SOME METABOLITES

OF

ASPERGILLUS FLASCHENTRAEGERI

A THESIS PRESENTED BY

ROSS DONALD LYON REID

TO THE UNIVERSITY OF GLASGOW

FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

THE CHEMISTRY DEPARTMENT

NOVEMBER, 1975

Summary

A new member of the group of compounds known as the cochlioquinones has been isolated from extracts of the culture filtrates of *Verticillium lamellicola*. It is probable that this new pigment is the dehydro analogue of one of the earlier members of the group, cochlioquinone B.

The biosynthesis of the pimarane-type diterpene antibiotic traegeric acid, isolated from extracts of the culture filtrates of *Aspergillus flaschentraegeri*, has been shown by ^{13}C-n.m.r. spectroscopy to follow the expected path, via mevalonic acid.

The yellow pigment physcion anthrone has been reisolated from mycelial extracts of the same fungus by a novel method which has resulted in greatly increased yields of the metabolite. The biosynthesis of this polyketide-derived pigment has been studied by ^{13}C-n.m.r. spectroscopy. An interesting conformational effect has been observed in the C-10 acetyl derivative of physcion anthrone triacetate. The 10,10' oxidatively coupled dimer of physcion anthrone has been isolated.

A novel metabolite, asperflaschin, has been isolated and its biosynthesis studied. The isolation of this compound has necessitated revision of earlier ideas on the biosynthesis of 2-(γ,γ-dimethylallyl)-physcion, which has also been reisolated. In this context, the isolation of a novel metabolite, physcion-2-(γ,γ-dimethylallyl)-emodin-10,10'-bianthrone may also be of relevance.

I should like to express my gratitude to my supervisor, Dr. N.J. McCorkindale, for his constant encouragement and advice during the course of this work and for his guidance in the presentation of this thesis.

I should also like to thank the Science Research Council for maintenance during the last three years and Professor G.W. Kirby for providing the opportunity and facilities for research in this department.

I am also indebted to Mrs. M. Tate and her staff of the Mycology department for technical assistance in the preparation and separation of fungal extracts. Thanks are also due to Mrs. W. Harkness and her staff (microanalyses), to Mrs. F. Lawrie and her staff (infra-red spectra), Mr. A. Ritchie (mass spectra) and to Mr. J. Gall and Dr. D.S. Rycroft (n.m.r. spectra). I am grateful to my colleagues for their helpful discussions and advice.

Finally, I wish to thank Mrs. M. Reid, my typist, for her patient assistance in the preparation of this thesis.

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Contents

The Fungi

Fungi, together with algae and bacteria, are usually regarded as members of a sub-section of the plant kingdom, the Thallophyta. Those organisms which belong to this sub-section have no true roots, stems or leaves and, as they lack the chlorophyll necessary for the promotion of photosynthesis, require a carbon source other than carbon dioxide.

The characteristic vegetative body of a fungus is the mycelium (an interwoven system of branching tubes called hyphae, the walls of which enclose multinucleate protoplasm). These hyphae are sometimes divided into sections by transverse walls and, when this is the case, the mycelium is said to be septate. When no such sectioning of the constituent hyphae is present, the mycelium is described as aseptate. The mycelium can be involved in taking up nutrients from the substrate by absorption over the whole of its surface (vegetative mycelium) or, alternatively, the mycelium may serve in the more specialised process of spore-formation (aerial mycelium). Spore formation may be described as sexual (meiotic spore formation from two different hyphae) or asexual (mitotic spore formation from a single hypha) and it is on the basis of both whether or not the mycelium is septate and on the type of spore formation (sexual or asexual), that fungi are classified.

The Phycomycetes are the most primitive of fungi, and this class is distinguished from others in having an aseptate mycelium (when present). They are simple organisms, often unicellular, and often parasitic (especially on plants), eg. Phytophthora.

A second class, the Ascomycetes, have a septate mycelium. They may bear their spores, which are sexual, in a sac-like vessel

(ascus), as in truffles. Many Ascomycetes are parasites of plants eg. Claviceps purpurea.

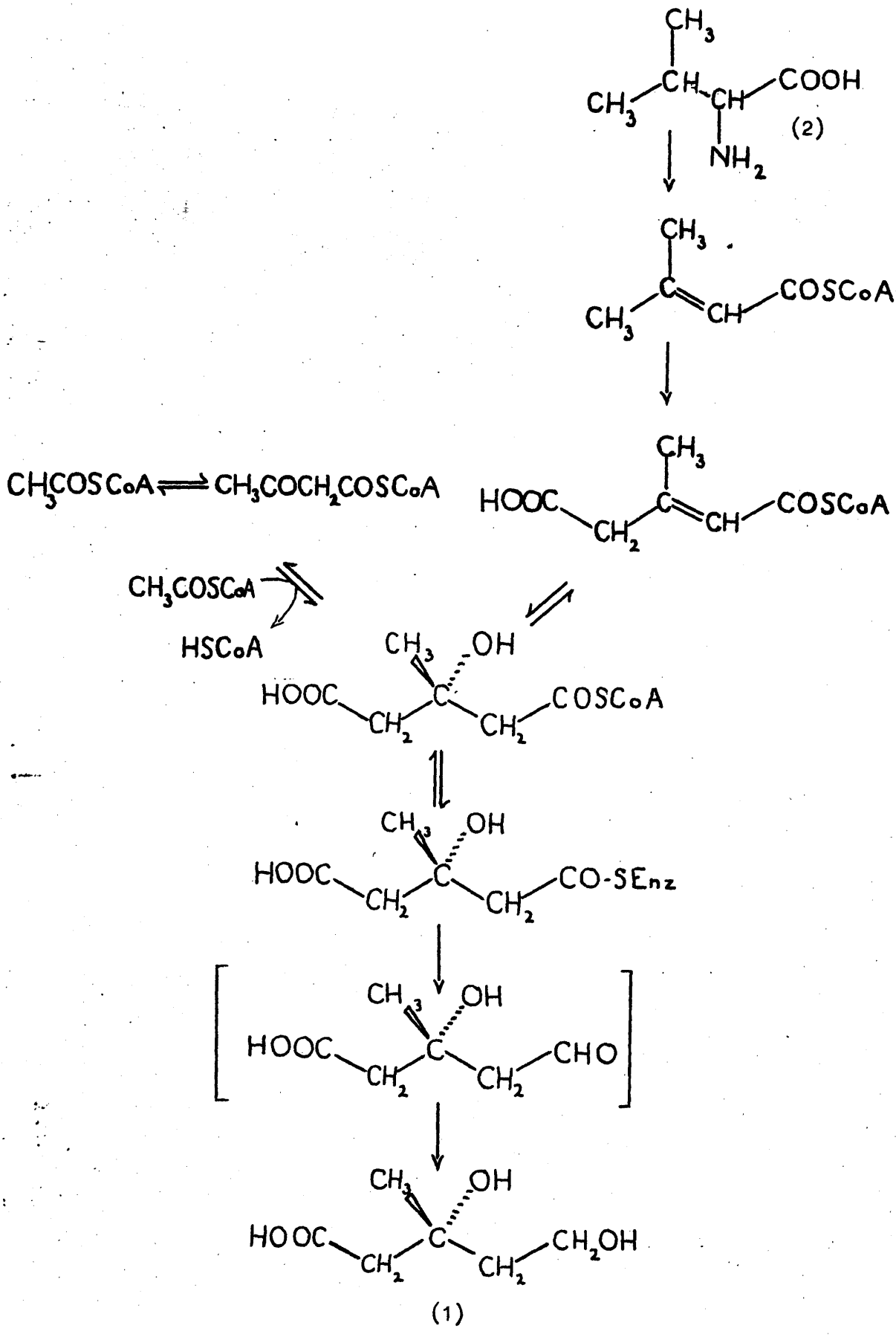
Another class with septate mycelium and sexual spores is the Basidiomycetes, but here the spores are borne on basidia which are often associated in large numbers into organised fruiting bodies, as in the mushrooms.

The Fungi Imperfecti is a fourth, rather artificial class of fungi. The mycelium is septate but asexual spores, only, are formed. This class includes the genera Penicillia and Aspergilli, which are often responsible for the grey-green growth sometimes observed on food. It is now recognised that many fungi imperfecti are actually conidial stages of perfect fungi.

The processes of primary metabolism, necessary for energy production and general maintenance of cell function, are present in fungi as in every other living organism. However, in common with higher plants and bacteria, fungi produce secondary metabolites, compounds which have no obvious role in the maintenance of life in the organisms. Many of these secondary metabolites are species specific but the elucidation of their biosynthesis and the recognition that these metabolites can all be derived by a few basic pathways constitute significant achievements in this field of research in modern organic chemistry.

INTRODUCTION PART A

Figure A.1.


CH3
CH
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COOH
CH3
NH2
(2)
CH3
C
CH
COSCoA
CH3
CH3COSCoA ⇌ CH3COCH2COSCoA
HOOC
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HSCoA
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COSCoA
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CH3
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HOOC
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CH2
CH2OH
(1)

Tricyclic Fungal Diterpenes and Their Biosynthesis[2]

1) Biosynthesis of acyclic precursors

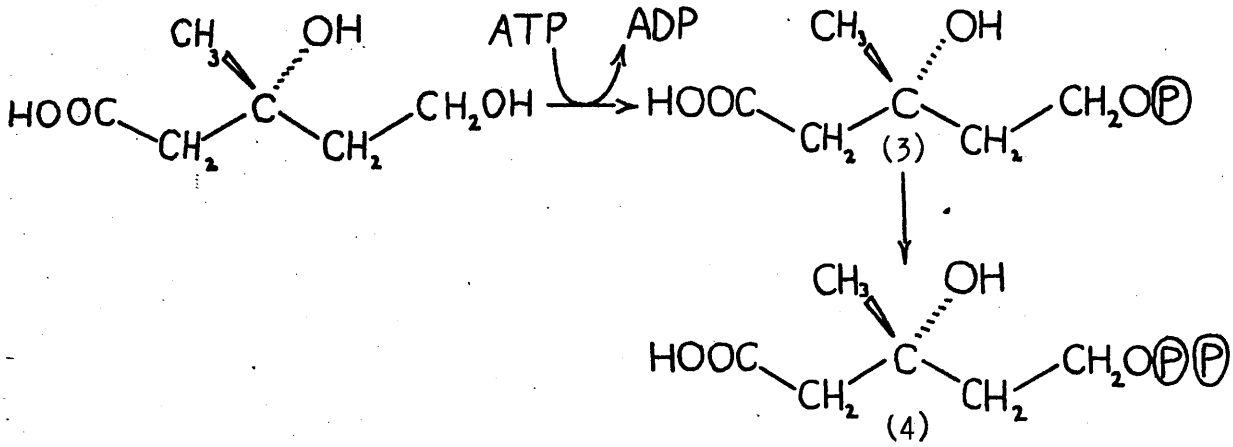
A certain regularity of structural features in terpenoid substances was first noted by Wallach[3] and it was suggested that terpenes were derived from polymerisation of a C_5 (isoprene) unit.

Ruzicka's 'Biogenetic Isoprene Rule'[4] was a modification of these earlier proposals as it was recognised that many terpenes did not fit into the previous pattern and did not have structures derived logically from simple polymerisation of such C_5 units. An 'active isoprene unit' was invoked and terpene structures could derived from condensation of such units either directly, with head-to-tail linkages, or by way of stereospecific dimerisations, cyclisations or rearrangements of combinations of isoprene units.

While the derivation of cholesterol from acetate had been demonstrated by Bloch[5], the identity of the isoprenoid precursor proved elusive until mevalonic acid (MVA, 1), a compound isolated from brewers' solubles, was found to be a better precursor of cholesterol than acetate[6]. Stages in the biosynthesis of MVA are outlined in Figure A.1. The route from leucine (2) is utilised in certain plants and micro-organisms and there is evidence[7] of another route involving malonyl-coenzyme A but this, like the route from leucine, is a minor pathway only, the main pathway being from condensation of acetyl-coenzyme A with acetoacetyl-coenzyme A. The absolute configuration shown for MVA is that of the natural isomer (R) -MVA. The sole known biological role of (R)-MVA is in steroid and terpenoid synthesis; the (S)-isomer is inactive[8].

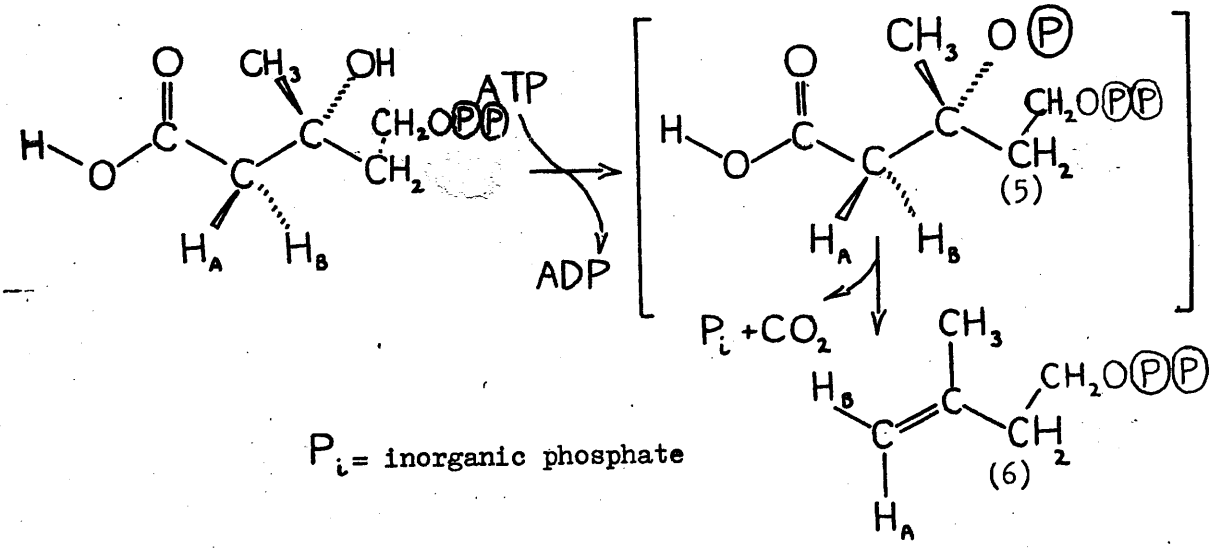
The first two processes leading from MVA to terpenoids are both phosphorylations, which successively produce mevalonic acid 5-phosphate (3) and mevalonic acid 5-pyrophosphate (4). The terminal phosphoryl group in ATP (adenosine triphosphate) is in each case the phosphate source (figure A.2.). The two enzymes responsible have

Figure A.2.



Ⓟ Ⓟ = pyrophosphate

Figure A.3.



P_i = inorganic phosphate

Figure A.4.

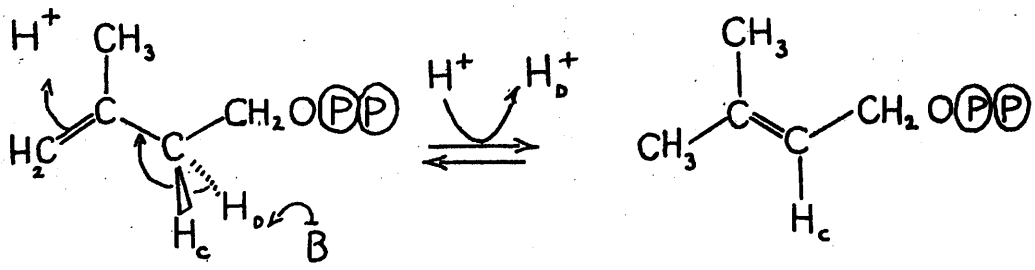


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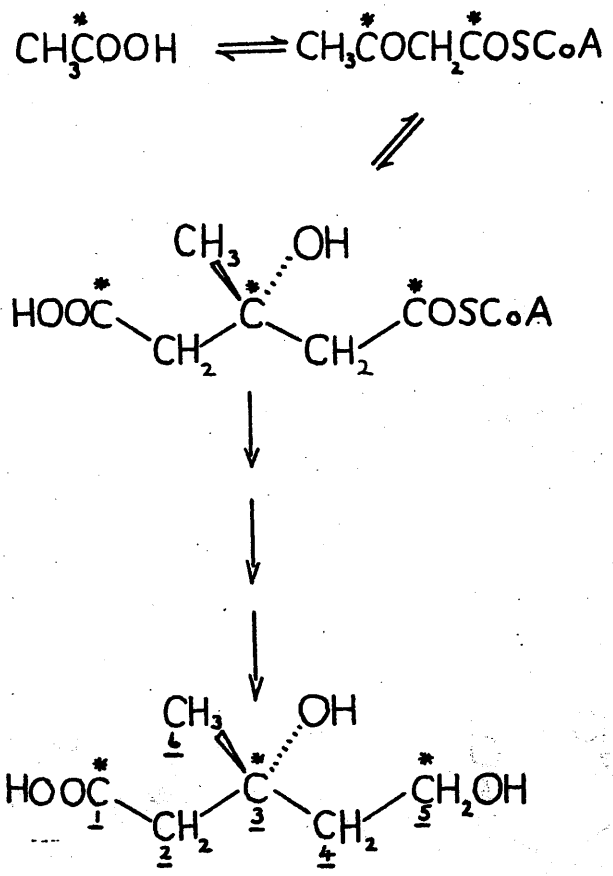
Figure A.5.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CH3COCH2COSC·A
CH3
OH
HOOC
CH2
C
CH2
COSC·A
OH
HOOC
CH2
CH2
CH2OH

been purified from yeast[9,10] and from liver[11,12], especially that which produces the monophosphate. Only (R)-MVA is utilised by this latter enzyme[9,13]. Mevalonic acid 5-pyrophosphate reacts on the enzyme with ATP to give ADP (adenosine diphosphate), carbon dioxide and isopentenyl pyrophosphate (ipPP,6) Experiments with [3 -^{18}O]-MVA have shown that the oxygen atom from the tertiary hydroxyl group is found in inorganic phosphate after the reaction[14]. This suggests that phosphorylation of this hydroxyl group by ATP occurs before elimination, but the inferred intermediate (5) has not been detected. This process has been shown[15] to involve <u>trans</u> - elimination (Figure A.3).

Catalysed by an enzyme (a 'prenyl isomerase') which has been obtained in a partially purified state from yeast and from pig liver[16,17,18], and also from a cell - free preparation from <u>Pinus radiata</u>[19], isopentenyl pyrophosphate is then converted by a prototropic shift into dimethylallyl pyrophosphate (7) It has been shown[19,19a] that this elimination is a stereospecific process with the pro-4S hydrogen (H_d) of isopentenyl pyrophosphate being eliminated. The addition of a proton to the methylene group has been shown to be a stereospecific process[19,19b] and the relationship between the stereochemistry of the two processes was that which would be expected of a concerted reaction in which the new double bond is formed as the old one is saturated (Figure A.4.). Chemically, a substance with a relatively unreactive phosphoryl group and a nucleophilic double bond is converted into a highly reactive electrophilic allyl pyrophosphate.

The addition of [1-^{13}C]- sodium acetate to a system capable of the biosynthesis of (R)-MVA would result in mevalonic acid with the labelling pattern shown in Figure A.5. If [1,2-^{13}C]-sodium acetate were added instead then, in the ^{13}C-n.m.r. spectrum of the resulting mevalonic acid, ^{13}C-^{13}C coupling would be observed between the carbon atom pairs C-1 and C-2, C-3 and C-6 and C-4 and C-5. In the proton-

Figure A.6.

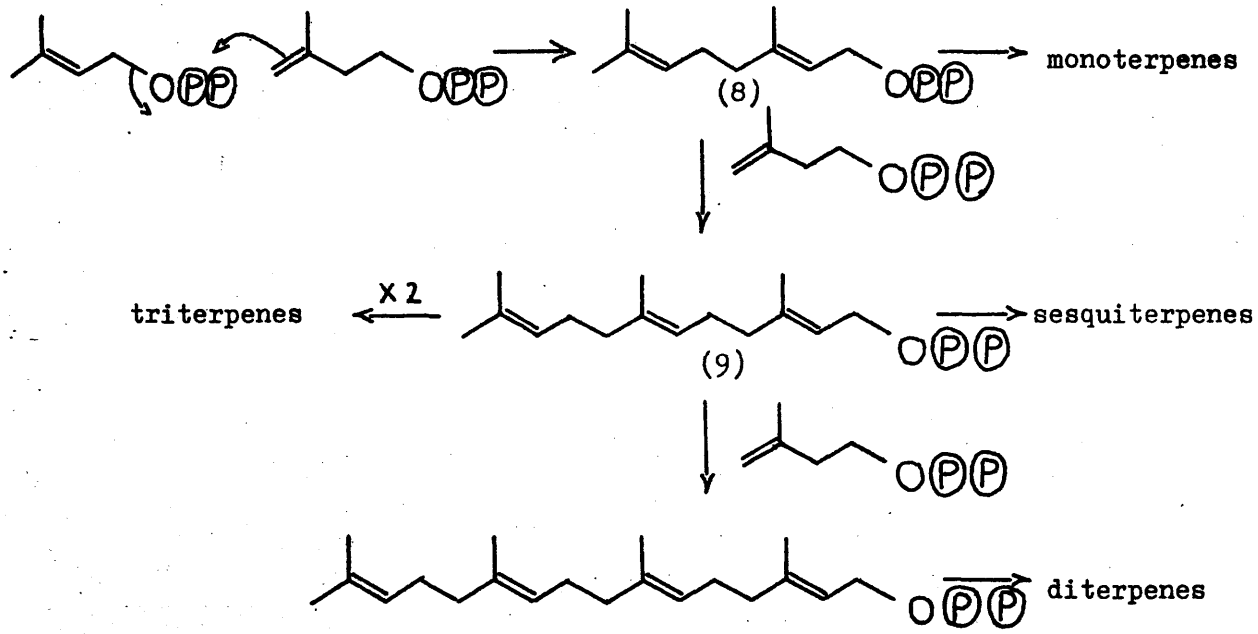
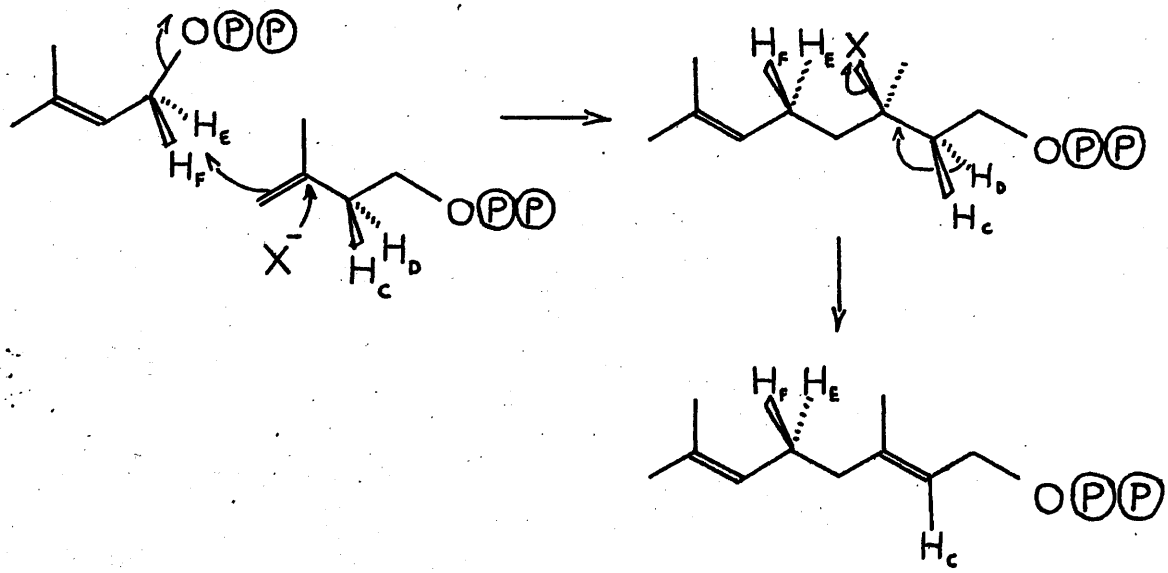


Figure A.7.

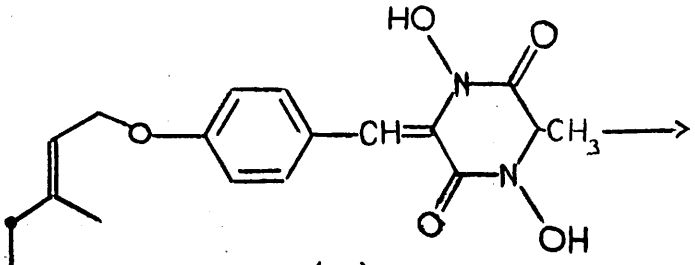


noise-decoupled ^{13}C-n.m.r. spectrum of the labelled compound, this coupling would be manifested by the signals appearing as triplets, the centre peaks representing the non-coupled natural abundance peaks while the satellite peaks are due to $^{13}C-^{13}C$ spin-spin splitting. This $^{13}C-^{13}C$ coupling is observed with the labelled compound along the C-C bonds which formed the precursor acetate molecule. Each pair of coupled carbon atoms thus represents the incorporation of a molecule of acetate into (R)-MVA. The coupling is not observed through the C-C bonds formed by condensation of acetate units as, statistically, within any one molecule of (R)-MVA, labelled acetate units will not occur in adjacent positions.

In the formation of isopentenyl pyrophosphate from (R)-MVA, the C-1 carbon atom of (R)-MVA is lost as carbon dioxide (Figure A.3). Thus C-2 has lost what was its neighbour in the original acetate unit and so no $^{13}C-^{13}C$ coupling is observed for C-2. The signal for this carbon atom then appears as a singlet in the proton-noise-decoupled ^{13}C-n.m.r. spectrum of (6)

Dimethylallyl pyrophosphate and isopentenyl pyrophosphate are joint substrates for an enzyme (a 'prenyl transferase') catalysing their condensation to yield geranyl pyrophosphate (8)(Figure A.6.). The transferase from liver has been partially purified [20,21,22] and no indication has been found that a different enzyme catalyses the further addition of isopentenyl pyrophosphate to give farnesyl pyrophosphate (9). However, this enzyme is incapable of using farnesyl pyrophosphate as a substrate for the further addition of C_5 units. In contrast, the transferase from pumpkin fruit has been isolated[23] and it has been shown that this enzyme catalyses the condensation of isopentenyl pyrophosphate with dimethylallyl pyrophosphate, geranyl pyrophosphate and with farnesyl pyrophosphate. Other workers have shown that the transferase catalysing the condensation of dimethylallyl pyrophosphate and isopentenyl pyrophosphate can use as substrates, on the one hand, dimethylallyl pyrophosphate or at

Figure A.8.



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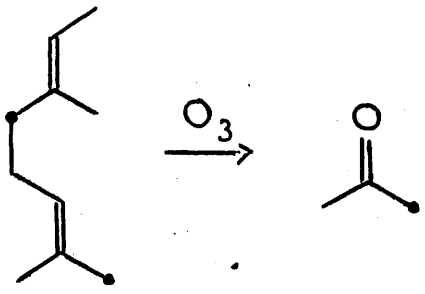
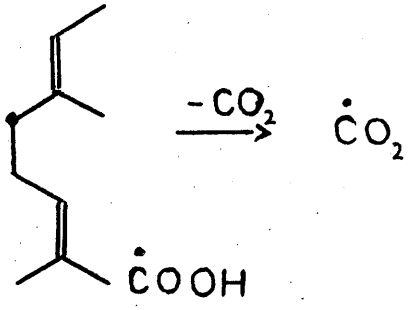
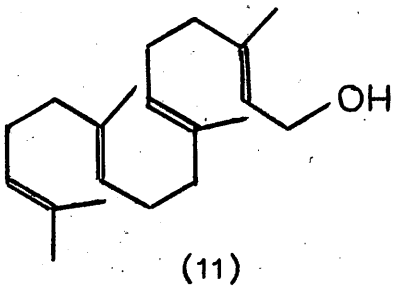


RABBIT





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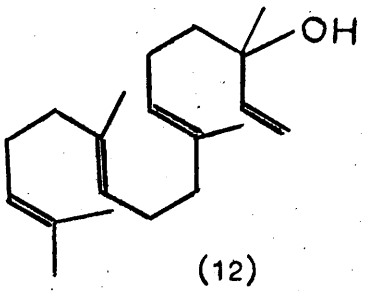

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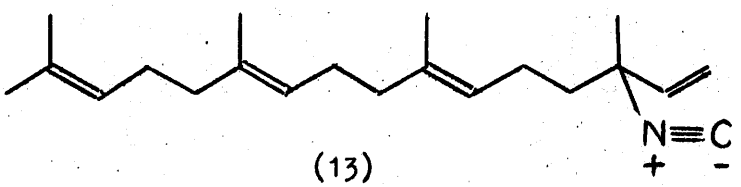

(13)

least a closely related homologue which possesses a tri-substituted double bond[24] and, on the other hand, isopentenyl pyrophosphate (3-methyl -3-butenyl pyrophosphate) or its 3-ethyl homologue, but no higher homologue.[25]

The stereochemistry of the coupling of the C_5 units has been extensively studied using substrates asymmetrically labelled with hydrogen isotopes[26] and it has been established that the formation of the new carbon to carbon bond is accompanied by complete inversion of configuration at the allylic carbon atom. Also, it has been suggested, from the fixed stereochemical relation between addition of the allylic C_5 unit and elimination of the hydrogen ion (added and lost, respectively, from the same side of the isopentenyl pyrophosphate molecule), that the enzymic process proceeds in two distinct steps[26]: (i) trans -addition of the allylic unit and of a nucleophilic group X to the double bond of isopentenyl pyrophosphate and (ii) trans -elimination of X and the proton (H_d) (figure A.7.). As yet, X is unspecified: it may be a group such as a water molecule bonded to an enzyme, or perhaps an oxygen atom of the pyrophosphate leaving group[27].

As a result of the stereospecificity of the prototropic shift in going from isopentenyl pyrophosphate to dimethylallyl pyrophosphate, C-2 of MVA becomes the trans methyl of dimethylallyl pyrophosphate and the trans methyl of the isopropylidene group of geranyl pyrophosphate. Thus, in the ^{13}C-n.m.r. spectrum of isopentenyl pyrophosphate, the signal for the carbon of the trans methyl, derived from C-2 of MVA when this has been biosynthesised from $[1,2-^{13}C]$-acetate, will appear as a singlet, as does C-4 of (6). This retention of individuality of the geminal methyl groups was first demonstrated[28] in the case of mycelianamide (10) and further cases cited later suggest this may be general. Mycelianamide (10) was labelled with $[2-^{14}C]$-mevalonate. The part of the molecule that had been labelled with $[2-^{14}C]$ -M.V.A. was obtained by degradation and then fed to a rabbit and the stereospecifically

Figure A.9.

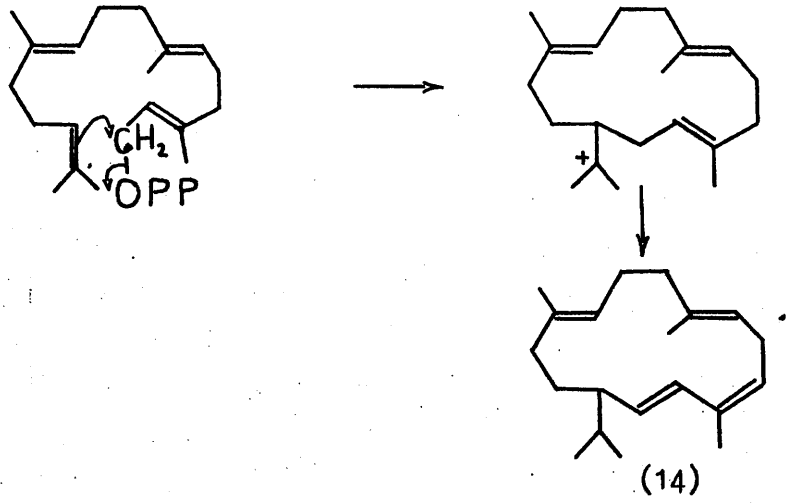

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Figure A.10.

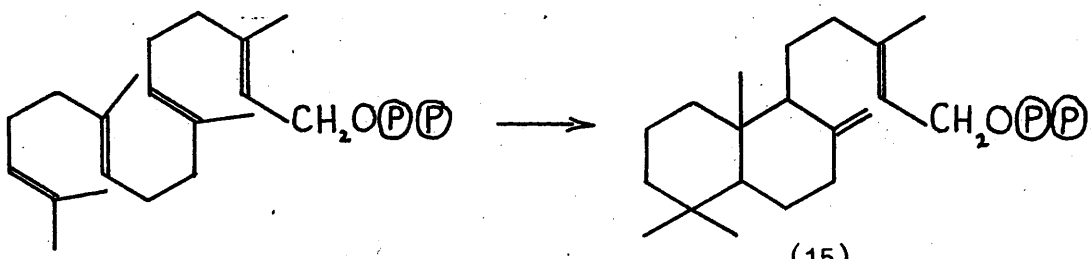

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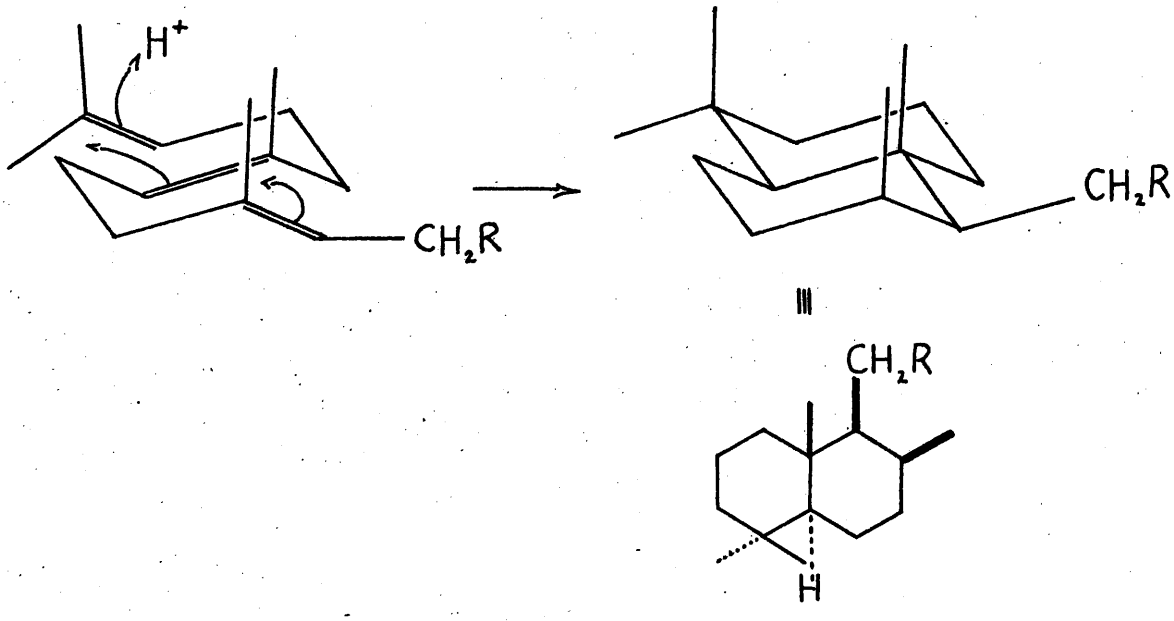
Figure A.11.



oxidised product, in which all the tracer resided in the carboxyl group, was isolated from the animal's urine (Figure A.8).

(ii) Biosynthesis of Tricyclic Diterpenes

Cyclic terpenes can be considered to arise from cyclisation of appropriate acyclic terpenoid species. Thus geranyl-geranyl pyrophosphate is believed to be the progenitor of diterpenes, the parent geranylgeraniol (11) and its isomer geranyl linalool (12) having been isolated from natural sources[29,30]. The isolation, from a marine sponge, of the unusual isonitrile analogue of geranyl linalool (13) has also been reported[31].

Direct cyclisation of geranylgeranyl pyrophosphate (by the terminal pyrophosphate leaving group generating a carbonium ion which may then attack the starting isopropylidene group) is common in lower terpenes but rare in diterpenes, with only a few examples of this mechanism known, e.g. cembrene (14) (Figure A.9.).

The major mode of cyclisation involves the formation of perhydronaphthalene and perhydrophenanthrene derivatives. Protonation of the double bond of the starter isopropylidene unit initiates cyclisation (Figure A.10). The first stage in such a cyclisation process is believed[32] to involve the formation of the bicyclic labdane-intermediate, copalyl pyrophosphate (15). Implicit in such a process in the possibility of forming two enantiomeric series of compounds with 'normal' (5α,10β) and 'antipodal' (5β, 10α) trans A/B ring junctions. The former optical series includes the steroids while the gibberellins are important examples of the latter. The nature of this ring junction is a function of the relative orientation of the cyclising double bonds and may be visualised as arising through different modes of coiling the open-chain precursors on an enzyme surface (Figure A.11). The 'anomolous' cis-fused bicyclic diterpenes must arise by a different mode of cyclisation and thus a recent report[33] of the co-occurrence of cis and trans - fused clerodane-type diterpenes in the same tissue

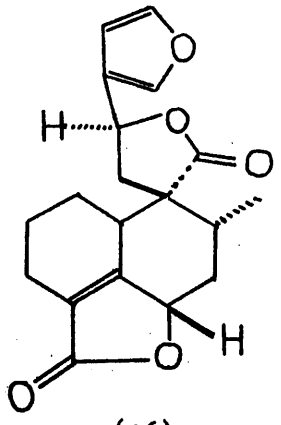


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Figure A.12.

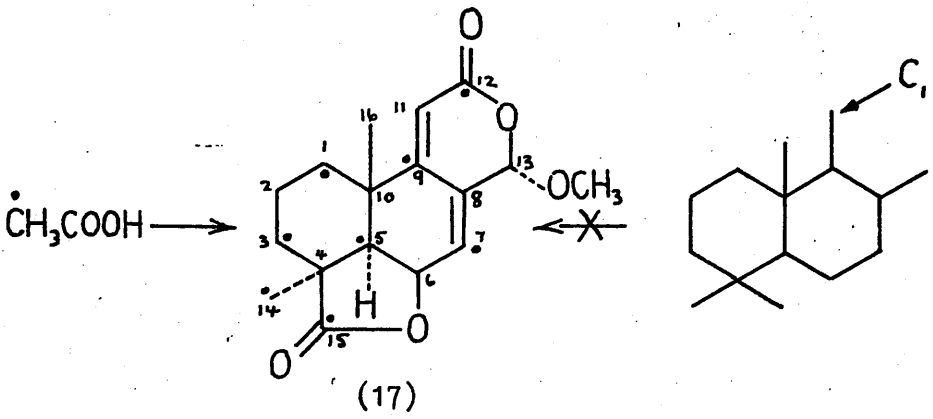

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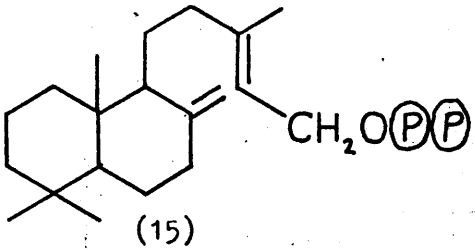
Figure A.13.



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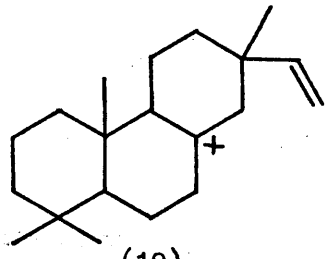

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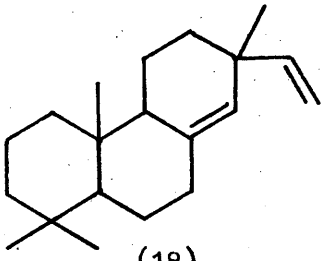

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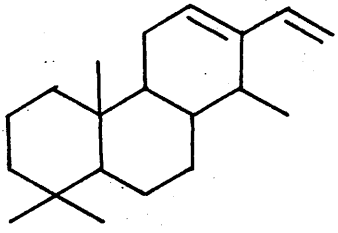

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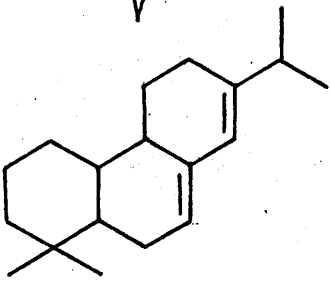

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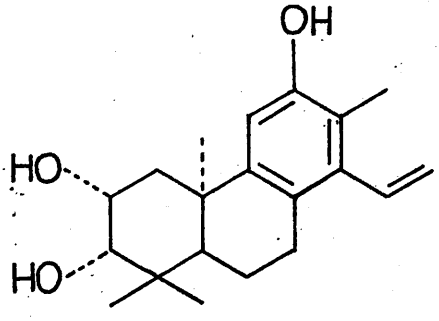

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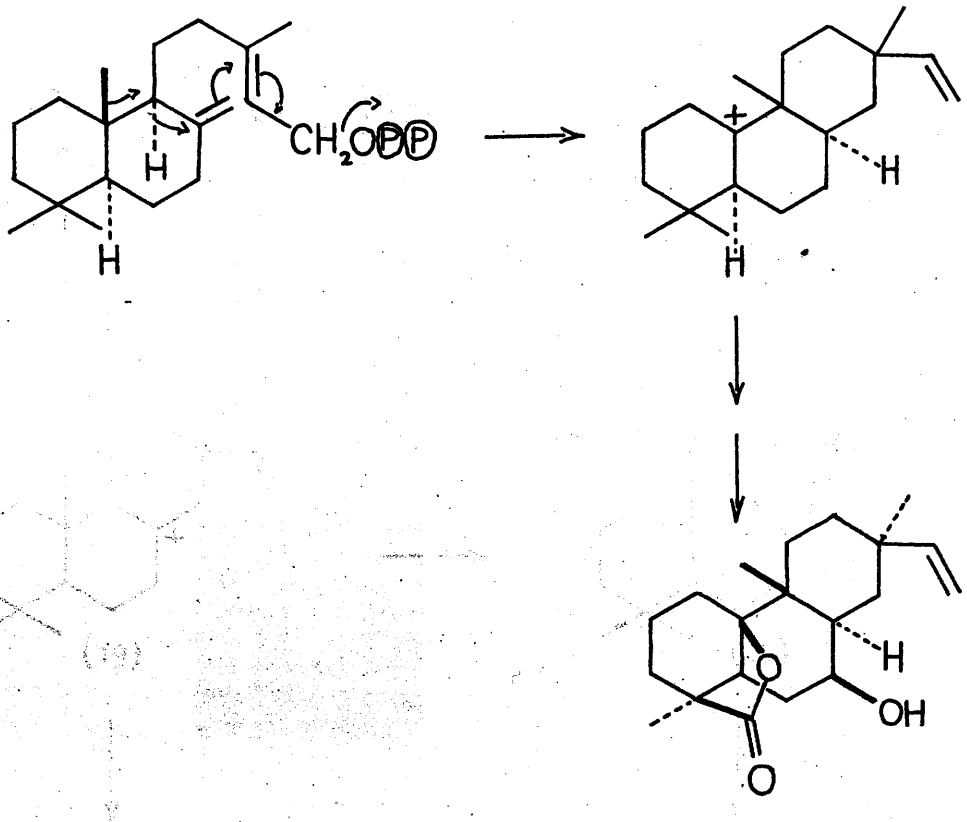
Figure A.14.



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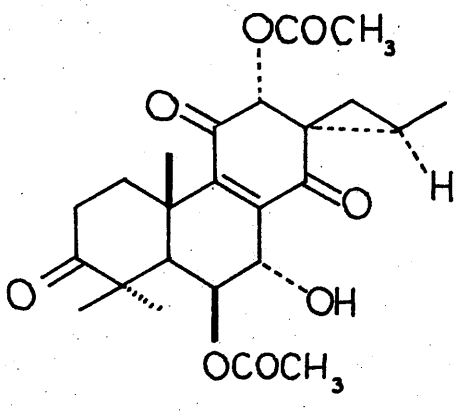

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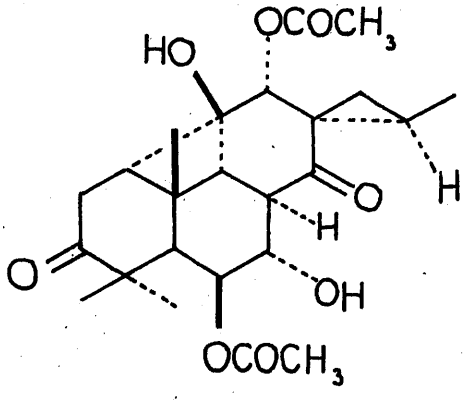

(25)

of a Solidago species is of interest.

