupling between C-12.

In the Fourier Transform ^{13}C n.m.r. spectrum of mollisin obtained from doubly labelled acetate, ^{13}C-^{13}C coupling was observed with C-3 and C-4, C-6 and C-7, C-2 and C-12, and C-13 and C-14 but not with C-11. Therefore, the operation of either pathway (a) or (b) in the biosynthesis is excluded and another pathway e.g. (c) may be involved in the formation of mollisin (10). This was an early example which showed the undoubted capabilities of this technique, by

establishing how the polyketide was arranged before cyclisation.

Another example of this technique was shown in the biosynthesis of multicolic acid (11) from Penicillium multicolor. The biosynthesis was studied by incorporations of singly and doubly ^{13}C-labelled acetate into multicolic acid (11). The observations of ^{13}C-^{13}C couplings and more significantly their absence from the ^{13}C resonances of certain carbons led to the proposal that multicolic acid (11) and related tetronic acids from Penicillium multicolor[27] were biosynthesised via oxidative cleavage of an intermediate containing a benzenoid ring. These proposals were subsequently



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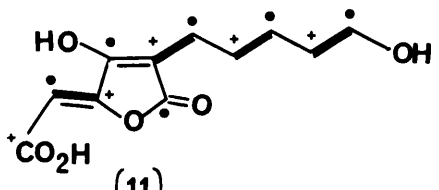
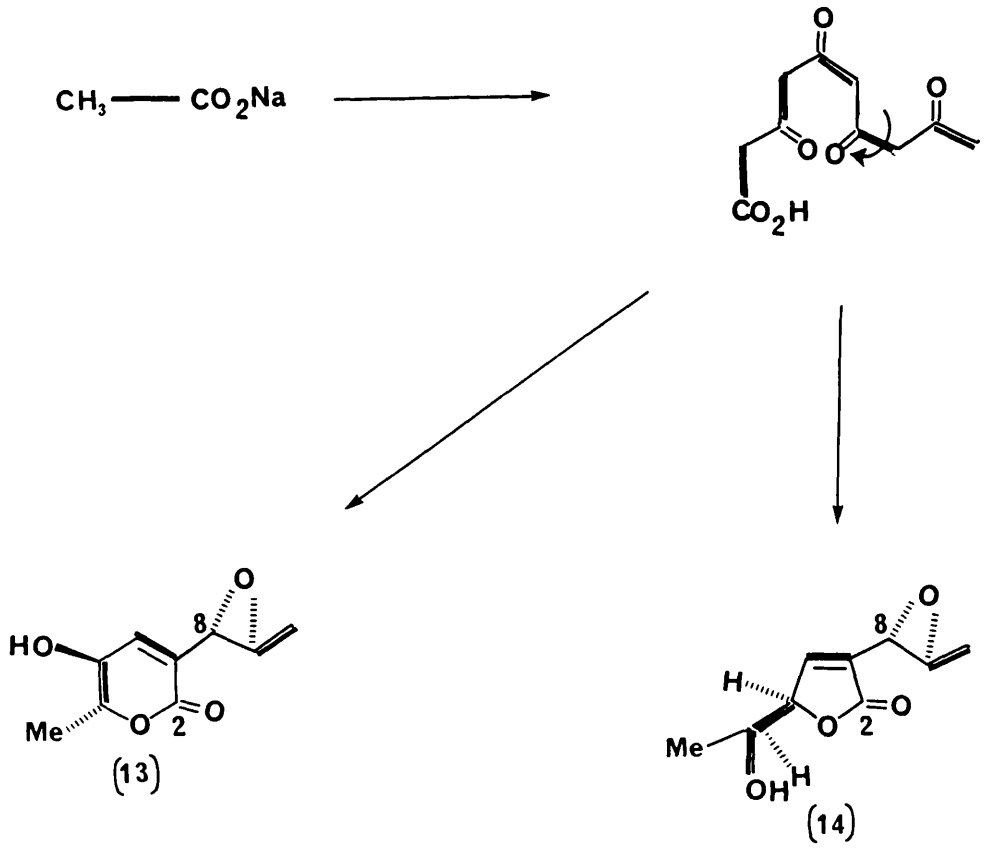


(11)

confirmed by the incorporation of 6-pentylresorcylic acid (12)[28] into multicolic acid and from ^{18}O-labelling studies.[29]

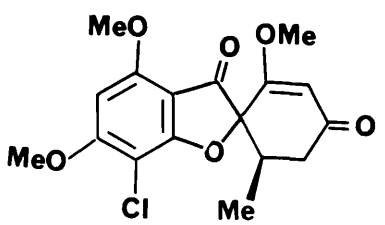
The use of doubly labelled acetate can also reveal whether an intramolecular 1,2-migration of a bond has taken place during the biosynthesis of a metabolite. An intramolecular 1,2-migration may be seen by using a precursor which is isotopically substituted at adjacent atoms with carbon-13. 1,2-Migrations would result in the metabolite containing a two bond coupling rather than a one bond coupling. This has been observed in the biosynthesis of aspyrone (13)[30] and asperlactone (14)[31] in Aspergillus melleus (see Scheme 1.3).

SCHEME 1.3



The incorporation of ^{13}C from sodium [1,2-$^{13}C_2$]-acetate results in the appearance of a two-bond coupling of 6.2Hz between C-2 and C-8 in both (13) and (14) as well as the indicated one-bond couplings from intact acetate units. The lack of other coupling between carbon nuclei of adjacent units is a critical requirement in this type of experiment to establish completely that both C-2 and C-8 come from the same molecule of acetate. This was the first observation of such a coupling in biosynthetic studies.[30,31]

At about this time the idea was conceived that the involvement of a symmetrical intermediate at any stage in a biosynthetic pathway would result in a randomisation of labelling. Griseofulvin (15) was chosen as a model to test this hypothesis. [1,2-$^{13}C_2$]Acetate was incorporated using a high



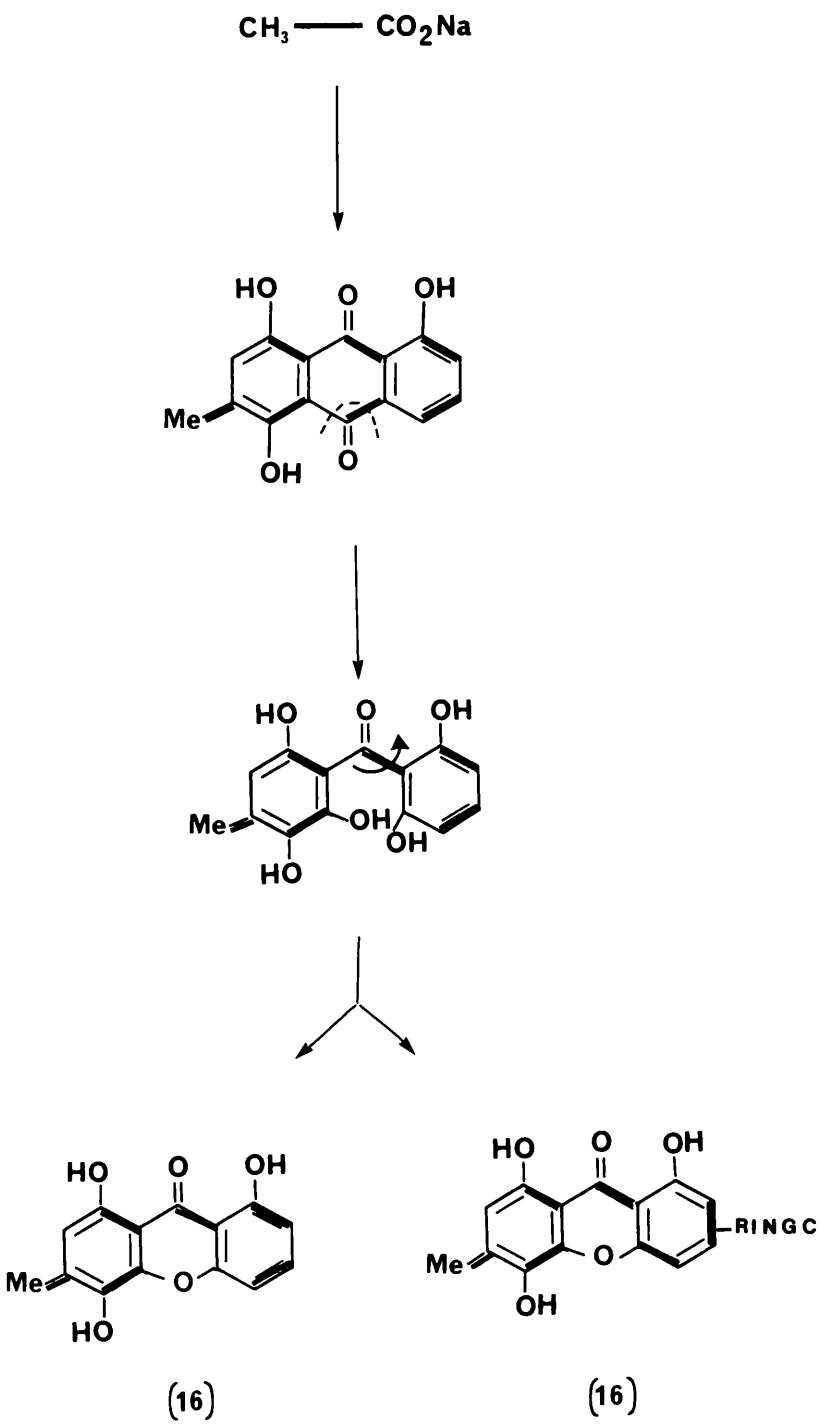
(15)

yielding commercial strain of Penicillium patulum. The

results obtained in this study turned out to be very complicated. Rapid metabolic turnover of exogenous acetate resulted in multiple labelling of individual molecules and 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 C1 pool into the methoxyl carbons.[32]

However, the hypothesis was soon proven to be correct when a study on the xanthone, ravenelin (16), was carried out. Incorporation of [1,2-$^{13}C_2$]acetate into ravenelin in cultures of Helminthosporium ravenelii resulted in the predicted randomization of ^{13}C-^{13}C couplings in ring C. This is consistent with the intermediacy of a symmetrical benzophenone intermediate (Scheme 1.4), which is derived from cleavage of an anthraquinone.[33] This type of information has subsequently found use in biosynthetic studies.

SCHEME 1.4



(16) (16)

1.4 Isotope-Induced Shifts in ^{13}C n.m.r.

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 origins and fates of the hydrogen and oxygen atoms. Hydrogen and oxygen can be monitored directly by using n.m.r. active isotopes ^{2}H, ^{3}H, or ^{17}O.[10,34] However, all of these have distinct disadvantages and a great advance has been made by the use of ^{13}C as a reporter nucleus in labelling studies. The presence of ^{2}H alpha or beta to a ^{13}C atom can be deduced from the appearance in the proton noise decoupled ^{13}C n.m.r. spectrum of ^{13}C-^{2}H coupling and/or an isotope induced shift. Similarly the presence of ^{18}O alpha to a ^{13}C atom can be detected by an isotope induced shift in the ^{13}C n.m.r. spectrum. Before discussing these methods some general points to note are:[35]

a) Substitution by a heavy isotope shortens the average length of the bond holding the isotope, and, less so, the remaining bonds of the molecule. This almost always shifts the n.m.r. signal of neighbouring nuclei to higher field, although the magnitude of the shift is inversely dependent on the remoteness of the substitution.

b) The shift is largest where the fractional change in mass is largest and is also proportional to the number of atoms substituted.

c) The magnitude of the shift decreases with an increase in 's' character of the bond holding the isotope and depends also on the resonant nucleus, correlating with the range of shifts observed for that nucleus.

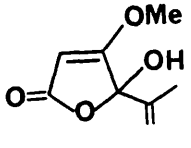
1.5 Tracing the fate of hydrogen atoms

1.5.1 Tritium n.m.r.[36]

The nuclear properties of tritium are ideal for detection by n.m.r. spectroscopic methods.[10] The magnetogyric ratio is the highest known for any isotope. The nuclear spin of ½ results in proton like spectra with narrow width lines being obtained. Chemical shift values are very similar to those of the equivalent proton. Hence signals can be readily assigned by reference to the ^{1}H n.m.r. spectrum. Coupling constants are also easily evaluated as the ratio of coupling constants $J(^{3}H-^{1}H)/J(^{1}H-^{1}H)$ is proportional to the ratio of magnet ogyric constants (1.067). The Nuclear Overhauser Effect (n.O.e.) observed with tritium is small and negative, the

theoretical maximum being 47% compared with 199% with ^{1}H decoupling.[37] Tritium n.m.r. spectra may therefore be integrated to give a measure of isotopic content at each site which has been found to agree reasonably well (±10%) with tritium analysis by conventional counting methods. Its extremely low natural abundance further enhances the sensitivity of detection of tritium label. Unfortunately, the relatively high radioactivity that is associated with the levels of tritium that are observable by ^{3}H n.m.r. spectroscopy discourages its use. It is not uncommon for well in excess of 100μCi to be required at a single site if it is to be detected, even with high field n.m.r. instruments.

The first reported biosynthetic application of ^{3}H n.m.r. was a study of [^{3}H]acetate into penicillic acid (17)[38] a metabolite of Penicillium cyclopium which has been studied extensively with carbon isotopes.[39]



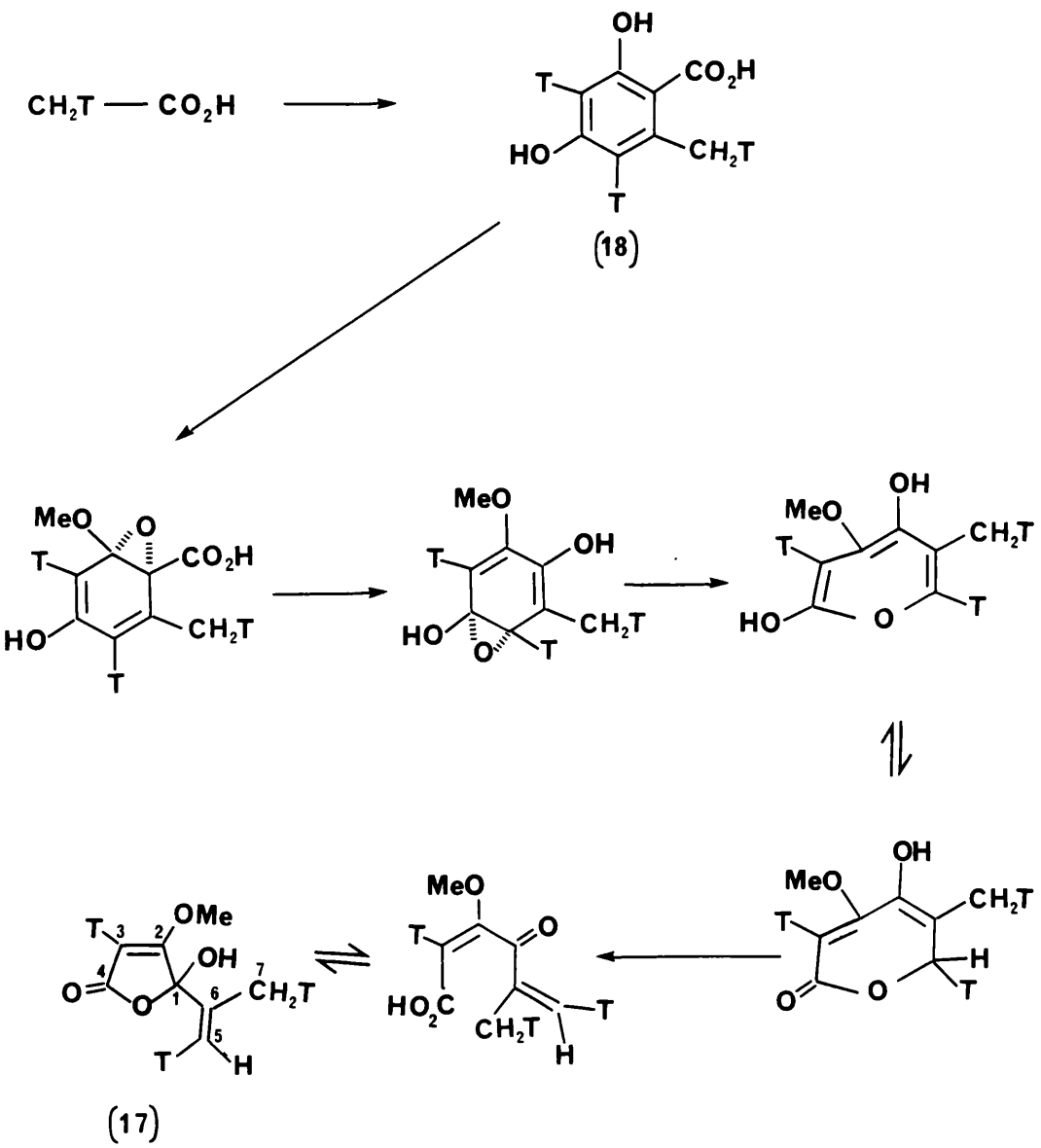
(17)

In fact, more information came directly from the use of tritium labelled precursors and ^{3}H n.m.r. spectroscopy than from the isotopic carbon studies.[40] This was because of the ability of isotopic hydrogen to distinguish between non-equivalent hydrogen atoms at sp^2 methylene groups.

The ^{3}H n.m.r. spectrum of the penicillic acid (17) was assigned by reference to the corresponding ^{1}H n.m.r. spectrum. Tritium was found to be present at the 3-, 5- and 7-positions, consistent with the mode of biosynthesis as outlined in Scheme 1.5.

The 7-position showed greatest incorporation of tritium (least exchange loss of label), with the 3- and 5-positions having progressively less tritium. This is in agreement with chain initiation by an acetate methyl group rather than an activated chain building methylene group derived from malonate via acetate. The 5-methylene group in penicillic acid (17) had preferentially been labelled <u>trans</u> to the 7-methyl group as can be seen from Scheme 1.5. Subsequent incorporation studies using [$^{3}H_2$]malonate led to labelling of only the 3- and 5-positions of penicillic acid. In addition [3,5-$^{3}H_2$]orsellinic acid (18) was established as an advanced precursor by being incorporated into penicillic acid with the same

SCHEME 1.5


CH2T — CO2H
OH
T
CO2H
HO
CH2T
T
(18)
MeO
O
T
CO2H
HO
CH2T
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OH
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MeO
CH2T
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OMe
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OH
4
O
O
1
7
CH2T
6
5
T
H
MeO
T
O
HO2C
CH2T
T
H
(17)

distribution of label between the 5-trans and 5-cis positions as from simple precursors.

1.5.2 Deuterium n.m.r.

The magnetic properties of deuterium and the advantages and drawbacks of ^{2}H n.m.r. spectroscopy in biosynthetic applications have been reviewed.[10] The major disadvantages are the inherently low sensitivity for detection of n.m.r. (relative to ^{1}H or ^{3}H). The chemical shift values obtained in deuterium n.m.r. spectra are closely similar to those of the equivalent protons; however the scale in Hz is only 15% that of the proton n.m.r.[10] Coupling constants $J(^{2}H-^{1}H)$ are approximately one sixth the value of $J(^{1}H-^{1}H)$. The relaxation behaviour of deuterium is dominated by a quadrupole exchange mechanism and this results in extensive line broadening. The two major limitations of deuterium n.m.r. are spectral crowding and poor resolution, but there is one major advantage over other n.m.r. techniques. The short relaxation times combined with the absence of an n.O.e. effect, minimise the possibility of partial saturation. The extent of enrichment in a partially deuteriated molecule may therefore be determined accurately by integration.

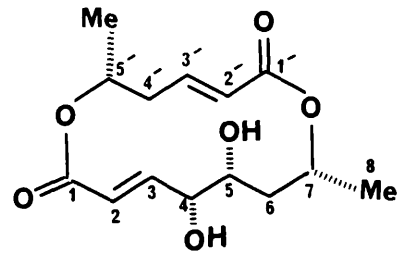
Other advantages of using deuterium n.m.r. in

biosynthetic study are, firstly, that it is an inexpensive tracer which, unlike tritium, does not require special handling. Secondly, compared with ^{13}C (natural abundance 1.1%), the low natural abundance (0.016%) enables the incorporation of deuterium-labelled precursors to be positively identified even after 6600 fold dilution of the precursor in the metabolic pool.[14] The incorporation of molecules singly labelled with ^{13}C can only be detected with confidence at dilution levels of less than 100 fold. Thus, ^{2}H n.m.r. is effectively sixty times more sensitive than ^{13}C n.m.r. when applied to biosynthetic study.

Deuteriated precursors that are used in biosynthetic study now employ some form of multiple labelling with another isotope (usually ^{13}C). However, direct ^{2}H n.m.r. is often the most reliable way to determine the actual content of deuterium at a particular site. In the biosynthesis of polyketides, a starter acetate unit (incorporated without the intermediacy of malonate) usually retains most of the deuterium of [$^{2}H_3$]-acetate. However, extensive loss of the hydrogen label, by exchange with the medium, often occurs at subsequent units along the chain. The number of deuterium atoms at a particular site, their relative stereochemistry, and the extent of exchange can provide

valuable clues to the process of assembly of polyketides. Even though a majority of studies now employ detection of ^{2}H by ^{13}C n.m.r. (see Section 1.6.1), deuterium n.m.r. provides an important check on deuterium content and is often the most direct means to obtain stereochemical information.

Recent work on the formation of colletodiol (19) produced by a Cytospora species illustrates that ^{2}H induced β-isotope shifts in ^{13}C n.m.r. spectra (Section 1.6.1) can have values close to zero. This may have given the impression that deuterium is absent



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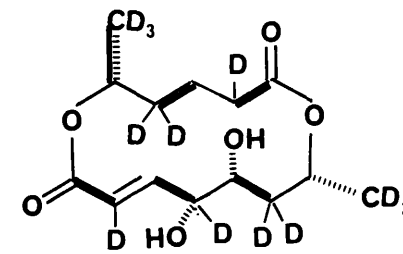
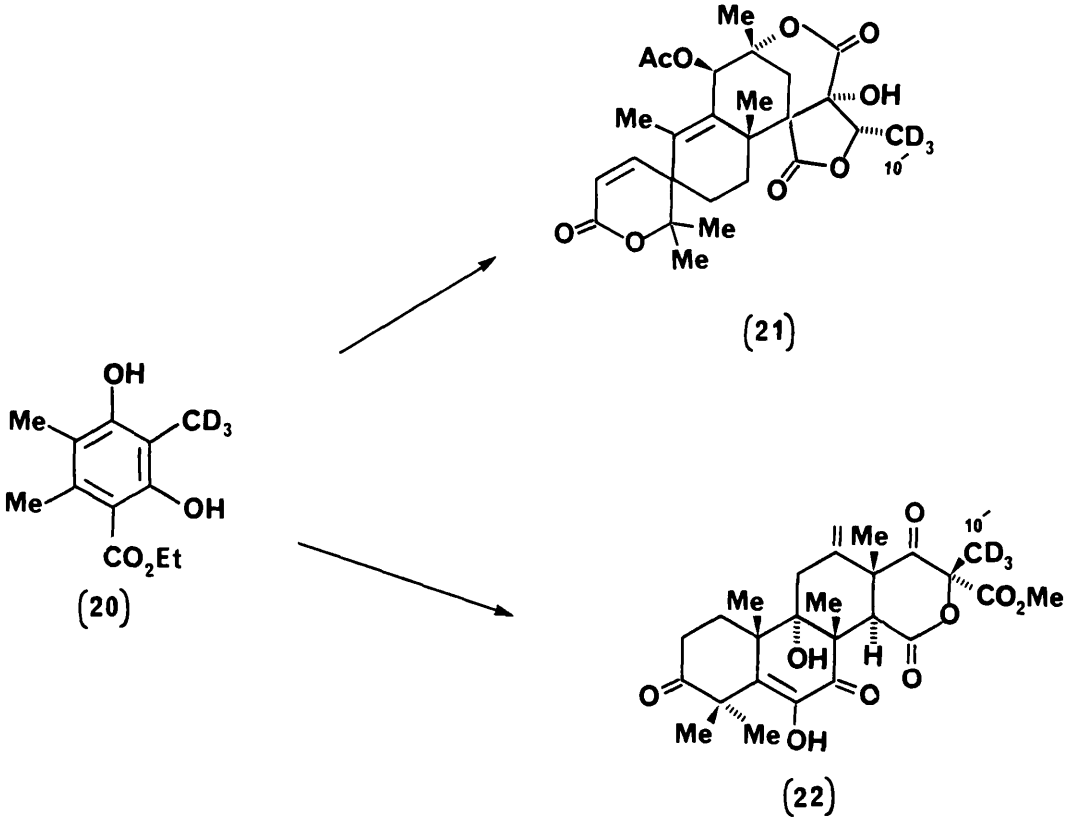


at an adjacent site. However, the use of direct ^{2}H n.m.r. revealed that deuterium was there.[41] [1-^{13}C, $^{2}H_3$]Acetate was fed and administered to cultures of Cytospora Sp. (ATCC, 20502). No ^{2}H isotope induced shifts could be observed for C-1 or C-11 in the proton noise decoupled ^{13}C n.m.r. spectrum of colletodiol. However, carbonyl groups are known to be poor reporter groups[42] for ^{2}H shifts and the presence of ^{2}H label at both C-2 and C-2' was shown by ^{2}H n.m.r. analysis of the enriched metabolite.

The low natural abundance of deuterium, makes it a very sensitive probe for studying the incorporation of advanced precursors into secondary metabolites. Simpson et al[43] whilst working on the formation of austin (21) from Aspergillus ustus and of terretonin (22) from Aspergillus terreus fed a sample of ethyl-3,5-dimethylorsellinate (20), bearing three deuterium atoms in the methyl group at C-3, to the cultures. The ^{2}H spectra of the two enriched metabolites (21) and (22) each consisted of only one signal, corresponding in chemical shift to the hydrogen atoms of the methyl group C10´.



1.6 Detection of Deuterium by ^{13}C n.m.r.

1.6.1 Deuterium that is directly attached to ^{13}C

Tracing the fate of hydrogen is an important aspect of biosynthesis which allows detailed information to be gained concerning the pathway under investigation.

Both of the direct n.m.r. methods discussed in the previous two sections allow the average degree of enrichment at each labelled site to be measured. They therefore yield evidence for the retention or transfer of hydrogen, from which it is possible to deduce information about transformations in the carbon skeleton. An attractive alternative is to substitute deuterium for hydrogen that is directly attached to a ^{13}C nucleus. This is an extremely powerful tool for detection of the label and can be seen to great effect in the case of complex polydeuteriated molecules. The high dispersion of values of chemical shifts that is available in the carbon spectrum can overcome the problems of overlap of peaks that are common with ^{2}H n.m.r. or ^{1}H n.m.r. methods. In addition, double labelling of a precursor with ^{2}H and ^{13}C can be used as a marker to show that a bond has remained intact, in a fashion analogous to labelling with two ^{13}C nuclei.

The effects on n.m.r. spectra of the substitution of deuterium for hydrogen that is attached to carbon-13 are described in reviews by Garson and Staunton[10] and by Stothers.[44] Each deuterium atom shifts the position of resonance of a directly attached nucleus upfield by 0.3 to 0.6 p.p.m.[45] The presence of each additional deuterium shifts the signal a further 0.3 to 0.6 p.p.m. upfield and increases the multiplicity of the resonance. This is because deuterium has a nuclear spin of one, thus ^{13}CD appears as a triplet of lines

of equal intensity, a $^{13}CD_2$ group produces a quintet (1:2:3:2:1) and a $^{13}CD_3$ produces a septet resonance (1:3:6:7:6:3:1). The different signals for a methyl group overlap to a certain extent and are also superimposed upon the normal signal for protonated nuclei. A partially deuteriated methyl thus gives rise to a complex signal comprising lines from $-CD_3-$, $-CD_2H-$, $-CDH_2-$ as well as $-CH_3$ labelled species.

The spectral analysis can be simplified by re-running the spectrum with deuterium decoupling instead of proton decoupling. Interference from protonated nuclei is greatly reduced in this mode of operation because of the loss of n.O.e.

$^{13}C-^{2}H$ n.m.r. is limited by the relative weakness of the $^{13}C-^{2}H$ n.m.r. signal.[46] This is the result of three factors

1) $^{13}C-^{2}H$ relaxation is much less efficient than $^{13}C-^{1}H$ relaxation, since the rate of relaxation is proportional to the square of the magnetogyric ratio.

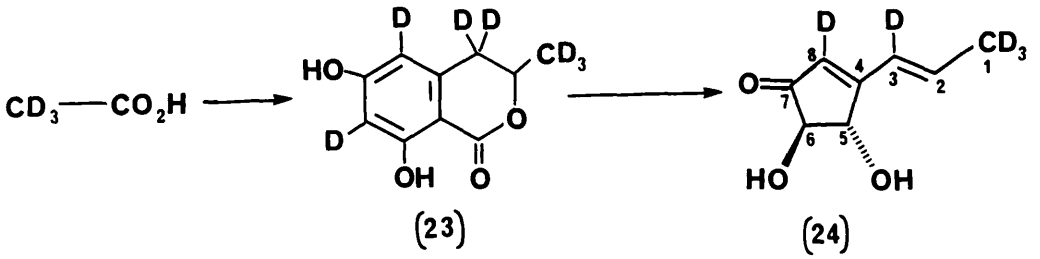
2) The replacement of hydrogen by deuterium is accompanied by loss of n.O.e. enhancement.

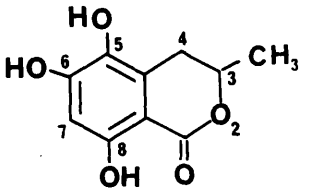
3) Increased multiplicity causes loss of signal strength in individual lines.

1.6.2 Application to polyketide biosynthesis

Often much of the hydrogen label is lost by exchange from the activated methylenes of the polyketide chain. However, the possibility remains that more than one isotopic label may be retained at positions derived from the methyl group of acetate. The number of deuteriums detected at a given site can be used to identify the chain starter unit or to distinguish between possible biosynthetic intermediates. This can give information about the mode of biosynthesis. The extent of deuterium retention may be correlated to the amount of hydrogen exchange during biosynthesis. This may provide clues to the complex mechanisms involved in the construction of the carbon skeleton.

An example of this technique used in studies of polyketide biosynthesis was completed in 1977 with two reports on terrein biosynthesis.[47,48] Terrein (24), a metabolite of Aspergillus terreus, has been extensively studied with carbon isotopes.[49] The biosynthesis is known to proceed through the condensation of five acetate units to give, after aromatisation, the dihydroisocoumarin (23) which then undergoes an interesting ring contraction.





In the proton-decoupled ^{13}C n.m.r. spectrum of terrein enriched with [2-^{13}C, 2-$^{2}H_3$]acetate, the signal for C-5 was enriched five-fold relative to that for C-6 which was unlabelled. Using C-5 as internal standard (since the proton at this position is derived from the medium) the signals for C-3, C-8 and C-1 were less intense than expected, indicating the presence of a deuterium. The signal for C-1 was a broad envelope of peaks from which signals at 18.4 and 18.7 p.p.m., indicated respectively, molecules containing $-CH_3$ and $-CH_2D$ at this position.

A more detailed analysis was undertaken after

re-running the spectrum with deuterium decoupling. They showed that the C-1 was present as CD_3 as well as CD_2H. The detection of some molecules with three deuteriums at C-1 confirmed that this methyl is derived intact from C-2 of acetate and is therefore part of a chain starter unit. This technique provides a useful alternative to the standard procedure of using either ^{14}C or ^{13}C-labelled malonate to pick out the chain starter unit. It was also shown that after deuterium decoupling two sharp singlets were seen at C-3 and C-8 thus indicating that both carbons carry deuterium. The retention of some deuterium at C-8 rules out the involvement of a 5-hydroxyderivative (25) in terrein biosynthesis.

1.6.3 Deuterium that is two or more bonds away from ^{13}C

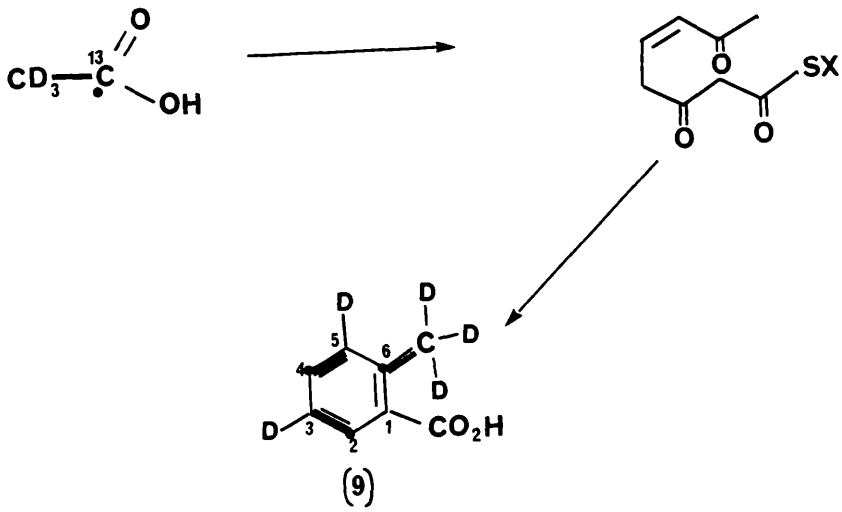
For complex polydeuteriated molecules, though deuterium spectroscopy is very sensitive, the ^{2}H n.m.r. often include broad overlapping signals. The peaks in the ^{13}C n.m.r. spectra from carbon atoms that are directly attached to deuterium suffer from poor relaxation, increased signal multiplicity, and loss of n.O.e. The spectra can be further complicated by the overlap of signals due to co-occurrence of different

isotopic species at a particular site.

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. The isotope shift, although reduced, is still observable. The β-hydrogens only contribute markedly to the relaxation of non-protonated ^{13}C nuclei. Therefore the shifted signals otherwise retain any n.O.e. effect also experienced by the unshifted signals over proton decoupling. Geminal carbon-proton couplings are generally small,[15] and carbon-deuterium couplings are over six times smaller again: thus the shifted signals are effectively singlets, even without deuterium decoupling. This gives a further increase in the signal to noise ratio compared with the corresponding α-shift experiment.

The method was first applied by Abell and Staunton[50] to biosynthesis in a study of 6-methylsalicylic acid (9).

[1-^{13}C, 2-$^{2}H_3$]acetate was fed to cultures of Penicillium griseofulvum. The proton noise decoupled ^{13}C n.m.r. spectrum of the methyl ester of the resulting 6-methylsalicylic acid showed shifted signals for C-2, C-4 and C-6. This corresponded to the presence of deuterium label at positions 3, 5 and 7

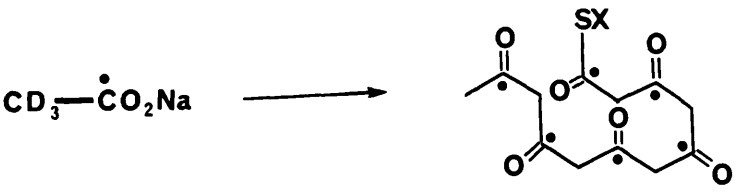


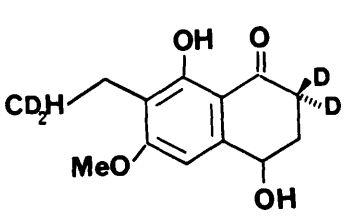
(9)

respectively. Thus this method permits the detection of two adjacent bonds which have remained intact during biosynthesis.

The β-isotope shifts that are observed are most frequently upfield (0.01 to 0.1 p.p.m. per deuterium) but they can also be downfield or zero especially if next to a carbonyl.[41,51-53] Simpson and Stenzel[53] showed β-shifts to be additive whilst working on O-methylasparvenone (26a), a metabolite from Aspergillus parvulus.

[1-$^{13}C_2$,$^{2}H_3$]Acetate was incorporated into





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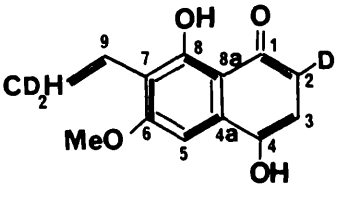


(26a)

O-methylasparvenone (26a). The resulting proton noise decoupled ^{13}C n.m.r. spectrum showed that C-9 had two isotopically shifted signals corresponding to the presence of one and two deuteriums on the adjacent methyl carbon. The furthest shifted signal was (+)0.16 p.p.m. (upfield) for C-9 which corresponds to (+)0.08 p.p.m. per deuterium and clearly shows that the β-shift is additive. However C-1 showed a downfield shifted resonance ((-)0.06 p.p.m.) with the intensity being much lower than that expected from the observed level

of incorporation of deuterium to the 2-axial position. The amount of deuterium label found at the 2-axial position was determined by ^{2}H n.m.r. In addition, as a consequence of a high level of multiple labelling of individual molecules from the precursor, the ^{2}H at the 2-axial position resulted in a small isotopically shifted resonance ($^{(+)}$ 0.13 p.p.m.) being observed for C-3.

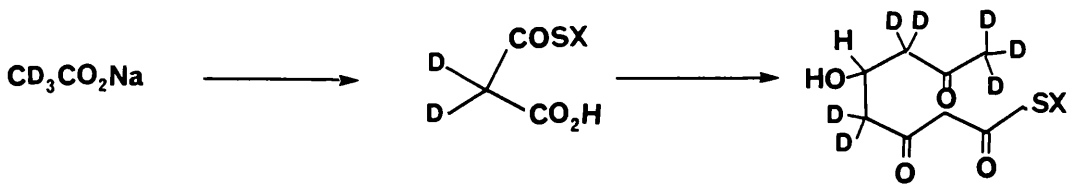
[2-$^{2}H_{2}$]-O-Methylasparvenone (26b) was prepared by base-catalysed exchange (NaOMe in Me$O^{2}H$). The p.n.d. ^{13}C n.m.r. spectrum showed isotopically shifted resonances at $^{(-)}0.08$ and $^{(+)}0.17$ p.p.m. for C-1 and C-3 respectively. This suggests that either the induced isotope shifts are non-additive or that equatorial ^{2}H has a much smaller effect than axial ^{2}H. They examined this further by carrying out a second exchange experiment in equimolar MeOH and Me$O^{2}H$. Deuterium n.m.r. spectroscopy of the product confirmed that equal exchange of the axial and equatorial hydrogen had occurred. The p.n.d. ^{13}C n.m.r. spectrum for C-3 showed three isotopically shifted resonances at $^{(+)}0.03$, $^{(+)}0.13$, and $^{(+)}0.17$ p.p.m. of similar intensities. This was as expected for the statistical mixture of molecules labelled at the 2-equatorial and 2-axial positions separately, and both 2-equatorial and 2-axial positions. C-1 also showed three isotopically shifted resonances at $^{(-)}0.02$, $^{(-)}0.06$, and $^{(-)}0.08$

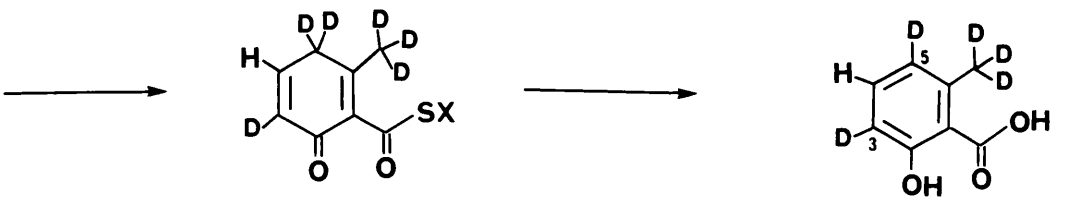
p.p.m., but their intensities decreased as the number of deuterium atoms increased. These results show that the β-isotope shifts are additive and moreover are dependent on the stereospecificity of labelling. A further example of this is found in Chapter 2 when [1-^{13}C, 2-$^{2}H_3$]acetate was fed to Periconia macrospinosa and the metabolites isolated showed similar results.

To ensure that all deuterium labels have been detected correctly, results from the β-isotope shift technique are often checked by another method, usually ^{2}H n.m.r.

The incorporation of [1-^{13}C, 2-$^{2}H_3$]acetate into polyketides has provided a wealth of information about possible intermediate oxidation states and mechanisms of cyclisation. The incorporation of mono- and tri-deuteriated acetate precursors into 6-methylsalicylic acid (9) has been studied in order to investigate the degree of stereocontrol in the aromatisation sequence.[54] A possible sequence of late enzyme-bound intermediates on the pathway to (9) is outlined in Scheme 1.6 and the consequence of using [$^{2}H_3$]acetate which would produce [2-$^{2}H_1$] malonate are compared. In the actual experiments, the relative amounts of ^{2}H label retained at C-3 and C-5 were compared using both direct ^{2}H n.m.r. and indirect ^{13}C n.m.r. analysis (β-isotope shifts) using

SCHEME 1.6





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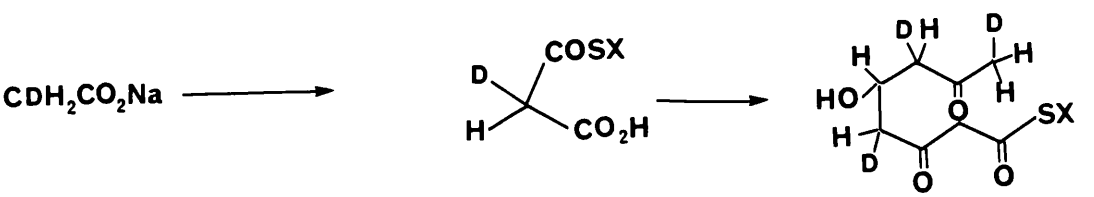
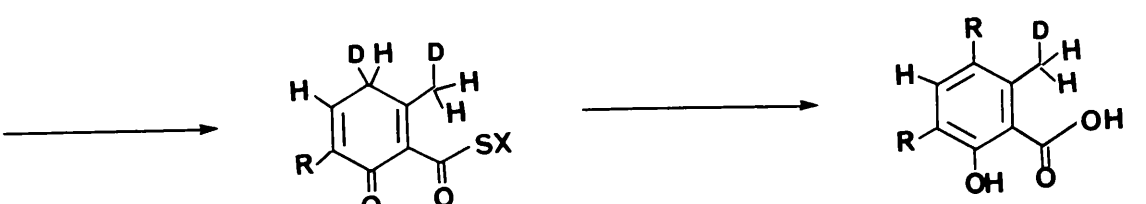




(9)

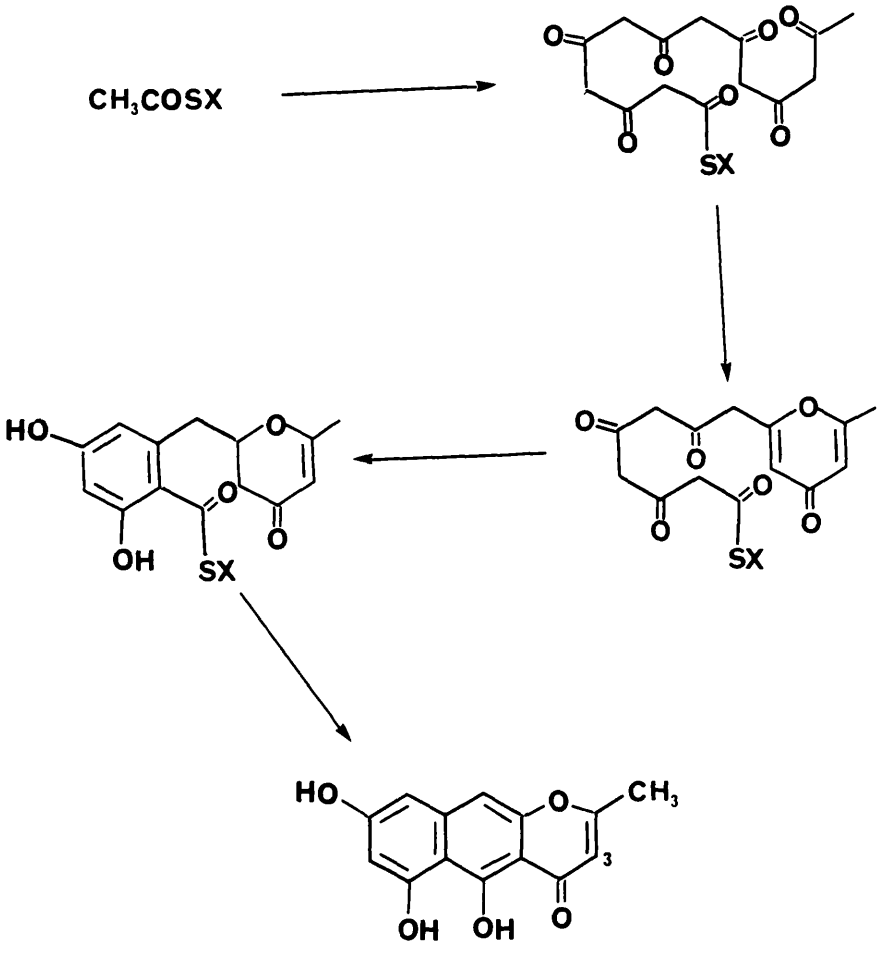
R = H or D

mono- and tri-deuteriated acetates. In both cases, the relative amounts of ^{2}H retention at both C-3 and C-5 were the same. The ^{13}C n.m.r. experiment indicated that the necessary hydrogen removal from the C-3 and C-5 methylenes during aromatisation occurs with no isotope effect. Overall, the results are therefore consistent with stereospecific hydrogen removal at both C-3 and C-5 in the aromatisation sequence. This in turn indicates that aromatisation occurs in a chiral environment and so the steps required are under enzymatic control. This experiment provided the first direct evidence to support this long held assumption.

The biosynthesis of the naphthopyrone rubrofusarin (27) from Fusarium culmorum has been studied.[55] Incorporation of both [1,2-$^{13}C_2$] and [1-^{13}C, 2-$^{2}H_3$]acetates substantiate the folding pattern as shown in Scheme 1.7.

The important point to note is that the β-isotope shift has shown an unusual folding pattern, which contrasts with the folding pattern assumed for this and several other metabolites in many textbooks. Deuterium n.m.r. analysis of [$^{2}H_3$]acetate enriched rubrofusarin (27) revealed a surprisingly high retention of ^{2}H label at C-3 when compared to similar γ-pyrone positions in previous studies. This led to the proposal that the closure of the γ-pyrone ring occurs early in the pathway as shown in Scheme 1.7. As for 6-methyl-

SCHEME 1.7

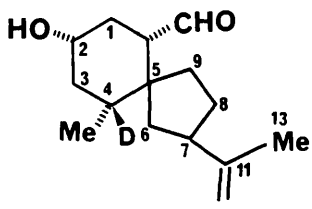


(27)

salicylic acid (9) discussed above, on feeding [$^2H_1$]acetate and comparing the relative enrichments with those observed when feeding [$^2H_3$]acetate, no significant differences were observed. This provides more support for the theory that the removal of the extra hydrogens from methylene sites during

aromatisation must be under enzymatic control.

The detection of deuterium-induced β-isotope shifts in ^{13}C n.m.r. is an excellent tool for observing the migrations of hydride that commonly occur during the biosynthesis of terpenoids. These migrations can also be detected by using advanced doubly labelled precursors in conjugation with ^{2}H n.m.r. The 'β-hop' technique of Stoessl and Stothers[56,57] provides a very attractive alternative in cases where there is severe overlap of ^{2}H resonances. These workers studied the incorporation of sodium [2-^{13}C, 2-$^{2}H_3$]acetate into potato tubers that were being stressed with Monilinia fruticola and isolated the resulting phytoalexins lubimin (28), hydroxylubimin (29), and rislutin (30).



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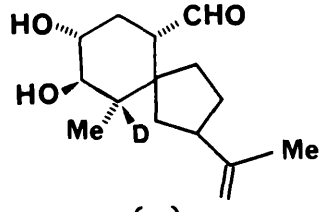

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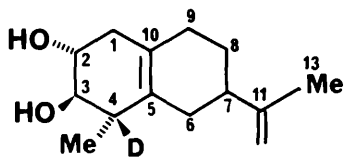


(30)

In each of these compounds, deuterium was originally directly attached to ^{13}C at C-5 of an intermediate cationic species. This underwent a 1,2-migration of hydride to C-4, thereby causing a β-isotope shift at C-5 in the ^{13}C n.m.r. spectrum.

1.7 Determining the Source of Oxygen Atoms

1.7.1 Labelling with Oxygen-17

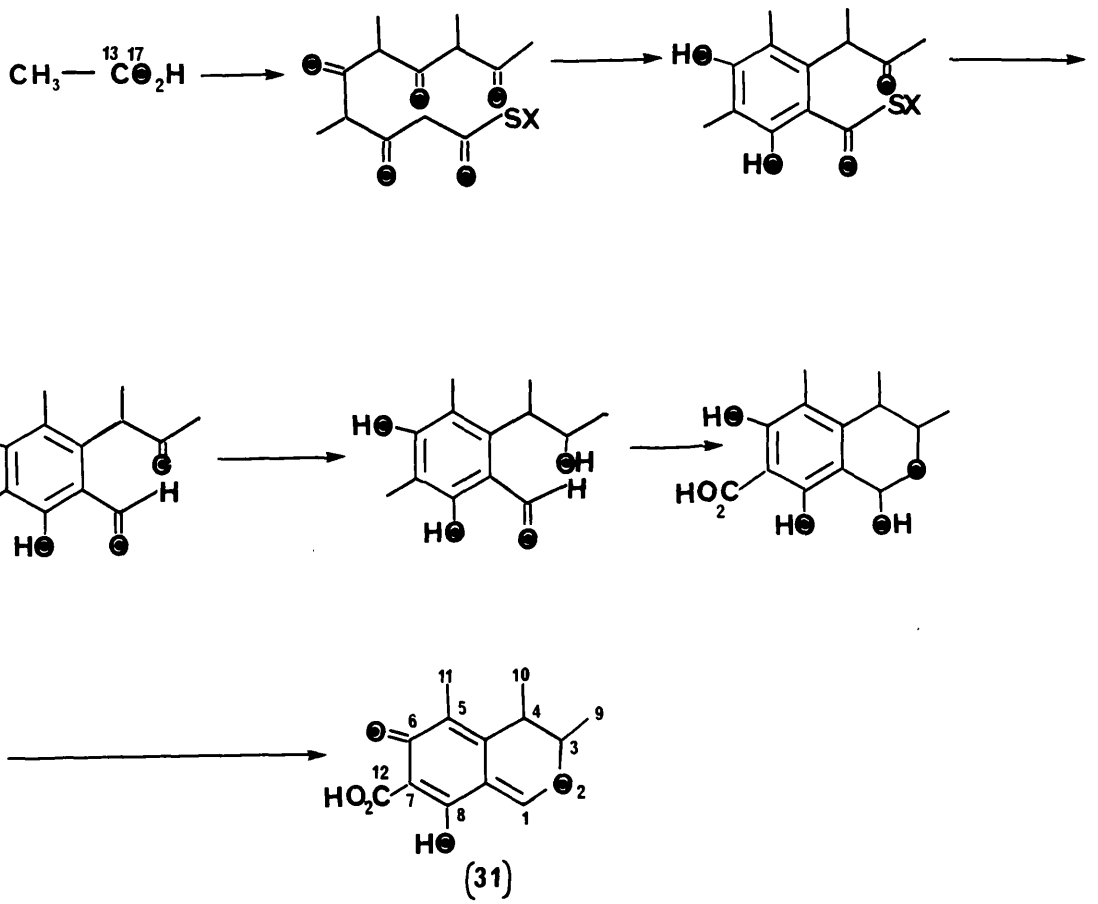
Two less abundant isotopes of oxygen are readily available for labelling studies. These are oxygen-17 (natural abundance 0.037%) and oxygen-18 (natural abundance 0.204%). Both are routinely detected by mass spectrometry,[58] but only oxygen-17 has a nuclear spin (5/2) and can be directly observed by n.m.r. spectroscopy.[59,60] Extensive reviews of ^{17}O n.m.r. techniques are available.[57,58] The use of ^{17}O in biosynthetic studies has been partly limited by its expense. However the major problems have been low n.m.r. sensitivity and very broad lines in its n.m.r. spectra due to its quadrupole. Although the range of chemical shifts is very large (>1,500 p.p.m.), complex polyoxygenated molecules with ^{17}O labels at multiple sites can show extensive overlap of resonances in their n.m.r. spectra. However, the common contamination of relatively inexpensive ^{18}O-labelled

compounds by ^{17}O [61] and the possibility of using lanthanide shift reagents[62] may encourage the greater use of ^{17}O n.m.r. in biosynthetic work.

The first successful application of ^{17}O n.m.r. to biosynthetic work was performed by Sankawa *et al*[34,63] when studying the biosynthesis of citrinin (31) a metabolite from *Aspergillus terreus* (Scheme 1.8).

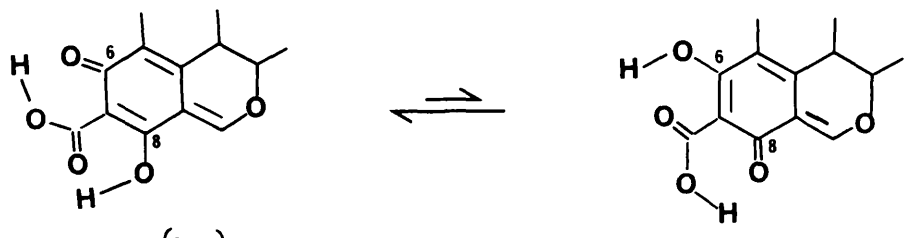
Different feedings were carried out on the metabolite. [2-^{13}C, 2-$^{2}H_3$] and [1-^{13}C, $^{18}O_2$]acetate were fed to *Aspergillus terreus* and the results obtained are consistent for the proposed pathway (Scheme 1.8). The feeding of [1-^{13}C, ^{17}O]acetate also gave positive results. In the ^{17}O n.m.r. spectrum, three signals of the labelled citrinin (31) were obtained and were tentatively assigned according to published data.[64,65] ^{17}O chemical shift values are very sensitive to the changes of electron density on oxygen atoms. The chemical shift value of C-6 oxygen (279 p.p.m.) is infact smaller than those of normal carbonyl groups. The shifts of C-8 oxygen and O-2 (179 and 148 p.p.m. respectively) are larger than those observed for phenol or enol groups.[65,66] These facts may be explained by keto-enol tautomerism of citrinin (31a and 31b).

SCHEME 1.8



(31)

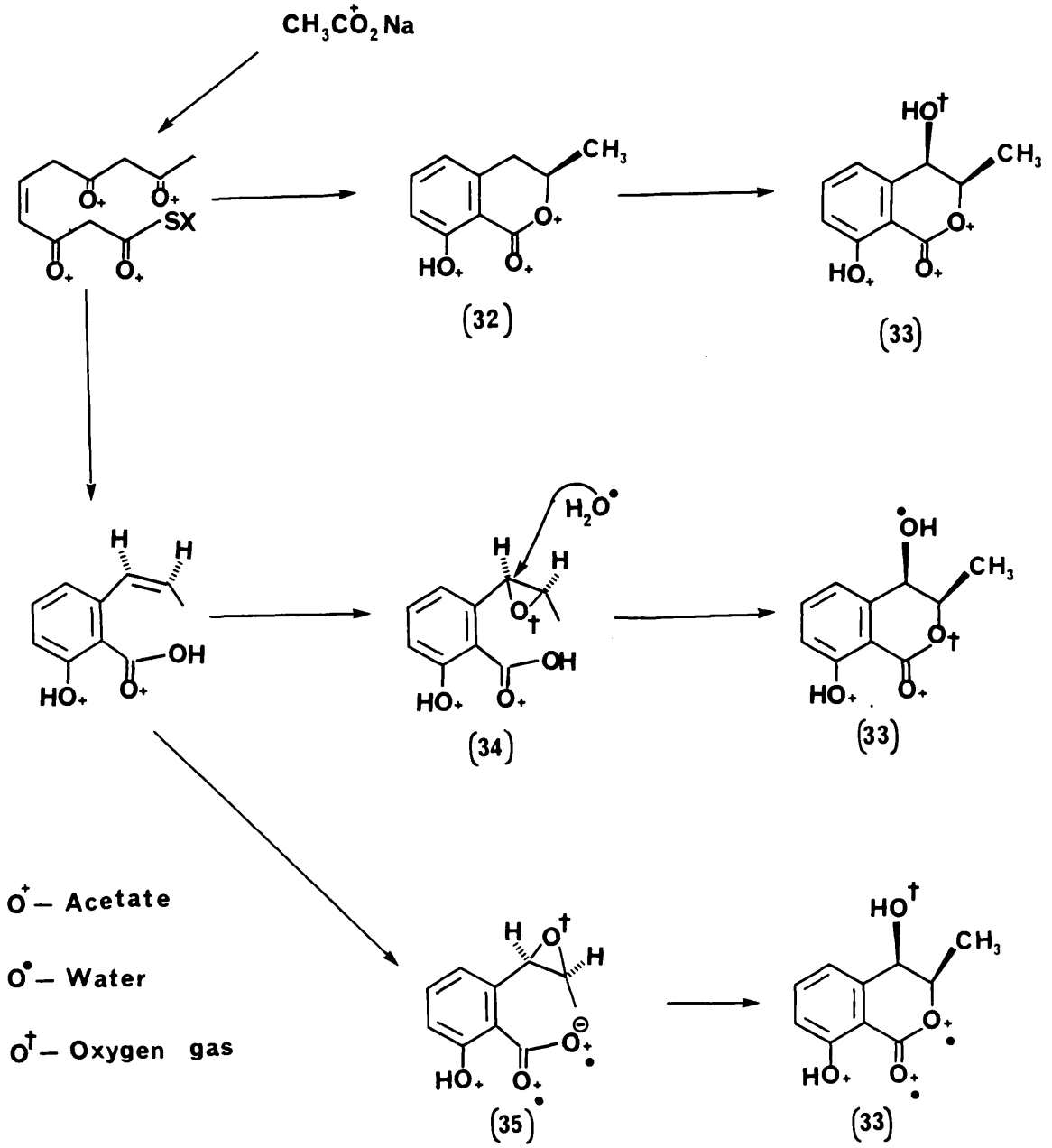
A recent application of the use of ^{17}O n.m.r. in biosynthesis has been performed by Staunton et al[67]



(31a) (31b)

who studied the biosynthesis of hydroxymellein (33) from Aspergillus melleus. The origin of the four oxygen atoms in hydroxymellein was studied using ^{17}O n.m.r. spectroscopy. The four resonances were assigned

SCHEME 1.9



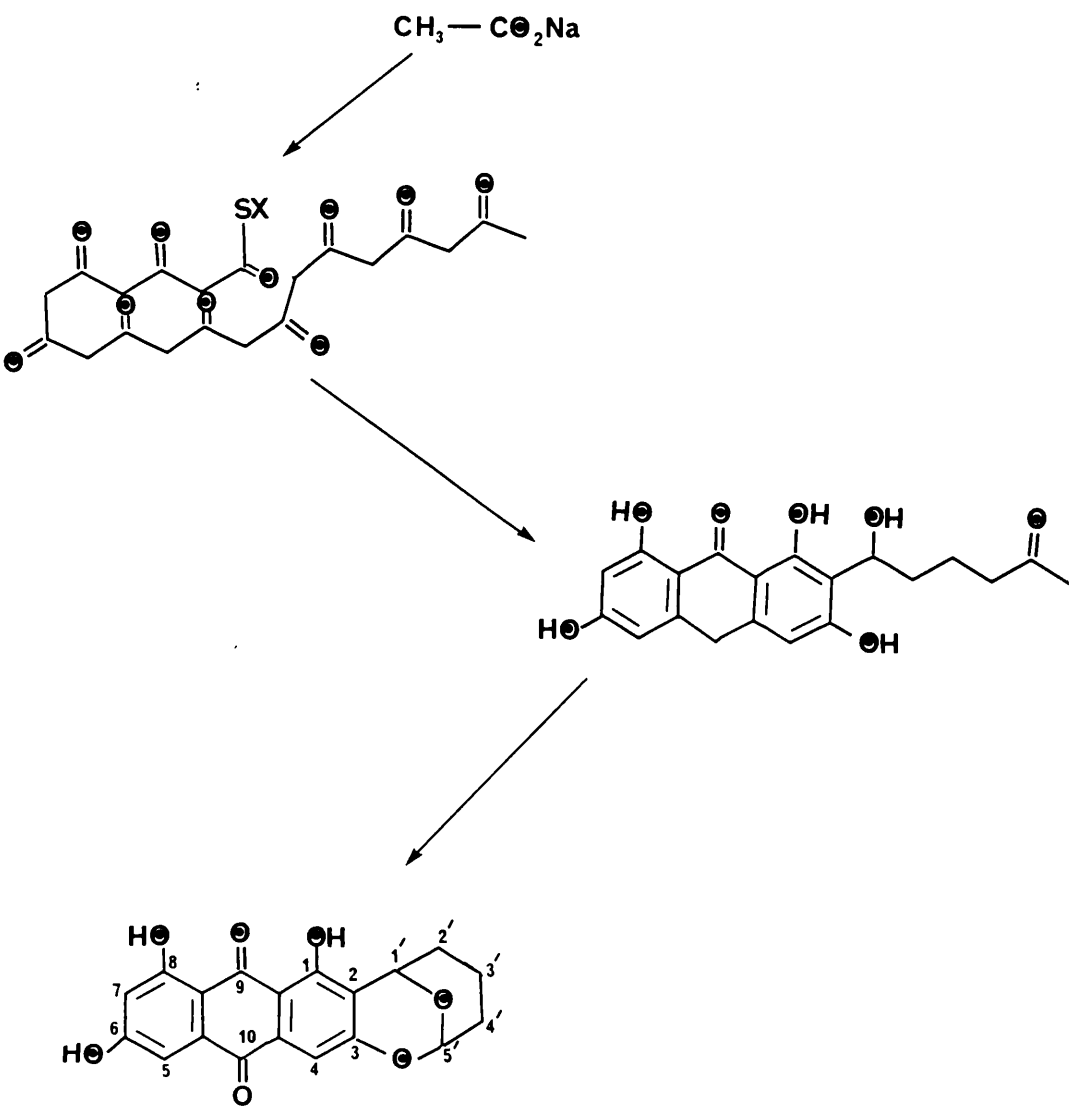
on the basis of chemical shift[59] to the carbonyl oxygen, O-2, the phenol oxygen and the C-4 hydroxyl group. The spectrum obtained after incorporation of [^{17}O] acetate into hydroxymellein (33) showed incorporation from acetate into all the oxygens except the C-4 hydroxyl group. They grew the fungus in a closed system under an atmosphere of oxygen, 20% enriched with ^{17}O, this yielded hydroxymellein labelled uniquely and unambiguiously in the C-4 hydroxy group. These results are consistent with (33) being derived by stereospecific hydroxylation of mellein (32). These results also ruled out the possibility of epoxides (34) and (35), Scheme 1.9.

1.6.2 Labelling with Oxygen-18, Detection by ^{13}C n.m.r.

The indirect detection of oxygen-18 by ^{13}C n.m.r. spectroscopy is a powerful means by which the position and the extent of labelling in complex polyoxygenated molecules can be directly detected. Investigations by Boyer and co-workers,[68] Risley and Van Etten,[69] Davensbourg et al,[70] and Vederas[71] confirmed Jameson's prediction[72] that isotopic substitution with oxygen-18, induced an upfield shift in the position of nuclear magnetic resonances of a directly attached carbon-13 atom. The magnitudes of these isotopic shifts range from about 0.010 to 0.035 p.p.m.

for singly bonded oxygen atoms and from about 0.030 to 0.055 p.p.m. for doubly bonded oxygen atoms. Their sizes have been tabulated and structural influences on this effect have been reviewed.[45] The first application in biosynthesis was reported in 1980.[73]

SCHEME 1.10



(36)

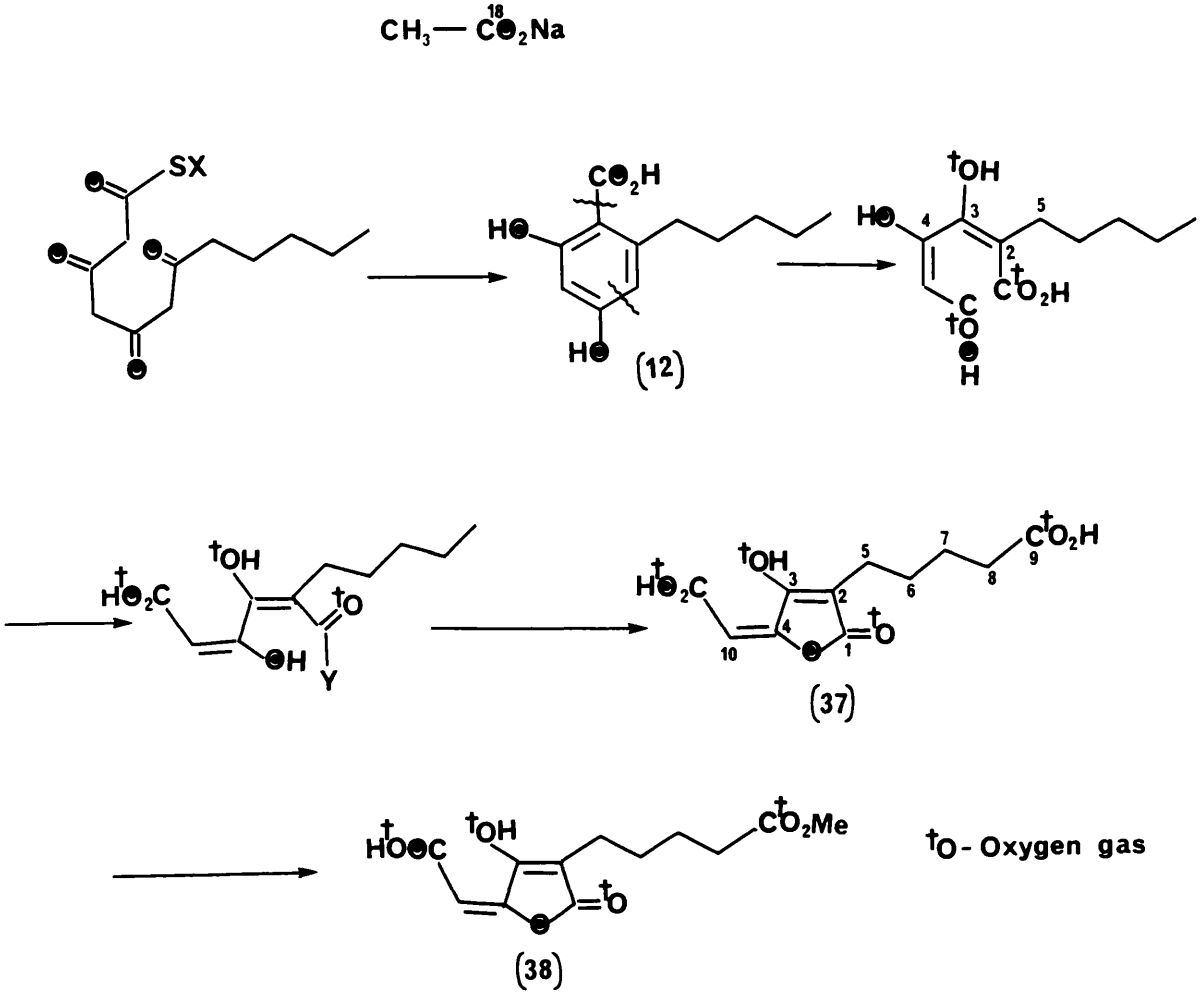
Sodium [1-^{13}C, $^{18}O_2$]acetate administered to a mutant of Aspergillus parasiticus gave labelled averufin (36). It was shown that there was an intact incorporation of acetate derived carbon-oxygen bonds at all positions except at C-10. The oxygen at C-10 was shown to originate from oxygen gas by allowing the fermentation to take place in an atmosphere that contained $^{18}O_2$ (Scheme 1.10).

The use of stable isotopes in biosynthetic studies can yield a wealth of information about possible intermediates on the pathway to the secondary metabolites which could not be obtained from radioactive isotopes.

An example of this is the metabolite multicolosic acid (37) produced by Penicillium multicolor. Incorporation experiments with sodium [1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, [1-^{13}C, $^{18}O_2$] acetate and 6-pentyl-resorcylic acid (12) have indicated that (37) is a polyketide metabolite derived from oxidative cleavage of an aromatic precursor (Scheme 1.11)[28,29]

In particular, labelling studies with [1-^{13}C, $^{18}O_2$]acetate have shown that the bond between C-4 and the lactone oxygen remains intact during the biosynthesis of (37). Also such an intact bond can be found in the C-11 carboxy group. Recently,

SCHEME 1.11



studies were carried out by Holker et al[19] who monitored incorporations with $^{18}O_2$ gas which defined the origin of all the oxygen atoms in multicolosic acid (37).

Incorporation of ^{18}O was detected by observation of upfield ^{18}O-induced shifts in the ^{13}C n.m.r. spectrum of (38). All carbons bearing oxygen, except C-4 showed α isotope shifts. Unexpectedly large

upfield β-isotope shifts (7.2, 22.3, 29.9 p.p.b.) were observed for C-2 which bears no oxygen. Since such isotope shifts are additive,[74] the 29.9 p.p.b. shift is caused by molecules of (38) bearing ^{18}O at both C-1 and C-3.

Generally ^{18}O-induced β-isotope shifts are less than 10 p.p.b.[74,75] Recently two other examples have been found in which an olefinic carbon two bonds away from an enolic oxygen displays a very large β-isotope shift.[76] On this basis it would appear that the 22.3 p.p.b. shift at C-2 results from molecules of (38) bearing ^{18}O at C-3 and only ^{16}O at C-1. Hence the 7.2 p.p.b. shift at C-2 is due to molecules bearing ^{16}O at C-3 and ^{18}O at the C-1 carbonyl oxygen. No β-shifts were found for C-10 or C-8.

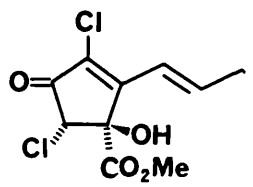
In the following chapters many of the techniques discussed above are used to study the biosynthesis of the metabolites cryptosporiopsinol, microline and dechloromicroline. The different methods are dealt with in the appropriate chapters.

CHAPTER 2

THE BIOSYNTHESIS OF CRYPTOSPORIOPSINOL

2.1 Introduction

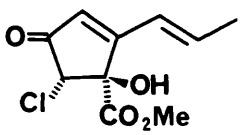
In 1969 the isolation of cryptosporiopsin (39) was first reported from the coprophilous fungus Sporomia affinis[77] and a Cryptosporiopsis sp. isolated from the yellow birch Betula alleghaniensis.[78] The structure of cryptosporiopsin (39) was demonstrated by chemical degradation, spectroscopic analysis and by an x-ray analysis.



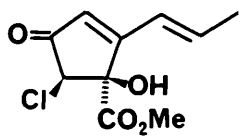
(39)

Thereafter, cryptosporiopsin was detected in extracts of Periconia macrospinosa.[79]

A number of chlorine containing metabolites are found with cryptosporiopsin. In Sporomia affinis it occurs with dechlorocryptosporiopsin (40) and its C-5 epimer (41) as well as the dihydroisocoumarins (42), (43) and (44).[80]



(40)



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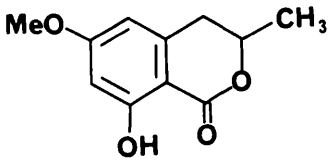

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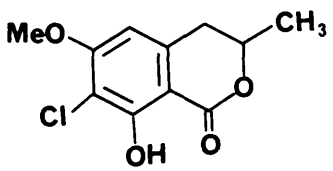

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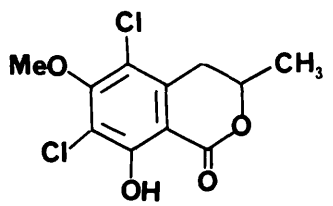

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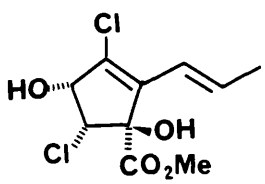

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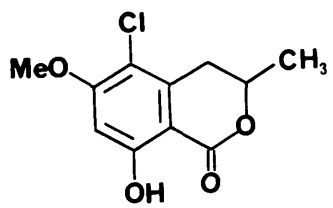

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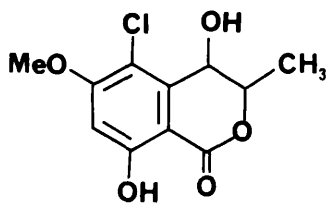

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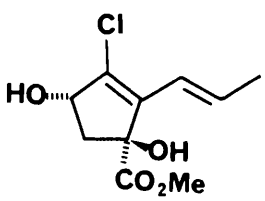

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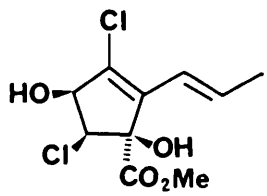
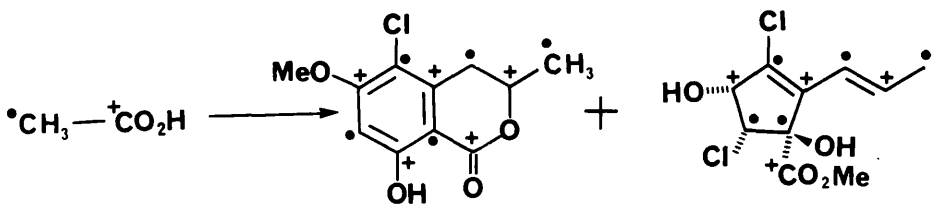


(49)

In Periconia macrospinosa cryptosporiopsinol (45) and the dihydroisocoumarin (46) are the major metabolites. Small amounts of other metabolites have also been detected (47) (48) and (49).[81]

2.2 Early Studies Done on the Biosynthesis of Cryptosporiopsinol (45)

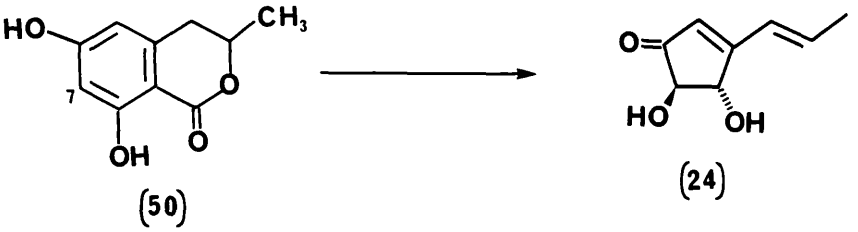
The first investigation to be carried out into the biosynthesis of cryptosporiopsinol (45) was done by Holker and Young in 1975.[81] Cultures of Periconia macrospinosa were supplemented with [1-^{13}C], [2-^{13}C] and [1,2-^{13}C]acetate. By assignment of the peaks in the ^{13}C n.m.r. spectra of the metabolites (45) and (46), the compounds were found to be enriched in the positions shown.



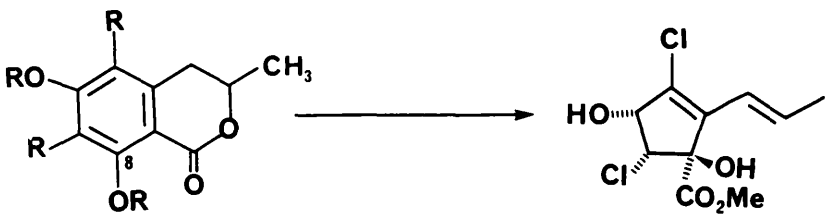
(46) (45)

From his doubly labelled acetate feedings and related work done on the biosynthesis of terrein (24)[47,48],

Holker proposed that cryptosporiopsinol was derived by a ring cleavage of a dihydroisocoumarin precursor.



(50) (24)



(51) (45)

From Holker's ^{13}C-acetate feeding results in cryptosporiopsinol, the ring cleavage of the dihydroisocoumarin (51) must involve extrusion of C-8 from the aryl ring. This contrasts with the formation of terrein (24) in which C-7 is lost from the aryl ring (50).

Another feature to be taken into account is the introduction of chlorine into cryptosporiopsinol.

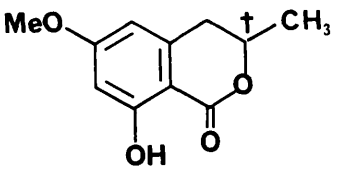
2.3 Introduction of Chlorine into Cryptosporiopsinol

Little is known about the mechanism of biological chlorination. The process is thought to involve the oxidation of chloride to chlorine cations or some equivalent. This could be accomplished by a peroxidase enzyme (a chloroperoxidase) together with a suitable oxidising agent. Such an enzyme has been isolated from the mould Caldariomyces fumago.[82] The purified preparation was shown to catalyse the formation of carbon-halogen bonds in the presence of hydrogen peroxide.

Details of the structure of the enzyme are known: it contains one mole of ferriprotoporphyrin IX as a prosthetic group and has a molecular weight of 42,000 of which 25-30% is carbohydrate. However, details of the structure of the active site and the mechanism by which the halogen atom is transferred to the substrate is lacking. Specifically, the nature of the group to which the halogen atom is added during transfer is not known. In studies of the Caldariomyces fumago

chloroperoxidase, the relative non-specificity of the enzyme was noted: it catalysed the chlorination, bromination and iodination of a number of cyclic or acyclic 1,3-dicarbonyl compounds.

With these considerations in mind Henderson[80] synthesised the eight dihydroisocoumarins (42), (43), (44), (46), (50), (52), (53), and (54) with a ^{14}C-label at C-3 and fed them to Periconia macrospinosa as their sodium salts. Of the eight labelled dihydroisocoumarins only (42), (50) and (52) were incorporated into the dihydroisocoumarin metabolite (46) at significant levels, but each of the eight was incorporated into cryptosporiopsinol (45). Degradation showed that the compounds were being incorporated intact. Considering the incorporation into the dihydroisocoumarin metabolite (46), Henderson



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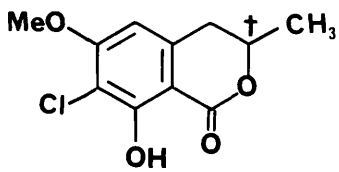

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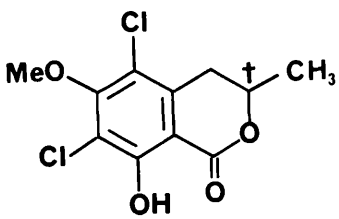

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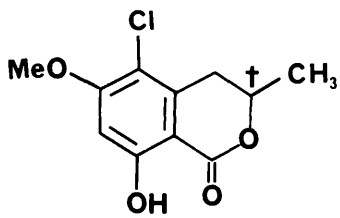

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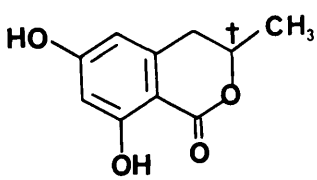

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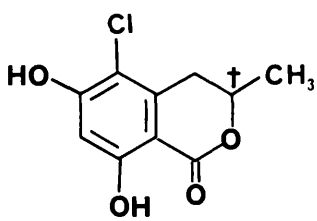

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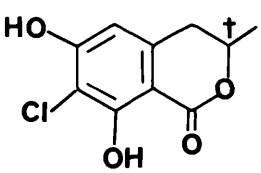

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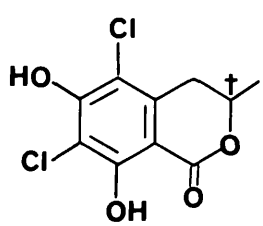


(54)

TABLE 2.1

Incorporations of [3-^{14}C] Dihydroisocoumarins into 5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (46)

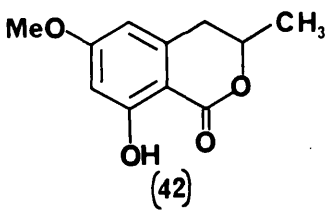
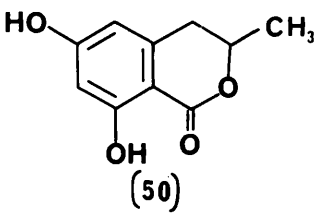
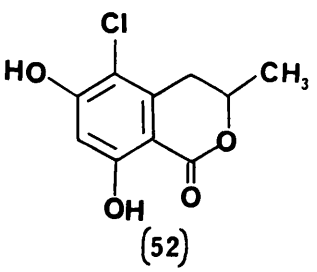
COMPOUND FED	INCORP INTO (46) %	% ACTIVITY AT C-3
 (42)	1.66	89
 (50)	17.60	83
 (52)	16.20	85

TABLE 2.2

Incorporations of [3-^{14}C] Dihydroisocoumarins into Cryptosporiopsinol (45)

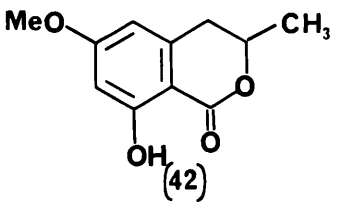
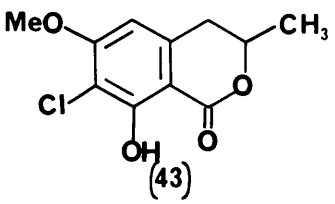
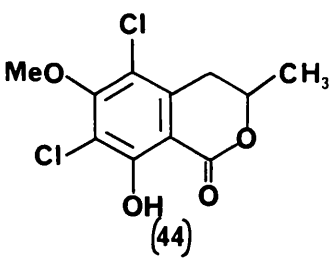
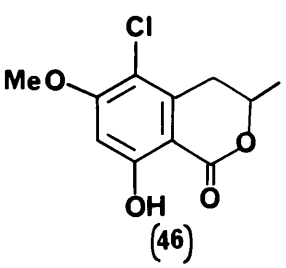
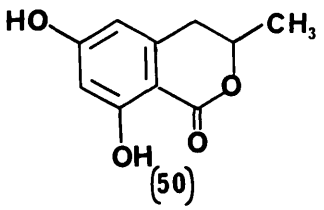
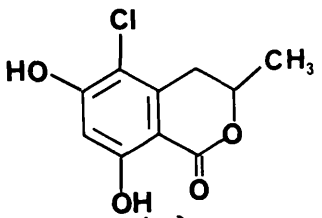
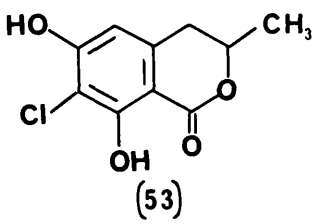
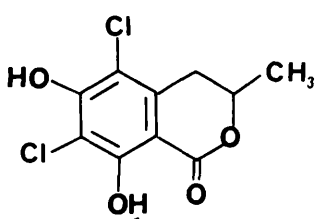
COMPOUND FED	INCORP INTO (45) %	% ACTIVITY AT C-2
 (42)	0.97	85
 (43)	1.75	96
 (44)	2.30	87
 (46)	0.13	-

TABLE 2.2 (cont)

Incorporations of [3-^{14}C] Dihydroisocoumarins into Cryptosporiopsinol (45)

COMPOUND FED	INCORP INTO (45) %	% ACTIVITY AT C-2
 (50)	4.60	89
 (52)	5.0	82
 (53)	8.0	84
 (54)	17.60	81

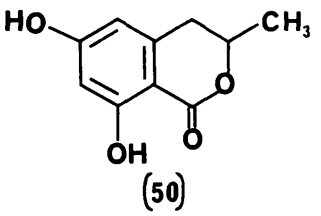
postulated that, since the demethylated dihydroisocoumarins (50) and (52) were incorporated far more efficiently (17.6 and 16.2% respectively) than the O-methyl derivative (42) (1.66%), chlorination of the ring occurs after aromatisation but before O-methylation (see Table 2.1). The fact that the O-methyl derivative was incorporated was explained as due to the relatively low specificity of the chlorinating enzyme. The enzyme was able to accept substrates having both hydroxyl and methoxyl groups at the 6-position.. Alternatively, the 6-methoxy derivative (42) could be incorporated via O-demethylation to the 6,8-dihydroxy derivative (50), followed by chlorination and subsequent O-methylation. Biological O-demethylations are known to occur.[83-85]

The incorporations of the 6-O-methyl dihydroisocoumarins (42), (43), (44) and (46) into cryptosporiopsinol (45) were found to be significantly less than those of the corresponding 6-hydroxy analogues (50), (52), (53) and (54) (Table 2.2). Henderson thus proposed the 6-O-methyl derivatives were unlikely to be on the direct pathway to cryptosporiopsinol (45).

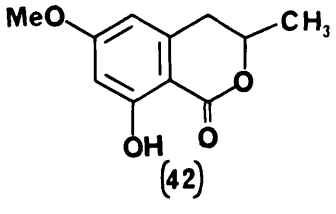
To prove that the dihydroisocoumarins (53) and (54) were true intermediates, precursor trapping experiments were performed.The unlabelled 7-chloro-dihydroisocoumarin (53) and the 5,7-dichlorodihydroisocoumarin (54) were fed in large quantities in separate experiments along with sodium [1-^{14}C]acetate to the organism. Radioactive isocoumarins (53) and (54) were recovered from the fungus. By increasing the metabolite pool of a potential precursor and feeding radioactive labelled acetate, the proposed intermediate may be 'trapped' in radiolabelled form if it is produced by the organism and if it is derived from acetate. Thus the production of these metabolites in Periconia macrospinosa was established and since they are both incorporated into cryptosporiopsinol, they are probably both intermediates.

Interestingly, on growing cultures of Periconia macrospinosa without a chloride ion source (normally present as potassium chloride), no reduction in mycelium growth was observed. On work-up no ring cleaved products were detected and the dihydroisocoumarins (50) and (42) were isolated.

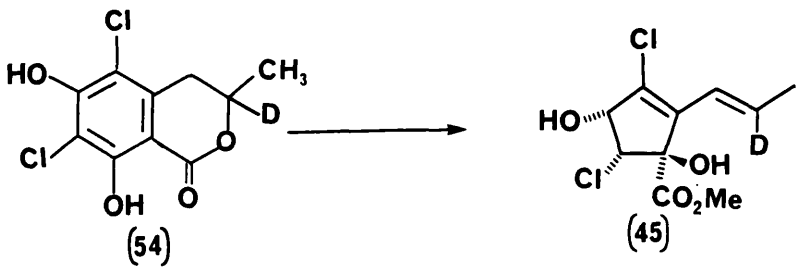
To test the fate of the proton at C-3, Henderson made the 5,7-dichloro-6,8-dihydroxy-3,4-dihydro-3-methyl-isocoumarin (54) with a deuterium label at C-3 and



(50)



(42)

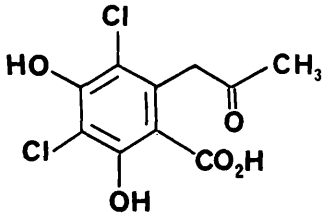


(54) (45)

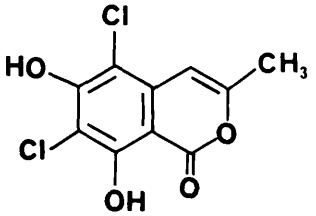
fed it to the fungus.

Using ^{13}C n.m.r. spectroscopy and applying the β-shift technique, the position of the deuterium atom was detected in the isolated cryptosporiopsinol (45) in the allyl group.

Thus Henderson showed that the proton at C-3 was retained throughout the biosynthesis of cryptosporiopsinol (45) and other possible precursors such as (55) and (56) could be disregarded.

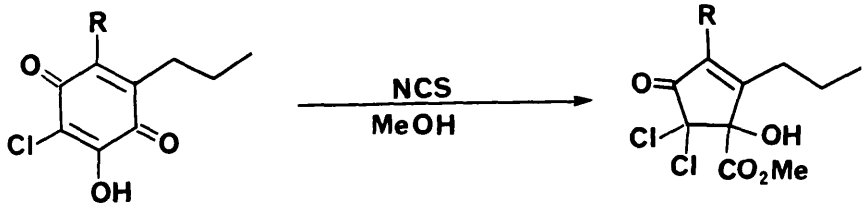


(55)



(56)

In order to test the possible involvement of hydroxy quinones to affect the ring-contraction reaction of a six-membered system to the cyclopentenone system of cryptosporiopsin, Henderson made the hydroxyquinones (57) and (58) and showed that on treatment with N-chlorosuccinimide in methanol at room temperature they cleaved to give the cyclopentenone ring system (59) and (60).



R = H (57)

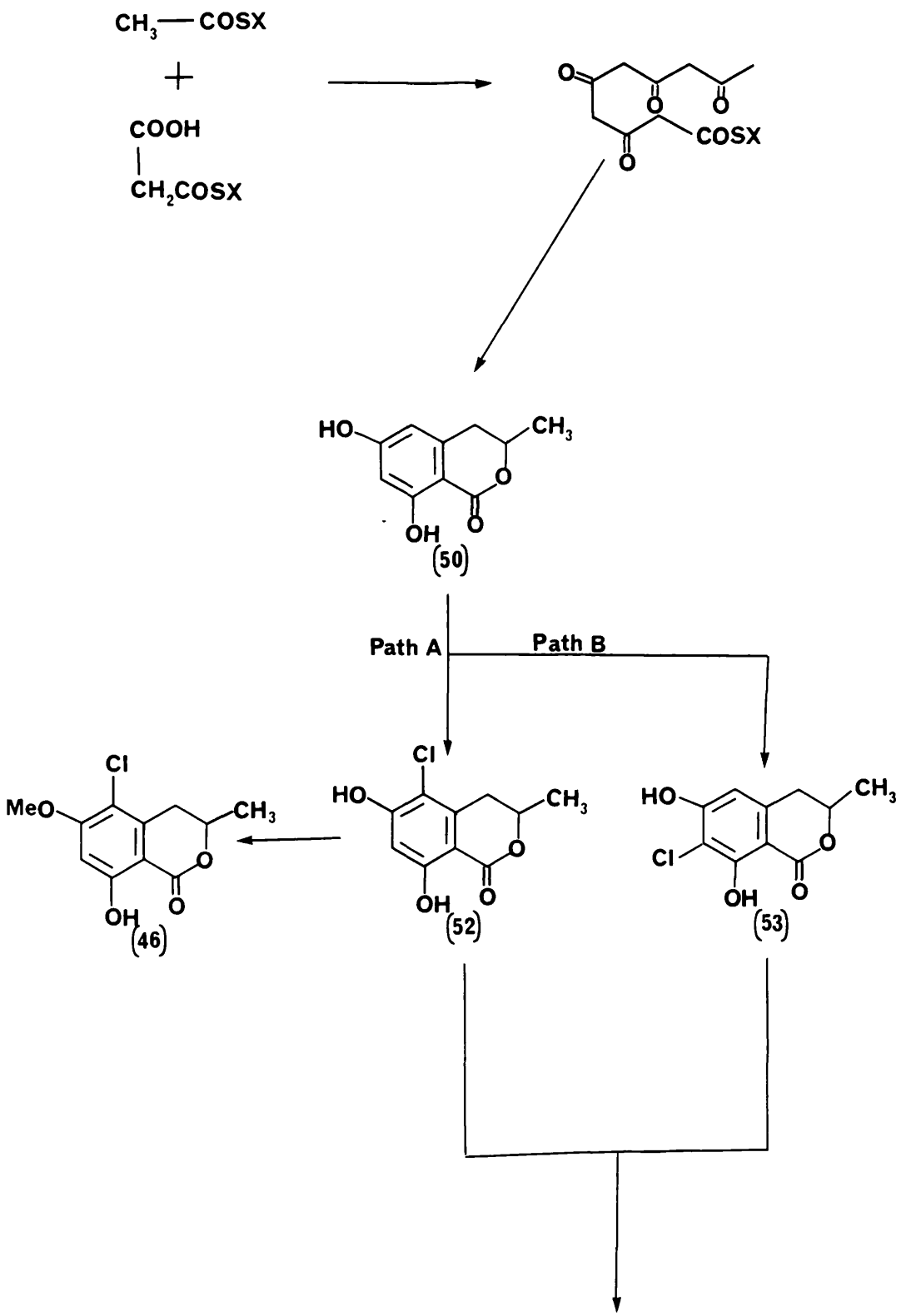
R = Cl (58)

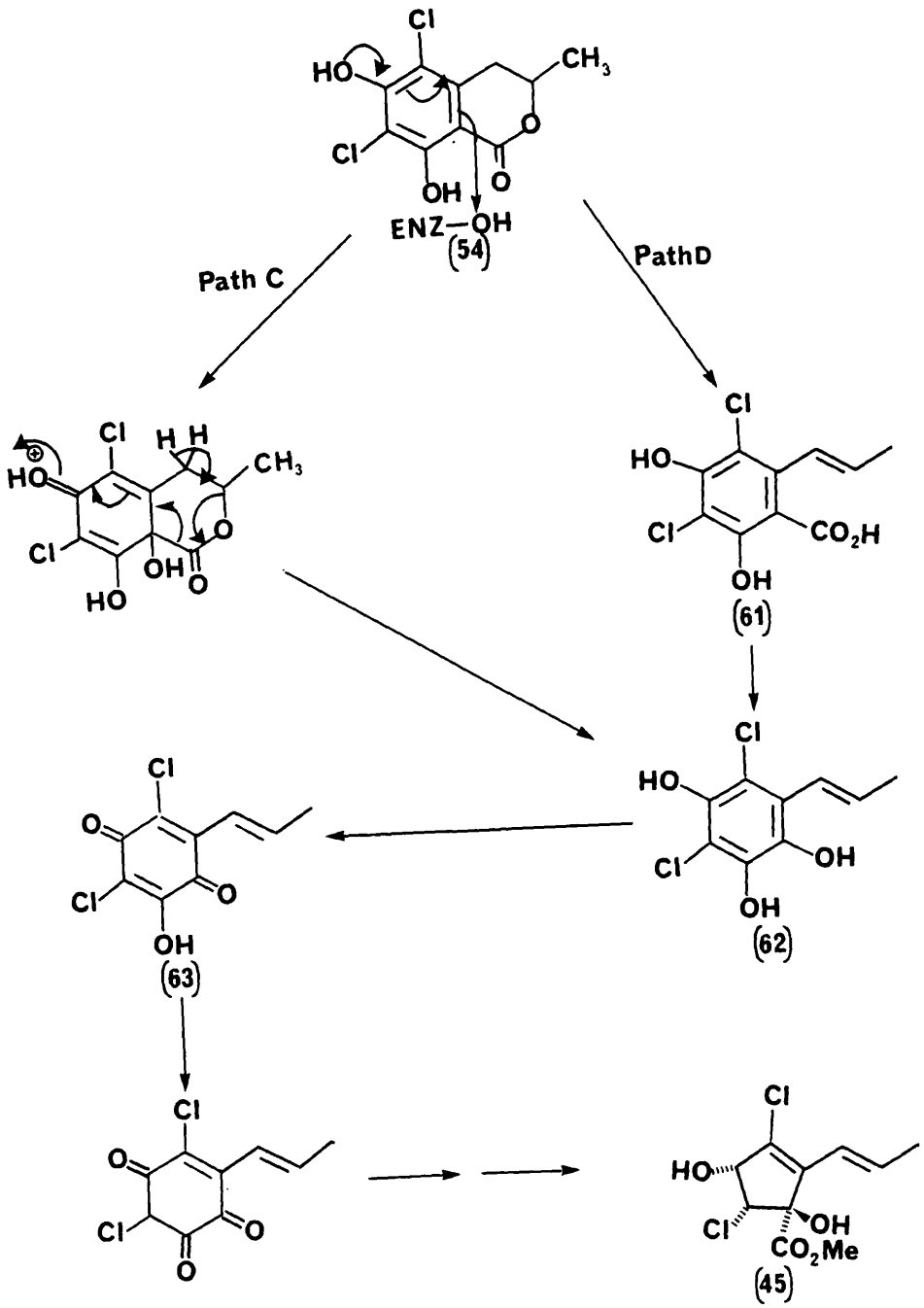
R = H (59)

R = Cl (60)

Armed with these results Henderson proposed the following scheme for the biosynthesis of cryptosporiopsinol (Scheme 2.1). The first step involves cyclisation of a pentaketide chain to give the dihydroisocoumarin (50).

SCHEME 2.1


CH3—COSX
+
COOH
CH2COSX
COSX
HO
CH3
OH
(50)
Path A
Path B
Cl
MeO
CH3
OH
(46)
Cl
HO
CH3
OH
(52)
HO
CH3
Cl
OH
(53)


Cl
HO
CH3
Cl
O
O
OH
ENZ—OH
(54)
Path C
PathD
Cl
H H
HO
CH3
Cl
O
OH
O
HO
Cl
HO
Cl
CO2H
OH
(61)
Cl
HO
Cl
OH
OH
(62)
Cl
O
Cl
O
OH
(63)
Cl
O
Cl
O
O
Cl
HO
Cl
OH
CO2Me
(45)

The intermediate (50) is chlorinated at the 5-position (PATH A) or at the 7-position (PATH B). Either of these intermediates can be chlorinated to give the 5,7-dichloro-6,8-dihydroxy-3,4-dihydro-3-methyl-isocoumarin (54).

Consideration of the functional changes which the dihydroisocoumarin precursor (54) must undergo leads to the recognition that at least 3 operations must take place: formation of the allyl group; oxidation and decarboxylation at C-8a, and ring cleavage.

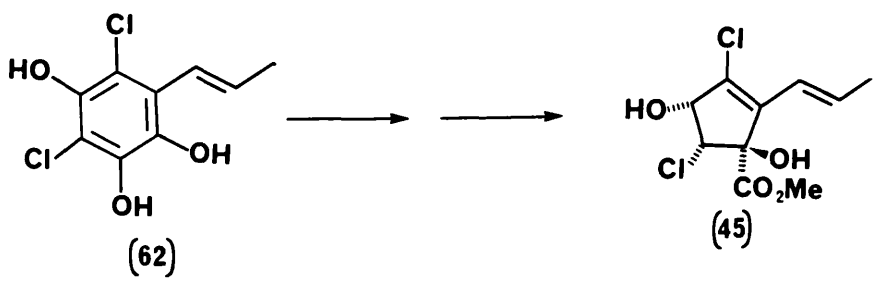
A plausible mechanism for the formation of the allyl goup and oxidative-decarboxylation involves initial enzymic oxidation at C-8a and subsequent loss of C-1 as carbon dioxide to give the trihydroxyphenylprop-1-ene (62). This could occur in a concerted manner (path C) or via the acid (61). The trihydroxyphenylprop-1-ene (62) could be oxidised to the quinone (63) which could undergo ring contraction, O-methylation and reduction to give cryptosporiopsinol (45).

2.4 Feeding of Phenylprop-1-enes to Periconia Macrospinosa

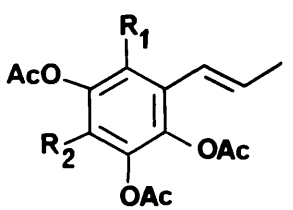
As a continuation of the study of the biosynthesis of

cryptosporiopsinol Maclachlan[86] investigated the plausible intermediacy of the dichlorotrihydroxyphenyl-prop-1-ene (62). Attempts to synthesise and purify the postulated precursor (62) were met with difficulty as it was found that compounds of this nature were difficult to handle due to oxidation and polymerisation.

Thus it was thought that the desired acetate (67) which was easier to purify and was more stable, could be fed to Periconia macrospinosa and would be incorporated via acetate hydrolysis in the fungal broth. Hence all the possible chlorinated derivatives (64), (65), (66) and (67) were synthesised and fed to the fungus. This was to study the significance of chlorination at the 4- and 6-positions of the aryl ring in the ring contraction reaction to form cryptosporiopsinol (45).



(62) (45)



(64) $R_1 = R_2 = H$
(65) $R_1 = Cl$ $R_2 = H$
(66) $R_1 = H$ $R_2 = Cl$
(67) $R_1 = R_2 = Cl$

All the triacetoxyphenyl propenes were fed with a deuterium at the C-2 position of the propene system. Detection of the label in the final metabolite could be accomplished by making use of the β-shift technique. That is, if any of these compounds were incorporated into cryptosporiopsinol (45) then the deuterium would appear at C-2 in the propene system of cryptosporiopsinol (45). Hence in the ^{13}C n.m.r. spectrum of cryptosporiopsinol, shifted β-signals should be observed for C-1, and C-3 of the propene system.

On feeding the four deuterium labelled triacetoxyphenyl propenes (64), (65), (66) and (67) it was found that the non-chlorinated compound (64) inhibited the production of cryptosporiopsinol (45). The remaining chlorinated triacetoxyphenylprop-1-enes (65), (66) and (67) did not block cryptosporiopsinol production but were not incorporated into cryptosporiopsinol to any detectable degree.

Henderson had shown 5,7-dichloro-6,8-dihydroxy-3,4-dihydro-3-methylisocoumarin (54) to be a true precursor of cryptosporiopsinol. Therefore, it was postulated that the dichlorotrihydroxyphenyl propene (62) formed by oxidation and decarboxylation of (54) would have been incorporated well into cryptosporiopsinol. Thus

the non-incorporation of the chlorinated compounds (65), (66) and (67) began to throw some doubt on the proposed biosynthetic pathway (Scheme 2.1).

There may be several reasons why (65), (66) and (67) were not incorporated into cryptosporiopsinol (45)

1) They may not have been hydrolysed in the aqueous fungal medium. However, there was no trace of the triacetoxyphenylpropenes in the work up of the fungus.

2) They may have been hydrolysed to the corresponding trihydroxyphenylpropenes, but were then oxidised or polymerised.

3) They may not be true precursors of cryptosporiopsinol.

However, these results are inconclusive and the feeding of the free phenols should be carried out. Maclachlan did not have the available time to carry out the feeding of the free phenols.

2.5 Application of β-shift Technique to Cryptosporiopsinol Biosynthesis

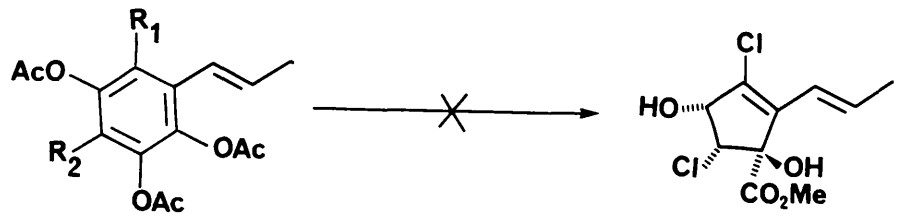
As mentioned earlier the β-shift method is a new technique which is being used more and more nowadays in obtaining information about biosynthetic pathways to metabolites. Therefore, in order to throw some light on the biosynthesis of cryptosporiopsinol this technique was applied.

Two feedings were carried out:

1) $CD_3{}^{13}CO_2Na$ was fed to Periconia macrospinosa
2) $CH_3{}^{13}C{}^{18}O_2Na$ was fed to Periconia macrospinosa

The fungus was also grown in an $^{18}O_2$ atmosphere to determine which oxygen atoms were derived from acetate and which were derived from the atmosphere.

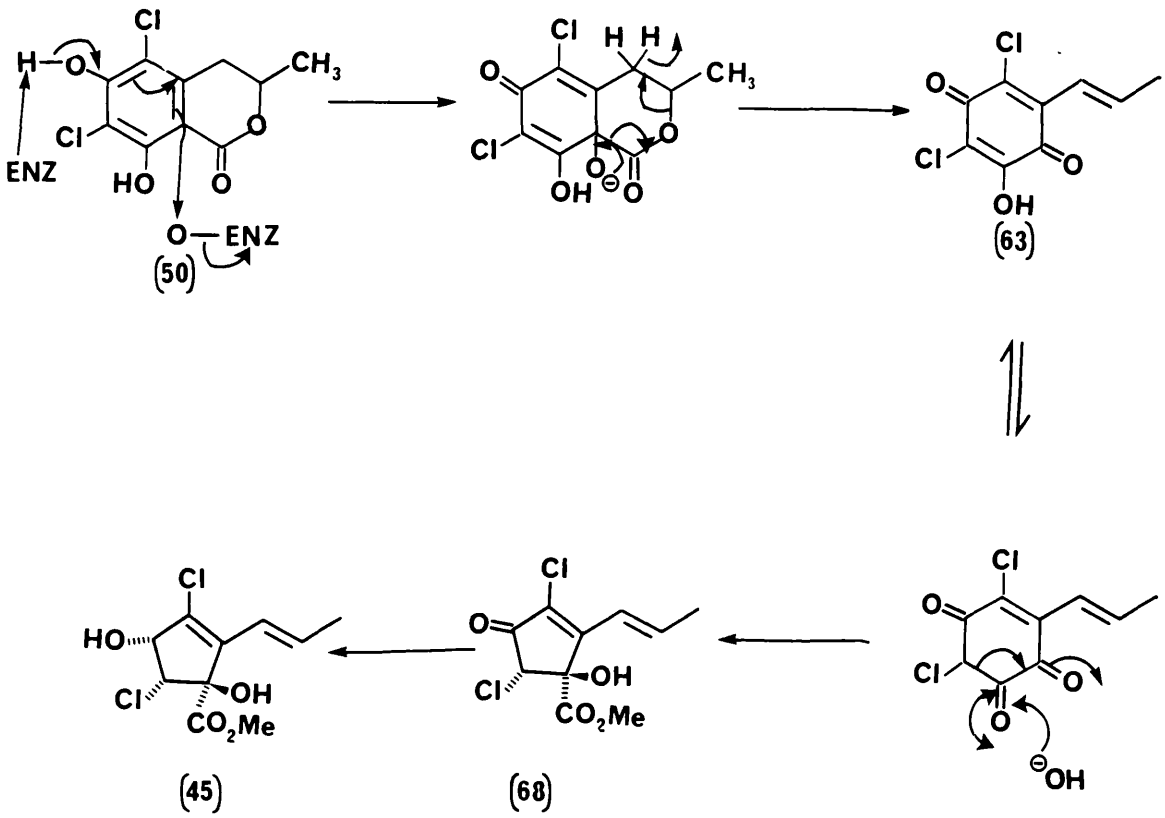
The fact that the chlorinated derivates (65), (66) and (67) were not incorporated into cryptosporiopsinol (45) may suggest that the pathway outlined in Scheme 2.1 was not altogether correct.



(45)

R_1-Cl R_2=H (65)
R_1=H R_2=Cl (66)
R_1=R_2=Cl (67)

SCHEME 2.2



A possible pathway may be as shown in Scheme 2.2 starting from the isocoumarin (50). It may be oxidised in the manner shown, the oxygen may come from the atmosphere and be enzymatically bound in the active site. Formation of the carbonyl, loss of carbon dioxide and hydride or some equivalent would produce the quinone (63). The quinone (63) being the oxidised form of the trihydroxyphenyl propene (62), could be the key intermediate and not the fully aromatic compounds as earlier suggested by Henderson. This quinone (63) could then undergo the same ring contraction as shown in Scheme 2.1 giving the acid (68) which could be esterified and reduced giving cryptosporiopsinol (45).

Thus it was hoped that the $^{18}O_2$ and $CH_3{}^{13}C^{18}O_2Na$ experiments would give some information about the ring-contraction from the 6-membered to the 5-membered ring.

2.5.1 Feeding of $CD_3{}^{13}CO_2Na$ to Periconia macrospinosa

$CD_3{}^{13}CO_2Na$ was fed to Periconia macrospinosa on day 14 and the broth was extracted on day 21. The β-shifts were obtained from the ^{13}C proton noise decoupled spectrum of the isocoumarin (46) and are given in Table 2.3. Likewise the β-shifts were obtained for cryptosporiopsinol (45) and are also given in Table 2.3.

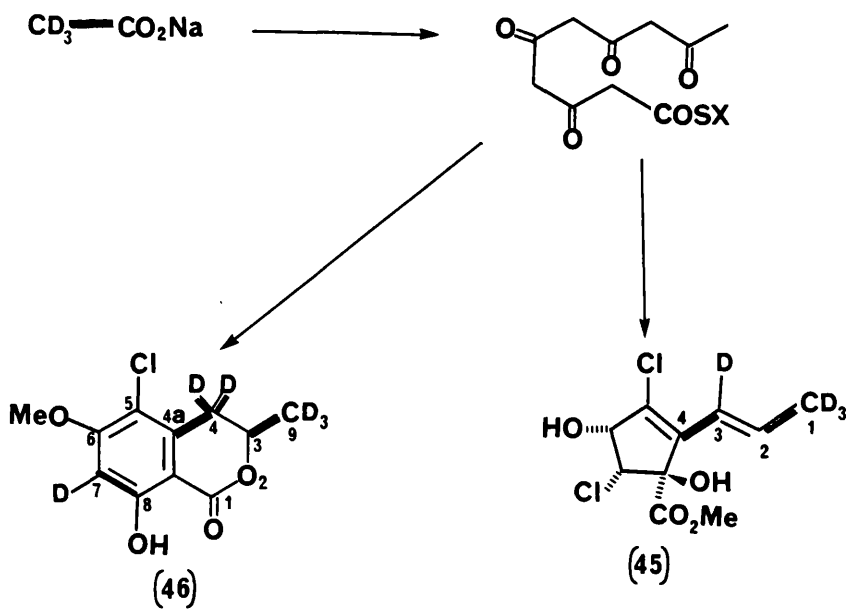


TABLE 2.3

ISOCOUMARIN (46)		CRYPTOSPORIOPSINOL (45)	
CARBON	β-SHIFT (ppm)	CARBON	β-SHIFT (ppm)
3	0.04, 0.08, 0.12	2	0.04, 0.08, 0.12
4a	0.03, 0.07, 0.1	4	0.04
8	0.04		

For the isocoumarin (46) carbon 3 shows shifts of 0.04 p.p.m., 0.08 p.p.m., and 0.12 p.p.m. corresponding to carbon 9 having one, two, or three deuteriums attached

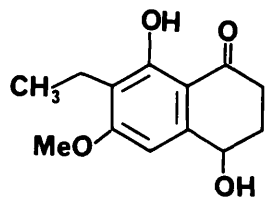
to it respectively. Thus at carbon-3 if carbon-9 has one deuterium attached a shift of 0.04 p.p.m. is observed. If carbon-9 has two deuteriums a shift of 0.08 p.p.m. is observed, and if carbon-9 has three deuteriums attached a shift of 0.12 p.p.m. is observed. Therefore each deuterium gives an additive shift value of 0.04 p.p.m.

An interesting result occurs for C-4a of the isocoumarin. Here there are three observed shifted signals:

1) Deuterium at the equatorial site on C-4.
2) Deuterium occupying an axial site on C-4.
3) Two deuteriums attached to C-4.