Subsequent modification of (15) may lead to further bicyclic diterpenoids including those with the conversion of the side chain into a furan ring. Loss of one carbon atom is also possible and both types of modified structure are seen in the nor-clerodane, teucvin (16)[34]. Degradation is one form of modification of the precursor (15) which has been suggested[35] for the biosynthesis of the antibiotic LL-Z12710α (17) which has a C_{16} skeleton. Evidence in support of the postulated diterpene origins of this compound was provided by feeding experiments using [2-^{13}C]-sodium acetate and [2-^{14}C,5-$^{3}H_2$]-MVA[36]. The labelling pattern, as determined by ^{13}C-n.m.r. (Figure A.12) and the enrichment observed in the signals for both C-12 and C-15, indicated that both of these carbons were derived from mevalonate, so precluding the possibility that the compound might be a methylated drimenol-type sesquiterpene (Figure A.12). The results obtained from feeding doubly labelled MVA were consistent with the incorporation of four, rather than three, molecules of MVA accompanied by loss of four tritium atoms.

Further cyclisation of (15), with pyrophosphate acting as leaving group, leads to the tricyclic diterpenes eg. pimaradiene (18), again with the possibility of differences in stereochemistry giving the epimeric series (5α, 9α, 10β and 5β, 9β, 10α) of diterpenes with further epimers at C-13 possible. Rearrangements of the carbonium ion (19) can lead [37] into the abietane, cassane and cleistanthane series, eg. abietadiene (20), cassaine (21) and cleistanthanol (22) (Figure A.13). An alternative mode of cyclisation may lead[38] into the rosane skeleton, eg. rosenolactone (23) (Figure A.14). The isolation of the cyclopropane-ring containing cogeners, barbatusin (24) and cyclobutatusin (25), from leaves of Colleus barballus may imply that the pimarane-abietane conversion by migration of the C-17 methyl group may not be a simple, one-step process[39,40].

In vitro syntheses of pimaradienes[41] and rosadienes[41,42] from

Figure A.15.

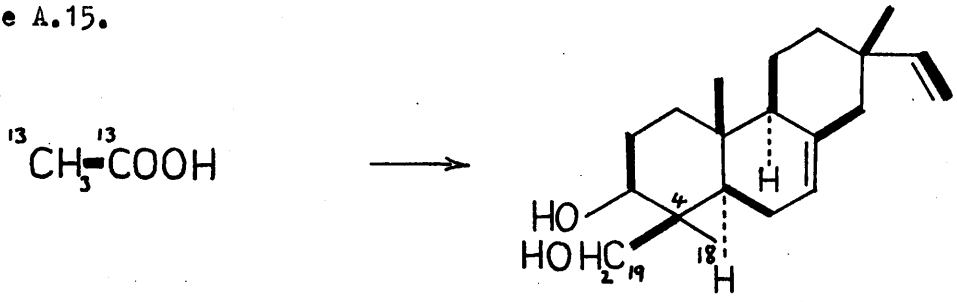

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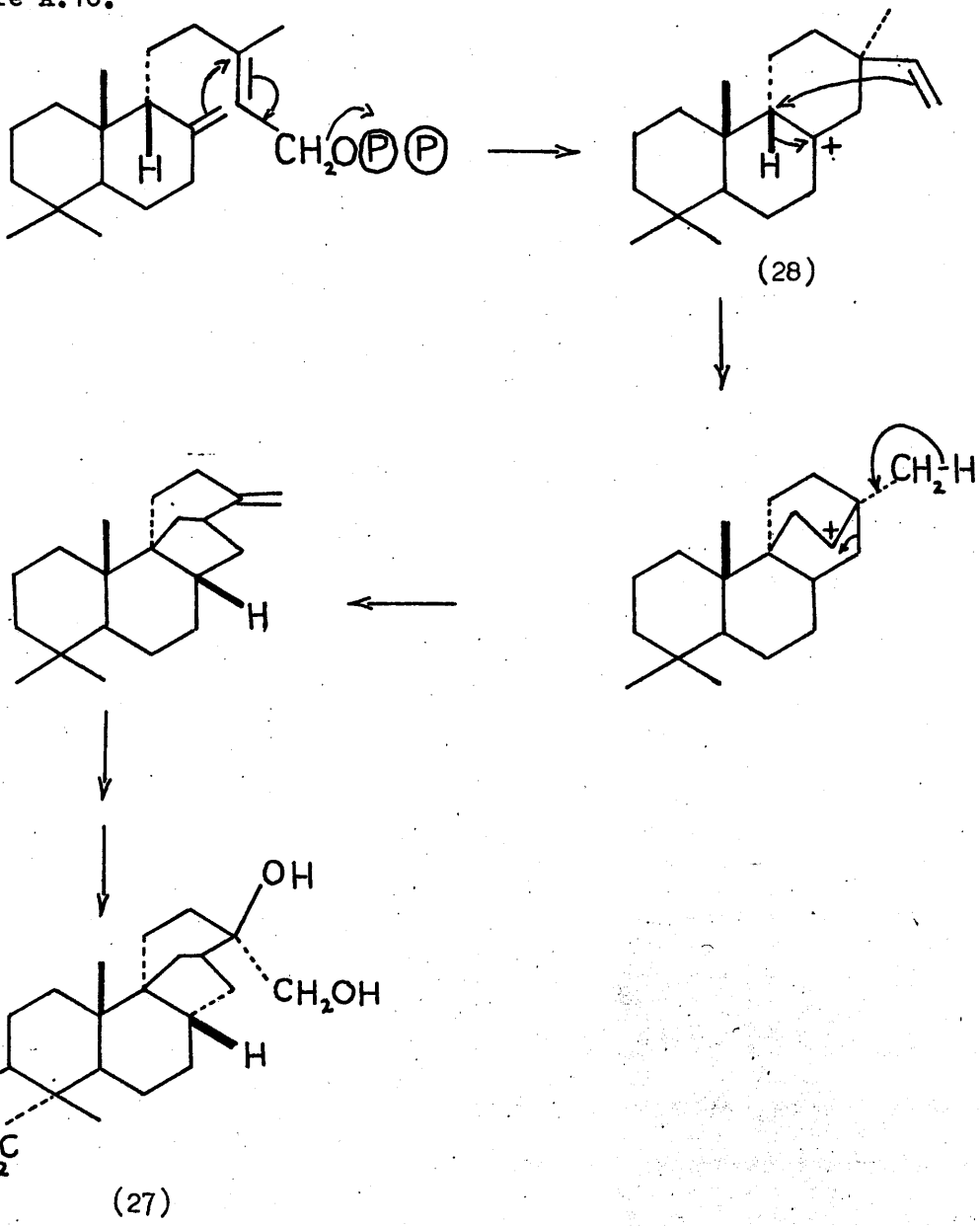
Figure A.16.



(28)

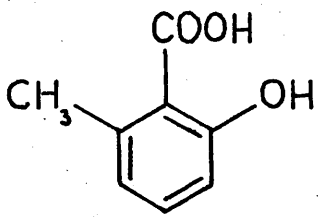
(27)

labdane-type precursors and a cleistanthane from a pimarane type precursor[43] have been accomplished. However, attempts to induce a model compound of the pimarane type to rearrange to a cassane-type skeleton under a variety of conditions proved unsuccessful[44].

The retention of stereochemical individuality of the angular methyl groups of cyclised terpenes during their formation from acyclic precursors has been demonstrated in the case of the tricyclic fungal diterpenes, the virescenols. Addition of [1,2-^{13}C]-sodium acetate to cultures of Oospora virescens[45] resulted in an enriched singlet, in the ^{13}C-n.m.r. spectrum of virescenol B(26), for the C-18 methyl group while the geminal C-19 carbon showed satellites arising from ^{13}C-^{13}C coupling with C-4. C-18 thus originated from C-2 of MVA and no scrambling of label between the geminal carbon atoms had taken place (Figure A.15.).

Similarly, the biosynthesis of the antibiotic aphidicolin (27) has been studied using [1,2-^{13}C]-acetate and 4R-[4-^{3}H,2-^{14}C]-MVA[46]. By feeding these precursors to cultures of Cephalosporium aphidicola, it was shown that (27) could originate from the cation (28), as had been suggested[47] (Figure A.16).

INTRODUCTION PART B



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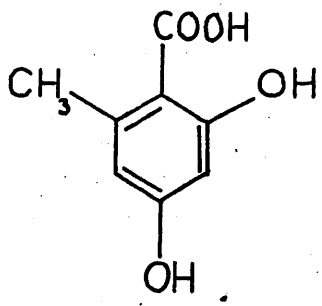

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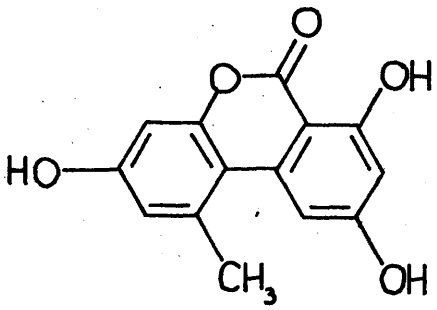


(31)

Figure B.1. Biosynthesis of Fatty Acids

$CH_3COSCoA \longrightarrow CH_3COS\text{-}A.C.P.$

$CH_3COSCoA \xrightarrow{CO_2} CH_2(COOH)COSCoA$

$CH_3COS\text{-}A.C.P. \longrightarrow CH_3COS\text{-}Enz$

$CH_2(COOH)COSCoA \longrightarrow CH_2(COOH)COS\text{-}A.C.P.$

$CH_3COS\text{-}Enz + CH_2(COOH)COS\text{-}A.C.P. \longrightarrow CH_3COCH_2COS\text{-}A.C.P. + CO_2 + Enz\text{-}SH$

$CH_3COCH_2COS\text{-}A.C.P. \xrightarrow{NADPH} CH_3CH(OH)CH_2COS\text{-}A.C.P.$

$CH_3CH(OH)CH_2COS\text{-}A.C.P. \longrightarrow CH_3CH = CHCOS\text{-}A.C.P. + H_2O$

$CH_3CH = CHCOS\text{-}A.C.P. \xrightarrow{FMNH} CH_3CH_2CH_2COS\text{-}A.C.P.$

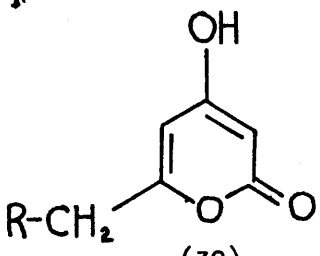
$CH_3CH_2CH_2COS\text{-}A.C.P. \longrightarrow CH_3CH_2CH_2COS\text{-}Enz$

$CH_3CH_2CH_2COS\text{-}Enz + CH_2(COOH)COS\text{-}A.C.P. \longrightarrow CH_3CH_2CH_2COCH_2COS\text{-}A.C.P.$

Polyketide Biosynthesis

The hypothesis that the structures of large numbers of natural products could be derived from cyclisation of chains of acetate units, combined in head-to-tail fashion, was originally put forward by Collie in 1907[48]. This concept lay dormant until re-investigated by Birch and Donovan in 1953[49], when they related the oxygenation patterns observed in a large variety of naturally-occurring aromatic compounds to the earlier hypothesis and postulated the involvement of poly-β-ketomethylene units in the biosynthesis of these compounds. Birch later provided the first experimental evidence in support of the theory with the incorporation of [1-^{14}C] and [2-^{14}C]-sodium acetate into 6-methylsalicylic acid (29) with cultures of Penicillium griseofulvum[50]. An experiment by Gatenbeck and Mosbach[51] demonstrated that the phenolic oxygens of orsellinic acid (30) were derived from the oxygen of acetate which was labelled with ^{18}O. Similar results have now been obtained with many plant and fungal metabolites.

It is now recognised that there is, in the biosynthesis of polyketide compounds, some similarity with the processes involved in the biosynthesis of fatty acids. Experiments with cell-free systems have shown that the building blocks of polyketide biosynthesis are, as in fatty acid biosynthesis, active acetyl and malonyl-coenzyme A units. The biosynthesis of 6-methylsalicylic acid (29)[52] and alternariol (31)[53] have been studied with such systems. It has been shown[54] that polyketide biosynthetic activity is associated with what appears to be a multi-enzyme complex, the physical properties of which closely resemble fatty-acid synthase enzyme complexes. Further evidence of similarity in biosynthesis is provided by the observation that 6-methylsalicylic acid and fatty acids co-produced in the presence of [1-^{14}C,2-^{3}H]-acetate have distributions of radioactivity consistent with their formation by the same condensation processes[55]. While the



(32)

R = H

R = CH_3

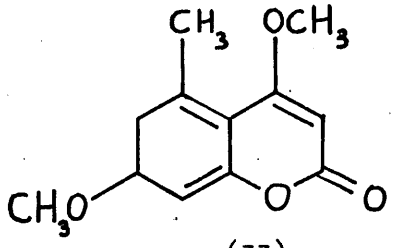


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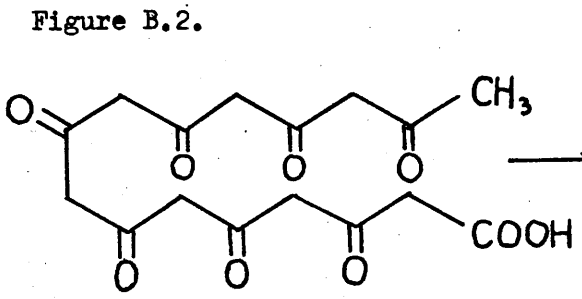
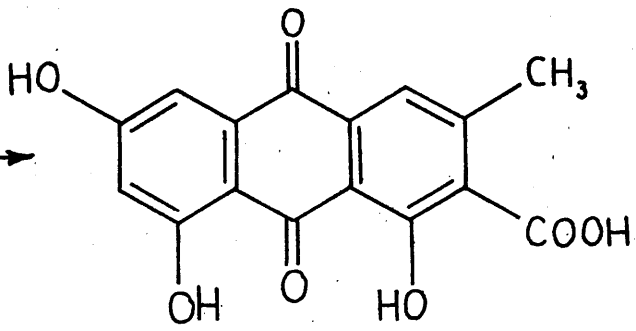
Figure B.2.





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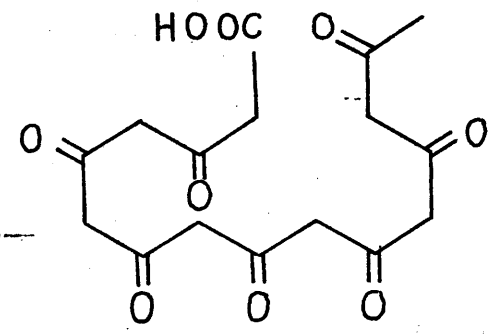

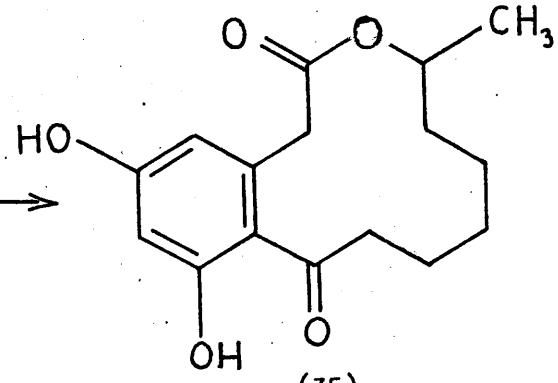

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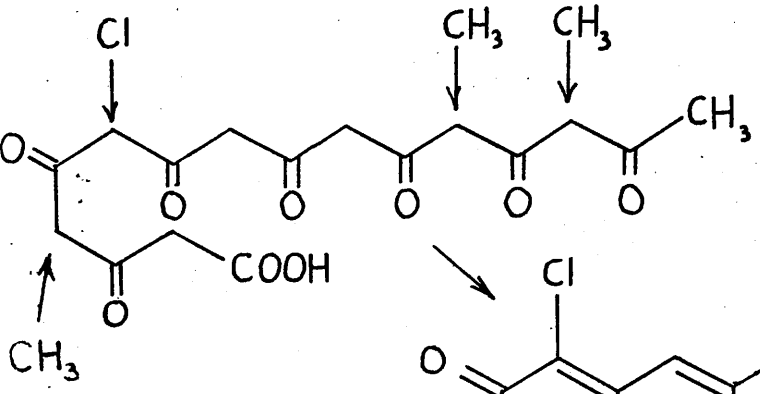
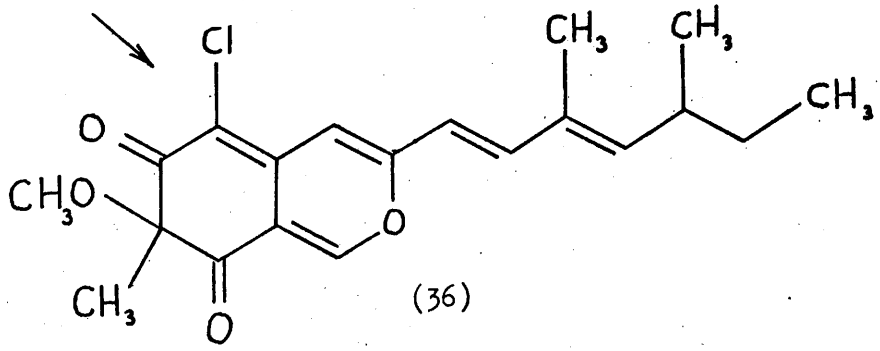


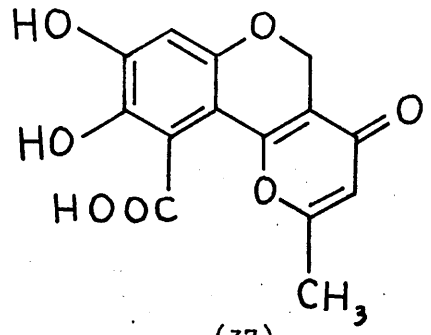


(36)

details of fatty acid biosynthesis have been elucidated using purified enzyme systems[56], the individual enzymes involved in polyketide biosynthesis have not been obtained in anything like the same numbers. Stages in the biosynthesis of fatty acids are shown in Figure B.1.

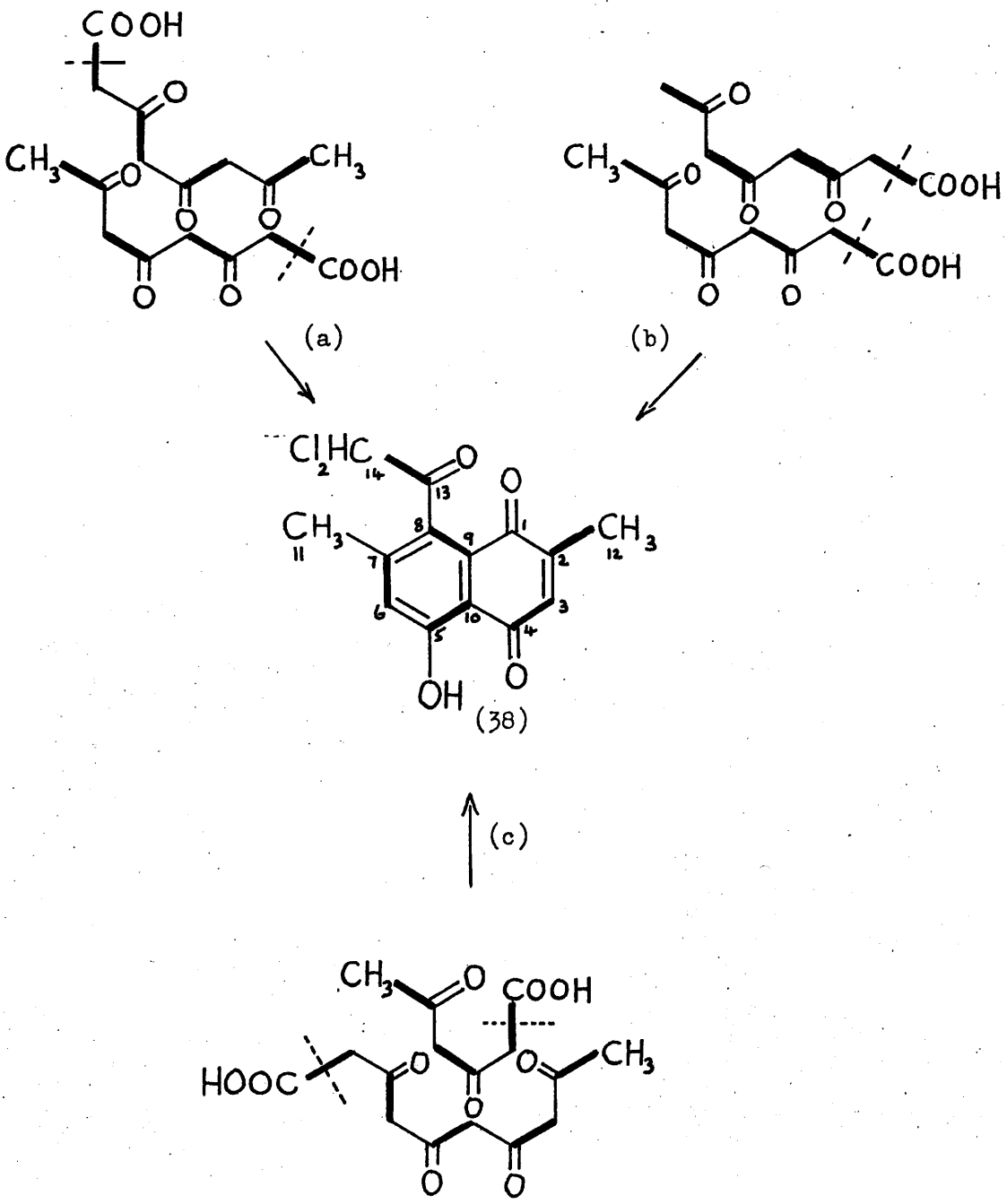
An acetyl residue is transferred from coenzyme A to 'acyl carrier protein' (A.C.P.) and then to an enzyme (Enz). A second acetylcoenzyme A moiety is carboxylated by a biotin-dependent process and the newly-formed malonyl group transferred from coenzyme A to acyl carrier protein. Condensation of the acetyl and malonyl units results in the formation of acetoacetyl-A.C.P. which, still bound to the carrier protein, undergoes successive reduction (NADPH), dehydration and reduction to give butyryl-A.C.P. This is then transferred back onto the enzyme and condensed with another malonyl-A.C.P. unit. This sequence continues until the correct length of chain is reached and the fatty acid then released from the protein. In polyketide biosynthesis, the sequential reduction and dehydration steps following each two-carbon elongation are withheld until final cyclisation reactions of the polyketide are attained.

Fatty acid synthases have been used to demonstrate that the postulated polyketide-producting reactions are possible. In the absence of NADPH, synthases from pigeon liver[57] and yeast[58] are able to carry out the addition of two or three malonyl units to acetyl-SEnz, but with reduction no longer possible, tri and tetra-acetic acid lactones (32) are the principal products. These lactones may be regarded as stabilised polyketide chains and their formation highlights the inherent instability of polyketide thiol esters. It is believed that growing polyketide chains remain bound to enzyme surfaces, so stabilising them, until they have reached their final length and are not released until they become self-stabilised through cyclisation and aromatisation. It has been suggested[59] that metal chelation may play a part in the stabilisation of poly-enol intermediates and in specificity of cyclisation during polyketide biosynthesis. The fact that lactones (32)



(37)

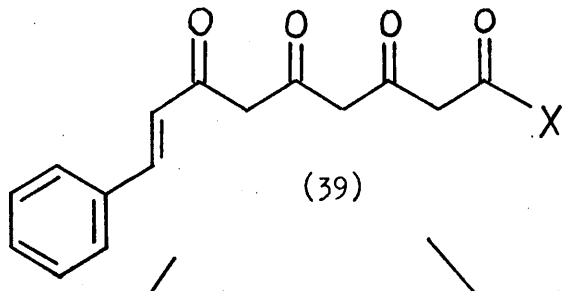
Figure B.3.



do not serve as intermediates in the biosynthesis of other polyketides *in vivo*[60] emphasises the continued binding of enzyme and substrate without the release of partially cyclised intermediates. In recently reported biogenetic-like syntheses, methods such as chelation by metal ions and the use of partially protected precursors have been employed, *inter alia*, in an attempt to mimic the control exerted by enzymes *in vivo*[61]. It has been suggested that the modes of cyclisation which a polyketide chain of a particular length can undergo depend upon the topography of the enzyme to which it is bound[62]. The body of evidence accumulating from metabolic studies suggests that most possible modes of cyclisation to form six-membered rings are exemplified. However, Turner has pointed out that, from existing evidence, uncyclised residues from the methyl ends of polyketide chains are never shorter than the residues from the carboxyl end of the chain and he suggests that this restriction on cyclisation types is a result of the mechanism of formation of aromatic compounds from polyketide precursors[1]. The recent discovery of siderin (33), a metabolite of *Aspergillus variecolor*[63], would present the first example of an exception to Turner's rule, if this metabolite is polyketide-derived. Some of the possible modes of cyclisation of an octaketide chain are seen in such diverse structures as endocrocin (34), curvularin (35) and sclerotiorin (36)(Figure B.2).

In accord with this acetate-polymalonate theory of polyketide biosynthesis, it has been found that acetate is more efficiently incorporated into the first two carbon atoms of polyketide chains as these atoms are derived directly from acetate, the rest of the chain coming via malonate[64,52]. This 'starter effect' has been put forward as evidence for the biosynthesis, from two chains, of several metabolites, eg. citromycetin (37)[65] and mollisin (38)[66]. ^{14}C-tracer experiments had suggested formation of mollisin from two chains and the two biosynthetic pathways (a) and (b) were proposed, pathway (a) being favoured since this route uses an activated methylene for the chlorination step. When mollisin was obtained from cultures of *Penicillium cyclopium*

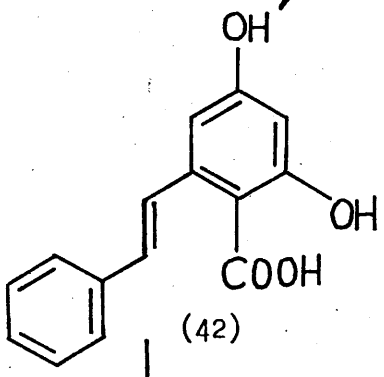
Figure B.4.



(39)

ALDOL

CLAISEN



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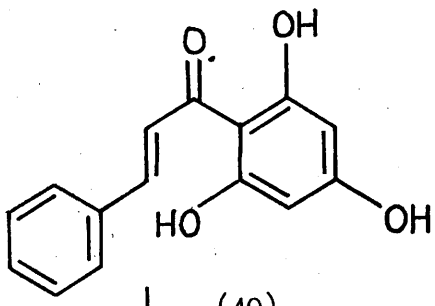

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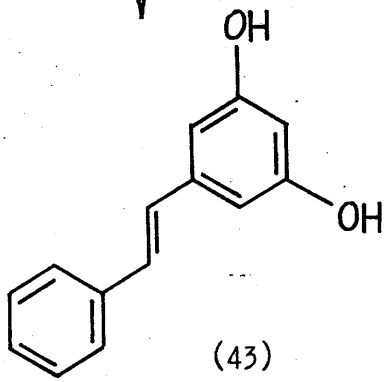

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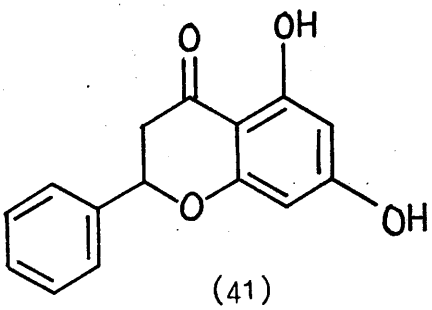

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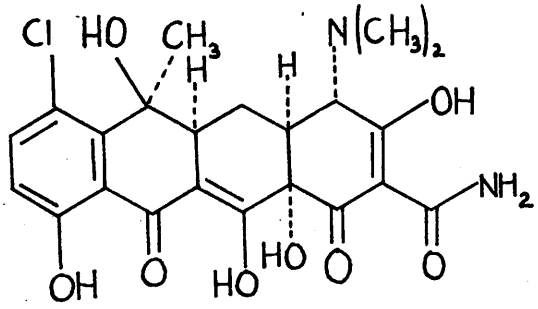

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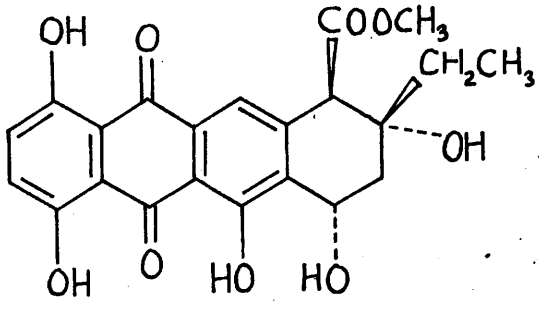

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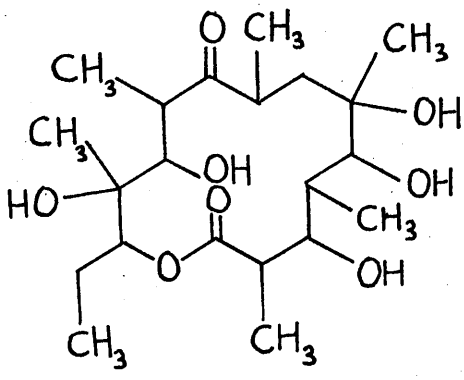

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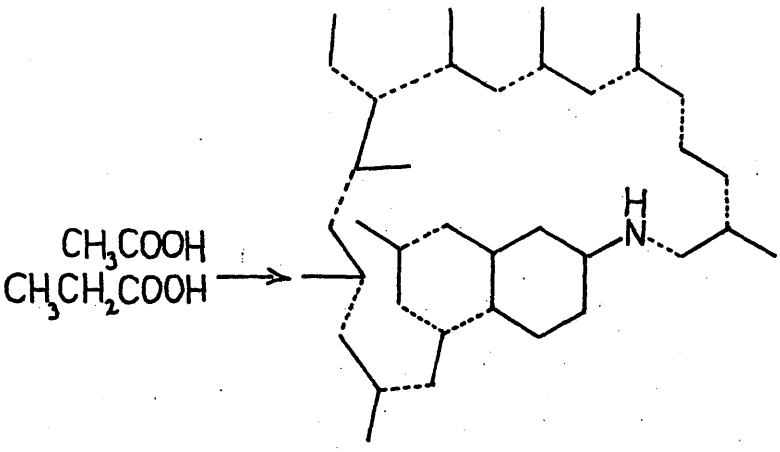
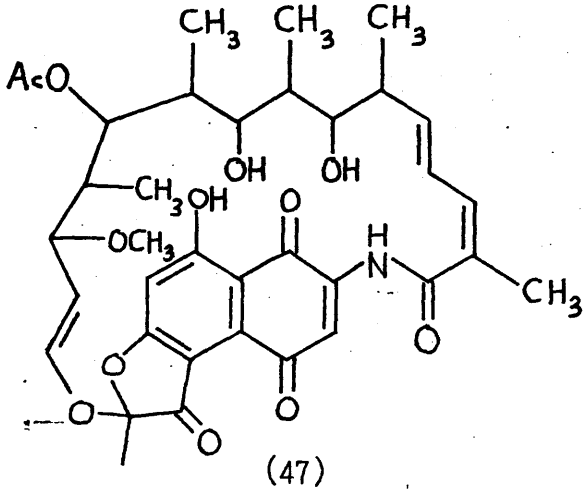
Figure B.5.





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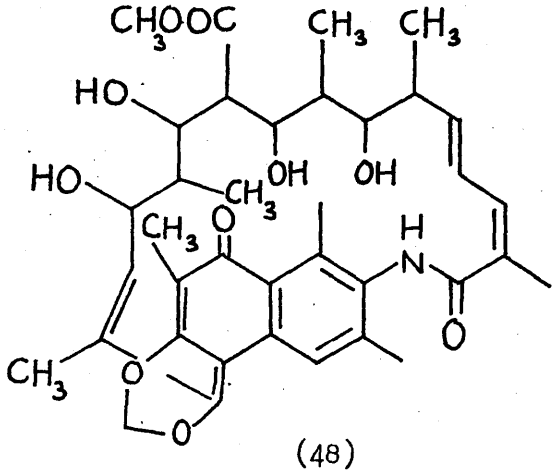

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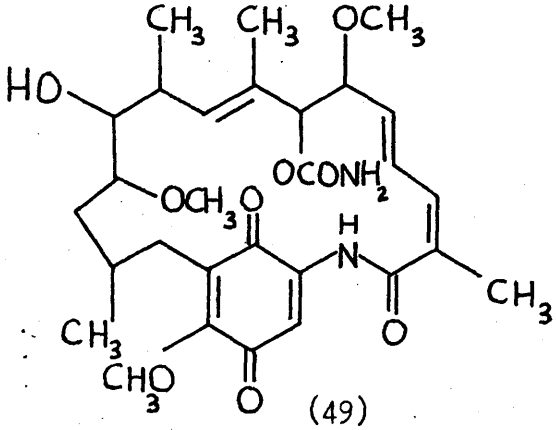

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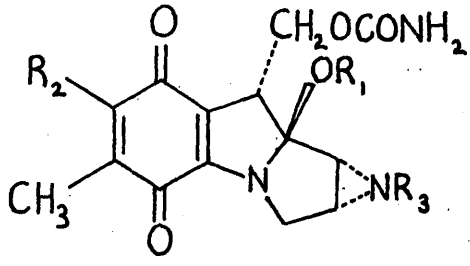
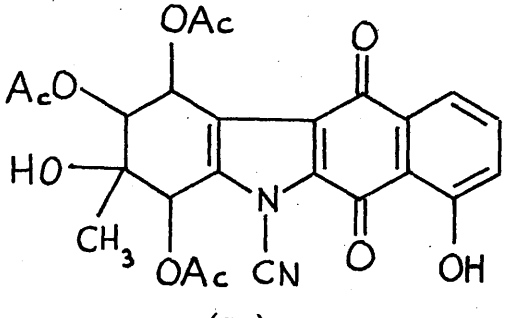


(50)

A $\begin{cases} R_1 = Me \\ R_2 = OMe \\ R_3 = H \end{cases}$

B $\begin{cases} R_1 = H \\ R_2 = OMe \\ R_3 = Me \end{cases}$



(51)

to which [1,2 - ^{13}C]-acetate had been added, ^{13}C - ^{13}C coupling was observed in the ^{13}C-n.m.r. spectrum of mollisin for the signal for C-12, so excluding route (b) and the signal corresponding to the carbon of the C-11 methyl group appeared as a singlet, so excluding route (a)[67]. Pathway (c) was proposed (Figure B.3).

In higher plants and bacteria, the biosynthesis of polyketides may often involve starter units other than acetyl coenzyme A and chain-extending units other than malonyl coenzyme A. In plants addition of three malonyl units to a shikimic acid derived starter unit could produce the polyketide (39) which would give the chalcone (40) and compounds of the flavonoid series such as flavonone (41), by Claisen condensation and compounds of the stilbene series such as pinosylvic acid (42) or pinosylvin (43) by Aldol condensation (Figure B 4).

In the tetracyclines, eg. 7-chlorotetracycline (44), elaborated by Streptomyces spp., the starter unit is probably malonyl coenzyme A, while propionate is the starter unit in the bacterial metabolite ∈-pyrromycinone (45). In bacteria, not only can propionate replace acetate in initiating chain formation but, with methyl malonate, it can replace malonate in the chain extending process as exemplified by erythronolide (46), the aglycone of the macrolide antibiotic erythromycin. An as yet unidentified starter unit, which has seven carbon atoms and one nitrogen atom in its skeleton, serves as the initiator in the biosynthesis of the ansamycin antibiotics rifamycin S (47)[68], from Nocardia mediterranei, and streptovaricin D (48)[69] from Streptomyces spectabilis (Figure B.5). The starter unit is located within the naphthalenic chromophore, the remainder of the chromophore originating from propionate and acetate. The same starter unit, with the characteristic feature of the 1,3-arrangement of the one-carbon side chain and amino group, is probably involved in the biosynthesis of a related member of this group of antibiotics, geldanamycin (49)[70] and it was recently pointed out[71] that this same C_7 unit is present in other antibiotics, eg. as the aromatic

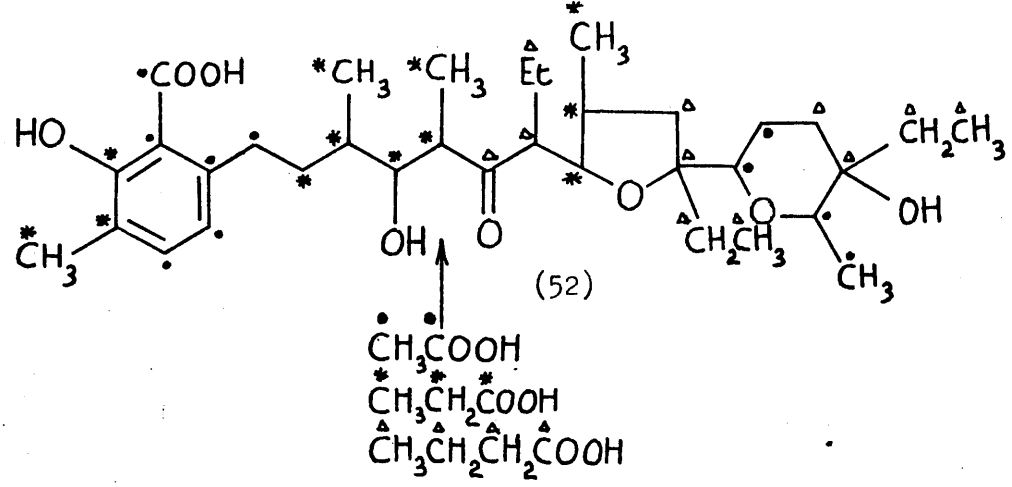


Figure B.6.

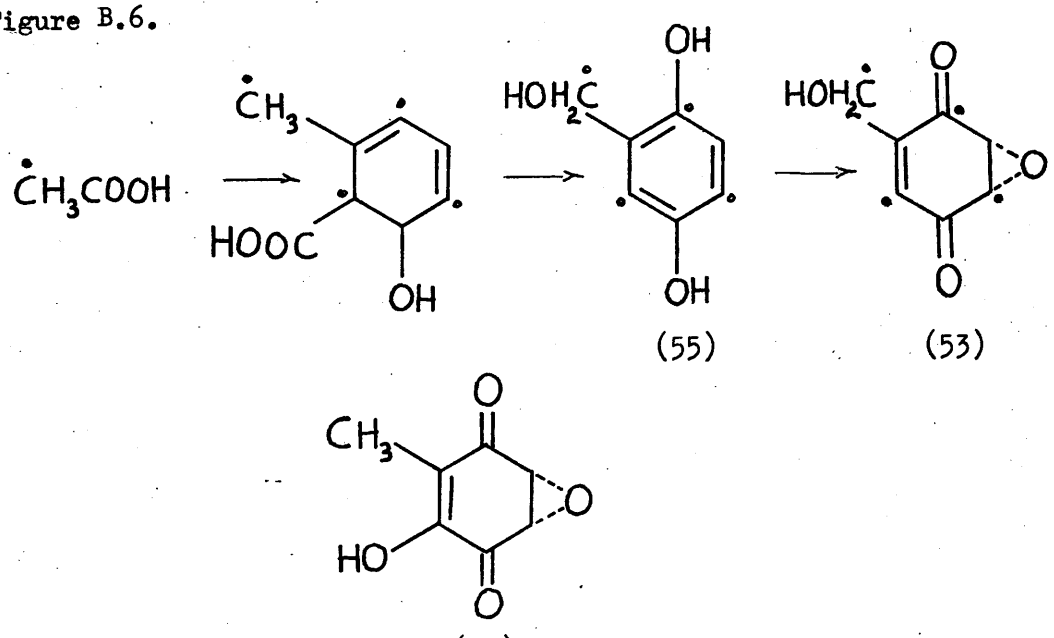
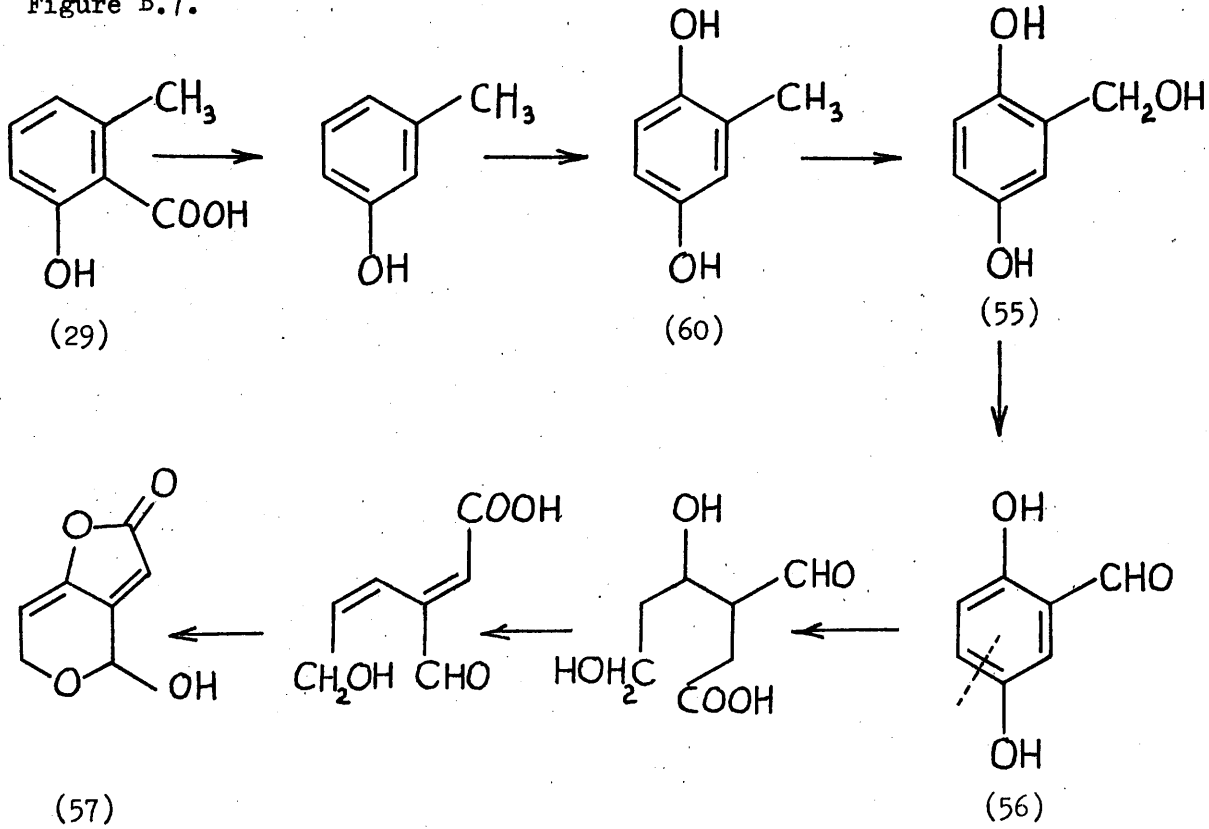


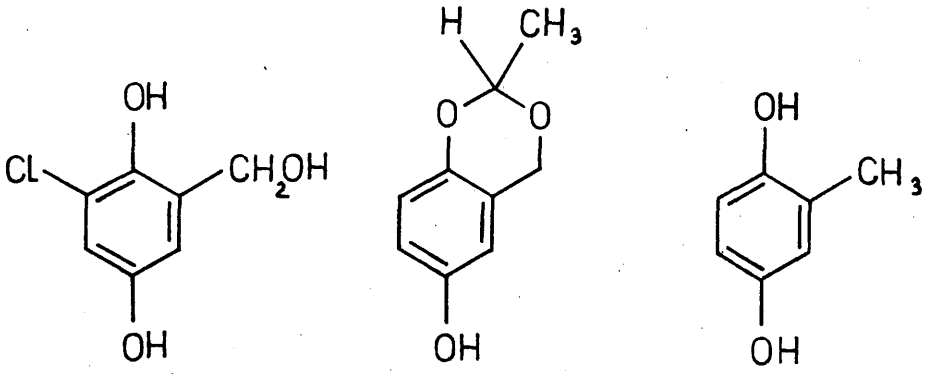
Figure B.7.



unit[7] in the mitomycins (50)[71], and in kinamycin (51)[72]. It was suggested that the C_7 units have a common biogenetic origin from carbohydrate metabolism. The ansamycins also illustrate another interesting feature - the co-occurrence of propionate and acetate, as established by feeding studies[68,69], as polyketide chain-extending units within the ansa chain. It has been suggested[73] that the co-occurrence of more than one type of acyl chain extending unit (other than the carboxy derivative of the starter unit) within the same polyketide chain be described as 'heteropolyketide' biosynthesis. The normal fungal, acetate-polymalonate pathway would then be an example of 'homopolyketide' biosynthesis. Other examples of metabolites with heteropolyketide chains are aureothin[74], nonactinic and homononactinic acids[75] and antibiotic X-537A (52)[76], all from Streptomyces spp.