From studies carried out on the β-shifts arising from deuterium attached at various carbons on the isocoumarin (46) (see Chapter 5) it has been shown that the lowest shifted signal (0.03 p.p.m.) is due to the equatorial deuterium attached to C-4. The shifted signal 0.07 p.p.m. is due to the axial deuterium. Where two deuteriums appear on the same carbon a shifted signal of 0.1 p.p.m. is observed. This again shows the additive feature of the β-shifts. These findings are similar to results obtained by Simpson and Stenzel[53] when they studied the biosynthesis of

O-methylasparvenone (26) (see Chapter 1).

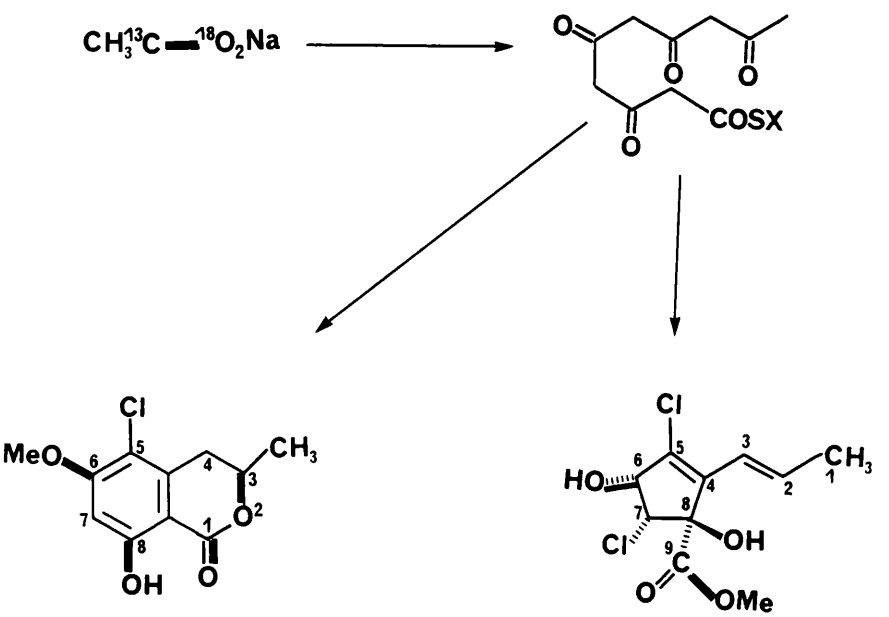


(26)

The β-shifted signals could also be observed for cryptosporiopsinol (45) i.e. carbon 2 showed shifted signals which were also additive corresponding to one, two or three deuterium atoms being attached to carbon 1 of the propene chain.

2.5.2 Feeding of $CH_3{}^{13}C-{}^{18}O_2Na$ to Periconia Macrospinosa

$CH_3{}^{13}C{}^{18}O_2Na$ was fed to Periconia Macrospinosa on day 14 and the broth was extracted on day 21.

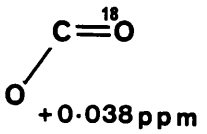


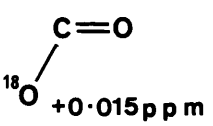
(46) (45)

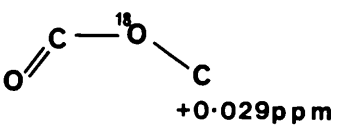
TABLE 2.4

ISOCOUMARIN (46)		CRYPTOSPORIOPSINOL (45)	
CARBON	SHIFTED SIGNAL	CARBON	SHIFTED SIGNAL
1	0.035	6	0.016 p.p.m.
3	0.034	9	0.017 p.p.m.
6	0.017		
8	0.010		

The ^{18}O isotope effect on ^{13}C nuclear shielding has been studied.[69,70,87-89] The findings are as follows: (i) For unhindered alcohols and ethers the value of the α-shift is 0.025 p.p.m. (ii) For aldehydes and ketones the α-shift is 0.050 p.p.m. (iii) For carboxylic acid derivatives the double bonded oxygen gives a higher α-shift value than that for a single bonded ^{18}O. Also the effects observed in phenols are smaller than in aliphatic alcohols.[71,89]







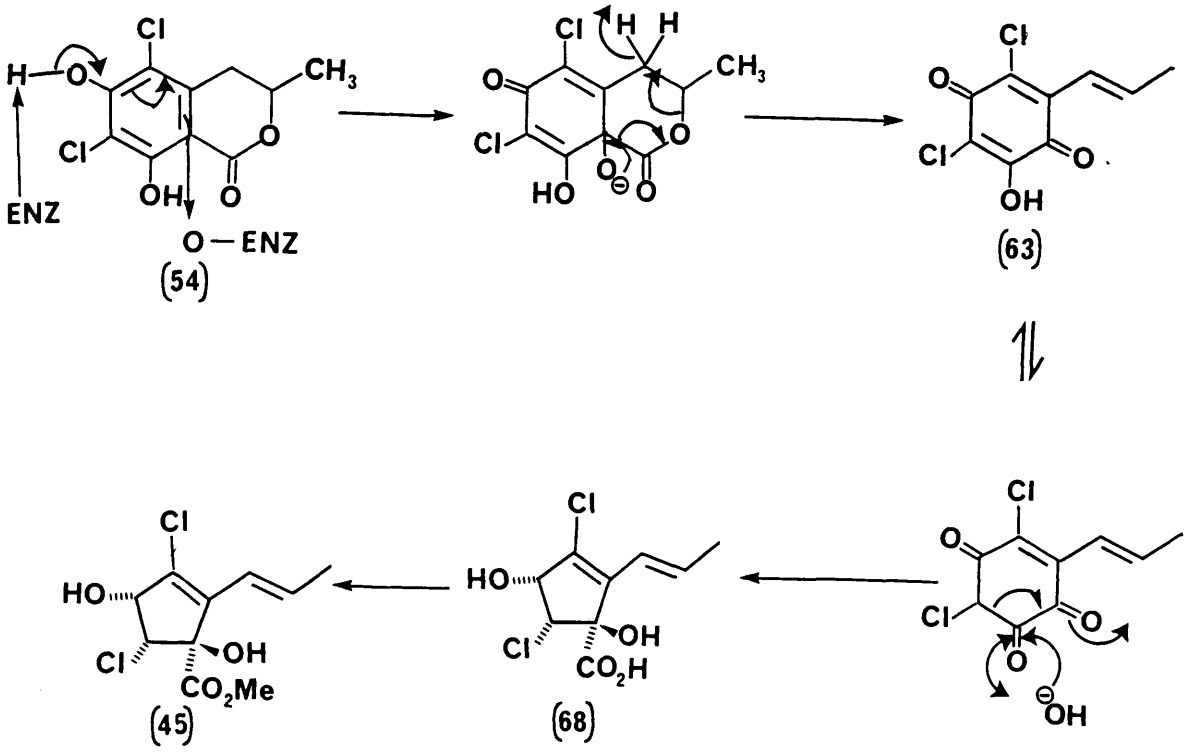
Vederas[71] has observed for carbonyl compounds the following trend in α-shift values: ketones > aldehydes > esters > amides and that conjugation reduces the effect by 0.002-0.006 p.p.m.

The results obtained for the isocoumarin (46) were in agreement with those observed by Holker.[81] The largest shifted signal found was that at carbon-1 which implied the carbonyl had been incorporated as an intact unit, and the pentaketide moiety folds as shown.

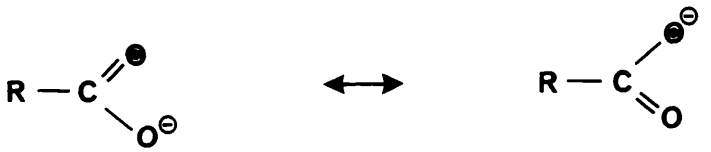
However, the acetate feeding results for

cryptosporiopsinol (45) are in contrast to those anticipated. The carbon-6 of cryptosporiopsinol has retained its oxygen which can be visualised as being derived from the carbon-6 oxygen bond of the isocoumarin (46). The only other shifted signal is that due to carbon-9 (0.017 p.p.m.) which is in agreement for a carbon-oxygen single bond. This now suggests that the pathway outlined in Scheme 2.2 is not altogether correct.

SCHEME 2.2

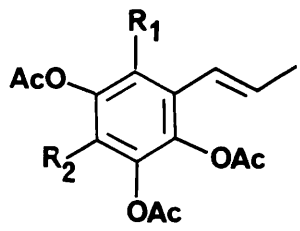


By considering Scheme 2.2 it was envisaged that the quinone intermediate (63) would undergo a ring contraction mechanism in the manner shown to give the acid (68). If this is the correct pathway, then the carbonyl oxygen of the acid (68) will be derived from the 8-hydroxyl group of the isocoumarin (54). Thus if the acid (68) were an intermediate then the label would be attached to the carbonyl group of the ester in cryptosporiopsinol (45). It may be possible that the acid (68) exists as the carboxylate anion. If this were so then a scrambling of the label could occur



giving a 50:50 mixture of the label on the carbonyl and on the single bond oxygen. Further manipulation of the intermediate (68) would give cryptosporiopsinol which should contain scattering of the ^{18}O label. However, the cryptosporiopsinol (45) isolated from the feeding of [1-^{13}C, $^{18}O_2$]acetate had no label in the carbonyl group of the ester. This rules out the probability of the acid (68) being an intermediate on the pathway to cryptosporiopsinol.

These results also help to clarify the findings of MacLachlan[84] who fed the chlorinated triacetoxyphenyl propenes (65), (66) and (67) and found they were not incorporated. One suggestion was that the acetate

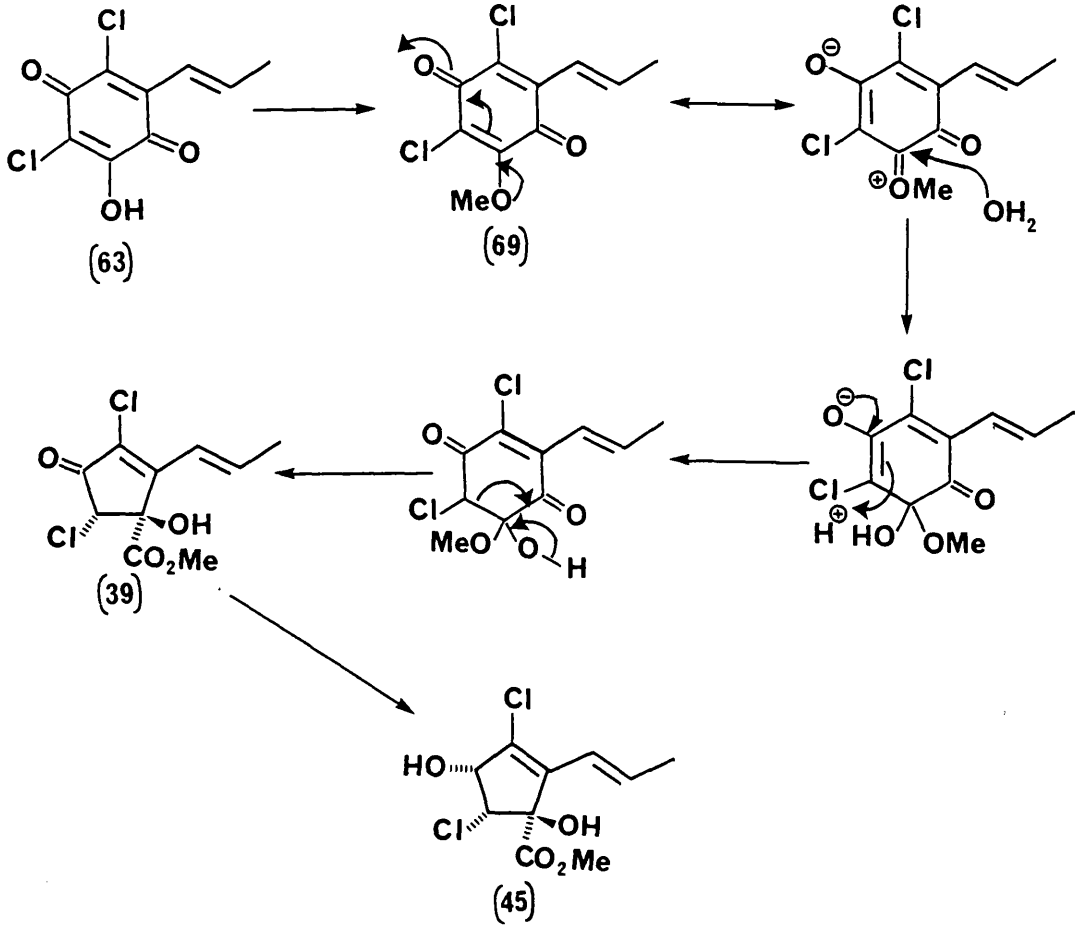


R=Cl R=H (65)
R=H R=Cl (66)
R=R=Cl (67)

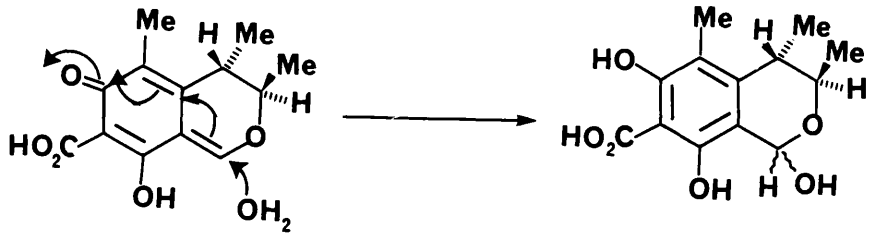
groups were not hydrolysed in the fungal broth. However, it now seems unlikely that these compounds are not true intermediates. The pathway would seem more likely to follow that of Scheme 2.3 in which the quinone (63) is the key intermediate. The ring contraction must go via another process and one plausible way is as shown in Scheme 2.3.

One way to test this hypothesis would be to synthesise the quinone (63) and methylate with $^{14}CH_3I$ to give the radiolabelled species (69).Feeding this intermediate (69) and checking for radioactive cryptosporiopsinol would confirm whether the quinone (69) was a true intermediate or not.

SCHEME 2.3



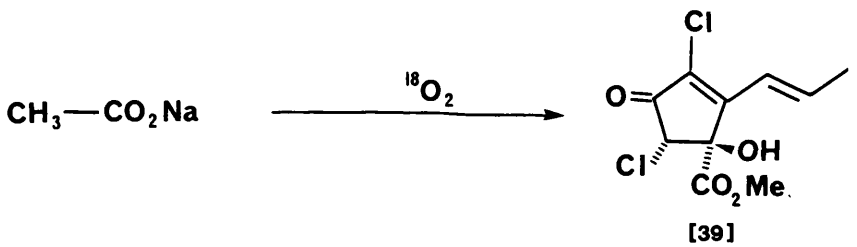
Evidence to support the Michael addition of water to a fungal metabolite was reported recently by Barber et al.[90] These workers have shown by spectroscopic means that citrinin (31) in aqueous solution at physiological pH exists not as the quinone methide (31) but as a diastereomeric mixture of hydrates (70).



It was thought that the pathway could be more fully understood if the origins of the other two oxygen atoms in cryptosporiopsinol could be established.

2.6 $^{18}O_2$ Gas Experiment

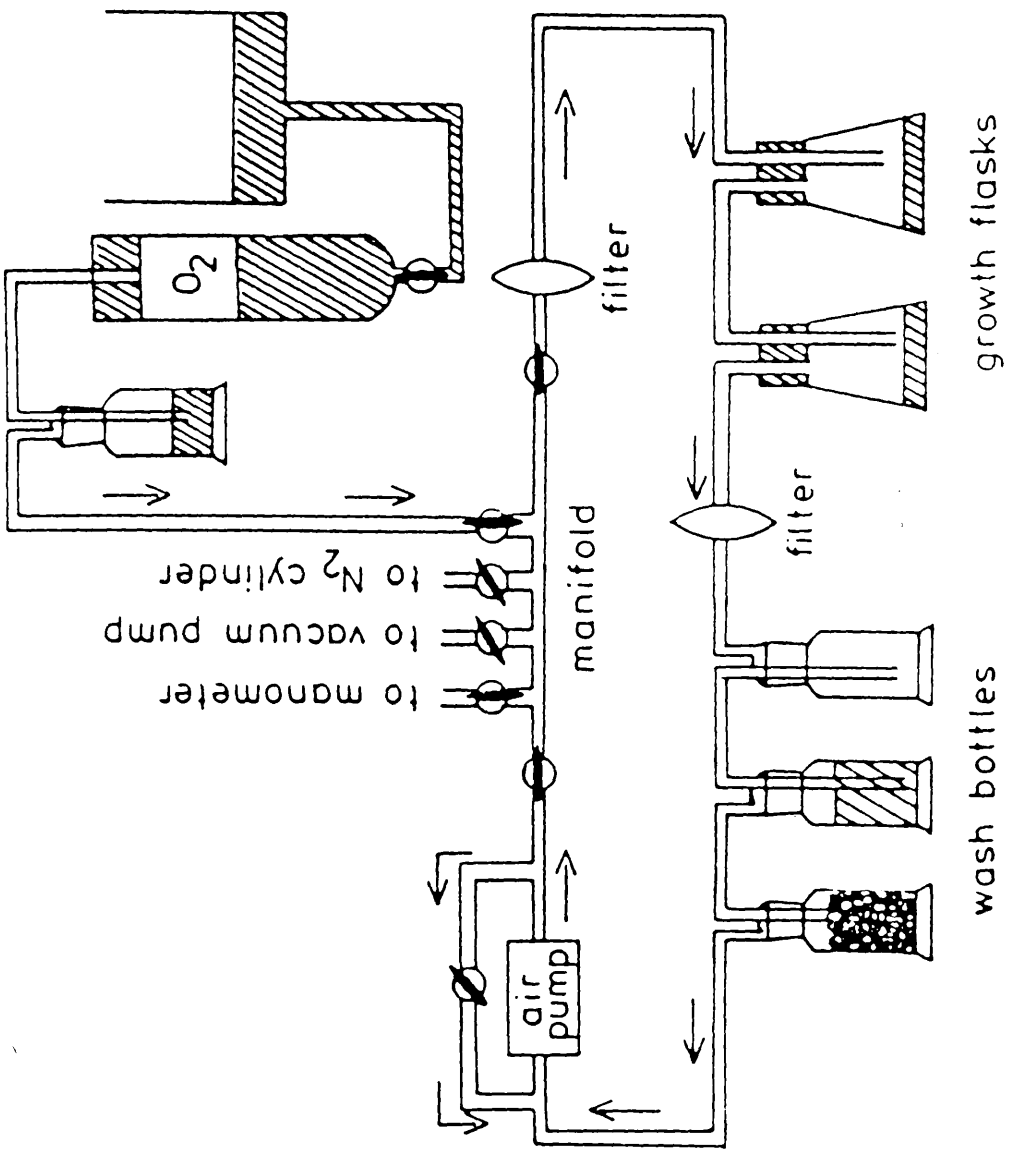
Periconia macrospinosa was grown in an $^{18}O_2$ atmosphere for 21 days. However, after work up of the fungal broth, there was only a small amount of metabolite produced. The ^{13}C n.m.r. revealed the compound obtained to be cryptosporiopsin (39), the oxidised form of cryptosporiopsinol. Unfortunately it was difficult to tell which atoms had been shifted due to the small amount of material obtained. There was not enough time to repeat this experiment during the course of this work.



[39]

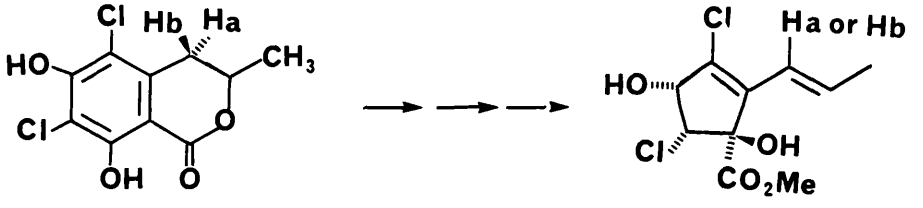
DIAGRAM 5.1

$^{18}O_2$ GAS EXPERIMENT



2.7 Feeding of Labelled Isocoumarins

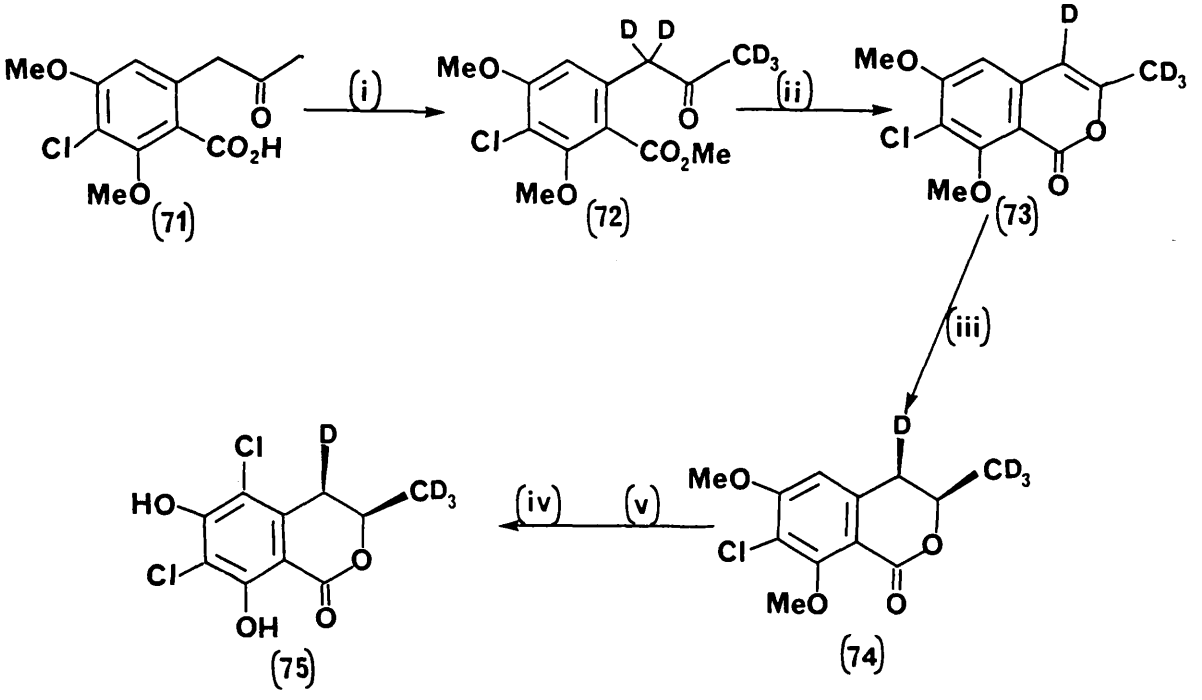
In the biosynthesis of cryptosporiopsinol (45) Henderson[80] has already established that various isocoumarins are incorporated into this metabolite. Indeed the best incorporated was the isocoumarin (54). As well as studying the nature of the ring contraction from the 6-membered to the 5-membered ring it is also of interest to establish which proton is lost in the conversion of the isocoumarin (54) to cryptosporiopsinol (45).



54 45

Thus to show which hydrogen is lost a synthesis is required which will give two isocoumarins, one having an α-deuterium and one having a β-deuterium at the 4-positon.

SCHEME 2.4



Reagents: (i) Na, D_2O, (ii) $HClO_4$, Ac_2O, EtOAc,
(iii) H_2/Pd, (iv) BBr_3, (v) SO_2Cl_2

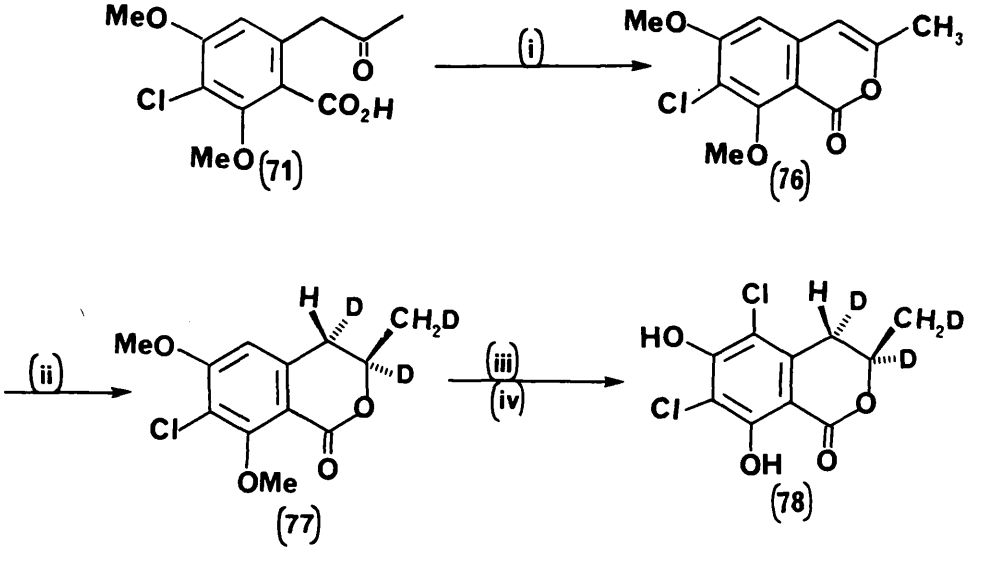
2.7.1 Synthesis of Deuterio-isocoumarin (75)

A synthesis of the keto acid (71) is outlined in chapter 3. Dissolving sodium in deuterium oxide and refluxing for 2 hours exchanged all the α-hydrogens, giving the pentadeuterio compound (72). Cyclising to the isocoumarin (73) was performed as before (chapter

3) by standing in a solution of perchloric acid and acetic anhydride for 10 minutes. Hydrogenation of the double bond using palladium on charcoal as catalyst afforded the required 4β-deuterioisocoumarin (74). This is because cis reduction of the double bond places the deuterium on the same side as the methyl group. The methyl group will occupy an equatorial site, and therefore in order for the deuterium to be on the same side it will occupy an axial site. Demethylation with boron tribromide and chlorination with sulphuryl chloride afforded the desired deuterioisocoumarin (75).

2.7.2 Synthesis of Deuterio-isocoumarin (78)

SCHEME 2.5



(i) $HClO_4$, Ac_2O, EtOAc ; (ii) D_2; (iii) BBr_3 ; (iv) SO_2Cl_2

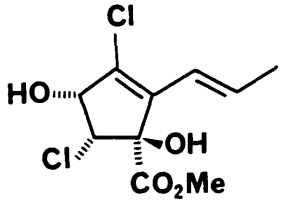
Reduction of the isocoumarin (76) with deuterium gas, formed by the dropwise addition of deuterium oxide onto lithium metal afforded the isocoumarin (77). By ^{1}H n.m.r. there was no proton at the 3-position and the 4-position integrated solely as one proton. However, integration of the methyl group revealed that one of the protons had been exchanged with a deuterium.

2.7.3 Feeding Results

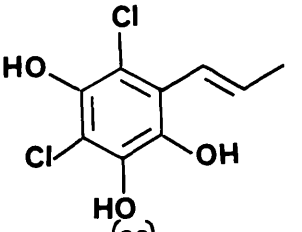
The deuterio-isocoumarins (75) and (78) were fed as their sodium salts to Periconia macrospinosa. Several metabolites were produced from these feedings. The fungus appeared to have changed its metabolite production by the time this work was performed. It was not possible to obtain meaningful results from the ^{13}C n.m.r. and ^{2}H n.m.r. spectra of the metabolites due to insufficient quantities of cryptosporiopsinol obtained. There was not sufficient time to repeat this experiment with a new Periconia macrospinosa culture.

2.8 Conclusions

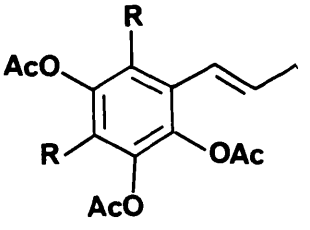
The feeding of $CH_3{}^{13}C{}^{18}O_2Na$ to Periconia macrospinosa has given more information about the biosynthesis of cryptosporiopsinol (45). The ^{13}C

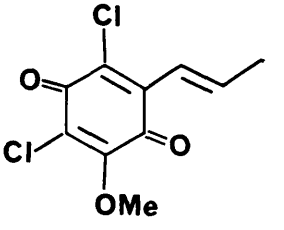


(45)



(62)





(69)

R = R = H (64)

R = Cl R = H (65)

R = H R − Cl (66)

R = R = Cl (67)

n.m.r. spectrum obtained for the metabolite revealed that only the carbon-oxygen single bond of the ester showed an isotope shift. This result means that it is unlikely that the trihydroxyphenylprop-1-ene (62) is an intermediate as had been believed. Therefore, the finding by MacLachlan that the triacetoxyphenylpropenes (64), (65), (66) and (67) were not incorporated into

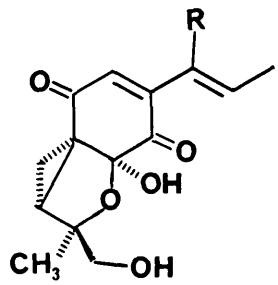
cryptosporiopsinol (45) is because they are probably not true intermediates on the biosynthetic pathway. The proposed pathway outlined in Scheme 2.1 would now seem unlikely. A more plausible route is outlined in Scheme 2.3 and the quinone (69) would now appear to be the key intermediate.

3. BIOSYNTHESIS OF MICROLINE AND DECHLOROMICROLINE

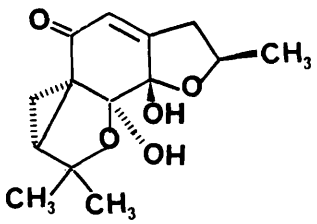
3.1 Introduction

Microline (79) is the most complex of a family of isoprenoid-polyketide metabolites isolated from the fungus Gilmaniella humicola and an ectendomycorrhizal fungus found on the roots of Monotropa hypopitys. These metabolites have antifungal properties and some antibacterial action; presumably the metabolites are produced in self-defence. The metabolites or their analogues also may be of interest as fungicides for crop protection. Little is known about the biosynthesis of this class of metabolite, although there has been some speculation in the literature. The metabolites isolated from Gilmaniella humicola include microline (79),[91,92] dechloromicroline (80),[91,92] gilmicolin (81),[93] mycorrhizinol (82) and 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50).[91]

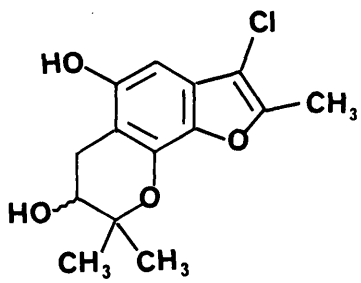
The biosynthesis of microline (79) and dechloromicroline (80) and similar metabolites may formally arise through the intermediacy of a polyketide chain and an isoprene unit as in Scheme 3.1. The simultaneous occurrence of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50)[94]



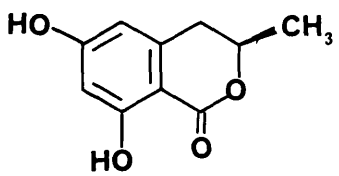
R = Cl (79)
R = H (80)



(81)



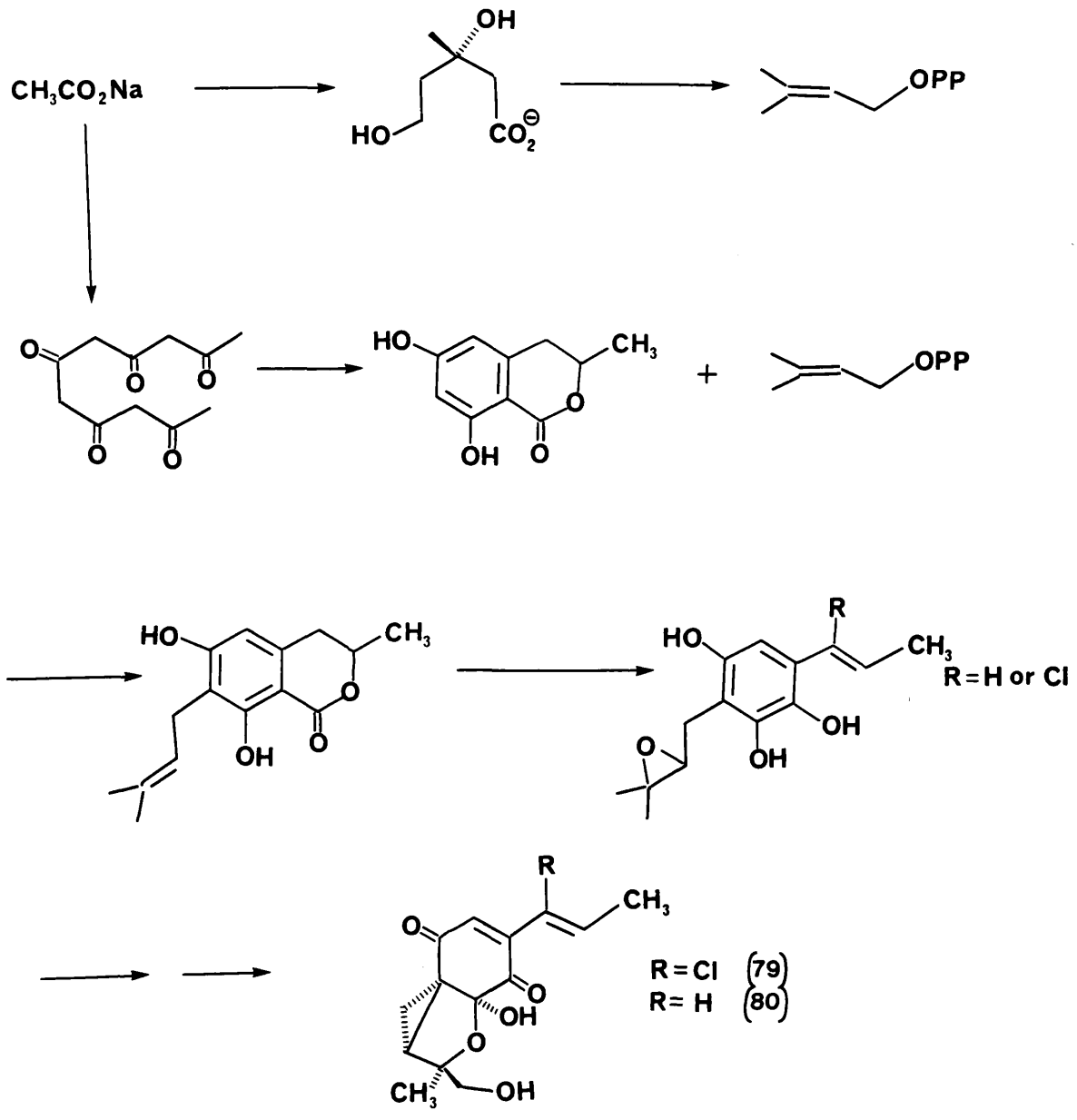
(82)



(50)

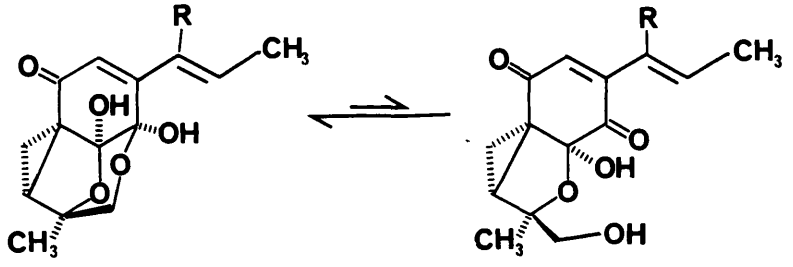
and other biogenetically related metabolites tend to support this hypothesis.[95]

SCHEME 3.1



Before carrying out various feeding experiments with ^{13}C-labelled precursors Chexal and Tamm assigned

the ^{13}C n.m.r. spectra of microline and dechloromicroline.[96] The X-ray structure of microline (79) had been obtained and shown to be (79a). However, in solution microline (79) and dechloromicroline (80) exist as a 3:1 ratio of their tetracyclic (79a, 80a) and tricyclic structures (79b, 80b).



R = Cl (79a)
R = H (80a)

R = Cl (79b)
R = H (80b)

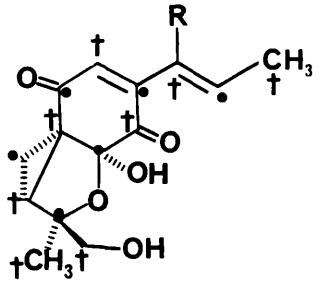
Chexal and Tamm studied the incorporation of a number of ^{14}C-labelled precursors into dechloromicroline.[96] The non-incorporation of phenylalanine ruled out the possibility of prephenic acid/shikimate intermediates in the biosynthetic pathway.

The labelling pattern observed on supplementing the

media with sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]acetates is shown below.

• = [1-^{13}C] Acetate

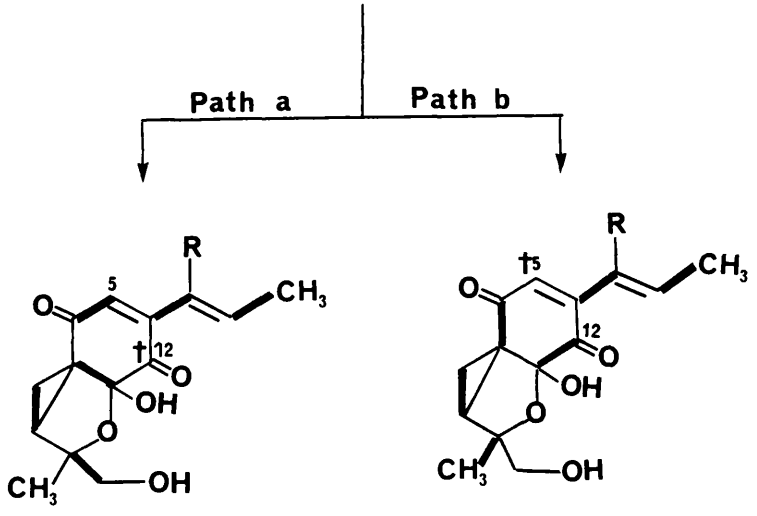
† = [2-^{13}C] Acetate



R=Cl (79)

R=H (80)

[1,2-^{13}C] Acetate



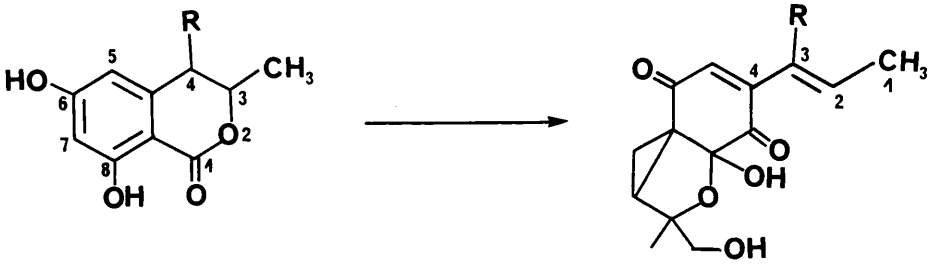
The alternate labelling of carbon atoms with [1-^{13}C] and [2-^{13}C]acetates supports the polyketide mode of biogenesis. The origin of C(8), C(9), C(10), C(13) and C(14) from an isoprene unit is also confirmed. In the ^{13}C n.m.r. spectrum of di-O-acetylmicroline (exists exclusively as the tricyclic structure) labelled by [1,2-^{13}C]acetate 12 of the 14 signals showed a doublet. This is due to ^{13}C-^{13}C coupling in the doubly labelled acetate unit - in addition to the usual singlet arising for ^{13}C present at natural abundance. Thus C(1) - C(2), C(3) - C(4), C(5) - C(6), C(7) - C(11), C(8) - C(9) and C(10) - C(13) all showed coupling. However, the signals for C(12) and C(14) appeared as singlets in the spectrum.

The appearance of C(12) as a singlet and C(5) - C(6) as a doublet (Jcc 54 Hz) in the ^{13}C n.m.r. spectrum can only be accommodated if the biosynthesis of microline (79) and dechloromicroline (80) proceeds according to pathway a. If pathway b were correct then C(5) would have appeared as a singlet and C(11) - C(12) would have exhibited C-C couplings. Similarly, the appearance of C(14) as a singlet and C(10) - C(13) as a doublet clearly defined the origin of C(13) from the methyl group attached to C(3) in the mevalonate precursor.

Thus Chexal and Tamm using singly and doubly labelled

^{13}C-acetate precursors have elucidated the biosynthetic pathway of microline (79) and dechloromicroline (80). However, the nature of the intermediates still has to be proven. An investigation of one of these postulated intermediates 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50) in the biosynthesis of microline and dechloromicroline was undertaken.

The occurrence of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50) along with microline (79) and dechloromicroline (80) in Gilmaniella humicola led to the belief that the aforementioned isocoumarin (50) may be a true intermediate in the pathway to these metabolites.



R = Cl (83)
R = H (50)

R = Cl (79)
R = H (80)

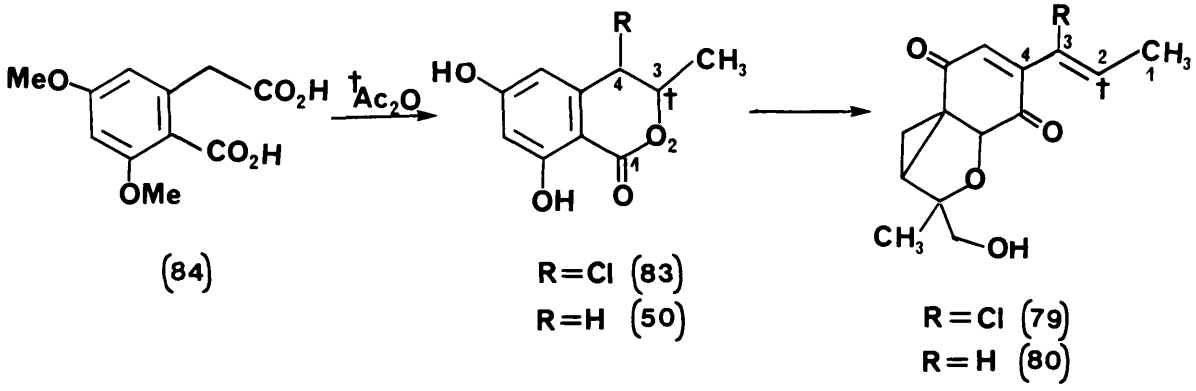
It was thought that the 4-chloroisocoumarin (83) may

also be an intermediate, and if this were so, it would establish that chlorination of microline (79) occurred at an early stage in the biosynthesis. Thus to confirm or disprove this, a synthesis of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50) and 4-chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (83) was required which would allow a label to be introduced at a position in the isocoumarins (83) and (50) which would be detectable in the final metabolites (79) and (80) respectively.

3.2 Synthesis of labelled dihydroisocoumarins

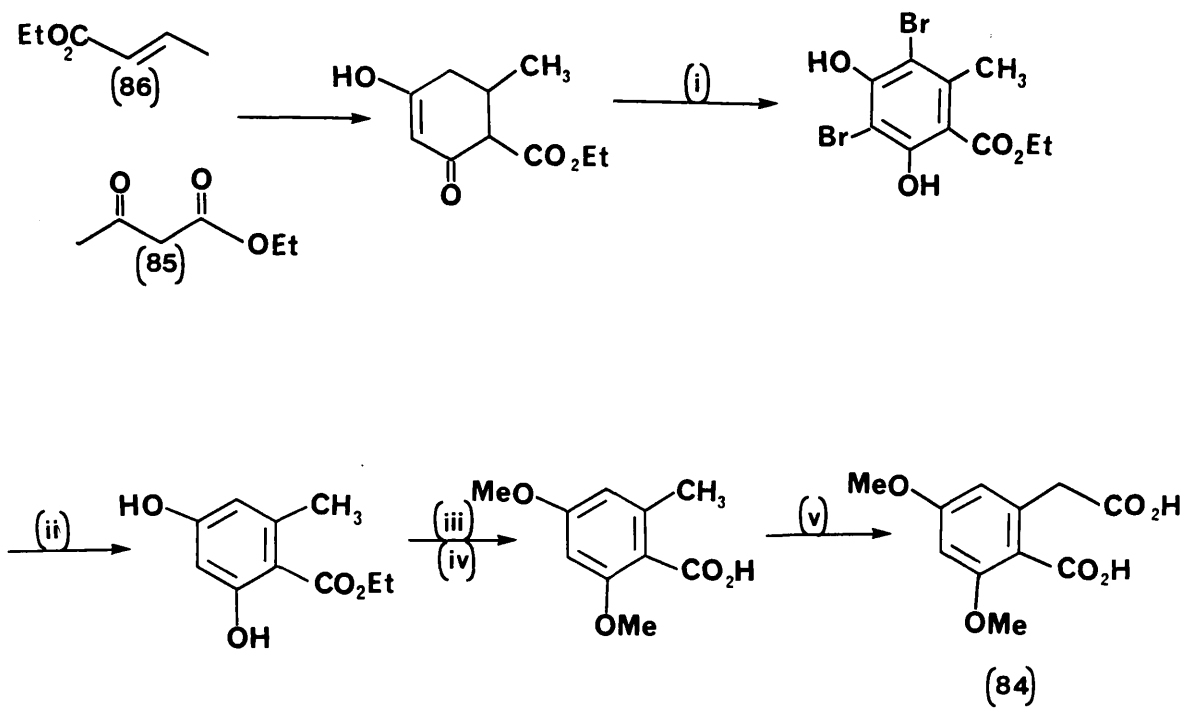
There are several syntheses of dihydroisocoumarins to be found in the literature.[97,98] However, since labelled dihydroisocoumarins were sought, a synthesis was required which would allow the label to be introduced at as late a stage as possible. 3,5-Dimethoxyhomophthalic acid (84) was the key intermediate since there are only a few steps from this compound to the dihydroisocoumarin (50). This is a key intermediate because reacting the homophthalic acid (84) with [1-^{14}C]acetic anhydride would introduce the radioactive label at the 3-position of the isocoumarin. This corresponds to the 2-position of microline (79) or dechloromicroline (80) if the

anticipated mode of incorporation is correct.



A recent synthesis[80] of 3,5-dimethoxyhomophthalic acid (84) is outlined in Scheme 3.2.

SCHEME 3.2



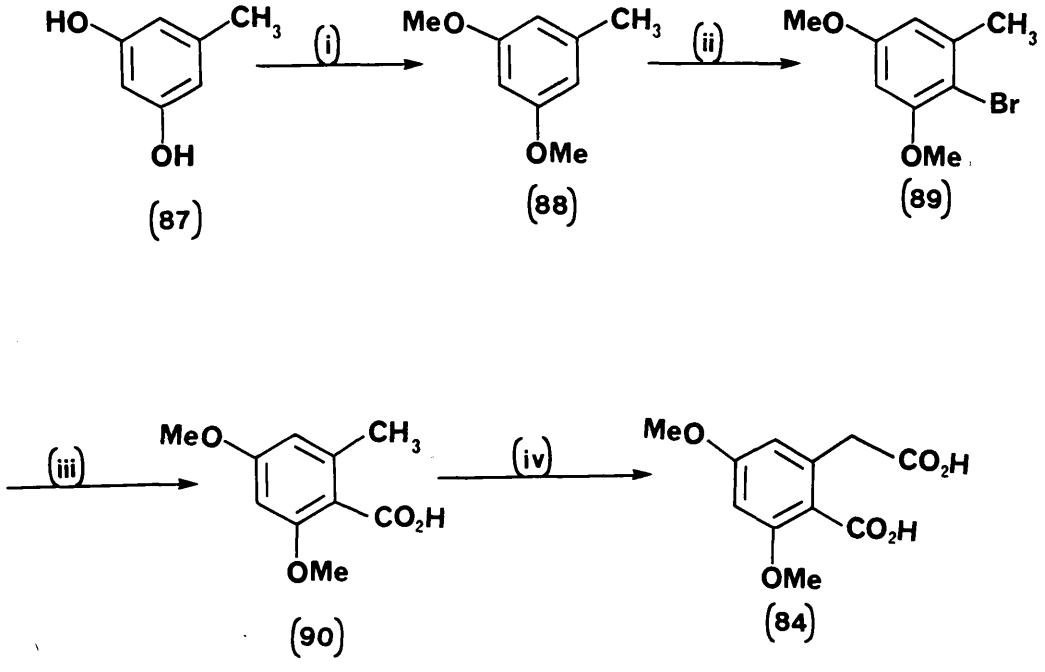
Reagents: (i) Br_2, HOAc; (ii) Raney Nickel, KOH;

(iii) Me_2SO_4, K_2CO_3, $(CH_3)_2CO$; (iv) KOH;

(v) LDA, Me_2CO_3, H_2O.

The synthesis of 3,5-dimethoxyhomophthalic acid (84) was that employed by Henderson.[80] The route had moderate yields, although the condensation between ethyl acetoacetate (85) and ethyl crotonate (86) proceeded with a yield of 45%. An improved route to 3,5-dimethoxy- homophthalic acid (84) was developed, see Scheme 3.3.

SCHEME 3.3



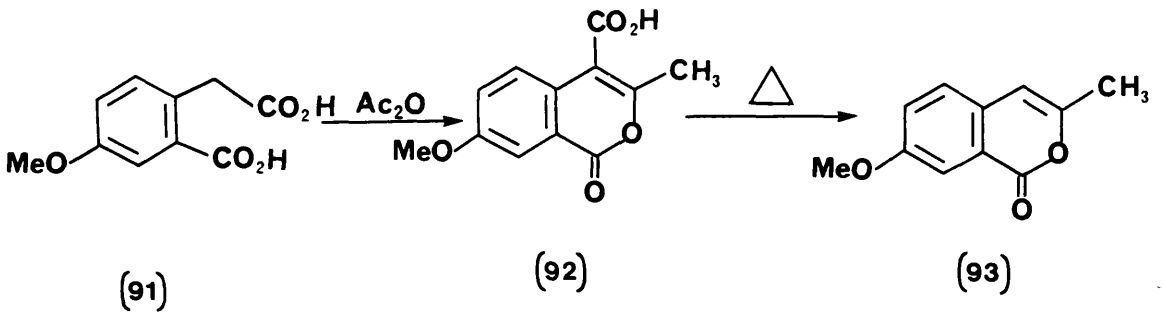
Reagents: (i) Me_2SO_4, K_2CO_3, $(CH_3)_2CO$;

(ii) Br_2, $Cl\text{-}CH_2CH_2\text{-}Cl$; (iii) nBuLi, CO_2

(iv) LDA, Me_2CO_3, H_2O.

Methylation of orcinol (87) proceeded in good yield to give the dimethyl ether (88). Treatment of the dimethyl ether (88) with one equivalent of bromine in 1,2-dichloroethane afforded 2-bromo-3,5-dimethoxytoluene (89) in 75% yield. This was confirmed by mass spectroscopy which gave parent ions m/z 196 and 198. In the ^{1}H n.m.r. spectrum the aromatic multiplet associated with (88) collapsed giving two doublets (J 2 Hz) which is consistent for a meta coupling between two aromatic protons. Treatment of the bromo derivative (89) with n-butyllithium afforded the 2-lithio species which reacted with carbon dioxide to give 6-methyl-3,5-dimethoxybenzoic acid (90). This again was confirmed by mass spectroscopy which had a parent ion m/z 194. The ir spectrum showed absorptions at 1650 and 3300 cm^{-1} which are consistent for a carboxylic acid. When the acid (90) was treated with lithium diisopropylamide and dimethyl carbonate, it gave 3,5-dimethoxyhomophthalic acid (84) after hydrolysis and work up.

Homophthalic acids have been converted into isocoumarins by a number of routes.[99] Tirodkar and Usgaonkar [100] reported that 4-methoxyhomophthalic acid (91) could be converted into 4-carboxy-7-methoxy-3-methylisocoumarin (92) on treatment with acetic anhydride and pyridine.

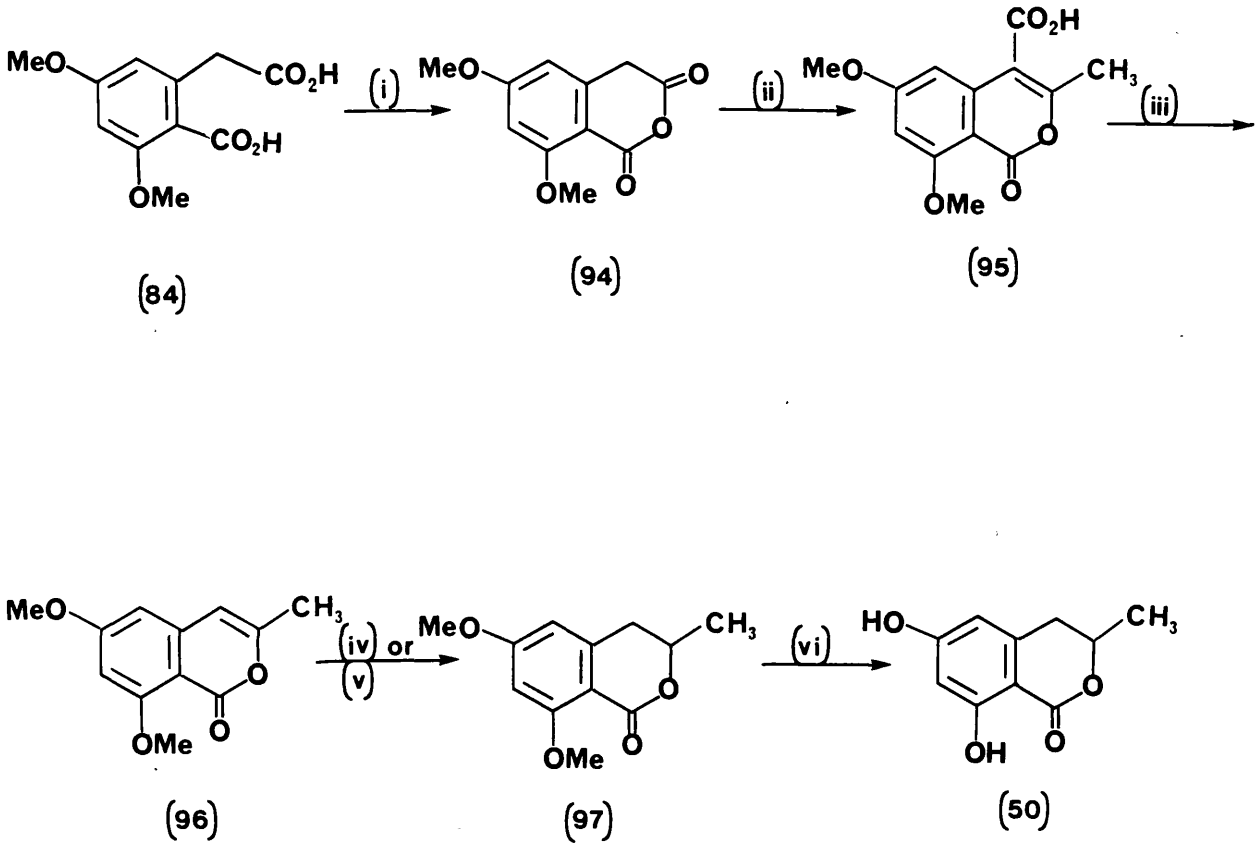


(91) (92) (93)

Decarboxylation afforded 7-methoxy-3-methylisocoumarin (93). Thus it was decided to adopt this approach for the synthesis of these substituted 3-methyl-isocoumarins. Henderson[80] found that a mixture of products was obtained when the homophthalic acid (84) was treated with acetic anhydride and pyridine. He found that is was better to isolate the intermediate homophthalic anhydride (94) which could thus be converted to the desired isocoumarin (95) in better yield (Scheme 3.4). The synthesis of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50) is as outlined in Scheme 3.4. Treatment of 3,5-dimethoxyhomophthalic acid (84) with acetyl chloride afforded 3,5-dimethoxyhomophthalic anhydride (94). Acylating the homophthalic anhydride with acetic anhydride and pyridine gave 4-carboxy-6,8-dimethoxy-3 -methylisocoumarin (95). Heating at 160°C resulted

in loss of carbon dioxide affording 6,8-dimethoxy-3-methylisocoumarin (96).