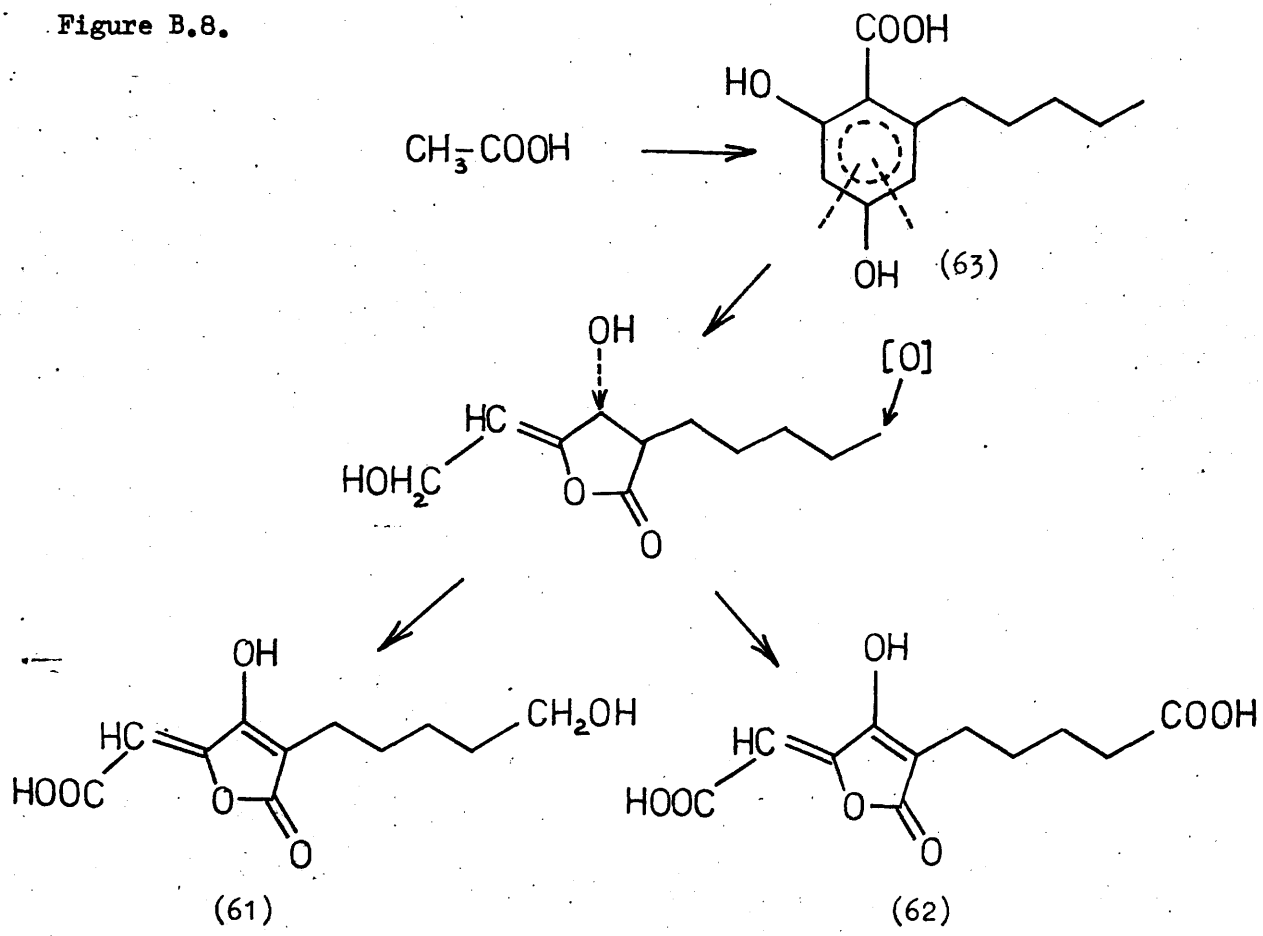
Many polyketide-derived metabolites have structures which are the result of further modifications of the basic polyketide skeleton. These secondary transformations include such processes as oxidation, reduction, decarboxylation, the introduction or removal of heteroatoms, skeletal rearrangements and electrophilic substitution.

Numerous metabolites, eg. epoxydon (53) and terreic acid (54), are derived from a tetraketide chain yet the parent compound, orsellinic acid (30), is not accepted as a precursor. In contrast, 6-methylsalicylic acid (29), which possesses one oxygen atom less than the prototypical polyketide chain, is incorporated. Experiments with a purified 6-methylsalicylic acid synthetase, isolated from P. patulum, suggest that, in view of the predictable chemical difficulty in removing a phenolic hydroxyl group, the oxygen atom is lost at a pre-aromatic stage by reduction and dehydration before the polyketide chain is stabilised and detached from the enzyme[77]. Thus, 6-methylsalicylic acid (29) is the proposed precursor of epoxydon (53) which has been shown by labelling studies with [1-^{13}C] and [2-^{13}C]-acetate to be derived

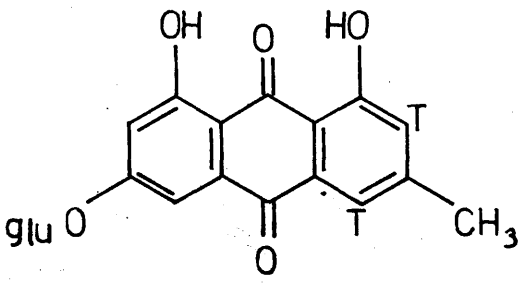


(58) (59) (60).

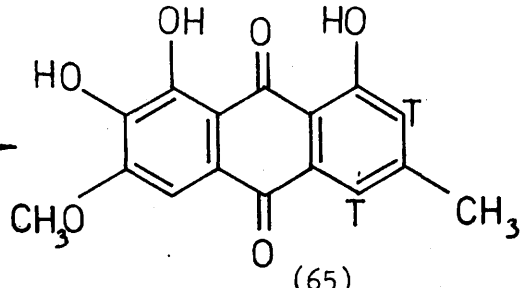
Figure B.8.



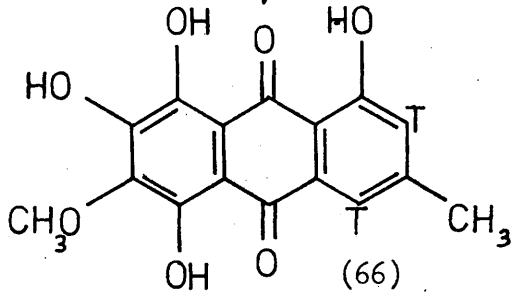
(61) (62)



(64)



(65)



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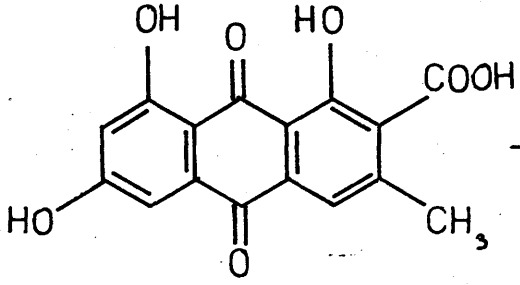

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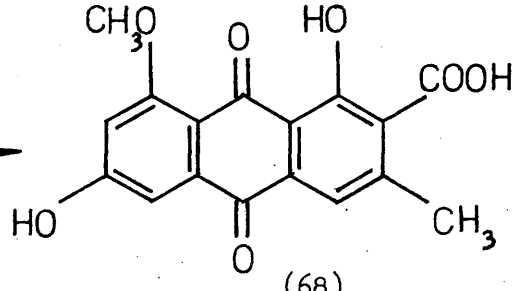

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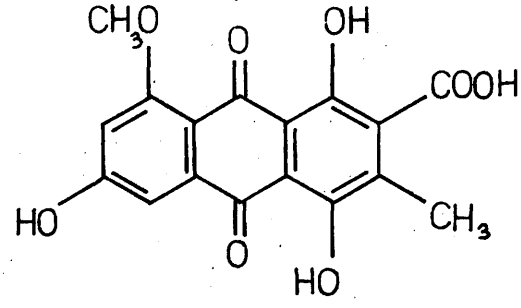


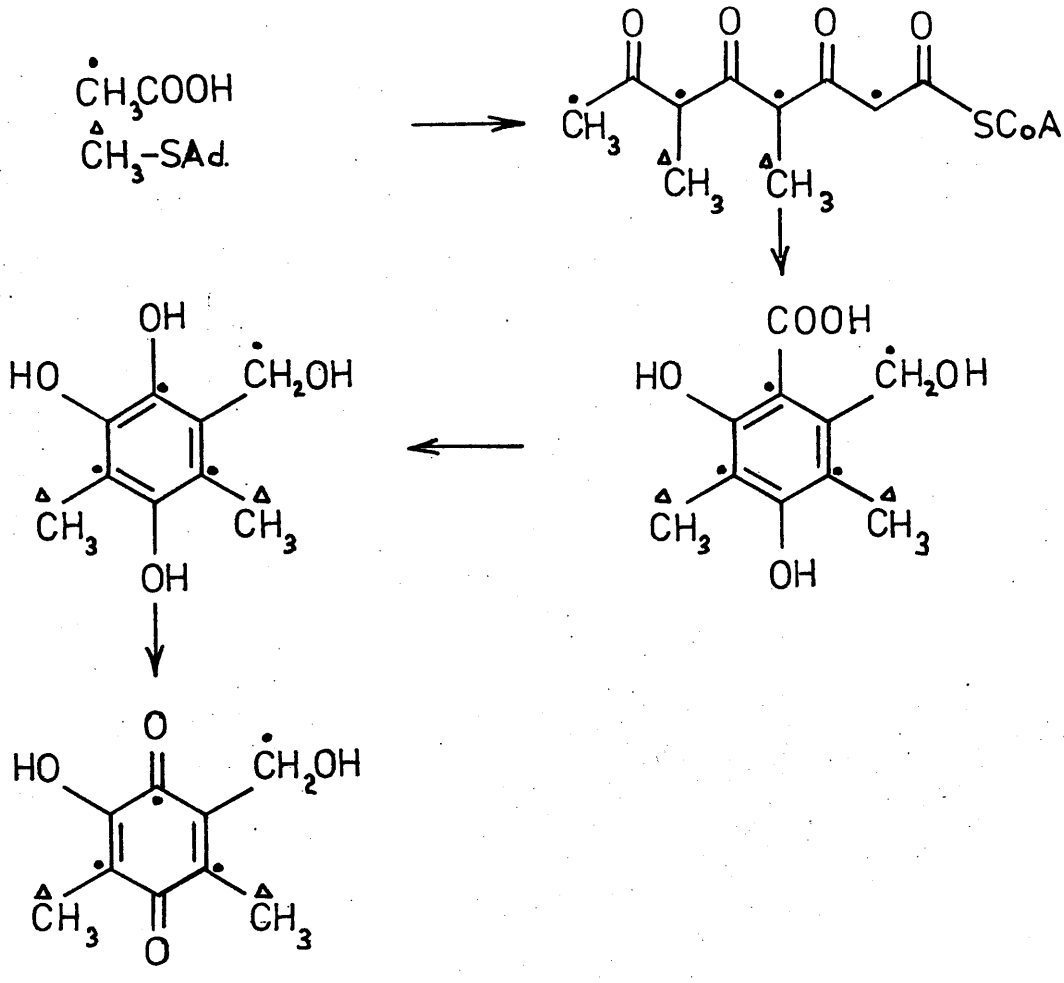
(69)

from acetate[78]. In addition, gentisyl alcohol (55) was incorporated (25%) into (53). The labelling patterns, as determined by ^{13}C-n.m.r. spectroscopy, indicated that the carbon of the alcohol group originated in the methyl of the starter group (Figure B.6.) 6-Methylsalicylic acid has also been shown to be a precursor for terreic acid (54)[79]. The oxygenation to form the epoxide was shown to occur by way of molecular oxygen, as deduced from experiments with $^{18}O_2$. The introduction of oxygen in this way was probably catalysed by a mixed function oxygenase[1].

The isolation by Scott et al.[80] from P.patulum of a partially purified NADPH-dependent aromatic dehydrogenase which was capable of the reversible conversion of gentisyl alcohol (55) into gentisaldehyde (56), lends support to the biosynthetic sequence leading to patulin (57) which was proposed on the basis of earlier labelling studies (Figure B.7).[81] Further evidence for the ready interconvertibility of aldehydes and alcohols is provided by the co-occurrence, in a Phoma spp.[82] of gentisyl alcohol (55), chlorogentisyl alcohol (58), gentisyl acetal (59) and toluhydroquinone (60).

Decarboxylation with extensive skeletal modification has been shown, by labelling studies with [1,2-^{13}C]-acetate, to occur in the biosynthesis of multicolic acid (61) and its co-metabolite multicolosic acid (62) from a common hexaketide precursor (63) in P. multicolor[83] (Figure B.8). Biosynthetic experiments, also relating to the loss of the terminal carboxyl group, in anthraquinones, have been undertaken by Steglich et al., using intact sporophores of the mushroom Dermocybe sanguinea[84]. The 6-mono-β-D-glucoside of emodin (64), labelled with tritium, was well converted into dermoglaucin (65) and dermocybin (66) whereas [9,carboxy-^{14}C]-labelled endocrocin (67) was converted into dermolutein (68) and dermorubin (69). No decarboxylation of endocrocin (67) to give the neutral compounds was observed suggesting that decarboxylation may occur at a pre-aromatic stage. In the case

Figure B.9.



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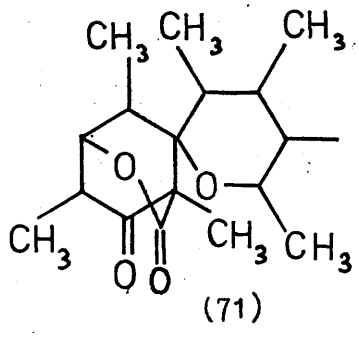
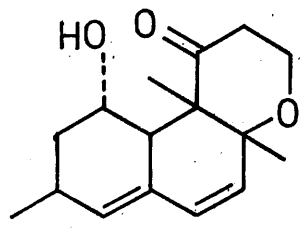


(71)



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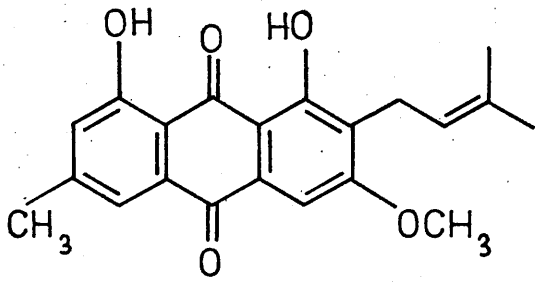
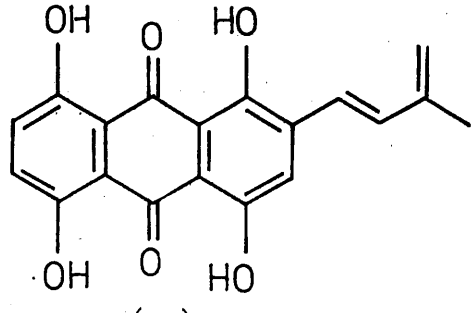


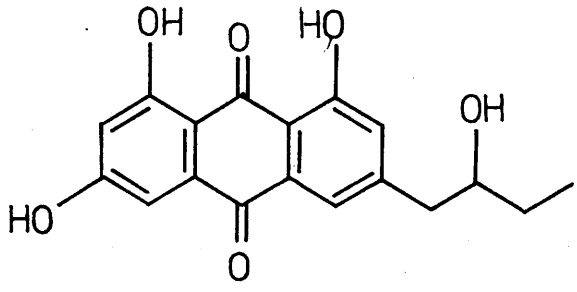
(73)



(74)

of some metabolites, eg. orsellinic acid (30)[85] and 6-methylsalicylic acid (29)[86], the enzymes which catalyse the decarboxylation of the polyketide substrates have been isolated.

Another common modification of polyketide skeletons involves the introduction of electrophilic alkyl groups which are not biogenetically part of the chain. The source of the methyl groups (in fungi) is almost invariably S-adenosyl methionine in which the methyl group of methionine has been 'activated' such that it is prone to nucleophilic attack. Prenyl units are introduced probably via prenyl pyrophosphates. The introduced units are almost always found at positions corresponding to the enolisable methylene groups of the polyketide chain. Thus, shanorellin (70) has been found to be acetate-derived and both methyl groups are derived from methionine[87]. Other phenols were found as co-metabolites but these did not include such presumptive cogeners as 6-methylsalicylic acid, orsellinic acid or 3-methyl orsellinic acid as would be expected if methylation were a post-aromatic process. Thus it was suggested that methylation occurred prior to aromatisation and it was established that the alcohol group was formed by oxidation of the starter methyl group (Figure B.9). The structure of portentol (71) suggested a propionate derivation but it was found to be formed from methylation of an acetate-derived precursor[88]. The fungal metabolite (72)[89] has, in common with (71) the feature, rare amongst fungal polyketide metabolites, of tertiary methyl groups. The biosynthesis of (72) is presumably analogous to that of (71). The introduction of dimethylallyl groups into anthraquinones is virtually unknown, but 2-(γ,γ-dimethylallyl)-physcion (73) was recently isolated from Aspergillus flaschentraegeri[90] and the isolation of tectoleafquinone (74) from teak leaves has been reported[91]. An anthraquinone with a five-carbon side chain (75) was reported to have been isolated from a marine species, the crinoid Comanthus bennetti, but it was suspected that (75) originated by way of a C-polyketide with loss of one carbon[92].



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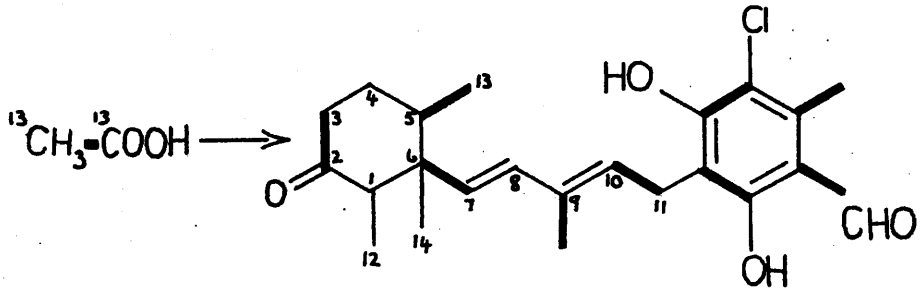
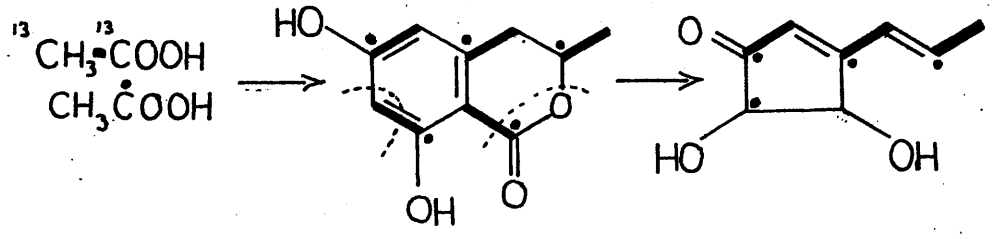


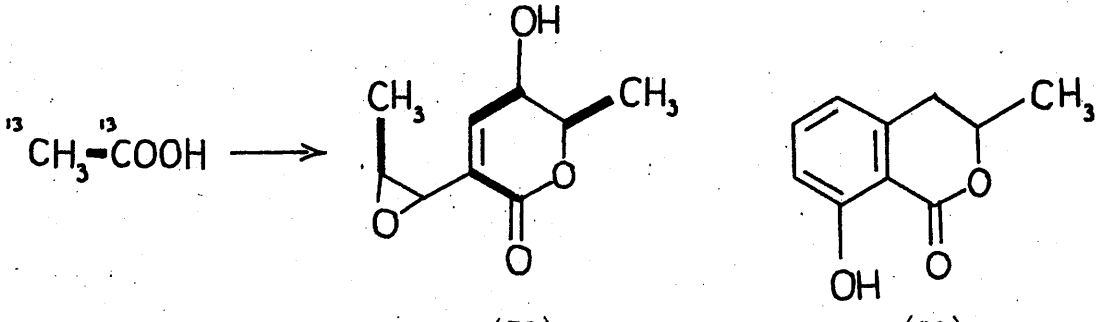
(76)

Figure B.10.

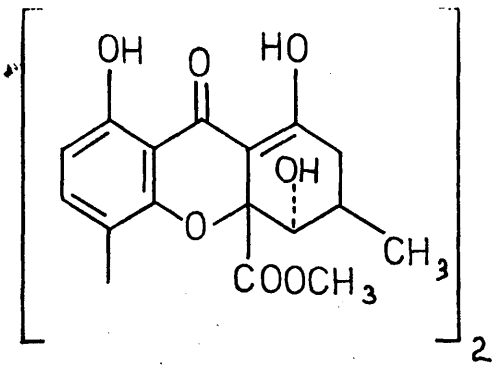


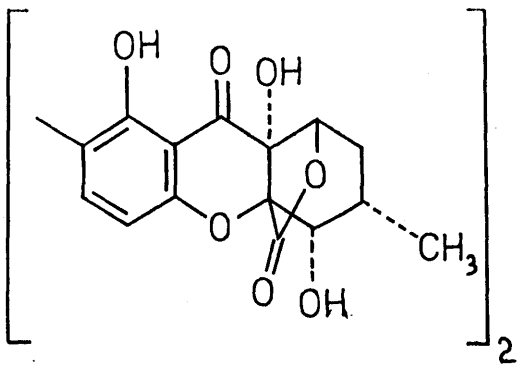
(78) (77)

Figure B.11.



(79) (80)





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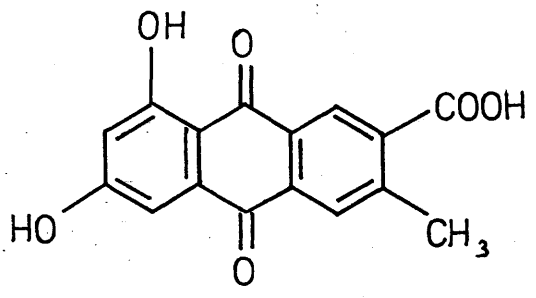

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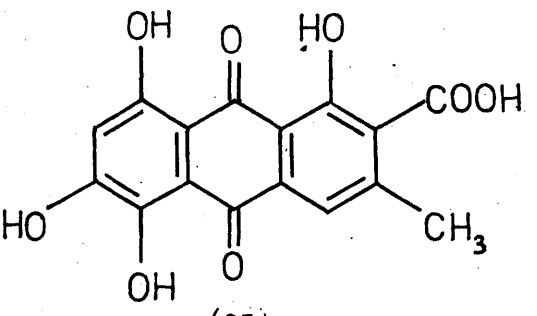

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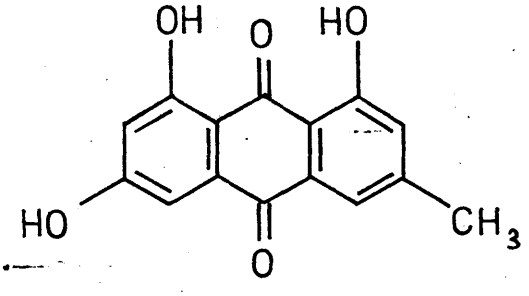

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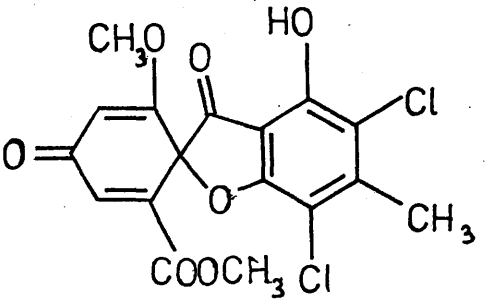

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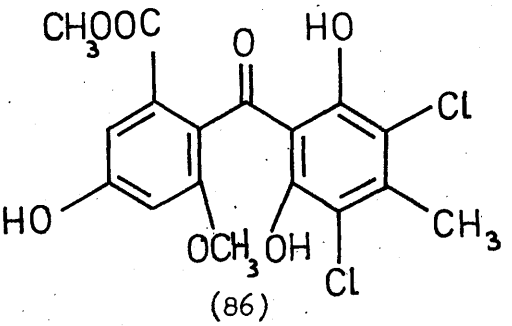

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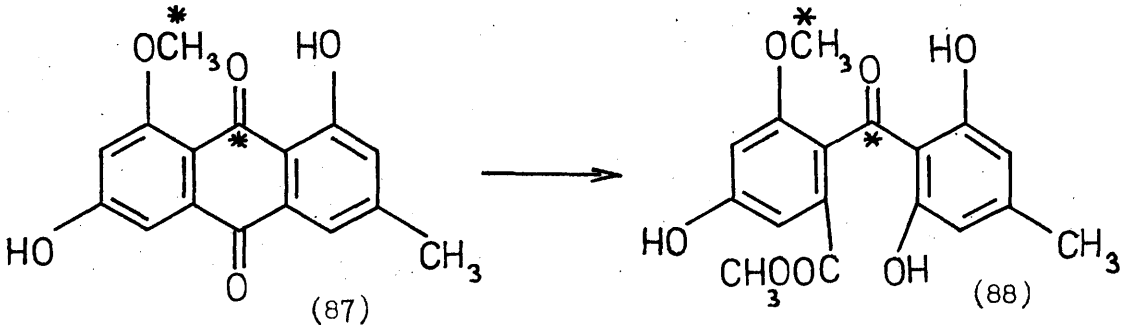


(87) (88)

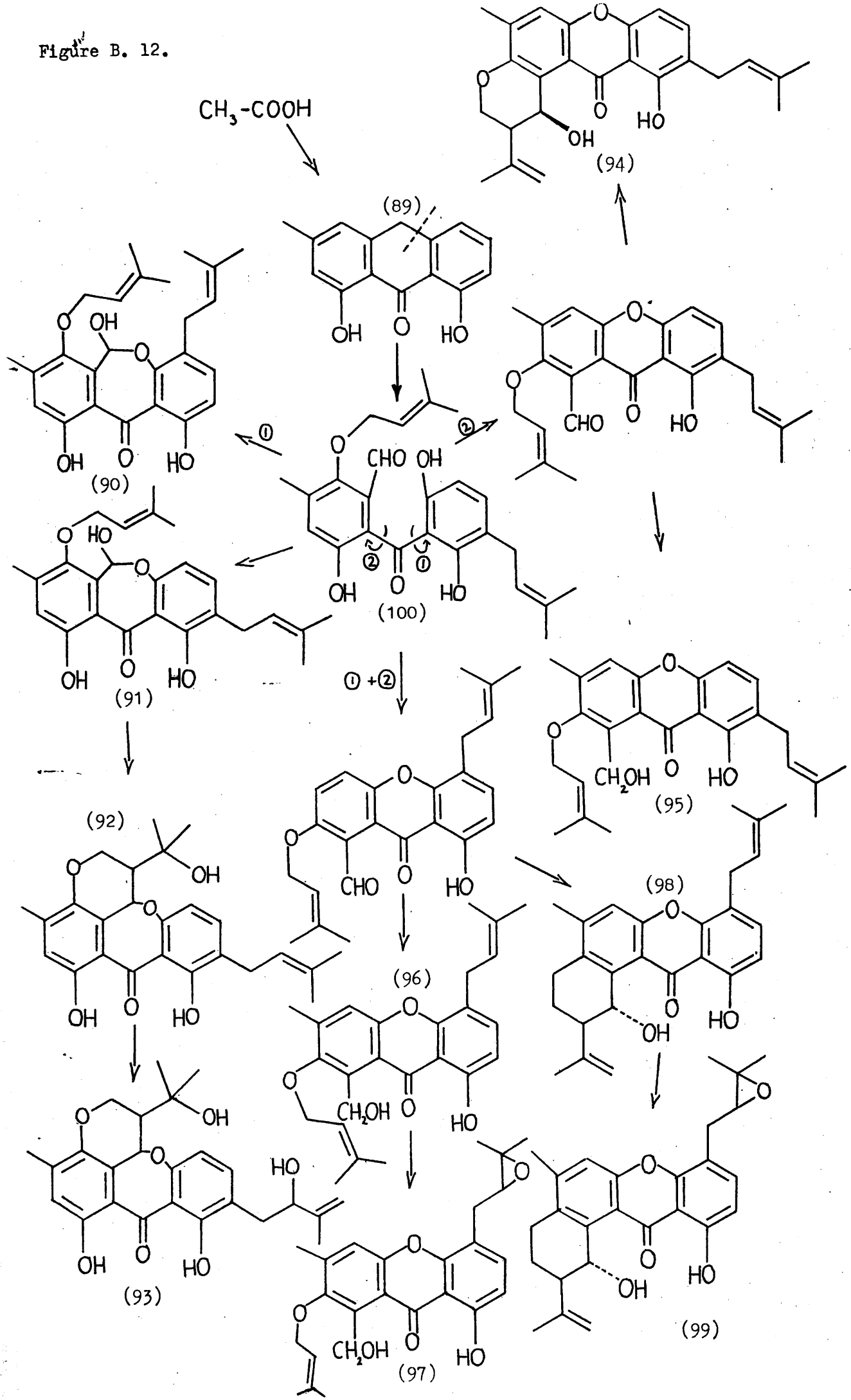
Ascochlorin (76) is an example of a polyketide precursor which has been alkylated, probably with farnesyl pyrophosphate. The terpenoid origins of the aliphatic portion of the molecule were established by ^{13}C-n.m.r. spectroscopy after [1,2-^{13}C]-acetate had been added to cultures of Nectria coccinea[93]. It was established that the farnesyl unit had been modified by migration of the C-14 methyl group from C-1 to C-6. The signals for carbon atoms 4,12 and 15 appeared as enriched singlets indicating their origins from C-2 of mevalonate.

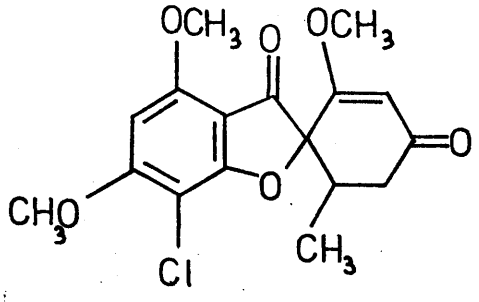
The loss of a carbon atom other than by a decarboxylation process is indicated in the biosynthesis of the fungal metabolite terrein (77)[94]. The separate additions of [1-^{13}C] and [1,2-^{13}C]-acetate to cultures of A. terreus and examination in each case of the ^{13}C-n.m.r. spectra of the isolated (77), indicated the loss of the terminal carboxyl group and one of the carbons of the polyketide chain derived from C-2 of acetate during biosynthesis. Evidence for the proposed biosynthesis from a pentaketide precursor (78) (Figure B.10) was provided by the appearance of two enriched singlets corresponding to the signals for the two hydroxyl-bearing carbon atoms of the cyclopentene ring. This result is in accord with evidence from previous labelling studies[95] and earlier suggestions[95] for terrein biosynthesis.

The addition of [1,2-^{13}C]-acetate to cultures of A. melleus has, in the same way, allowed the elucidation of the biosynthesis of the pyrone (79), a co-metabolite of mellein (80)[96]. The use of doubly labelled acetate has highlighted in (79) the unusual feature of a head-to-head linkage of acetate units, only previously observed in aflatoxins and related compounds (Figure B.11).

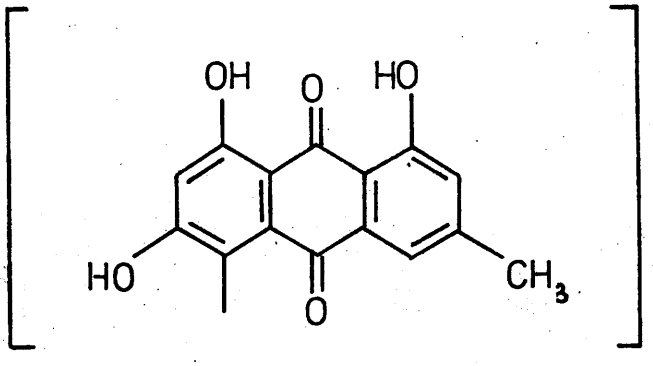
The co-occurrence of the ergochromes (81) and anthraquinones (82) and (83) in cultures of Claviceps purpurea suggested that the former might be formed by oxidative cleavage of an anthraquinone precursor. Radiotracer studies by Franck et al. have shown that emodin (84) is a precursor not only of these compounds[97], but also the chlorine-

Figure B. 12.

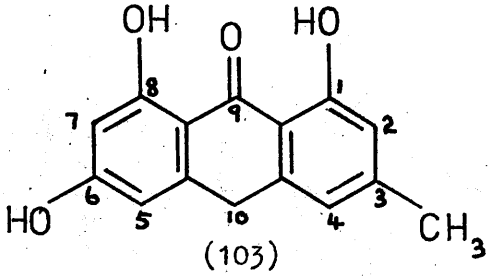



OCH3
O
OCH3
O
CH3O
O
CH3
Cl

(101)


OH
O
HO
HO
CH3
O
2

(102)


OH
O
HO
8
1
9
7
2
6
3
HO
5
10
4
CH3

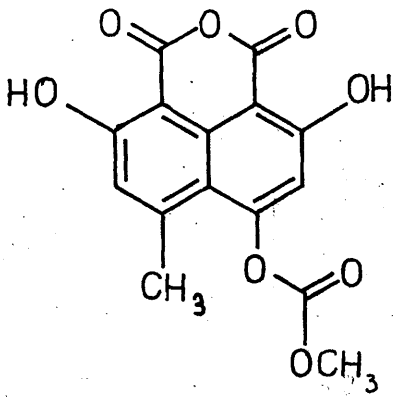
(103)

containing netabolites geodin (85) and dihydrogeodin (86)[98]. Alternative modes of ring cleavage were suggested which would lead to the two classes of compound.

A similar cleavage mechanism to that proposed for geodin biosynthesis has been advanced by Hassal et al. for the biosynthesis of sulochrin (88) from questin (87)[99]. In the same way, oxidative ring cleavage of a postulated precursor, chrysophanol anthrone (89), has been suggested by Holker et al. as the key biosynthetic step which would allow rationalisation of the structures of various metabolites (90-99) co-occurring in some Aspergillus spp.[100]. By a combination of prenylations, ring cleavages, rotations about carbon-carbon bonds and ring-closures, it was suggested that formation of these co-metabolites could be all be related to a common precursor (100)(Figure B.12). Some of the xanthones (90,91,94,95,98,) isolated by Holker's group have also been isolated from mutant strains of A. nidulans in these laboratories[101].

--- The ergochromes (81) illustrate , as well as oxidative ring cleavage, a further feature which is quite common in fungal and plant metabolites, phenol-oxidative coupling. This may be intra-molecular in nature, as has been suggested in the formation of the ether link in griseofulvin (101)[102], or inter-molecular in nature, as in the formation of skyrin (102). Although the monomer, emodin (84) is a co-metabolite of skyrin (102) and incorporated into (102), it was suggested that coupling occurs at a stage prior to quinone formation, possibly at the level of the intermediate anthrones[103]. The higher incorporation of emodin anthrone (103) into (102) tends to confirm this proposal[103].

DISCUSSION



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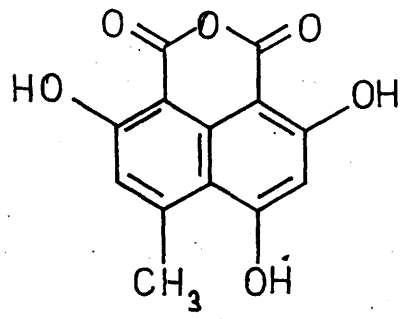

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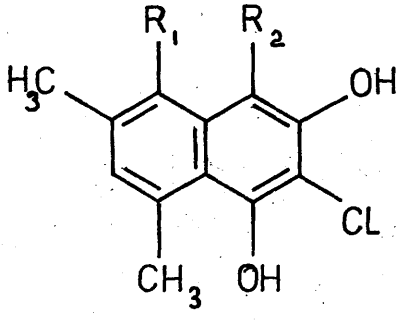
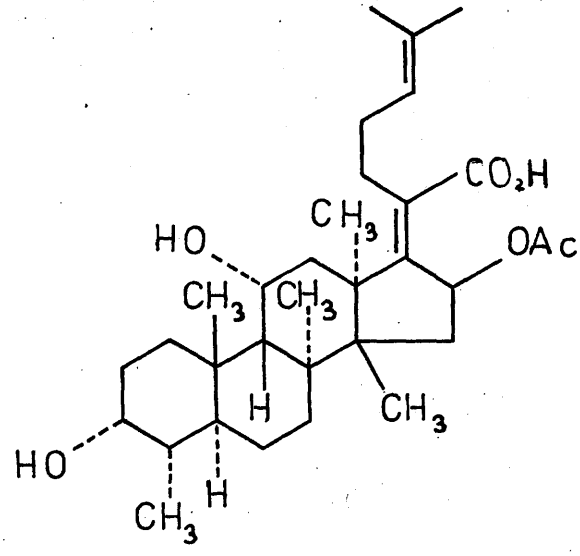


(106)

R_1 = $COOCH_3$, R_2 = COOH

or R_1 = COOH, R_2 = $COOCH_3$.



(107)

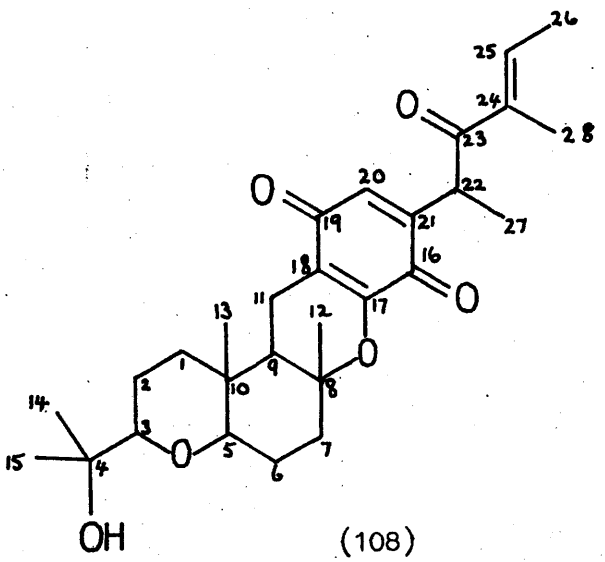
Chapter 1

Culture filtrates of the fungus Verticillium lamellicola have been observed to possess antibacterial activity.[104] In a previous investigation[101] methylene chloride extracts of the broth were examined in an attempt to isolate and identify the active constituent(s).

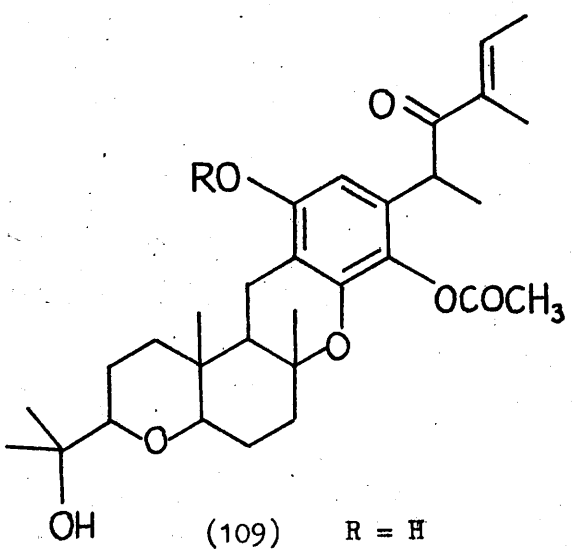
One substance, obtained by chromatographic fractionation of the extract, was identified as 4-O-carbomethoxy lamellicolic anhydride (104), the first reported natural product containing a methyl carbonate unit.

More polar chromatographic fractions provided a crude 'active fraction' with enhancement of actitity over that of the crude extract. Fractional crystallisation of this fraction afforded the major, though inactive, component of the mixture, lamellicolic anhydride (105). Further chromatography of the residue after removal of (105) gave a chlorine - containing metabolite (106), structurally related to (105). The antibacterial activity of the extract remained in the residual gummy mixture but the active component could not be isolated. There was some evidence, though, that the active constituent might be fusidic acid (107) which has been reported as a metabolite of strains of V.lamellicola.[105]

In 1972 heavy bacterial contamination of the fungus under study necessitated the acquisition of fresh stocks of the fungus. The samples obtained (from Centraalbureau Voor Schimmelcultures, Holland; No. 11625) were reported to be morphologically the same as the previous stocks. During column chromatography of the methylene chloride extract of the broth of the new sample, however, a yellow pigment was eluted which had not been observed in extracts of the previous culture. This pigment, which has been assigned the trivial name lamelliquinone, was obtained, after extensive chromatography, as a yellow oil ($C_{28}H_{38}O_6$, parent ion at $^m/_e$ = 470 with an apparent molecular ion at $^m/_e$ = 472). In the present work, this compound has been re-isolated and its structure elucidated.



(108)



(109) R = H

(110) R = $COCH_3$

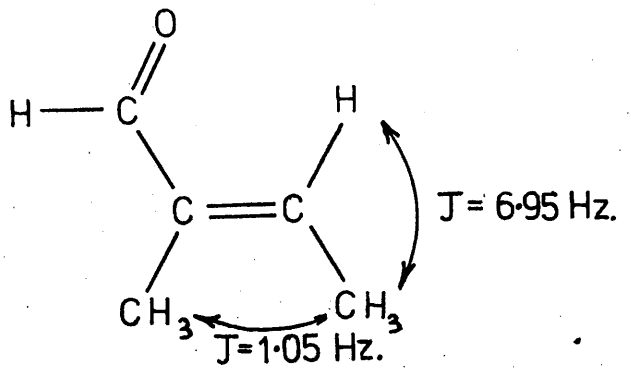
The structure (108) for lamelliquinone was deduced on the basis of both spectral and chemical data. A weak band at 401 nm. (ϵ = 711) and a strong band at 259 nm. (ϵ = 7,969) in the u.v. spectrum of (108) indicated the presence of a p-quinonoid chromophore[106] while a strong band at 232 nm. (ϵ = 12,350) was interpretated as being due to an enone function.[107]

Evidence from the i.r. spectrum of (108) was in accord with this with bands at 1673, 1653 and 1647 cm.$^{-1}$(ν_{co}) and 1605 cm.$^{-1}$ ($\nu_{c=c}$) for the quinone moiety,[113] and strong absorptions at 1665 cm.$^{-1}$ (ν_{co}) and 1605 cm.$^{-1}$($\nu_{c=c}$) for the enone group.

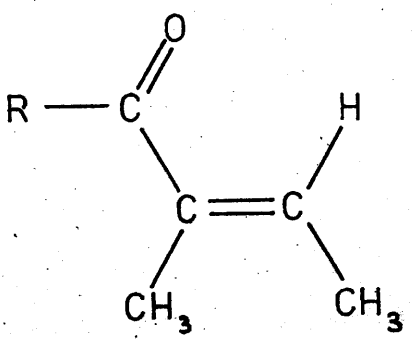
Chemical evidence for the existence of the p-quinone group was obtained from treatment of (108) with zinc dust in acetic anhydride - pyridine[108] at room temperature. In both the resulting mono-acetate (109) (ν_{OH}3540 - 3100 cm.$^{-1}$ ν_{co}1773 cm.$^{-1}$) and the di-acetate (110) (ν_{co}1775 cm.$^{-1}$), a hydroxyl group in the parent compound (ν_{OH}3580 cm.$^{-1}$; τ 7.40) remained unchanged, so suggesting the incorporation of a hindered or tertiary hydroxyl group within the structure of (108).

Attempts to provide chemical evidence for presence of the enone function, by reduction of this group to a saturated ketone using lithium in liquid ammonia,[109] were unsuccessful, no single product being isolable from PLC of the complex mixture of reaction products. However, the n.m.r. spectrum of (108) was useful in establishing the presence of the enone group.

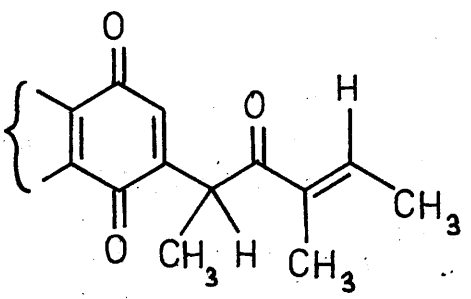
A 1 H low field quartet (τ 3.13, J = 7Hz) was attributable to a vinylic proton geminal to a methyl group and possibly part of an α,β - unsaturated system. The 7 Hz coupling observed compares well with the vicinal coupling (6.95 Hz) between the geminal methyl



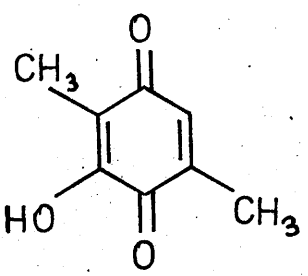
(111)



(112)



(113)



(114)

group and proton in tiglaldehyde (111).[110] Further support for this relationship was obtained from double resonance experiments. Irradiation at τ 3.13 resulted not only in collapse of a broad 3 H doublet at 8.15 (J = 7 Hz), representing the geminal methyl group, but also in sharpening of a second broad 3 H doublet at τ 8.21 (J = 1 Hz); corresponding to a second methyl group. The 1 Hz coupling observed in the signal for this methyl group is the result of homoallylic coupling[111] between this methyl and the methyl, geminal with the proton, above. The absence of further coupling with any of these protons pointed to further substitution of the double bond, possibly by a carbonyl function. A plausible partial structure at this stage was (112). The chemical shifts and coupling constants agree fairly well with those observed for tiglaldehyde (111).[110] The 1 Hz homoallylic coupling observed between the methyl groups in (108) suggested as cis arrangement for these substituents, as a coupling of this size is also observed in(111). Thus the stereochemistry about the double bond was tentatively assigned as in (112).