SCHEME 3.4



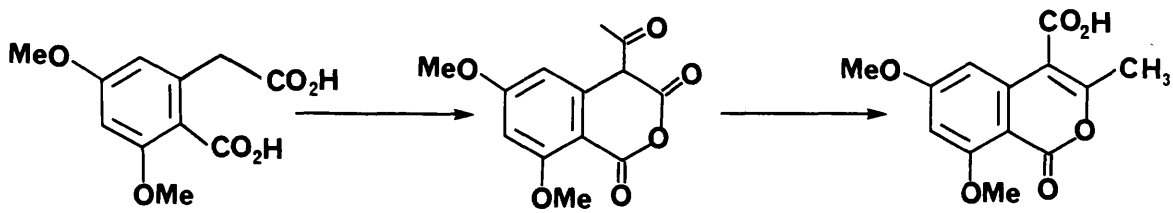
Reagents: (i) $CH_3\overset{O}{\overset{\|}{C}}Cl$; (ii) Ac_2O, pyr; (iii) Δ ;
(iv) $NaBH_4$; (v) H_2, Pd; (vi) BBr_3

Reduction of the 3,4-double bond to give (97) can either be achieved by hydrogenation in the presence of a palladium catalyst, or by treatment with sodium borohydride in refluxing aqueous sodium hydroxide solution. Demethylation of (97) was achieved by treatment with boron tribromide which gave the desired 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50) in 67% yield.

However, the main problem encountered in the synthesis was the conversion of 3,5-dimethoxyhomophthalic anhydride (94) to the 4-carboxyisocoumarin (95). The procedure adopted by Henderson[80] involved stirring the anhydride (94) in a solution of acetic anhydride and pyridine in dry THF for 1h followed by refluxing for 1h. Variable yields were obtained when this was performed. Varying conditions and amounts of reagents failed to produce 4-carboxy-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (95) in good yield.

There have been several reports published[100-103] concerning the synthesis of various 4-carboxyisocoumarins starting from the related homophthalic acids. The synthesis involves treatment of the homophthalic acids with acetic anhydride and pyridine obtaining a chroman-1,3-dione which when treated with concentrated sulphuric acid at 0°C

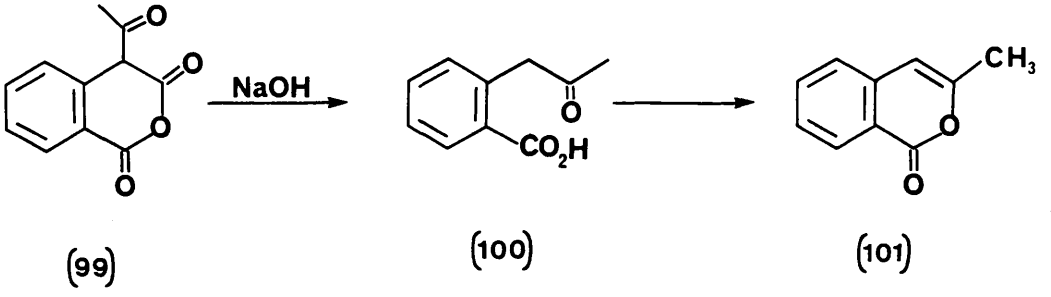
readily rearranges to give the desired 4-carboxy-isocoumarins.



(84) (98) (95)

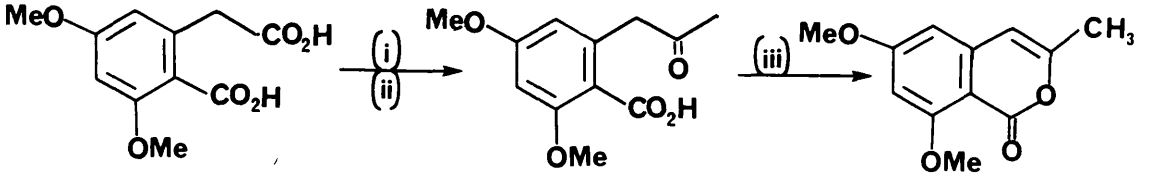
The aforementioned reactions were applied to 3,5-dimethoxyhomophthalic acid (84). Treatment of the acid (84) gave a crystalline compound (98) which when treated with concentrated sulphuric acid at 0°C gave a poor yield of 4-carboxy-6,8-dimethoxy-3-methylisocoumarin (95). Attempts to improve the yield of this intermediate failed. Due to the low yields in the formation of the 4-carboxyisocoumarin (95), another route was required which would give a better yield of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50).

Tirodkar and Usgaonkar[100] had reported that treatment of the intermediate (99) with aqueous sodium hydroxide



(99) (100) (101)

gave the keto-acid (100), which on treatment with acid cyclised to the isocoumarin (101). Therefore, this synthetic strategy was applied to 3,5-dimethoxy-homophthalic acid (84).



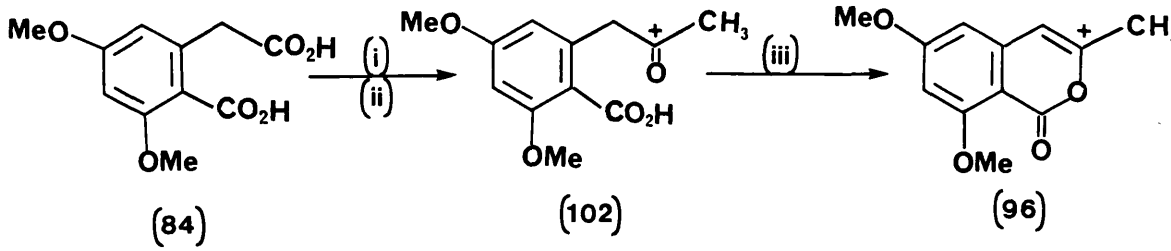
(84) (102) (96)

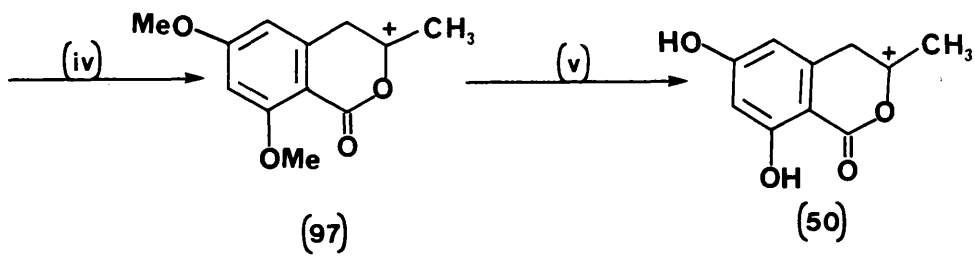
Reagents: (i) Ac_2O, pyr; (ii) NaOH; (iii) Ac_2O, $HClO_4$, EtOAc

Thus treatment of the homophthalic acid (84) with acetic anhydride and pyridine at room temperature afforded an intermediate which was not isolated. This was refluxed with sodium hydroxide solution, and after work-up gave the keto compound (102) in 70% yield. Ring closure to the isocoumarin (96) was achieved by standing in a solution of ethyl acetate, acetic anhydride and perchloric acid as reported by Edwards and Rao.[104] Reduction and demethylation gave the dihydroisocoumarin (50). Therefore an efficient route was now obtained which permitted a label to be introduced into the desired isocoumarin (50).

The synthesis of radioactive 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (50) was achieved as outlined in Scheme 3.5. If the radioactive isocoumarin were incorporated into microline and dechloromicroline then a degradation would be required to show that the label had been incorporated at the expected position in the final metabolite. An alternative method of investigating the incorporation of the isocoumarin precursor (50) is to use deuterium labelling as in the pentadeuterioisocoumarin (103). Deuterium n.m.r. would reveal where the deuterium atoms were situated in the metabolites microline (79) and dechloromicroline (80). Thus a degradation of the metabolites would not be required. The pentadeuterioisocoumarin (103) was

SCHEME 3.5



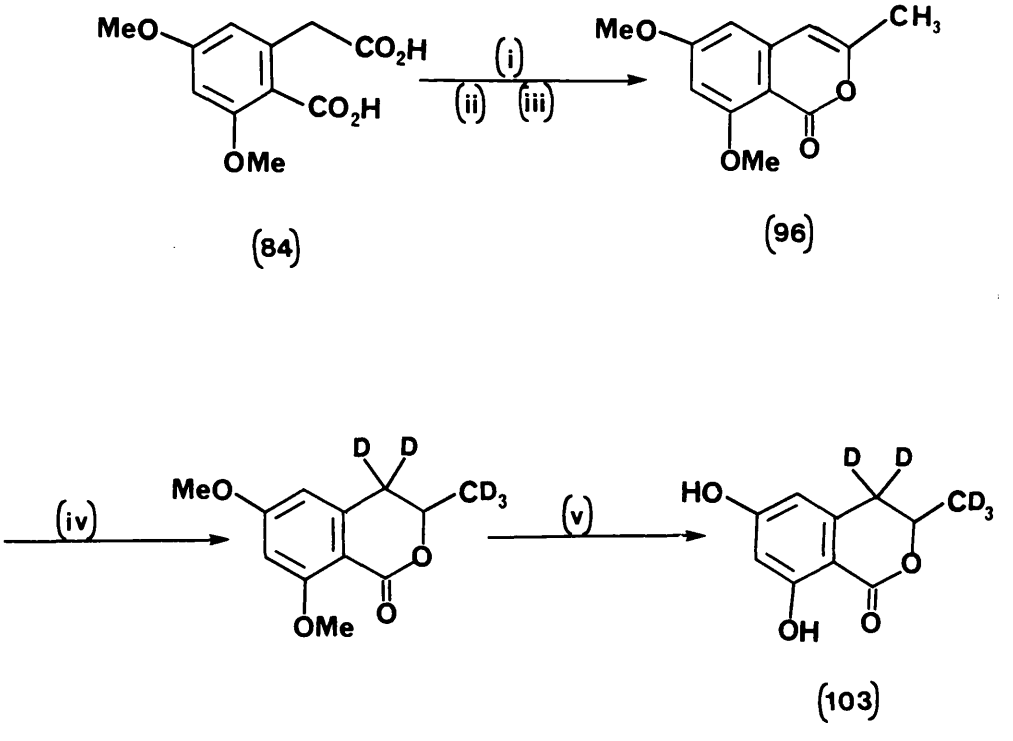


Reagents: (i) $(CH_3^{\dagger}CO)_2O$, pyr; (ii) NaOH;
(iii) $HClO_4$, Ac_2O, EtoAc; (iv) H_2-Pd; (v) BBr_3

synthesised by the route outlined in Scheme 3.6. Refluxing the isocoumarin (96) in sodium deuteroxide exchanges all the acidic protons and after treatment

with sodium borohydride and cyclisation affords the pentadeuterioisocoumarin. This is then demethylated with BBr_3 to give (103).

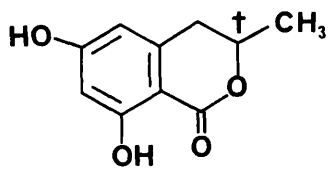
SCHEME 3.6



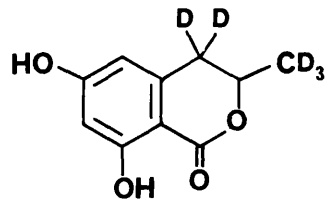
Reagents: (i) Ac_2O, pyr; (ii) NaOH; (iii) $HClO_4$, Ac_2O, EtoAc; (iv) NaOD, $NaBH_4$; (v) BBr_3

3.3 Feeding of labelled 3,4-dihydro-6,8-dihydroxy-3-methyl isocoumarins (50) + (103)

Growth of Gilmaniella humicola, on the medium used by Bollinger et al[91] gave no detectable quantities of microline, however, large quantities of dechloromicroline were obtained. The biosynthetic studies were performed on this compound. The two isocoumarins (50) and (103) were fed as their sodium salts to Gilmaniella humicola on day 4 of fungal growth.



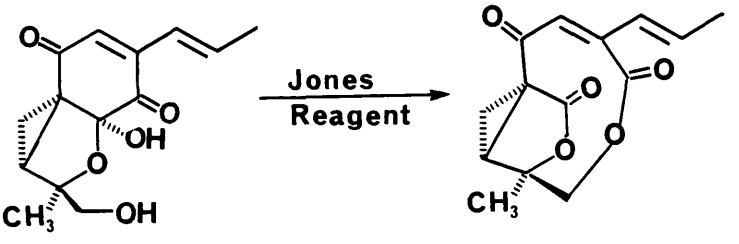
(50)



(103)

The fungus was extracted on day 7 as described in the experimental section(Chapter 6). Dechloromicroline was isolated as an oil. It was necessary to convert the dechloromicroline (80) into a crystalline derivative

which could be recrystallised to constant activity for the radioactive sample. Chexal and Tamm[96] converted dechloromicroline (80) into the crystalline bislactone (104) by oxidation using Jones reagent. The dechloromicroline obtained from each feeding experiment was oxidised to the bislatone (104).



(80) (104)

3.3.1 Feeding results from isocoumarin (50)

The bislactone (104) was recrystallised to constant activity. The percentage incorporation of the dihydroisocoumarin (50) into the bislactone (104) and hence dechloromicroline (80) can be expressed as:

$$\% \text{ Incorporation} = \frac{\text{counts from bislactone (104)}}{\text{counts from isocoumarin (50)}} \times 100\%$$

From the results obtained (see experimental section) it was found that 8.2% of the radioactivity from the isocoumarin (50) was found in the bislactone (104). Therefore, the isocoumarin (50) was converted into dechloromicroline with an incorporation of 8.2%. The radioactive technique is a good method of determining percentage incorporation of postulated precursors into metabolites. However, it does not give any information about bonds that have been broken or remained intact in the conversion of the precursor to the metabolite. It was hoped that the results obtained from the feeding of the pentadeuterioisocoumarin (103) would give more information about the nature of the construction of dechloromicroline (80) and hence microline (79).

3.3.2 Feeding results from the isocoumarin (103)

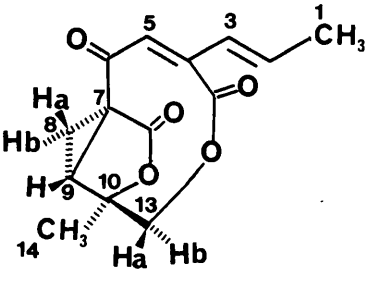
Before any feedings were carried out a 360 MHz 'H n.m.r. of the compound (104) was obtained and the protons were assigned as shown in Table 3.1.

The singlets which appear at δ1.45 p.p.m. (3H) and δ6.06 p.p.m. (1H) were assigned to the protons at carbons 14 and 5 respectively.

The two doublets which appear at δ5.22 p.p.m. and δ4.07 p.p.m. were assigned to the two protons at carbon-13 since they show a geminal coupling constant of 11.45 Hz. The proton which appears at δ4.07 p.p.m. was assigned H-13a whilst the proton that appears at lower field was assigned H-13b. This is because H-13b occupies a region which is close to the oxygen atom between carbon-10 and carbon-11. This causes a deshielding of this proton and thus accounts for its appearance at lower field. The signal at δ1.87 p.p.m. (3H) which has coupling constants of 0.8 Hz and 6.4 Hz was assigned to the protons attached to carbon-1. The larger value of J 6.4 Hz was due to coupling with H-2 and the smaller value J 0.8 Hz was attributed to allylic coupling with H-3. The signals centred at δ6.13 p.p.m. and δ6.09 p.p.m. were assigned to H-3 and H-2 respectively on the basis of the splitting pattern

Table 3.1

Assignment of 'H n.m.r. spectrum



Proton	δ
CH_3-1	1.87 (3H, dd, J 0.8 and 6.4 Hz)
H-2	6.09 (1H, dq, J 6.4 and 15.8 Hz)
H-3	6.13 (1H, dq, J 0.8 and 15.8 Hz)
H-5	6.06 (1H, s)
H-8a	1.76 (1H, dd, J 5.5 and 8.0 Hz)
H-8b	1.10 (1H, dd, J 4.9 and 5.5 Hz)
H-9	2.34 (1H, dd, J 4.9 and 8.0 Hz)
H-13a	4.07 (1H, d, J 11.4 Hz)
H-13b	5.22 (1H, d, J 11.4 Hz)
CH_3-14	1.45 (3H, s)

and their coupling constants. The signal centred at δ6.09 p.p.m. has coupling constants of 15.8 Hz and 6.4 Hz. The larger coupling constant J 15.8 Hz was due to coupling across the trans double bond with H-3 and the smaller coupling constant J 6.4 Hz was attributed to vicinal coupling between this proton and the methyl group. Similarly, the signal centred at δ6.13 p.p.m. showed coupling of J 15.8 Hz and J 0.8 Hz. The smaller value was due to the allylic coupling with the methyl group. The signals at δ1.10 p.p.m. and δ1.76 p.p.m. were attributed to the protons at carbon-8 since both signals exhibit a coupling constant of 5.5 Hz which is characteristic of a geminal coupling in a three membered ring. Therefore, the signal centred at δ 2.34 p.p.m. was due to the proton attached to carbon-9. The assignment of the individual protons at carbon-8 was based on coupling constants between these protons and H-9. The larger value (J 8 Hz) arises through coupling between H-9 and H-8a. These two adjacent protons are almost eclipsed which corresponds to a very small dihedral angle. The observed coupling constant is found to be in agreement to the value suggested for a dihedral angle of 0^{o} from the Karplus equation.[105]

A ^{2}H n.m.r. of the compound (104) was obtained after the feeding of the pentadeuterioisocoumarin (103). The chemical shift values in ^{2}H n.m.r. correlate directly

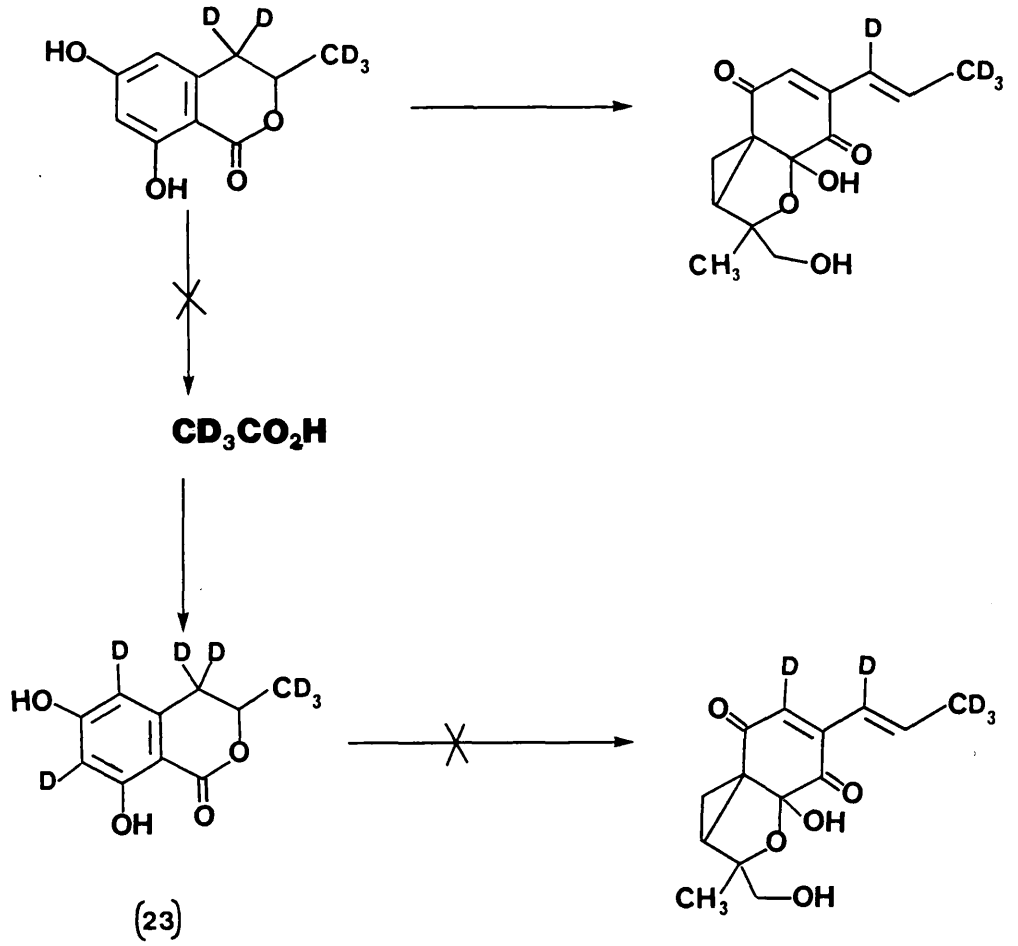
with those in 'H n.m.r., therefore it is possible to know which protons had been substituted by deuterium in the final compound (104), on the basis of chemical shift.

The ^{2}H n.m.r. contained two peaks in the spectrum. The two peaks were found at δ1.83 p.p.m. and δ6.13 p.p.m. which corresponds to the three protons on carbon-1 and to H-3 respectively. This is obtained from comparison of the 'H n.m.r. spectrum of (104) with the ^{2}H n.m.r. spectrum of the same compound. There was no signal found at δ6.06 p.p.m. in the ^{2}H n.m.r. spectrum which corresponds to H-5 and therefore shows that (103) had not been broken down to CD_3CO_2H and then incorporated into (23). The isocoumarin (103) is therefore incorporated into dechloromicroline intact.

These results support the biosynthetic pathway proposed in Scheme 3.1.

The isocoumarin (50) is an early intermediate in the biosynthesis of microline and dechloromicroline. Much work has to be done to establish latter intermediates which would help solve many of the questions associated with dechloromicroline biosynthesis.

The use of different isotopes of carbon has highlighted



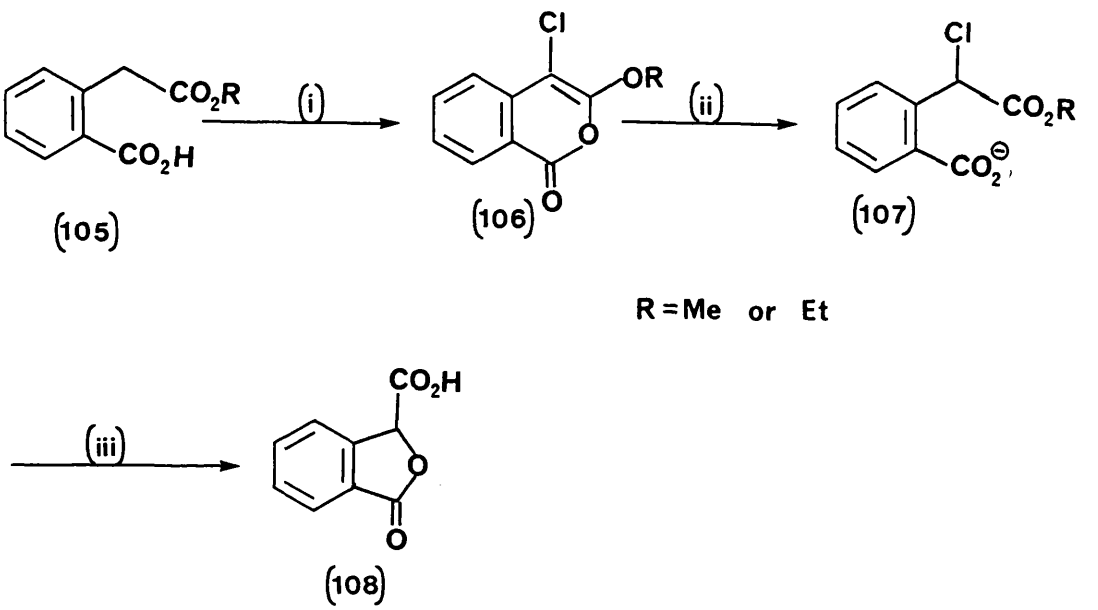
(23)

the benefits available to organic chemists as they try to understand biosynthetic pathways. The radioactive isotope gave a quantitative measure of how well the precursor (50) was incorporated into dechloromicroline (80). The use of stable isotopes e.g. ^{13}C and ^{2}H showed that the molecule had not been broken down and was incorporated intact.

3.4 Attempted Synthesis of 4-Chloroisocoumarins

The main problems encountered in synthesising 4-chloroisocoumarins or indeed any 4-substituted isocoumarins are discussed in chapter 4.

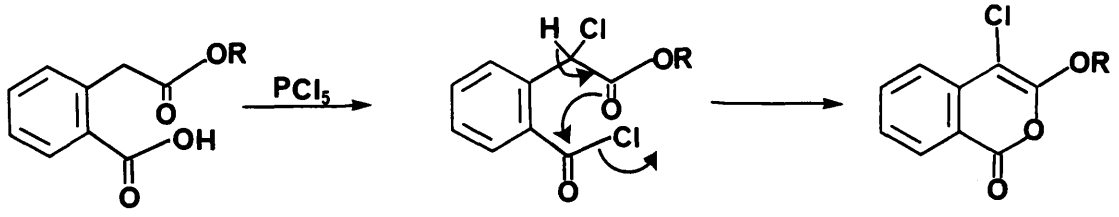
A report by Tirodkar and Usgaonkar[106] stated that when the ester (105) was treated with 2 moles of phosphorus pentachloride the 3-alkoxy-4-chloroisocoumarin (106) was formed.



Reagents: (i) PCl_5; (ii) NaOH; (iii) H^+

They found that treating the isocoumarin (106) with aqueous sodium hydroxide solution gave the carboxylate ion (107)

which cyclised under acidic conditions to give phthalide-3-carboxylic acid (108). The mechanism of the cyclisation to the isocoumarin (106) from the half-ester (105) is thought to take place as follows:

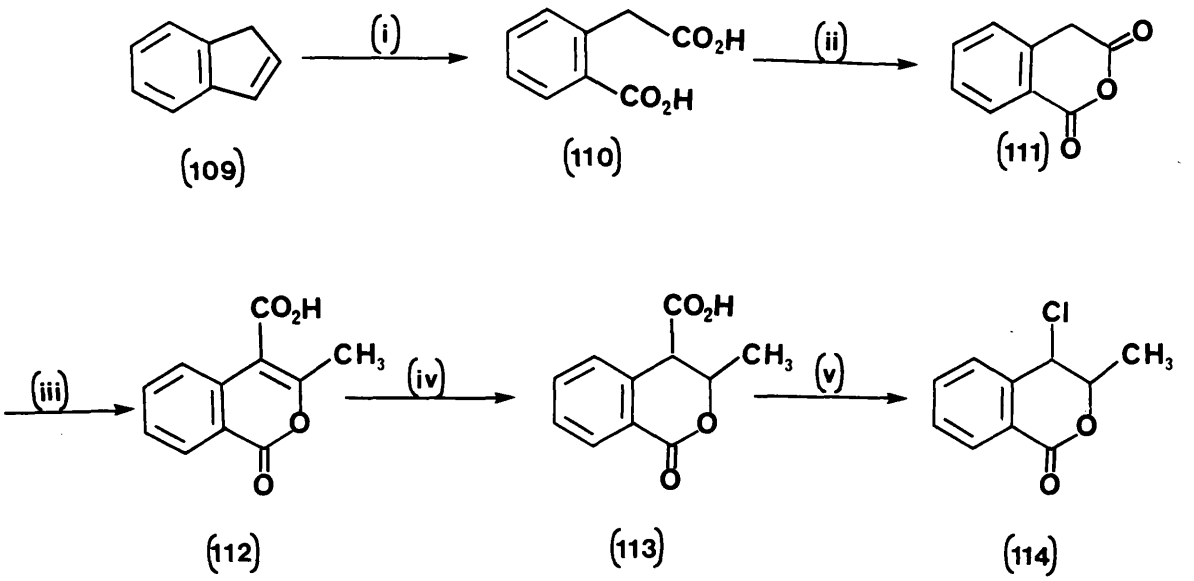


(105) (106)

Homophthalic acid (110) was used in a model study towards the synthesis of 4-chloroisocoumarins. This was obtained by oxidation of indene (109) using chromic acid.[107]

A report by Becker et al[108] states that chlorination of saturated carboxylic acids can be achieved in good yield using lead tetraacetate. Therefore, it was anticipated that the conversion of 4-carboxy-3,4-dihydro-3-methylisocoumarin (113) to 4-chloro-3,4-dihydro-3-methylisocoumarin (114) could be accomplished by using lead tetraacetate. The 4-carboxyisocoumarin (113) was prepared by the analogous route used for 3,5-dimethoxyhomophthalic acid (84).

SCHEME 3.7



Reagents: (i) CrO_3; (ii) $CH_3\overset{O}{\overset{\|}{C}}Cl$ (iii) Ac_2O, pyr (iv) H_2-Pd; (v) $Pb(OAc)_4$, NCS, DMF.

The reaction conditions employed were identical to those used for the corresponding dimethoxy-derivatives. As found with the 3,5-dimethoxy-series the unsubstituted homophthalic anhydride (111) was not converted to 4-carboxy-3-methylisocoumarin (112) in good yield. Attempts to improve the yield of the synthesis of this isocoumarin (112) were not successful. Hydrogenation of isocoumarin (112) reduced the double bond to give the 3,4-dihydroisocoumarin

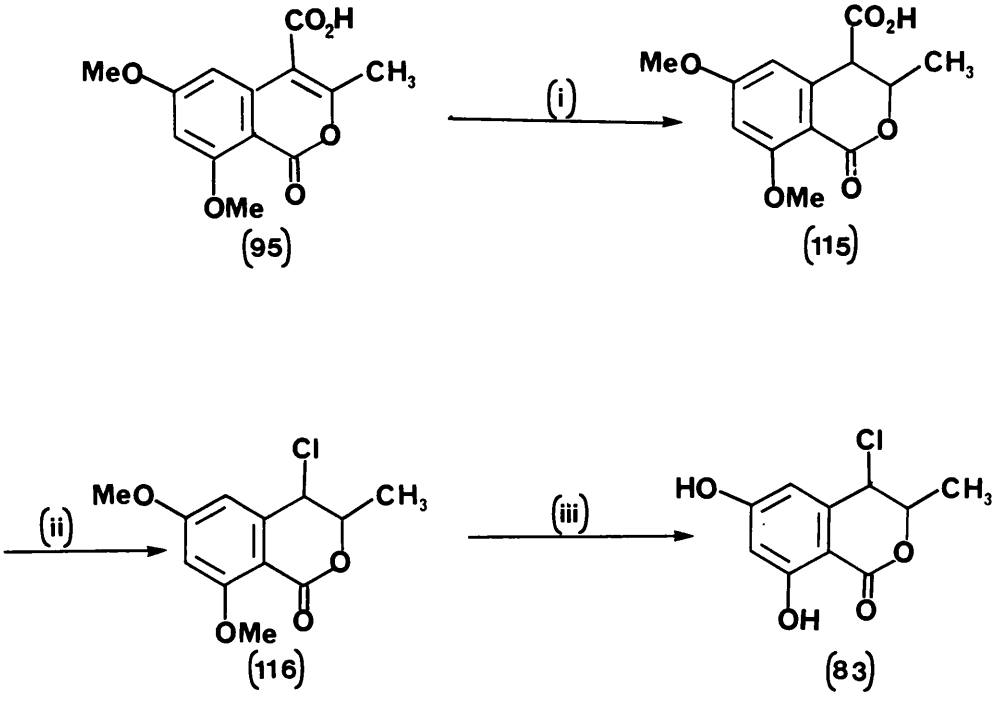
(113). Replacement of the acid group was achieved by treatment of the isocoumarin (113) with N-chlorosuccinimide and lead tetraacetate in a solution in dimethyl formamide yielding the 4-chloroisocoumarin (114).

Encouraged by these results obtained for the model compounds, it was envisaged that this synthetic strategy could be used to produce 4-chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (116). Demethylation using boron tribromide would yield the desired 4-chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (83). The synthetic strategy analogous to the model study is shown in Scheme 3.8.

However all attempts to produce the 4-carboxydihydro derivative (115) failed. The hydrogenation reaction gave a variety of products and after much effort this route was abandoned.

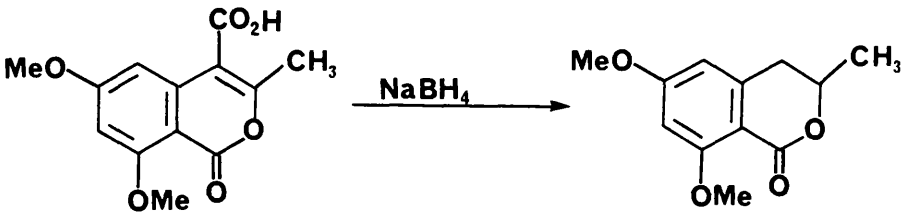
Attempts to reduce the double bond using sodium borohydride resulted in decarboxylation producing 3,4-dihydroisocoumarin (97).

SCHEME 3.8



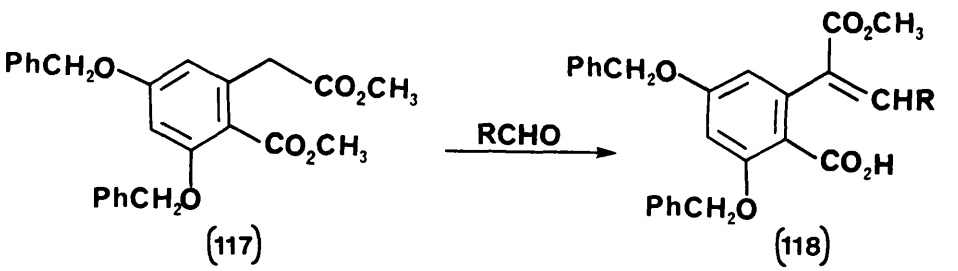
Reagents: (i) H_2-Pd; (ii) $Pb(OAc)_4$, NCS, DMF; (iii) BBr_3

Due to difficulties encountered in the reduction of the double bond of the 4-carboxyisocoumarin (95) and also the problems encountered with the actual synthesis of the 4-carboxy derivative (95) it was decided to investigate alternative syntheses of 4-carboxy-3,4-dihydroisocoumarins.

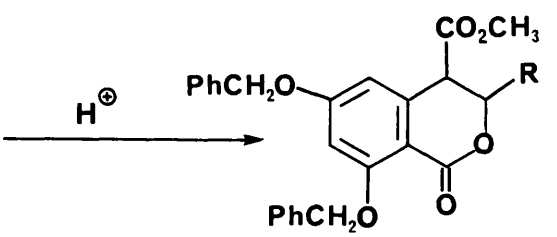


(95) (97)

Hurd and Shah[109] reported Stobbe condensations of dimethyl 3,5-bis (benzyloxy)homophthalate (117) with a variety of aliphatic aldehydes in the presence of sodium hydride to give the Stobbe half-esters of type (118). Treatment of these half-esters with acid gave the corresponding isocoumarins (119).

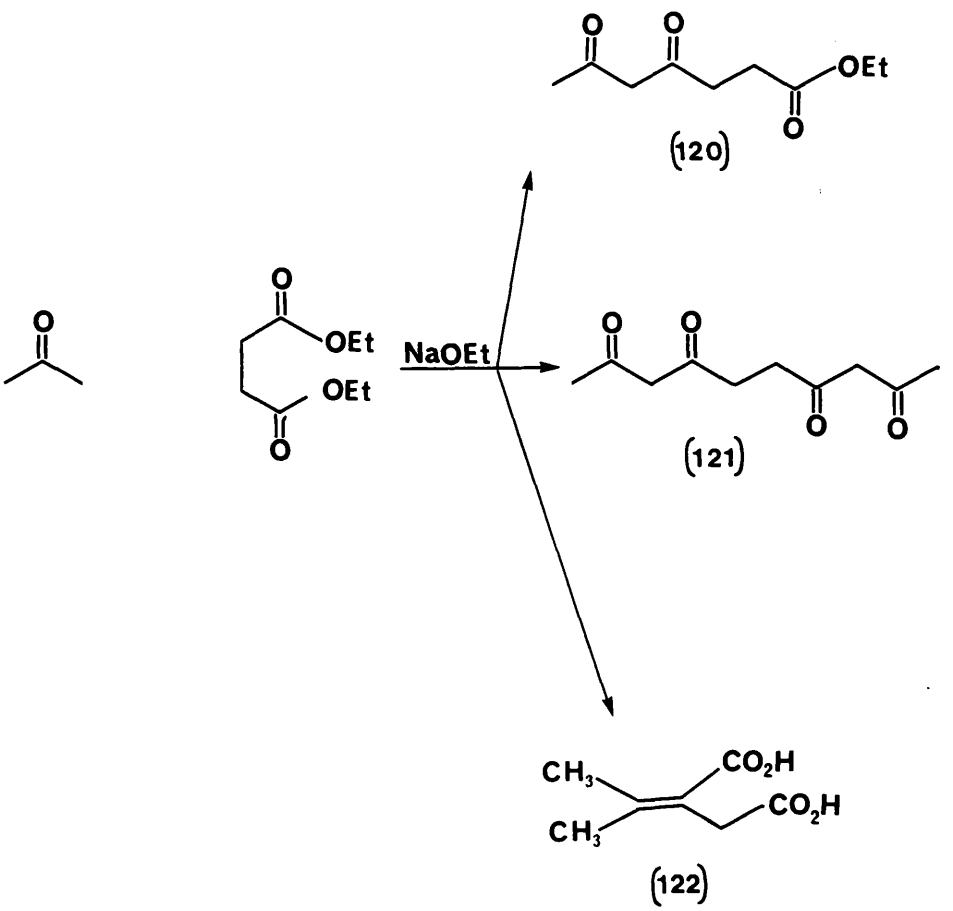


(117) (118)

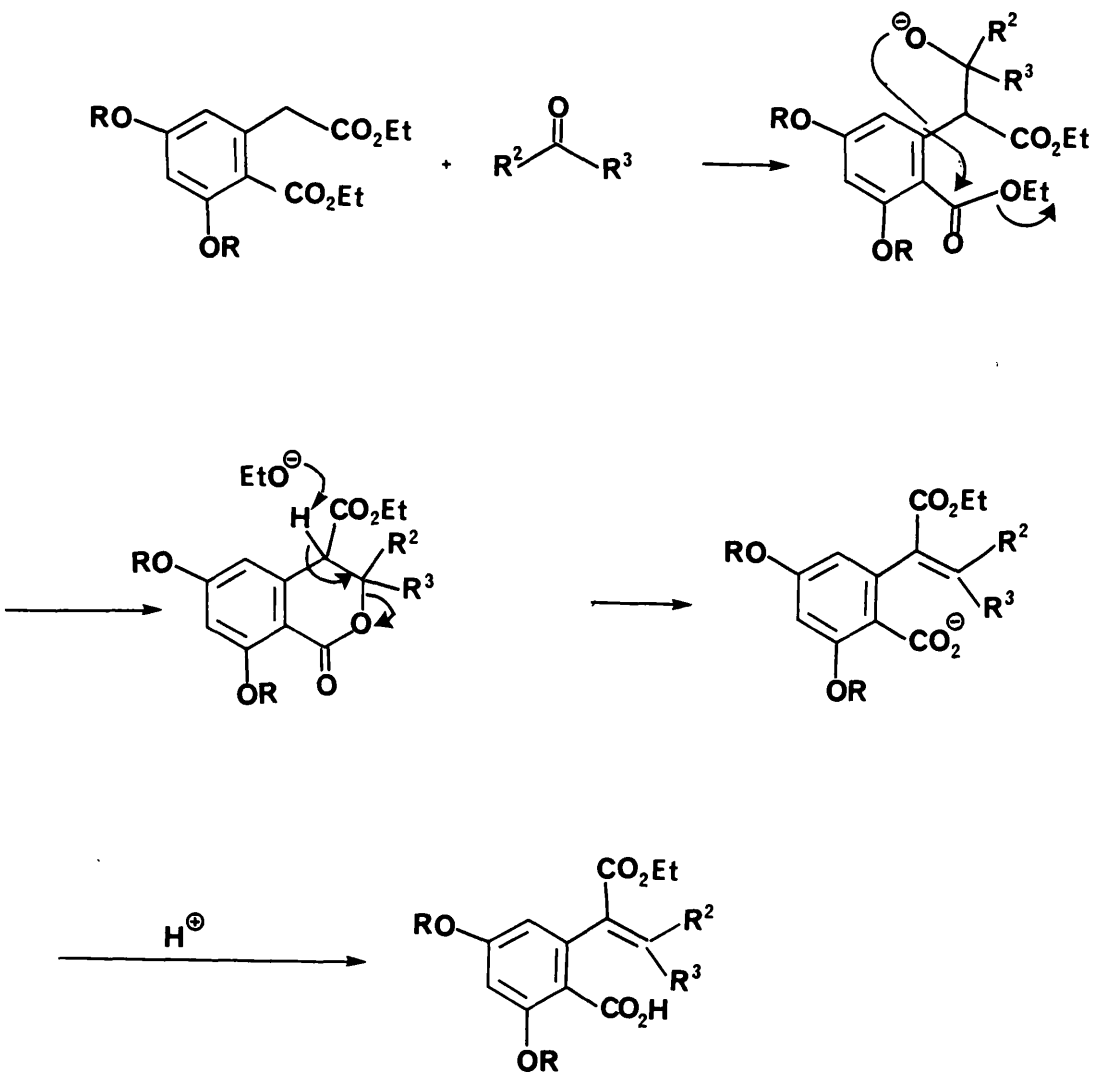


(119)

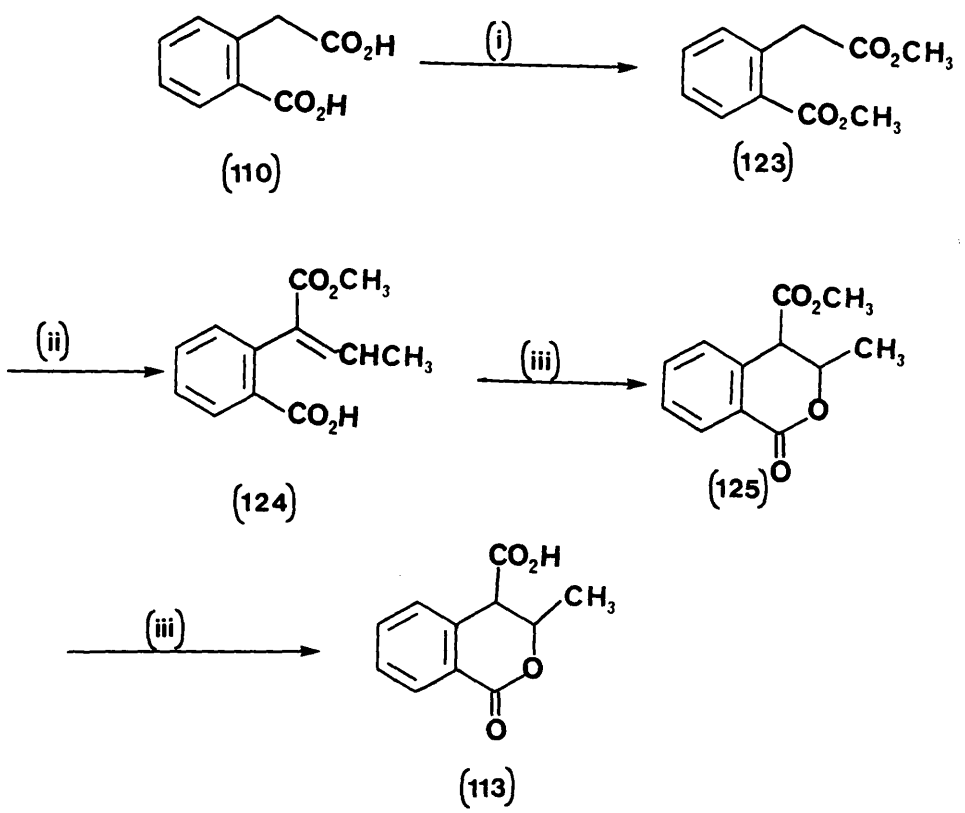
In 1893 Stobbe[110] had demonstrated that when acetone and diethyl succinate were treated with sodium ethoxide, the expected acetoacetic ester type of condensation to give a β-diketo compound (120) or (121) did not occur. The main reaction product was the acid (122) formed by aldol condensation between the carbonyl group of the ketone and an α-methylene group of the ester.



The success of the Stobbe condensation is not attributed to a high reactivity of the α-methylene group of succinic esters as shown by the failure of diethyl malonate to condense with benzophenone. The specificity of succinic esters in this reaction may be associated with the juxtaposition of a carbethoxy group



for ring formation. This would explain why esters of homophthalic acid were found to undergo the Stobbe condensation with a variety of aldehydes. The ester group is situated in such a way that ring formation occurs readily. This synthetic strategy was applied with a view to making the 4-carboxy-3,4-dihydroisocoumarin (115).

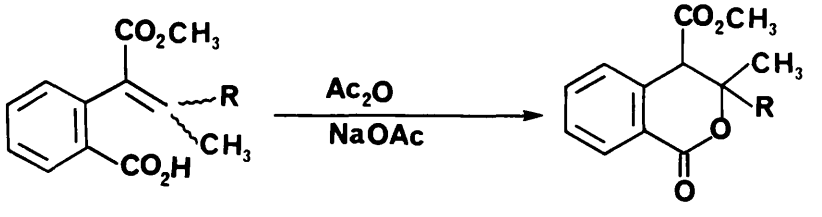


Reagents: (i) MeOH/$SOCl_2$; (ii) CH_3CHO, NaH; (iii) H^+

Dimethylhomophthalate (123) was obtained by treatment of the corresponding acid with thionyl chloride and methanol. Reaction of this ester (123) with acetaldehyde should give the intermediate (124) which on treatment with acid will cyclise to the isocoumarin (125).

However, the condensation between dimethyl homophthalate (123) and acetaldehyde did not proceed smoothly and the desired intermediate (124) was obtained in poor yield. Attempts to improve the yield of this reaction were unsuccessful. A report in the literature stated that these condensations proceed in high yield if a few drops of ethanol are added.[111] This also proved fruitless.

Awad et al[112] reported that treatment of the half-ester (126) with acetic anhydride and fused sodium acetate gave the corresponding isocoumarin (127).

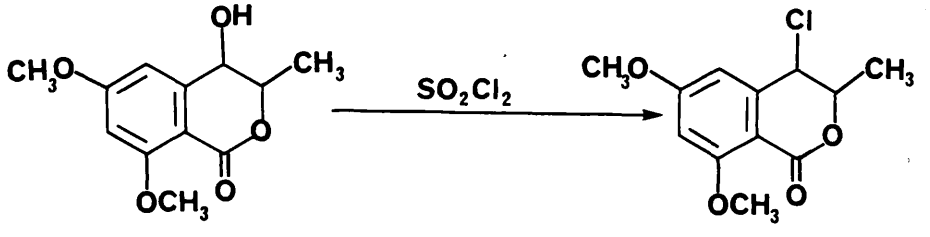


R = H (124)
R = Ar (126)

R = H (125)
R = Ar (127)

However, treatment of the half-ester (124) with acetic anhydride and sodium acetate gave a number of compounds by t.l.c. Again due to the low yielding reactions and uncertainty about some of the steps, this route was abandoned.

Thus it was thought that the way to the 4-chloro-isocoumarins would be via the 4-hydroxyisocoumarins, via treatment with sulphuryl chloride.

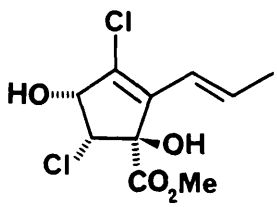


The synthesis of 4-hydroxyisocoumarins is discussed in chapter 4. Unfortunately a route to this type of compound was not developed and likewise a synthesis of 4-chloroisocoumarins was not obtained. Therefore, the question of whether chlorination occurs early or not in the biosynthesis of microline (79) and dechloro-microline (80) remains unanswered.

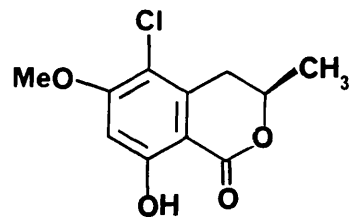
4. THE STRUCTURE OF PERIMACOL

4.1 Introduction

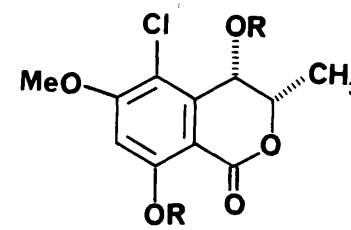
Cryptosporiopsinol (45) and 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (46) are the main metabolites produced by the fungus Periconia macrospinosa. Henderson[80] isolated three new chlorine containing metabolites (47), (48) and (49) in minor quantities.



(45)

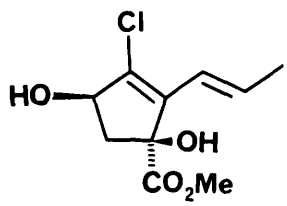


(46)

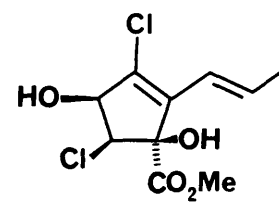


R = H (47)

R = CH_3 (128)

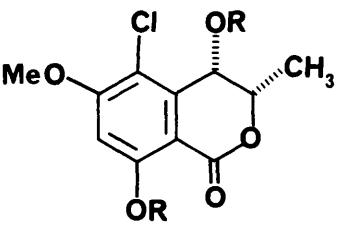


(48)



(49)

On the basis of spectroscopic data they were assigned the structures as shown. Structure (49) being the diastereomer of cryptosporiopsinol (45). The metabolite (47) was given the trivial name perimacol and after spectroscopic tests the structure was assigned. Henderson showed that perimacol gave a



R=H (47)

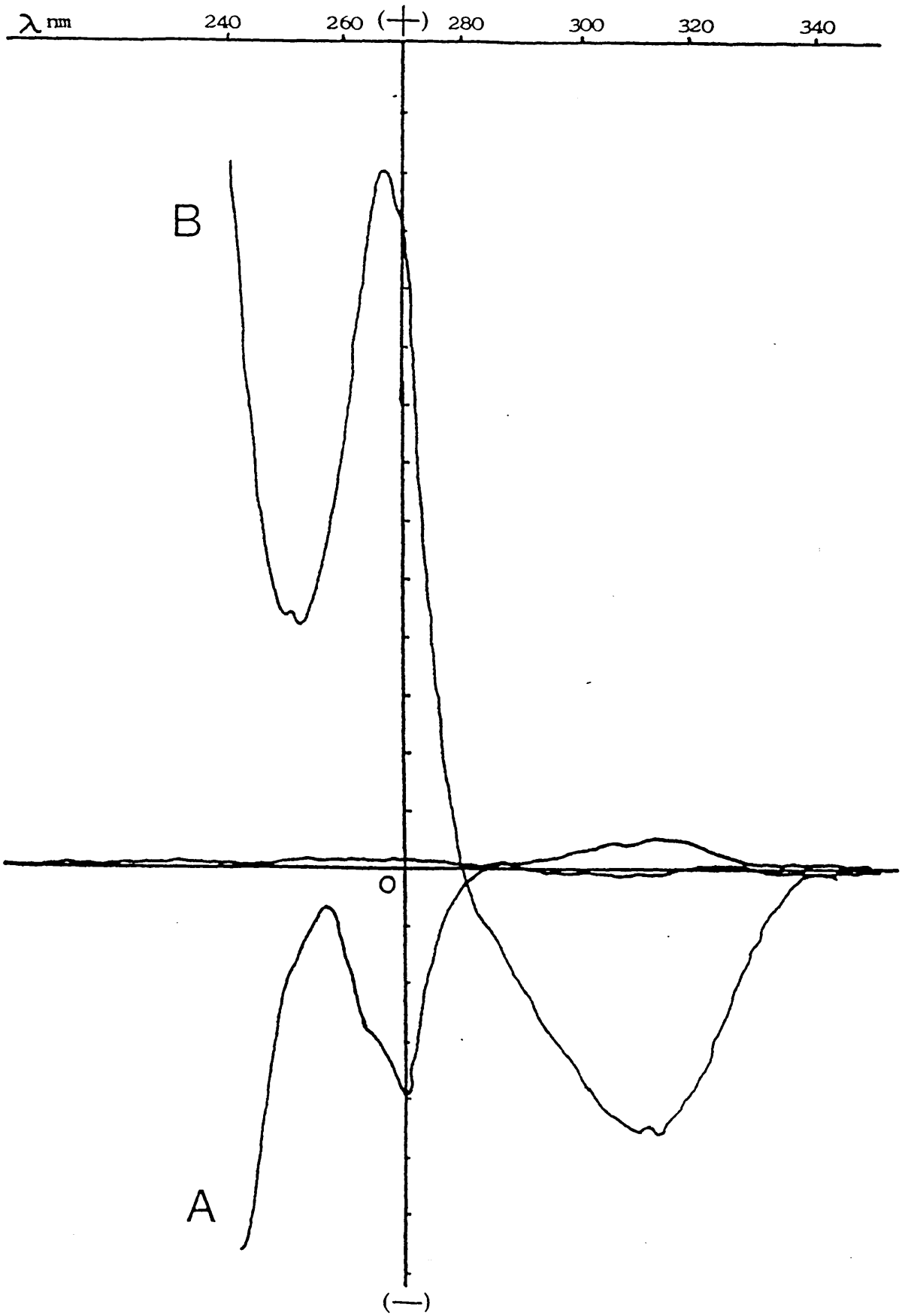
$R=CH_3$ (128)

positive ferric chloride test and that it formed a dimethyl derivative (128) when treated with methyl iodide and silver oxide. This derivative was not ferric chloride active. There are two interesting features to note about the structure of perimacol (47)

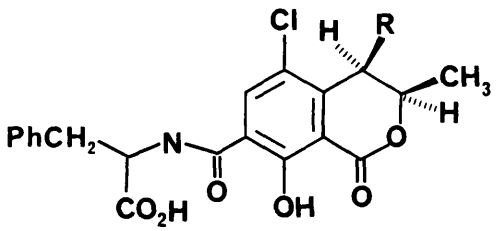
1) the S-configuration at the 3 position
2) the cis orientation between the hydroxyl and methyl group at carbons 4 and 3 respectively.