Additional features in the n.m.r. spectrum of (108) included a further 1 H broad quartet (J = 7 Hz) at τ 5.40 and an associated 3 H doublet at τ 8.69, which collapsed to a singlet upon irradiation at τ 5.40 A second hydrogen - geminal methyl arrangement was thus indicated. The broadening in the resonance for the single hydrogen at 5.40 was removed on irradiation at τ 3.50, this latter signal appearing as a 1 H doublet. This doublet was taken as representing a proton on the quinone ring which was coupled only to the proton at τ 5.40 (irradiation at τ 5.40 resulted in collapse to a singlet). The absence of further coupling indicated that the quinone proton was adjacent to a

Table 1.1. N.M.R. spectral data for (108), (109), and (110).

τ (CCl_4):	(108)	(109)	(110)
H-25	3.20	3.37	3.50
H-20	3.58	3.97	3.77
H-22	5.40	5.63	5.73
H-3,5	ca.6.93	ca.6.93	ca.7.00
H-11	ca.7.53	ca.7.30	ca.7.47
Me-26	8.10	8.20	8.27
Me-28	8.23	8.30	8.33
Me-27	8.69	8.73	8.77
Me-12,14,15	8.80,8.95	8.83,8.90	8.87,8.93
Me-13	9.30	9.30	9.37

Table 1.2 U.V. absorption maxima for (108) and (114)

	λ_{max} (nm.)	log ϵ
(108)	259	3.90
	401	2.85
(114)	266	4.24
	402	2.94

Figure 1.1.

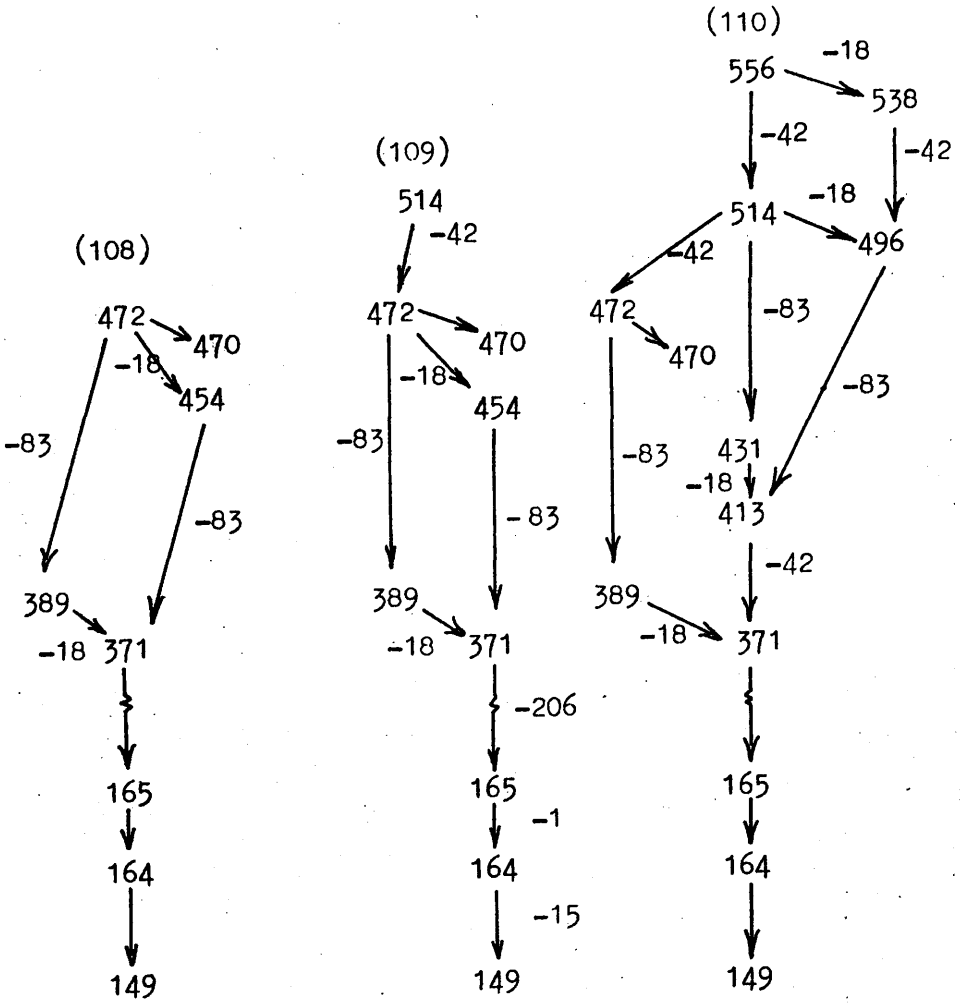
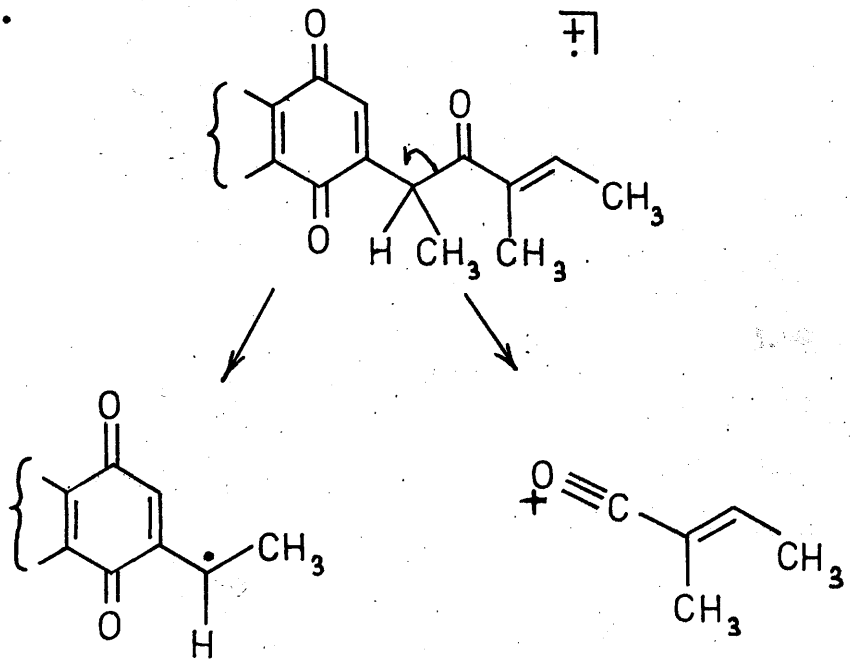


Figure 1.2.



substituted position, most probably by the side chain as in (113). On acetylation, the resonance for this quinone ring proton moved upfield (τ3.58 → 3.97, measured in CCl_4) (Table 1.1), attributable to its transformation into a true aromatic proton with shielding by an ortho phenolic hydroxyl in the mono-acetate (109). This shielding was reduced on further acetylation (τ3.77) to give the di-acetate (110).

The signals observed for the second geminal methyl-proton grouping corresponded to the proton and methyl α to the enone carbonyl group in (113). The proton at τ5.5 is deshielded by the adjacent carbonyl group and vinylogously by the remote quinonoid carbonyl group. Both of these types of protons experienced upfield shifts (τ 5.40 → 5.63 → 5.73; τ 8.69 → 8.73 → 8.77) on conversion of the quinone to the aromatic system (Table 1.1).

The lack of any other low-field protons, aromatic methyls or methoxyls suggested that the quinone moiety was most likely a benzoquinone substituted in three of the four available positions. The absence of a shoulder or maximum around 430 nm. in the u.v. spectrum of (108) suggested that the quinone ring carried an oxygen substituent.[112] A model compound[113] with u.v. absorption maxima very similar to those observed for (108) is 2 - hydroxy - 3, 6 - dimethyl benzoquinone (114)(Table 1.2).

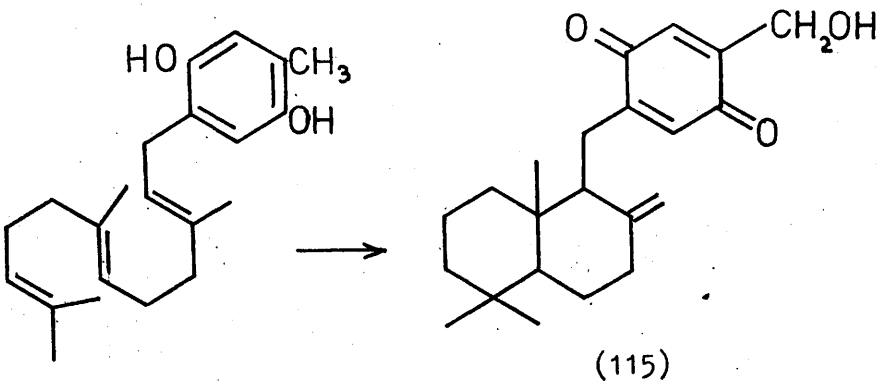
Further evidence for this partial structure in (108) was provided by the fragmentation pattern in the mass spectra of (108),(109) and (110) (Figure 1.1). A prominent peak common to all three spectra appeared at $^{m}/_{e}$ = 83, corresponding to loss of the enone side chain by an α-cleavage process (Figure 1.2). Also, the appearance of an ion at M + 2 in the spectrum of (108) has ample precedent in the mass spectra of p-benzoquinones[114,115]. This is attributable to the two oxygen atoms of the quinone group

each'picking up' a hydrogen atom within the spectrometer source. (Evidence for this process was obtained in one case[115] by flushing the spectrometer source with D_2O prior to obtaining the spectrum. The corresponding peaks appeared at M + 4 in the resulting spectrum).

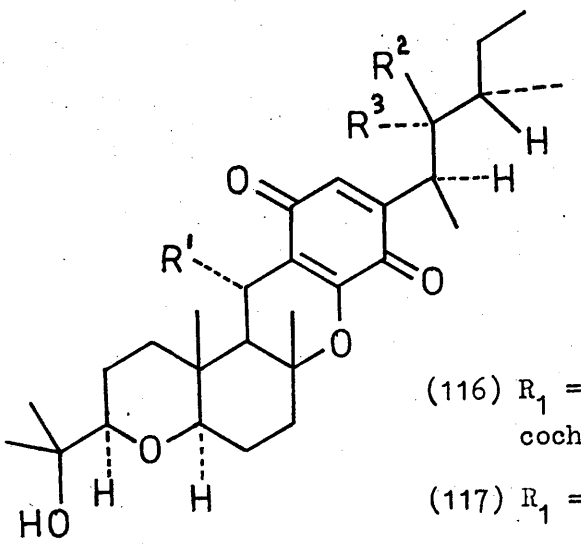
Also in the n.m.r. spectrum of (108), a broad 3 H multiplet corresponding to both the tertiary hydroxyl proton (exchangeable with D_2O) and to benzylic protons (H - 11) appeared at τ 7.40. On conversion of the quinone to the diphenolic mono-acetate (109), the signal for the benzylic protons underwent a downfield shift of 0.23 ppm. (τ 7.53 → 7.30, in CCl_4)(Table 1.1). Apparently a free phenolic group causes peri - deshielding of adjacent benzylic protons. This effect was largely removed (τ 7.47) upon forming the di-acetate (110).

Other features in the n.m.r. spectrum of (108) included a mass of unresolved signals in the region τ 8.0 - 9.0 with sharp singlets at τ 8.80, 8.82, 8.85 and 9.26. This was similar to the complex absorption 'envelope', surmounted by sharp singlets, typical of steroid and terpenoid systems, the broad envelope attributable to methylene groups and hydrogen atoms directly attached to the ring skeleton and the sharp singlets to angular methyl groups[116]. Thus, four methyl groups of this type were indicated in the spectrum of (108). The pattern of signals observed in the high field region of the spectrum pointed to at least a portion of the molecular structure of (108) which was probably terpenoid in origin.

In the partial structure (113), the quinone - enone oxygenation pattern suggests polyketide origins for this grouping, with methylation of the precursor at the positions α to the enone carbonyl group. The fact that (108) appears to be part terpenoid and part quinonoid is reminiscent of

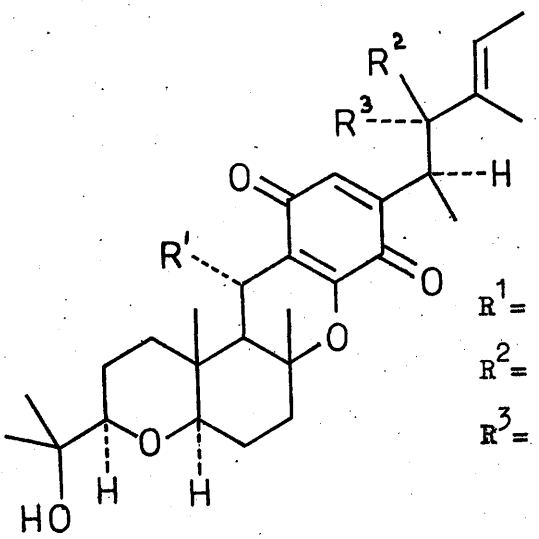


(115)



(116) R_1 = OH, R_2 = H, R_3 = $OCOCH_3$: cochlioquinone A

(117) R_1 = H, R_3R_2 = O: cochlioquinone B



R^1 = OH

R^2 = H

R^3 = $OCOCH_3$

(118)

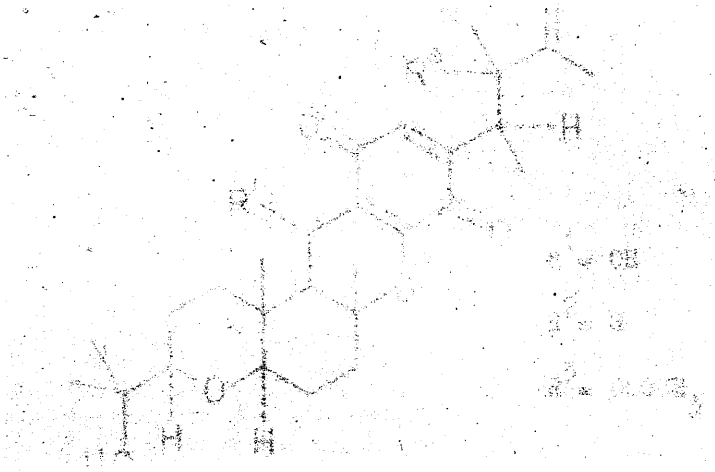
Table 1.3. Resonances of identifiable groups in lamelliquinone (108) in solutions with Eu(dpm)3*

(τ) Group	H-3	Me-14	Me-15	H-5	Me-13	H-20	H-22	H-25	Me-27	Me-28	Me-26
concn 0	6.87	8.80	8.82	6.87	9.26	3.50	5.40	3.13	8.69	8.21	8.15
0.2m	5.02	7.13	7.22	6.32	8.86	3.27	5.27	3.03	8.61	8.10	8.06
0.4m	(2.90)	5.02	5.40	(5.63)	8.38	2.99	5.06	2.88	8.50	8.00	8.03
0.6m	(0.60)	1.80	2.11	(4.58)	7.62	2.41	4.80	2.00	8.28	7.80	7.95
*gradient	9.93	9.45	8.55	3.10	2.20	1.20	0.83	0.60	0.48	0.45	0.15

Values in brackets represent chemical shifts predicted from graph but not identifiable. These signals lay underneath others.

* A graph of change in resonance values ($\Delta\delta$) against reagent: substitute molar concentration ratio was drawn and gradients measured for each identifiable group in the molecule.

A large gradient for particular group indicates that the group is close to the site of complex formation with the shift reagent.



tauranin (115). The terpenoid portion in this molecule is a sesquiterpene and the quinonoid portion is derived mainly from acetate, with the addition of one C-methyl group from S-adenosyl methionine.[117] Recently, however, a new group of compounds of mixed biogenesis, namely the cochlioquinones (116, 117), were isolated from the fungus Cochliobolus miyabeanus[118], having molecular weights closer to that of lamelliquinone (108). Comparison of data for the cochlioquinones and for a further member of the group, stemphone (118), subsequently isolated from Stemphylium sarcinaeforme[112], allowed the full structure (108) to be proposed for lamelliquinone.

Evidence in support of the full structure (108) was obtained when the n.m.r. spectra of lamelliquinone were recorded in solutions of the lanthanide shift reagent, $Eu(dpm)_3$. The signals attributable to H - 3, Me - 14 and Me - 15 exhibited large changes in chemical shift, as would be expected if the $Eu(dpm)_3$ reagent were co-ordinating with its substrate at the tertiary C - 4 hydroxyl group. H - 5 and Me - 13 showed slightly smaller changes as would be predicted on the grounds of distance of these groups from the site of complex formation[119] (Table 1.3).

Further evidence in support of the proposed structure for lamelliquinone was obtained from its ^{13}C - n.m.r. spectrum. Assignments were made on the basis of values reported for model compounds[120] and in particular stemphone (118)[112] and from residual splittings and multiplicities of peaks in off - resonance-decoupled (O.R.D.)^{13}C - n.m.r. spectra of (108) (Table 1.4).

The signal at lowest field (δ 200.6) was assigned

Table 1.4. Assignments of ^{13}C-n.m.r. signals for lamelliquinone (108)

Carbon chemical shift δ_C ppm.	Proton chemical shift δ_H ppm.	Residual coupling in O.R.D. spectrum J_{res} (Hz)	Assignment
200.6	—	s. —	23
186.3	—	s. —	19
180.9	—	d. 5.2	16
153.0	—	s. —	17
146.4	—	s. —	21
138.4	6.87	d. 81.4	25
136.8	—	s. —	24
133.4	6.50	d. 83.5	20
118.9	—	s. —	18
84.5	ca.3.13	d. 36.0	5
83.5	ca.3.13	d. 34.8	3
78.9	—	s. —	4
71.7	—	s. —	8
43.5	?	d. 14.6	9
37.7	4.60	d. 46.2	22
36.8	?	t. 15.6	2,6,7.
26.9	1.20	q. 13.1	14
26.0	1.18	q. 12.4	15
23.8	1.15	q. 12.3	12
23.6	—	s. —	10
21.6	?	t. 15.1	1
16.2	1.31	q. 14.7	27
16.2	?	t. 27.3	11
15.0	1.85	q. 21.3	26
11.5	1.79	d.q. 24.6 3.0	28
11.5	0.74	q. 7.8	13

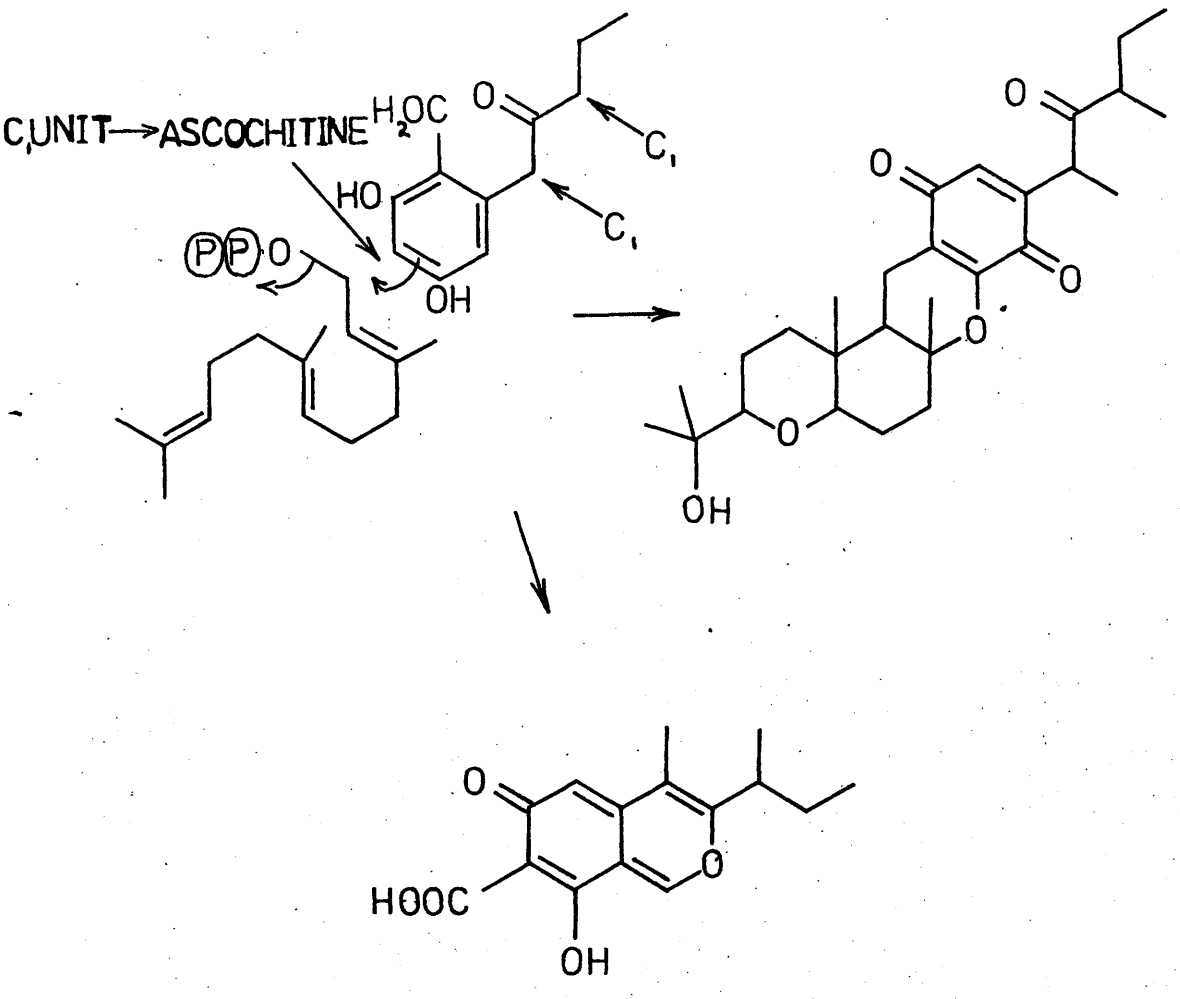
to the carbonyl carbon of the enone system, while two other low-field resonances, at δ 186.3 (singlet in O.R.D. spectrum) and δ 180.9 (doublet, with residual coupling, J_{RES} = 5.2 Hz), were assigned to C-19 and C-16, respectively. The doublet observed for C-16 was the result of transoid vicinal coupling with H - 20. There was no discernible cisoid vicinal coupling between H - 20 and C - 22. This result is in accord with the relative magnitudes ($^3J_{TRANS} > {}^3J_{CIS}$) of the residual couplings observed between the carbonyl carbon and the cis and trans hydrogens in acrolein and acrylic acid.[121] Similarly transoid vicinal coupling was observed between H - 25 and the carbon C - 28 of the vinyl methyl group which appeared as a double quartet (residual couplings $^1J_{C,H-28}$ = 24.6 Hz, $^3J_{C,H-25}$ =3.0 Hz) at δ 11.5. No cisoid coupling of H - 25 with the carbonyl carbon of the enone system (δ 200.6) was observable.

A doublet at δ 133.4 (J_{RES} = 83.5 Hz) was assigned to the hydrogen - bearing carbon of the quinone ring, while singlets at δ 118.9, 146.4 and 153.0 were assigned to the remaining members of the quinone ring - the quaternary carbons C - 18 and C - 21, and the oxygen - bearing carbon C - 17, respectively. These assignments are in good agreement with those for the corresponding carbons in the model, stemphone (118).

A doublet, at δ 138.4 (J_{RES} = 81.4 Hz), was assigned to the olefinic carbon C- 25, while a singlet at δ 136.8 was attributed to the remaining olefinic carbon, C - 24. This last assignment is close to the value for the corresponding carbon in stemphone (132.1).

Upfield, doublets at δ 84.5 (J_{RES} = 36 Hz) and δ 83.5 (J_{RES} = 34.8) were assigned to C - 5 and C - 3, although

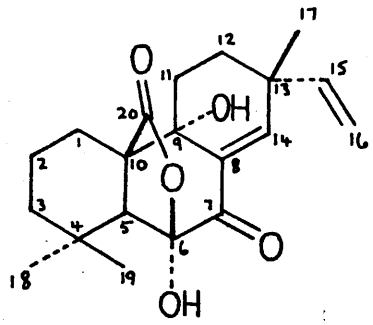
Figure 1.3.



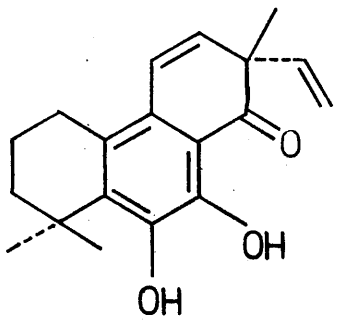
(119)

these assignments may be reversed. A singlet at δ 78.9 was assigned to C - 4 and another at δ 71.7 to C - 8. These resonances all lie within the spectral region expected for a quaternary oxygen - bearing sp^3 - hybridised carbon[120]. Other assignments were made on the basis of multiplicities and residual couplings in the O.R.D. ^{13}C - n.m.r. spectrum of lamelliquinone.

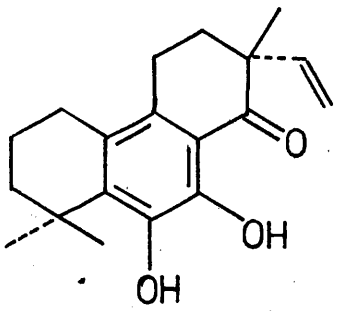
In experiments relating to the biosynthesis of cochlioquinones A and B (116,117), Canonica et al. have established the terpenoid origins of rings A, B and part of C (15% incorporation of [2 - ^{14}C] - mevalonolactone into cochlioquinone A, 3% into cochlioquinone B).[122] It was suggested by this group that the polyketide unit in (116) and (117) had been assembled from two propionate units and four acetate units but they subsequently established that the two methyl groups (Me - 27 and Me - 28) originated from methionine, with the incorporation of [Me - ^{14}C] - methionine into (116) and (117) (1.59% and 0.86%, respectively). Thus cochlioquinone biosynthesis appears to involve the addition of a farnesyl unit onto an aromatic acid derived from a hexaketide and two C_1 units (Figure 1.3). The postulated aromatic precursor is possibly the same as that involved in the biosynthesis of ascochitine (119), isolated from Ascochita fabae.[123] The biosynthesis of lamelliquinone presumably follows a similar course.



(120)



(121)



(122)

Figure 2.1.

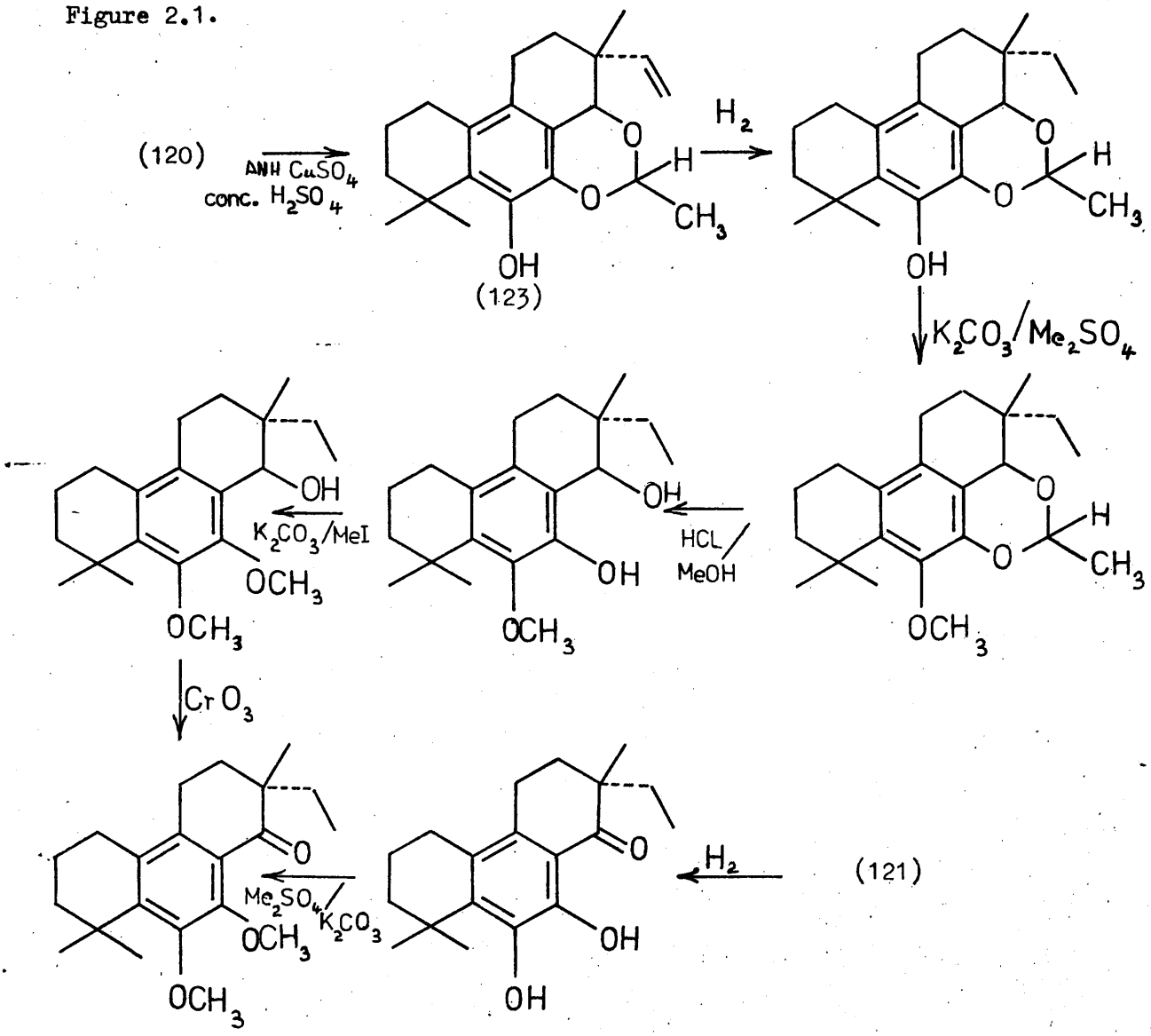
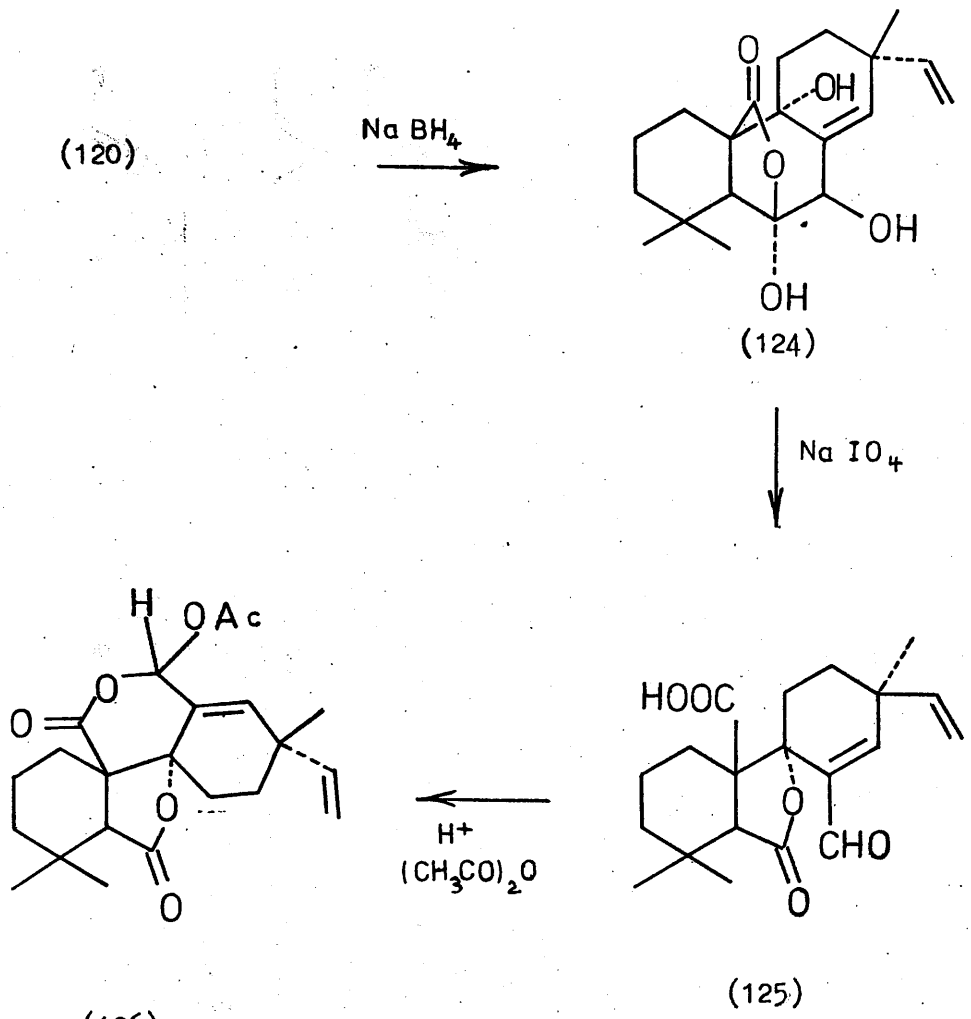


Figure 2.2.


(120)
Na BH4
O
OH
O
OH
OH
(124)
Na IO4
H
OAc
O
O
O
O
H+
(CH3CO)2O
HOOC
O
CHO
O
(126)
(125)

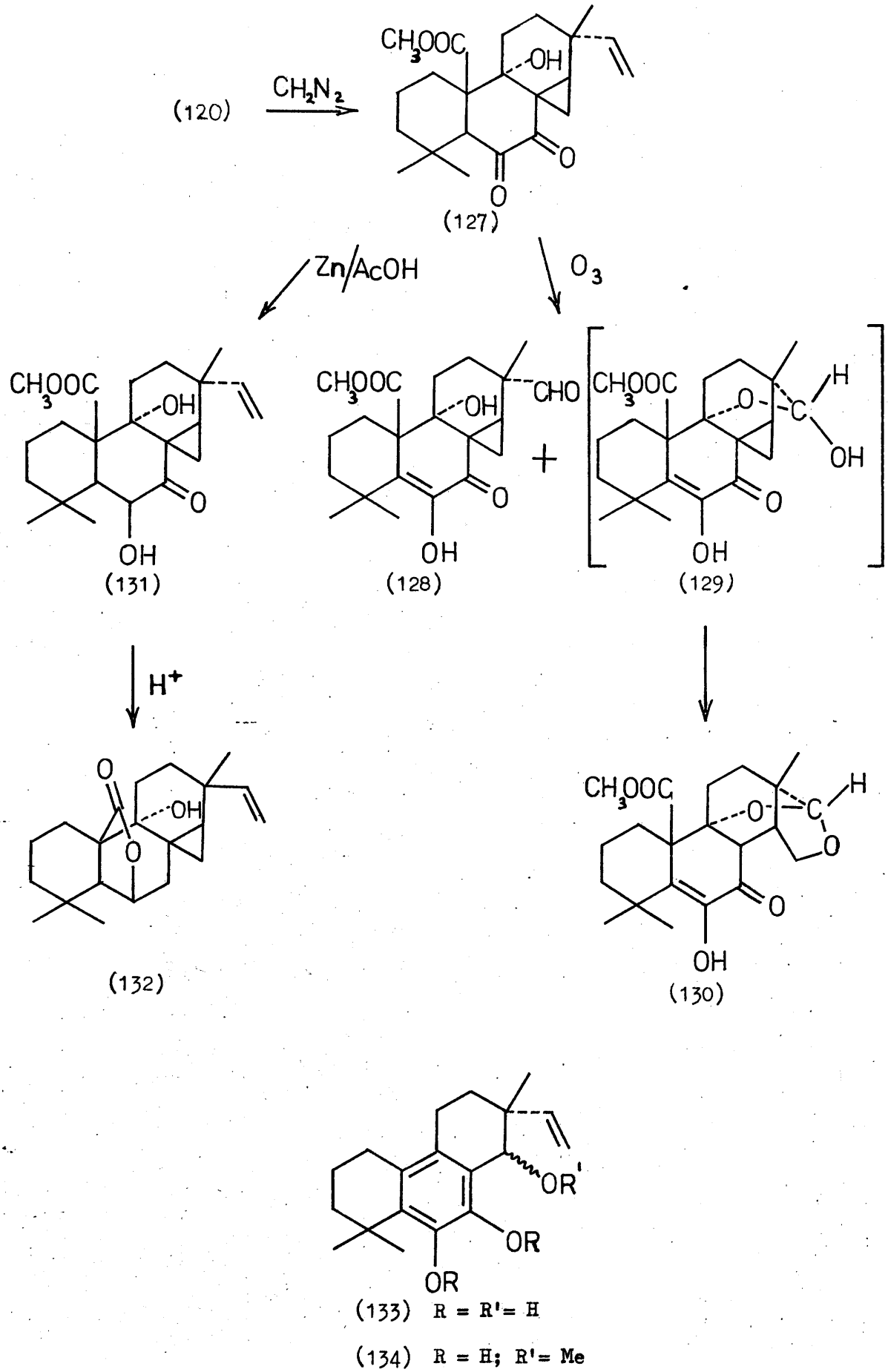
Chapter 2

Culture filtrates of the fungus *Aspergillus flaschentraegeri* are known to possess antibacterial and antifungal properties. The active principle has been isolated[124] and identified[125] as the pimarane - type diterpene, traegeric acid (120). Evidence for terpenoid character in this molecule was provided by the incorporation of $[2 - {}^{14}C]$ - mevalonolactone and formulation as a diterpene indicated by its empirical formula and molecular weight ($C_{20}H_{26}O_5$, M^+ at $^m/_e$ = 346), although other possibilities could not be excluded.

Co-occurring with traegeric acid (120) were the closely related pigments flascherone (121) and dihydroflascherone (122)[125]. The structure (120) for traegeric acid was deduced by establishing an interrelationship between the product (123), obtained from (120) under dehydrating conditions (ethereal copper sulphate - concentrated sulphuric acid), and the transformation products derived from flascherone (121) by the reaction sequence outlined in Figure 2.1[125]. From this it can be seen that the dehydration product (123) played a major role in finally elucidating the structure of the antibiotic.

Previous work with this metabolite has established the relative stereochemistry at C - 5, C - 6, C - 9, C - 10 and C - 13 to be that shown in (120)[125]. Sodium periodate treatment of (124), the product of sodium borohydride reduction of (120), resulted in cleavage of the glycol but the product isolated was the lactone (125) (Figure 2.2). Treatment of (125) with acetic anhydride and a catalytic amount of p-toluenesulphonic acid gave (126)[126]. The formation of (126) established the *trans* relationship of the C - 10 and C - 9 substituents, as inspection of models shows that a C - 10: C - 9 *cis* arrangement would

Figure 2.3.


(120)
CH_2N_2
CH_3OOC
OH
O
O
(127)
$Zn/AcOH$
O_3
CH_3OOC
OH
O
OH
(131)
CH_3OOC
OH
CHO
O
OH
(128)
+
CH_3OOC
O
C
H
OH
O
OH
(129)
H^+
O
OH
O
(132)
CH_3OOC
O
C
H
O
O
OH
(130)
OR'
OR
OR
(133) R = R'= H
(134) R = H; R'= Me

not allow closure between the C - 10 carboxyl and C - 7 aldehyde functions. Further evidence of this trans relationship was indicated by the facile loss of carbonic acid from C - 9 and C - 10 in the formation of the ethylidene acetal (123) during dehydration of (120).

The cis relationship of the C - 9 hydroxyl and C - 13 vinylidene group was established [125] by ozonolysis of the cyclopropyl derivative (127), the unusual product of treatment of (120) with diazomethane (Figure 2.3). The expected aldehyde (128) was isolated along with a second, unexpected product (130). The formation of (130) was explained by inferring attack of the hydroxyl group of the presumed intermediate acetal (129) on the cyclopropyl ring. It was supposed that quantitative conversion of the appropriate diastereoisomer of (128) into the acetal (129) had occurred during work-up of the ozonolysis reaction and, in confirmation, it was found that the hydroxy aldehyde (128) isolated, comprising the opposite 8,14-diastereoisomer, could not be induced to form (130).

Treatment of (127) with zinc and acetic acid gave the dihydro compound, (131). The observed coupling constant between H - 5 and H - 6 of 11 Hz might be interpreted as indicating a trans diaxial relationship between these hydrogens. This would be possible only if the A/B ring junction were cis and the OH at C - 6 β or, alternatively, the A/B ring junction were trans and the OH at C - 6 α . Since reduction of the carbonyl group can be presumed to occur from the least hindered side and in view of the ready lactonisation of (131) to (132), the OH at C - 6 would appear to be β , suggesting that the A/B ring junction is cis.

However, the observed size of $J_{5,6}$ is also compatible with a trans ring junction if ring B adopts a conformation such that H - 5 and H - 6 lie at a dihedral angle of near 0°.

In the derived lactones (132), the dihedral angle for H - 5 and H - 6 would be 45° if the A/B ring junction were cis and 90° if the ring junction were trans. Since the observed $J_{5,6}$ in this compound was zero, a trans A/B ring junction was indicated.

The structures (120) and (124) have been reported by an American group for two co-occurring antibiotics (LL - S491β and LL - S491γ, respectively), isolated from a strain of Aspergillus chevalieri[128]. Here evidence for the carbon skeleton and masked α- diketone system in (120) was obtained by treatment of (120) with methanol - hydrochloric acid, giving the catechols (133) and (134). The relative stereochemistry at the A/B ring fusion was deduced from a downfield shift, in the n.m.r. spectrum of (120), of 0.56 ppm. for H - 5 when run in pyridine rather than $CDCl_3$. This result necessitated a 1,3 - cis diaxial relation between the C - 9 hydroxyl and H - 5 [129]. The relative stereochemistry at C - 10 and C - 9 was determined by the same method employed in these laboratories and this, taken together with the evidence for a cis relationship between the C - 9 hydroxyl and H - 5, indicated a trans A/B ring fusion.

The relative and absolute stereochemistries at C - 5, C- 6, C - 9, C - 10 and C - 13 in LL - S 491β and C - 5, C - 6, C - 7, C - 9, C - 10 and C - 13 in LL - S 491γ were established by comparison of the C.D. spectra of these two metabolites with those of model compounds of known absolute stereochemistry, and are as shown in (120) and (124). Similar optical data for the metabolite

isolated in these laboratories means that this metabolite also has the absolute stereochemistry shown in (120). With the structure of traegeric acid now established, attention was directed at elucidating the biosynthesis of the metabolite.

Early workers isolated and purified metabolites from culture filtrates of A. flaschentraegeri by a tedious and laborious procedure involving solvent extraction of the broth with acetone or chloroform and preliminary separation of metabolites in the extract by column chromatography, followed by PLC of appropriate fractions. Recently, however, it was found that extraction of the broth with ethyl acetate and fractional crystallisation of the extract with suitable solvent systems (e.g. ether - light petroleum) afforded traegeric acid (120) more easily and in better yield than before (e.g. 3g. crude traegeric acid, compared with 1.5g. previously, from 15g. extract)[126]. Further, the change in solvent also allowed a more selective extraction, with only two metabolites present in the ethyl acetate extract in any quantity, a major component (traegeric acid) and a minor component which behaved on TLC in a similar manner to the sodium borohydride reduction product (124). This component corresponds to the metabolite LL - S 491 γ isolated by the American group.

Since the diterpene fraction produced consisted of the two closely related compounds (a ketone and the corresponding alcohol), it was considered that a higher yield of purer metabolite would be obtained if the mixture were reduced with an excess of sodium borohydride and the resulting dihydrotraegeric acid (124) isolated.

Prior to this, however, it was necessary to assign the signals in the proton - noise-decoupled ^{13}C - n.m.r. spectrum of (124). To this end, a sample of traegeric acid (120) was

Table 2.1. ^{13}C-n.m.r. data for traegeric acid (120)

C atom	δ[1] ppm.	Multiplicity and $J_{res.}$	Int. n.a. spectrum	Int. $[1-^{13}C]$ spectrum	Normalised[2] int. $[1-^{13}C]$ spectrum	% Peak enhancement	Int. centre peak $[1,2-^{13}C]$ spectrum	Normalised[3] int. centre peak	% Peak enhancement	$^{1}J_{C-C}$ Hz.
1	22.7	t 12.3	169	91	177	5%	181	215	32%	—
2	17.9	t 14.0	148	151	294	99%	133		41%	33.0
3	40.4	t 10.6	133	77	150	13%	136		43%	32.8
4	32.2	s —	127	109	212	67%	108		35%	ca.33.2
5	51.1	d 29.6	161	92	179	11%	166		36%	39.1
6	103.6	s —	75	51	99	32%	50		29%	39.5
7	191.6	s —[5]	56	20	39	-30%	49	58	8%	—
8	132.7	s —	61	52	101	66%	44		36%	69.0
9	71.1	d 3.7[4]	84	44	86	2%	72		21%	39.5
10	57.1	s —	72	67	130	81%	72		14%	47.9
11	26.8	t 18.1	157	164	319	103%	134		42%	39.2
12	29.3	t 17.5	147	83	162	10%	202	240	70%	—
13	39.4	s —[5]	103	92	179	74%	76		54%	ca.32.8
14	152.4	d 78.7	164	80	156	-5%	128		40%	68.5
15	144.3	d 65.6	113	62	121	7%	82		48%	70.0
16	113.3	t 60.6	203	206	401	98%	144		50%	70.1
17	22.4	q 11.9	128	66	129	0.4%	126		ca.50%	ca.35.0
18	32.4	q 12.1	118	67	130	11%	171	204	79%	—
19	24.3	q 11.4	118	66	129	9%	118		56%	33.9
20	174.6	s —	52	27	53	1%	28		59%	46.4

1) Downfield from Me_4Si.

2) Normalisations achieved by comparing intensities of (predicted) non-enriched positions with those of the corresponding positions in the spectrum of unlabelled material, calculating a "scaling factor" for each position, and so an average scaling factor. All experimental intensities were then multiplied by this scaling factor, giving normalised intensities.

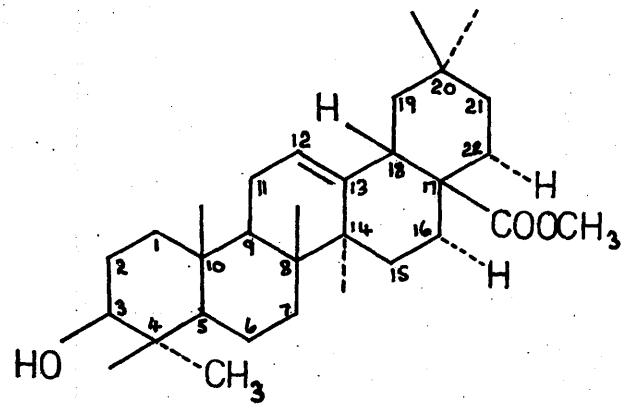
3) Only those signals with no satellite peaks require normalisation. The intensities of the centre peaks were compared with the intensities of the peaks in unlabelled material and an average scaling factor calculated. This was used, as before, to calculate normalised intensities, but, this time, for these four peaks only.

4) Vicinal (^{3}J) coupling between H-14 and C-9.

5) Drop in signal intensity relative to intensity of signal for C-10.

Table 2.2. ^{13}C-n.m.r. data for (124)

C atom	ppm.	Multiplicity and $J_{res.}$ Hz.
1	24.2	t ca. 15.0
2	18.8	t 15.9
3	41.3	t 10.7
4	32.2	s —
5	52.6	d 31.1
6	?	?
7	74.9	d 54.1
8	136.9	s —
9	71.4	d 3.7[4]
10	59.2	s —
11	28.3	t 20.2
12	30.5	t 10.8
13	37.8	s —[5]
14	131.3	d 72.0
15	147.6	d 66.5
16	110.7	t 55.4
17	21.7	q 12.3
18	32.2	q ca. 12.6
19	23.9	q 8.0
20	176.4	s —



(135)

treated with sodium borohydride and the ^{13}C - n.m.r. spectrum of the product (124) obtained in a $CDCl_3/d_5$ - pyridine solvent mixture. The ^{13}C - n.m.r. spectrum of (120) was also obtained, in $CDCl_3$.

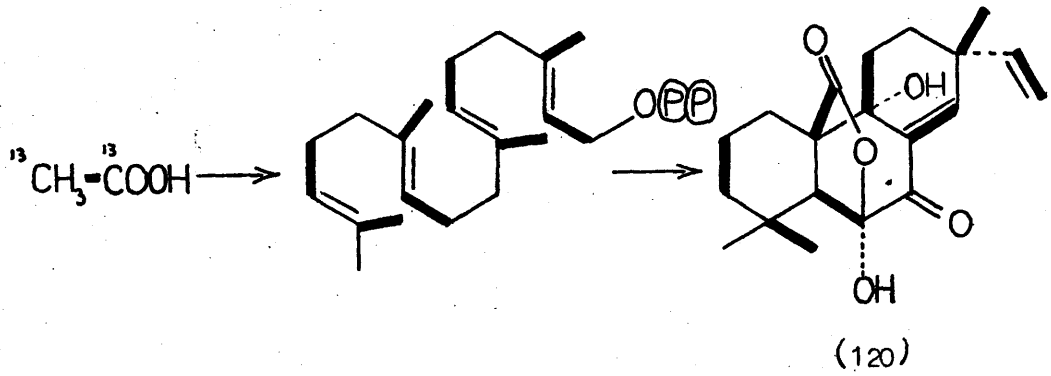
Partial spectral assignment followed from chemical shift data[120] and values reported for suitable model compounds (e.g. pimaradiene[130] and virescenols A and B[131]) and the multiplicities and residual couplings of peaks in O.R.D. ^{13}C - n.m.r. spectra (Tables 2.1 and 2.2). One feature of particular interest in the O.R.D. ^{13}C - n.m.r. spectra of both (120) and (124) was the transoid vicinal coupling between H - 14 and C - 9. The residual coupling constant ($^3J_{RES}$) was in each case 3.7 Hz. Cisoid vicinal coupling was less obvious with only a drop in signal intensity for C - 7, relative to C - 10, due to H - 14: C - 7 coupling. This type of coupling has been reported in the case of the olean - 12 - enes (135)[132]. In these compounds, residual splittings were always observed for the quaternary C - 14 resonance as a distinct doublet in the O.R.D. ^{13}C - n.m.r. spectra of these compounds. These results parallel the relative magnitudes ($^3J_{TRANS} > {}^3J_{CIS}$) of couplings observed[121] in acrolein and acrylic acid, as described earlier. Vicinal coupling was also observed in (120) and (124) between C - 13 and the C - 16 olefinic protons. In this case, however, the coupling was not so distinct and, in the O.R.D. ^{13}C - n.m.r. spectra of both (120) and (124), was manifested only by a drop in the signal height for C - 13 relative to that for the quaternary carbon - 10.

Examination of the proton - noise-decoupled ^{13}C - n.m.r. spectra of (120) and (124) showed that there were in the spectrum of(120) twenty clearly distinguishable peaks, while in the spectrum of (124) only nineteen definite signals were discernible. A small

signal at δ 136.1 was visible but it was considered that this was not a reliable enough signal to be definitely assigned to the twentieth carbon atom. It was concluded that either this very small peak represented the signal for the twentieth carbon atom or that the 'missing' signal accidentally coincided with another and was not distinguishable even in the O.R.D. ^{13}C - n.m.r. spectrum of (124). Because of the relative difficulty so encountered in the assignment of peaks in (124) compared with (120), this approach - via (124) - to elucidating the biosynthesis of (120) was abandoned and efforts were concentrated on studying (120) itself. In later work it was found that samples of (120) could be obtained in sufficient quantity and purity to enable spectral examination to be carried out without recourse to reduction to (124).

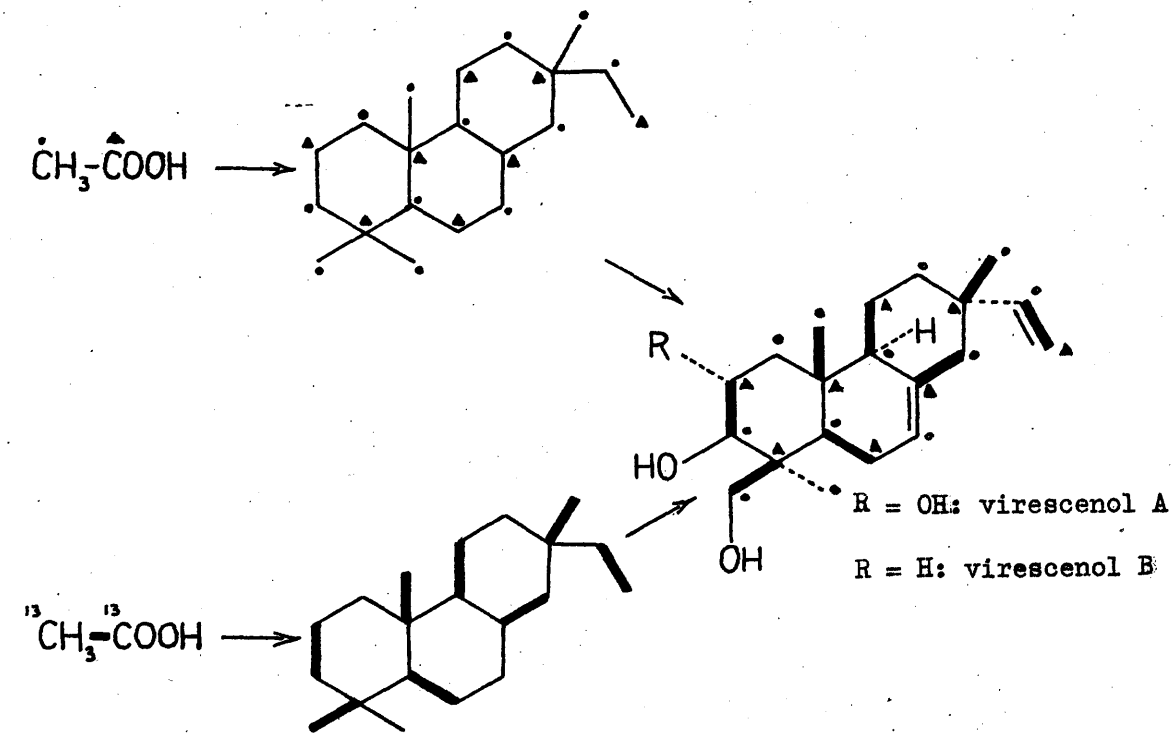
Complete spectral assignment of (120) was aided by the additions of $[1 - ^{13}C]$ and $[1,2 - ^{13}C]$ - acetate to cultures of A. flaschentraegeri and isolation of the appropriately labelled samples of (120). $[1 - ^{13}C]$ - sodium acetate was added to 5 - day old cultures of the fungus (0.1 mg.ml.$^{-1}$ of culture fluid) and again, at the same concentration, 24 hours later. This procedure was adopted in order to avoid possible detriment to fungal growth by the presence of large concentrations (> 200μg. ml.$^{-1}$) of acetate [68b] and also because it was found that the addition of precursors in 'pulses' resulted in a more efficient incorporation of precursor into the metabolite under study. In the proton-noise- decoupled spectrum of traegeric acid (120) isolated (0.03 mg. ml.$^{-1}$) from these cultures, peak enhancements averaging ca. 80% compared with the peak intensities of unlabelled material, were observed for 8 signals corresponding to the carbons derived from positions C - 3 and C - 5 in each of four molecules of the precursor mevalonate.

Figure 2.4.



(120)

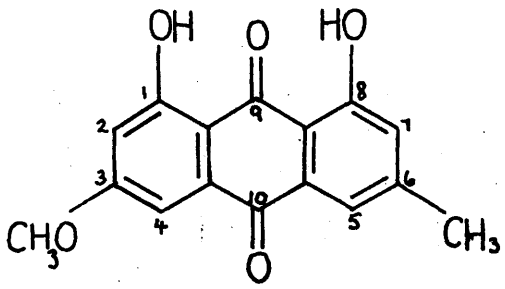
Figure 2.5.



In the addition of $[1,2-{}^{13}C]$ - acetate, suitably labelled traegeric acid (120) was obtained by feeding a 2:1 mixture of unlabelled and $[1,2-{}^{13}C]$ - acetate to cultures of the fungus 96 hours after innoculation (0.09 mg. $ml.^{-1}$ culture fluid) and in five further pulses of equal concentration at 12 - hour intervals. Labelled traegeric acid (120) was recovered (0.02 mg. $ml.^{-1}$ culture fluid) after a further twelve hours. In the proton-noise - decoupled ^{13}C - n.m.r. spectrum of (120) labelled in this way, sixteen signals with satellites due to $^{13}C - {}^{13}C$ coupling were observed indicating the incorporation (via mevalonate) of eight molecules of acetate intact. In addition, four enriched singlets were observed each of which corresponded to the carbon atom derived from C - 2 of the precursor mevalonate, indicating that(120) is assembled from four MVA units(Figure 2.4). $^{13}C - {}^{13}C$ coupling constants in the spectrum of (120) labelled with $[1,2-{}^{13}C]$ - acetate were useful in assigning, unambiguously, those peaks which could not be definitely assigned previously on the basis of literature values and comparison with model compounds.

Overall examination of the spectra of traegeric acid (120) led us to conclude that the biosynthesis of (120) followed the pattern expected for a pimarane - type diterpene as outlined in Figure 2.4. One result, which supports the previously outlined mechanism of terpenoid biosynthesis (see introduction, part A) is the absence of scrambling of label between the carbon atoms of the C - 18 and C - 19 gem - dimethyl groups. The sharp singlet, in the noise - decoupled spectrum of the $[1,2-{}^{13}C]$ - acetate labelled traegeric acid, for C - 18, as for C - 1, C - 7 and C- 12, indicated quite clearly the biosynthetic origins of these carbon atoms from C - 2 of MVA. A similar unambiguous result was reported in studies related to the biosynthesis of other fungal diterpenes,

the virescenols (from Oospora virescens).[133] Here, too, no scrambling of label was observed between, in this case, the hydroxyl - bearing C - 19 and the C - 18 methyl group carbon (Figure 2.5).



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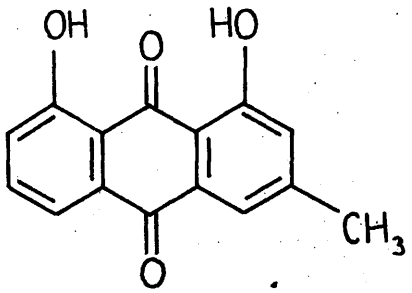

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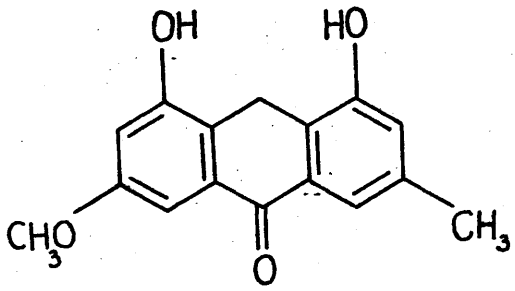
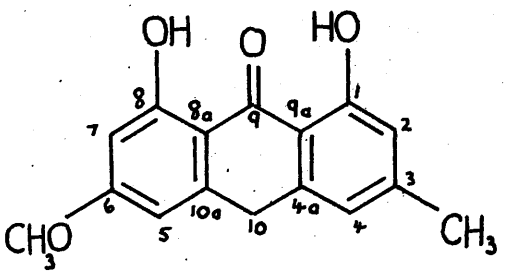


(138)



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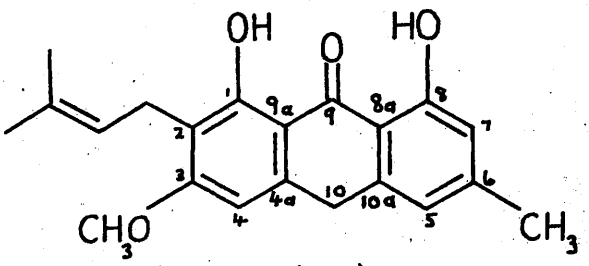


(140)

Chapter 3

Physcion (136) is an orange pigment widely distributed in nature, having been isolated from various fungi, lichens and higher plants.[133] It frequently occurs as its glycoside and often in association with related compounds such as emodin (84) and chrysophanol (137).

Physcion was isolated from the root bark of the Indian shrub Ventilago madraspatana by Perkin and Hummel in 1894.[134] These authors also reported the isolation, in addition to physcion, of two other compounds - 'physcion anthrone A' (138) and 'physcion anthrone B' (139) - both of which they converted into physcion by oxidation. The biosynthetic importance in the co-occurrence of such closely related metabolites was noted by these workers.

The isolation of physcion anthrone B, together with a related metabolite 'dehydroemodin anthrone monomethyl ether', from chrysarobin, a crystalline exudation from the wood of various Andira spp., was reported by Tutin and Clewer in 1912[135], but no further reports of the isolation of physcion anthrone A (138) appeared until in 1939, along with (139) and several other pigments, Raistrick et al.[136] obtained a dull yellow compound with similar properties to those reported for (138), from mycelial extracts of various Aspergillus spp.

Recently, physcion (136) and a related pigment 2 - (γ,γ - dimethylallyl) - physcion (73) (chapter 4) were isolated from extracts of the broth of Aspergillus flaschentraegeri.[125] The latter metabolite has also been obtained[137] from mycelial extracts of the same fungus along with the related pale yellow metabolite physcion anthrone (139). In addition, there was in this last investigation evidence for the presence of 2 -

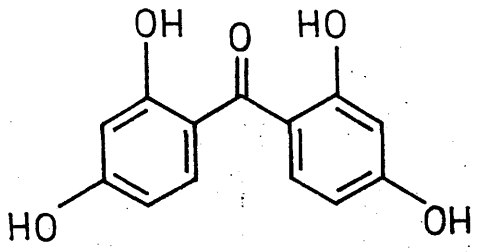
(γ, γ - dimethylallyl) - physcion anthrone (140), the anthrone analogue of (73).

The present work originated as an attempt to establish the biogenetic relationship between the above mentioned metabolites, in particular the order in which prenylation, methylation and oxidation occur. While this goal has not in fact yet been reached, several of the above metabolites have been reisolated and a number of other metabolites - some novel, some not previously isolated from this fungus - have been obtained. The presence of one of these metabolites (assigned the trivial name asperflaschin) may have important implications in the biosynthesis of 2 - (γ, γ - dimethylallyl) - physcion (73) (chapter 4).

Physcion (136) was obtained as an orange amorphous solid, m.p. 206 - 8°C (lit.,[133] 207°) from fractions eluted (90% chloroform - 10% light petroleum) from a column of ethyl acetate extract of the mycelium of A. flaschentraegeri. Its identity was established by comparison (TLC, m.p., spectral data) with an authentic sample of physcion.

Examination of the strongly orange - coloured column fractions (21 - 38) and (39- 58), as likely sources of physcion and related pigments, was undertaken but the isolation of any of these compounds in a pure state was greatly hampered by the presence in these fractions of large quantities of fatty material. However, repeated fractional crystallisation of these mixtures did eventually afford samples of 2 - (γ, γ - dimethylallyl) - physcion (chapter 4).

At about this time, a report appeared [138] describing the isolation of anthraquinones from the mycelium of Pyrenophora avenae by immersing the mycelium in light petroleum (b.p. 100 - 120°C)



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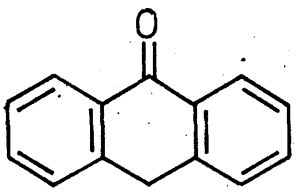

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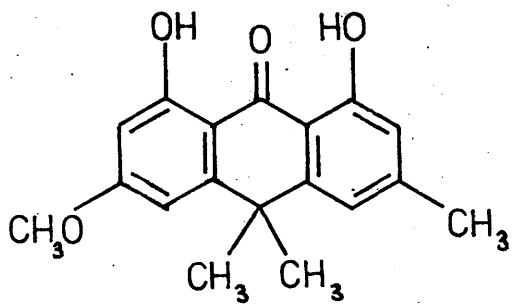

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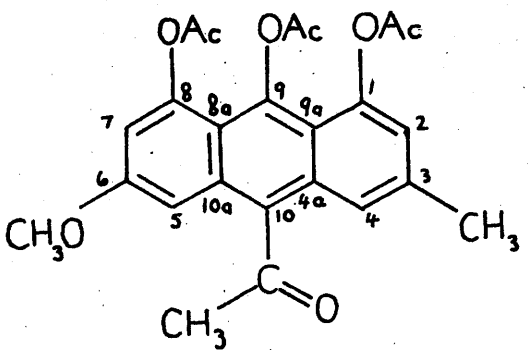


(144)

for one day and evaporation of the solvent to leave a deposit of crude anthraquinones. It was reported that obtaining anthraquinones from live mycelium in this way was unlikely to favour artifact formation and that successive samples could be obtained from a single batch of mycelium, as light petroleum (b.p. 100 - 120°C) had been found to be harmless to living mycelium. By application of this procedure in a modified form, the isolation of physcion anthrone (139, $C_{16}H_{14}O_4$, M^+ at $^m/_e$ = 270) as pale yellow fluffy crystals, m.p. 185 - 7°C (lit.,[136] 181 - 2°), was achieved more conveniently and in far higher yield than before (typically 200 mg.l.$^{-1}$ compared with 10 mg.l.$^{-1}$ previously[137]). (Physcion anthrone could also be obtained as one component of a mixture isolated as an orange band, R_f 0.50 (50% chloroform - light petroleum) by PLC of crude (ethyl acetate) mycelial extract. However, the great difficulty encountered in attempting to separate this metabolite from the other component of the mixture, physcion (136), precluded this method as a practicable route to the isolation of pure physcion anthrone. This similarity in physcial properties between the two metabolites and the concomitant difficulty in separating them has been noted before[136]).

The u.v. data recorded for the present metabolite are in good agreement with those reported [137,139] for 'physcion anthrone B'. The long wavelength absorption (352nm. in ethanol) is close to the absorption maximum reported [140] for the tetrahydroxybenzophenone (141) and this band undergoes an irreversible bathochromic shift (515 nm.) on basication, probably due to the formation of the quinone by autoxidation.

In the n.m.r. spectrum of (139), signals for an aromatic methyl group (3H, τ 7.64), aromatic methoxyl group (3H, τ 6.16)

and chelated hydroxylprotons (2H, τ - 2.59, - 2.29) were evident. In addition a broad 2H singlet (τ 5.81), absent in the product obtained by treatment with Jones' reagent, was taken to indicate the presence of an anthrone methylene group. The corresponding signal in the parent compound (142) appears at τ 5.68.[137] Two broad 2H singlets at τ 3.34 and τ 3.64 correspond to the aromatic protons H - 2,4 and H - 5,7 respectively. The upfield signal represents the two protons in the di-oxygenated aromatic ring.

Evidence for the substitution pattern was obtained from nuclear Overhauser effect (N.O.E.) studies on the pigment. Irradiation at τ 7.64 resulted in an 8% enhancement in peak intensity of the signal at τ 3.34 and irradiation at τ 6.16 produced an 18% enhancement of the upfield signal τ 3.64. The absence, in each case, of enhancement of the signal intensity of the other peak confirms the respective signal assignments above.

In addition, irradiation at τ 5.81 resulted in a 13% enhancement in signal intensity for the peak at τ 3.64 and a 16% enhancement of the signal at τ 3.34. This result now precludes the structure (138) for 'physcion anthrone B' (m.p. 181 - 2°) as in this structure there are no aromatic protons <u>peri</u> to the anthrone methylene protons, these positions being substituted by hydroxyl groups. The substitution pattern in (139) also follows from these results. This structural assignment for physcion anthrone B is in accord with previous results.[139]

Treatment of (139) with potassium carbonate and excess methyl iodide in acetone gave the gem-dimethyl compound (143), as indicated by loss of the signal for the anthrone methylene protons and the appearance of a 6H singlet (τ 8.38) for the gem - dimethyl protons in the n.m.r. spectrum of (143)[141].

Thus C - methylation, instead of the intended O- methylation, had taken place, The relative acidity of these bridge protons is probably attributable to them being both 'doubly benzylic' and, in a vinylogous sense, α to the remote carbonyl group.

This reactivity was emphasised when (139) was treated with pyridine - acetic anhydride. While treatment of (139) with refluxing acetic anhydride gave only a complex mixture of products, overnight stirring with pyridine - acetic anhydride gave the acetyl derivative (144). This compound ($C_{25}H_{22}O_9$, M^+ at $^m/e$ = 438) was obtained as a pale yellow amorphous solid, m.p. 279-80°C (lit.,[139] 275 - 6°)

Evidence for structure (144) for this compound came from i.r. ($\nu_{C=O}$, acetates, 1765 cm. $^{-1}$, $\nu_{C=O}$ ketone, 1695 cm. $^{-1}$) and mass spectral data (ions at $^m/e$ = 396, 354 and 312, corresponding to successive losses of 42 mass units - ketene).

In the n.m.r. spectrum of (144) signals for aromatic methoxyl (3H, τ 6.14) and methyl (3H, τ 7.53) groups were evident and for the acetate methyls (9H, τ 7.60, 7.72) and ketone methyl (τ 7.24). Signals for four aromatic protons at τ 2.66 (m., H-4), τ 3.08 (br.d. J = 1.5 Hz, H - 2), τ 3.18 (d. J = 2Hz, H - 7) and τ 3.25 (d. J = 2Hz, H - 5) were also visible. These assignments for the aromatic protons and the methyl group were confirmed by irradiating at τ 7.53, resulting in sharp doublets (J = 1.5Hz) for the two signals at τ 3.08 and τ 2.66. Evidence for the unequal coupling existing between each of these protons and the aromatic methyl group was seen in the multiplet nature of the lower field signal (τ 3.08) which appeared as a broadened doublet, only. Also, in an expansion of the region of the spectrum containing the aromatic methyl signal, this peak appeared as a broadened doublet. Irradiation at τ 3.08 produced only a sharpened

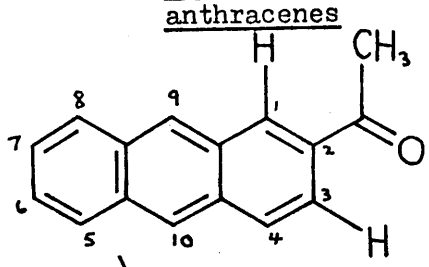
doublet (J = 1.1 Hz) while irradiation at τ 2.66 resulted in collapse of the doublet to a broad singlet.

Further corroborative evidence for the above assignments was provided by irradiation at the chemical shift of the aromatic methoxyl protons (τ 6.14) resulting in enhancements (N.O.E.) of 28% and 17% in the signal intensities at τ 3.25 and 3.18, respectively. The relative magnitudes of these enhancements are in agreement with previous results, which were related [142] to the greater double bond character within the aromatic nucleus for the 5,6 bond compared with the 6,7 bond.

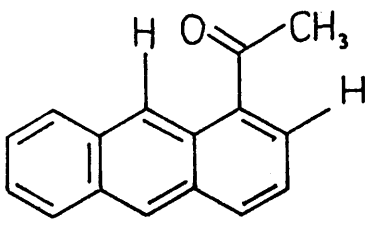
The chemical shift for H - 5 is, compared with that for H - 4, abnormally high considering that both protons are peri to the acyl group at C - 10. At first sight it is not immediately obvious why there should be such a large difference in chemical shift between H - 5 and H - 4.

Gore and Thadani [143] have argued, from comparison of the position of the long wavelength absorption maxima of monoacetyl anthracenes (Table 3.1), that an acetyl group in the 1-position, but more especially in the 9-position, of anthracene cannot be coplanar with the aromatic ring. It was found that the positions of the long wavelength absorption maxima in 1 - , 2 - and 9 - acetyl anthracenes were in the reverse order to the known conjugative powers of these anthryl positions and they attributed this to an acetyl group in the 2 - position being relatively free from interaction with the peri hydrogen atoms of the anthracene nucleus, so enabling it to adopt a conformation in which it is coplanar with the aromatic ring. Thus the acyl group in this compound serves to extend the chromophore (λ_{MAX} 395nm.) In contrast, in the 9 - substituted compound severe steric interaction with the peri protons prevents the acetyl group

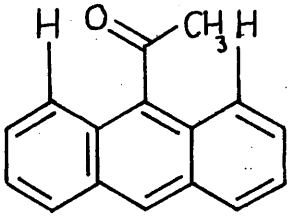
Table 3.1. Long wavelength absorption maxima of acyl-substituted anthracenes



λ_{MAX} 395 nm.



λ_{MAX} 385 nm.



λ_{MAX} 380 nm.

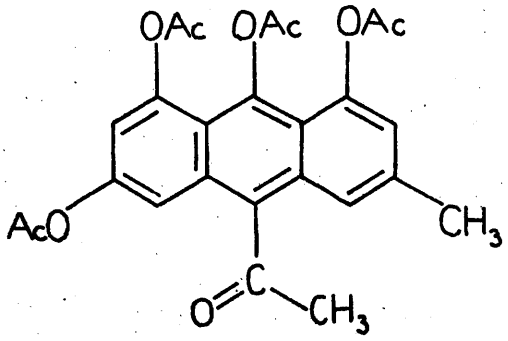
Table 3.2 Chemical shifts of aromatic protons in (144) and (145)

τ ($CDCl_3$):	(144)	(145)
H - 2	3.08	2.97
H - 7	3.18	2.97
H - 5	3.25	2.57
H - 4	2.66	2.57

from assuming a conformation in which it is coplanar with the aromatic ring and the absorption maximum correspondingly appears at a shorter wavelength (λ_{max} 380nm.). In 1-acetyl anthracene an intermediate situation is found. Here the restriction on coplanarity is less than in the case of the 9-substituted derivative but is still sufficient to allow only partial conjugation of the carbonyl group and the aromatic ring. The absorption maximum accordingly appears at an intermediate wavelength (λ_{max} 385nm.).

Evidence from the n.m.r. spectra of 9-formyl and 9-acetyl anthracenes supported this hypothesis. In 9-formyl anthracene the resonances for the peri (H-1, H-8)protons (τ 1.08) appeared 0.99 ppm. downfield compared with H-4,H-5 (τ 2.07); in 9-acetyl anthracene H-1,H-8,H-4 and H-5 had rather similar shifts (τ 2.26,complex multiplet). It was argued that these results showed how the CHO group can approach more closely to the peri (H-1, H-8) protons than the bulky $COCH_3$ group.

In the present compound (144), the situation is slightly different as the resonances for one of the two hydrogen atoms peri to the C-10 acetyl group is deshielded (τ 2.66) while the resonance for the second peri proton lies considerably upfield from the first. It is suggested that this difference, which is too large to be attributable merely to the difference in π-electron densities of the respective aromatic rings,is the result of a preferential orientation of the C-10 acetyl group such that the "carbon end" of the C=O dipole is directed towards the di-oxygenated ring which is, due to the greater electron-donating ability of a methoxyl substituent compared with a methyl group, a relatively "electron-rich" ring compared to the methyl-substituted ring. With such a situation, it is now possible to attribute the shielding experienced by H-5 to a field effect produced by the carbonyl group, H-5 existing within the



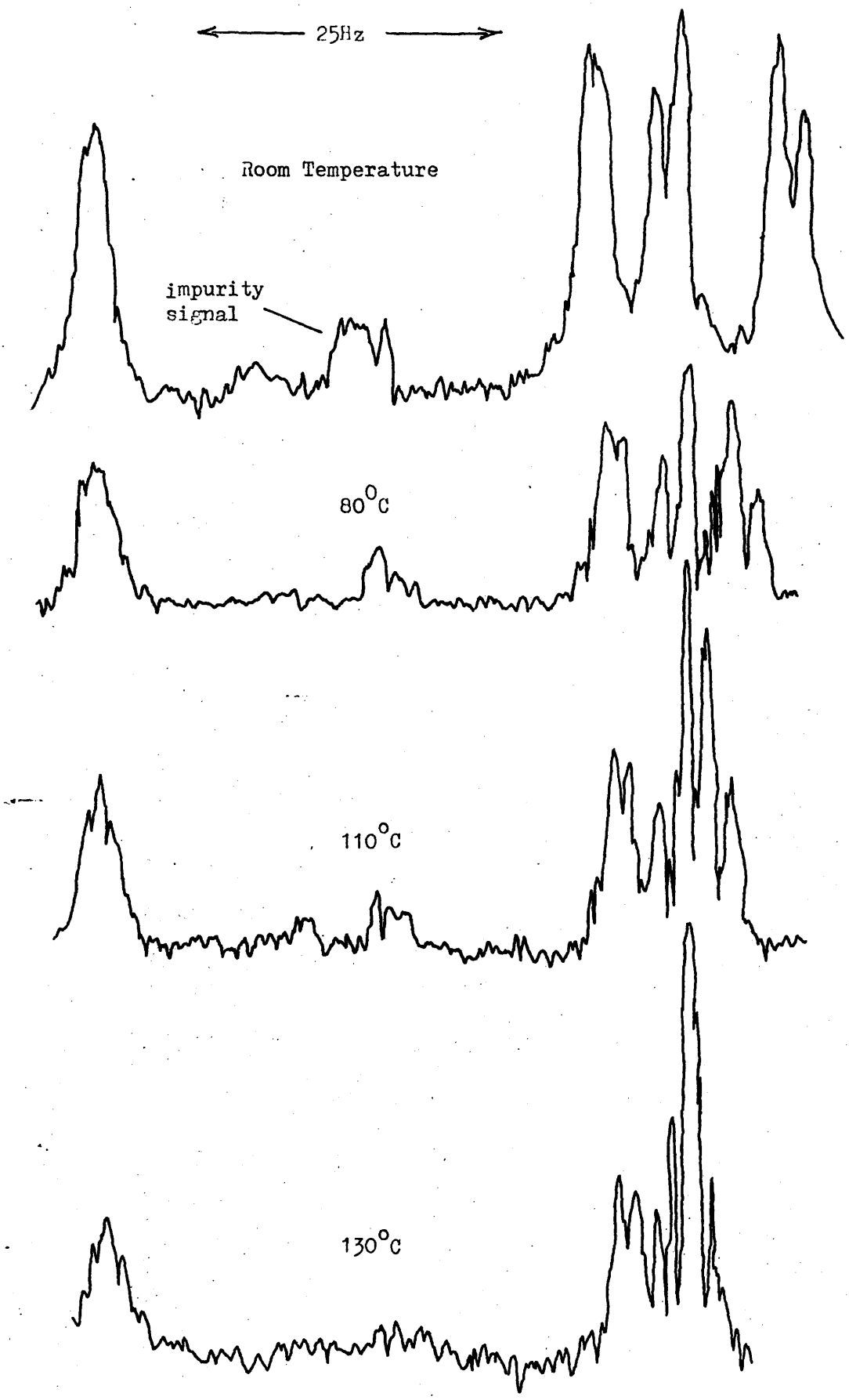
(145)

space near the carbon end of the C = O bond which experiences shielding by this group.[144] A similar situation is found in cyclobutanone where the protons of the β - methylene group have exactly the same chemical shift as those of cyclobutane.[145] The effect of the electric dipole of the carbonyl group would be to deshield these protons by 0.5 ppm.[144] so that to cancel out this effect a long-range shielding effect of the same order of magnitude must be present. Inspection of molecular models shows that the H - C distance between H - 5 and the acetyl carbonyl carbon in (144) is approximately the same as the corresponding distance in cyclobutanone, so that an effect of this magnitude in (144) would seem possible.

The critical factor in this hypothesis is the preferential orientation of the acetyl carbonyl group and the factors responsible for this. In a corresponding compound in which the methoxyl group is replaced by a less powerful electron-donating group, this preferential orientation should be absent and both peri hydrogens deshielded to an equal extent. This was indeed found to be the case in the acyl tetra-acetate of emodin anthrone (145), which was formed from emodin anthrone (103) - itself prepared either by demethylation of physcion anthrone (139) or reduction of emodin (84) - by treatment with pyridine and acetic anhydride.

In the n.m.r. spectrum of the acyl tetra-acetate (145) the signals for the aromatic protons appeared as two 2H doublets (τ 2.57, J = 2Hz; τ 2.97, J = 2Hz) representing H - 4,5 and H - 2,7 respectively. Thus the carbonyl group now seems to have no preferred orientation and deshields both peri protons (H-4,5) by an equal amount (Table 3.2). The difference in appearance of the pattern of signals in the aromatic region of the n.m.r. spectra of these two compounds seems to be attributable simply to replacement of an acetoxyl group with a methoxyl substituent.

Figure 3.1. Aromatic region of 100MHz n.m.r. spectrum of (144) recorded at room temperature, 80°,110° and 130°C.



As a further test of this presumed preferential orientation of the carbonyl group, the n.m.r. spectrum of (144) was recorded at room temperature and again at several higher temperatures (Figure 3.1). It was observed that the signals for H - 7 and H - 5 at room temperature had the appearance of a typical AB quartet and that, as the temperature was raised, the signal for H - 5 began steadily to 'move' downfield until, at 130°C, the signals for H - 5 and H - 7 coalesced. The signal for H - 4 does not, however, undergo a corresponding upfield shift, as would be expected if completely free rotation of the C - 10 acetyl group were taking place. These results may be interpreted as indicating that a temperature high enough to permit completely free rotation had not actually been reached and that the acetyl group is only 'wobbling' about the C-C bond, the preferential orientation of the carbonyl group being retained in some measure. However, as the temperature is raised, H - 5 will exist in an environment which increasingly is outside the region of space shielded by the carbonyl group, and consequently a downfield movement in the chemical shift of this proton is observed. Presumably, if the temperature of the system were raised sufficiently, the signal for H - 5 would continue to move downfield and that for H - 4 would begin to move upfield until an equilibrium situation was reached at which the two signals would appear at chemical shifts consistent both with the individual electronic environments in their respective rings and with equal deshielding by the C - 10 acetyl group.

In spite of the difficulty experienced in interpreting the observed chemical shifts of the aromatic protons in (144), the formation of this derivative of physcion anthrone (139) did have the effect of removing the accidental coincidence

Table 3.3 ^{13}C-n.m.r. data for physcion anthrone (139)

Exp. chem. shift δ ppm.	Cal. chem. shift δ	Assignment	Intensity nat. abund. spectrum (1)	Intensity nat. abund. spectrum (2)	Intensity [1-^{13}C] acetate actual	Intensity [1-^{13}C] acetate normalised	% enhance.	Intensity [1,2-^{13}C] acetate nat. abund. peak	$\frac{\Sigma \text{Satellites}}{\text{nat. abund.}}$ %	J Hz.
22.0	——	CH_3	61	46	40	53.8	-12%	47	55%	43.3
147.2	143.8	C-3	33	32	150	201.8	+511%	82	45%	42.8
119.6	122.6	C-4	106	100	73	98.2	-7%	260	50%	60.3
141.1	140.2	C-4a	44	46	115	154.7	+252%	77	35%	61.4
32.8	——	C-10	132	129	111	149.3	+13%	93	49%	41.4
143.6	141.3	C-10a	35	45	114	166.8	+377%	69	41%	41.5
106.2	107.5	C-5	113	101	84	113.0	0	302	42%	67.2
165.7	166.3	C-6	31	40	112	150.7	+389%	78	46%	67.1
99.4	98.5	C-7	119	103	83	111.7	-6%	346	45%	70.6
165.4	157.8	C-8	49	43	143	192.4	+293%	88	38%	71.8
110.0	110.9	C-8a	20	22				66	44%	58.7
191.0	——	C-9	29	24	76	162.2	+253%	46	36%	58.9
113.5	115.7	C-9a	18	30				79	51%	62.5
162.7	156.7	C-1	48	48	151	203.1	+323%	85	38%	62.9
115.8	113.6	C-2	101	82	70	94.2	-7%	418	singlet 55%	——
55.5	——	OMe	127	114	86	115.7	-9%	74	——	——

Table 3.4. ^{13}C-n.m.r. data for (144).

δ	Assignment	J_{c-c}	$J_{res.}$ (Hz)	δ(H)
122.7	2	—	not measured	6.92
137.4	1	45	—	—
133.3	9a	44.4	—	—
145.7	9	65	—	—
129.7	8a	64	—	—
157.6	8	74.3	—	—
99.2	7	74.2	not measured	6.82
146.9	6	80 ?	—	—
115.8	5	78.9	not measured	6.75
115.5	10a	?	—	—
141.6	10	?	—	—
116.5	4a		—	—
121.1	4	57.7	not measured	7.34
127.6	3	43.8	—	—
21.9	Ar-CH_3	43.6	ca. 17.0	2.47
55.7	OCH_3	—	30.0	3.86
33.6	ketone methyl	—	19.3	2.76
	acetate methyl	—	ca. 17.0	2.40,2.38
169.1	acetate carbonyl	—	—	—
169.5	acetate carbonyls	—	—	—
207.7	ketone carbonyl	—	—	—

in chemical shifts for H - 2 and H - 4 and for H - 5 and H - 7 observed in the n.m.r. spectrum of (139). In order to study the biosynthesis of (139) using ^{13}C - n.m.r., definite assignment of the resonances corresponding to C - 2 and C - 4 was considered essential. Since the resonances corresponding to H - 2 and H - 4 could be unambiguously assigned in (144) unlike those in(139), it was considered that the ^{13}C - n.m.r. spectrum of (144) would be more useful than that of (139) itself since firm assignment of C - 2 and C - 4 might be possible by the use of selective proton decoupling.

In the proton - noise - decoupled spectrum of (139-$C_{16}H_{12}O_4$) sixteen signals were visible while in the spectrum of (144-$C_{24}H_{22}O_8$) only twenty signals were discernible. Assignment of signals as far as possible, in the spectrum of (139) was made on the basis of reported chemical shift data[120] while partial spectral assignment of (144) followed from reported chemical shift data[120] and residual couplings and multiplicities of signals in the O.R.D. ^{13}C - n.m.r. spectrum of this compound (Tables 3.3 and 3.4).

Towards our goal of elucidating the biosynthesis of (139) a solution of $[1-^{13}C]$ - acetate (300mg., 0.1mg.ml^{-1} culture fluid) was added to the 5 - day old cultures of A.flaschentraegeri and a second addition of the same amount and at the same concentration was made 24 hours later. Inspection of the proton-noise-decoupled ^{13}C - n.m.r. spectrum of (139) isolated after a further three days' growth of the fungus showed that, although enrichments of the predicted carbon atoms were apparent, the level of peak enhancement (20%) could not be regarded as outwith the bounds of experimental error. They were, however, in keeping with

Table 3.5. Incorporation of [1-^{14}C,2-^{3}H] sodium acetate into physcion anthrone (2 pulses,5-9)

Activity of precursor	Activity of physcion anthrone	% incorporation
^{14}C : 0.2009 mCi(4.46×10^{8} dpm.)	0.0019 mCi(4.19×10^{6} dpm.)	0.94
^{3}H : 4.0941 mCi(9.09×10^{9} dpm.)	0.0214 mCi(4.74×10^{7} dpm.)	0.52
Precursor ^{3}H/^{14}C = 20.4:1	Final ^{3}H/^{14}C = 11.3:1	

Table 3.6 Time study of incorporation of [1-^{14}C] sodium acetate into physcion anthrone.*

Feeding period (days)	Total activity fed. mCi	Total activity fed. dpm.($\times 10^{8}$)	Weight physcion anthrone isolated (mg.)	Specific activity of isolated physcion anthrone (dpm./mM $\times 10^{6}$)	% incorp.
3-5	0.1010	2.242	28.2	8.63	0.41
3-6	0.0999	2.218	23.0	4.62	0.18
3-7	0.0989	2.196	30.0	3.41	0.18
4-6	0.1003	2.226	27.1	14.90	0.67
4-7	0.1010	2.242	30.2	10.60	0.53
5-7	0.1004	2.223	44.4	5.03	0.37
5-7	0.1013	2.248	90.8	10.13	1.52
5-9	0.1009	2.239	106.8	3.64	0.64
5-11	0.1007	2.236	84.7	3.41	0.48
7-9	0.1026	2.278	66.7	2.97	0.32
7-11	0.1018	2.260	84.7	2.44	0.34
9-11	0.1013	2.249	82.0	2.28	0.31

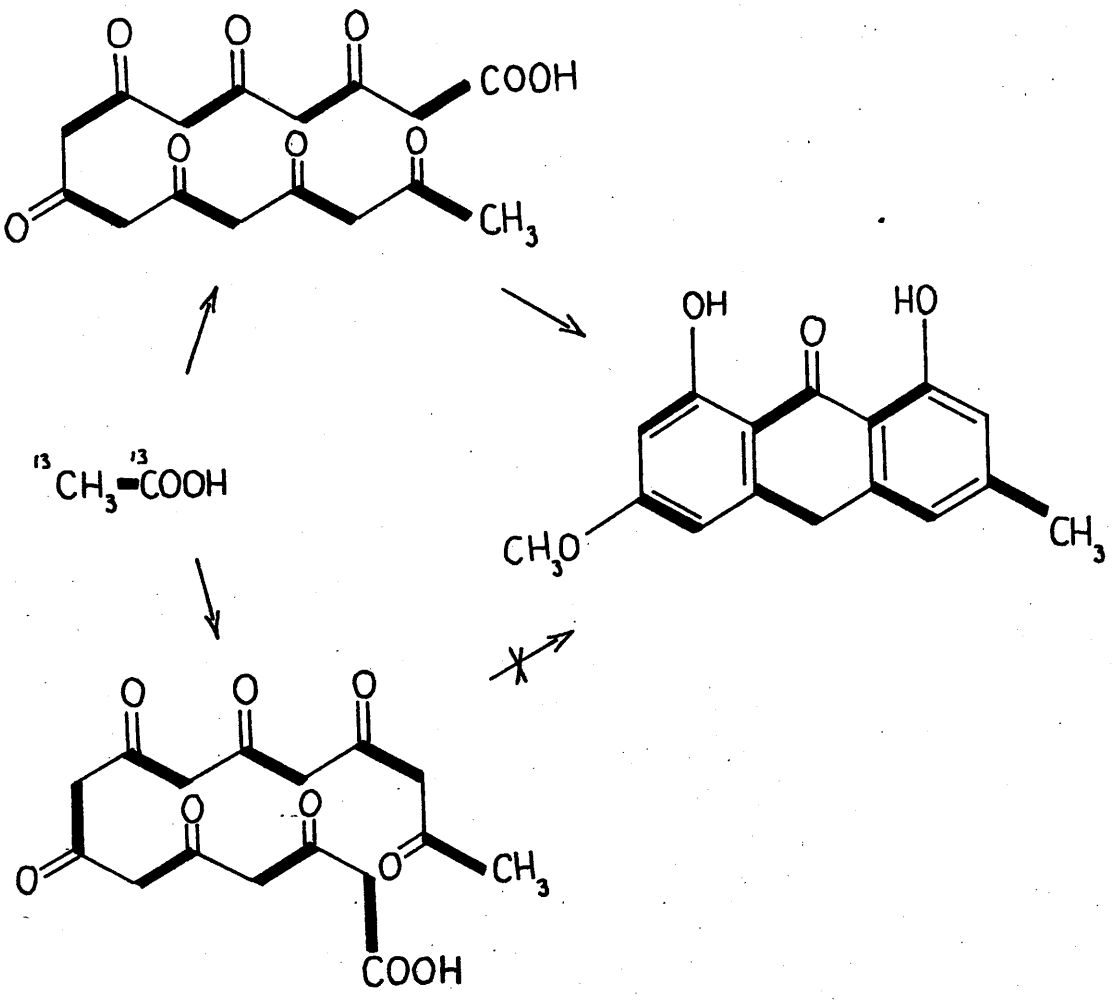
* Study was carried out on two separate batches of fungus. The (5-7) experiment was repeated to give some point of comparison of results from the two batches of fungus.

the low level of incorporation (0.9%) of $[1\text{-}^{14}C,\ 2\text{-}^{3}H]$ - sodium acetate fed in a parallel experiment (Table 3.5).