FIGURE 4.1

5-Chloro-3,4-dihdro-8-hydroxy-6-methoxy-3-methylisocoumarin (Curve A)
perimacol (Curve B)



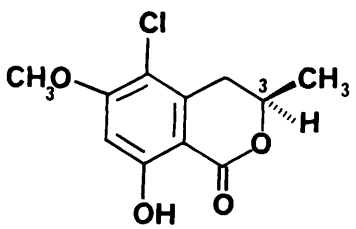
The absolute stereochemistry at C-3 was determined by utilising the technique of optical rotary dispersion (ORD). Much information has been obtained about structure, conformation, and configuration of organic compounds from measurements of optical rotation as a function of wavelength (i.e. optical rotatory dispersion). Like other phenomena involving interactions between electro-magnetic radiation and organic molecules, as in infrared, ultraviolet and n.m.r. spectroscopy, optical rotatory dispersion curves are quite sensitive to small changes in structure. Arakawa[113] showed that dihydroisocoumarins having a 3R-substituent gave rise to a negative Cotton effect near 260 nm. Steyn[114] used the ORD technique to determine the absolute stereochemistry of 4-hydroxyochratoxin A (130), a metabolite of Penicillium viridicatum. The configuration at C-3 had also been established by degradation studies. The ORD curves of perimacol (47) and the major dihydroisocoumarin metabolite (46) of Periconia macrospinosa are shown in Figure 4.1.The ORD curve of the dihydroisocoumarin (46) shows a negative extremum at 269 nm and is similar to the curve obtained by Arakawa[113] for a number of 3R substituted



R = H (129)

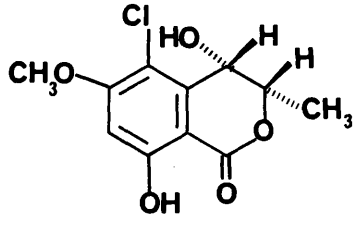
R = OH (130)

dihydroisocoumarins. Similar curves were obtained by Steyn[114] for ochratoxin A (129) and 4-hydroxyochratoxin A (130). On the basis of this result the dihydroisocoumarin (46) was assigned the R configuration at the 3-position.



(46)

The ORD curve for perimacol (47) shows a positive extremum at 267 nm and is almost the mirror image of the curve obtained for the major dihydroisocoumarin metabolite (46) of Periconia macrospinosa. On the basis of this result perimacol would appear to have the S-configuration at the 3-position. Henderson postulated that the small coupling constant between H-3 and H-4 (J 2 Hz) in the ^{1}H n.m.r. spectrum was consistent with a dihedral angle of approximately 60°. This agrees well with a cis orientation.[105] Therefore, the structure proposed by Henderson is shown and is (3S, 4S)-5-chloro-3,4-dihydro-4,8-dihydroxy-6-methoxy-3-methylisocoumarin (47).



(47)

It would have been preferable to determine the absolute configuration of the new metabolite by degradation. However, owing to small amounts of material obtained, Henderson was unable to perform any degradation

experiments on the metabolite. It is unusual that two similar metabolites perimacol (47) and the dihydroisocoumarin (46), of the same fungus, Periconia macrospinosa appear to have different configurations at the 3-position. The only structural difference is that perimacol (47) has a hydroxyl group at C-4. There are two possible explanations for the opposite ORD curves obtained.

1) The ORD technique is not rigorous. Although two opposite ORD curves were obtained for similar metabolites indicating opposite configurations, the stereochemistry may be the same.

2) In the biosynthesis of perimacol (47), the enzyme responsible for the hydroxylation at C-4 may require the methyl group to have an S-configuration to allow the molecule to sit in the active site of the enzyme.

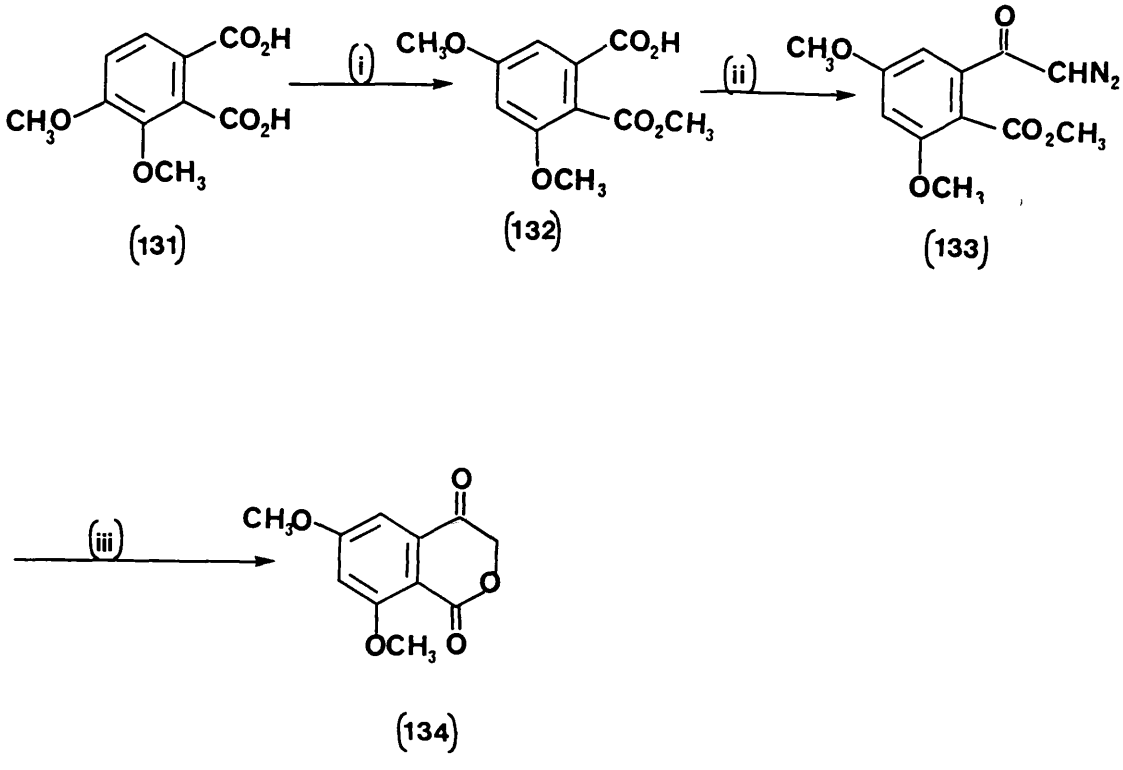
To unambiguously determine the structure, a synthesis of perimacol was thus investigated.

4.2 Synthesis of 4-Hydroxydihydroisocoumarins

Consultation with the literature revealed that the synthesis of 4-hydroxydihydroisocoumarins has been little studied. Thus as well as a synthesis of

perimacol (47) being undertaken, it was hoped that a general synthesis of 4-hydroxydihydroisocoumarins could be found. Chatterjea et al[115] reported a synthesis of 4-oxodihydroisocoumarins. This synthesis, starting from 3,4-dimethoxyphthalic acid (131) is shown in Scheme 4.1.

SCHEME 4.1

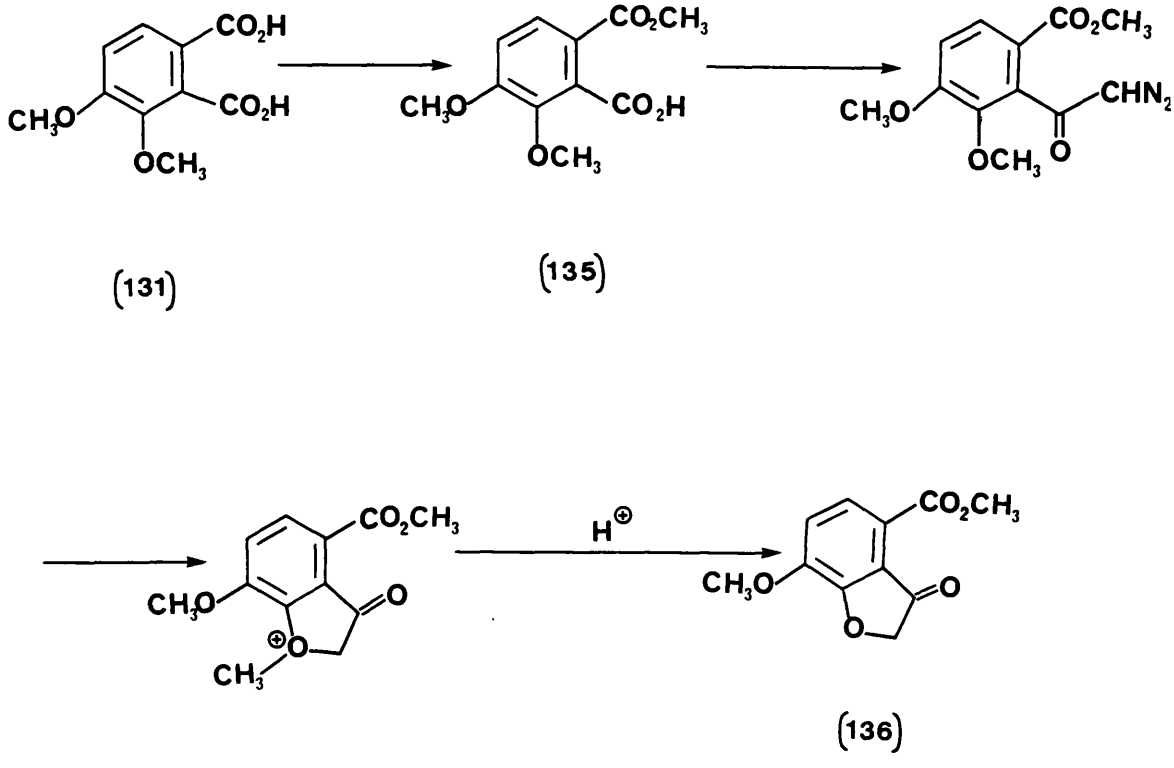


Reagents (i) (a) Ac_2O, (b) MeOH (ii) $(COCL)_2$, CH_2N_2

(iii) H^+

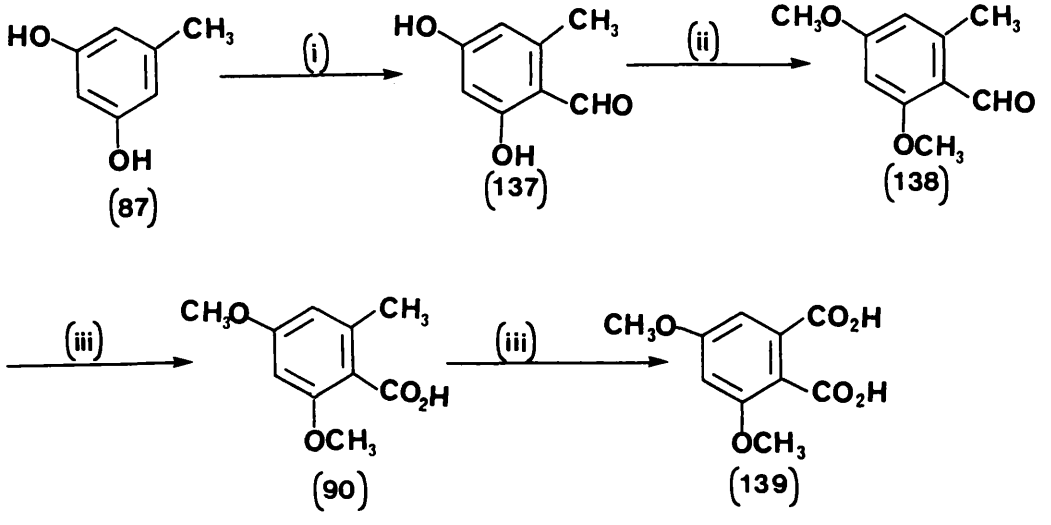
Treatment of the half-ester (132) with oxalyl chloride and then diazomethane afforded the diazoketone (133). Heating the diazoketone (133) in dilute acid yielded 7,8-dimethoxy-4-oxodihydroisocoumarin (134). However, applying the same reaction sequence on the half-ester (135) the isocoumarin (134) is not formed. The ketone (136) is formed instead (Scheme 4.2).

SCHEME 4.2



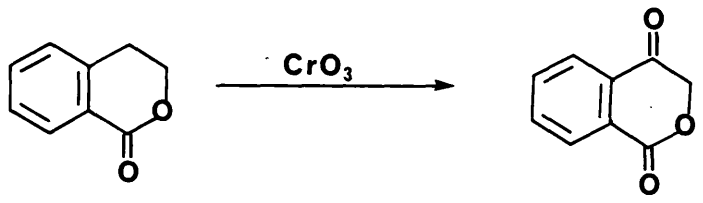
Substituting diazoethane for diazomethane it was anticipated that a synthesis of 4-oxo-3-methyl-dihydroisocoumarins could be obtained. The acid (90) was obtained by the route outlined in Scheme 4.3.[116] A Vilsmeier-Haack formylation of orcinol (87) gave the benzaldehyde derivative (137). This was protected as the dimethyl ether (138) by treatment with dimethyl sulphate and was subsequently oxidised to give the acid (90). Oxidation of this acid (90) with potassium permanganate gives the corresponding phthalic acid (139). However, after very low yields of the acid (139) were obtained it was decided to investigate another route for the synthesis of 4-hydroxyisocoumarins.

SCHEME 4.3

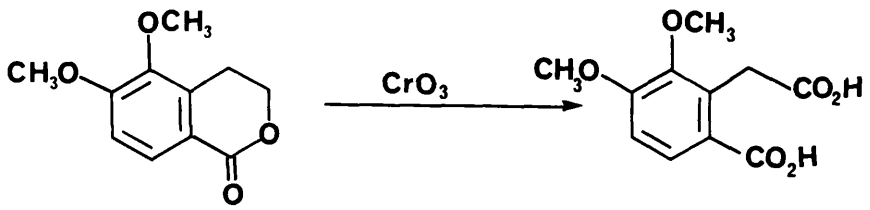


Reagents: (i) $POCl_3$, DMF; (ii) Me_2SO_4, K_2CO_3, $(CH_3)_2CO$; (iii) $KMnO_4$

Chatterjea et al[115] also reported that treatment of 3,4-dihydroisocoumarin (140) with chromic acid gave 3-hydro-4-oxoisocoumarin (141) in poor yield. However, treatment of 3,4-dihydro-5,6-dimethoxyisocoumarin (142) by the same method gave 2,3-dimethoxyhomophthalic acid (143).



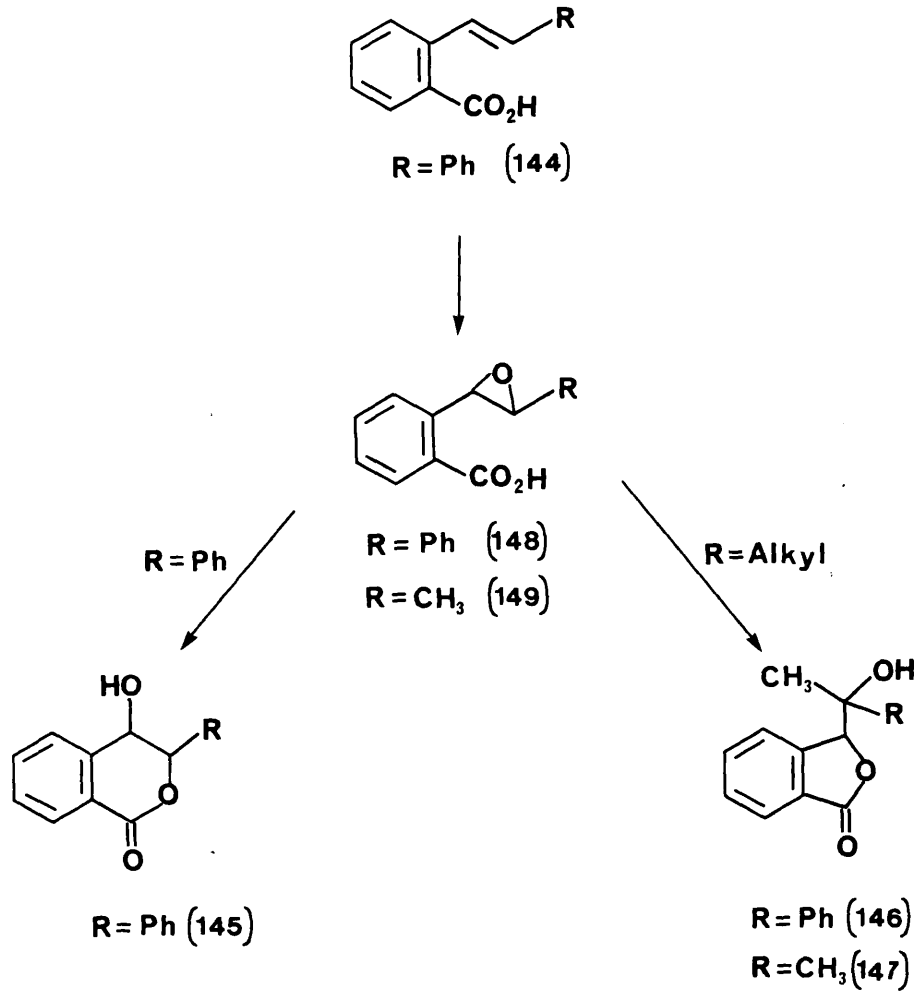
(140) (141)



(142) (143)

Berti[117] investigated the reaction of trans and cis stilbene-2-carboxylic acid (144 R=Ph) with organic peroxides and discovered that the 3,4-dihydro-4-hydroxy-3-phenylisocoumarins (145 R=Ph) were formed. No evidence was obtained for the formation of the corresponding epoxides. It was found that treatment with base opened the lactone which

recyclised to give the phthalide derivative (146 R=Ph) under acidic conditions.



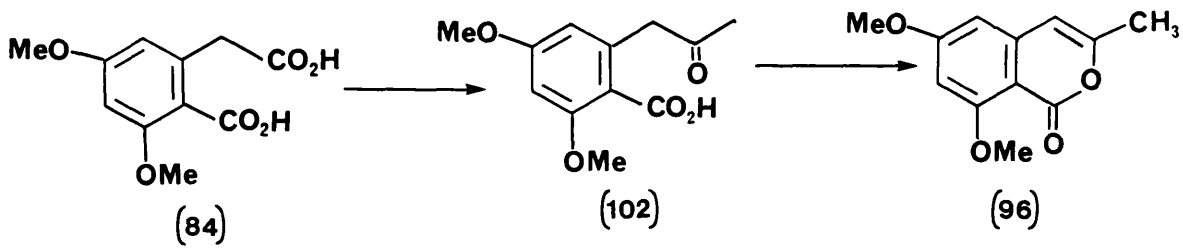
However, when R was alkyl, no formation of the 4-hydroxyisocoumarin was observed. The phthalide derivative (147 R=Me) was produced solely. Since it is believed[117] that the compounds (145) and (147) are formed from their epoxides (148 R=Ph), (149 R=Me), these results suggest that for 4-hydroxyisocoumarin

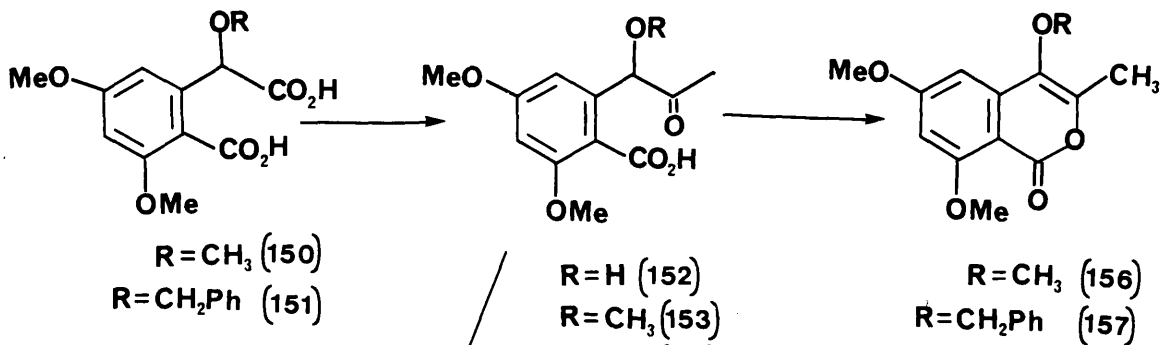
formation, R needs to be a group capable of supporting a positive charge. It can be seen that this route could not be used in a synthesis of perimacol (47). Therefore a new synthesis of 4-hydroxyisocoumarins was investigated.

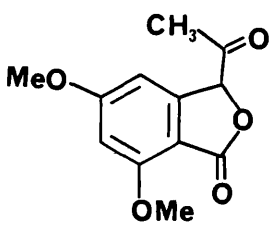
4.3 Synthesis of 4-Alkoxyisocoumarins

6,8-Dimethoxy-3-methylisocoumarin (96) is formed by the treatment of the keto-acid (102) with acid. This keto-acid (102) is synthesised from 3,5-dimethoxyhomophthalic acid (84) as described in Chapter 3.

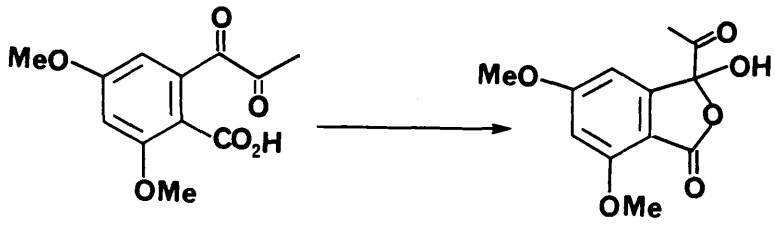
By synthesising the corresponding keto-acids (153) and (154) from their homophthalic acids (150) and (151) it was hoped that the desired isocoumarins (156) and (157) could be formed. Grove[118] has shown that the diketone compound (158) ring closes to the phthalide derivative (159). In a similar manner it was anticipated that the keto-acid (152) would also ring close giving the phthalide derivative (155). It was hoped that by forming the more stable ether derivatives (153) and (154) the possibility of cyclising to the phthalide derivative (155) under acidic conditions would be reduced. The required isocoumarins (156) and (157) would be formed instead.





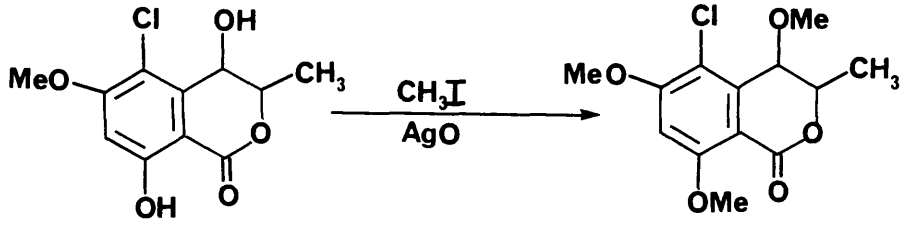


Henderson[80] synthesised the dimethyl ether of perimacol (128) by treatment of perimacol (47) with methyl iodide and silver oxide. Converting the methyl ether derivative (150) to the 4-methoxyisocoumarin (156) followed by chlorination would give the dimethyl ether of perimacol. A comparison could be made between

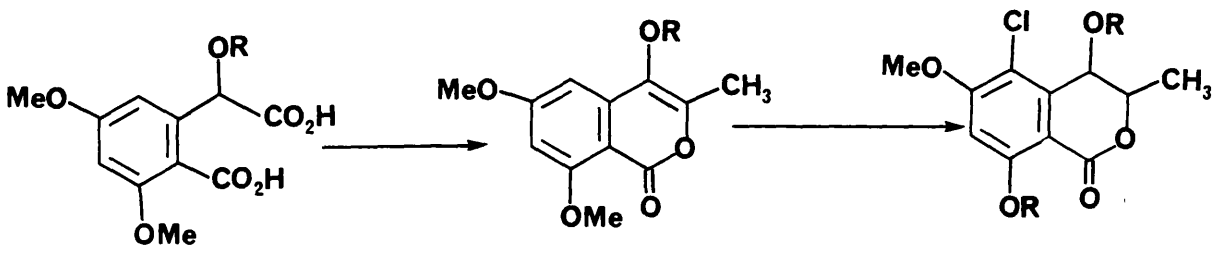


(158) (159)

synthetic product and the naturally derived product.



(47) (128)



R=CH_3 (150)
R=CH_2Ph (151)

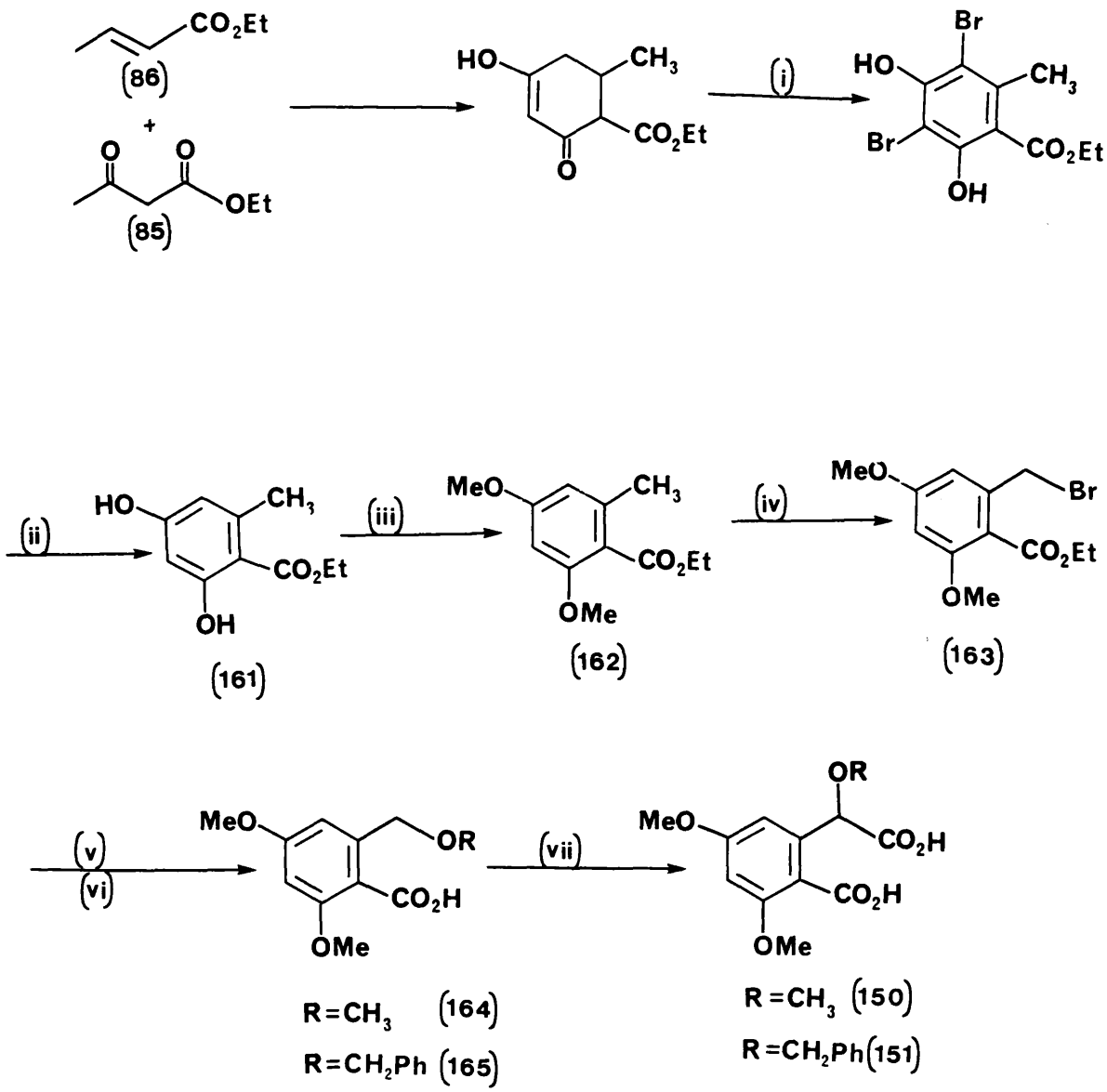
R=CH_3 (156)
R=CH_2Ph (157)
R=H (160)

R=CH_3 (128)
R=H (47)

By synthesising the benzyl ether (151) and converting to the 4-benzyloxyisocoumarin (157) followed by removal of the protecting group it was anticipated would give the 4-hydroxyisocoumarin (160). Selective demethylation of the dimethoxy-4-hydroxyisocoumarin (160) and chlorination would give perimacol (47). Thus it was hoped that this synthetic strategy could be used as a general route towards the synthesis of 4-hydroxyisocoumarins.

The strategy proposed for the synthesis of the substituted homophthalic acid compounds (150) and (151) is outlined in Scheme 4.5. The synthesis of ethyl orsellinate (161) is outlined in Chapter 5. The key step in the synthesis of the required compounds (150) and (151) is the monobromination of the dimethyl ether of ethyl orsellinate (162) to give (163). The ether

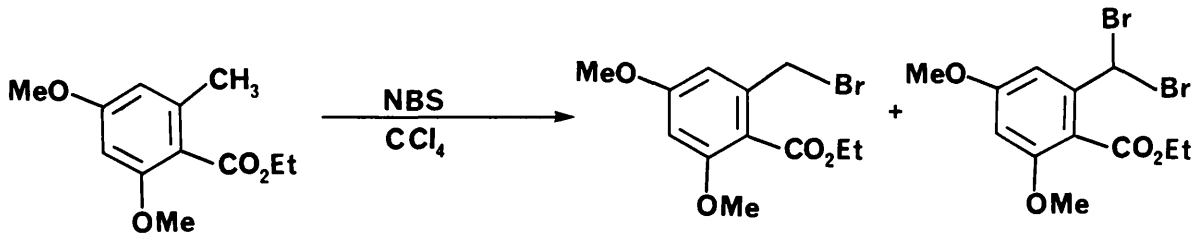
SCHEME 4.5



Reagents: (i) Br_2, HOAc; (ii) Raney Nickel; (iii) Me_2SO_4, K_2CO_3, $(CH_3)_2CO$; (iv) NBS, CCl_4; (v) MeOH or $PhCH_2OH$ (vi) KOH (vii) LDA, Me_2CO_3

(162) is obtained by treatment of ethyl orsellinate (161) with dimethyl sulphate and potassium carbonate. It was thought that nucleophilic substitution of the bromide by a suitable alkoxide ion followed by hydrolysis would give the appropriate ethers (164) and (165). These ethers it was envisaged could be converted to the required substituted homophthalic acid derivatives (150) and (151) by treatment with lithium diisopropylamide and dimethylcarbonate followed by hydrolysis.

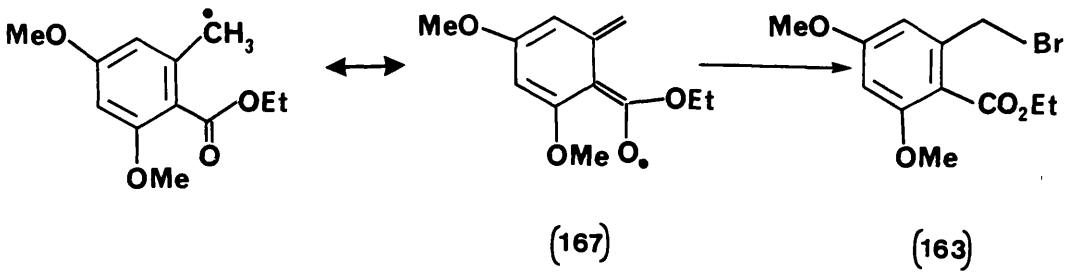
However, when the dimethyl ether of ethyl orsellinate (162) was refluxed with N-bromosuccinimide (NBS) in carbon tetrachloride under the irradiation of U.V. light a mixture of products was formed. Using exactly one equivalent of NBS, there was a mixture of the required monobromo compound (163), the dibromo compounds (166) and starting material (162). Varying the reaction conditions always resulted in a mixture of products. The bromination of compounds using NBS is known to proceed via a radical type mechanism.[119] Generally, it is known that in compounds with an active nucleus, side-chain bromination predominates in the presence of a catalyst such as dibenzoyl peroxide. Similarly, nuclear bromination predominates in the absence of a catalyst.[119] In a review by Pizey[119] several examples are given where di- or



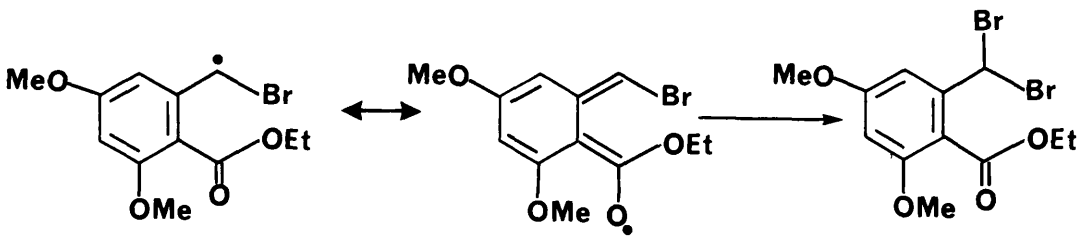
(162) (163) (166)

poly-bromination occurs. For the dimethyl ether of ethyl orsellinate (162) side chain bromination occurs predominately. Therefore, the occurrence of the mono- and dibromo- compound (163) and (166) as well as starting material must be related to the similar stability of the radical intermediates involved (167) and (168). Due to the low yield obtained for the monobromo compound (163), it was decided to investigate an alternative route to the required homophthalic acid derivatives (150) and (151).

The sequence of reactions involved in the synthesis of the homophthalic acid derivatives is outlined in Scheme 4.6. The starting material for the synthesis is the commercially available 3,5-dihydroxybenzoic acid (169). Methylation with dimethyl sulphate and potassium carbonate gave methyl 3,5-dimethoxybenzoate



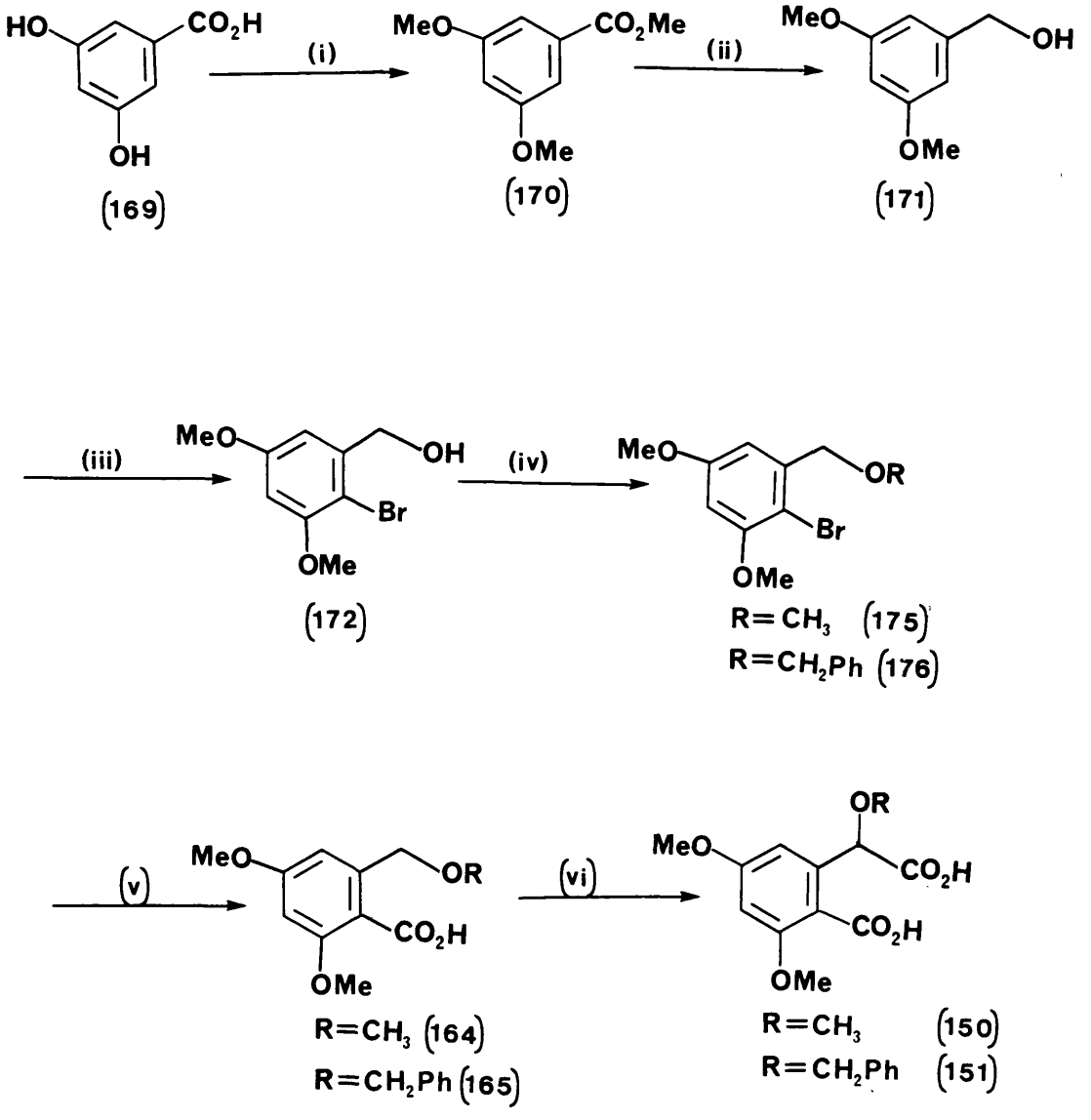
(167) (163)



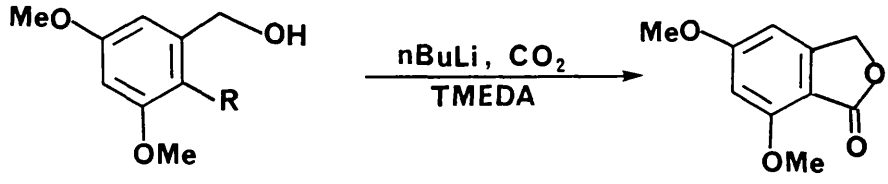
(168) (166)

(170). Reduction with lithium aluminium hydride in anhydrous THF gave 3,5-dimethoxybenzyl alcohol (171). Trost et al[120] showed that treatment of 3,5-dimethoxybenzyl alcohol (171) with n-butyl lithium in the presence of tetramethylethylene diamine followed by carboxylation afforded 5,7-dimethoxyphthalide (173) in moderate yield. When this reaction was repeated by Noire and Franck, the phthalide (173) was produced in

SCHEME 4.6



Reagents: (i) Me_2SO_4, K_2CO_3, $(CH_3)_2CO$; (ii) $LiAlH_4$; (iii) Br_2, $ClCH_2CH_2Cl$; (iv) MeI or $PhCH_2Cl$, NaH (v) 1)nBuli, 2)CO_2, (vi) LDA, Me_2CO_3, H_2O.

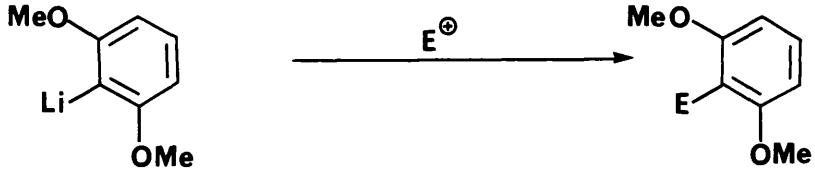


R=H (171)
R=Br (172)

(173)

8% yield.[121] However, they found that treatment of 2-bromo-3,5-dimethoxybenzyl alcohol (172) with n-butyl lithium followed by carboxylation gave the phthalide derivative (173) in 75% yield. The increase in yield is due to direct metal-halogen exchange of the lithium for the bromine which is known to occur rapidly at low temperatures.[122-124]

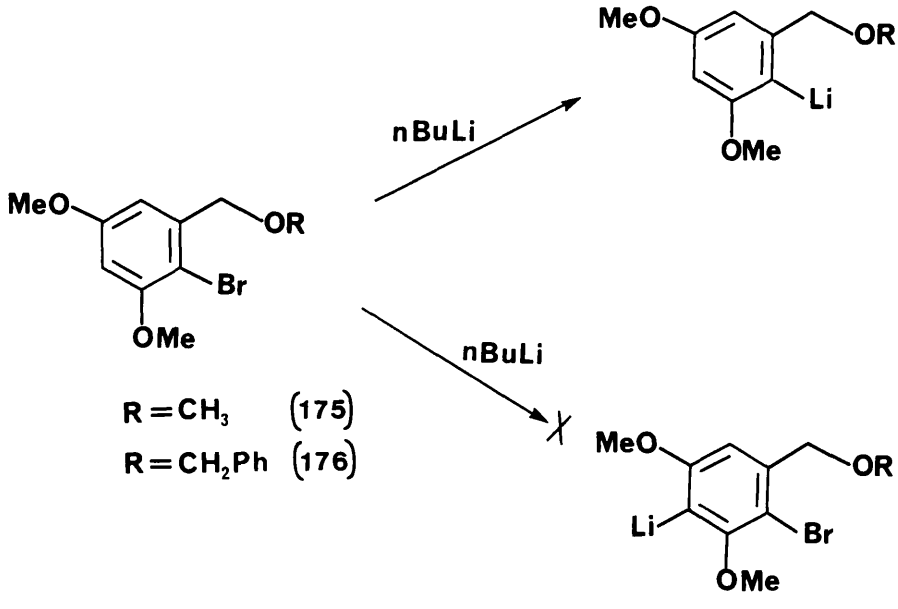
It has been shown that lithium exchange can occur between two methoxyl groups[125] giving rise to an intermediate such as (174) which can be further treated with a variety of electrophiles giving required substitution between the methoxyl groups.



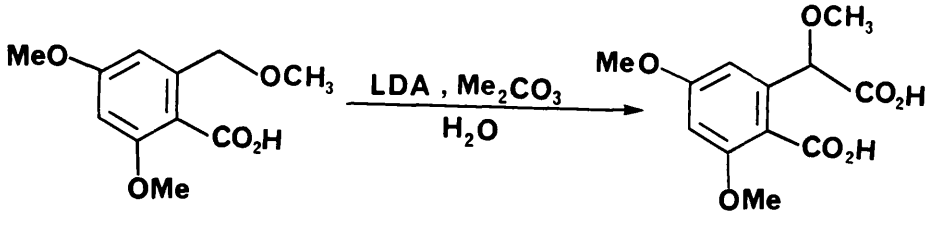
(174)

In the proposed synthesis of the acids (164) and (165), the bromo ethers (175) and (176) were thought to be key intermediates. Treatment with n-butyl lithium would lead to direct metal halogen exchange thus reducing the possibility of lithium exchange between the two methoxyl groups. Regioselectivity could be effected by treatment of these lithiated species with carbon dioxide.

These bromo-ethers were synthesised in two steps from 3,5-dimethoxybenzyl alcohol (171). Treatment of 3,5-dimethoxybenzyl alcohol (171) with bromine in 1,2-dichloroethane gave 2-bromo-3,5-dimethoxybenzyl alcohol (172) in 70%. When the benzyl alcohol (172) was treated with either methyl iodide or benzyl



chloride and sodium hydride the required ethers (175) and (176) were formed respectively. Treatment of these benzyl ethers (175) or (176) with n-butyl lithium at -78 °C followed by quenching with carbon dioxide gave the corresponding acid derivatives (164) or (165)



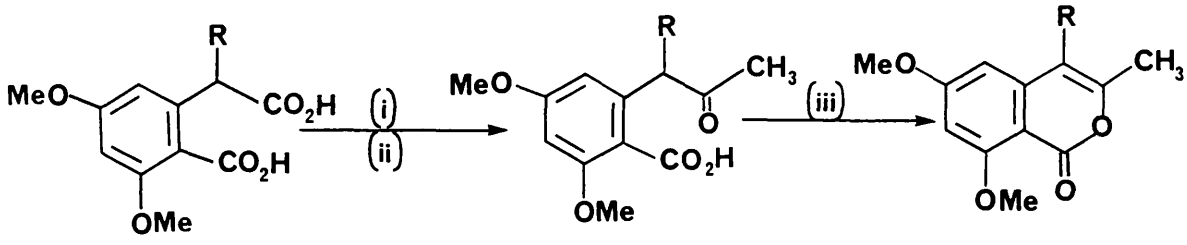
(164) (150)

respectively in 75% yield. Treatment of acid (164) with lithium diisopropylamide and dimethyl carbonate followed by hydrolysis afforded the desired α,3,5-trimethoxy homophthalic acid (150). This compound was used in the synthesis of the dimethyl ether of perimacol.

4.4 Synthesis of the Dimethyl Ether of Perimacol

As described in Chapter 3, treatment of 3,5-dimethoxy homophthalic acid (84) with pyridine and acetic anhydride followed by base hydrolysis gave after work-up the keto-acid (102). This was cyclised to the isocoumarin (96) by treatment with perchloric acid and acetic anhydride in ethyl acetate. Therefore, applying this synthetic strategy to the homophthalic acid derivative (150) should give the 4-methoxyisocoumarin (156) via the keto acid (153).

However, when α,3,5-trimethoxyhomophthalic acid (150) was treated with acetic anhydride and pyridine at room temperature followed by base hydrolysis, only a small amount of the required keto-acid (153) was produced. The major component was starting material. A small amount of another product was present which was shown to be 4,6,8-trimethoxyhomophthalic anhydride (177).



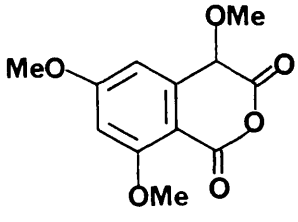
R=H (84)
R=OCH3 (150)

R=H (102)
R=OCH3 (153)

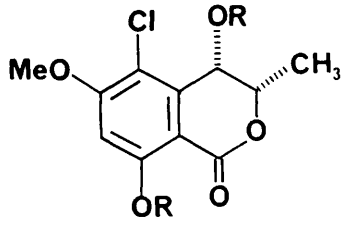
R=H (96)
R=OCH3 (156)

Reagents: (i) Ac_2O, pyr; (ii) NaOH, reflux;
(iii) $HClO_4$, Ac_2O; EtOAc.

4,6,8-Trimethoxy-3-methylisocoumarin (156) was formed in 64% directly from α,3,5-trimethoxyhomophthalic acid by refluxing in a solution of acetic anhydride and pyridine.

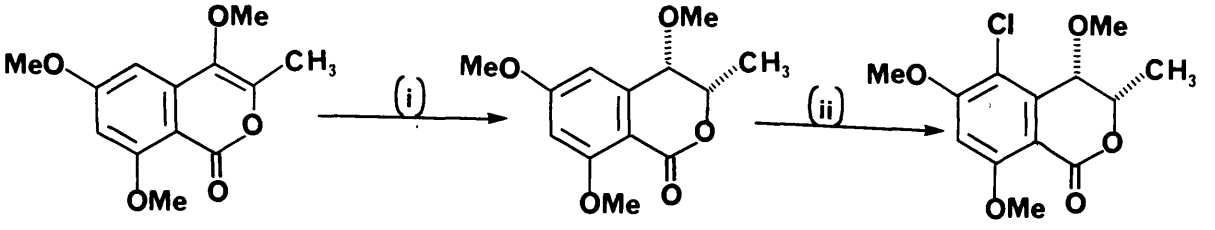


(177)

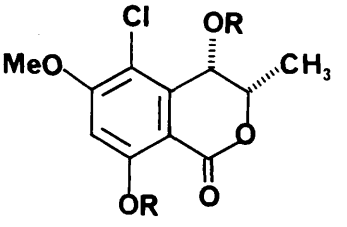


R=H (47)
R=CH3 (128)

Henderson assigned the structure of perimacol (47) and its dimethyl ether (128) as shown. Reduction of the double bond of 4,6,8-trimethoxy-3-methylisocoumarin (156) by catalytic hydrogenation would give the required relative cis stereochemistry between the methyl and methoxyl groups. This was achieved in very good yield. Chlorination of 3,4-dihydro-4,6,8-trimethoxy-3-methylisocoumarin (178) with sulphuryl chloride yielded the synthetic perimacol dimethyl ether (179) with relative cis stereochemistry.



(156) (178) (179)



R=H (47)
R=CH3 (128)

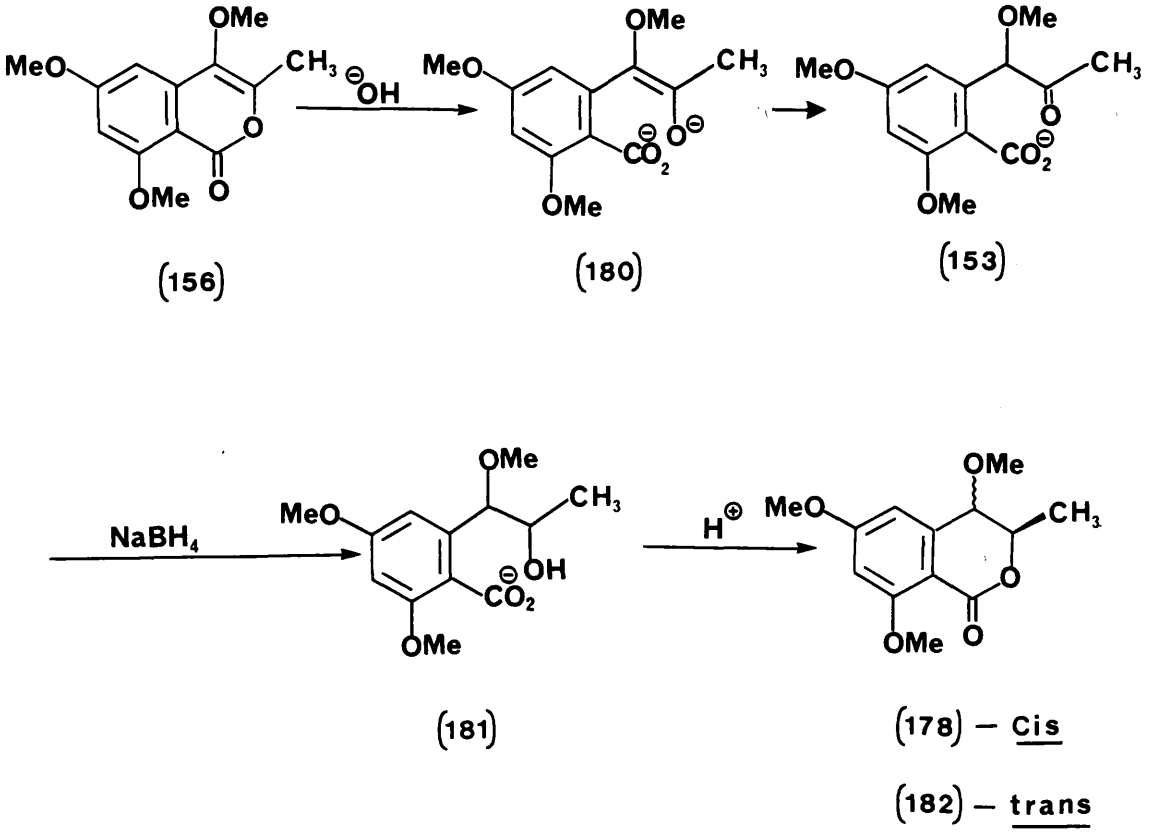
Reagents: (i) H_2-Pd; (ii) SO_2Cl_2

Comparing the ^{1}H n.m.r. of the synthetic dimethyl ether of perimacol (179) with that from the natural product (128) revealed that they were different. Although the two n.m.r.'s were very similar the major difference was found to be the chemical shift of the methyl doublet. In the synthetic product (179) the value was δ1.53 p.p.m. whereas in the dimethyl ether of the natural product (128) the methyl doublet appeared at higher field at δ1.14 p.p.m.[80] This suggests that the methoxyl and methyl groups of the natural product do not have a cis configuration for the substituents at carbons 4 and 3 respectively.

The spectroscopic data of the synthetic compound (179) is consistent with the structure proposed for the dimethyl ether of perimacol (128). However, the chemical shift of the methyl doublets of the two compounds differed by 0.4 p.p.m. Therefore, it is proposed that a trans arrangement exists between the methyl and methoxyl groups at carbons 3 and 4 respectively.

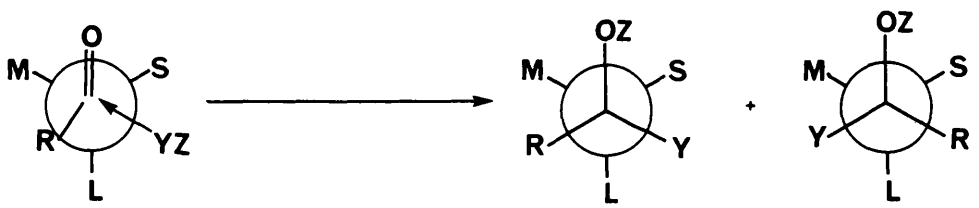
Synthetically a trans arrangement between the methyl and methoxyl groups at carbons 3 and 4 could be achieved by reduction of the isocoumarin (156) with sodium borohydride in aqueous sodium hydroxide solution. When 4,6,8-trimethoxy-3-methylisocoumarin (156) was reduced with sodium borohydride the ^{1}H

n.m.r. spectrum revealed that a 2:1 mixture of trans:cis isocoumarins (182) and (178) had formed.



In basic solution the lactone of the isocoumarin (156) opens to give the acid derivative (180). This derivative tautomerises to give the ketone (153) which is reduced by sodium borohydride to yield the alcohol (181). On treatment with acid cyclisation occurs forming the dihydroisocoumarins (178) and (182).

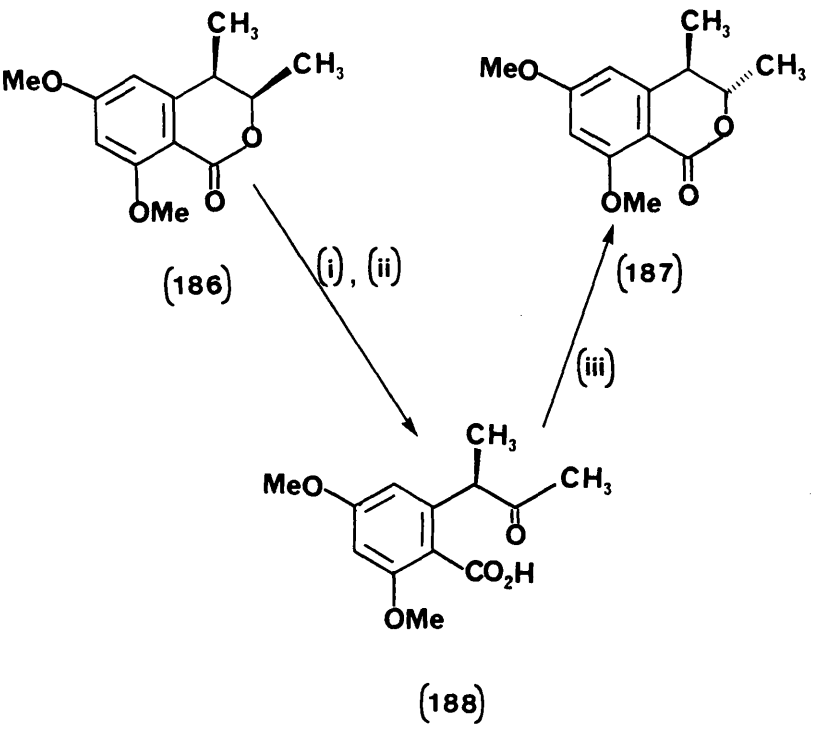
The reduction of ketones using either sodium borohydride or lithium aluminium hydride in most cases gives the trans product as predicted by Cram's rule.[126,127] If the molecule is observed along its axis it may be



(183) (184) (185)

represented as in (183), where S, M and L stand for small, medium, and large substituents respectively. The oxygen of the carbonyl orientates itself between the small and the medium sized groups. The hydride attacks from the less hindered side i.e. the side containing the small group. Thus (184) will be formed as the major component and (185) as the minor component. In some cases the reduction is stereospecific. The stereochemical outcome of the reduction of ketones is generally known as the Rule of Steric Control of Asymmetric Induction.