In view of this relatively meagre incorporation rate, a time study (using $[1\text{-}^{14}C]$ - acetate) was initiated in order to determine the optimum time of feeding precursor and the most suitable time for harvesting the cultures. It was found from this study that feeding on the fourth day of growth and harvesting on the sixth gave the highest rate of incorporation (Table 3.6). However, because of the large quantities of acetate which required to be added to achieve any significant enhancement of signal intensity in the ^{13}C - n.m.r. spectrum of the labelled (139) and the deleterious effects which the presence of large amounts of acetate have on fungal growth,[68b] the addition of label would necessarily have to be made in 'pulses' and a sufficient period of time would have to elapse between the administration of the last pulse and the harvesting of the fungus to permit efficient uptake of precursor. In view of these requirements, it was therefore decided that commencing feeding on the fourth day of growth and harvesting on the seventh would be more suitable.

Thus $[1\text{-}^{13}C]$ - acetate (75mg.) was added on the fourth day of growth to cultures of A.flaschentraegeri ($0.125mg.ml.^{-1}$ culture fluid) and in five further pulses of equal concentration at twelve hour intervals. Labelled (139) was isolated ($225mg.\ l.^{-1}$) after harvesting the fungus on the seventh day of growth. Peak enhancements averaging ca. 350% (compared with the observed intensities of the corresponding signals in the spectrum of unlabelled (139), run under the same experimental conditions as far as possible) were observed for seven signals in the spectrum of the labelled sample. This was interpreted as indicating

Figure 3.2. Possible modes of biosynthesis of (139) from a single polyketide precursor.



the incorporation of eight acetate units into the polyketide precursor of (139). The eighth labelled carbon atom is presumably lost as CO_2 during biosynthesis, by decarboxylation of the polyketide precursor.

The enhancements observed for these signals were useful in the complete assigment of signals in the spectrum of (139). It was noted that a much larger than average peak enhancement (511%) was produced in the signal for C - 3. This is a clear demonstration, in the biosynthesis of this molecule, of the 'starter effect', C - 3 corresponding to the carbon derived from the carboxyl group of the starter acetyl group, the aromatic methyl being the other half of this acetate unit. There was no evidence for the presence of two starter units, indicating that (139) had been formed from a single polyketide chain (Figure 3.2).

The unambiguous assignment of signals in the spectrum of (139) followed from the addition of $\left[1,2 - {}^{13}C\right]$ - acetate to cultures of the fungus. Suitably labelled physcion anthrone (139) was obtained by feeding a 3:1 mixture of unlabelled and $\left[1,2 - {}^{13}C\right]$ - acetate to cultures of A. flaschentraegeri (0.125mg.ml.$^{-1}$ culture fluid) on the fourth day of growth and in five further pulses of equal concentration at twelve hour intervals. Labelled physcion anthrone was recovered in the usual way and, in the proton - noise - decoupled ^{13}C - n.m.r. spectrum of this material, fourteen signals with satellites due to ^{13}C - ^{13}C coupling were observed, indicating the incorporation, intact, of seven molecules of acetate. The signal for the methoxyl carbon (derived presumably from methionine) appeared as a singlet as expected, as did the signal for C - 2, the single carbon remaining form the eighth acetate molecule by decarboxylation during biosynthesis. By analysis of the ^{13}C - ^{13}C coupling

constants in physcion anthrone labelled in this way, it was possible to differentiate between the two possible modes of cyclisation of the polyketide precursor chain (Figure 3.2). The crucial assignments were those for C - 2 and C - 4. One signal appears as a singlet while the other exhibits $^{13}C - ^{13}C$ coupling with the signal at δ 141.0. The assignment of this signal to C - 4 a seems reasonable, as the alternative assignments of this signal to the oxygen-bearing aromatic C - 1 and the signal at lower field (δ 162.7) to the quaternary aromatic C - 4 a appear untenable on the basis of recorded chemical shift data[120].

Thus the biosynthesis of physcion anthrone (139) appears to involve, as expected,[1] the cyclisation of a single octaketide chain (Figure 3.2). The biosynthesis of this metabolite from a single octaketide chain in this way is comparable with the biosynthesis of islandicin as established by experiments with $[2 - ^{14}C]$ - malonate.[146]

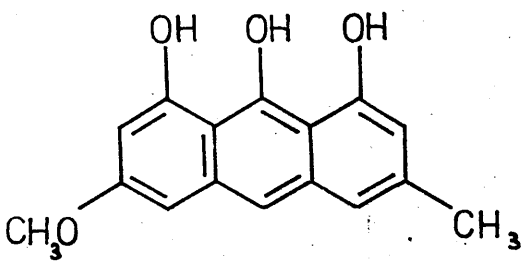
Although, in the event, it was found unnecessary to resort to acetylation of labelled samples of (139) to make a complete assignment of signals in the spectrum of (144), and indirectly (139), a sample of (139) labelled with $[1,2 - ^{13}C]$ - acetate was acetylated and this helped to clarify some of the assignments in the spectrum of (144). The proton-noise-decoupled and O.R.D. ^{13}C - n.m.r. spectra of labelled (144) were useful in making possible some further spectral assignments, but a low signal - to - noise ratio and similarity in sp^2 character of some of the quaternary (ring junction) and oxygen-bearing carbon atoms made the unambiguous assigment of every signal impossible. This applied especially to the signals at δ 115.5 and δ 141.6 in which low signal intensities precluded the

measurement of $^{13}C - ^{13}C$ coupling constants. Thus some of the assignments in Table 3.4 are interchangeable.

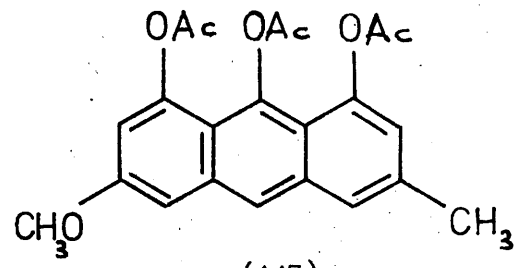
A further metabolite, apparently closely related to (139), was isolated by PLC of both crude (ethyl acetate) mycelial extracts of A. flaschentraegeri and column fractions (21 - 57). This compound was obtained as a dull yellow amorphous solid, melting above 250°C, and giving a "parent" ion at $^{m}/e$ = 270 and fragmentation pattern in its mass spectrum similar to that of (139). It was only sparingly soluble in most organic solvents.

The pattern and position of bands in the region 1650 - 1550 cm^{-1} in the i.r. spectrum of this metabolite (1638, 1617, 1592, 1569 cm^{-1}) closely resembled those in the spectrum of physcion anthrone (1644, 1618, 1596, 1568 cm^{-1}) and the long wavelength absorption maximum (362 nm. in $CHCl_3$) in its u.v. spectrum suggested the presence of the same tetrahydroxybenzophenone - like chromophore as in physcion anthrone (355 nm. in $CHCl_3$).

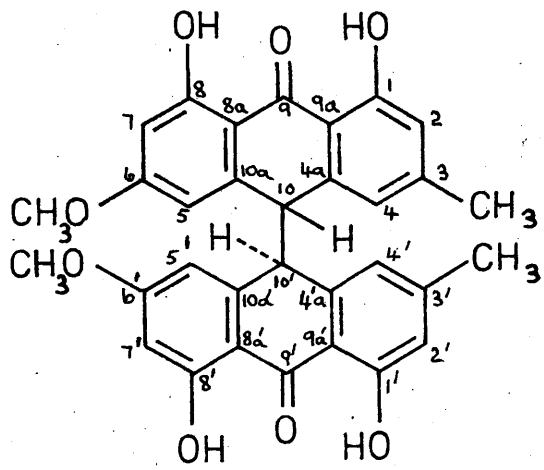
In view of these facts and in keeping with considerations of the presence of intramolecular hydrogen bonding or its absence, it was thought that this compound — more polar (R_f 0.20 in 50% chloroform - light petroleum) than physcion anthrone (R_f 0.57) — might be the isomeric 'physcion anthrone A' (138). The melting point and extremely low solubility in common organic solvents are consistent with the physical properties reported previously [136] for this compound. In (138), the absence of hydrogen - bonding between the carbonyl function and the hydroxyl groups would be expected to result in a much more polar compound than its isomer (139) with its strongly chelated carbonyl and hydroxyl functions. However a greater difference in carbonyl absorption


OH
OH
OH
CH3O
CH3

(146)


OAc
OAc
OAc
CH3O
CH3

(147)


OH
O
HO
CH3O
CH3O
H
H
CH3
CH3
OH
O
HO

(148)

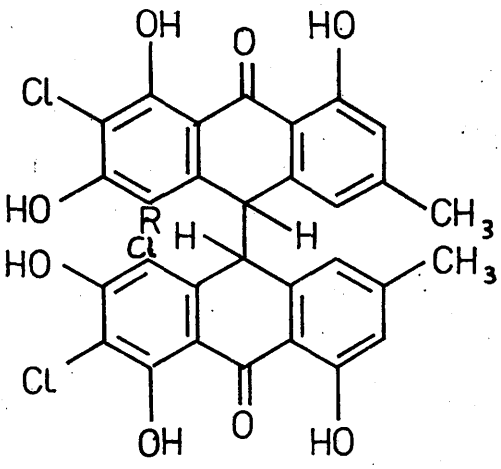
frequencies than observed would also be predicted on the same grounds.

Before further spectral characterisation of the metabolite had been carried out it was suggested [147] to us that 'physcion anthrone A' was, in fact, the triphenolic tautomer (146) of (139) and that the two tautomeric forms were stable with respect to each other. In an attempt to test this hypothesis, by converting some of the form (139) into (146), physcion anthrone was dissolved in aqueous potassium hydroxide under nitrogen (to avoid possible oxidative phenol-coupling) but, even after refluxing, only starting material was recovered when the solution was cautiously neutralised and there was no indication by TLC of any conversion to (146).

A second line of approach to (146) was the use of sodium borohydride to reduce the triacetate (147) to (146), a method based on the report of a similar conversion [148] of a phenolic acetate to a free phenol. However, none of the required intermediate triacetate (147) could be obtained, as acetylation of physcion anthrone with acetic anhydride gave only a very complex mixture of products, as described earlier.

However, with evidence obtained subsequently from the n.m.r. spectrum of the isolated polar yellow metabolite, it became clear that the structure (138) for this metabolite was no longer tenable - and that this compound was actually the dimer (148).

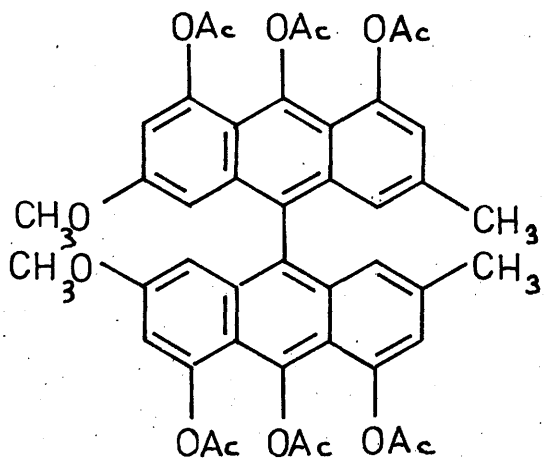
A sharp singlet at τ 6.18 suggested the presence of an aromatic methoxyl while a rather broad singlet at τ 7.68 was ascribed to an aromatic methyl group. A sharp singlet at τ 5.66 was taken as evidence for presence of anthrone methylene hydrogen(s). These signals were in positions very similar to the corresponding signals in physcion anthrone (139). However,



(149)

R = H: flavoobscurin A

R = Cl: flavoobscurin B



(150)

it was downfield in the aromatic region and in the region of the spectrum normally associated with chelated hydroxyl protons, that striking differences between the spectra of (139) and this polar metabolite were observed and where most evidence for the structure (148) was obtained.

In the spectrum of (148) two signals at τ 3.32 and 3.65 were very close to those for the aromatic protons in physcion anthrone (τ 3.34, 3.64). There were, however, two additional signals in the spectrum of (148) considerably upfield (τ 3.89, 4.03) from the first two. The signals at τ 3.32 and τ 3.89 were identified as corresponding to the protons of the methyl - substituted aromatic rings by irradiating at τ 7.68 (the aromatic methyl resonance), whereupon both signals were observed to sharpen. The appearance of signals for aromatic protons considerably upfield from the region of the spectrum normally associated with such hydrogens has been reported previously in the n.m.r. spectra of bianthrones (eg. flavoobscurins A and B, 149[149]). The upfield chemical shift of these signals has been attributed [149] to anisotropic diamagnetic shielding of the corresponding protons in each half of the dimer by the aromatic ring of the other half.

Strong confirmatory evidence for the proposed dimeric structure (148) was provided when the molecular weight of the compound was found by osmometric methods to be that of the dimer (m.w. = 538). The absence of any peak at $^{m}/e$ = 538 in the mass spectrum of (148) and the appearance of an ion at $^{m}/e$ = 270 (the molecular weight of physcion anthrone) can be attributed to cleavage of the 10, 10' linkage within the

spectrometer. The pattern and position of peaks in the rest of the spectrum were the same as those observed for physcion anthrone (139). No ions other than those attributable to the fragmentation of physcion anthrone were observed, thus indicating the identity of each of the constituent monomers in (148). A similar fragmentation into the constituent monomers was reported [149] in the case of the flavoobscurins, with the molecular ion also absent. In an attempt to stop this cleavage, by conversion of the anthrone ring systems into completely aromatic anthracene systems which might be less prone to cleavage of the dimeric link, acetylation of the dimer to give the bis-triacetylanthracene (150) was attempted using pyridine-acetic anhydride. However, T.L.C. indicated that no reaction had taken place, unreacted starting material only being recovered. This result is in accord with a previous result when it was found that neither acetic anhydride nor acetyl chloride would give an acetate of this compound.

The present dimer (148) is optically active with an experimental $[\alpha]_D = +24^\circ$ but, because of the extremely low solubility of the compound in all common solvents, this specific rotation must be regarded as an approximate value only. However, the compound is optically active and this suggests that the dimeric structure is not an artifact, formed during isolation. Other naturally - occurring bianthrones have been found to be optically active (eg. flavoobscurins A, B_1 and B_2 [149], sennidins A [151] and C [152]) and, with the finding [153] that bianthrones (in particular the glycosides of emodin bianthrone and palmidin C) are found in fresh Rhamus frangula bark while anthrone and anthraquinone glycosides were obtained

Table 3.7. ^{13}C - n.m.r. data for (148)

Chem. shift δ ppm.	Assignment	Corresponding shift in physcion anthrone
22.0	CH_3	22.0
146.9	C-3	147.2
120.8	C-4	119.6
140.6		141.1
140.3	C-4a	
56.6	C-10	32.8
143.3		143.6
142.9	C-10a	
107.8	C-5	106.2
169.3	C-6	165.7
100.3	C-7	99.4
165.3	C-8	165.4
164.7		
111.0	C-8a	110.0
190.4	C-9	191.0
114.3	C-9a	113.5
162.1	C-1	162.7
162.0		
117.1	C-2	115.8
55.7	OMe	55.5

from aged bark, it has been suggested [154] that the quinones were actually derived from the dimeric compounds (cf. the relationship between emodin (84), emodin anthrone (103) and skyrin (102) - see introduction, part B).

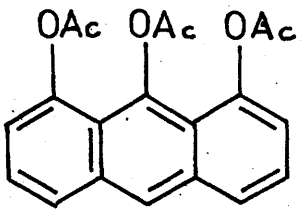
Evidence which might be interpreted as indicating the presence of a second isomer - possibly the optically inactive meso form - in the sample isolated was obtained from the proton-noise-decoupled ^{13}C - n.m.r. spectrum of (148) (Table 3.7). Several signals appear at values close to those recorded for the corresponding carbon atoms in physcion anthrone (139). However, the appearance of doublets for C - 1, C - 8, C - 10 and C - 4 may indicate the presence of more than one diastereoisomer of (148) in the sample isolated. Exactly whether this would account for the observed pattern of signals or not, is not clear but this is one possible explanation.

An attempt to synthesise the dimer (148) from physcion anthrone (139) by passing air through an alkaline solution of the latter[151] met with limited success on a trial scale but, on a preparative scale even after the addition of potassium ferricyanide to promote oxidative coupling, no identifiable product could be isolated from the reaction mixture. Dimerisation did inadvertently take place, however, when (139) was treated with ethereal diazomethane in an attempt to methylate the phenolic hydroxyl groups in that compound. After partial recovery of unreacted starting material it was found, from the n.m.r. spectrum of the product, that quantitative conversion of the remaining physcion anthrone to the dimer (148) had taken place. In an attempt to discover exactly how this conversion

had taken place, physcion anthrone (139) was allowed to stand overnight in chloroform, methanol and methanol-chloroform solutions, as these solvents had been used in the earlier reaction, but, in the absence of diazomethane, no coupling was observed to take place. Presumably diazomethane has the effect of promoting the formation of radicals, so giving the observed products.

The structure (148) has been reported[150] for the revised structure of one component ("dehydroemodin anthrone monomethyl ether") isolated[135,150] from chrysarobin, as described earlier. Also described earlier were the isolations of 'physcion anthrones A and B' from the root bark of V. madraspatana[134] and from several Aspergillus spp.[136]. 'Physcion anthrone B' has also been obtained from chrysarobin.[150] In view of the similarity in physical properties (m.p., colour, low solubility) and the fact that they both are co-metabolites of 'physcion anthrone B', it is suggested that the compound described as 'physcion anthrone A' may in fact have been the compound isolated here, i.e. physcion bianthrone (148). As found by Raistrick[136], this compound would not be expected to be formed by zinc and acetic acid reduction of physcion (136). Further, the structure (148) for physcion anthrone A is more acceptable, on biogenetic grounds, than the previously reported[136] structure (138).

The dimer (148) has also been isolated[155], both as a diglycoside and as the free aglycone, in the pods of Cassia occidentalis, a species long known in the tropics for its theraupeutic applications. It has been noted[156] that in natural purgatives based on anthraquinone derivatives, cathartic action increases in the order quinone < anthrone < bianthrone, that glycosides are more effective than aglycones and that the mixture



(151)

of glycosides found in Cassia leaves and pods in the most effective of all. It has also found[157] that triacetyl esters of the type (151), related to (144), have some application in the treatment of psoriasis.

Table 4.1. U.v. absorption data for physcion (136) and dimethylallyl physcion (73).

λ_{max}(nm.)	(136)	(73)
	224	221
	inf. 248	inf. 247
	253.5	
	266	
		277
	287	
	301.5	304
	432	435
	sh. 452	452

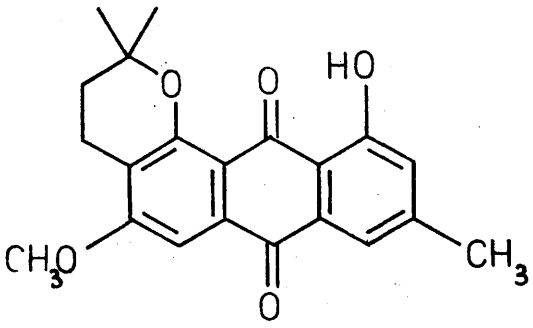
Table 4.2. Aromatic Proton Resonances of (136) and (73).

τ ($CDCl_3$):	(136)	(73)
H-5	2.42 } broad singlets	2.42 } broad singlets
H-7	2.95	2.97
H-4	2.67 } $J_{2,4}$ = 2Hz	2.65 singlet
H-2	3.37	—

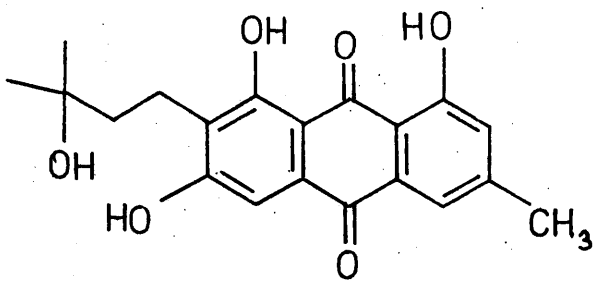
Chapter 4

The orange pigment 2 - (γ,γ - dimethylallyl) - physcion (73) was isolated by trituration and fractional crystallisation of the fat - contaminated column fractions (21 - 58), as described earlier (chapter 3). It generally seemed to be the case that this metabolite co-occurred with large amounts of fatty material as samples obtained in other instances both by PLC and column chromatography of mycelial extracts were similarly contaminated. However, it was found that chilling and trituration with ether, followed by crystallisation from ethyl acetate, afforded pure samples of (73), m.p. 215 - 7°C (lit., [125] 210 - 2°), in a yield of ca. 20mg.l^{-1}.

The structure (73) for isolated compound was deduced by comparison of u.v. data (Table 4.1) and n.m.r. data (Table 4.2) for this metabolite with those of physcion (136). The absence, in the n.m.r. spectrum of (73), of the upfield signal (τ 3.37) for the aromatic proton H - 2 in (136) and the presence of signals characteristic of a 3,3 - dimethylallyl grouping[158] were strong evidence for the structure (73) for this metabolite. Also, comparison of data for the present metabolite and that reported previously [125] for this compound confirmed the identity of the two. Evidence for the relative orientation of substituents in (73) was provided in the earlier investigation by the demonstration of a nuclear Overhauser effect in the n.m.r. spectrum of the metabolite, irradiation at the methoxyl group resonance resulting in a 25% enhancement of signal intensity for H - 4 only. Corroborative evidence for this structure was provided, during the present work, by overnight treatment of (73) with trifluoroacetic acid, resulting in cyclisation of the 3,3 -



(152)



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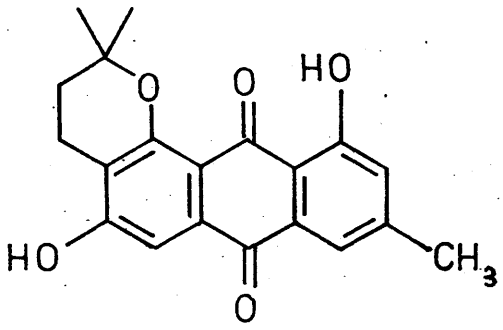
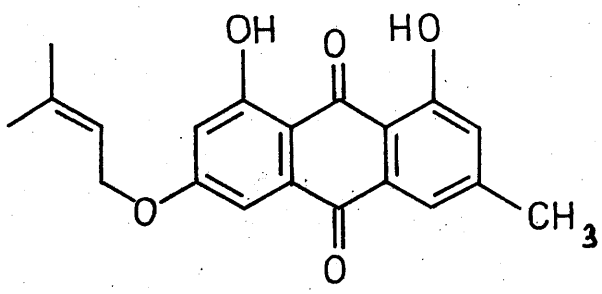


(154)



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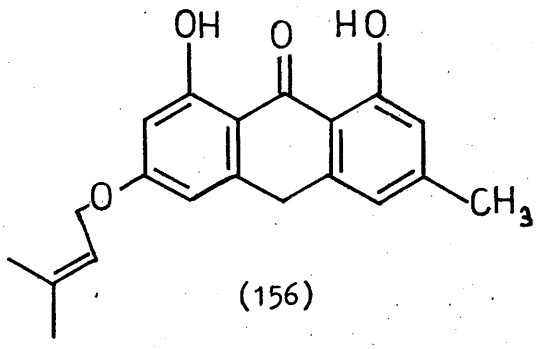

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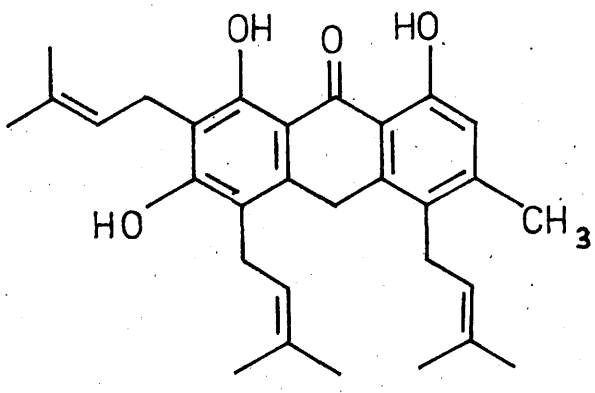

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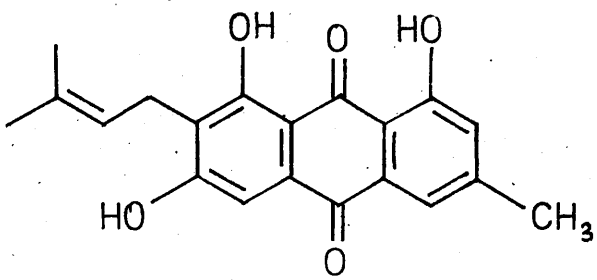


(158)

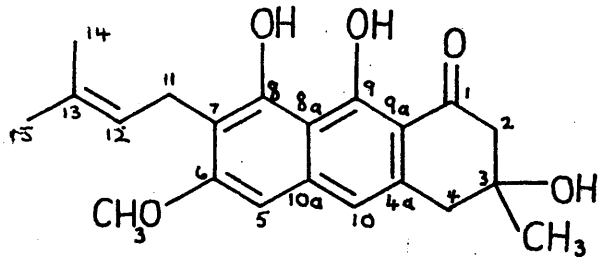
dimethylallyl side chain to give the dihydropyran derivative (152). The formation of this derivative was indicated by the loss in its n.m.r. spectrum of the signals for the 3,3 - dimethylallyl group and the appearance of a 6H singlet (τ 8.55) for the gem-dimethyl group and two 2H triplets (τ 7.29, J = 7Hz and τ 8.17, J = 7Hz) for the dihydropyran ring methylene groups. Also the signal for one of the chelated hydroxyl protons was now absent and a downfield movement (τ - 3.33) in the chemical shift of the other reflected an increase in hydrogen bonding between this remaining proton and the carbonyl group.

The anthraquinone (73) is one of very few known, fungal anthraquinones with this C - dimethylallyl substituent. The isolation of the prenylated emodin derivative (153) or its cyclised analogue (154) from A. flaschentraegeri has been reported[159] but, these apart, possibly the only other related anthraquinone isolated from natural sources is tectoleafquinone (74), from teak leaves, with its modified dimethylallyl grouping[91]. The isolation of the O - prenylated anthraquinone madagascin (155) along with its anthrone analogue (156), from the bark of Harungana madagascariensis has, however, been reported[160]. The C - prenylated harongin anthrone (157) was also isolated from the same source.[160]

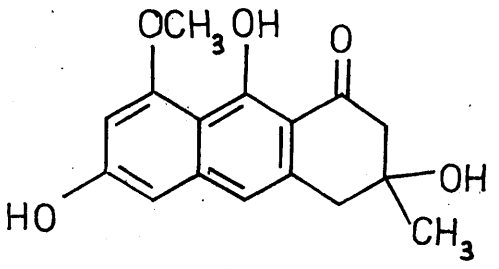
2 - (γ, γ - Dimethylallyl) - emodin (158), the desmethyl analogue of 2 - (γ, γ - dimethylallyl) - physcion (73), was thought to be a possible biogenetic precursor of (73). It was, therefore, considered that the complete characterisation (particularly the polarity on TLC) of (158), synthesised by demethylation of (73), would facilitate the detection of (158) if it were present in fungal extracts. In order to avoid possible

cyclisation of the 3,3 - dimethylallyl group, demethylation methods which involved the use of acidic media were avoided and, of several methods available which might effect demethylation of a phenolic ether in basic media, the method of choice involved treatment of (73) with the sodium salt of ethanethiol in D.M.F.[161]. In the n.m.r. spectrum of the major product, obtained as an orange bond by PLC of the crude reaction mixture, the signals for the chelated hydroxyl groups were absent and two sets of signals attributable to ethoxyl groups (τ 5.73, 6.05, quartets, J = 7 Hz and τ 8.57, 8.60, triplets, J = 7Hz) were present. It seemed possible that ethylation of the chelated hydroxyl groups had occurred. It also seemed that partial demethylation had taken place with signals which could be ascribed to an aromatic methoxyl (τ 5.95) and a free phenolic hydroxyl group (τ 0.05) both present. However the mixture seemed to be inseparable by PLC and it appeared that the required compound had not been formed

As well as 2 - (γ,γ - dimethylallyl) - physcion (73), there was on one occasion evidence for the presence in the orange band obtained by PLC of mycelial extracts of 2 - (γ,γ - dimethylallyl) - physcion anthrone (140), the anthrone analogue of (73). This occurred when the normal final recrystallisation of (73) from ethyl acetate was not carried out. N.M.R. spectral examination of the crude semi-crystalline material obtained from this particular band indicated the presence in this sample of a large amount of fatty material (broad signal for aliphatic methylene groups of alkyl chain, τ 8.70), but there was also clear evidence for the presence of both (73) (τ - 2.39 and - 2.10 chelated OH's; τ 2.40, 2.63 and 2.96 aromatic protons; τ 6.00, 7.54



(159)



(160)

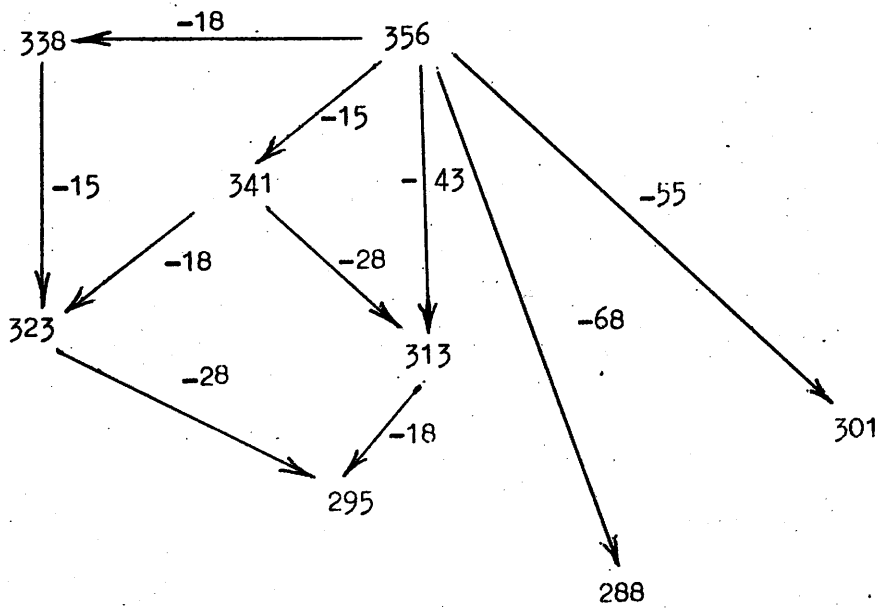
Table 4.3. U.V. absorption data for (159) and (160).

λ_{max}(log ϵ):	(159)	(160)
	231 (4.06)	230 (4.13)
	279 (4.23)	269 (4.51)
	317 (3.52)	317 (3.64)
	331 (3.44)	335 (3.40)
	402 (3.58)	392 (3.91)

aromatic methoxyl and methyl groups; τ 4.78, 6.64, 8.16, 8.27 dimethylallyl group) and the anthrone analogue (140) (τ - 2.64, - 2.37 chelated OH's; τ 3.35, 3.61 anthrone methylene group; same signals for dimethylallyl group as in quinone; τ 6.10 and 7.63 aromatic methoxyl and methyl groups). By analysis of the integrated signal intensities, it appeared that the two compounds were present, within the limits of experimental error, as a 1 : 1 mixture. This ratio may be purely accidental or may be interpreted as indicating that the two compounds co-exist in some form of complex. On no other occasion in the present investigation was there any evidence for the presence of (140) although in a previous investigation [137] it was reported that this compound had been detected in mycelial extracts of the same fungus. The isolation of this material from extracts of A. flaschentraegeri and its complete characterisation would provide significant evidence in respect of the order in which methylation and anthrone oxidation in the biosynthesis of (73) occur.

One factor which may very probably have some bearing on this point—and which necessitated some revision of earlier theories on the biosynthesis of (73)—was the isolation of the closely related compound (159) which was assigned the trivial name asperflaschin. This compound was isolated, in the first instance, by fractional crystallisation of the light petroleum (b.p.100 - 120°) mother liquors of physcion anthrone (139) but, subsequently, it was found that this metabolite could be obtained more conveniently in a yield of ca. 30 mg.l.$^{-1}$, by repeated PLC of crude mycelial extracts. It was obtained as yellow-green needles, m.p. 179 - 81°C, $[\alpha]_D$ = + 54°, which analysed for $C_{21}H_{24}O_5$ (M^+ at $^m/e$ = 356).

Figure 4.1. Mass spectralfragmentation pattern for (159)

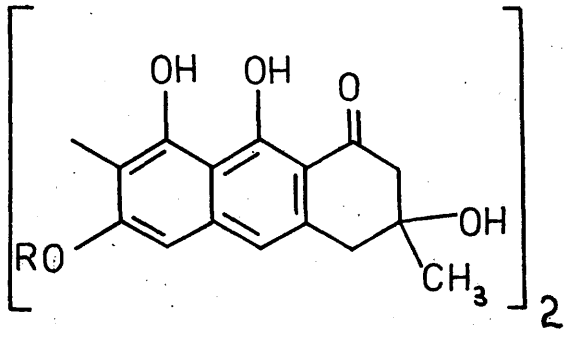


The structure (159) for this material was deduced, in part, from a comparison of data for the present metabolite with that for the related metabolite asperflavin (160), isolated from extracts of the culture filtrates of Aspergillus flavus[139]. The similarity between (159) and (160) is evident from their u.v. spectra (Table 4.3), both being consistent[139] with the presence of a 1,8 - dihydroxynaphthalenic chromophore conjugated with the carbonyl group which lies in the third carbocyclic ring.

The i.r. spectrum of (159) included a band (1632cm.$^{-1}$) attributable to a strongly chelated carbonyl group and the appearance of a very low field signal (τ - 6.12), in the n.m.r. spectrum of (159), for the chelated hydroxyl proton was consistent with this. A second non-chelated phenolic hydroxyl group was indicated by the appearance of a signal (τ 0.06) which disappeared on shaking with D_2O.

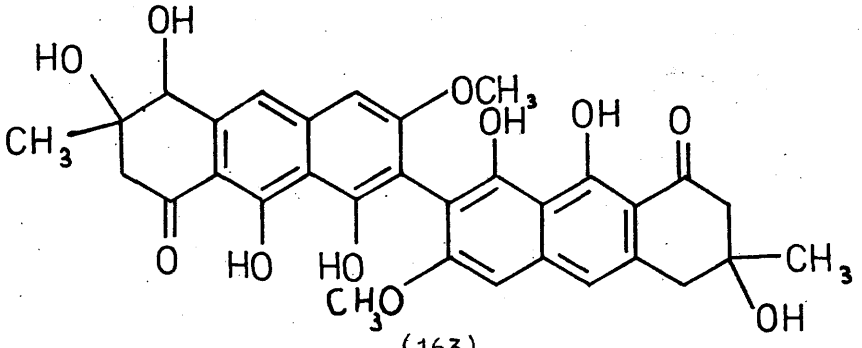
The mass spectrum pointed to the presence of a 3,3- dimethylallyl grouping. Normal fission[162] of this substituent was evident from the appearance of fragment ions at $^{m}/_{e}$ = 301 (M - 55), 313 (M - 43) and 288 (M - 68) arising from benzylic cleavage with loss of C_4H_7, rearrangement and elimination of C_3H_7 and complete fission and loss of C_5H_8, respectively (Figure 4.1)

The presence of this group in (159) was confirmed by the appearance in the n.m.r. spectrum of the signals characteristic[158] of a 3,3 - dimethylallyl substituent. Asperflaschin also contained a methoxyl group (τ 6.09) and two aromatic protons, one a sharp singlet (τ 3.49), the other with a slightly broader signal (τ 3.18). The presence of two methylene groups was indicated by a singlet at τ 7.18 and a slightly broader signal at τ 6.98, both in the region associated with benzylic protons.

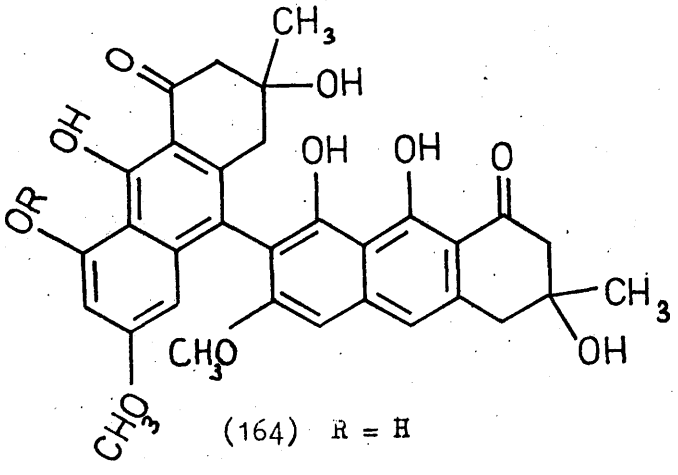


(161) R=H

(162) R=CH_3



(163)



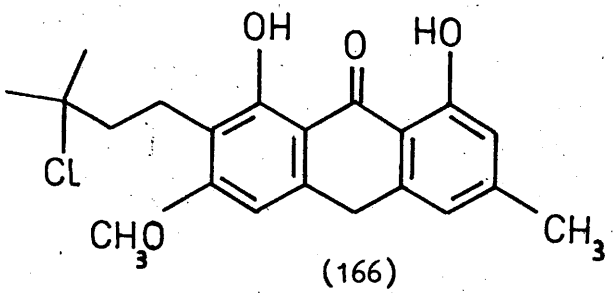
(164) R = H

(165) R = CH_3

The true benzylic nature of the latter methylene group (τ 6.98) was demonstrated when irradiation at the broad, lower field aromatic resonance (τ 3.18) resulted in sharpening of the signal for the methylene group. Irradiation at τ 6.98 had a reciprocal effect on the signal at τ 3.18.

Analysis of the data at this stage showed that all of the positions around the anthracene nucleus had been accounted for. The absence of coupling between the two benzylic-like methylene groups meant that they could not exist in adjacent positions in the carbocyclic ring and some intervening group, which did not couple with either, was required. A sharp singlet at τ 8.55 suggested the presence of a tertiary methyl group, possibly contained in the partial structure R^1R^2C (OH) Me. A signal at τ 8.21, which disappeared on shaking with D_2O, was ascribed to the tertiary hydroxyl group. All the above data could be accomodated in the structure (159).

The same type of 1-keto-tetrahydroanthracene nucleus present in asperflaschin (159) and asperflavin (160) is found in several other fungal metabolites. One of these compounds, flavomannin, with the 7,7′ - dimeric structure (161), is a yellow pigment isolated from Penicillium wortmanii[163]. It can be seen that (161) is a dimer of emodin anthrone (103) hydrated in ring C. Closer to the structure of asperflaschin is the 6,6′- dimethyl ether (162) of flavomannin, isolated by Steglich et. al. from several Dermocybe spp.[164] while the enantiomers, 4 - hydroxy-flavommanin - 6,6′ - dimethyl ethers A_1 and B_1, (163), were isolated[165] from Cortinarium vitellinus and Tricholoma sulfureum, respectively. These authors also reported the isolation from Cortinarius odorifer of phlegmacin(164)[166]



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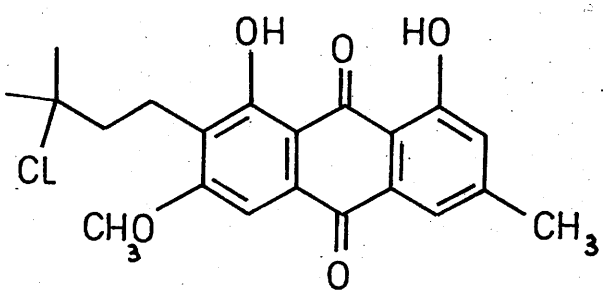


(167)

and its 8' - methyl ether (165)[167] in which the 7,7' link in (162) has been replaced by 7,10' linkages.

There is in the structure (159) an obvious relationship with the structure (140) of 2 - (γ,γ - dimethylallyl) - physcion anthrone and, in an attempt both to provide chemical evidence for the proposed structure for asperflaschin and to demonstrate the relationship between (140) and (159), asperflaschin was treated with concentrated hydrochloric acid in acetic acid to dehydrate (159) to (140)[139]. This operation did have the desired effect of dehydrating the β - hydroxy ketone system to the required anthrone nucleus (anthrone methylene protons τ 5.79, aromatic methyl group τ 7.63, aromatic protons τ 3.34 and 3.61) but this transformation was accompanied by the addition of HCl across the double bond of the dimethylallyl group (appearance of two 2H triplets, τ 7.15 and 8.03, J = 7Hz, for methylene groups) and the actual product was the chloro-derivative (166). Although the desired product was not obtained from this reaction, the compound isolated (166) was useful in providing evidence for the positioning of the dimethylallyl group in (159), as oxidation of (166) with Jones' reagent gave a compound (167) identical in all respects with that obtained by treatment of 2 - (γ,γ - dimethylallyl) - physcion (73) with hydrochloric acid in acetic acid. As the positioning of the dimethylallyl group in (73) was firmly established with the formation of the dihydropyran derivative (152) the positioning of this substituent within (159) necessarily follows. Additionally, treatment of (159) with aqueous sodium hydroxide in air[168] effected transformation of (159) into 2- (γ,γ - dimethylallyl) - physcion (73), as established by n.m.r. spectroscopy..

Other possible routes to (140) from (159) were explored, including treatment of (159) with thionyl chloride in pyridine (used[169] in the dehydration of tertiary alcohol systems in steroid syntheses) and heating (159) as an intimate mixture with potassium bisulphate[170]. In the former case no reaction was detectable by TLC after 48 hours but TLC of the latter reaction mixture did suggest this as a possible dehydration method. The extremely low yield encountered with this method, however, led us to abandon it as a practicable synthetic route to (140).

A more promising method appeared, initially, to be provided in the treatment of (159) with dicyclohexylcarbodiimide (D.C.C.) under nitrogen, in refluxing toluene. A preliminary treatment of (159) with D.C.C. in refluxing methylene chloride resulted in no detectable (TLC) conversion and it was thought that this might be attributable to the relatively low boiling point of methylene chloride (41^{0}C). Accordingly the reaction was repeated in the higher boiling (110^{o}C) solvent toluene. By monitoring the progress of the reaction by TLC, it was found that no reaction had taken place after 48 hours with two equivalents of D.C.C. and so excess D.C.C. (11 equivalents) was added. After overnight reflux, no starting material remained. Treatment with dilute acetic acid to remove any unreacted D.C.C.[171] and PLC gave the product as a dull yellow amorphous solid (in 11% yield), virtually insoluble in most organic solvents.

Evidence from the mass spectrum of the product seemed to indicate that the desired product had been formed, with a molecular ion at $^{m}/e = 338$, the molecular weight of (140). The result from an exact mass measurement was also

consistent with this. Because of the low solubility of the product in most organic solvents, its n.m.r. spectrum was far from satisfactory, but several features in it indicated that the product did not have the structure (140) but some type of dimeric structure; in particular the doubling of chelated hydroxyl signals (τ - 2.65, - 2.37, - 2.33 and - 1.97) and the appearance of signals upfield from the region of the spectrum normally associated with aromatic protons. The characteristic signals for the 3,3 - dimethylallyl grouping were present as was a signal (τ 5.77) for the anthrone methylene protons.

In the u.v. spectrum of this product the same long wavelength absorption maximum (λ_{max} = 360nm.) attributable to the tetrahydroxbenzophenone - like chromophore in physcion anthrone (352 nm.) and physcion bianthrone (362nm.) was present. The extremely low solubility of this material in common organic solvents is one other point of similarity with physcion bianthrone. Further characterisation of this product was not possible due to lack of material but it seemed clear from the existing data that the desired reaction had not taken place.

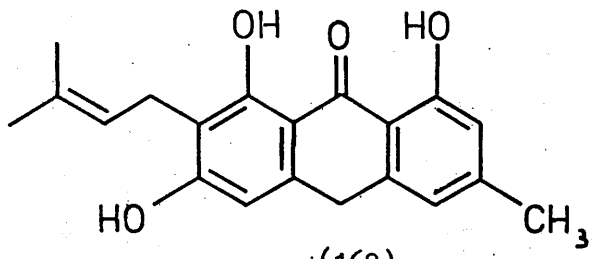
This interpretation was subsequently confirmed by the properties of authentic (140) obtained in high yield (77%) by dehydration of (159) in refluxing collidine. The n.m.r. spectrum of this product showed two signals (τ - 2.65, - 2.35) for the chelafed hydroxyl groups, an aromatic methyl (τ 7.65), a broad singlet (τ 5.85) for the anthrone methylene and two signals (τ 3.36, 2H and τ 3.63, 1 H) representing the aromatic protons in the methyl and methoxyl - substituted rings, respectively. The characteristic signals for the 3,3 - dimethylallyl group were also evident. The appearance of two signals for chelated hydroxyl groups in (140) means that

Table 4.4. Nmr. data for (139), (140)

τ ($CDCl_3$)	(139)	(140)
-OH	-2.59	-2.65
-OH	-2.29	-2.35
H-5,7	3.34	3.36
H-4	3.64	3.63
β -H	—	4.80
anthrone-CH_2	5.81	5.85
Ar-OMe	6.16	6.13
α -CH_2	—	6.67
Ar-CH_3	7.64	7.65
γ -Methyls	—	8.20,8.32.

Table 4.5. U.V. data for (139), (140)

λ_{max} nm.	(139)	(140)
	224.5	226
		sh.232
	inf. 252	
	256	
	272	273.5
		284.5
	303	307
	352	358



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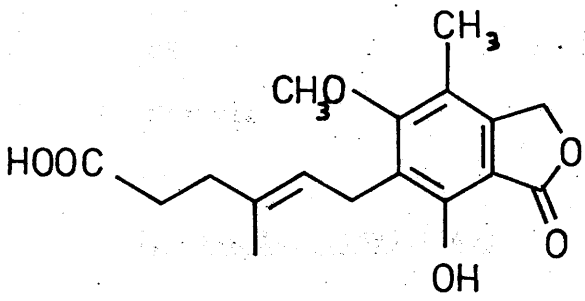

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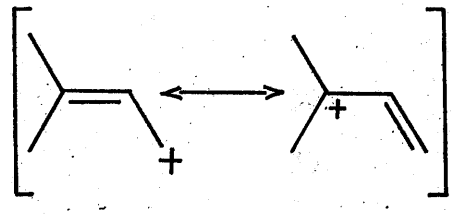


(170)

the methoxyl group in this compound cannot be in the same position as in asperflavin (160) and in this way the formation of (140) helps to establish the orientation of this substituent in (159).

Comparison of n.m.r. and u.v. spectral data for (140) with those of physcion anthrone (Tables 4.4 and 4.5) pointed to the close relationship between the two compounds and the appearance of an ion at M - 55 in the mass spectrum of the former was interpreted as confirming the presence in this product of the 3,3-dimethylallyl group.

The n.m.r. data for this derivative (140) agreed very closely with that recorded earlier for the material detected as a co-metabolite of 2 - (γ,γ - dimethylallyl) - physcion (73) during PLC of mycelial extracts.

This conversion of asperflaschin (159) into (140) made available two compounds of possible importance in the biosynthesis of 2 -(γ,γ - dimethylallyl) - physcion (73). A third compound which might be a precursor of both (73) and (140) was 2- (γ,γ - dimethylallyl) - emodin anthrone (168) and, in view of this, attempts to synthesise (168) were initiated.

The first general approach to (168) was by the introduction of a dimethylallyl group into the anthrone nucleus, this reaction being attempted with a variety of reagents and under several sets of experimental conditions.

Previously, the synthesis of 2 - (γ,γ - dimethylallyl) - physcion (73) had been attempted[137] by the introduction of a dimethylallyl group into physcion (136) using dimethylallyl bromide and silver oxide. The procedure used on this occasion

followed that employed by Canonica et al.[172] in the synthesis of mycophenolic acid (169). It had been reported by Canonica's group that C - alkylation took place only when there were two free hydroxyl groups ortho to the site of alkylation. The lack of success in alkylating physcion (136) was attributed in the same way to insufficient activation being provided by the methylated phenolic grouping. It was also thought that the presence of the second quinone carbonyl group para to the site of alkylation in physcion was not conducive to alkylation at this position. Thus, it was considered that there was a better chance of success with the non-methylated anthrone, emodin anthrone (103).

It was found that (103) could be conveniently synthesised by reduction of emodin (84) with hydriodic acid in acetic acid[173] or by demethylation of physcion anthrone (139), with the same reagent. Attempts to demethylate physcion anthrone with pyridine - hydrochloride[99] yielded only an intractable black residue.

In general there are three methods of introducing 3,3 dimethylallyl groups into positions ortho to phenolic hydroxyl functions, and all three were attempted.

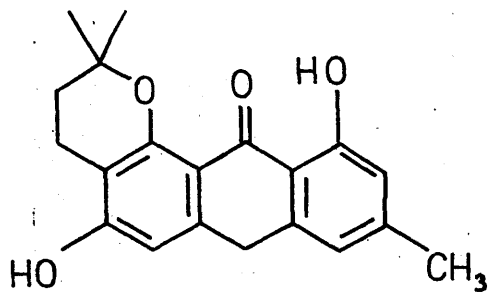
The first approach entails the direct alkylation of phenols with allylic halides (eg. dimethylallyl bromide) in basic media. The same method as used in the attempted synthesis of (73) - silver oxide, dimethylallyl bromide - was again attempted but no reaction was detectable by TLC. The same result was obtained when pyridine was used as the base.

A second approach, modelled on proposed biogenetic routes to the introduction of C - isopentenyl groups (either by attack of the phenol on the resonance stabilised cation (170)[174], or S_n2- type displacement of a pyrophosphate leaving group[175])

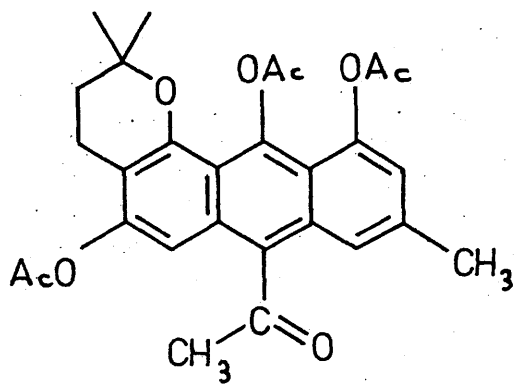
involved generation of the required mesomeric cation (170) by treatment of 2 - methylbut - 3 - en - 2 - ol with aqueous formic acid at room temperature.[176] It was reported [176] that further cyclisation of dimethylallyl units to the corresponding chromans took place if the reaction were maintained at high temperatures over a long period of time, but when the reaction was carried out at room temperature the corresponding C-prenylated compounds could be obtained in moderate yield. However, following this procedure no reaction could be detected with the present compound (103) even after 48 hours.

The third route attempted was reported[177] to be more effective than either of the foregoing methods, involving partial hydrogenation of the α,α - dimethylpropargyl ether of a phenol to the corresponding α,α - dimethylallyl ether, followed by thermal rearrangement[178]. This required the initial formation of the propargyl ether and in the literature description[178] this was achieved by base - catalysed attack of the phenol on propargyl chloride (itself synthesised[179] by treatment of the corresponding alcohol with cuprous chloride in concentrated hydrochloric acid). It was found however, that, under the conditions described, dialkylation of (103) had occurred (M^+ at $^m/_e$ = 338 with successive losses of 67 (C_5H_7) mass units). Further this alkylation appeared to be occurring at the anthrone methylene position - reflecting earlier observations on the reactivity of this position - with no signals for these protons visible in the n.m.r. spectrum of the product. There was no sign of the desired mono-alkylated compound among the reaction products.

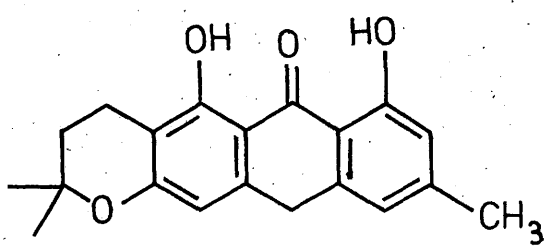
In an attempt, therefore, to moderate the alkylation of (103), the reaction was repeated at a lower temperature, but only a complex mixture of products resulted from which no



(171)



(172)



(173)

single pure product was isolable by PLC. In view of these disappointing results, this general approach was abandoned and attention focussed on methods based on dehydration and demethylation of the already prenylated potential precursor asperflaschin (159).

It was hoped that treatment of (159) with lithium iodide in refluxing collidine[180] might effect not only dehydration but also demethylation to the desired product (168). In spite of several attempts, however, a complex mixture was in practice the only result.

Asperflaschin (159) was also treated with hydriodic acid in acetic acid under the conditions used to demethylate physcion anthrone. It was believed that this procedure would either result in the desired product (168) or, alternatively, the cyclised derivative (171). While the formation of this latter compound was not the immediate object of the synthesis, it was considered that this product would be useful in extending our studies on the conformation of the C - acetyl group in compounds of the type (144). The C - acetyl compound (172) would be useful in this context. In the event, although treatment of(159) with hydriodic acid - acetic acid did effect dehydration and demethylation, these transformations were accompanied by an alternative mode of cyclisation of the dimethylallyl substituent to give the gem-dimethyl dihydropyran (173) — absence in the n.m.r. spectrum of the signals for the methoxyl and dimethylallyl groups, presence of signals for two chelated hydroxyl groups τ - 2.92, - 2.40, signals for anthrone methylene τ 5.85 and dihydropyran ring methylenes τ 7.27, 8.17, (triplets J = 7Hz) and gem - dimethyl protons τ 8.65. While neither of the expected products was obtained the formation of (173) did provide corroborative evidence for the earlier pattern of substitution in asperflaschin.

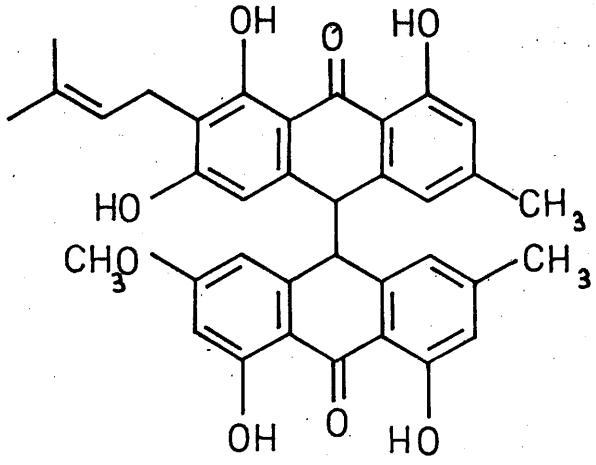
Table 4.6. I.R. absorption data for (148),(174) and "D.C.C. dehydration product"

	(148)	(174)	D.C.C. product
$\nu_{co}(cm^{-1}.)$	1617	1618	1619
	1592	1597	1596
$\nu_{c=c}(cm^{-1}.)$	1638	1630	1633

Table 4.7.

U.V. absorption maxima for (148),(174) and D.C.C. product.

$\lambda_{max.}^{nm.}(\log \epsilon)$:	(148)	(174)	D.C.C. product
	inf.259	—	—
	280	279	278
	362	353	360



(174)

Some indication that 2 - (γ,γ - dimethylallyl)-emodin anthrone (168) did occur naturally was provided by the isolation in one instance of a small amount of a metabolite with physical properties (amorphous fawn-coloured solid, low solubility in common organic solvents) very similar to those of physcion bianthrone (148) (chapter 3). This second insoluble metabolite was more polar (TLC) than (148) and co-occurred with (148) in fractions from a column of asperflaschin-rich portions of mycelial extract.

The ir. and uv. spectral data were very similar to those for physcion bianthrone (148) and for the suspected dimeric product, referred to earlier, from the reaction between D.C.C. and asperflaschin (Tables 4.6 and 4.7).

In the mass spectrum, the 'parent' ion appeared at $^{m}/e = 324$, the molecular weight of (168) and the base peak appeared at $^{m}/e = 270$, the molecular weight of (139).

The low solubility of this polar metabolite in common organic solvents again resulted in a rather poor n.m.r. spectrum but several features were visible which pointed to the dimeric structure (174) for this compound. There were present the signals (τ 4.73, 6.65, 8.18, 8.25) typical of a dimethylallyl group and examination of the integrated signal intensities showed that there was only one such group in the molecule. Downfield there were visible the characteristic 'doubled' signals for chelated hydroxyl protons at τ - 2.47 ($\frac{1}{2}$H) and - 2.43 ($\frac{1}{2}$H), τ - 2.13 ($\frac{1}{2}$H) and - 2.06($\frac{1}{2}$H), and τ - 1.83(1H) and - 1.78(1H). There were also signals for one methoxyl group (τ6.17, $^{3}/2H$ and τ6.23, $^{3}/2H$) and two methyl groups (τ 7.70, $^{6}/2H$ and τ 7.77, $^{6}/2H$). In the aromatic proton region of the spectrum, a broad 2H singlet (τ 3.35) corresponded to H - 7 and H - 7' while a 1 H broad singlet (τ 3.66) represented H - 2'. Both of these signals were at values very close to the corresponding

resonances in 2 - (γ,γ - dimethylallyl) - physcion anthrone (140). Upfield a complex 4H multiplet (τ 3.87 - 4.18) was taken as representing the aromatic protons H - 4,4', 5 and 5', shielded by the aromatic rings of each half of the molecule. No signal was visible for the C - 3 hydroxyl group.

The observed signal intensities might be interpreted as indicating that the sample isolated was a mixture of two isomeric forms of the metabolite, possibly rotamers. Thus the signal which appears at τ - 1.83 represents the two chelated phenolic protons, present in the methylated rings of the two monomeric units, in one rotameric form of the molecule and the signal at τ - 1.78 the same two protons in the second rotameric form. Together, the two signals have the integrated peak intensity required for two protons. Also the signals (each integrating for ½H) at τ - 2.47 and - 2.43 represent one of the two remaining chelated hydroxyl protons in each of the two rotameric forms, the signals at τ - 2.13 and 2.06 representing the other. It is not possible to say which of these pairs of signals represents which chelated proton. In the same way, two signals (τ 6.17, 6.23) which together integrate for 3 H represent the one methoxyl group present, in the two rotameric forms of the molecule. Probably the same interpretation can be applied to the two 3H signals for the methyl groups, representing these substituents in the two rotameric forms of the molecule. In all cases, the difference in chemical shift between the two signals representing each particular group in the two rotameric forms lies within the range 0.04 - 0.07 ppm. In the case of the aromatic protons, the signals are all broad and this may represent an

Table 4.8. ^{13}C - n.m.r. data for asperflaschin (159)

Carbon atom	δ	$J_{res.}$	$\delta(H')$	$J_{13\,13\,C-C}$	Int. nat. ab.	Int. Cr(acac)$_3$	Int.[1,2-^{13}C] centre peak.	Int.[1,2-^{13}C] norm.	% enh.
2	51.0	t. 24.5	2.82*	—	181	47	348	271.5	50
1	201.6	—	—	55.6	86	39	119		30
9a	108.1	—	—	55.4	56	68	65		49
9	156.0	—	—	74.9	66	44	116		46
8a	114.7	—	—	75.8	93	48	137		33
8	165.8	—	—	66.1	75	44	119		29
7	108.1	—	—	65.7	56	68	129		49
6	161.9	—	—	69.5	65	41	92		36
5	97.8	d. 65.1	6.51	69.5	195	58	267		37
10a	138.9	—	—	57.3	86	47	124		34
10	117.6	d. 68.6	6.82	56.2	155	48	259		34
4a	134.2	—	—	41.3	96	39	127		42
4	43.3	t. 25.6	3.02	41.6	186	36	283		41
3	71.0	—	—	39.9	138	30	211		36
$-CH_3$	28.8	q. 12.5	1.45	40.7	130	32	201		45
11 (α-CH_2)	22.0	t. 29.0	3.45	43.1	206	91	304		65
12 (β-CH)	122.3	d. 51.2	5.23	43.4	182	63	267		65
13	131.7	—	—	42.5	85	52	145		50
14 (<u>cis</u> Me)	17.8	br.q. 17.3	1.84	42.2	73	50	130		76
15 (<u>trans</u> Me)	25.8	br.q. 13.1	1.72*	—	125	55	294	187.5	50
OMe	55.6	q. 37.6	3.91	—	179	57	297	—	—

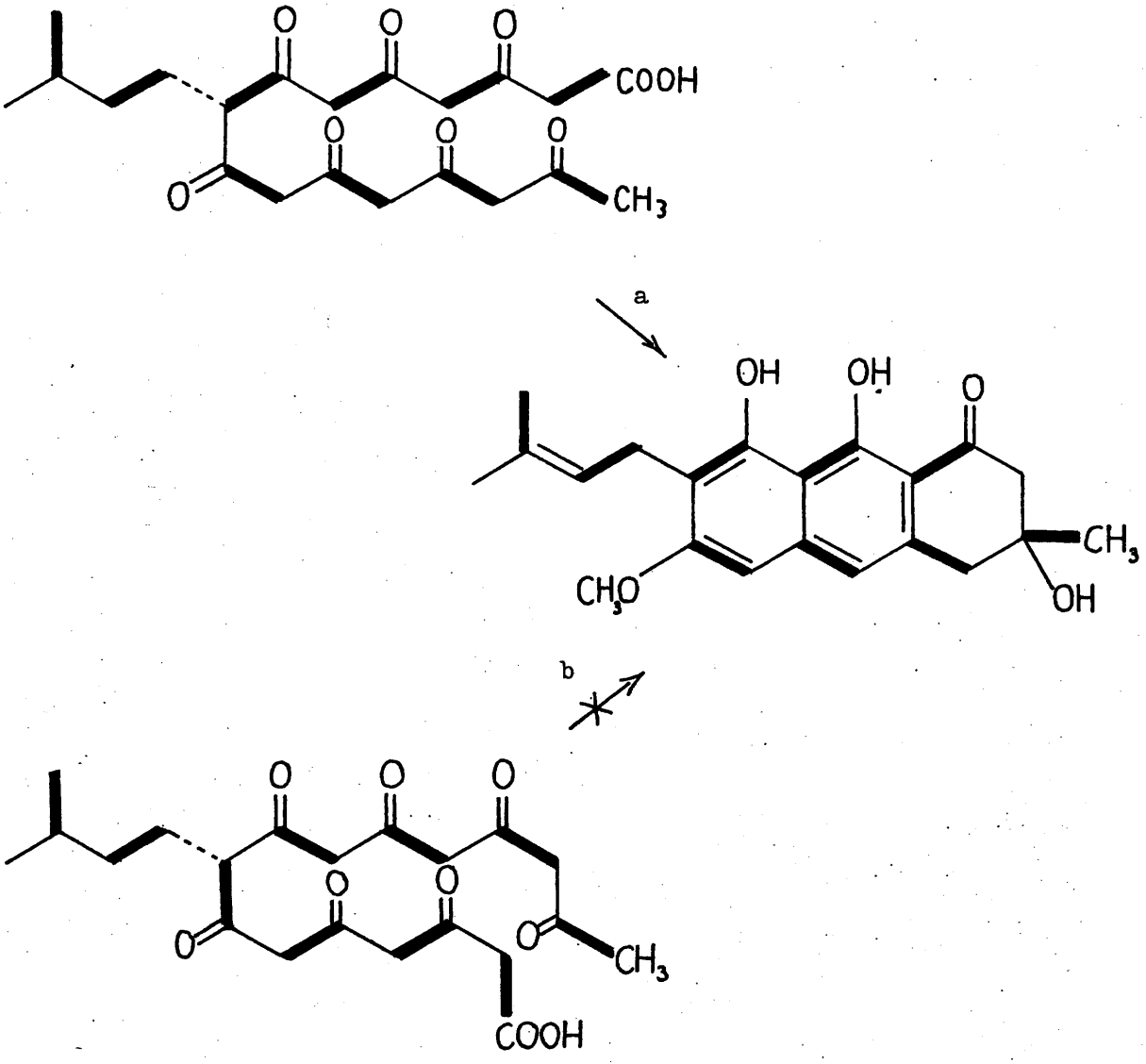
*Enrichment calculated by same method as non-coupled positions in (120).

effect, similar in nature but different in degree, to that observed for the other groups.

Elucidation of the biosynthesis of asperflaschin (159) was approached in the same way as with previous metabolites. In the proton-noise-decoupled ^{13}C-n.m.r. spectrum of unlabelled asperflaschin ($C_{21}H_{24}O_5$) only twenty signals were visible (although the addition of the paramagnetic relaxation reagent tris (acetylacetonato) - chromium (III), did suggest the 'missing' signal coincided with another at δ 108.1), but partial spectral assignment was possible on the basis of chemical shift data[120] and residual couplings and multiplicities of peaks in the O.R.D. spectrum of (159). Complete spectral assignment followed from the spectrum of a sample biosynthesised in the presence of $[1,2 - ^{13}C]$ - acetate, added to 4 - day old surface cultures of A. flaschentraegeri. Labelled asperflaschin was isolated ($38 mg.l^{-1}$) along with labelled traegeric acid (chapter 2). In the proton-noise-decoupled ^{13}C - n.m.r. spectrum, eighteen signals with satellites due to $^{13}C - ^{13}C$ coupling, were visible together with a further three singlets. Thus signals for all of the carbons in the molecule were now visible with the suspected duo of signals at δ 108.1 now clearly separable as a result of their slightly different $^{13}C - ^{13}C$ coupling constants (Table 4.8).

In analysing these results and deciding which of the two postulated modes of cyclisation of the precursor polyketide chain would give the observed labelling pattern, it is clear that differentiating between the assignments of signals corresponding to C - 2 and C - 4 is of crucial importance as one of these methylene carbons appears as a singlet in the spectrum of (159) labelled with $[1,2 - ^{13}C]$ - acetate, and so represents the C - 2 carbon of the terminal acetate unit in the polyketide chain.

Figure 4.2.



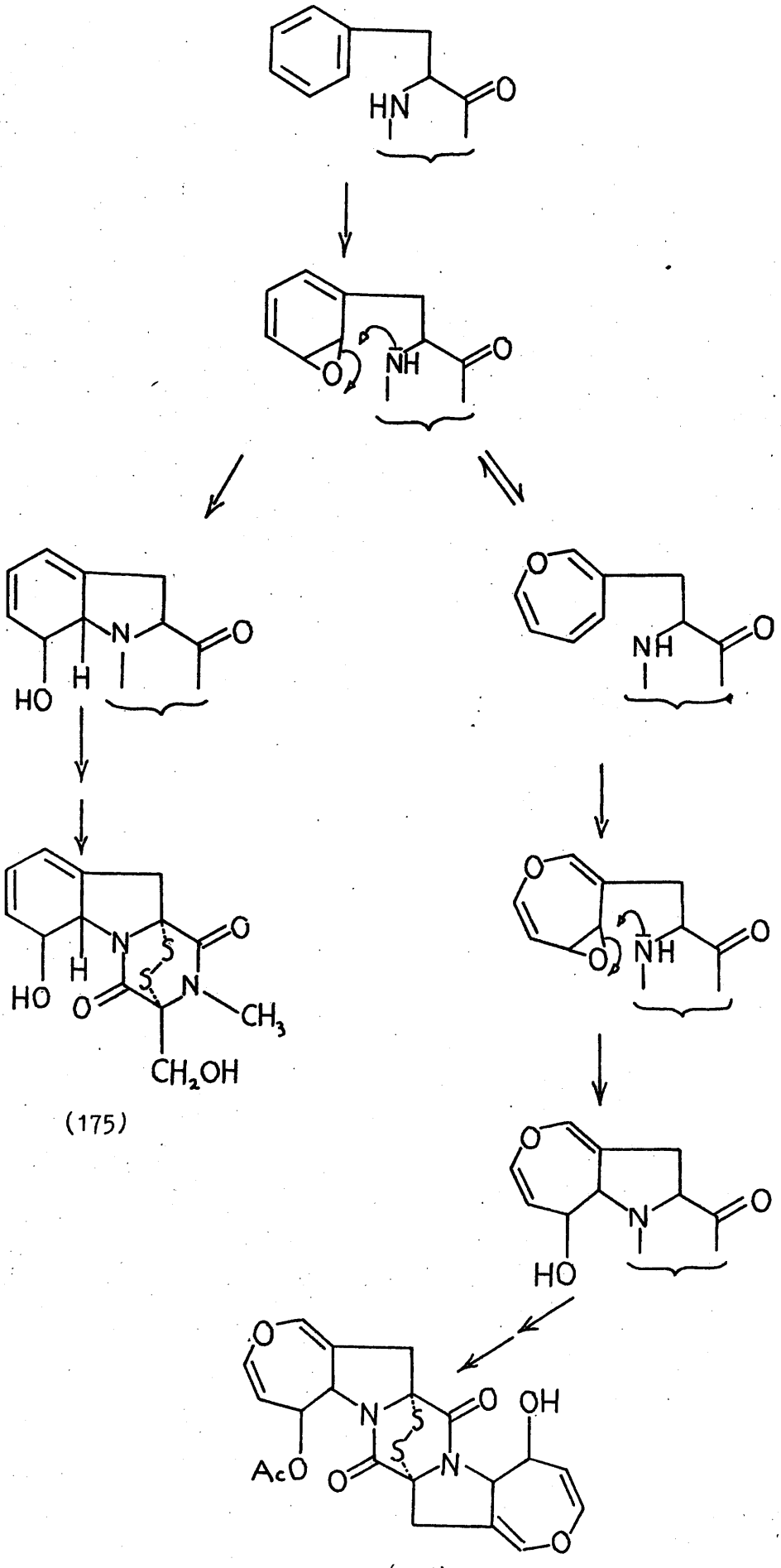
The above assignments (Table 4.8) were made, in part, on the basis of the size of the residual couplings observed in the O.R.D. spectrum of (159).

As irradiation at the resonance (τ 3.18) for H - 10 in the ^{1}H - n.m.r. spectrum of (159) resulted in sharpening of the signal for a methylene group (τ 6.98), this latter signal was ascribed to the benzylic protons H - 4 in (159) and, as this is the more downfield of the signals for the two methylene groups in the carbocyclic ring, the residual coupling observed for this carbon in the O.R.D. ^{13}C - n.m.r. spectrum of (159) should be larger than for the remaining methylene carbon. The fact that this is indeed the case in the above assignments, means that these assignments are correct and that the biosynthesis of asperflaschin occurs by route (a) in Figure 4.2.

One further feature of interest from these results is the demonstration here of the retention of individuality between the gem-dimethyl carbons of the (mevalonate derived) dimethylallyl side chain in (159). The signal for C - 15 appears as a singlet in the spectrum of (159) labelled with [1,2 - ^{13}C] - acetate, this carbon having originated from C - 2 of mevalonate, while the signal for C - 14 (C - 6 of precursor mevalonate) appears with satellite signals due to coupling with C - 13 (C - 3 of precursor mevalonate).

Although the experiments directed towards the elucidation of the biosynthesis of physcion anthrone (139) and asperflaschin (159) have demonstrated the modes of cyclisation of their respective polyketide precursors, these experiments have not helped to establish the order in which these compounds appear on the biosynthetic pathway(s) leading to 2-(γ, γ - dimethylallyl) - physcion (73),

Figure 4.3. Possible relationship of compounds related to gliotoxin (175) and aranotin (176) via a benzene oxide-oxepin rearrangement.



if indeed this is the end product of the pathway. However, with the isolation of asperflaschin (159) and the detection of 2 - (γ,γ - dimethylallyl) - physcion anthrone (140), it seems likely that prenylation occurs prior to oxidation to the level of the quinone and, with the detection of the derivative (174) of 2 - (γ,γ - dimethylallyl) - emodin anthrone (168), that prenylation precedes methylation. In contrast, the isolation of the derivative (153) of 2 - (γ,γ - dimethylallyl) - emodin suggests either that at least one other biosynthetic pathway is in operation in parallel with the first as the isolation of this compound is apparently in contradiction to the above evidence, or that the anthraquinones with their chemically very stable structures are not the true end products of the biosynthetic processes but are merely by-products formed by autoxidation or are artifacts resulting from the isolation procedure. It is possible, for instance, that asperflaschin (159) is the true biosynthetic end - product and that 2 - (γ,γ - dimethylallyl) - physcion anthrone is a precursor of (159) through hydration of the methyl-substituted aromatic ring in (140). Although this process does not look very feasible to the practised eye of a synthetic organic chemist, it does have some precedent in the enzymically - controlled chemistry of natural products in the suggestion, by Neuss *et al.*[181], put forward to explain how compounds with gliotoxin (175) and aranotin (176) type ring systems could be formed from a common benzene - oxide intermediate (Figure 4.3).

However, it is clear that, in the case of the metabolites isolated from *A.flaschentraegeri*, experiments involving the feeding of labelled samples of, for example, (103), (139), (159) and (140) are required before the present questions can be answered conclusively.

EXPERIMENTAL

EXPERIMENTAL

INSTRUMENTATION

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Ultraviolet spectra were obtained on a Unicam SP800 recording specrophotometer. Infra-red spectra were measured with Perkin-Elmer SP220 and SP257 spectrometers. Proton nuclear magnetic resonance spectra were recorded with Varian T-60 and HA-100 spectrometers using tetramethylsilane as an internal standard. ^{13}C-nuclear magnetic resonance spectra were recorded on a Varian XL-100 spectrometer at 25.2 MHz with tetramethylsilane as internal standard, using 10 mm. tubes.

Routine mass spectra were obtained with an A.E.I. MS-12 mass spectrometer and high resolution spectra on an A.E.I. MS-9 mass spectrometer.

THIN LAYER CHROMATOGRAPHY (T.L.C.)

Rf values were obtained from elution on 0.25mm. layers of Kieselgel HF_{254}, the compounds being located under ultraviolet light (λ=254nm.) and by spraying with a 1% solution of ceric ammonium nitrate in 5N sulphuric acid and heating. The colour developed by this spray is typically recorded thus:

T.L.C. Rf 0.62 (50% chloroform-light petroleum); Ce^{4+}: red.

Numerical values quoted are indications of relative polarity, only, and are not quantititative reproducible results.

Preparative layer chromatography (P.L.C.) was carried out on 1mm. layers of Kieselgel HF_{254} bands being located by irradiation with ultraviolet light (λ = 254 and 357nm.)

COUNTING OF RADIOACTIVE MATERIALS

Radioactive assays were carried out with a Phillips liquid

COMPOSITION OF SCINTILLATOR SOLUTION

2,5-Diphenyloxazole	4.0 g.
1,4-Bis-2-(4-Methyl-5-Phenyl Oxazolyl)-Benzene	0.1 g.
Toluene	1.0 l.

scintillation counter. Where doubly-labelled (^{14}C and ^{3}H) samples were counted, the external channels ratio method was used.

Solid samples were weighed on aluminium foil and transferred to Packard scintillation vials. The samples were each counted in a mixture of diluter solvent (D.M.F., 1ml.) and scintillator solution (14mls.).

Radioactive substances were assayed by weighing out and counting two separate samples from a particular crystallisation and the average activity calculated accordingly.

GENERAL

Diazomethane was prepared by the method of Moore and Reed[182] from bis-(N-methyl-N-nitroso)-terephthalimide. 'Jones reagent' refers to a solution containing chromium trioxide (266mg./ml.). Organic extracts were routinely washed with saturated brine solution and dried over anhydrous sodium sulphate. Solvents were removed under vacuum using a rotary thin film evaporator. Unless otherwise indicated, light petroleum refers to a light petroleum fraction, b.p. 60-80°C.

In reporting spectral data, the following abbreviations were used:

a) (in N.M.R. data) s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; d.d., double doublet; br.s., broad singlet.

b) (in I.R. data) s, strong; m, medium; w, weak.

c) (in U.V. spectral data) inf, point of inflection; sh, shoulder.

In reporting U.V. data, molar extinction coefficients (ϵ) for each absorption maximum are recorded as the logarithm, ($\log\epsilon$) and are presented in brackets after each maximum.

In the following typical description of N.M.R. spectral data:

τ 3.01 (1H, d. J = 4, irr. 7.30→s, H-3); irradiation at τ 7.30 has resulted in the collapse of the 1H doublet, with coupling constant 4 Hz, at τ 3.01 to a singlet.

Composition of Czapek-Dox medium (g./l.)

Sodium Nitrate	2.00 g.
Potassium Chloride	1.00 g.
Magnesium Sulphate	1.00 g.
Dipotassium Hydrogen Phosphate	0.50 g.
Ferrous Sulphate	0.01 g.
Glucose	50.00 g.

Growth of surface cultures of Verticillium lamellicola

The strain of Verticillium lamellicola studied (C.B.S. No. 11625) has been cultured in the Glasgow University Joint Mycology Laboratories since 1972. The fungus was subcultured onto 2% malt - agar slants and seed grown in the same medium in Roux bottles (15 x 9 cm.). Typically, a spore suspension prepared from 12 of these bottles and distilled water (2 l.) was used to innoculate 100 Roux bottles which had previously been sterilised and contained 200 ml. each of the medium described opposite. The fungus was grown at 75% humidity and 25°C and the cultures were usually harvested after 10 days.

Extraction of the broth and separation of broth metabolites

After the prescribed period of growth the broth was decanted off, filtered and extracted with methylene chloride in a continuous liquid - liquid extraction apparatus for 48 hours and the solvent evaporated to give the crude broth extract (0.5g. from 100 Roux bottles). Accumulated crude broth extract (6g.) was applied to a column of silica (600g., 5 x 60 cm.) set up in 100% chloroform (1 l.). Separation of metabolites was achieved by stepwise elution with 1, 2½, 5, 10, 50, 100% ethyl acetate - chloroform mixtures. Fractions (300 ml.) were collected

A yellow substance was eluted from the column with 2½ - 5% ethyl acetate - chloroform mixtures. The crude fractions containing this yellow pigment were combined and repeated chromatography (20% ethyl acetate - chloroform) afforded a dull yellow oil (0.050g., 0.25mg.l^{-1}), identified by spectral data as a new member of the cochlioquinone group of compounds and assigned the trivial name lamelliquinone (108).

Chapter 1

Lamelliquinone (108)

This compound was isolated as described earlier as a dull yellow oil.

T.L.C.: R_f 0.45 (20% ethyl acetate-chloroform); Ce^{4+} : dark brown.

I.R. ν_{max} (CCl_4) $cm.^{-1}$: 3580(w), 2975(s), 2915(s), 2875(m), 2845(m), 1678(s), 1665(s), 1653(s), 1647(s), 1637(s), 1605(s).

U.V. λ_{max} (EtOH)nm.: 230(4.09), ca. 259(inf.,3.90), ca. 295(inf.,3.29), 401(2.85).

(EtOH-NaOH)nm. : 270(3.94), ca.310(inf.,3.53), 401(2.85).

Does not revert to original spectrum on neutralisation.

N.M.R. (100 MHz) τ ($CDCl_3$): 3.13 (1 H, br.q. J =7, H-25); 3.50(1 H, d. J =2, irr. 5.40 ⟶ s, H-20); 5.40(1 H, br.q. J=7, irr.3.50 ⟶ sharp q., H-22); ca. 6.87(2 H,m, H-3,5); ca. 7.40(3 H,m, partly exchanges with D_2O , H-11 and C-4 OH); 8.15(3 H, br.d. J =7, irr. 3.13 ⟶ br.s., Me-26); 8.21(3H, d. J =1, irr. 3.13 ⟶ sharpening, Me-28); 8.96(3 H, d. J =7, irr. 5.40 ⟶ s, Me-27); 8.80(3 H,s, Me-14): 8.82(3 H, s, Me-15); 8.85(3 H, s, Me-12); 9.26(3 H, s, Me-13).

Spectrum was also obtained in CCl_4 (Table 1.1).

Spectrum was also obtained in solutions of the paramagnetic shift reagent tris-(dipivalomethanato)-europium, with $Eu(dpm)_3$: substrate concentration ratios 0.2, 0.4 and 0.6 molar (Table 1.3).

A graph of change in resonance values against reagent:substrate ratio was drawn and gradients measured for each identifiable group in the molecule.

^{13}C-n.m.r.: See assignments in Table 1.4.

M.S.: M^+ (apparent) at $^m/e$ =472(0.10); also 470(0.06), 454(0.06), 389(0.11), 371(0.10), 370(0.22), 250(0.17), 182(0.17), 167(0.19), 165(0.14), 149(0.50), 105(0.29), 83(0.84), 77(0.36), 76(1.00).

$C_{28}H_{38}O_6$ requires m.w. = 470.

Accurate M^+(apparemt) at $^m/_e$ = 472.2831; $C_{28}H_{40}O_6$ requires 472.2824. Also accurate M^+ at $^m/_e$ = 470.2667; $C_{28}H_{38}O_6$ requires 470.2668.

Attempted reduction of lamelliquinone to dihydrolamelliquinone.

Lithium metal (2 molar excess) was cautiously added to freshly distilled liquid ammonia (ca. 25ml.) in a flask fitted with a dry-ice condenser. The blue solution was allowed to stir for about 5 minutes. Lamelliquinone (143 mg.) in dry ether (5ml.) was slowly added. The solution was allowed to stir for a further 20 minutes then tertiary butanol (excess) was cautiously added to the mixture.

The ammonia was allowed to evaporate at room temperature, ethyl acetate (25 ml.) added to the mixture, the solution washed to neutrality with brine, dried and the solvent evaporated, giving a black oil (125 mg.). PLC (20% ethyl acetate - chloroform) afforded only a very complex mixture of products.

Reaction of Lamelliquinone with zinc in acetic anhydride - pyridine

Freshly activated zinc dust (24.4 mg., 0.375 m.mole) was added to a solution of lamelliquinone (108, 117,5 mg., 0.25 m.mole) in acetic anhydride (5 ml.) and pyridine (1 ml.). The solution was stirred at room temperature for 1½ hours, dilute hydrochloric acid (10mls.) added, the mixture extracted with ethyl acetate, the organic layer then washed with brine to neutrality, dried and the solvent evaporated to give a virtually colourless oil (102 mg.). PLC (20% ethyl acetate - chloroform) yielded the monoacetate (109,34mg., R_f 0.26) and the diacetate (110, 34 mg., R_f 0.41) of lamelliquinone.

Dihydrolamelliquinone monoacetate (109)

Chilling (acetone - dry ice) and trituration of

the more polar fractions with pentane afforded the monoacetate (109) as an off-white solid (6mg., 5%) m.p. 95-99°C.

T.L.C.: R_f 0.26 (20% ethyl acetate-chloroform); Ce^{4+}: pink.

I.R. $\nu_{max}(CCl_4)cm.^{-1}$: 3550(w), 3540 — 3100(broad,s), 2976(s), 2935(s), 2876(s), 2850(s), 1773(s), 1672(m), 1661(s), 1649(m), 1632(m), 1600(m), 1215(m), 1190(s).

U.V. λ_{max}(MeOH)nm.: 209.5(4.47), ca.222.5(inf.,4.28), ca.279(inf.,3.36).
(MeOH-NaOH)nm.: 218(4.89), ca.235(sh.,4.39), ca.280(inf.,3.87).
Reverts to original spectrum on neutralisation.

N.M.R. (60MHz) τ (CCl_4): ca.3.37(1H,m,H-25); 3.97(1H,br.s.,H-20); 5.63(1H, q.J=7,H-22); ca.6.93(2H,m,H-3,5); ca.7.30(3H,m,C-4 hydroxyl proton and H-11); 7.73(3H,s, acetate Me); 8.20(3H, d.J=6,Me-26); 8.30(3H, br.s.,Me-28); 8.74(3H,d.J=7, Me-27); 8.83(3H,s, Me-14); 8.90(6H,br.s., Me - 15, Me - 12); 9.30(3H,s,Me - 13).

M.S. : M^+ at $^m/_e$ = 514(0.16); also 496(0.05), 472(0.66), 454(0.22), 389(0.75), 371(0.26), 249(0.22), 231(0.06),223(0.22), 165(0.41),121(0.16), 83(1.00).

$C_{30}H_{42}O_7$ requires m.w. = 514.

Dihydrolamelliquinone Diacetate (110)

Chilling and trituration of the less polar fraction with pentane afforded the diacetate (110) as a solid which crystallised from chloroform - light petroleum as off-white granules (21 mg., 16%), m.p.119-22°C.

T.L.C.: R_f 0.41 (20% ethyl acetate - chloroform); Ce^{4+}: pink.

I.R. ν_{MAX} (CCl_4) cm^{-1}:3585(w),2980(m),2937(m),2877(w), 2850(w),1775(s),1677(m),1650(w),1631(w),1204(s),1184(s).

U.V. λ_{MAX} (MeOH)nm.:211(4.41),ca.222(inf.,4.33),ca.274.5 (inf.,3.39),280.5(3.41).
(MeOH-NaOH)nm.:220(5.23),ca.235(sh.,4.40),ca.280(inf.,3.82).
Reverts to original spectrum on neutralisation.

N.M.R. (60MHz) τ (CCl_4):ca.3.50(1H,m,H-25);3.77(1H,br.s.,H-20); 5.73(1H,q.J=7,H-22); ca.7.0(2H,m,H-3,5);ca.7.47(3H,m,C-4 hydroxyl,H-11);7.78(6H,s,acetate Me's); 8.27(3H,d.J=5,H-26); 8.33(3H,br.s.,Me-28); 8.77(3H,d.J=7,Me-27); 8.87(3H,s,Me-14); 8.93(6H,s,Me-15,Me-12); 9.37(3H,s,Me-13).

M.S.: M^+ at $^m/_e$=556(0.13); also 538(0.03),514(0.18),496(0.08),472(0.08), 456(0.07),431(0.38),413(0.11),389(0.04),372(0.04),371(0.04),291(0.05), 249(0.07), 231(0.04), 224(0.06),165(0.12),164(0.25),121(0.12),83(1.00).

$C_{32}H_{44}O_8$ requires m.w. = 556.

Culture medium for Aspergillus flaschentraegeri

Glucose	1000 g.
Ammonium Tartrate	56 g.
Potassium Dihydrogen Phosphate	100 g.
Magnesium Sulphate	20 g.
Sodium Chloride	20 g.
Difco Yeast Extract	20 ml.
Essential Mineral Concentrate	20 ml.
Distilled Water	to 2 l.

The resulting fluid medium has pH = 5.

Growth of Surface cultures of A. flaschentraegeri

Aspergillus flaschentraegeri (Commonwealth Mycological Institute No. 101,651) was grown on surface culture using the same procedure as described for the growth of V. Lamellicola. The growth medium used was that detailed opposite.

Normally, cultures were harvested after 10 days' growth. (Previous work had established that this growth period was that required for optimum antifungal and antibacterial activity, corresponding to the optimum concentrations of the diterpenoid broth metabolite, traegeric acid (120). The present work has, however, established that other growth times are more suitable for other metabolites, particularly mycelial metabolites). When required, other growth times were used.

Extraction of the broth and separation of broth metabolites

A typical procedure for the extraction of a 10 - day culture was as follows. The broth was decanted off from the mycelial mats, filtered and extracted with ethyl acetate in a continuous liquid-liquid extraction apparatus for 24 hours. The extract was then dried and the solvent evaporated, leaving a dark brown oil (8.5 g. from 100 Roux bottles).

This oil was taken up in ether as far as possible, filtered, the filtrate triturated with pentane, filtered again and evaporated to dryness. The filtrate was successively triturated in this way until a pale yellow oil was obtained. A few drops of benzene were added to the oil and allowed to stand for 5 minutes. The resulting solid crystallised from benzene-light petroleum, giving colourless needles of traegeric acid (up to ca. 2g. from 100 Roux bottles, 100 mg.$l.^{-1}$) m.p.181-3^{0}C (lit.,[128] 180-5^{0}).