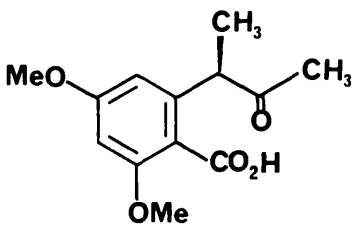
A report by Regan and Staunton[128] stated that a mixture of diastereomeric isocoumarins (186) and (187) could be converted to the isocoumarin (187) by a three



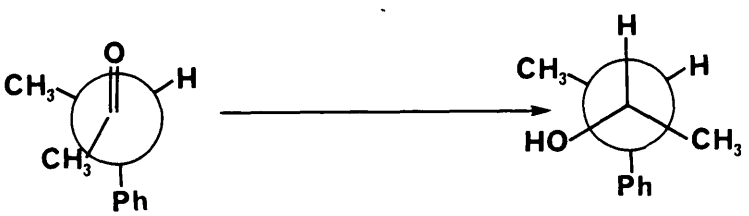
Reagents: (i) LiOH(aq); (ii) PDC; DMF; (iii) $NaBH_4$, MeOH

step sequence. The key reaction is the reduction of the ketone (188) with sodium borohydride giving the solely trans product (187). The mixture was first hydrolysed to give the corresponding alcohols which were then oxidised to give the ketone (188). Stereoselective reduction of this ketone gave the trans isocoumarin (187). Applying Cram's rule to this ketone (188) the predicted product of reduction is in fact the

isocoumarin with the two methyl groups trans to each other.



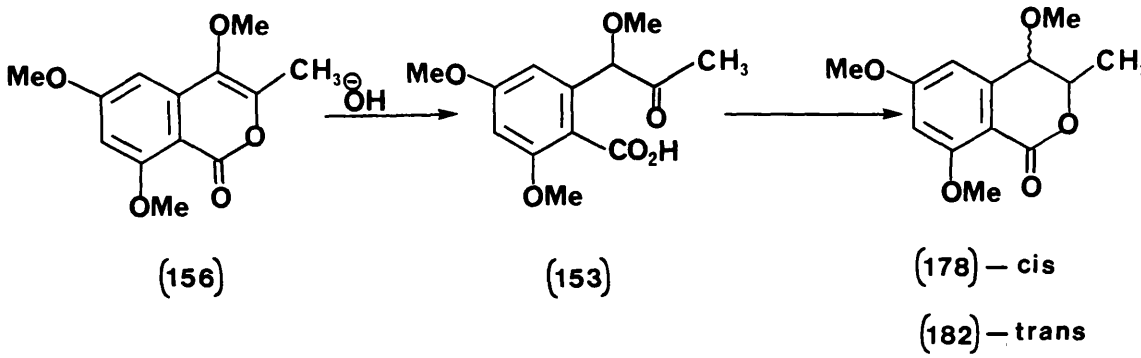
(188)



(188) (187)

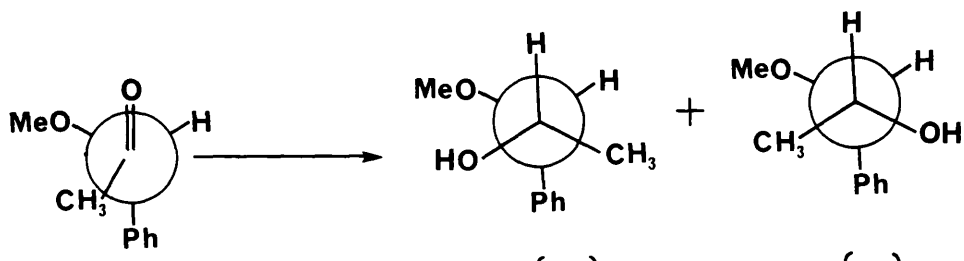
However, borohydride reduction of the α-methoxy-ketone (156) followed by cyclisation gave a 2:1 mixture of trans:cis substituted isocoumarins (178) and (182).

The rule of asymmetric induction usually requires modification when the α-carbon bears polar substituents such as OH, or NH_2. The assumption that only torsional strain and steric interactions determine the favoured transition state is no longer valid.



(156) (153) (178) — cis

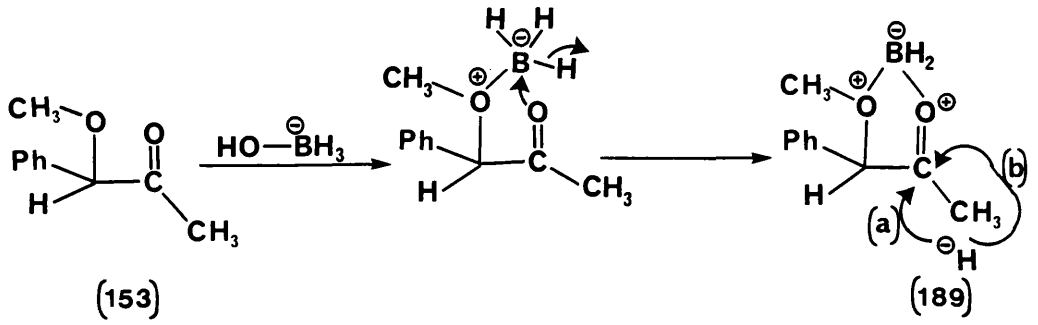
(182) — trans



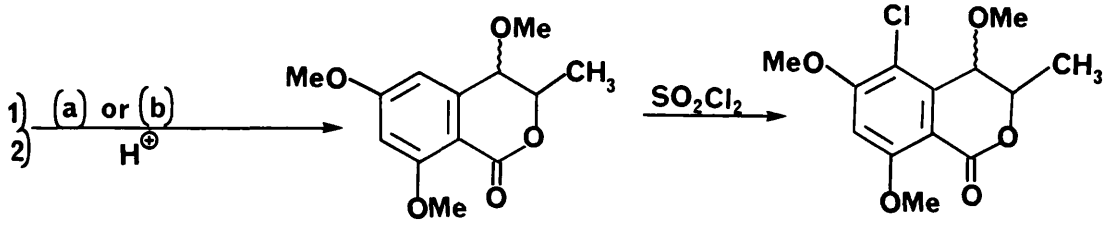
(153) (182) (178)

2 : 1

During the reduction of the α-methoxyketone (153) with borohydride, the oxygen of the methoxyl group can form a complex (189) with the reagent. The boron atom is now co-ordinated between the oxygen of the methoxyl group and the oxygen of the carbonyl group. The incoming hydride ion can attack from either side of the molecule. The most favoured mode of attack gives the trans product (182). The α-carbon is not sterically congested. Therefore, there is little differentiation between the planes of the molecule and the hydride can attack from either side. This results in an unusually



(153) (189)



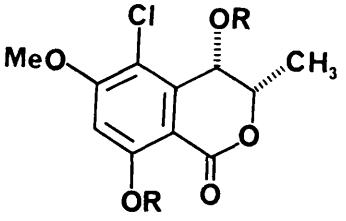
(178) — cis
(182) — trans

(179) — cis
(190) — trans

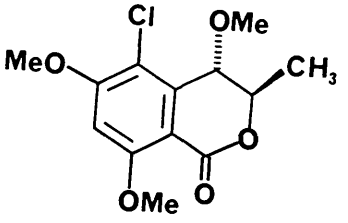
large amount of cis compound being formed.

The two diastereoisomers could not be separated, and therefore the mixture was chlorinated in the same manner as before using sulphuryl chloride giving the chlorinated isocoumarins (179) and (190). Similarly, this mixture of diastereoisomers could not be separated.

The ^{1}H n.m.r. of the synthetic mixture was obtained. Subtracting the peaks that could be assigned to the cis compound (179) enabled a direct comparison to be made



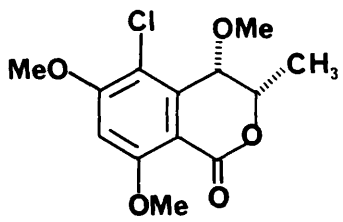
R=H (47)
R=CH3 (128)



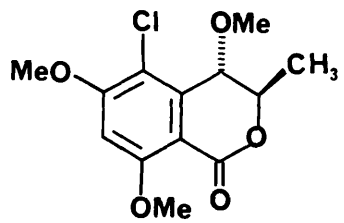
(190)

with the trans isocoumarin (190) and the dimethyl ether of the natural product (128). The ^{1}H n.m.r. of the synthetic product (190) matched the ^{1}H n.m.r. of the dimethyl ether of the natural product (128). Therefore, it has been shown synthetically that there exists a trans configuration between the methyl and methoxyl groups at carbons 3 and 4 respectively in the dimethyl ether of the natural product (128). Likewise, a trans configuration must also exist between the methyl and hydroxyl groups in perimacol (47). Thus the original structures proposed by Henderson for perimacol (47) and its dimethyl ether (128) in which a cis configuration between the groups at carbons 3 and 4 was suggested has now been shown to be incorrect.

Examining the chemical shifts between the methyl doublet of the relative cis compound (179) and the methyl doublet of the relative trans compound (190) revealed a significant difference. The chemical shift



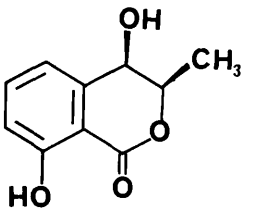
(179)—cis



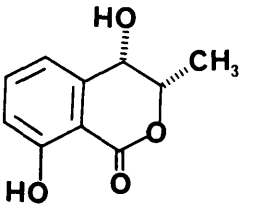
(190)—trans

obtained for the cis compound agrees with other similar isocoumarins with a cis configuration between substituents at carbons 3 and 4. For natural products with a cis configuration, the methyl group adopts a pseudo-equatorial site. Therefore, for the relative cis isocoumarin (179) which has a similar chemical shift value of its methyl doublet to other cis isocoumarins (33),[67,129] (191),[130] (192),[132] (193),[132] it is reasonable to assume that the methyl group will adopt a pseudo-equatorial site.

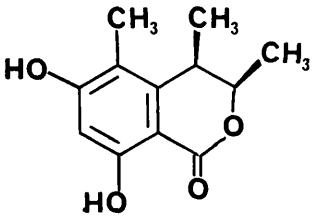
For the relative trans isocoumarin (190) however, the methyl doublet at δ1.14 occurs at slightly higher field which suggests that the methyl group is experiencing a



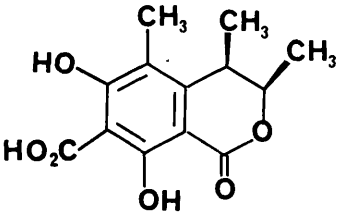
(33)



(191)



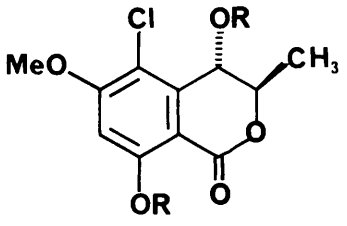
(192)

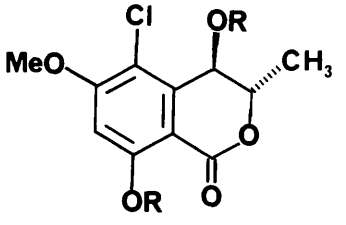


(193)

slight shielding effect. For this to occur the methyl group must be adopting a pseudo-axial conformation. When in the pseudo-axial conformation, the methyl group can occupy a region in space which overlaps the shielding ranges for a carbonyl group and an aromatic ring. This causes the methyl group to experience a slight shielding effect. Hence, the <u>trans</u> compound appears at slightly higher field. Therefore, the difference in the chemical shift values obtained for the methyl doublets in the <u>cis</u> and <u>trans</u> compounds can be explained by the methyl group adopting different conformations.

Now that a trans arrangement has been shown to exist between the two groups at carbons 3 and 4, the structure of perimacol can be either (194) or (195).



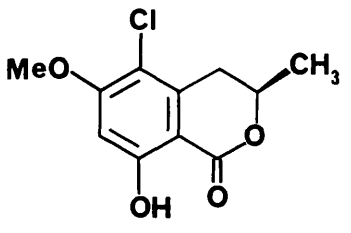


(194) R = H
(190) R = Me

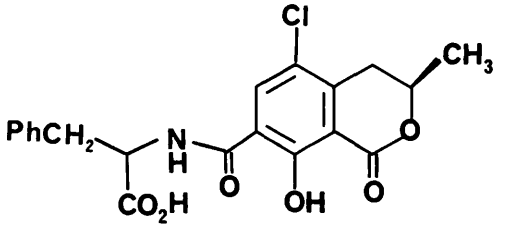
(195) R = H
(196) R = Me

In a similar manner the structure of the dimethyl ether can be (190) or (196). As mentioned earlier in this chapter Henderson[80] had assigned the stereochemistry at carbon-3 to be S because this compound had an opposite ORD curve to the isocoumarin (46). The sign of the ORD curve of isocoumarin (46) is equal to that of ochratoxin A (129). Ochratoxin A was shown to be R at carbon-3 by degradative studies. Therefore, the isocoumarin (46) was assigned R at this centre. On

this basis the structure would be (195) and its dimethyl ether (196). However, ORD is not a rigorous technique and it will be shown that the correct structure of perimacol is (194) in which there is R stereochemistry at carbon-3.



(46)



(129)

An alternative way of using ORD data makes use of the octant rule[133] This is a generalisation which is essentially qualitative and relates the sign of the ORD curve with the configuration or conformation of a carbonyl compound.

Three mutually perpendicular axes, x, y, and z divide the region of the carbonyl group into eight octants. The carbonyl group is the x-axis with the two other axes drawn through the midpoint of the double bond see

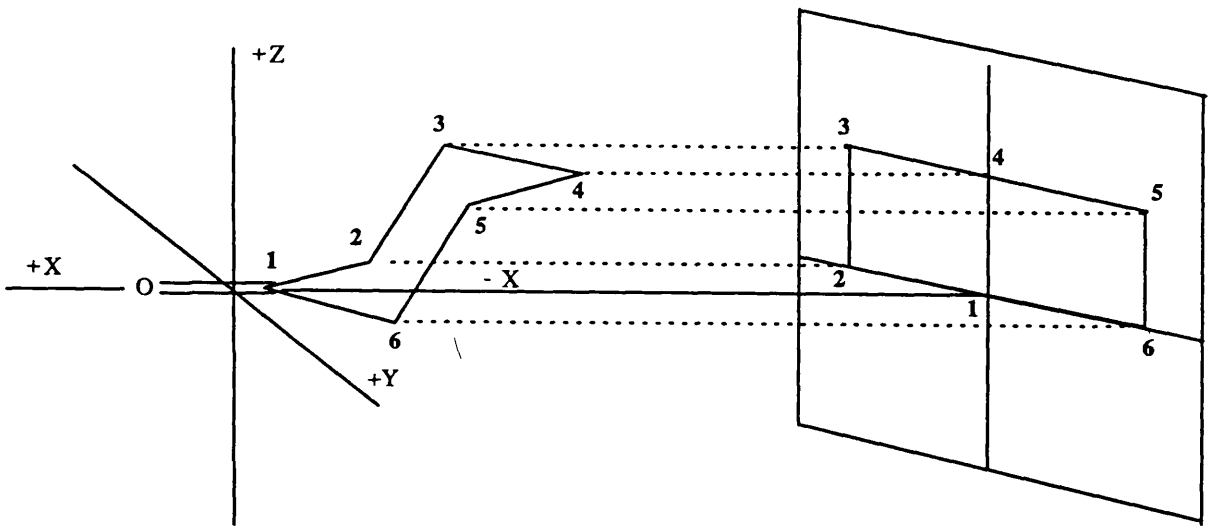
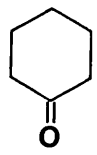
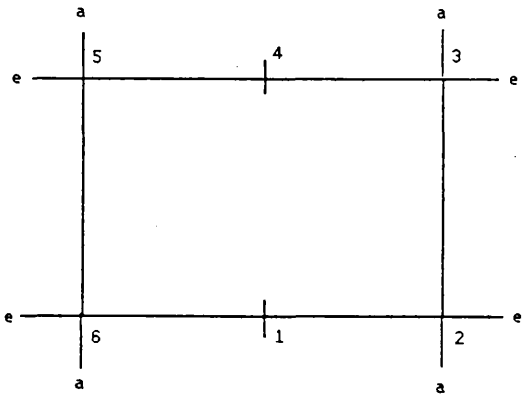


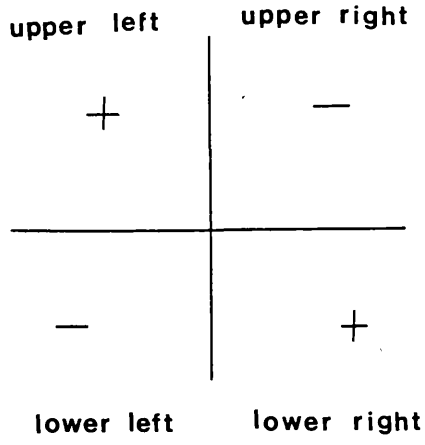
fig 4-1

Fig. 4.1. The molecule is then viewed from the side nearest the oxygen atom. The octants are a result of the three planes dividing the region of the carbonyl group. The front four octants are ignored since usually no atoms lie in front of the carbonyl group. Therefore, in the octant rule it is only the back four octants which are considered.

It is useful to consider the projection when cyclohexanone is drawn with the carbonyl at the bottom of the molecule. This can be seen in figure 4.2a. in which the projections of the axial and equatorial bonds are also shown. The signs obtained for the octants in Fig. 4.2b. are a result of the product of a given atoms





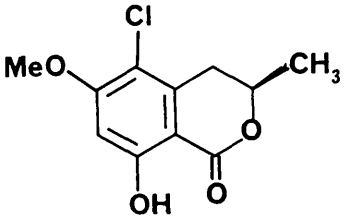


4·2a 4·2b

co-ordinates. Thus atoms lying in the upper left and lower right make positive contributions to the ORD curve. Atoms in the upper right and lower left make a negative contribution. Hydrogen atoms are ignored since their contributions are insignificant. Equatorial substituents on either α-carbon (with respect to the carbonyl carbon) make no contribution since they lie on the y-axis and one of its co-ordinates is equal to zero.

Applying the octant rule to the compounds (46) and (190) the following observations were made. In the

isocoumarin (190) the methoxyl group at carbon-4 whether axial or equatorial will lie on the z-axis (see figure 4.3). Therefore, the product of its co-ordinates will



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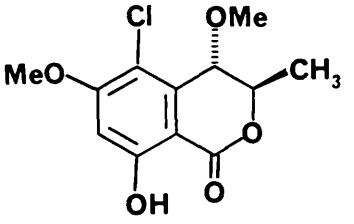
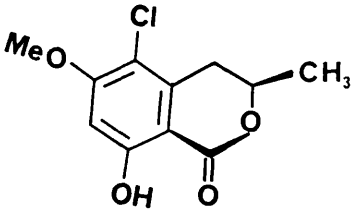
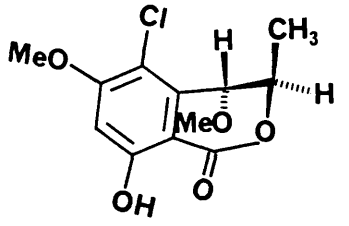
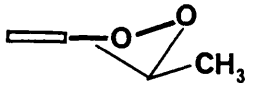
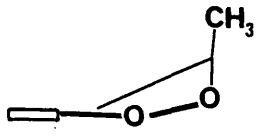


(190)









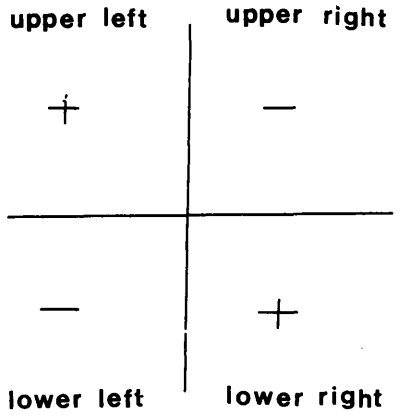
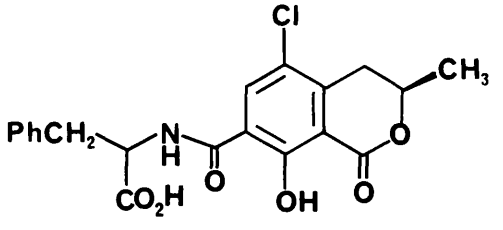


fig 4·3

be zero. This means that the methoxyl group will make no contribution to the sign of the ORD curve. Similarly, since the aromatic ring is planar and lies along the y-axis this also makes no contribution to the sign of the ORD curve. Therefore, the sign of the ORD curve will be determined by the methyl group at carbon-3.

For the isocoumarin (46), the ORD curve has the same sign as the ORD curve for ochratoxin A (129) which from

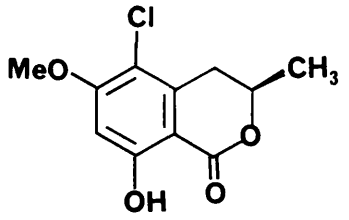


(129)

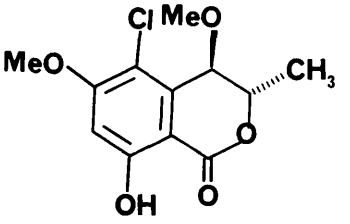
degradation studies has been shown to have the R-configuration at C-3. Therefore, the isocoumarin (46) has an R-configuration at C-3 and also the methyl group adopts a pseudo-equatorial conformation at this carbon. Applying the octant rule to this molecule reveals that the methyl group occupies the bottom right hand octant.

For the trans compound (190) the ^{1}H n.m.r., chemical shift and coupling constant (J 2Hz) strongly suggest that the methyl group is occupying a pseudo-axial site. If the octant rule is applied to this compound with the R-stereochemistry at C-3 maintained (as in (190) with the methyl group adopting a pseudo-axial conformation) it can be seen from diagram 4.3 that the methyl group occupies the top right octant. This predicts that structure (190) will have an opposite ORD curve to that of the isocoumarin (46). Compounds (46) and (190) have 3R-configurations but different conformations at carbon-3. Application of the octant rule predicts that these compounds should have opposite ORD curves. This agrees with the ORD curves obtained experimentally.

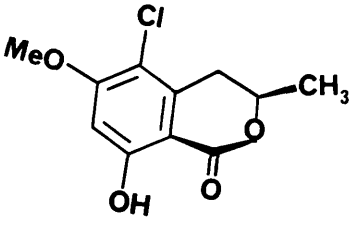
The ^{1}H n.m.r. data is consistent with the methyl group adopting a pseudo-axial conformation. If the octant rule is applied to the isocoumarin (196) with S-configuration at C-3 and with the methyl occupying a pseudo-axial site the following observation was found. The methyl group of compound (196) occupied the same octant as the methyl group of the isocoumarin (46). Isocoumarin (46) has 3R-configuration with the methyl group pseudo-equatorial. If the isocoumarin (47) isolated had the S-configuration at C-3 with the methyl group adopting a pseudo-axial site then the ORD curves

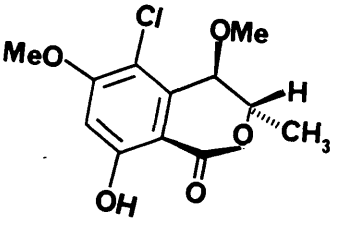


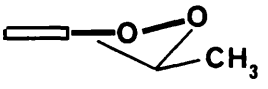
(46)

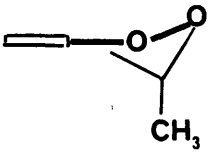


(196)



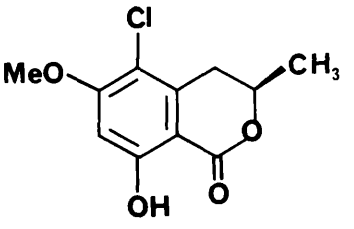






of this isocoumarin (47) and the natural product (46) would have been the same. The fact that different ORD curves are obtained is because the isocoumarins (46) and (190) have the same configuration at C-3 but have different conformations at this carbon. Hence the structure of perimacol is (194) and likewise the structure of the dimethyl ether is (190).

Further evidence for these conclusions is contained in a report by Okunu et al[134] who were studying



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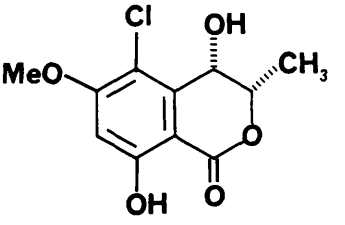
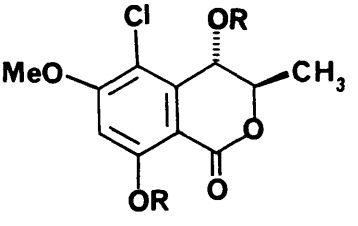


(47)

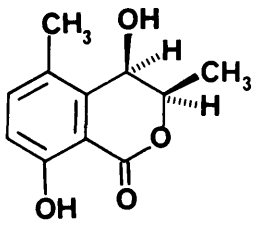
metabolites produced by the fungus Valsa ceratosperma. They isolated the new isocoumarins (197) and (198) and observed similar chemical shifts for the methyl



R = H (194)

R = Me (190)

doublets as had been observed for the corresponding doublets in the cis dimethyl ether (179) and the trans dimethyl ether (190). Opposite ORD curves were obtained for the isocoumarins (197) and (198). The cis isocoumarin (197) had a similar ORD curve to the



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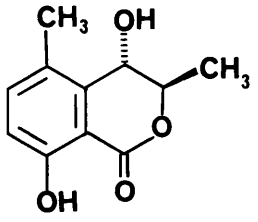

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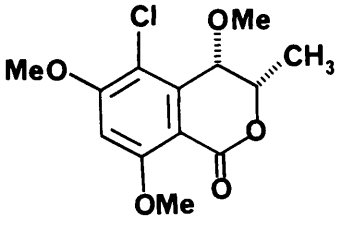

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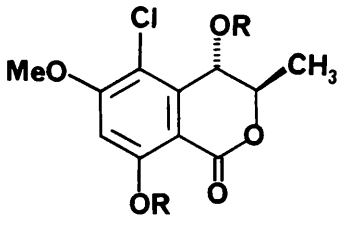
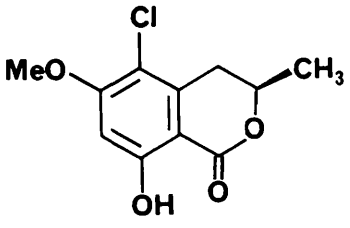
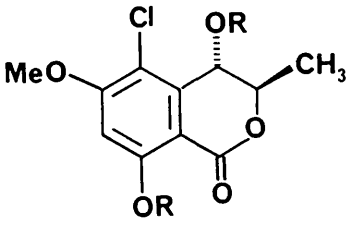


R = Me (190)
R = H (194)



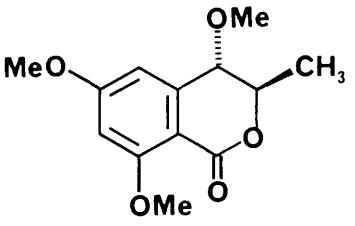
(46)

isocoumarin (46) and the trans isocoumarin had a similar ORD curve to perimacol (194). This is further evidence that for a trans isocoumarin, opposite ORD curves can be obtained which can be related to the conformation of the methyl substituent at carbon-3, whilst the configuration is the same.



R = H (194)

R = CH_3 (190)



(182)

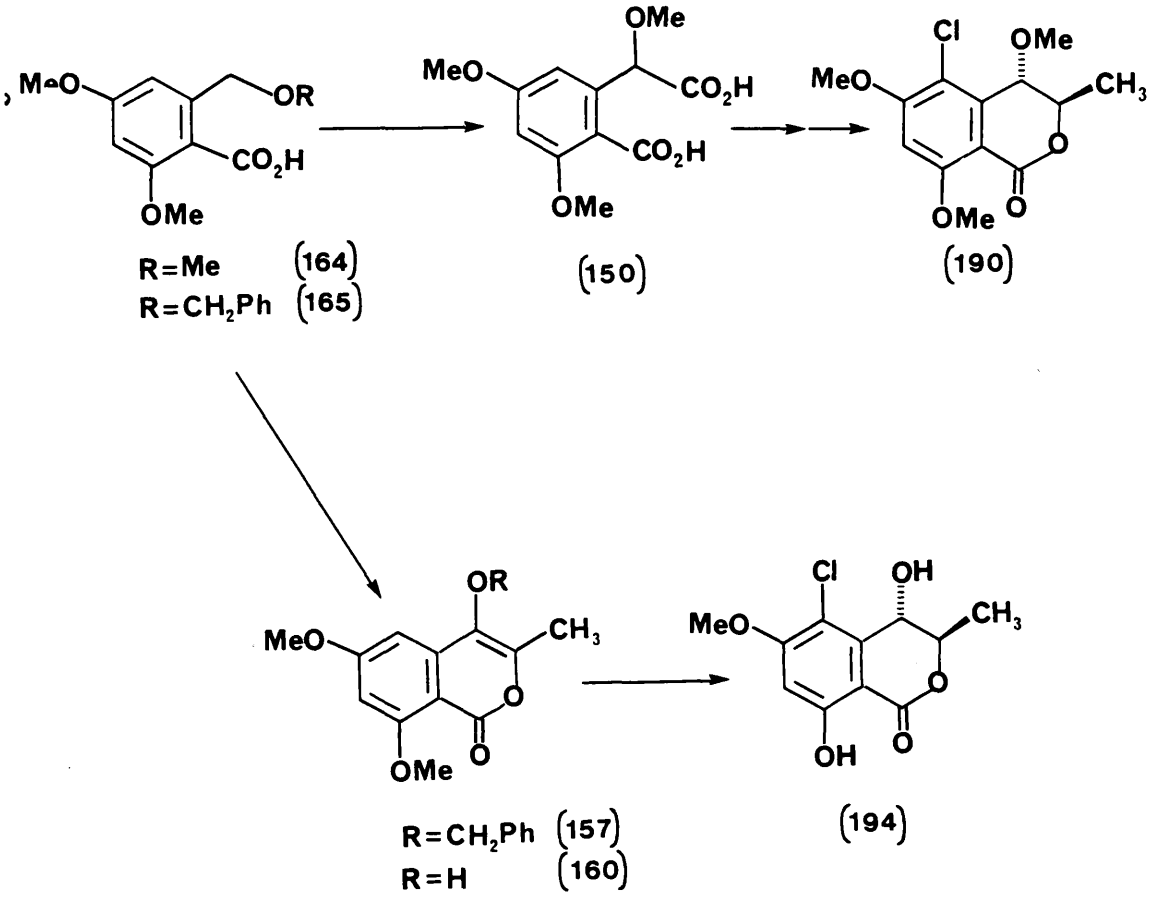
The small coupling constant between H-3 and H-4 in the dimethyl ether of perimacol (190) and hence perimacol (194) can be explained.

The coupling constant between H-3 and H-4 (J 7Hz) in the isocoumarin (182) agrees well with the Karplus equation[105] for a dihedral angle of approximately 150°. However, the introduction of a bulky chlorine atom at carbon-5 in (190) causes a conformational change of the molecule. The dihedral angle between H-3 and H-4 is reduced to approximately 60° thereby giving a smaller coupling constant. The conformational change that has occurred is due to the steric interaction between the methoxyl group at carbon-4 and the chlorine atom at carbon-5. The chlorine atom forces the methoxyl group into a more

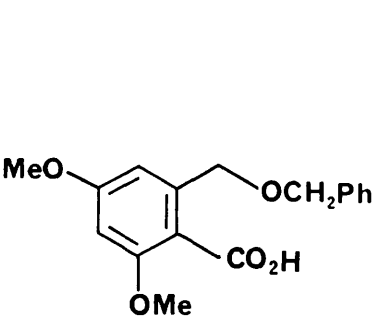
axial-like conformation thereby causing H-4 to move into a more equatorial like conformation. The dihedral angle is reduced resulting in a smaller coupling between H-3 and H-4.

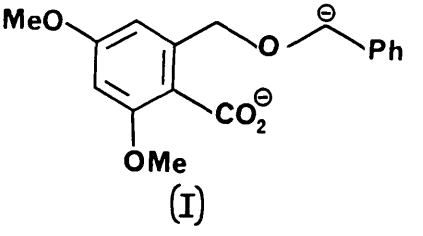
4.5 Attempts to Synthesise 4-Hydroxyisocoumarins

A synthesis of the ethers (164) and (165) was reported earlier in this chapter. The methyl ether (164) was converted to the homophthalic acid derivative (150) and subsequently converted to the dimethyl ether of perimacol (190). It was anticipated that application of the same reactions to the benzyl ether (165) would lead to the isocoumarin (157). This could be converted to the 4-hydroxyderivative (160) which after further reduction, demethylation and chlorination would yield the synthetic perimacol (194). However, when the benzyl ether (165) was treated with LDA and dimethyl carbonate no clean isolable product was obtained. A number of products may be formed from the dianion species (I) which could react with the dimethyl carbonate. It was thought that the species (II) would be the more stable since the negative charge can be delocalised by the aromatic ring and the carboxyl group. Due to the number of products formed, it is suggested that competing reactions are taking place.

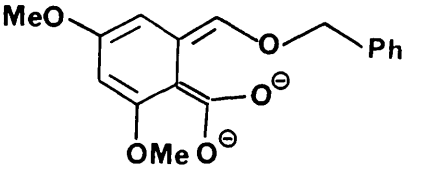


It was hoped that the 4-hydroxyisocoumarin (160) could still be synthesised by employing a different protecting group. The t-butyldimethylsilyl (TBDMS) protecting group was chosen due to its stability in basic conditions. Treatment of 2-bromo-3,5-dimethoxybenzylalcohol (172) with t-butyldimethyl-silylchloride and imidazole in DMF yielded the TBDMS





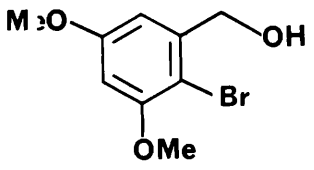
(I)



(II)

ether (199). This was treated with n-butyllithium followed by carbon dioxide to give the acid (200). An n.m.r. of the crude product suggested that the desired compound had been formed. However, after column chromatography, the acid cyclised to the undesired phthalide derivative (173). This highlights the main problem in synthesising 4-hydroxyisocoumarins.

Although the 4-hydroxyisocoumarin (160) was not synthesised via the homopthalic acid route, it appears to be the most promising synthetic route to these types of compound. By the choice of a suitable protecting



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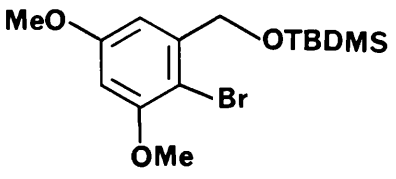

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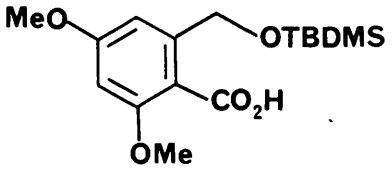

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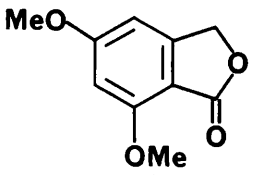


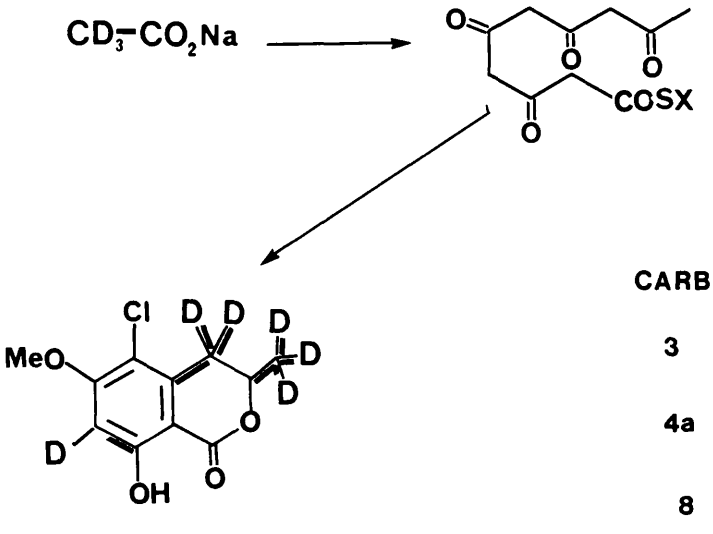
(173)

group, this route could be employed to synthesise a number of novel 4-hydroxyisocoumarins.

5. ISOTOPE SHIFT STUDIES

5.1 Introduction

The isocoumarin (201) isolated after the feeding of CD_3-$^{13}CO_2Na$ to Periconia macrospinosa gave an interesting β-shift result.



CARBON	β – SHIFT
3	0·04, 0·08, 0·12
4a	0·03, 0·07, 0·10
8	0·04

(201)

In the ^{13}C n.m.r. spectrum of the isocoumarin (201), the signals for carbons 3, 4a and 8 showed upfield shifted signals due to the presence of β-deuterium atoms. These shifted signals are commonly called

β-shifts. β-Shifts were observed for the signal due to carbon-3 of 0.04, 0.08 and 0.12 p.p.m. indicating the presence of 1, 2 and 3 deuterium atoms at carbon 9 respectively. A β-shift was also observed for the signal from carbon-8 which was due to a deuterium being present at carbon-7. Interestingly β-shifts observed for carbon-4a showed shifts of 0.03 p.p.m., 0.07 p.p.m. and 0.10 p.p.m. This result suggests that a deuterium adopting an axial conformation is producing one shift and a deuterium adopting an equatorial conformation is producing another. The third shift (0.10 p.p.m.) is the additive value of the other two shifts and is observed when there are two deuterium atoms present at carbon-4. Thus it was decided to investigate this further. In particular, to assign unambiguously the shifted signals arising from the equatorial and axial deuterium atoms causing the shift.

5.2 Isotope Effects of Nuclear Shielding

Isotope effects have been known for a long time and several reviews exist.[135-142] It was found that the size of the shift is related to the distance the isotope is from the nucleus under observation and to

the shielding range of the resonant nucleus. Therefore, it is observed that the largest isotope shifts occur when there is a large change in mass upon substitution. In general the size of the isotope nuclear shielding is related to the number of atoms which have been isotopically substituted. Isotope effects can occur over one, two or three bonds, giving rise to the α, β or γ shifts respectively. The shifts can be observed over more than three bonds, but it is generally a rare occurrence.

In this study, the effects of deuterium on the chemical shifts of ^{13}C nuclei have been studied. The size of the isotope effect depends on the hybridization of atoms, i.e. sp^2 or sp^3, involved at the observed carbon and at the carbon bearing deuterium. In the following sections the hybridization at the observed carbon and the carbon bearing deuterium are signified by the notation Csp^3D Csp^3 etc.

5.3 Deuterium Isotope Effects on ^{13}C Nuclear Shielding

5.3.1 $Csp^3D\ Csp^3$ systems

5.3.1.1 α-shifts in $Csp^3D\ Csp^3$ systems

Generally it has been found for deuteriated methanes that an isotope effect of +0.187 p.p.m. per deuterium is common. These values have been shown to be additive.[143] An effect of +0.36 p.p.m. per deuterium has been observed for straight chain $-CD_2-$groups.[144] In the case of cyclohexanes larger values have been observed.[145,146] Interestingly, an axial and an equatorial deuterium have been shown to give different α-shift values in cyclohexane. It has been demonstrated that the smaller shift is due to the deuterium occupying the equatorial site.[146,147]

5.3.1.2 β-Shifts in $Csp^3D\ Csp^3$ systems

The effect over two bonds - known as the β-shift - is smaller than the corresponding α-shifts and is less than 0.1 p.p.m. The line broadening that is caused by unresolved carbon deuterium coupling is in most cases negligible as $^2J(C-^1H)$ and hence $^2J(C-D)$ are small. For saturated open chain hydrocarbons β-shift

values are approximately +0.08 p.p.m.[145] For cyclic hydrocarbons, the values are slightly greater than +0.1 p.p.m. with the exception of cyclopropane which is +0.064 p.p.m.[145] In cyclohexane, a difference has been observed in the β-shift value arising from an equatorial deuterium and the value arising from an axial deuterium.[146] Gorin[148] has found a relationship between the size of the β-shift and the substituent on the β-carbon. The smaller the β-shift value observed, the more electronegative is the substituent at the carbon under observation.

5.3.1.3 γ-Shifts in $Csp^3D\ Csp^3$ systems

The shift values observed over three bonds - γ-shifts - are quite small and in many cases are not resolved under the experimental conditions used. The values are generally close to +0.02 p.p.m.[135]

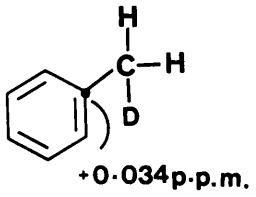
5.3.2 $Csp^2D\ Csp^3$ systems

This type of effect is observed in alkyl-substituted aromatics and carbonyl compounds.

5.3.2.1 β-shifts in $Csp^2D\ Csp^3$ systems

For deuteriated toluenes (202), an additive β-shift value of +0.034 p.p.m. per deuterium has been observed.

Ethylbenzene-α-d (203) (+0.0296 p.p.m.) and isopropylbenzene-α-d,(204), (+0.0296 p.p.m.) show comparible effects. The β-shift value obtained for



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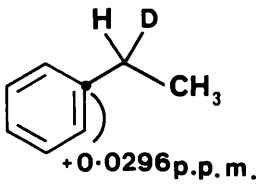

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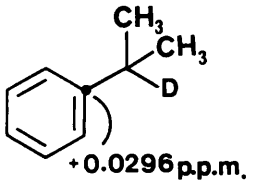
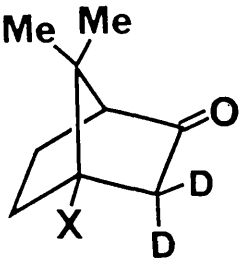


(204)

acetone-D_6 (-0.28 p.p.m.) was the first negative isotope value to be reported.[149] Negative values have been reported for substituted camphors (205).[150] The β-shift values observed at carbon-4 in a variety of substituted camphors (205) all showed positive shifts. However, the values observed at carbon 2 in the same derivatives all showed negative shifts. Negative shift values have been partly attributed to hyperconjugation.

Isotope effects are caused by differences in



X=Me
=Cl
=NO_2

(205)

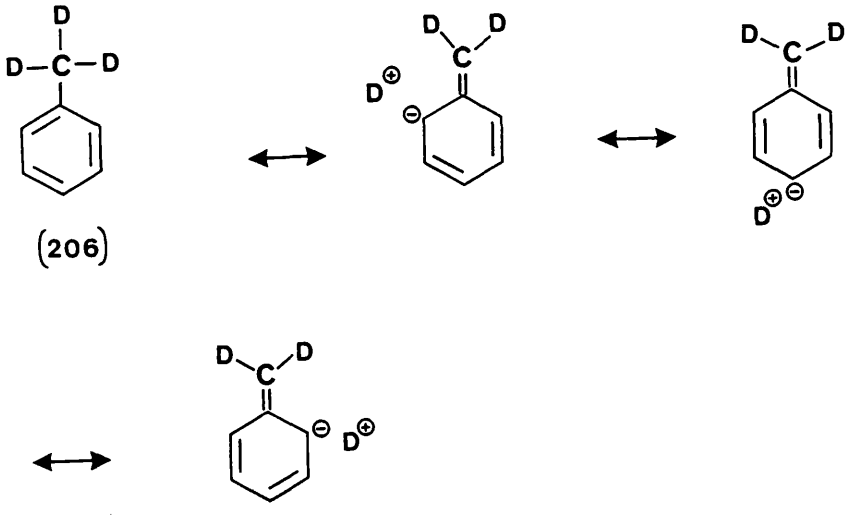
vibrational zero-point energy. These differences are related to the inductive and hyperconjugative effects of the substituents.[151,152] The observation of isotope effects can be explained by two phenomena:

1) the more electron-donating ability of a deuterium atom compared to that of a proton

2) the reduced hyperconjugative ability of a C-D bond compared to that of a C-H bond

Long range isotope effects have been observed in toluene.[153] For toluene d_1, d_2 and d_3 the effects are additive as mentioned earlier. Interestingly, for toluene d_3 are the effects that are observed over three (γ-shifts) and over five bonds

(ε-shifts). These two values are negative, γ-shift (-0.0016 p.p.m.), ε-shift (-0.0121 p.p.m.) whereas the δ-shift value (over four bonds) is zero.

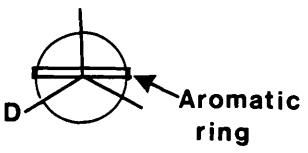


(206)

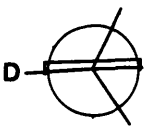
In toluene d_3 (206) the negative isotope effects occur at carbons where hyperconjugative interactions might contribute. The γ-shift being less than the ε-shift may be due to the inductive effect of the C-D bond adding to the value of the γ-shift.

Further evidence that hyperconjugation is important can be found when the γ-shift values of ethylbenzene-α-d_1 (3) (+0.002 p.p.m.) and isopropylbenzene-α-d_1 (+0.0143 p.p.m.) are compared.[154] For ethylbenzene and isopropylbenzene the ortho effect becomes

increasingly positive and the para effect is diminished.



A



B

Comparing with toluene, it was found that negative isotope shifts were observed at the ortho and para positions. Without knowing the population of the different rotational isomers present it can be assumed that the Cα-D bond for ethylbenzene and isopropylbenzene occupies a position less favourable for hyperconjugation. This is indicated by the Newman projections A and B. Since hyperconjugation is associated with a negative value, this is somewhat diminished and a more positive value is observed.

5.3.3 $Csp^2D\ Csp^2$ systems

These are found in olefins and aromatics including heteroaromatics.

5.3.3.1 α-Shifts in Csp^2D Csp^2 systems

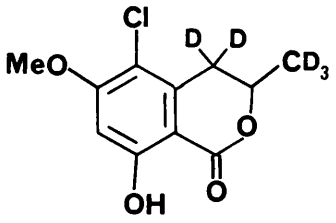
The α-shift value often found in olefins is approximately +0.2 p.p.m. However, the α-shift value for aromatic hydrocarbons is between +0.24 p.p.m. and +0.31 p.p.m.

5.3.3.2 β-Shifts in Csp^2D Csp^2 systems

For olefins and aromatics the β-shifts observed are of similar magnitude and are approximately +0.1 p.p.m. For polycyclic aromatics the β-shifts can vary from +0.05 p.p.m. to +0.12 p.p.m.[155] The size of the β-shift is controlled by the effects that the substituents have on the vibrational modes of the aromatic carbons.[135]

5.4 Synthesis of Deuterium-labelled Isocoumarins

A number of deuterium-labelled isocoumarins (207), (208), (209), (210) and (211) were synthesised. This was to determine whether the larger shift observed at carbon-4 for the isocoumarin (201) was caused by the axial or equatorial deuterium.



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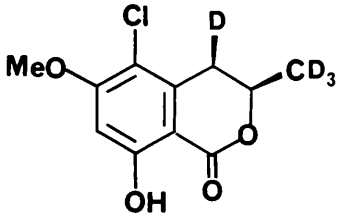

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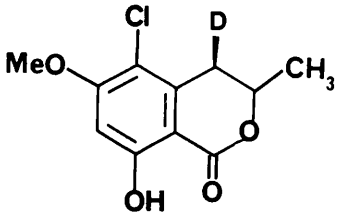

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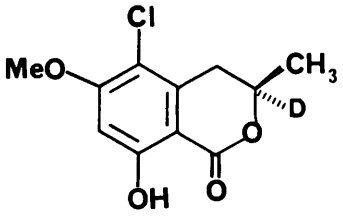
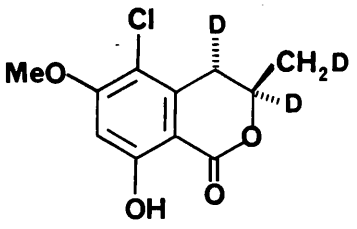


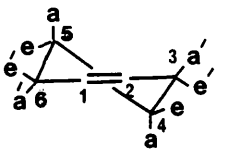
(210)



(211)

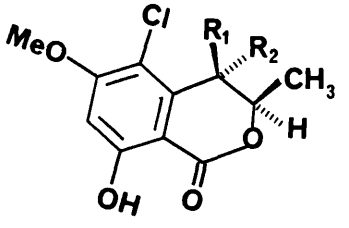
These studies would also check that the conclusions as to the origins of the three shifted signals arising from carbon-4a are correct.

When a cyclohexane ring contains trigonal carbon atoms its shape is distorted from that of a normal chair. By considering cyclohexene (212) it can be seen that the ethylenic carbons and the two adjacent allylic carbons (i.e. 3 and 6) are in a plane. However, the atoms on the other side (i.e. 4 and 5) are normally staggered as in cyclohexane. The hydrogen (or other) atoms attached



(212)

to atoms 4 and 5 occupy normal equatorial and axial positions. Those attached to atoms 3 and 6 are imperfectly staggered and do not take up the normal equatorial and axial positions. These atoms are said to occupy pseudo-equatorial and pseudo-axial positions. The 6-membered ring in (213) is not a true cyclohexane shape due to the presence of the sp^2



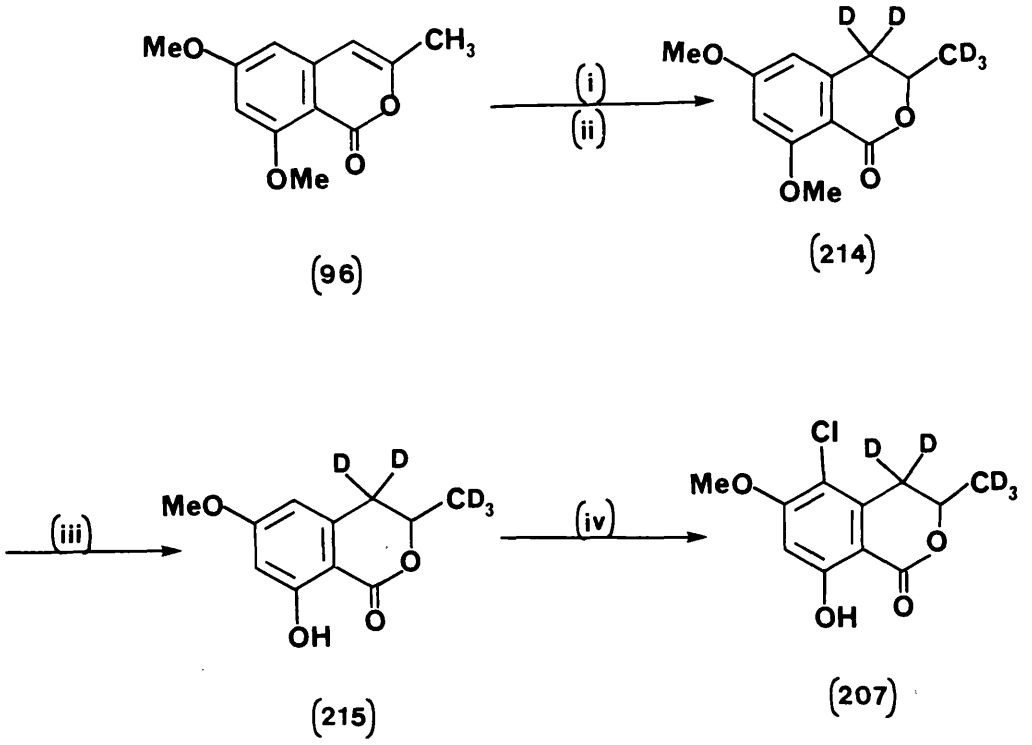
(213)

hybridised carbons. Therefore, substituents at carbons

3 and 4 will occupy pseudo-equatorial and pseudo-axial sites. The methyl group at carbon-3 being the only substituent occupies a pseudo-equatorial position. Therefore, a substituent at carbon 4 which is cis to the methyl group will occupy a pseudo-axial site. Similarly, a substituent that is trans to the methyl group will occupy a pseudo-equatorial site.

5.4.1 Synthesis of [4,4,9,9,9-$^2H_5$]-4-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (217)

The synthesis of the isocoumarin (96) is described in Chapter 3. The pentadeuterioisocoumarin (214) was obtained in good yield from the isocoumarin (96) by refluxing in a mixture of sodium deuterioxide, deuterium oxide and sodium borohydride. Selective demethylation gave (215) which was chlorinated using sulphuryl chloride which afforded the desired labelled isocoumarin (207).

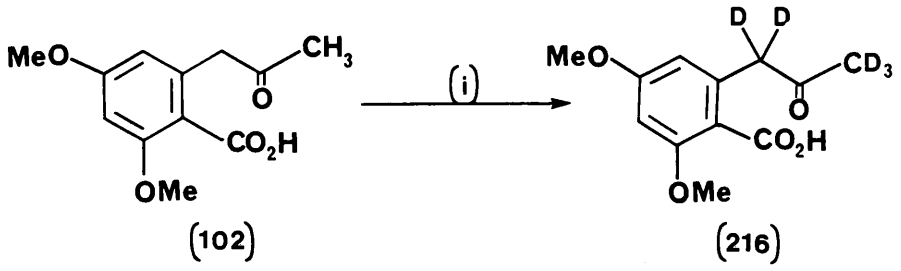


(96) (214) (215) (207)

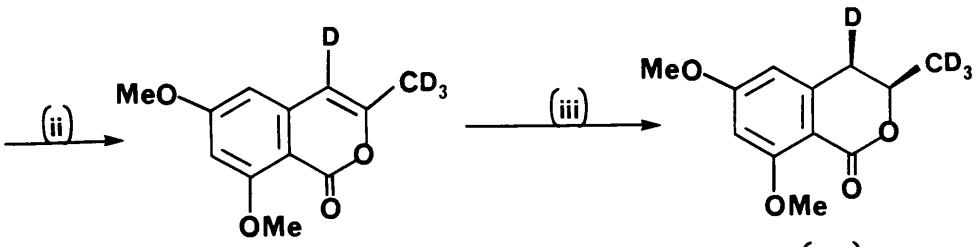
Reagents: (i) NaOD, D_2O; (ii) $NaBH_4$;
(iii) $AlCl_3$,$PhNO_2$; (iv) SO_2Cl_2

5.4.2 Synthesis of [3R*,4R*][4,9,9,9,-$^2H_4$]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (208)

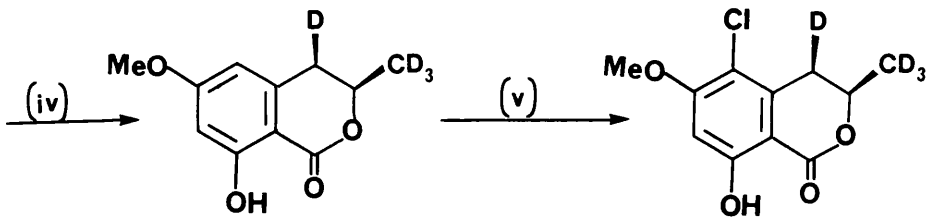
A synthesis of the keto-acid (102) is described in Chapter 3. The keto-acid (102) was heated at reflux in a solution of sodium deuterioxide and deuterium oxide



(102) (216)



(217) (218)



(219) (208)

Reagents: (i) $NaOD$, D_2O; (ii) Ac_2O, $EtOAc$, $HClO_4$;

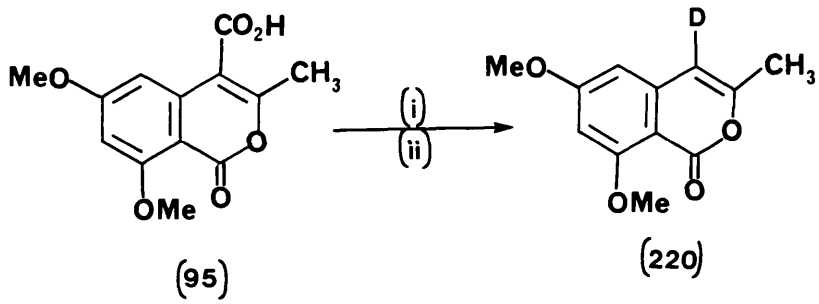
(iii) H_2/Pd; (iv) $AlCl_3$, $PhNO_2$;

(v) SO_2Cl_2

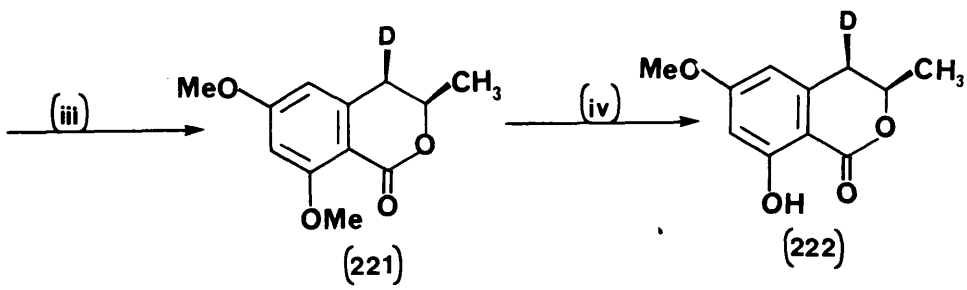
which gave the penta-deuterio keto-acid (216). The isocoumarin (217) was formed by acid treatment of the keto-acid (216). The dihydroisocoumarin (218) was obtained by hydrogenation of (217) using a palladium catalyst. This gave the relative cis stereochemistry between the CD_3 group at carbon-3 and the deuterium at the 4-position. Demethylation using aluminium chloride gave the mono hydroxyisocoumarin (219) which was chlorinated as before yielding the desired isocoumarin (208).

5.4.3 Synthesis of [3R*,4R*][4-^{2}H]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (209)

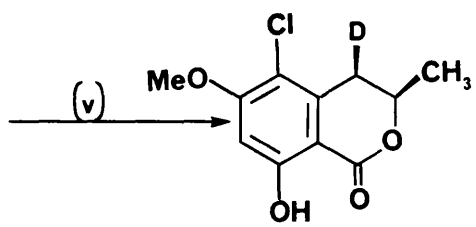
A synthesis of 4-carboxy-6,8-dimethoxy-3-methyl-isocoumarin (95) is described in Chapter 3. Treatment of the 4-carboxyisocoumarin (95) with deuterium oxide resulted in exchange of the acid proton for a deuterium. Heating the isocoumarin (95) led to decarboxylation and retention of the deuterium at the 4-position which gave (220). The relative cis stereochemistry was obtained between the deuterium and methyl group by hydrogenation which resulted in (221). Demethylation with aluminium chloride afforded the 8-hydroxyisocoumarin (222) which was converted to the 5-chloroisocoumarin (209) as before using sulphuryl chloride.



(95) (220)



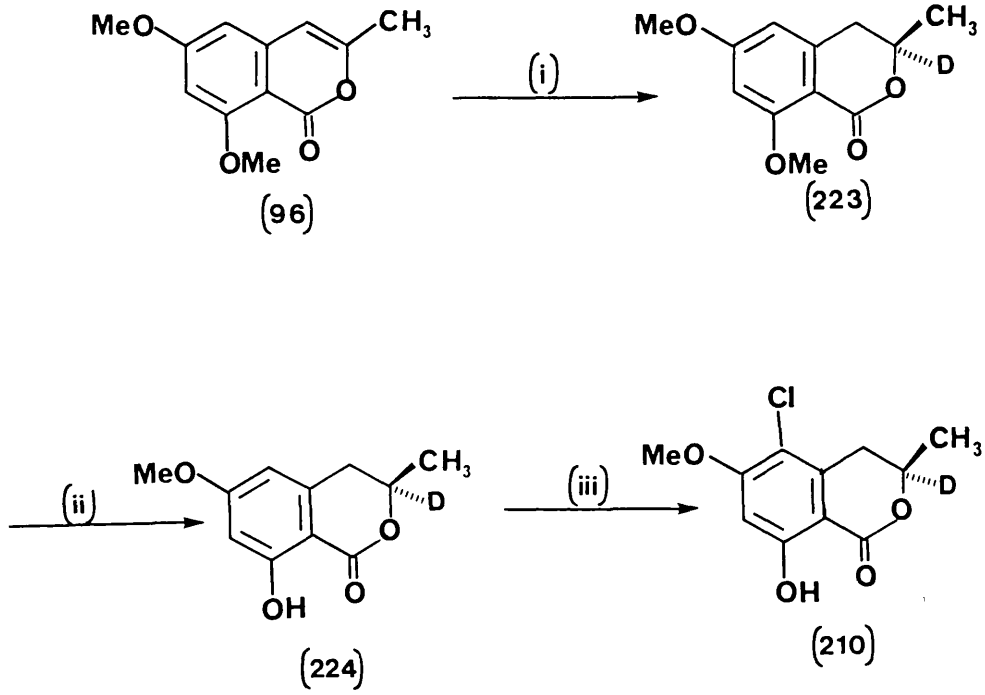
(221) (222)



(209)

Reagents: (i) D_2O; (ii) Δ ; (iii) H_2/Pd;
(iv) $AlCl_3$, $PhNO_2$; (v) SO_2Cl_2

5.4.4 Synthesis of [3-^{2}H]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (210)

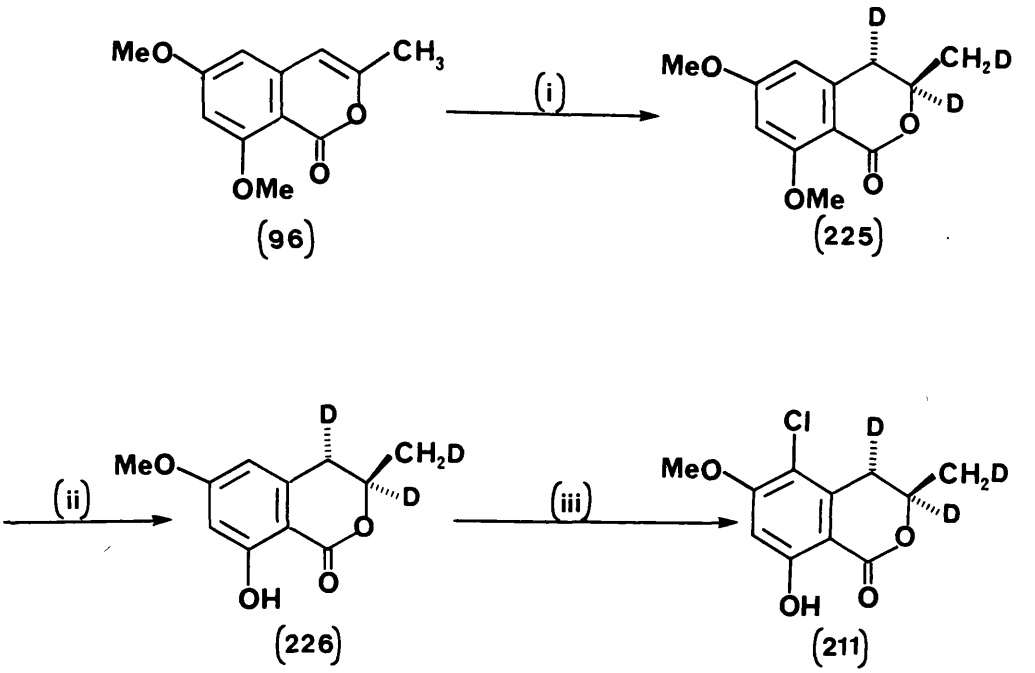


Reagents: (i) $NaBD_4$; (ii) $AlCl_3$, $PhNO_2$;
(iii) SO_2Cl_2

The synthesis of the isocoumarin (96) is described in Chapter 3. Reduction of the isocoumarin (96) with sodium borodeuteride afforded the dihydroisocoumarin (223). ^1H N.m.r. revealed that deuterium was present at carbon-3. Selective demethylation gave (224) which

was chlorinated with sulphuryl chloride to give the desired compound (210).

5.4.5 Synthesis of [3R*, 4S*][3,4,9-$^{2}H_3$]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methyl isocoumarin (211)



Reagents: (i) D_2; (ii) $AlCl_3$, $PhNO_2$;
(iii) SO_2Cl_2

Reduction of the isocoumarin (96) with deuterium gas, formed by the dropwise addition of deuterium oxide onto lithium metal, yielded the isocoumarin (225).

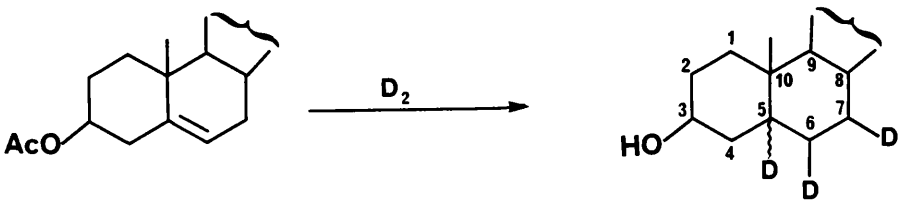
Selective demethylation afforded (226) which was chlorinated using sulphuryl chloride to give the desired isocoumarin (211).

The ^{1}H n.m.r., ^{2}H n.m.r. and mass spectrum of the final compound (211) revealed that a deuterium atom had also exchanged with a proton from the methyl group.

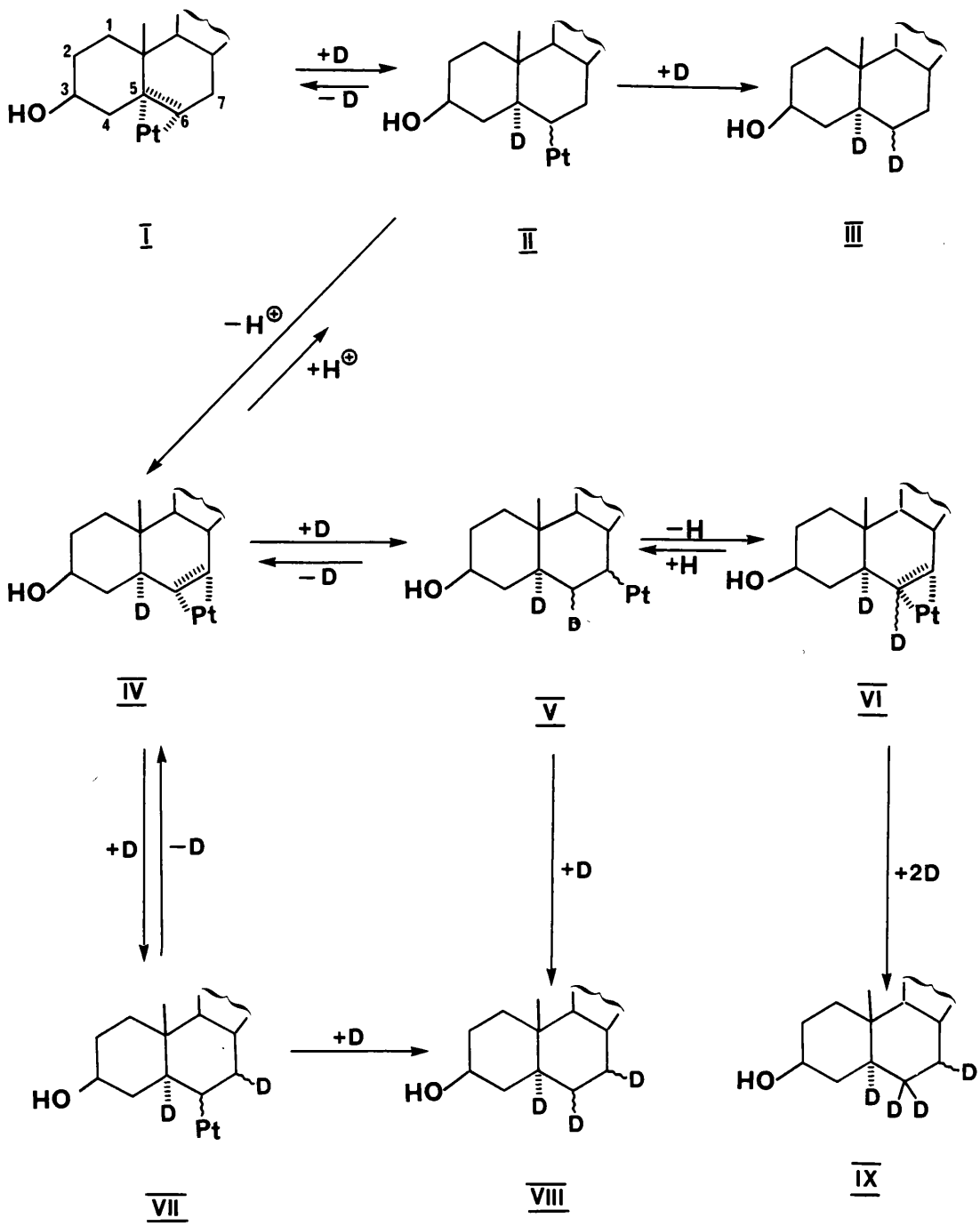
Fukushima and Gallagher[156] obtained a similar result when they were studying the catalytic reduction with deuterium on a variety of steroids. Hydrogenation and saponification of cholesterol acetate in the presence of deuterium gave cholestane-3β-ol which had 2.55D (gram atoms of deuterium per mole of compound).

Reducing a number of steroids in an analogous manner, they showed that the secondary alcohol group at C-3 did not have an influence on the reaction. It was found that the reduction of a tertiary carbon was not a controlling factor. Also, the long side chain of the sterols had no influence upon the reduction. This conclusion was drawn because all of these reductions gave compounds which had more than two deuterium atoms per mole of product.

They showed that 16% of the total isotope incorporated appeared at C-7, the carbon atom immediately adjacent to the double bond. It was also found that there was no isotopic substitution at C-4.



A mechanism has been proposed which allows for the introduction of more than two deuterium atoms. The first step in the reduction of cholesterol is the formation of the complex I. Allowing for steric considerations at the ring juncture, attack by a deuterium occurs at the 5-position giving the half-hydrogenated intermediate II. This intermediate II is then further reduced to give the species III, which is de-adsorbed from the catalyst surface. However, it is possible for the intermediate II to form complexes I or IV by dissociation of the hydrogen at carbon 5 or carbon 7 from the catalyst. The intermediate IV is in equilibrium with deuterium and other species of half-hydrogenated states V and VII. Reduction of either of these species with deuterium

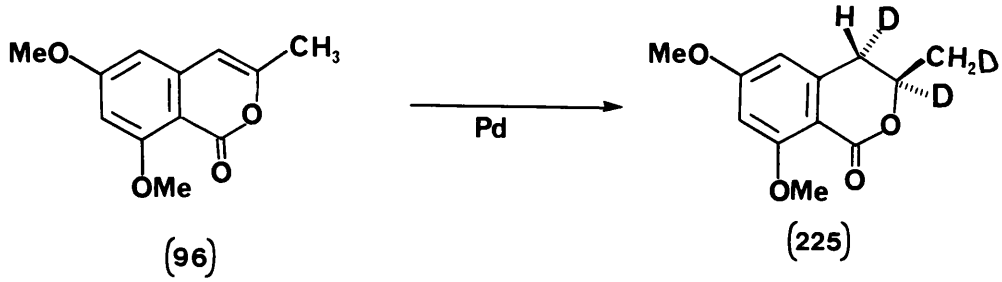

HO
Pt
+D
−D
D
+D
D
−H⊕
+H⊕
+D
−D
−H
+H
B
+D
−D
+D
+2D
+D
I
II
III
IV
V
VI
VII
VIII
IX

gives the product VIII which has deuterium atoms present at carbons 5, 6 and 7. It is also possible for the complex V to equilibrate with VI in which deuterium is found at carbon 5 and carbon 6. Further reduction of VI with deuterium gives the tetradeuteriated species IX in which deuterium is present at carbons 5, 6α, 6β and 7. A combination of species III, VIII and IX accounts for the isotope distribution that is found experimentally.

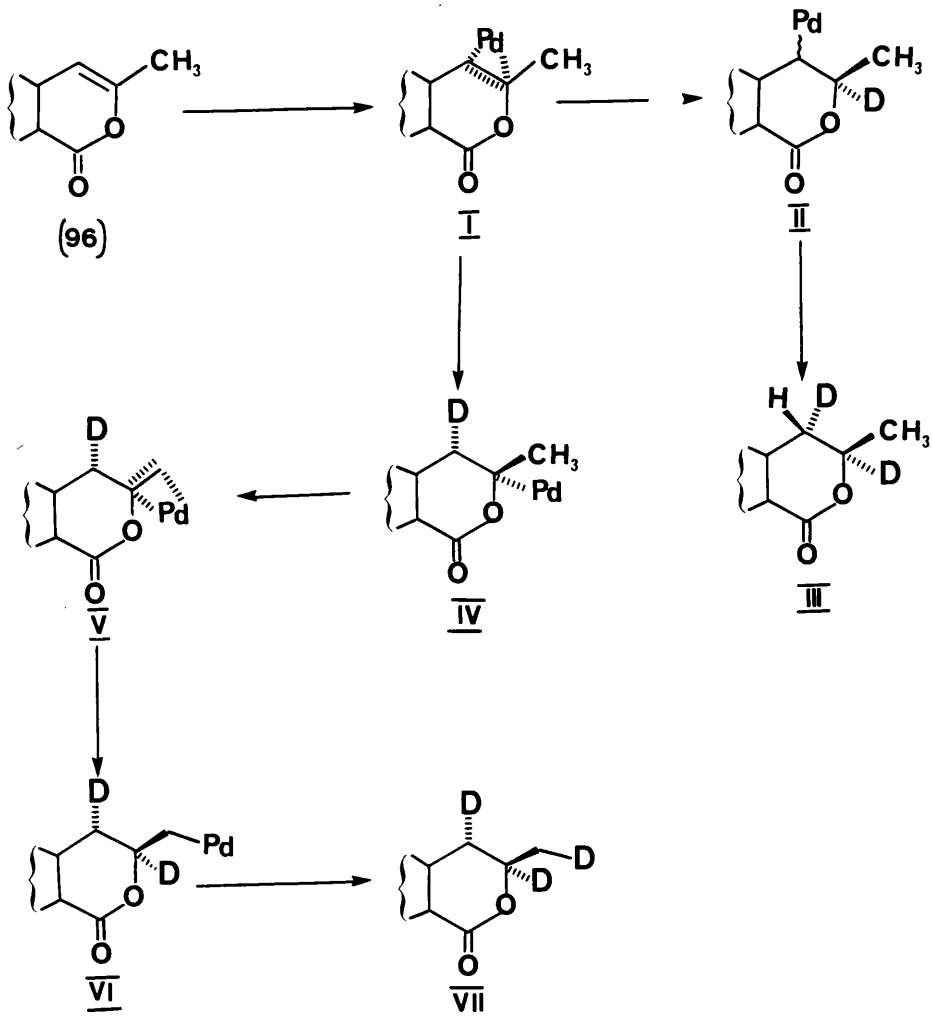
The same principle can be applied to the reduction of the isocoumarin (96) with deuterium gas giving the dihydro compound (225). This was found to contain some deuterium in the terminal methyl carbon.

Different catalysts were used for the reduction of the isocoumarin (96) and cholesterol. However, the results were similar and therefore it is possible to draw an analogy between the reduction of cholesterol with deuterium gas and the isocoumarin (96) with deuterium gas.

It is proposed that the first step is the formation of a complex I. This can then be attacked by deuterium at either carbon 3 or carbon 4 giving the different



(96) (225)



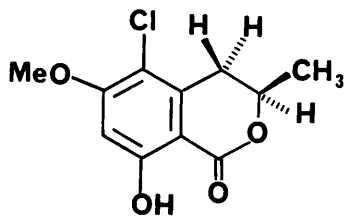
(96) I II III IV V VI VII

species II and IV. Since carbon 4a is a tertiary carbon, species II cannot lose hydrogen and form a different complex to I. Therefore, species II can be further reduced giving the product III which can be removed from the surface of the catalyst by de-adsorption. However, species IV may form the intermediate V by dissociation of hydrogen at carbon 9 or it may be further reduced also giving the product III. Reduction of this intermediate V would then give VI. Further reduction would lead to the formation of the trideuteriated isocoumarin VII. Interestingly, only a very small exchange of the 4β-hydrogen of compound (225) was observed.

5.5 Deuterium N.m.r.'s of Biosynthetic and Synthetic Isocoumarins

5.5.1 Introduction

The deuterium n.m.r. was obtained of the final synthesised isocoumarin to confirm that the deuterium atom was occupying the correct site. In the proton n.m.r. spectrum of the isocoumarin (46) the chemical shifts for the equatorial and axial protons are different. The shift of the equatorial proton is further downfield and appears at δ3.21 p.p.m. whereas

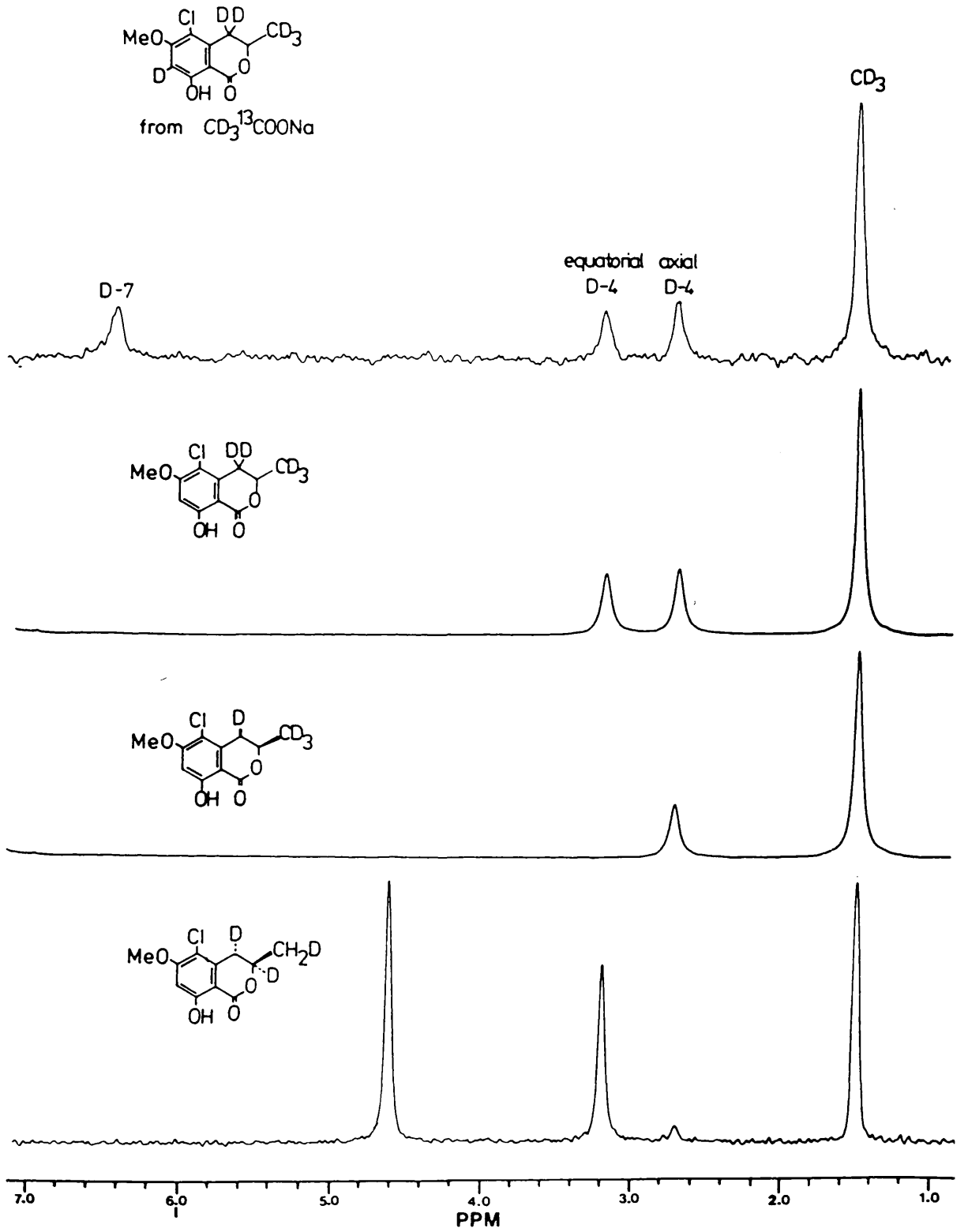


(46)

the axial proton appears at δ2.65 p.p.m. The chemical shifts in the deuterium n.m.r. correlate directly with the chemical shifts in proton n.m.r. Therefore, deuterium n.m.r. would unambiguously assign which proton had been exchanged by a deuterium atom in the final product (see Figure 5.1).

Figure 5.1 (d), the deuterium n.m.r. for compound (211), shows that some of the methyl hydrogens have been exchanged by deuterium. The peak at δ4.62 p.p.m. represents the presence of a deuterium at carbon 3. Thus it can be seen that approximately one hydrogen atom has been exchanged per molecule of isocoumarin at the methyl group. The deuterium n.m.r. shows that most of the deuterium at C-4 is present in the equatorial

FIGURE 5.1


Cl DD
MeO
CD3
D
O
OH O
from CD3 13COONa
CD3
D-7
equatorial D-4
axial D-4
Cl DD
MeO
CD3
O
OH O
Cl D
MeO
CD3
O
OH O
Cl D
MeO
CH2D
D
O
OH O
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM

site. However, a small amount is present at the axial site showing that a slight loss of stereoselectivity has occurred. This is probably due to an exchange mechanism.

5.9 ^{13}C N.m.r. and Shifted Peaks

^{13}C N.m.r. spectra of the various deuteriated isocoumarins were obtained on the Bruker 360 MHz spectrometer in Edinburgh. The shifted values listed in Table 5.8 for the deuteriated isocoumarins (201), (207), (208), (209), (210) and (211) show that the effect of each deuterium atom on the magnitude of a particular shift value is approximately additive in agreement with earlier conclusions.[135]

5.9.1 Carbon-3 Shifts

Carbon-3 in the biosynthetic isocoumarin (201) shows β-shift values of 0.04, 0.08 and 0.12 p.p.m. due to CDH_2, CD_2H and CD_3 respectively. Each deuterium atom contributes 0.04 p.p.m. to the shift value. In the synthetic isocoumarin (207), a shifted value of 0.21 p.p.m. is observed. This is accountable to a total of 5 deuteriums which arise from 4-D_2 + CD_3. From the biosynthetic experiment it is known that the

deuterium atoms at carbon-9 give an additive shift value of 0.12 p.p.m. Subtraction shows that the two deuterium atoms at carbon-4 give an additive β-shift value of 0.09 p.p.m. at carbon-3.

For the isocoumarin (208), shifts of 0.13 and 0.17 p.p.m. are observed. These shifts correspond to 4-axial D + CD_2H and 4-axial D + CD_3 respectively. It was shown by ^1H n.m.r. that some exchange of a methyl deuterium by a hydrogen atom had taken place. When three deuterium atoms are at carbon-9 an effect of 0.12 p.p.m. is observed. Therefore, the 4-pseudo-axial deuterium causes a shift of 0.05 p.p.m. at carbon-3. Further support that the pseudo-axial deuterium causes a shift of 0.05 p.p.m. can be found from compound (209). A β-shift of 0.048 p.p.m. is observed which is caused by the sole deuterium at carbon-4 which is pseudo-axial. The additive value of the two deuterium atoms at carbon 4 is 0.09 p.p.m. From the results obtained it has been shown that the pseudo-axial deuterium gives a shift of 0.05 p.p.m. Therefore, the pseudo-equatorial deuterium must cause a shift of 0.04 p.p.m. The observation at carbon-3 for the isocoumarin (211) is more complex. Here β-shift values of 0.035 and 0.08 p.p.m. were found. The value of 0.035 p.p.m. corresponds to the presence of the pseudo-equatorial deuterium at carbon-4. The value of 0.08 p.p.m.

corresponds to the presence of the pseudo-equatorial deuterium plus CH_2D. Each deuterium at carbon-9 causes a shift of 0.04 p.p.m. Therefore, it is evident that the pseudo-equatorial deuterium is having an effect of 0.04 p.p.m. at carbon-3.

For the isocoumarin (210) an α-shift of 0.40 p.p.m. is observed at carbon-3 which has a coupling constant of J 23 Hz. This was also found to be in agreement for the isocoumarin (211). Although slightly more complex this compound (210) showed an α-shift of 0.39 p.p.m. and a coupling constant of J 23 Hz. Further shifts of 0.425 p.p.m. and 0.47 p.p.m. were observed at C-3 in the isocoumarin (211). These are the additive values of the α-shift plus each of the different β-shifts, i.e. (0.39 + 0.035), (0.39 + 0.08 p.p.m.).

5.9.2 Carbon-4 shifts

For the isocoumarins (209) and (208) which have pseudo-axial deuteriums at the 4-position, α-shifts of 0.37 p.p.m. and 0.44 p.p.m. were recorded respectively. For the isocoumarin (208) the presence of the deuterium atoms at carbon-9 has an effect at carbon-4. A γ-shift of 0.07 p.p.m. is observed. Therefore, the value of 0.44 p.p.m. is the α + γ additive shift value. Subtracting the γ-shift value

gives a shift of 0.37 p.p.m. which is in agreement with the value obtained at C-4 for the isocoumarin (209). These α-shifts recorded at the benzylic positions correspond well with the α-shift observed at the same position in ethylbenzene (203) (0.35 p.p.m.).[154] A γ-shift of 0.025 p.p.m. is observed from carbon-9 to carbon-4 for the isocoumarin (211) which has one deuterium present at the methyl carbon.

For isocoumarins (210) and (211) β-shifts were observed at carbon 4 and were 0.125 p.p.m. for both compounds. This is unusually large for a β-shift.

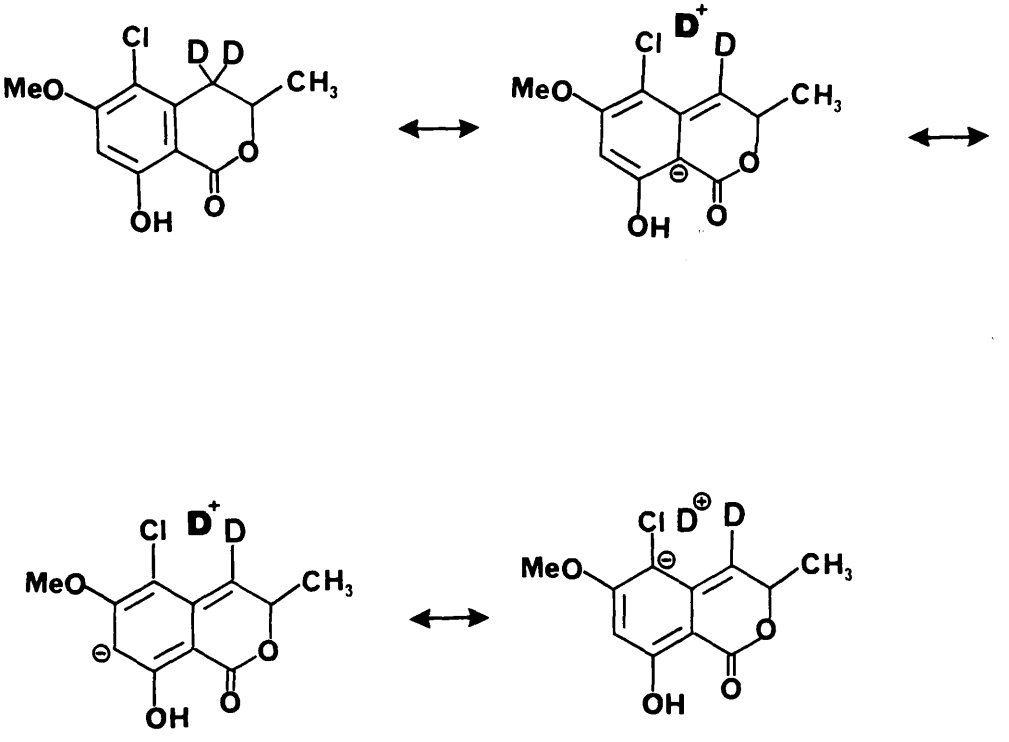
5.9.3 Carbon 4a shifts

From the biosynthetic experiment, three shifts were observed at carbon-4a in the isocoumarin (201) of 0.03, 0.07 and 0.1 p.p.m. The differences were thought to arise when the deuterium is pseudo-axial or pseudo-equatorial. The value of 0.1 p.p.m. is thought to occur when both deuteriums are present at carbon 4.

Interestingly, for the synthetic isocoumarin (207) the additive shift value obtained at carbon 4a was 0.09 p.p.m. However, for the isocoumarin (201) the value obtained from the biosynthetic feedings was 0.10 p.p.m. In the synthetic isocoumarins (208) and (209)

the deuterium atom is pseudo-axial at C-4. It was found that for the isocoumarin (208) a β-shift value at carbon-4a was 0.06 p.p.m. A β-shift recorded at the same carbon in the isocoumarin (209) gave a value of 0.072 p.p.m. The only difference between the two compounds is the presence of the deuterium atoms at carbon-9 in compound (208). Therefore it is concluded that these deuterium atoms have an overall effect of -0.01 p.p.m. at carbon 4a. This would explain the observed difference between the additive shift value of 0.09 p.p.m. for the synthetic isocoumarin (207) and the biosynthetic derivative (201) of 0.1 p.p.m. Similarly, the difference between compounds (208) and (209) can be explained in this way. For the isocoumarin (211) in which the deuterium at carbon -4 is pseudo-equatorial a shift of 0.03 p.p.m. was observed. Therefore, it was found that two different deuterium atoms each gave a different shift value. The values obtained were in close agreement with the values obtained from the biosynthetic experiment. Thus it can be noted that the shift of 0.03 p.p.m. arises from the pseudo-equatorial deuterium. Likewise, the shift of 0.07 p.p.m. arises from the pseudo-axial deuterium. A similar observation has been found by Simpson and Stenzel[53] who also found that an axial deuterium caused a larger shift.

5.9.4 Carbon-5 shifts



No shifts were observed for the isocoumarins (201), (210) and (211). The shifts that were observed for the isocoumarins (207) (208) and (209) were all γ-shifts and were all negative. The sign of the shift can be explained in terms of hyperconjugation (see Section 5.3.2.1). These shifts occur at positions where hyperconjugation interactions may contribute. A similar observation occurred with toluene α-d_3 where negative isotope effects were observed at the

γ-position. For the isocoumarins (208) and (209), in which the deuterium at carbon 4 is pseudo-axial, a γ-shift of -0.04 p.p.m. was observed. However, for the isocoumarin (207) in which there are two deuterium atoms at carbon 4, a γ-shift of -0.03 p.p.m. was observed. This suggests that the pseudo-equatorial deuterium is having an effect of +0.01 p.p.m. at carbon 5. This difference may be due to the equatorial deuterium causing a slight change in conformation thereby reducing the γ-isotope shift.

5.9.5 Carbon 8a shifts

Only the isocoumarins (207) and (208) show a shift at carbon-8a. Surprisingly, no shift was observed at carbon-8a for the isocoumarins (209) and (211) which both had a deuterium present at carbon 4. The shifts observed at carbon 8a were γ-shifts and were negative. The negative sign can be explained as above in terms of hyperconjugation. The values obtained for the isocoumarins (207) and (208) of -0.0024 p.p.m. and -0.0022 p.p.m. respectively were similar. It would appear that for carbon 8a the presence of the pseudo-equatorial deuterium has no effect on the size of the shift. Insufficient data was obtained for this carbon to make any definite conclusions.

5.9.6 Carbon 9 shifts

Only two isocoumarins (210) and (211) showed isotope effects at carbon 9. The isocoumarin (210) gave a β-shift of 0.13 p.p.m. This is also observed for the isocoumarin (211), and is an unusually large β-shift. The presence of the deuterium attached to carbon 9 gives an α-shift of 0.40 p.p.m. which has a coupling constant of J 20 Hz.

5.10 Conclusions

From these results it can be seen that the deuterium shift values of the various deuteriated isocoumarins are largely understood. In particular, the carbon-4a β-shift values for the biosynthetic isocoumarin (201) have been assigned. The shift values of 0.03 and 0.07 are due to the presence of a pseudo-equatorial and a pseudo-axial deuterium at carbon-4 respectively. The shift value of 0.10 p.p.m. is due to the presence of two deuteriums at carbon-4.

These results imply that deuterium from $CD_3{}^{13}CO_2Na$ is incorporated into position 4 of the isocoumarin(201) at both pseudo-equatorial and pseudo-axial sites. Loss of deuterium at this position appears to be non-stereospecific suggesting that a non-enzymic exchange has taken place.

TABLE 5.8

C - carbon; S - size of shift; T - type of shift and no. of deuterium causing shift

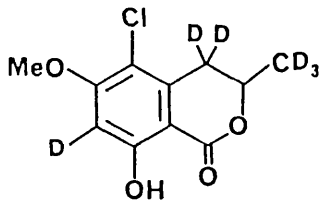
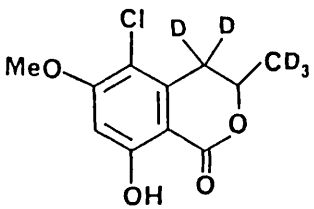
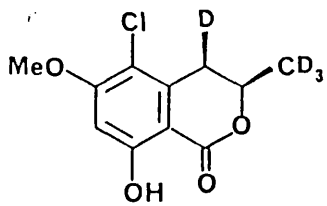
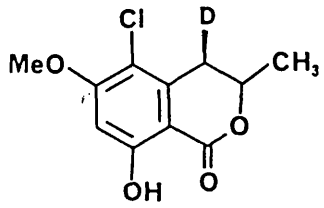
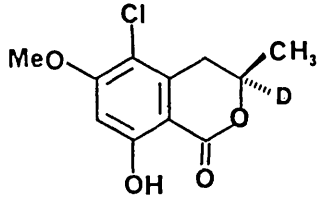
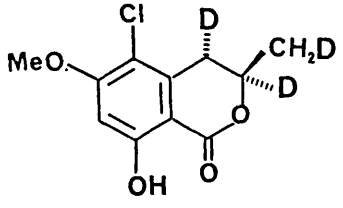
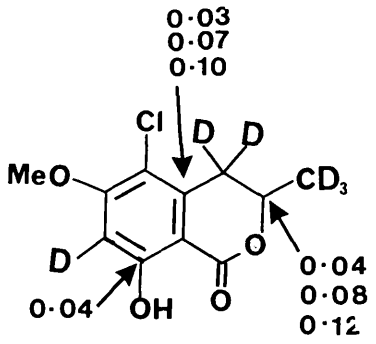
Compound	C	S	T	C	S	T	C	S	T	C	S	T	C	S	T	C	S	T
 (201)	3	0.04 0.08 0.12	β(1D) β(2D) β(3D)	4	-	-	4a	0.03 0.07 0.10	β^1(1D) β^2(1D) β (2B) ($\beta^1+\beta^2$)	5	-	-	8	0.04	β(1D)	8a	-	-
 (207)	3	0.21	β(5D)	4	-	-	4a	0.09	β(2D)	5	-0.03	γ(1D)	8	-	-	8a	-0.02	γ(1D)
 (208)	3	0.13 0.17	β(3D) β(4D)	4	0.07 0.44	γ(3D) (α+γ) α J20Hz	4a	0.06	β(1D)	5	-0.04	γ(1D)	8	-	-	8a	-0.02	γ 1D

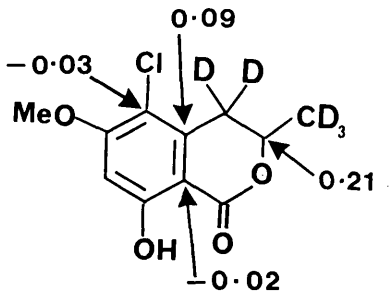
TABLE 5.8 (cont)

C - carbon; S - size of shift; T - type of shift and no. of deuterium causing shift

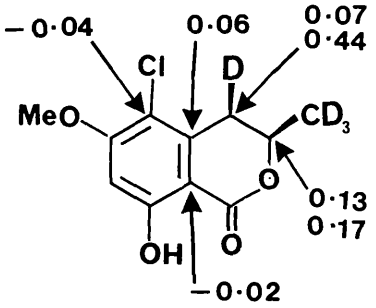
Compound	C	S	T	C	S	T	C	S	T	C	S	T	C	S	T	C	S	T
 (209)	3	0.048	β(1D)	4	0.37	α J 20Hz	4a	0.072	β (1D)	5	-0.04	γ (1D)						
 (210)	3	0.40	α J 23Hz	4	0.125	β	4a	0.01	γ	5	-		9	0.13	β			
 (211)	3	0.035 0.08 0.39 0.425 0.47	β^1(1D) β^2(2D) α J 23Hz α+β^1 α+β^2	4	0.015 0.125 0.15 0.42	γ β γ+βγ) α J=20Hz	4a	0.033	β				9	0.135 0.40	β α J 20Hz			



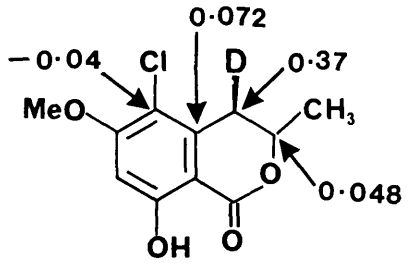
(201)



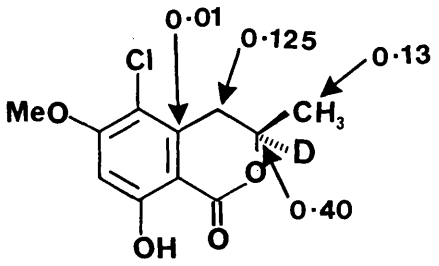
(207)



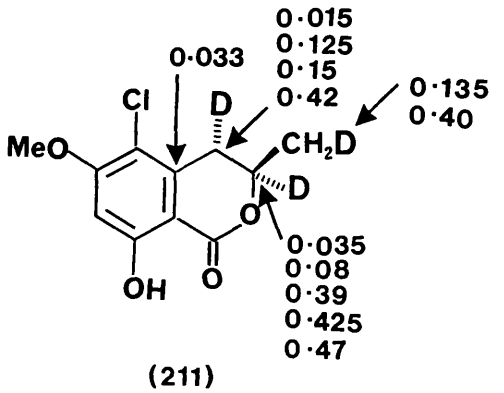
(208)



(209)



(210)



(211)

EXPERIMENTAL

General Procedures

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infra-red spectra were recorded for potassium bromide discs (unless otherwise stated) on a Perkin-Elmer 580 spectrometer. The following abbreviations are used: s - strong, m - medium, w - weak and br - broad.

Proton nuclear magnetic resonance spectra were determined on a Perkin-Elmer RB spectrometer using deuterio chloroform as solvent (unless otherwise stated). Me_4Si was used as internal standard. The following abbreviations are used: s - singlet, d - doublet, t - triplet, q - quartet, m - multiplet, dd - double-doublet, dq - double quartet and br - broad. Carbon nuclear magnetic resonance spectra were determined on a Bruker WP 360 spectrometer in the pulsed F. T. mode using deuterio chloroform as solvent and Me_4Si as internal standard.

Mass spectra were obtained with a MS12 or MS902 mass spectrometer.

All apparatus and solutions involved in the growth of the micro-organism were sterilised before and after use in an autoclave.

Preparative thin layer chromatography was carried out using Merck Kieselgel GF254. Analytical thin layer chromatography was carried out on commercial plates with a 0.25 mm layer of the same silica gel.

Organic solutions were evaporated on a rotary evaporator under reduced pressure; solutions in organic solvents were dried over anhydrous magnesium sulphate.

Culture and harvesting of Periconia macrospinosa

Stock cultures of Periconia macrospinosa (CMI 24411) were maintained on potato-dextrose agar slopes. The slopes were inoculated with a spore inoculum from the master strain (or from earlier sub-cultures) and incubated at 25 °C for 14 days after which time they were used as inocula for large-scale cultures or stored at 4 °C.

Large-scale cultures of P. macrospinosa were grown as surface cultures in Roux bottles on a solution of glucose (50 g), sodium nitrate (2 g), potassium chloride (1 g), magnesium sulphate (1 g), dipotassium hydrogen orthophosphate (0.5 g), ferrous sulphate (0.01 g), yeast extract (1 g) in distilled water (1 L). After 21 days incubation at 25 °C (during which time the pH of the culture medium increased from 5 to 7.5) the mould had formed a thick black surface mat and the medium had become cloudy and dark brown in colour. The mycelium was separated from the culture medium and dried over P_2O_5. The culture medium was continuously extracted with ethyl acetate for 24 hours.

Isolation of methyl-2-allyl-3,5-dichloro-1,4-dihydroxy-cyclopent-2-eneoate (cryptosporiopsinol)

The ethyl acetate extract of P. macrospinosa culture medium was dried and evaporated to leave a brown gum (typically 1 g/L of medium). The extract was first passed through a

short column of silica gel (2.5 x 1 cm) under suction (water pump), using ethyl acetate as eluent, to remove highly polar resinous material and then subjected to preparative t.l.c. using chloroform as eluent. Cryptosporiopsinol (R_F 0.2) was isolated as a colourless oil which crystallised on standing (typical yield: 0.5 g/L of medium). Recrystallised from dichloromethane/hexane as cubes, m.p. 120-121 °C (lit., [79] 121-122 °C). νmax. 3 500s, 3 460s 1 710s, 1 655w and 1 608w cm^{-1}; $\delta(CDCl_3)$ 6.10 (2 H, m, Ha and Hb), 4.47 (2 H, m, H_4 and H_5), 3.90 (3 H, s, OMe), 2.90 (1 H, br, 6-OH), 1.78 (3 H, m, $-CH_3$) p.p.m; $\delta_c(CDCl_3)$ 172.22, 137.24, 134.49, 132.91, 120.61, 87.38, 75.50, 66.10 54.37, 19.41 p.p.m; m/z 266/268/270 (M^+).

Isolation of 5-chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

The dried mycelium (typical dry weight: 1 g/L of medium) was powdered and extracted in a soxhlet apparatus (ethyl acetate; 24h). The ethyl acetate extract was evaporated to give an oily residue which was subjected to preparative t.l.c. (chloroform/ petroleum ether 40-60 then chloroform). The dihydroisocoumarin was obtained as a colourless crystalline residue (typical yield 90 mg/g dry mycelium). Recrystallised from ethyl acetate/petroleum ether 40-60 as needles, m.p. 122-123 °C (lit., [79] 123-124 °C); ν max. 1 655s, 1 620s, 1 580m, cm^{-1}; $\delta(CDCl_3)$ 6.43 (1 H, s, Ar-H), 4.65 (1 H, m, 3-H), 3.92 (3 H, s, -OMe), 3.25 (1 H, dd, J 3

and 17 Hz, $-CH_2-$), 2.75 (1 H, dd, J 3 and 17 Hz, $-CH_2-$), 1.49 (3 H, d, J 6 Hz, $-CH_3$) p.p.m; $\delta_c(CDCl_3)$ 176.44, 162.93 161.02 137.62 124.12 111.45 101.63 74.90 57.45 32.13 20.63 p.p.m; m/z 242/244(M^+).

Harvesting of Gilmaniella humicola

Spores of Gilmaniella humicola NRNL 5487 were grown on a medium consisting of glucose (20 g), malt extract (2 g), yeast extract (2 g), peptone (2 g) potassium dihydrogen phosphate (2 g) and magnesium sulphate (2 g) in distilled water (1 L). Erlenmeyer flasks (500 ml) containing 150 ml of the sterile medium were inoculated with 1 ml of spore suspension and shaken on a rotary shaker at 27 °C. After 6 days the mycelium and the medium were extracted with ethyl acetate and the organic layer washed with water. The organic extract was dried ($MgSO_4$) and evaporated giving a brown residue.

Isolation of Dechloromicroline

The extract was first passed through a short column of silica gel (2.5 x 1 cm) under suction (water pump), using ethyl acetate as eluent to remove highly polar resinous material then subjected to preparative t.l.c. using ether as eluent. Dechloromicroline (R_F0.4) was isolated as an oil, typical yield ~45 mg/L. νmax. 3 500 br, 1 670 m, cm^{-1}; $\delta(CDCl_3)$ 6.10-6.60 (3 H, m, H-2,3,5) 4.50 (1 H, br, OH) 3.42 (2 H, m,

H-13) 1.45-2.10 (3 H, m, H-8, 9) 1.92 (3 H, d, J 6.7 Hz H-1), 1.39 (3 H, s, $-CH_3$) m/z 264 (M^+).

Oxidation of Dechloromicroline

A cold solution of dechloromicroline (40 mg) in acetone (5 ml) was treated with Jones reagent (CrO_3/H_2SO_4) (0.3 ml). After 10 mins ethanol (2 ml) was added to destroy excess reagent and the reaction mixture then diluted with dichloromethane (50 ml). The organic solution was washed with water (3 x 20 ml), brine (2 x 10 ml) dried and evaporated giving the crude product. This was purified by preparative t.l.c. eluting with hexane/ethyl acetate 3:1, (15 mg 37%) m.p. 153-156 °C (lit.,[96] 153-158 °C); νmax. 1 780m, 1 740m, 1 680m cm^{-1}; $\delta(CDCl_3)$ (360 MHz) 6.13 (1 H, dq, J 0.8 and 15.8 Hz H-3) 6.09 (1 H, dq, J 6.4 and 15.8 Hz, H-2) 6.06 (1 H, s, H-5) 5.22 (1 H, d J 11.4 Hz, H-13b) 4.07 (1 H, d, J 11.4 Hz, H-13a) 2.34 (1 H, dd, J 4.9 and 8.0 Hz, H-9) 1.87 (3 H, dd, J 0.8 and 6.4 Hz, CH_3) 1.76 (1 H, dd, J 5.5 and 8.0 Hz, H-8a) 1.45 (3 H, s, H-14) 1.10 (1 H, dd, J 4.9 and 5.5 Hz, H-8b) p.p.m. m/z 260 (M^+).

FEEDING EXPERIMENTS

1. Periconia macrospinosa

Feeding experiments to Periconia macrospinosa were carried out on day 14 and the fungal broth was

extracted on day 21. The isocoumarins (75) and (78) were fed to the fungus as their sodium salts. The isolation of labelled 5-chloro-3,4-dihydro-6-hydroxy-8-methoxy-3-methylisocoumarin (46) and cryptosporiopsinol (47) was as described for the unlabelled metabolites. The results from the feeding experiments are described in chapter 2.

2. Gilmanella humicola

The isocoumarins (50) and (103) labelled with ^{14}C and ^{2}H respectively were fed as their sodium salts to Gilmaniella humicola on day 4. The fungus was grown as described above and the broth and mycelium were extracted after day 6. The dechloromicroline was purified and oxidised to the crystalline derivative (104) as described for the unlabelled material. The derivative (104) was recrystallised to constant activity. The results are discussed in chapter 3.

Data for Radioactive Feeding

wt of isocoumarin (50) fed = 50 mg Total activity = 1654 Bq

wt of bislactone (104) recovered = 9.2 mg Total activity = 135.6 Bq

Therefore % incorporation = $\frac{135.6}{1654} \times 100 = \underline{8.2\%}$

Ethyl 1,2-dihydro-o-orsellinate[157]

Sodium (23 g) was dissolved in dry ethanol (300 ml); ethyl acetoacetate (124 ml) and ethyl crotonate (112 ml) were added and the mixture was refluxed for 2 h then stirred overnight at room temperature. The mixture was acidified with 5% sulphuric acid and then filtered. The filtrate was then diluted with water (200 ml) and extracted with chloroform. The organic extracts were dried and evaporated to leave an oily product which was chilled until crystallisation began, diluted with hexane, then left standing at 0 °C overnight. The product was collected by filtration and recrystallised from benzene/hexane. (90 g, 46%), m.p. 90 °C (lit.,[158] 89-90 °C); δ ($CDCl_3$) 9.68 (1 H, s, OH), 5.51 (1 H, s, 3-H), 4.26 (2 H, q, J 7 Hz, $OC\underline{H}_2CH_3$), 3.08 (1 H, d, J 10 Hz, 1-H), 2.8-2.00 (3 H, m, 5H and 6H), 1.28 (3 H, t, J 7 Hz, $OCH_2C\underline{H}_3$), 1.08 (3 H, d, J 6 Hz, $-CHC\underline{H}_3$) p.p.m.; m/z 196 (M^+).

Ethyl-3,5-dibromo-o-orsellinate [159]

A solution of bromine (71 g, 23 ml) in glacial acetic acid (30 ml) was added with stirring to a solution of ethyl 1,2-dihydro-o-orsellinate (25 g) in glacial acetic acid (200 ml) at such a rate that the temperature of the reaction mixture remained above 60 °C. The reaction mixture was stirred for 16 h then poured onto iced water (1 L); a white precipitate of ethyl-3,5-dibromo-o-orsellinate separated and was collected by filtration, washed with water and air dried.

A portion was recrystallised from dichloromethane/hexane. (26 g, 58%), m.p. 104 °C (lit.,[159] 105-106 °C); δ($CDCl_3$), 4.45 (2 H, q, J 8 Hz, $-CH_2CH_3$), 2.67 (3 H, s, $Ar-CH_3$), 1.41 (3 H, t, J 8 Hz, $-CH_2CH_3$) p.p.m; m/z 352, 354 (M^+).

Ethyl-o-orsellinate

Ethyl-3,5-dibromo-o-orsellinate (20 g) was dissolved in sodium hydroxide solution (2 M; 240 ml) and Raney nickel alloy (20 g) was added portionwise with stirring at 0 °C. After complete addition of the alloy the mixture was stirred at 0 °C for a further 1 h then filtered through celite. The filtrate was poured onto ice-cold concentrated hydrochloric acid (150 ml) and the acidic solution extracted with ether (3 x 50 ml). The ethereal extracts were dried and evaporated to give the product which was recrystallised from aqueous acetic acid as needles. (7 g, 67%), m.p. 129-130 °C (lit.,[160] 131-133 °C); ν max. 3 360br, 1 640s, 1 580m cm^{-1}; δ($(CD_3)_2CO$) 6.26 (2 H, m, Ar-H), 4.39 (2 H, 9, J 7 Hz, $-OCH_2CH_3$), 2.47 (3 H, s, $Ar-CH_3$) 1.38 (3 H, t, J 7 Hz, $-CH_2CH_3$) p.p.m; m/z 196 (M^+).

Ethyl-3-chloro-o-orsellinate

A solution of chlorine (17 g) in glacial acetic acid (200 ml) was added with stirring and ice cooling to a solution of ethyl-1,2-dihydro-o-orsellinate (24 g) in glacial acetic acid (100 ml) in a closed system. The reaction mixture was

stirred at room temperature for 0.5 h then at 60 °C for 3 h and then poured onto iced water (1 L). The precipitate was separated by filtration, washed with water and dried. A portion was recrystallised from dichloromethane/hexane as needles. (13 g, 46%), m.p. 120-122 °C (lit.,[80] 120-124 °C); νmax. 3 375br, 1 640s, 1 600m cm^{-1}; $\delta(CDCl_3)$ 6.43 (1 H, s, Ar-H), 4.43 (2 H, q, J 7 Hz, $-OC\underline{H}_2CH_3$), 2.50 (3 H, s, $Ar-CH_3$), 1.40 (3 H, t, J 7 Hz, $OCH_2C\underline{H}_3$) p.p.m; m/z 230 232 (M^+).

2,4-Dimethoxy-6-methylbenzoic acid

To a solution of ethyl-o-orsellinate (8 g) in anhydrous acetone (160 ml) was added dimethylsulphate (12 ml) and anhydrous potassium carbonate (20 g). The mixture was heated at reflux for 8 h with stirring. On cooling, the reaction mixture was filtered and the precipitate washed with acetone (3 x 50 ml). The combined organic solutions were evaporated leaving a dark brown oily residue. The residue was dissolved in ether (100 ml) and washed with aqueous ammonia solution (10%, 3 x 30 ml), sodium hydroxide solution (10%, 3 x 25 ml), and water (3 x 20 ml). The ether solution was evaporated to give a pale oil. The oil was suspended in potassium hydroxide solution (10%, 100 ml) and heated at reflux for 6 h. On cooling, the basic solution was washed with ether (3 x 20 ml). The basic solution was acidified with concentrated hydrochloric acid at 0 °C and then extracted with ethyl acetate (4 x 25 ml). This was dried

and evaporated giving the desired compound which was recrystallised from dichloromethane/hexane as prisms (6.5 g, 81%), m.p. 140 °C (lit.,[80] 140 °C; νmax. 1 680s, 1 600s cm^{-1}; δ ($CDCl_3$) 6.40 (2 H, m, 2Ar-H), 3.93 (3 H, s, OMe) 3.83 (3 H, s, OMe), 2.53 (3 H, s, Ar-CH_3) p.p.m.; m/z 196 (M^+).

3-Chloro-2,4-dimethoxy-6-methylbenzoic acid

Prepared by the methylation and hydrolysis of ethyl-3-chloro-o-orsellinate (8 g) by the foregoing method. Recrystallised from dichloromethane/hexane as prisms. (6.3 g, 78%); m.p. 152-153 °C (lit.,[161] 154-156 °C); νmax. 1 690s, 1 585s cm^{-1}; δ($CDCl_3$) 10.90 (1 H, br, OH), 6.59 (1 H, s, Ar-H), 3.96 (3 H, s, OMe), 3.91 (3 H, s, OMe), 2.45 (3 H, s, Ar-CH_3) p.p.m.; m/z 230 232 (M^+).

3,5-Dimethoxyhomophthalic acid

n-Butyllithium (54 ml; 1.5 M in hexane) was added to a solution of diisopropylamine (8.2 g, 11.36 ml) in dry tetrahydrofuran (THF) (25 ml) under nitrogen at 0 °C with stirring. After 10 mins the solution was cooled to -78 °C and a solution of 2,4-dimethoxy-6-methylbenzoic acid (4 g) and dimethyl carbonate (4.4 g, 4.12 ml) in THF (25 ml) was added dropwise during 15 mins. The cooling bath was removed and the solution allowed to warm to room temperature. After 4 h, water (30 ml) was added and the suspension stirred for

16 h. The organic solvents were removed by evaporation and the resulting aqueous solution was washed with ether (3 x 30 ml). After acidification with dilute hydrochloric acid, the solution was extracted with ethyl acetate (3 x 30 ml). The combined extracts were dried and evaporated to give 3,5-dimethoxyhomophthalic acid which was recrystallised from acetone/hexane as prisms. (3.6 g, 74%) m.p. 170-171 °C (lit.,[162] 172-173 °C); νmax. 3 000-2 700br, 1 680s, 1 600s, 1 585m cm^{-1}; $\delta((CD_3)_2CO)$ 6.59 (2 H, s, Ar-H), 3.91 (3 H, s, OMe), 3.87 (3 H, s, OMe), 3.79 (2 H, s, $-CH_2$) p.p.m.; m/z 240 (M^+).

4-Chloro-3,5-dimethoxyhomophthalic acid

Prepared by treatment of 3-chloro-2,4-dimethoxy-6--methylbenzoic acid (5.5 g) by the foregoing method.The product was recrystallised from dichloromethane/hexane (5.1 g, 78%) m.p. 143-144 °C (lit.,[80] 141-146 °C); νmax. 3 000-2 600br, 1 720s, 1 610s, 1 590m, cm^{-1}; $\delta(CD_3OD)$ 6.82 (1 H, s, Ar-H), 3.88 (3 H, s, OMe), 3.85 (3 H, s, OMe), 3.75 (2 H, s, $-CH_2$) p.p.m.; 274 276 (M^+).

Homophthalic acid[162]

Indene (18 ml) was added dropwise to a stirred solution of potassium dichromate (60.7 g) and concentrated sulphuric acid (180 ml) in water (900 ml) at such a rate that the temperature of the reaction was maintained at 65 °C ± 2 °C. After the addition was complete the reaction was stirred at 65 °C for a further 2 h. The flask was cooled to 20 °C then further cooled by use of an ice-bath to 0 °C. The flask was then stirred at 0 °C for a further 5 h. The product was collected by filtration, washed with water and dried (20 g, 72%) m.p. 140-141 °C (lit., [162] 140-142 °C); $\delta(CD_3)_2CO$ 7.88 (1 H, dd, J 2 and 8 Hz, H-8) 7.40 (3 H, m, Ar-H), 4.06 (2 H, s, CH_2) p.p.m. m/z 178 (M^+).

3,5-Dimethoxyhomophthalic anhydride

A solution of 3,5-dimethoxyhomophthalic acid (4 g) in acetyl chloride (20 ml) was heated at reflux for 0.5 h, by which time the solution was homogenous. After cooling, the solution was diluted with light petroleum (b.p. 40-60 °C) until the product began to crystallise from solution. The product was isolated by filtration, washed with light petroleum (b.p. 40-60 °C) and air dried. (3.1 g, 84%), m.p. 159-160 °C (lit., [163] 160-162 °C); νmax. 1 715s, 1 660s, 1 610s, 1 580m cm^{-1}; $\delta(CDCl_3)$ 6.46 (1 H, d, J 2 Hz, Ar-H), 6.34 (1 H, d, J 2 Hz, Ar-H), 3.98 (2 H, s, Ar-CH_2), 3.95 (3 H, s, OMe), 3.90 (3 H, s, OMe) p.p.m.; m/z 222 (M^+).

Homophthalic anhydride

Prepared by treatment of homophthalic acid (1 g) by the foregoing method. (820 mg, 80%) m.p. 138-139 °C (lit., [164] 139-140 °C); δ ($CDCl_3$) 7.94 (1 H, dd J 1 and 8 Hz, H-8), 7.30 (3 H, m, Ar-H), 3.87 (2 H, s, CH_2) p.p.m.; m/z 162 (M^+).

4-Carboxy-6,8-dimethoxy-3-methylisocoumarin

To a solution of 3,5-dimethoxyhomophthalic anhydride (250 mg) in anhydrous THF (30 ml) was added acetic anhydride (0.15 ml) and dry pyridine (0.2 ml). The solution was stirred at room temperature for 40 mins then a further portion of acetic anhydride (0.2 ml) was added and the solution was heated at gentle reflux for 1 h. After cooling, the solution was evaporated and the residue dissolved in dilute aqueous sodium hydrogen carbonate. The basic solution was washed with ether (3 x 25 ml) then acidified cautiously with dilute hydrochloric acid and extracted with ethyl acetate (3 x 25 ml). The combined extracts were dried and evaporated to give the product as a white crystalline compound which was recrystallised from ethyl acetate/hexane (200 mg, 67%); m.p. 147-148 °C (lit., [80] 145-147 °C) (Found: C, 59.22; H, 4.52%; M^+, 264.062 $C_{13}H_{12}O_6$ requires C, 59.09; H, 4.58%; M, 264.0636); ν max. 1 745s, 1 705s, 1 650s, 1 605m, 1 570m cm^{-1}; δ ($(CD_3)_2CO$) 6.57 (1 H, d, J 1 Hz,

Ar-H), 6.41 (1 H, d, J 1 Hz, Ar-H), 3.98 (3 H, s, -OMe), 3.94 (3 H, s, -OMe) 2.59 (3 H, s, $-CH_3$) p.p.m.

4-Carboxy-3-methylisocoumarin

Prepared by treatment of homphthalic anhydride (250 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane (210 mg, 72%); ν max. 3 300br, 1 750s, 1 700s, 1 650s, 1 600m, 1 575m cm^{-1}; δ ($CDCl_3$) 8.23 (1 H, dd, J and 8 Hz, H-8), 7.50 (3 H, m, Ar-H), 2.63 (3 H, s, Me) p.p.m.; m/z 204 (M^+).

6,8-Dimethoxy-3-methylisocoumarin

4-Carboxy-6,8-dimethoxy-3-methylisocoumarin (250 mg) was heated to just below its m.p. (140-150 °C) for 10 mins by which time carbon dioxide evolution had ceased. The residue was purified by preparative t.l.c. (ethyl acetate as eluent) to give 6,8-dimethoxy-3-methylisocoumarin. Recrystallised from ethanol as plates (190 mg, 90%), m.p. 156-157 °C (lit.,[165] 157-158 °C); (Found: C, 65.57; H, 5.58%; M^+, 220.0736. $C_{12}H_{12}O_4$ requires C, 65.43; H, 5.49%; M, 220.0732); ν max. 1 710s, 1 665m, 1 600s, 1 570s cm^{-1}; δ($CDCl_3$) 6.46 (1 H, d, J 2 Hz, Ar-H), 6.34 (1 H, d, J 2 Hz, Ar-H), 6.12 (1 H, s, 4-H), 4.01 (3 H, s, OMe), 3.94 (3 H, s, OMe), 2.21 (3 H, s, Ar-CH_3) p.p.m.

3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin

6,8-Dimethoxy-3-methylisocoumarin (250 mg) was heated under reflux in aqueous sodium hydroxide solution (10%, 15 ml) for 1 h. Sodium borohydride (50 mg) was added with stirring and the mixture heated under reflux for 30 min; more sodium borohydride (50 mg) was added and heating was continued for a further 30 min. The hot solution was filtered and the filtrate was acidified with hydrochloric acid. The acidic solution was extracted with ethyl acetate, dried and evaporated to yield 3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin which was recrystallised from ether/hexane as needles (175 mg, 70%), m.p. 125-126 °C (lit.,[164] 125-126 °C; νmax. 1 710s, 1 660m, 1 600s, 1 575m cm^{-1}; $\delta(CDCl_3)$ 6.41 (1 H, d, J 2 Hz, Ar-H), 6.31 (1 H, d, J 2 Hz, Ar-H), 4.51 (1 H, m, 3-H), 3.92 (3 H, s, OMe), 3.87 (3 H, s, OMe), 2.83 (2 H, m, 4-H), 1.45 (3 H, d, J 7 Hz, $-CH_3$) p.p.m.; m/z 222 (M^+).

3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin

Palladium on charcoal (10%, 75 mg) was added to a solution of 6,8-dimethoxy-3-methylisocoumarin (250 mg) in ethyl acetate (20 ml) and the mixture hydrogenated at room temperature for 16 h. The catalyst was removed by filtering through celite. The ethyl acetate was evaporated to give the desired product which was recrystallised from ether/hexane as needles. (225 mg, 90%); m.p. 125-126 °C same data as above.

4-Carboxy-3,4-dihydro-3-methylisocoumarin

Palladium on charcoal (10%, 50 mg) was added to a solution of 4-carboxy-3-methylisocoumarin (250 mg) in ethyl acetate (25 ml). The mixture was then hydrogenated at room temperature and atmospheric pressure for 16 h. The catalyst was filtered through celite and washed with ethyl acetate. The organics were evaporated giving the title compound which was recrystallised from ether/hexane (220 mg, 88%); m.p. 142-143 °C; δ ($CDCl_3$) 8.12 (1 H, dd, J 2 and 8 Hz, H-8), 7.40 (3 H, m, Ar-H), 4.81 (1 H, dq, J 3 and 8 Hz, H-3) 3.46 (1 H, d, J 3 Hz, H-4) 1.56 (3 H, d, J 8 Hz, Me) p.p.m.; m/z 206 (M^+).

4-Chloro-3,4-dihydro-3-methylisocoumarin

Lead tetraacetate (2.63 g) was added to a solution of N-chlorosuccinimide (938 mg) and 4-carboxy-3,4-dihydro-3-methylisocoumarin (250 mg) in dry DMF (10 ml) and glacial acetic acid (2 ml) under nitrogen. The flask was warmed to 40-50 °C and stirred for 40 mins. The solution was allowed to cool and diluted with water (60 ml). The aqueous solution was extracted with ethyl acetate. The organic extracts were washed with water (3 x 15 ml), sodium bicarbonate solution (3 x 15 ml) and brine (2 x 15 ml). The organics were then dried and evaporated. The product was purified using preparative t.l.c. eluting with diisopropylether to give the desired compound. (25 mg, 40%);

δ($CDCl_3$) 8.16 (1 H, dd, J 2 and 8 Hz, H-8), 7.55 (3 H, m, Ar-H), 5.00 (1 H, d, J 1.5 Hz, H-4), 4.88 (1 H, dq, J 1.5 and 7 Hz, H-3), 1.67 (3 H, d, J 7 Hz, $-CH_3$) p.p.m.; m/z 196/198 (M^+).

3,5-Dimethoxytoluene[165]

Orcinol (12.4 g) in dry acetone (125 ml) containing anhydrous potassium carbonate (55 g) and dimethyl sulphate (19 ml) was heated at reflux for 8 h with stirring. After cooling, the solution was filtered and the residue washed with acetone (2 x 50 ml). The combined organic solutions were evaporated to give an oil which was dissolved in ether (100 ml). The organic layer was washed with ammonia solution (10%, 4 x 25 ml), sodium hydroxide solution (10%, 3 x 20 ml), and water 3 x 20 ml). The organics were dried and evaporated to give an oil which was purified by distillation (11.08 g, 73%), (60 ^{o}C/0.8 mm Hg); δ($CDCl_3$) 6.30 (3 H, s, Ar-H), 3.73 (6 H, s, 2 x OMe), 2.27 (3 H, s, $-CH_3$) p.p.m.; m/z 152 (M^+).

2-Bromo-3,5-dimethoxytoluene[166]

Bromine (1.6 ml) in 1,2-dichloroethane (10 ml) was added with stirring during 5 minutes to a solution of 3,5-dimethoxytoluene (5 g) in 1,2-dichloroethane (50 ml). Stirring was continued for a further 20 min. at room temperature. The organic solution was washed with water (3 x 20 ml) dried and evaporated to give an oil which

crystallised on standing. The product was recrystallised from methanol as cubes (5.3 g, 70%), m.p. 57 °C (lit.,[166] 57 °C); $\delta(CDCl_3)$ 6.29 (1 H, d, J 3 Hz, Ar-H), 6.19 (1 H, d, J 3 Hz, Ar-H), 3.78 (3 H, s, OMe), 3.69 (3 H, s, OMe), 2.30 (3 H, s, Ar-Me) p.p.m.

2,4-Dimethoxy-6-methylbenzoic acid

n-Butyllithium (5.4 ml, 1.5 M in hexane) was added to a stirred solution of 2-bromo-3,5-dimethoxytoluene (2 g) in THF (50 ml) at -78 °C under nitrogen. The solution was stirred at this temperature for 15 minutes and then poured onto an excess of crushed dry ice. The solution was allowed to warm to room temperature and water (25 ml) was added. The organic solution was evaporated and the aqueous solution was washed with ether (3 x 25 ml). The aqueous solution was then acidified with dilute hydrochloric acid. The acidic solution was extracted with ethyl acetate. The organic extracts were dried and evaporated yielding the desired compound which was recrystallised from dichloromethane/hexane (1.45 g, 85%) m.p. 140 °C same data as above.

2,4-Dimethoxy-6-(2-oxopropyl)benzoic acid

3,5-Dimethoxyhomophthalic acid (2 g) was added portionwise to a mixture of acetic anhydride (4 ml) and dry pyridine (1 ml). After 5 minutes, dry ether (15 ml) was added and stirring continued for 2 h. Sodium hydroxide solution (150 ml; 4 M)

was slowly added and the mixture refluxed until all the solid material had dissolved. The solution was cooled, washed with an equal volume of dichloromethane then cautiously acidified. The acidic solution was extracted with ethyl acetate (3 x 30 ml) which was dried and evaporated to give a solid which was recrystallised from acetone (1.4 g, 70%), m.p. 139-140 °C (Found: C, 60.49; H, 5.71%; M^+, 238.084; $C_{12}H_{14}O_5$ requires C, 60.48; H, 5.93%; 238.084); νmax. 1 710s, 1 680s, 1 600s cm^{-1}; $\delta((CD_3)_2CO)$ 6.48 (1 H, d, J 2 Hz, Ar-H, 6.39 (1 H, d, J 2 Hz, Ar-H) 3.99 (2 H, s, $-CH_2$), 3.85 (6 H, s, 2 x OMe), 2.29 (3 H, s, $-CH_3$) p.p.m.

3-Chloro-2,4-dimethoxy-6-(2-oxopropyl)benzoic acid

Prepared by treatment of 4-chloro-3,5-dimethoxyhomophthalic acid (2 g) by the foregoing method. The product was recrystallised from acetone as cubes (1.4 g, 70%), m.p. 143-144 °C (Found: C, 52.78, H, 4.85, Cl, 12.78%; M^+ 272.045, 274.042. $C_{12}H_{13}O_5Cl$ requires C, 52.85; H, 4.80; Cl, 13.00%; 272.043, 274.040). νmax. 1 710s, 1 680s, 1 600s cm^{-1}; $\delta((CD_3)_2CO)$ 6.60 (1 H, s, Ar-H), 4.05 (2 H, s, $-CH_2$) 3.90 (6 H, s, 2 x OMe) 2.05 (3 H, s, $-CH_3$) p.p.m.

6,8-Dimethoxy-3-methylisocoumarin

2,4-Dimethoxy-6-(2-oxopropyl) benzoic acid (500 mg) was dissolved in a solution of acetic anhydride and perchloric

acid in ethyl acetate (50 ml; made up by the method of Edwards and Rao[104]) and allowed to stand at room temperature for 10 minutes. The solution was then washed with water (3 x 50 ml) and sodium hydrogen carbonate solution (3 x 30 ml), dried and evaporated giving a brown solid. The product was purified by column chromatography eluting with ethyl acetate and recrystallised from ethanol (330 mg, 71%) m.p. 156-157 °C same data as above.

7-Chloro-6,8-dimethoxy-3-methylisocoumarin

Prepared by treatment of 3-chloro-2,4-dimethoxy-6-(2-oxopropyl)benzoic acid (500 mg) by the foregoing method. The product was recrystallised from dichloromethane/hexane as cubes. (300 mg, 64%) m.p. 182-190 °C (decomp.), (lit ., [80] 187-200 °C (decomp.) (Found: C, 56.56; H, 4.39; Cl, 13.90% $C_{12}H_{11}O_4Cl$ requires C, 56.59; H, 4.35; Cl, 13.92%); νmax. 1 715s, 1 670s, 1 590m, 1 556m cm^{-1}; $\delta(CDCl_3)$ 6.53 (1 H, s, Ar-H), 6.22 (1 H, s, 4-H), 3.98 (6 H, s, 2 x OMe), 2.21 (3 H, s, $-CH_3$) p.p.m.; m/z 254 256 (M^+).

7-Chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin

Palladium on charcoal (10%, 75 mg) was added to a solution of 7-chloro-6,8-dimethoxy-3-methylisocoumarin (250 mg) in ethyl acetate (20 ml) and the mixture hydrogenated at room temperature for 16 h. The catalyst was filtered through celite and the ethyl acetate evaporated to give the desired

product which was recrystallised from dichloromethane/hexane (225 mg, 90%), m.p. 155-156 °C (lit., [80] 154-157 °C); $\nu_{max.}$ 1 710s, 1 665s, 1 595m, 1 550m cm^{-1}; $\delta(CDCl_3)$ 6.59 (1 H, s, Ar-H), 4.55 (1 H, m, 3-H), 3.92 (6 H, s, 2 x OMe), 2.86 (1 H, br, s, 4-H), 2.76 (1 H, br, s, 4-H), 1.42 (3 H, d, J 7 Hz, $-CH_3$) p.p.m.; m/z 256 258 (M^+).

[1',1',3',3',3'-$^2H_5$]-2,4-Dimethoxy-6-(2'-oxopropyl)benzoic acid

2,4-Dimethoxy-6-(2-oxopropyl)benzoic acid (500 mg) was added to a solution of sodium (90 mg) in D_2O (15 ml) and the solution heated at reflux for 2 h. After cooling, the solution was acidified with dilute hydrochloric acid, then extracted with ethyl acetate (3 x 25 ml) dried and evaporated giving the desired deuterio compound which was recrystallised from acetone (490 mg, 95%), m.p. 139-140 °C; $\delta((CD_3)_2)CO)$ 6.46 (1 H, d, J 2 Hz, Ar-H, 6.37 (1 H, d, J 2 Hz, Ar-H), 3.85 (6 H, s, 2 x OMe) p.p.m.; m/z 243 (M^+) data identical with unlabelled material.

[1',1',3',3',3'-$^2H_5$]-3-Chloro-2,4-dimethoxy-6-(2'-oxopropyl)benzoic acid

Prepared by treatment of 3-chloro-2,4-dimethoxy-6-(2-oxopropyl)benzoic acid (500 mg) by the foregoing method (490 mg, 95%) data identical with unlabelled material.

$\delta((CD_3)_2CO)$ 6.60 (1 H, s, Ar-H) 4.05 (6 H, s, 2 x OMe) p.p.m.; m/z 277 279.

[4,α,α,α-$^2H_4$]-6,8-Dimethoxy-3-methylisocoumarin

[1',1',3',3',3-$^2H_5$]-3-Chloro-2,4-dimethoxy-6-(2'-oxopropyl)benzoic acid was cyclised to the isocoumarin by the same method described for the unsubstituted compound (300 mg, 66%) same data as unsubstituted compound. $\delta(CDCl_3)$ 6.40 (1 H, d, J 1 Hz, Ar-H), 6.27 (1 H, d, J 1 Hz, Ar-H), 6.05 (0.12 H, s, 4-H), 3.94 (3 H, s, OMe), 3.86 (3 H, s, OMe) p.p.m. m/z 224 (M^+).

[4,α,α,α-$^2H_4$]-7-Chloro-6,8-dimethoxy-3-methylisocoumarin

Prepared by cyclising [1',1',3',3',3'-$^2H_5$]-3-Chloro-2,4-dimethoxy-6-(2'-oxopropyl)benzoic acid to the isocoumarin by the foregoing method (300 mg, 66%) data identical with unsubstituted compound. $\delta(CDCl_3)$ 6.53 (1 H, s, Ar-H), 6.10 (0.26 H, s, 4-H), 3.97 (6 H, s, 2 x OMe); m/z 258 260 (M^+).

[4-$^2H_1$]-6,8-Dimethoxy-3-methylisocoumarin

A suspension of 4-carboxy-6,8-dimethoxy-3-methylisocoumarin (160 mg) in D_2O (10 ml) was stirred at room temperature for 15 minutes, then the D_2O was removed by evaporation. This compound was then heated at 140-150 °C for 15 minutes by which time carbon dioxide evolution had ceased. The residue

was purified by column chromatography eluting with ethyl acetate giving the desired [4-$^2H_1$]-6,8-dimethoxy-3-methylisocoumarin (110 mg, 82%) data identical with unsubstituted compound. $\delta(CDCl_3)$ 6.40 (1 H, d, J 1 Hz, Ar-H), 6.28 (1 H, d, J 1 Hz, Ar-H), 6.05 (0.2 H, s, 4-H), 3.94 (3 H, s, OMe), 3.88 (3 H, s, OMe), 1.99 (3 H, s, $-CH_3$) p.p.m.; m/z 221.

[4,4,α,α,α-$^2H_5$]-3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin

6,8-Dimethoxy-3-methylisocoumarin (200 mg) was added to a solution of sodium (40 mg) in D_2O (15 ml) and the solution heated under reflux with stirring for 1 h. Sodium borohydride (45 mg) was added portionwise and the mixture heated at reflux with stirring. After 30 minutes more sodium borohydride (45 mg) was added and reflux continued for a further 30 minutes. The solution was cooled and washed with ether (3 x 15 ml), acidified with dilute hydrochloric acid and then extracted with ethyl acetate (3 x 25 ml). This was then dried and evaporated leaving a crystalline compound which was recrystallised from ether/hexane (190 mg, 93%). Data identical with unsubstituted compound. $\delta(CDCl_3)$ 6.40 (1 H, d, J 1 Hz, Ar-H), 6.29 (1 H, d, J 1 Hz, Ar-H, 4.46 (1 H, br, s, 3-H), 3.87 (3 H, s, OMe), 3.84 (3 H, s, OMe) p.p.m.; m/z 227.

[4,α,α,α-$^2H_4$]-3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin

[4,α,α,α-$^2H_4$]-6,8-Dimethoxy-3-methylisocoumarin (300 mg) was hydrogenated by the same method used for the unsubstituted compound. The product was recrystallised from ether/hexane (260 mg, 86%). Data identical with unsubstituted compound. δ($CDCl_3$) 6.40 (1 H, d, J 1 Hz, Ar-H), 6.29 (1 H, d, J 1 Hz, Ar-H), 4.48 (1 H, br, s, 3-H), 3.88 (3 H, s, OMe), 3.85 (3 H, s, OMe), 2.76 (1.1 H, br, s, 4-H) p.p.m; m/z 226.

[4,α,α,α-$^2H_4$]-7-Chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin

[4,α,α,α-$^2H_4$]-7-Chloro-6,8-dimethoxy-3-methylisocoumarin (500 mg) was hydrogenated by the foregoing method. The product was recrystallised from ether/hexane (430 mg, 86%). Data identical with unsubstituted compound. δ($CDCl_3$) 6.65 (1 H, s, Ar-H), 4.77 (1 H, br, s, 3-H), 3.98 (6 H, s, 2 x OMe) 3.05 (1 H, s, 4-H) p.p.m; m/z 260 262.

[4-$^2H_1$]-3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin

[4-$^2H_1$]-6,8-Dimethoxy-3-methylisocoumarin (100 mg) was hydrogenated by the foregoing method. The product was recrystallised from ether/hexane. Data identical with unsubstituted compound. δ($CDCl_3$) 6.41 (1 H, d, J 1 Hz, Ar-H), 6.31 (1 H, d, J Hz, Ar-H), 4.50 (1 H, m, 3-H), 3.92

(3 H, s, OMe), 3.87 (3 H, s, OMe), 2.79 (1 H, br, s, 4-H), 1.95 (3 H, d, J 7 Hz, $-CH_3$) p.p.m; m/z 223 (M^+).

[3-$^2H_1$]-3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin

6,8-Dimethoxy-3-methylisocoumarin (250 mg) was heated under reflux in sodium hydroxide solution (10 %, 15 ml) for 1 h. Sodium borodeuteride (50 mg) was added with stirring and the mixture heated under reflux for 30 min; more sodium borodeuteride (50 mg) was then added and heating continued for a further 30 minutes. The hot solution was filtered and the filtrate acidified with dilute hydrochloric acid. The acidic solution was extracted with ethyl acetate (3 x 25 ml), dried and evaporated giving the required deuterio compound which was recrystallised from ether/hexane (170 mg, 68 %). Data identical with unsubstituted compound. δ ($CDCl_3$) 6.38 (1 H, d, J 1 Hz, Ar-H), 6.25 (1 H, d, J 1 Hz, Ar-H), 3.82 (6 H, s, 2 x OMe), 2.86 (2 H, br, s, $-CH_2$), 1.49 (3 H, s, $-CH_3$) p.p.m; m/z 223 (M^+).

[3,4, -$^2H_3$]-3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin

Deuterium gas, formed by the dropwise addition of D_2O onto lithium metal, was bubbled into a solution of 6,8-dimethoxy-3-methylisocoumarin (100 mg) in ethyl acetate (25 ml) with 10% palladium on charcoal (30 mg) under an atmosphere of nitrogen. The mixture was stirred at room temperature for 16 h and then filtered through celite. The organic solution

was evaporated giving the required deuterio compound, which was recrystallised from ether/hexane (92 mg, 91%). Data identical with unsubstituted compound. $\delta(CDCl_3)$ 6.40 (1 H, d, J 1 Hz, Ar-H), 6.28 (1 H, d, J 1 Hz, Ar-H), 4.51 (0.3 H, m, 3-H), 3.90 (3 H, s, OMe), 3.83 (3 H, s, OMe), 2.80 (1.3 H, m, 4-H), 1.41 (2 H, m, $-CH_2D$) p.p.m; m/z 224 (M^+).

[3,4,α-$^2H_3$]-7-Chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin

Prepared by the treatment of 7-chloro-6,8-dimethoxy-3-methylisocoumarin (200 mg) by the foregoing method. The product was recrystallised from dichloromethane/hexane (182 mg, 90%). Data identical with unsubstituted compound. $\delta(CDCl_3)$ 6.59 (1 H, s, Ar-H), 4.55 (0.35 H, m, 3-H), 3.94 (6 H, s, 2 x OMe), 3.10 (1.3 H, m, 4-H), 1.42 (2 H, m, $-CH_2D$) p.p.m; m/z 259 261 (M^+).

[4,4,α,α,α-$^2H_5$]-3,4-Dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

To a solution of [4,4,α,α,α-$^2H_5$]-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (200 mg) in freshly distilled nitrobenzene (16 ml) was added powdered aluminium chloride (600 mg). The solution was stirred at 50-60 °C for 6 h, then poured onto iced water and acidified with dilute hydrochloric acid. The acidic solution was extracted with ether (4 x 20 ml), and the combined organic solutions were extracted with sodium

hydroxide solution (5 %, 3 x 15 ml). The basic solution was washed with ether (3 x 10 ml) and then acidified. The acidic solution was extracted with ethyl acetate (4 x 20 ml), dried and evaporated leaving the title compound which was recrystallised from ether/hexane (152 mg, 80%) m.p. 95-96 °C. Data identical with unlabelled compound. $\delta(CDCl_3)$ 6.35 (1 H, d, J 1 Hz, Ar-H), 6.23 (1 H, d, J 1 Hz, Ar-H), 4.63 (1 H, br, s, 3-H), 3.81 (3 H, s, OMe) p.p.m; m/z 213 (M^+).

[4,α,α,α-$^2H_4$]-3,4-Dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

Prepared by the treatment of [4,α,α,α-$^2H_4$]-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (200 mg) by the foregoing method. The product was recrystallised from ether/hexane (150 mg, 80%). Data identical with unlabelled compound. $\delta(CDCl_3)$ 6.35 (1 H, d, J 1 Hz, Ar-H), 6.23 (1 H, d, J 1 Hz, Ar-H), 4.63 (1 H, br, 3-H), 3.80 (3 H, s, OMe), 2.80 (1.1 H, m, 4-H) p.p.m; m/z 212 (M^+).

[4-$^2H_1$]-3,4-Dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

Prepared by the treatment of [4-$^2H_1$]-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (100 mg) by the foregoing method. The product was recrystallised from ether/hexane (70 mg, 74%). Data identical with unlabelled compound. $\delta(CDCl_3)$ 6.38 (1 H, d, J 1 Hz, Ar-H), 6.25 (1 H, d, J 1 Hz,

Ar-H), 4.65 (1 H, br, 3-H), 3.81 (3 H, s, OMe), 2.85 (1 H, br, 4-H), 1.49 (3 H, d, J 7 Hz, $-CH_3$) p.p.m.; m/z 209 (M^+).

[3-$^2H_1$]-3,4-Dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

Prepared by the treatment of [3-$^2H_1$]-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (100 mg) by the foregoing method. The product was recrystallised from ether/hexane (70 mg, 74%). Data identical with unlabelled compound. $\delta(CDCl_3)$ 6.38 (1 H, d, J 1 Hz, Ar-H), 6.24 (1 H, d, J 1 Hz, Ar-H), 3.80 (3 H, s, OMe), 2.84 (2 H, m, 4-H), 1.49 (3 H, d, J 7 Hz, $-CH_3$) p.p.m.; m/z 209 (M^+).

[3,4,α-$^2H_3$]-3,4-Dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

Prepared by the treatment of [3,4,α-$^2H_3$]-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (200 mg) by the foregoing method. The product was recrystallised from ether/hexane (145 mg, 77%). Data identical with unlabelled compound. $\delta(CDCl_3)$ 6.35 (1 H, d, J 1 Hz, Ar-H), 6.25 (1 H, d, J 1 Hz, Ar-H), 4.66 (0.1 H, m, 3-H), 3.81 (3 H, s, OMe), 2.85 (1.1 H, m, 4-H), 1.49 (2 H, m, $-CH_2D$) p.p.m.; m/z 210 (M^+).

[4,α,α,α-$^2H_4$]-7-Chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin

To a solution of [4,α,α,α-$^2H_4$]-7-Chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (100 mg) in dichloromethane (25 ml) was added boron tribromide (0.5 ml, 1.0 M in dichloromethane) at -70 °C. The solution was allowed to warm to room temperature and then stirred for 16 h. Ether (5 ml) was added then water (5 ml) (cautiously!). The organic layer was dried and evaporated giving a crystalline compound which was recrystallised from ethyl acetate/hexane (55 mg, 62%) m.p. 193-195 °C. Data identical with unsubstituted compound.[80] δ((CD$_3$)$_2$CO) 6.39 (1 H, s, Ar-H), 4.74 (1 H, m, 3-H), 2.96 (1.2 H, m, 4-H) p.p.m.; m/z 232 234 (M^+).

[4,4,α,α,α-$^2H_5$]-3,4-Dihydro-6,8-dihydroxy-3-methylisocoumarin

Prepared by the treatment of [4,4,α,α,α-$^2H_5$]-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (200 mg) by the foregoing method. The product was recrystallised from acetone/hexane (165 mg, 80%). Data identical with unsubstituted compound. δ((CD$_3$)$_2$CO) 6.28 (2 H, s, Ar-H), 5.20 (2 H, br, 2 x OH), 4.66 (1 H, br, 3-H) p.p.m.

[3,4,x-$^2H_3$]-7-Chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin

Prepared by the treatment of [3,4, -$^2H_3$]-7-Chloro-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (100 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane (60 mg, 67%). Data identical with unsubstituted compound. $\delta((CD_3)_2CO)$ 6.39 (1 H, s, Ar-H), 4.73 (0.3 H, m, 3-H), 2.95 (1.3 H, m, 4-H), 1.44 (3 H, d, J 7 Hz, $-CH_3$) p.p.m.

[4,4,x,x,x-$^2H_5$]-5-Chloro-3,4-dihydro-6-hydroxy-8-methoxy-3-methylisocoumarin

To a solution of [4,4,x,x,x-$^2H_5$]-3,4-dihydro-6-hydroxy-8-methoxy-3-methylisocoumarin (100 mg) in anhydrous ether (40 ml) was added sulphuryl chloride (0.6 ml). The solution was kept in the dark for 1 h at room temperature, then the excess sulphuryl chloride was destroyed with water. The organic layer was dried and evaporated leaving a crystalline compound which was recrystallised from ethyl acetate/hexane (95 mg, 81%) m.p. 121-123 °C. Data identical with unsubstituted compound. $\delta(CDCl_3)$ 6.40 (1 H, s, Ar-H), 4.65 (1 H, m, 3-H), 3.92 (3 H, s, OMe) p.p.m; m/z 247 249 (M^+).

[4,α,α,α-$^2H_4$]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

Prepared by the treatment of [4,α,α,α-$^2H_4$]-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (50 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane (48 mg, 82%). Data identical with unsubstituted compound. δ($CDCl_3$) 6.42 (1 H, s, Ar-H), 4.64 (1 H, m, 3-H), 3.92 (3 H, s, OMe), 3.20 (1 H, m, 4-H) p.p.m.; m/z 246 248 (M^+).

[4-$^2H_1$]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin)

Prepared by the treatment of [4-$^2H_1$]-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (50 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane (45 mg, 78%). Data identical with unsubstituted compound. δ($CDCl_3$) 6.43 (1 H, s, Ar-H), 4.64 (1 H, m, 3-H), 3.91 (3 H, s, OMe), 3.20 (1 H, m, 4-H), 1.55 (3 H, d, J 7 Hz, $-CH_3$) p.p.m.

[3-$^2H_1$]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

Prepared by the treatment of [3-$^2H_1$]-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (50 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane

(45 mg, 78%). Data identical with unsubstituted compound. δ($CDCl_3$) 6.40 (1 H, s, Ar-H), 3.92 (3 H, s, OMe), 3.20 (1 H, d, J 3 Hz, H-4), 2.75 (1 H, d, J 3 Hz, H-4) 1.49 (3 H, s, $-CH_3$) p.p.m.

[3,4,α-$^2H_3$]-5-Chloro-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin

Prepared by the treatment of [3,4,α-$^2H_3$]-3,4-dihydro-8-hydroxy-6-methoxy-3-methylisocoumarin (50 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane (48 mg, 82%). Data identical with unsubstituted compound δ($CDCl_3$) 6.42 (1 H, s, Ar-H), 3.92 (3 H, s, OMe), 2.75 (1 H, m, H-4), 1.49 (2.1 H, m, CH_2D) p.p.m.

[4,α,α,α-$^2H_4$]-5,7-Dichloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin

Prepared by the treatment of [4,α,α,α-$^2H_4$]-7-Chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (150 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane (140 mg, 80%). Data identical with unsubstituted compound. δ($CDCl_3$), 4.64 (1 H, m, H-3), 3.91 (3 H, s, OMe), 3.20 (1 H, m, H-4) p.p.m.

[3,4,α-$^2H_3$]-5,7-Dichloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin

Prepared by the treatment of [3,4,α-$^2H_3$]-7-Chloro-3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (120 mg) by the foregoing method. The product was recrystallised from ethyl acetate/hexane (105 mg, 82%). Data identical with unsubstituted compound. 3.20 (1 H, m, H-4), 1.49 (3 H, s, $-CH_3$) p.p.m.

Methyl-3,5-dimethoxybenzoate

3,5-Dihydroxybenzoic acid (20 g) dissolved in dry acetone (500 ml) with anhydrous potassium carbonate (100 g) and dimethyl sulphate (40 ml) was heated at reflux for 8 h with stirring. After cooling, the solution was filtered and the residue washed with acetone (3 x 50 ml). The combined acetone solutions were evaporated and the brown residue was dissolved in ether (150 ml). The organic solution was washed with ammonia solution (10 %, 3 x 50 ml), water (2 x 50 ml), dried and evaporated. The residue crystallised on standing and was recrystallised from methanol (20.4 g; 78%), m.p. 40-41 ^oC (lit.,[167] 42 ^oC); $\delta(CDCl_3)$ 7.19 (2 H, m, J 1 Hz, 2 x Ar-H), 6.67 (1 H, m, J 1 Hz, Ar-H), 3.92 (3 H, s, OMe), 3.84 (6 H, s, 2 x OMe) p.p.m; m/z 196 (M^+).

3,5-Dimethoxybenzyl alcohol

Methyl-3,5-dimethoxybenzoate (10 g) was dissolved in dry THF (15 ml) and added dropwise to a stirred suspension of lithium aluminium hydride (2.4 g) in dry THF (250 ml) and heated at reflux for 5 h. After cooling, water (2.4 ml) was added cautiously followed by sodium hydroxide solution (15 %, 2.4 ml) and more water (7.2 ml) with stirring. The granular aluminium hydroxide was filtered through celite and washed with ether (150 ml). The organic solutions were evaporated to give a colourless oil which crystallised on standing and the product was recrystallised from diisopropylether as needles (7 g; 81%), m.p. 44-46 °C (lit.,[165] 47 °C); δ ($CDCl_3$) 6.52 (2 H, m, J 1 Hz, 2 x Ar-H), 6.39 (1 H, m, J 1 Hz, Ar-H), 4.62 (2 H, s, Ar-CH_2), 3.82 (6 H, s, 2 x OMe) p.p.m; m/z 168 (M^+).

2-Bromo-3,5-dimethoxybenzyl alcohol

N-Bromosuccinimide (5.27 g) was added portionwise over 10 minutes to a solution of 3,5-dimethoxybenzyl alcohol (5 g) in carbon tetrachloride (250 ml) and the flask warmed to 70 °C for 40 minutes. The reaction mixture was filtered whilst still warm and then diluted with ether (150 ml). The organics were then washed with water (3 x 50 ml) and saturated brine (2 x 50 ml) dried and evaporated to give a white crystalline compound which was recrystallised from acetone/hexane as fine needles (6.6 g, 90%) m.p. 96 °C

(lit.,[121] 96 °C); (Found: C, 43.58; H, 4.26; Br, 32.73%, M^+ 245.9888, 247.9879. $C_9H_{11}O_3Br$ requires C, 43.75; H, 4.49; Br, 32.34%, M 245.9888, 247.9868); δ ($CDCl_3$) 6.73 (1 H, d, J 2 Hz, Ar-H), 6.47 (1 H, d, J 2 Hz, Ar-H), 4.75 (2 H, s, Ar-CH_2), 3.90 (3 H, s, OMe), 3.84 (3 H, s, OMe) p.p.m.

2-Bromo-3,5-dimethoxybenzylmethyl ether

A solution of 2-bromo-3,5-dimethoxybenzyl alcohol (3.5 g) in dry THF (25 ml) was added dropwise to a stirred suspension of sodium hydride (1.02 g) and methyl iodide (1.77 ml) in dry THF (30 ml). The mixture was stirred for 1 h at room temperature after which time water (25 ml) was added cautiously to destroy the excess sodium hydride. The organics were evaporated and the resulting aqueous solution was extracted with ether (3 x 25 ml). The extracts were washed with water, brine, dried and evaporated leaving an oil which crystallised on standing. The crude product was recrystallised from ether as cubes (2.9 g, 78%) m.p. 57-58 °C; (Found: C, 45.93; H, 4.80; Br, 30.42%, M^+ 260.0037, 262.0025. $C_{10}H_{13}O_3Br$ requires C, 45.99; H, 5.01; Br, 30.60%, M 260.0044, 262.0024); δ($CDCl_3$) 6.61 (1 H, d, J 1 Hz, Ar-H), 6.44 (1 H, d, J 1 Hz, Ar-H), 4.53 (2 H, s, Ar-CH_2), 3.88 (3 H, s, OMe), 3.83 (3 H, s, OMe) 3.49 (3 H, s, OMe) p.p.m.

2-Bromo-3,5-dimethoxybenzylbenzyl ether

A solution of 2-bromo-3,5-dimethoxybenzyl alcohol (5 g) in dry THF (25 ml) was added dropwise to a suspension of benzyl chloride (3.5 ml) and sodium hydride (1.46 g) in dry THF (80 ml) and the mixture stirred at reflux for 16 h. After cooling, water (30 ml) was added cautiously and the organics evaporated. The resulting aqueous solution was extracted with ethyl acetate (3 x 30 ml), washed with water, brine, dried and evaporated. The crude product was distilled at high vacuum to remove any excess benzyl chloride and the resulting residue was recrystallised from ether (4.7 g; 69%) m.p. 49-50 ^{o}C (Found: C, 57.18; H, 5.33; Br, 23.31%, M^{+} 336.0365, 338.0347. $C_{16}H_{17}O_3Br$ requires C, 56.98; H, 5.08; Br, 23.70%, M 336.0356, 338.0336); ν max. 1 600m, 1 585s, 1 575s; δ ($CDCl_3$) 7.37 (5 H, m, Ar-H), 6.77 (1 H, d, J 2 Hz, Ar-H), 6.42 (1 H, d, J 2 Hz, Ar-H), 4.65 (4 H, s, Ar-CH_2), 3.87 (3 H, s, OMe), 3.81 (3 H, s, OMe) p.p.m.

2-Carboxy-3,5-dimethoxybenzylmethyl ether

n-Butyllithium (1 ml; 1.5M in hexane) was added to a stirred solution of 2-bromo-3,5-dimethoxybenzylmethyl ether (300 mg) in THF (20 ml) at -78 ^{o}C under nitrogen. After stirring for 20 minutes at this termperature the solution was poured onto an excess of dry ice. The solution was allowed to warm to room temperature and water (20 ml) was added. The organics were evaporated and the resulting aqueous solution was washed

with ether (3 x 15 ml) and then acidified with dilute hydrochloric acid. The acidic solution was extracted with ethyl acetate (3 x 20 ml) dried and evaporated to give a white compound which was recrystallised from ether (228 mg, 87%) m.p. 98-100 °C (Found: C, 58.41: H, 6.41%, M^+ 226.0844 $C_{11}H_{14}O_5$ requires C, 58.38; H, 6.24%, M 226.0837); $\nu_{max.}$ 3 300br, 1 680s, 1 600s; $\delta(CDCl_3)$ 6.95 (1 H, d, J 1 Hz, Ar H), 6.47 (1 H, d, J 1 Hz, Ar-H), 4.83 (2 H, s, Ar-CH_2), 3.99 (3 H, s, OMe), 3.89 (3 H, s, OMe), 3.49 (3 H, s, OMe) p.p.m.

2-Carboxy-3,5-dimethoxybenzylbenzyl ether

Prepared by treatment of 2-bromo-3,5-dimethoxybenzylbenzyl ether (500 mg) by the foregoing method. The product was recrystallised from ether as cubes (375 mg, 83%) m.p. 77-78 °C (Found: C, 67.56; H, 5.98% M^+ 302.1160, $C_{17}H_{18}O_5$ requires C, 67.54; H, 6.00% M 302.1149); $\delta(CDCl_3)$ 7.36 (5 H, m, Ar-H), 6.98 (1 H, d, J 1 Hz, Ar-H), 6.46 (1 H, d, J 1 Hz, Ar-H) 4.91 (2 H, s, Ar-CH_2), 4.64 (2 H, s, Ar-CH_2), 3.95 (3 H, s, OMe), 3.86 (3 H, s, OMe) p.p.m.

α,3,5-Trimethoxybenzoic acid

n-Butyllithium (4.3 ml; 1.5M in hexane) was added to a solution of diisopropylamine (1 ml) in dry THF (10 ml) under nitrogen at 0 °C with stirring. After 10 minutes the solution was cooled to -78 °C and a solution of 2-carboxy-

3,5-dimethoxybenzyl methyl ether (400 mg) and dimethyl carbonate (0.45 ml) in dry THF (15 ml) was added dropwise during 15 minutes. The cooling bath was removed and the solution allowed to warm to room temperature. After 4 h water (20 ml) was added and the suspension stirred at room temperature for 16 h. The organics were evaporated and the resulting aqueous solution was washed with ether (3 x 30 ml). After acidification with dilute hydrochloric acid, the product was extracted with ethyl acetate (3 x 20 ml), dried and evaporated giving a crystalline compound, which was recrystallised from chloroform (350 mg, 73%) m.p. 158 °C (Found: C, 53.42; H, 5.37%, M^+ 270.0720, $C_{12}H_{14}O_7$ requires C, 53.33; H, 5.22%, M 270.0735); ν max. 3 000-2 700br, 1 680s, 1 600s, 1 585m, cm^{-1}; $\delta(CD_3)_2CO$ 6.72 (1 H, d, J 1 Hz, Ar-H), 6.65 (1 H, d, J 1 Hz, Ar-H), 5.18 (1 H, s, -CH), 3.90 (3 H, s, OMe), 3.86 (3 H, s, OMe), 3.39 (3 H, s, OMe) p.p.m.

4,6,8-Trimethoxy-3-methylisocoumarin

α,3,5-Trimethoxyhomophthalic acid (150 mg) was dissolved in a solution of acetic anhydride (20 ml) and dry pyridine (1 ml) and the mixture was heated at reflux for 2 h. After cooling, the mixture was poured onto a suspension of ethyl acetate (30 ml) and ice/water (80 ml) and stirred at room temperature for 1 h. The aqueous solution was extracted with ethyl acetate (3 x 25 ml). The organic extracts were washed with sodium bicarbonate solution, brine, dried and

evaporated giving a crystalline compound which was recrystallised from ethyl acetate/hexane (95 mg, 68%) m.p. 122-123 °C (Found: C, 62.43; H, 5.59%, M^+ 250.0845, $C_{13}H_{14}O_5$ requires C, 62.38; H, 5.64%, M 250.0837); ν max. 1 735s, 1 720s, 1 670m, 1 600s, 1 570s cm^{-1}; $\delta(CDCl_3)$ 6.60 (1 H, d, J 1 Hz, Ar-H), 6.45 (1 H, d, J 1 Hz, Ar-H), 3.95 (3 H, s, OMe), 3.92 (3 H, s, OMe), 3.74 (3 H, s, OMe) p.p.m.

3S*4S*3,4-Dihydro-4,6,8-trimethoxy-3-methylisocoumarin

Palladium on carbon (10%, 20 mg) was added to a solution of 4,6,8-trimethoxy-3-methylisocourmarin (100 mg) in ethyl acetate (20 ml) and the mixture hydrogenated for 24 h. The catalyst was filtered through celite and washed with ethyl acetate. The combined organics were evaporated giving the crude product which was recrystallised from ethyl acetate/hexane (80 mg, 80%); ν max. 1 735s, 1 715s, 1 670m, 1 600s, 1 570s cm^{-1}; $\delta(CDCl_3)$ 6.50 (2 H, m, Ar-H), 4.53 (2 H, m, H-3 and H-4), 3.92 (3 H, s, OMe), 3.90 (3 H, s, OMe), 3.34 (3 H, s, 4-OMe), 1.43 (3 H, d, J 7 Hz, Me) p.p.m. m/z 252.

3R*4S*3,4-Dihydro-4,6,8-trimethoxy-3-methylisocoumarin

A solution of 4,6,8-trimethoxy-3-methylisocoumarin (200 mg) in sodium hydroxide solution (20 ml, 15%) was heated at reflux with stirring for 1 h. Sodium borohydride (30 mg) was added portionwise and the solution stirred at reflux for

30 mins. A further portion of sodium borohydride (30 mg) was added and the solution stirred at reflux for a further 30 mins. The solution was filtered hot and allowed to cool. The aqueous solution was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The organics were dried and evaporated giving the crude product which was purified as a mixture (trans: cis 2:1) using preparative t.l.c. eluting with ether. R_F 0.4 trans $\delta(CDCl_3)$ 6.50 (2 H, m, Ar-H), 4.10 (2 H, m, H-3 and H-4) 3.92 (6 H, s, 2 x OMe), 3.53 (3 H, s, 4-OMe), 1.38 (3 H, d J 7 Hz Me) p.p.m.

3S*4S*5-Chloro-3,4-dihydro-4,6,8-trimethoxy-3-methylisocoumarin

Sulphuryl chloride (0.3 ml) was added to a solution of 3S*4S*3,4-dihydro-4,6,8-trimethoxy-3-methylisocoumarin (40 mg and the solution was left standing in darkness for 1 h. Water (30 ml) was added and the organic layer separated. The organic layer was washed with a further portion of water, brine, dried and evaporated giving the crude product which was purified by preparative t.l.c. as an oil eluting with chloroform. $\delta(CDCl_3)$ 6.70 (2 H, m, Ar-H), 4.44 (2 H, m, H-3 and H-4), 4.00 (3 H, s, Ar-OMe), 3.98 (3 H, s, Ar-OMe), 3.40 (3 H, s, 4-OMe), 1.53 (3 H, d, J 8 Hz, Me) p.p.m; m/z 274 276.

3R*4S*5-Chloro-3,4-dihydro-4,6,8-trimethoxy-3-methylisocoumarin

Prepared by the treatment of 3R*4S*3,4-dihydro-4,6,8-trimethoxy-3-methylisocoumarin by the foregoing method. The product was obtained as an oil. $\delta(CDCl_3)$ 6.70 (2 H, m, Ar-H), 4.64 (2 H, m, H-3 and H-4), 4.01 (3 H, s, OMe), 3.97 (3 H, s, OMe), 3.41 (3 H, s, OMe), 1.16 (3 H, d, J 8Hz, Me) p.p.m; m/z 274 276.

2-Bromo-3,5-dimethoxybenzyltert-butyldimethylsilyl ether

tert-Butyldimethylsilyl chloride (785 mg) was added to a stirred solution of 2-bromo-3,5-dimethoxybenzyl alcohol (1 g) and imidazole (710 mg) in dry DMF (2 ml) at room temperature under nitrogen. The flask was stirred for 2 h at room temperature then water (50 ml) was added. The aqueous solution was extracted with ether (3 x 30 ml) washed with brine (2 x 15 ml) dried and evaporated. The product was purified by column chromatography eluting with hexane yielding an oil. (1.2 g, 61%); νmax. 1 600m, 1 585s; $\delta(CDCl_3)$ 6.75 (1 H, d, J 2 Hz, Ar-H), 6.40 (1 H, d, J 2 Hz, Ar-H) 4.65 (2 H, s, Ar-CH_2), 3.85 (3 H, s, oMe) 3.81 (3 H, s, oMe) 1.52 (9 H, s, $(CH_3)_3Si$) 0.21 (3 H, s, CH_3-Si) 0.19 (3 H, s, CH_3-Si) p.p.m.; m/z 360 362.

5,7-Dimethoxyphthalide

n-Butyllithium (1.2 ml; 1.5 M in hexane) was added to a stirred solution of 2-bromo-3,5-dimethoxybenzyltert-butyldimethylsilyl ether (400 mg) in dry THF (20 ml) at -78 °C under nitrogen. After stirring for 20 minutes at this temperature the solution was poured onto an excess of dry ice. The solution was allowed to warm to room temperature and water (20 ml) was added. The aqueous solution was extracted with ethyl acetate. The organics were dried and evaporated giving the crude product which after chromatography rearranged to 5,7-dimethoxyphthalide, m.p. 148-150 °C (lit., [120] 146-148 °C); ν max. 1 750m, 1 600s; δ($CDCl_3$) 6.52 (1 H, m, Ar-H), 6.49 (1 H, m, Ar-H), 5.17 (2 H, s, Ar-CH_2), 3.97 (3 H, s, OMe), 3.91 (3 H, s, OMe) p.p.m.; m/z 294.

[2'-^{4}C]2,4-dimethoxy-6-(2-oxopropyl)benzoic acid

Radioactive acetic anhydride (4.8 ul) was added to a stirred solution of 3,5-dimethoxyhomophthalic acid (170 mg) in dry THF (10 ml) at room temperature. After 5 mins acetic anhydride (0.4 ml) and pyridine (0.2 ml) were added and the flask was stirred at room temperature for 2 h. The solution was added to aqueous sodium hydroxide solution (1M: 100 ml) and the flask heated at reflux for 2 h. After cooling, the basic solution was washed with ehter (3 x 25 ml) and then acidified with dilute hydrochloric acid. The acidic

solution was extracted with ethyl acetate, dried and evaporated to give the desired compound which was recrystallised from acetone (120 mg, 71%). The reduction and demethylation was carried out as for the unlabelled isocoumarin.

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