Summary of chromatographic separation of A.flaschentraegeri mycelial metabolites

Fractions	Eluant	Weight	Constituents
1 - 13	100% light petroleum	0.228 g.	non-polar oils
14 - 17	20% chloroform-light petroleum	0.081 g.	unidentified mixture
18 - 20	20% chloroform-light petroleum	0.318 g.	unidentified mixture
21 - 38	30-40% chloroform-light petroleum	20.309 g.	dimethylallyl physcion + unidentified metabolites
39 - 58	50-70% chloroform-light petroleum	7.823 g.	dimethylallyl physcion + unidentified metabolites
59 - 66	80-90% chloroform-light petroleum	2.421 g.	unidentified mixture
67 - 71	90% chloroform-light petroleum	4.202 g.	physcion + unidentified metabolites
72 - 88	100% chloroform; 1-5% ethyl acetate-chloroform	12.252 g.	physcion, physcion anthrone, physcion anthrone dimer
89 - 112	10-100% ethyl acetate-chloroform	3.185 g.	unidentified polar mixture.

Extraction of the mycelium and separating of mycelial metabolites

a) Extraction with ethyl acetate

i) Routinely, the mycelial mats were separated from the broth as above, oven-dried (40°C) overnight and extracted with ethyl acetate in a Soxhlet apparatus for 24 hours. The solvent was then evaporated, leaving crude extract (ca. 0.2 - 0.3 g. per Roux bottle).

One separation procedure, as used initially, involved column chromatography of the crude ethyl acetate extract on silica gel. Crude extract (53.95g., accumulated from 3 batches of 100 Roux bottles each) was adsorbed on silica (100g.), dried under vacuum and introduced onto a column of silica gel. (2020g., 8 x 90 cm.)set up in light petroleum (column volume 3.25 l.). Separation was achieved by stepwise elution with 0, 20, 30, etc. to 100% chloroform-light petroleum, then 1, 5, 10, 25 and 100% ethyl acetate - chloroform mixtures, using one column volume of each solvent mixture. Fractions (450 ml.) were collected and monitored by TLC. Fractions which were shown to contain the same major components were bulked to give the fractions, as described in the table opposite.

Material (75 mg.) crystallising out of fraction 69 was found to be physcion (136), identified by comparison (TLC, m.p., spectral data) with authentic material.

Earlier fractions (21 - 38, 39 - 58), whose bright orange appearance suggested that they might be rich in pigments, were contaminated with undesirable fatty material. Removal of the non-polar components of the mixture (including the fats) was effected by trituration with ether. Further successive trituration of the sticky, orange, ether - insoluble portion with light petroleum, ethyl acetate and methanol afforded crops

of crude orange crystals, more of which were obtained by trituration of the ether insoluble portion with hot light petroleum. Recrystallisation (ethyl acetate) gave 2 - (γ, γ - dimethylallyl) - physcion (73, 50 mg.) as bright orange needles m.p. 215°C (lit., [125] 210 - 12°).

Bulked fractions (72 - 88) were further separated by PLC, eluting twice with 50% chloroform - light petroleum. An orange band (R_f 0.50) on extraction with ethyl acetate gave an orange gum which was shown by spectral data to be a mixture of physcion (136) and physcion anthrone (139).

A citrine-coloured band (R_f 0.20) gave the 10, 10' - dimer of physcion anthrone (148).

ii) Column chromatography was used on another occasion following a slightly different procedure. Mycelial extract was chromatographed on a silica column, as before. A crude separation of polar and non-polar material was achieved by repeated chilling and washing with light petroleum the column fractions (5.53g.) which had been shown by TLC to contain asperflaschin (see later). The insoluble portion was dried, giving a brown powder (2.76 g.). TLC showed this portion to contain the bulk of the asperflaschin. This powder was chromatographed on a column of silica (120 g.), set up in light petroleum. Stepwise elution with solvent mixtures ranging in polarity through 0, 5, 10% etc. chloroform - light petroleum was initiated, using one column volume of each. At 40% chloroform - light petroleum, asperflaschin was detected in the eluant along with two other less polar compounds, staining dull green (Rf 0.12) and bright green (Rf 0.20, 50% chloroform - light petroleum) with Ce^{4+}. Elution with solvent of this polarity was continued until 1.53 g. of material had been collected. Repeated PLC of

this material afforded asperflaschin (159, 324 mg.), physcion bianthrone (148, 39 mg. less polar green-staining compound) and the 10, 10' - dimer of physcion anthrone and dimethylallyl emodin anthrone (174, 28 mg., more polar green - staining compound).

iii) Generally, however, a more convenient separation procedure was direct PLC of mycelial extracts, eluting twice with 50% chloroform - light petroleum. Extraction of an orange - brown band (Rf 0.06) gave a crude brown gum which, on further PLC and crystallisation (ethyl acetate) afforded asperflachin (159) as yellow green needles, m.p. 179 - 81°C, $[\alpha]_D = +54°$ (c = 0.06, MeOH), in a yield of ca. 35 mg. l^{-1}.

Similarly, extraction of a citrine band (R_f 0.20) gave physcion bianthrone (148, ca. 30 mg. l^{-1}) and a strong orange - yellow band (R_f 0.47) was shown to be a mixture of physcion (136) and physcion anthrone (139), as described earlier. A thin orange band near the top of the plate (R_f 0.62) was shown to be 2- (γ,γ- dimethylallyl) - physcion, isolated earlier (ca 20 mg. l^{-1}).

b) Extraction with light petroleum (b.p. 100 - 120°)

An alternative extraction procedure, which was found to allow the selective isolation of physcion anthrone (139), involved soaking the mycelial mats in light petroleum (b.p.100 - 120°). After removal of the broth, the mycelial mat in each Roux bottle was covered with light petroleum (100 ml. per bottle) and allowed to stand overnight. The light petroleum solutions were then decanted off and replaced with fresh solvent. The mats were then shaken, with successive portions of solvent, for 2 days. The combined petroleum extracts were then dried and concentrated under vacuum until a pale yellow precipitate appeared, which was quickly filtered off, washed with chilled pentane and

recrystallised (ethyl acetate) yielding physcion anthrone (139), in an average yield of ca. 200 mg.l^{-1}, as pale yellow fluffy crystals, m.p. 185 - 7°C (lit.,[136] 181 - 2°). (On one occasion, repeated trituration of the mother liquors of (139) afforded asperflaschin (159), although in very low yield.)

The mycelial mats were dried in an oven overnight and then extracted overnight in a Soxhlet apparatus with light petroleum (b.p. 60 - 80°). Concentration of this extract as before afforded further crops (ca. 10 mg. per Roux bottle) of physcion anthrone (139).

Further extraction of the mycelium with ethyl acetate afforded a mixture which, together with all previous mother liquors, was routinely separated by PLC in the usual way.

During time studies (chapter 3), it was found that 7 days was a more suitable growth time for optimum incorporation of precursors into physcion anthrone (139). However, during routine work, the fungus was usually harvested after 10 days' growth.

Chapter 2

Conversion of traegeric acid (120) into the reduction product (124).

To traegeric acid (120, 1g.) in absolute ethanol (20ml.) was added pulverised sodium borohydride (220 mg.) in one portion. After stirring for three minutes more ethanol (20 mls.) was added and the suspension kept stirred at room temperature for 30 minutes. The reaction mixture was then diluted with water (200 ml.) and acidified with acetic acid to about pH 4. The crystalline suspension which formed was filtered off and washed with water giving crude (124). The filtrate and aqueous washings were extracted with ether (3 x 100 ml.) and the ether extracts combined with an ethereal solution of the crude filtered product, dried (Na_2SO_4) and evaporated to dryness. Recrystallisation from ether - chloroform - hexane gave (124) as off - white needles (0.72 g., 72%) identical (TLC, spectral data) with an authentic[126] sample.

Feeding of labelled precursors to cultures of A. flaschentraegeri

a) A sterilised solution of $[1 - {}^{13}C]$ - sodium acetate (90 atom % ^{13}C, 300 mg.) in distilled water (15 mls.) was added to the culture filtrates of a 5 day old surface culture of A. flaschentraegeri (15 Roux bottles). After a further 24 hours, a similar sterile solution of $[1 - {}^{13}C]$ - acetate in distilled water was added to the culture filtrates. The cultures were harvested after a further 72 hours. The broth was separated from the mycelium, filtered and extracted in 500 ml. portions with ethyl acetate - brine mixtures (3 x 200 ml., 3 x 300 ml, resp.). The combined ethyl acetate layers were washed with brine (3 x 100 mls.), dried and the solvent evaporated giving a brown oil (1.106 g.).

Trituration with ether and pentane in the usual way gave crude traegeric acid (120, 198 mg.). Successive crystallisations and treatment with animal charcoal afforded enriched traegeric acid (85 mg.). The ^{13}C - n.m.r. spectrum of this sample in $CDCl_3$ was compared with that of 85 mg. of unlabelled (120), determined under the same experimental conditions as far as possible (Table 2.1).

b) $[1,2 - ^{13}C]$ - sodium acetate (90 atom % ^{13}C, 375mg.) and unlabelled sodium acetate (750 mg.) were dissolved in distilled water (60mls.). This solution was divided into six solutions of equal volume and all were sterilised.

The first of these solutions was added to culture filtrates of a 4 day old surface culture of A. flaschentraegeri (For timing of feeding see chapter 3). After 12 hours, a second solution was administered in a similar manner. Additions of precursor solutions were continued at approximately 12 hour intervals until all six had been added.

The culture filtrates (2 l.), obtained in the usual way, were extracted with ethyl acetate in a liquid - liquid continuous extraction apparatus for 48 hours, the extract dried and the solvent evaporated, giving a brown residue (0.838 g.). Trituration with ether and pentane in the usual way produced crude grey solid (113 mg.). Recrystallisation (benzene - light petroleum) gave enriched traegeric acid (120, 52mg.). The ^{13}C - n.m.r. spectrum of this material was obtained in $CDCl_3$ (Table 2.1).

Chapter 3

Physcion (1,8 - dihydroxy - 6 - methyl - 3 - methoxy anthraquinone, 136)

Physcion was isolated from fraction 69 of a column of mycelial extract as described before and identified by comparison (m.p., spectral data) with authentic samples previously isolated[125] from this fungus in these laboratories.

Physcion was also isolated as one component of a mixture, with physcion anthrone (139), from a bright orange bond (R_f 0.50, 50% chloroform-light petroleum) during PLC of crude mycelial extract.

Physcion anthrone (1,8 - dihydroxy - 3 - methyl - 6 - methoxy - 10 - anthrone, 139)

Physcion anthrone was obtained, with physcion (136), from an orange band during PLC of crude mycelial extract, as described earlier. The pigment was also obtained, in a virtually pure form, by standing (or shaking) fresh mycelium from surface cultures of A. flaschentraegeri with portions of light petroleum (b.p. 100 - 120°) for about 3 days, as described earlier.

T.L.C: R_f 0.57 (50% chloroform - light petroleum); Ce^{4+}; olive green with yellow halo.

I.R. ν_{max} (KBr disc) $cm.^{-1}$: 1644(s), 1618(s)1596(s), 1568(s).

ν_{max} ($CHCl_3$) cm^{-1}: 2980(w), 1650(m), 1629(s), 1608(s).

U.V. λ_{max} (EtOH) nm.: 224.5 (4.37), ca.252(inf., 3.92),256(3.93), 272 (4.00), 303(3.93), 352(4.23).

(EtOH - NaOH) nm.:214.5 (5.03), 238(4.48), ca. 253(inf.,4.32),308(3.99), 408(3.70), 515(3.80). Did not revert to original spectrum on neutralisation.

N.M.R. (100MHz)τ($CDCl_3$): -2.59(1H,s,OH);-2.29(1H,s,OH); 3.34 (2H, br.s., H -2,4); 3.64(2Hbr.s., H-5,7);5.81(2Hbr.s.,anthrone CH_2);

6.16 (3H,s, Ar-OMe); 7.64(3H,s,Ar-CH_3).

Nuclear Overhauser effect: irr. 6.16 ⟶ 18% enhancement of signal at 3.64.

irr. 7.64 ⟶ 8% enhancement of signal at 3.34. irr. 5.81 ⟶ 13% enhancement of signal at 3.64, 16% enhancement of signal at 3.34.

^{13}C - n.m.r. See table 3.3

M.S. : M^{+} m/e = 270(1.00); also 255(0.25), 242(0.12),241(0.13), 227 (0.39), 149(0.38).

$C_{16}H_{14}O_4$ requires m.w. = 270.

Analysis: Found C: 71.05, H : 5.32; $C_{16}H_{14}O_4$ requires C:71.11, H:5.19.

Oxidation of physcion anthrone (139).

Physcion anthrone (139, 20mg.) in acetone (10mls.) at 0°C was treated with Jones reagent (ca. 5mls.) with stirring and left for 15 minutes at room temperature. The solvent was evaporated and ethyl acetate (20mls.) and water (20mls.) added. The aqueous layer was extracted with ethyl acetate (3x20mls.), the combined organic extracts washed with brine to neutrality and dried. The crude product was purified by PLC (50% chloroform-light petroleum). Crystallisation from ethyl acetate gave physcion (136, 15mg., 74%) identical with authentic material.

Attempted methylation of physcion anthrone (139)

a) Reaction with methyl iodide and potassium carbonate

Physcion anthrone (139, 105 mg.) in dry acetone (5ml.) was stirred and refluxed overnight with potassium carbonate (100 mg.) and excess methyl iodide under nitrogen. The solution was filtered and solvent evaporated giving an orange gum (129 mg.). PLC (50% chloroform-light petroleum) gave the 10, 10 dimethyl derivative (143) as an amorphous pale yellow solid (ethyl acetate-hexane), m.p.141-4°C

(52mg., 45%)

T.L.C. : R_f 0.50(50% chloroform-light petroleum); Ce^{4+} yellow

I.R. ν_{MAX} (KBr. disc)cm^{-1}:3000(w), 2920(w), 1633(s), 1605(s),1594(s).

U.V. λ_{MAX} (MeOH)nm.: 223(sh., 4.31),ca.229(sh.,4.24),ca.250(inf.,3.86), 255(3.88), ca. 265(inf.,3.72),274(3.95),307(3.97),364(4.20) (MeOH-NaOH) nm.: 216.5(4.87),255.5(4.07)273.5(4.02),302(4.00), 384(4.06). Reverted to original spectrum on neutralisation.

N.M.R. (60MHz) τ($CDCl_3$); -3.03(1Hs,-OH);-2.66(1H,s,-OH); 3.12(1H,d.J=2,H-4); 3.33(1H,d.J=2,H-2); 3.38(1H,d.J=2,H-5); 3.62(1Hd.J=2,H-7);6.14(3Hs,Ar-OMe); 7.62(3Hbr.s.,Ar-Me); 8.38(6H,s,gem-Me's).

M.S. : M^+ at $^m/e$ = 298(0.37); also 284(0.20), 283(1.00),269(0.07), 255(0.36), 240(0.11),212(0.20).

$C_{18}H_{18}O_4$ requires m.w. = 298

Analysis:Found C:72.44, H:6.18; $C_{18}H_{18}O_4$ requires C:72.47,H:6.08.

b) Treatment with diazomethane

Physcion anthrone (139,50mg.) in methanol (40ml.) was treated with an excess of diazomethane from nitrosan (385mg.) and the mixture left overnight. The solution was poured into chloroform(100mls.) and allowed to stand for 3 hours. Evaporation of solvents yielded a fawn-coloured solid (49mg.). TLC and spectral data showed this to be a mixture of unreacted (139) and the 10, 10' dimer (148).

In subsequent experiments, physcion anthrone was allowed to stand overnight in chloroform, methanol and a mixture of chloroform and methanol.Intheabsence of diazomethane, no reaction could be detected by TLC.

Attempted acetylation of physcion anthrone(139).

a) Reaction with acetic anhydride

Physcion anthrone (139, 30mg.) was refluxed with acetic

anhydride (5mls.) for 3 hours. Ethyl acetate (10 mls.) was added, the mixture washed with water (10mls.), brine (3 x 10mls.), dried and the solvents evaporated giving a brown oil (40mg.) from which no pure compound could be isolated by PLC. The i.r. spectrum of the major band (R_f 0.66, chloroform) showed the presence of ester groups (ν_{CO} 1770 $cm.^{-1}$). However, this material decomposed before any further characterisation was possible.

b) Reaction with acetic anhydride and pyridine

Physcion anthrone (139, 100 mg.) was stirred overnight at room temperature with acetic anhydride (5ml.) and dry pyridine (1 ml.) under nitrogen. Evaporation of solvents gave a pale yellow resin which crystallised from ethyl acetate, giving 1,8,9 - triacetoxy - 10 - acetyl - 6 - methoxy - 3 - methyl anthracene (144) as an amorphous pale yellow solid (84 mg., 51%), m.p. 279 - 80°C (lit.,[139] 275 - 6°).

T.L.C.: R_f 0.21 (50% chloroform - light petroleum); Ce^{4+}: yellow.

I.R. ν_{MAX} (KBr. disc)$cm.^{-1}$: 1765(s), 1695(s), 1627(s),1564(m).

U.V. λ_{MAX} (MeOH)nm. : ca.229(inf.,4.09), ca. 245(sh.,4.36), ca. 256(sh.,4.70), 264.5(4.97), 348(3.50),368(3.62),390(3.66),412(3.62). (MeOH-NaOH)nm. : 215.5(5.12),ca.249(inf.,4.40);270(4.55),ca.278 (sh., 4.51),363(3.94),382(4.07),ca. 495(inf.,3.50). Did not revert to original spectrum on neutralisation.

N.M.R. (100 MHz) τ ($CDCl_3$): 2.66(1H,m,irr.7.53 ⟶ d. J= 1.5, irr. 3.08 ⟶ q. J = 1, H - 4); 3.08(1H,br,d,,irr.7.53⟶d.J=1.5, irr. 2.66 ⟶ s, H - 2); 3.18(1H,d.J=2 H - 7); 3.25(1H,d.J=2,H-5); 6.14(3H,s,Ar-OMe); 7.24(3H,br.s., irr.2.66 ⟶ d.J =1.1,Ar-CH_3); 7.60, 7.72(9H's, acetate Me's).

Nuclear Overhauser effect: irr.τ 6.14 $\longrightarrow$28% enhancement of signal at τ 3.25, 17% enhancement of signal at τ 3.18.

^{13}C-n.m.r. : See Table 3.4.

M.S. : M^+ at $^m/_e$ = 438(0.12); also 396(0.12),354(0.21), 336(0.11), 312(1.00), 297(0.28), 283(0.09), 270(0.08), 255(0.09).

$C_{24}H_{22}O_8$ requires m.w. = 438.

Attempted synthesis of emodin anthrone (103)

a) Reaction of physcion anthrone (139) with pyridine-hydrochloride

Physcion anthrone (139,60mg.) and pyridine-hydrochloride(1g.) were heated at 175-80°C under nitrogen for 5½ hours. Water (50mls.) was added to the cooled mixture and the solution filtered, giving a green solid. This was dissolved in 5% sodium bicarbonate solution, filtered, the filtrate acidified with dilute hydrochloric acid and the resulting precipitate filtered off, yielding a black intractable residue.

b) Reaction of physcion anthrone(139) with hydriodic acid.

Physcion anthrone (139,200 mg.) was refluxed with aqueous hydriodic acid (47% v/v, 8ml.) and glacial acetic acid (18mls.) under nitrogen for 3 hours. On cooling, yellow crystals appeared which were removed, washed with water and dried. Recrystallisation from ethyl acetate afforded emodin anthrone (103) as yellow prisms(134mg., 71%) decomposing with melting at ca. 267°C.

T.L.C.: R_f 0.20 (chloroform); Ce^{4+} : grey-green.

I.R.ν_{max}(KBr.disc)cm.$^{-1}$: 3365(w), 3305(w), 1640(m), 1618(s), 1598(s).

U.V. λ_{max}(MeOH) nm.: 222(4.39) 251.5(3.89), 259.5(3.92), 271(3.96), 310(inf., 3.90), 356(4.21)

(MeOH-NaOH)nm.:210(5.05),237.5(4.49),257(4.29),286(4.22), 310(4.19),397(3.73), 526(3.83). Did not revert to original spectrum on neutralisation.

N.M.R(100MHz) τ (d_6-DMSO):-2.32(1H,s, exchanges D_2O, - OH); - 2.16(1H,s, exchanges D_2O,-OH); -0.71(1H,br.s., exchanges D_2O,- OH); 3.25(1H,br.s., H - 4); 3.35(1H,br.s., H-2);3.59 (1H,d.J=2, H-5); 3.68(1H,d.J=2, H-7); 5.72(2H,br.s.,$-CH_2-$); 7.66(3H,s,Ar-Me).

M.S. : M^+ at $^m/e$=256(1.00); also 241(0.23),228(0.12),227(0.13), 181(0.05), 152(0.06).

$C_{15}H_{12}O_4$ requires m.w. = 256.

Analysis: Found C:70.44,H:4.84;$C_{15}H_{12}O_4$ requires C:70.31,H:4.72.

c) Reaction of emodin (84) with hydriodic acid

Emodin (84,200mg.) was refluxed with hydriodic acid (47% v/v,10.2 mls.) and glacial acetic acid (24mls.) as in the previous experiment. Crystals deposited on cooling were filtered off, dried and recrystallised (ethyl acetate),giving emodin anthrone(103,165mg., 87%) as salmon - pink prisms with spectroscopic data identical to those of the product from the previous reaction.

Methylation of emodin anthrone(103)

Emodin anthrone(103,200mg.), in dry acetone (10mls.), was stirred and refluxed overnight with potassium carbonate (200mg.) and excess methyl iodide. The cooled solution was filtered and solvents evaporated, giving a brown gum (382 mg.). PLC (50% chloroform - light petroleum) gave a pale yellow product(86 mg., 38%), identical (i.r., n.m.r.,m.s.) to that obtained from methylation of physcion anthrone.

Acetylation of emodin anthrone(103)

Emodin anthrone (103,108mg.) was stirred overnight at room temperature with acetic anhydride (2ml.) and pyridine (0.5ml.). Evaporation of solvents and PLC (chloroform) gave the acetylated derivative (145,65mg., 20%) as an off-yellow amorphous powder(ethyl acetate-light petroleum), m.p. 227-9°C.

T.L.C.: R_f 0.38 (chloroform); Ce^{4+}: dull yellow.

I.R. ν_{MAX} (KBr. disc) $cm.^{-1}$: 1768(s), 1693(m) 1627(m), 1560(w), 1210(s), 1190(s).

U.V. λ_{MAX} (MeOH)nm.: 224.5(4.19) ca. 252(sh., 4.72), 262.5(4.98), ca. 344(3.45), 364(3.69), 381(3.81), 403(3.72).

(MeOH-NaOH)nm.; 216(5.61), 233(inf., 4.47), 254.5(inf., 4.28), 293(4.22), 310(4.23), 383(3.77), 525(3.91). Did not revert to original spectrum on neutralisation.

N.M.R. (60MHz) τ ($CDCl_3$) : 2.57(2H, d. J = 2, H-4,5); 2.97(2H, d. J = 2, H - 2,7); 7.23(3H, s, ketone Me); 7.53 (3H, br.s., Ar-Me); 7.61, 7.70(9H, acetate Me's).

M.S. M^+ at $^m/_e$ = 466 (0.10); also 424(0.08), 382(0.19), 364(0.19) 341(0.20), 340(1.00), 299(0.13), 298(0.78), 297(0.36), 283(0.23), 269(0.16), 255(0.08), 241(0.10), 226(0.10),

$C_{25}H_{22}O_9$ requires m.w. = 466

Analysis: Found C:64.55, H: 4.65; $C_{25}H_{22}O_9$ requires C: 64.38 H: 4.75.

Physcion - 10,10' - bianthrone(148)

This compound was isolated from mycelial extracts, by the methods described earlier, as an off - yellow amorphous solid $[\alpha]_D = 24^\circ$ (c = 0.04, $CHCl_3$), melting with decomposition above 250°C.

T.L.C.: R_f 0.20(50% chloroform - light petroleum); Ce^{4+}: bright green with yellow halo.

I.R. ν_{max} (KBr disc)$cm.^{-1}$: 3430(w), 1638(s), 1617(s), 1592(s), 1569(m).

U.V. λ_{max} ($CHCl_3$)nm.: ca. 259(inf., 4.13), 280(4.29), 362(4.43).

N.M.R. (100MHz) τ ($CDCl_3$): -2.12(1H, s, exchanges D_2O, - OH); -1.75 (1H, s, exchanges D_2O, - OH); 3.32(2H, br.s., irr.7.68 ⟶ sharpening, H-2,2');

3.65(2H, overlapping doublets, H-7,7'); 3.89(2H,br.s., irr. 7.68 ⟶ sharpening, H-4,4'); 4.03(2H, overlapping doublets,H-5,5'); 5.66(2H,br.s., H-10,10'); 6.18(6H,s,Ar- OMe); 7.68(6H,br.s.,Ar- CH_3).

^{13}C-n.m.r.: See table 3.7

M.S.: Similar to mass spectrum of physcion anthrone (139).

Accurate M^+ (apparent) at $^m/_e$ = 270.0873; $C_{16}H_{14}O_4$ requires m.w.= 270.0892.

Osmometric m.w. ($CHCl_3$) = 537; $C_{32}H_{26}O_8$ requires 538.

Attempted synthesis of the triphenolic tautomer (146) of physcion anthrone (139)

a) By treatment of (139) with aqueous potassium hydroxide

Physcion anthrone (139, 30mg.) was allowed to dissolve in aqueous potassium hydroxide (10mls.) under nitrogen. The red-orange solution was refluxed for 30 minutes then allowed to cool, still under nitrogen. The mixture was carefully brought to neutrality with 50% aquous acetic acid. A green-yellow precipitate which formed was filtered off. TLC showed no reaction had taken place.

b) By sodium borohydride reduction of the triacetate (147)

Physcion anthrone (139, 22mg.) was refluxed with acetic anhydride (20 ml.) under nitrogen until no starting material remained (TLC). Evaporation of solvents gave a brown oil (84mg.). No single product could be obtained from PLC (50% chloroform-light petroleum) of the crude mixture, however.

Attempted synthesis of physcion bianthrone (148)

A solution of physcion anthrone (139, 100 mg.) in dilute sodium hydroxide (20mls.) was stirred for 5 days, during which time air was bubbled through the solution. TLC of a sample removed at this point indicated no detectable conversion of (139) into (148). A catalytic amount (ca. 5 mg.) of potassium

ferricyanide was added to the mixture and the solution stirred for a further 2 days. The solution was then neutralised with dilute hydrochloric acid (ca. 25mls.), extracted with ethyl acetate (25mls.) and the organic layer washed with sodium bicarbonate solution (25mls.), then brine to neutrality and dried. Evaporation of solvents gave a grey-green solid. No dimerisation was detectable by TLC.

Attempted acetylation of physcion bianthrone (148).

Attempted acetylation of (148) with acetic anhydride-pyridine afforded only unchanged material as evidenced by T.L.C.

Feeding of labelled precursors to cultures of A.flaschentraegeri (biosynthesis of physcion anthrone, 139)

1) Preliminary experiments with radioactive precursors.

Initially, the determination of the activity of physcion anthrone (139) isolated from cultures of A. flaschentraegeri enriched with ^{14}C - labelled sodium acetate was attempted by counting several accurately weighed samples (each ca. 1mg.) of the 'hot' material directly (i.e. 1mg. in 1ml. D.M.F. and 14 mls. scintillator solution). It was found, however, that this procedure resulted in an unacceptably low (< 0.3) 'channels ratio', this value being the level below which the scintillation counter was incapable of accurately measuring the activity of a given sample. This effect was attributed to 'colour quenching' by the yellow pigment, itself, when present in counting solutions at this concentration. It was found, using standards of accurately known activity, that 0.5 mg. of the pigment was the maximum amount of sample which could be tolerated in 15 mls. of scintillation mixture in a single vial before colour quenching began to take place excessively.

Routinely, ca. 1-2 mg. of sample was weighed accurately

on aluminium foil and dissolved in 1 ml. D.M.F. and 14 ml. scintillator solution. Exactly 1 ml. of this solution (containing $^{1}/_{15}$ - $^{2}/_{15}$ mg. sample) was then removed, made up to 15 ml. with scintillator solution and counted, subsequent calculations being adjusted accordingly.

(a) A solution of $[1 - {}^{14}C]$ - sodium acetate (0.1007mCi) and $[2 - {}^{3}H]$ - sodium acetate (2.0618mCi) in distilled water (10mls.) was sterilised and added to the culture filtrates of a 5 day old surface culture of A. flaschentraegeri (5bottles). 24 hours later, a second solution (0.1002 mCi $[1 - {}^{14}C]$ - sodium acetate and 2.0323 mCi $[2 - {}^{3}H]$ - sodium acetate, in 10 mls. distilled water) was added in a similar manner. The cultures harvested after a further 72 hours' growth.

The broth and mycelium were separated and the mycelium shaken with portions (60ml. per bottle per day) of light petroleum (b.p. 100 - 120°) for 3 days. The combined petroleum extracts were concentrated and the pale yellow physcion anthrone (139) which separated (173 mg.) filtered off and dried. The mycelium was dried and extracted overnight (Soxhlet) with light petroleum (b.p. 60 - 80°). Concentration of this solution afforded more physcion anthrone (156 mg.). The results of this feeding are summarised in Table 3.5.

(b) In view of the relatively low incorporation of sodium acetate into (139) in the trial, 2-pulse, (5-9) day feeding experiment described above, a systematic time study was carried out to determine the optimum for incorporation of sodium acetate into physcion anthrone. This was achieved in two separate groups of experiments, using two different batches of surface cultures of A. flaschentraegeri.

In the first group, [1 - ^{14}C] - sodium acetate was added to 3 - day old cultures (0.1 mCi in 2 Roux bottles in each experiment) and the fungus harvested on the fifth, sixth and seventh days of growth to 4 day old cultures which were harvested on the sixth and seventh days and to a 5 day old culture which was harvested on the seventh day of growth.

In the second group, labelled acetate was added in a similar way (single pulse) to 5-day old cultures which were harvested on the seventh, ninth and eleventh days, to 7-day old cultures which were harvested on the ninth and eleventh days and to a 9-day old culture harvested on the eleventh day. (The repetition of the (5-7) day experiment served as an indicator on the consistency (or lack thereof) in incorpotation rates for this growth period between the two different batches of fungus).

In each experiment the times of feeding and harvesting were an exact number of hours after innoculation (eg. feeding 3-day old cultures involved the administration of precursor 72 hours after innoculation). Also, in each experiement physcion anthrone (139) was isolated by flooding the mycelium with light petroleum (b.p. 100 - 120°) (100mls. per Roux bottle per day) for 3 days. The combined light petroleum extracts were then concentrated and physcion anthrone isolated as described before. In all cases the material was 'counted' as isolated (if chemically) pure) without further crystallisation, as an approximate measurement, only, of activity was required. (To check that no gross distortion in the activity was being obtained, in one case further crystallisation was carried out, without significant change in the activity being observed).

The results from these experiments are summarised in Table 3.6.

2) Experiments with ^{13}C-labelled precursors.

(a) $[1 - ^{13}C]$ - sodium acetate (90 atom%, 453 mg.) in distilled water (36 mls.) was sterilised and added to 4 - day old surface cultures of A. flaschentraegeri (3bottles) in 6 approximately equal amounts (2mls. containing ca. 25mg., per bottle per pulse). The first pulse was administered exactly 96 hours after innoculation and the rest at approximately 12-hour intervals thereafter.

72 hours after the administration of the first pulse the fungus was harvested. The mycelium was extracted with light petroleum (b.p. 100 - 120°), (100 mls. per bottle per day) for 3 days in the usual way. Concentration of the combined light petroleum extracts afforded physcion anthrone (139, 81.4mg.).

The mycelium was then dried and extracted overnight (Soxhlet) with light petroleum (b.p. 60 - 80°). Physcion anthrone (94 mg.) which precipitated out was removed. Recrystallisation (ethyl acetate) of the combined samples afforded pure physcion anthrone (132 mg.).

The ^{13}C-n.m.r. spectrum of this material was obtained in $CDCl_3$. The spectrum of unlabelled material was also obtained under, as far as possible, identical conditions (Table 3.3).

(A radioactive feeding experiment was carried out in parallel with this ^{13}C - experiment in order to monitor the effect of pulse feeding over 3 days on the rate of incorporation of acetate into physcion anthrone. $[1 - ^{14}C]$ - sodium acetate (0.1 mCi) in distilled water (12 mls.) was added in 6 pulses to surface cultures of A.flaschentraegeri (2 bottles) at the same times as the additions of ^{13}C - precursor, above. Physcion anthrone (139, 95 mg.) was isolated by the same procedure described above and the activity of this material was determined

It was found that the rate of incorporation of acetate in (139) in this experiment was ca. 3.9%).

(b) [1,2 - ^{13}C] - sodium acetate (90 atom% 75 mg.) and natural abundance sodium acetate (225mg.) in distilled water (24 mls.) was divided into 6 approximately equal portions. The six solutions were sterilised and added to surface cultures of A. flaschentraegeri (2 bottles), the first addition 96 hours after innoculation, the other 5 at 12 hour intervals thereafter.

The fungus was harvested 72 hours after the first addition, and the mycelium extracted with light petroleum (b.p. 100 - 120^{0}) in the usual way. Recyrstallisation (ethyl acetate) of the physcion anthrone from the combined concentrated extracts afforded pure physcion anthrone (35mg.). The ^{13}C - n.m.r. spectrum of this material was obtained in $CDCl_3$ (Table 3.3).

2 - (γ,γ -Dimethylallyl)-physcion (1,8-dihydroxy-2(γ,γ dimethylallyl)-3-methoxy-6-methyl-anthraquinone, 73)

This compound was obtained from mycelial extracts by column chromatography but, more conveniently, by PLC as described earlier, as orange needles, m.p. 215-7°C (lit.,[125] 210-2°)

T.L.C.: Rf 0.62 (50% chloroform-light petroleum); Ce^{4+}: scarlet.

I.R. ν_{MAX}(KBr disc)cm.$^{-1}$: 3420(w),1668(w),1624(s) 1602(m), 1561(m).

U.V. λ_{MAX}(EtOH)nm.: 221(4.47),ca.247(inf.,4.08),277(4.44), ca. 304(inf., 3.93); 435(4.06),452(4.01).

(EtOH-NaOH)nm.: 217.5(4.50), ca. 233(inf., 4.53), 264(4.43), 312(4.12), 378(3.52), 522(3.99). Reverted to original spectrum on neutralisation.

N.M.R. (100MHz)τ ($CDCl_3$): -2.37(1H,s, exchanges D_2O,-OH); -2.10(1H,s, exchanges D_2O, - OH); 2.42(1H,br.d., irr.7.60→d.J=2, H-5); 2.65(1H,s,H-4); 2.97(1H,br.s., irr. 7.60→d.J=2, H-7); 4.82(1H,m,H-β); 6.01(3H,s,Ar-OMe); 6.60(2H,d.J=8,H-α); 7.60(3H,br.s., Ar-Me); 8.17(3Hbr.s., cis γ - Me); 8.28(3H,br.s. trans-γ-Me)

M.S.: M^+ at $^M/e$ = 352(0.45); also 337(0.21), 309(1.00),297(0.48), 284(0.12), 267(0.15) 241(0.09).

Accurate M^+ at $^m/e$ = 352.1313; $C_{21}H_{20}O_5$ requires 352.1311.

Analysis: Found C:71.55, H:5.82; $C_{21}H_{20}O_5$ requires C:71.58,H:5.72.

The ir., u.v. and n.m.r. data are very similar to those reported by Bell[125] for a yellow crystalline compound, plates m.p. 210-2°C, which analysed as $C_{21}H_{20}O_5 \cdot H_2O$.

Reaction of 2 - (γ,γ - dimethylallyl)-physcion (73)with trifluoroacetic acid.

2- (γ,γ - dimethylallyl)-physcion (73,63mg.) was allowed to dissolve in trifluoroacetic acid (2ml.) and stand overnight. P.L.C. (50% chloroform-light petroleum) gave the

dimethyldihydropyran (152) as red rosettes (ethyl acetate), m.p. 234 - 5°C (19mg., 30%).

T.L.C.: R_f 0.42(50% chloroform - light petroleum); Ce^{4+}:red.

I.R. ν_{MAX} (KBr. disc) cm^{-1}: 2970(w), 2935(w), 1657(w), 1632(s), 1571(s),1565(m).

U.V. λ_{MAX} (MeOH)nm.: 225(4.49), ca. 246(inf.,4.15),270(4.42), ca. 283(sh.,4.31), ca.302(inf.,3.96),424(3.97). (MeOH-NaOH)nm.:216.5(4.84),ca. 233(inf., 4.55), 267(4.47), 301(4.16), 368(3.75), 442(3.83). Reverted to original spectrum on neutralisation.

N.M.R. (60MHz)τ ($CDCl_3$): -3.33(1H,s, exchanges D_2O, - OH); 2.57 (1H,br.s., H-5); 2.85(1H,s.H-4); 3.06(1H,br.s.,H-7); 6.05(3H,s, Ar-OMe); 7.29(2H,t.J=7,α- CH_2); 7.62(3H,br.s.,Ar-Me); 8.17(2H,t.J=7, β-CH_2); 8.55(6H.s. γ - Me's).

M.S.: M^+ at $^m/e$ = 352(0.59); also 337(0.12), 323(0.07), 310(0.26), 309(0.59), 299(0.07), 298(0.30), 297(1.00), 267(0.22), 241(0.10). Accurate M^+ at $^m/e$ = 352.1306; $C_{21}H_{20}O_5$ requires 352.1311.

Analysis: Found C:71.55, H:5.82; $C_{21}H_{20}O_5$ requires C:71.58;H:5.72.

Reaction of 2-(γ,γ - dimethylallyl)-physcion(73) with hydrochloric acid - acetic acid.

2- (γ,γ- dimethylallyl)-physcion(73,20mg.) was heated with glacial acetic acid(3ml.) and concentrated hydrochloric acid (4 drops) at 100°C for 15 minutes. On cooling, crystals appeared which were removed, washed with water and dried. Recrystallisation (ethyl acetate) gave the chloro-derivative (167, 12mg., 54%) as orange needles, m.p. 194-7°C.

T.L.C.: R_f 0.55(50% chloroform - light petroleum); Ce^{4+}: scarlet.

I.R.: ν_{MAX} (KBr disc)$cm.^{-1}$: 2970(w),2937(w), 2840(w), 1669(m), 1620(s), 1598(s), 1557(m).

U.V. λ_{max} (MeOH)nm: 224.5(4.38), 245.5(4.07), 271.5(4.36), ca. 277(sh., 4.35), 306.5(3.89), 437(3.97), 460(3.90). (MeOH-NaOH)nm.:216(4.74), ca.231(inf.,4.50),264(4.40), 311(4.08), 382(3.51), 520(3.89). Reverted to original spectrum on neutralisation.

N.M.R. (100MHz)τ($CDCl_3$): - 2.60(1H,s, exchanges D_2O - OH); - 2.04(1H,s, exchanges D_2O - OH); 2.39(1H,br.s., irr. 7.53 ⟶ d. J= 2,H-5); 2.62(1H,s,H-4); 2.94(1H,br.s., irr. 7.53 ⟶ d.J= 2, H-7); 5.98(3H,s, Ar-OMe); 7.05(2H,t,J=8,α- CH_2); 7.53(3Hbr.s.,Ar-Me); 8.02(2H,t. J=8,β-CH_2); 8.28(6H,s, γ-Me's).

M.S.: M^+ (apparent)at $^m/_e$ = 352(0.50); also 337(0.11), 334(0.06), 323(0.06), 321(0.05), 319(0.05), 310(0.13), 309(0.54), 298(0.19), 297(1.00), 267(0.21), 241(0.13).

$C_{21}H_{21}O_5Cl$ requires m.w. = 388.5

Analysis: Found C: 64.63, H: 5.22, Cl: 9.28; $C_{21}H_{21}O_5Cl$ requires C:64.86, H: 5.41, Cl: 9.14.

This sample was identical in all respects to a sample prepared by oxidation of the chloro-derivative (166) as described later.

Attempted synthesis of 2-(γ,γ- dimethylallyl)-emodin(158)

Excess ethanethiol (1ml.) was added to a solution of sodium hydride (10.3mg.)in dry dimethylformamide (4mls.) and the mixture stirred under nitrogen until a clear yellow suspension formed. 2-(γ,γ - dimethylallyl) - physcion (73, 20mg.) in dry D.M.F. (5mls.) was added giving a purple solution. The mixture was refluxed for 3 hours, cooled, and 5% aqueous acetic acid solution added, giving an orange solution. This solution was extracted with ethyl acetate (35 mls.), the organic layer washed with aqueous sodium bicarbonate (20ml.), water (2x20 ml.), brine(2x20ml.) and dried. Evaporation of solvents

gave a dark red gum (65mg.). PLC (chloroform) gave an orange bond (R_f0.70) as main product.

N.M.R. (60MHz)τ($CDCl_3$): 0.65(br.s.,-OH); 2.35,2.60,2.93 (Ar-H); 5.73(q.J= 7 ,Ar-$O\underline{CH_2}CH_3$); 5.95(s,Ar-OMe);6.05(q.J=7, Ar-$O\underline{CH_2}CH_3$); 7.50(s,Ar-CH_3);8.57(t.J=7,Ar-$OCH_2\underline{CH_3}$); 8.60(t.J=7,Ar-$OCH_2\underline{CH_3}$)

Asperflaschin (2,4-dihydro-3,8,9-trihydroxy-7-(γ,γ- dimethylallyl)-3- methylanthracen - 1 (2H) - one, 159)

This compound was most conveniently obtained by PLC of mycelial extracts as described earlier, m.p. 179-81°C, $[\alpha]_D$ = 54° (c= 0.06,MeOH)

T.L.C.: R_f 0.06(50% chloroform- light petroleum Ce^{4+}: mottled orange

I.R.: ν_{max} (KBr disc)$cm.^{-1}$:3470(w),3370(w),1632(s),1598(s), 1580(s).

U.V. λ_{max} (EtOH)nm.:231(4.06),279(4.23),317(3.52),331(3.44),402(3.58) (EtOH-NaOH)nm.:210(4.36),ca.234(inf.,3.98),299.5(4.32) , ca.329(inf.,2.12), 404(3.79). Reverted to original spectrum on neutralisation.

N.M.R. (100MHz)τ($CDCl_3$):-6.12(1H,s,-OH);0.06(1H,s,H-5); 4.77(1H,br.t.J=6,irr.6.57 ⟶ br.s., irr. 8.16 ⟶ sharpening of triplet, irr. 8.28 ⟶ sharpening of triplet, H-β); 6.09(3H,s,Ar-OMe); 6.58(2H,br.d.,irr.4.77⟶br.s., irr. 8.16⟶sharpening of doublet, irr. 8.28⟶sharpening of doublet,α- CH_2);6.98(2H,br.s., H-4); 7.18(2H,br.s.,H-2);8.16(3H,br.s., irr.4.77⟶sharpening, cis γ -Me); 8.21(1J,s, exchanges D_2O, - OH); 8.28(3H.br.s., irr. 4.77⟶ sharpening, trans γ - Me); 8.55(3H,s,CH_3).

^{13}C- n.m.r.: See table 4.8.

M.S. M^+at $^m/_e$ = 356(0.72); also 341(0.39),338(0.31),323(0.25), 313(0.15), 302(0.25), 301(1.00),295(0.57).

Accurate M^+ at $^m/_e$ = 356.1619; $C_{21}H_{24}O_5$ requires 356.1624.

Analysis:Found C:70.55, H:6.77; $C_{21}H_{24}O_5$ requires C:70.77,H:6.79

C.D. data[183] λ_{MAX}(MeOH)($\Delta\epsilon$)nm : 266(+0.83),278(+0.70,292(-0.70), 320(-0.83),331(-1.39),370(+0.14),434(+0.19)

Attempted dehydration of asperflaschin(159)

a) Reaction with hydrochloric acid-acetic acid(Formation of the chloro-compound,166)

Asperflaschin (159,50mg.)was heated at 100°C with glacial acetic acid (2.5 ml.) and concentrafed hydrochloric acid (0.25 ml.) for 5 minutes. On cooling, yellow crystals formed which were removed, washed with water and dried. Recrystallisation from ethyl acetate afforded the chloro-derivative (166) as yellow needles (42mg., 80%), m.p. 197-9°C.

T.L.C. : R_f 0.60 (50% chloroform-light petroleum); Ce^{4+} orange.

I.R. ν_{MAX} (KBr disc)$cm.^{-1}$:3430(w),1623(s),1597(s),1561(w).

U.V. λ_{MAX}(MeOH) nm. : 220(4.49) ,ca.229(sh.,4.44),ca.265(inf.,3.83) 273(3.96),ca. 323(sh.,4.05),353(4.21).

(MeOH-NaOH)nm.:214(4.83),ca.234(inf.,4.42),263(4.31),ca.313 (inf.,3.90),366(3.93),ca.430(inf.,3.55),ca.480(inf.,3.50). Did not revert to original spectrum on neutralisation.

N.M.R.: (100MHz)τ($CDCl_3$):-2.67(1H,s, exchanges D_2O, -OH); - 2.35(1H,s, exchanges D_2O -OH);3.34(2H,br.s.,irr.7.63 —>sharpening, H-5,7); 3.61(1H,br.s., H-4); 5.79(2H,br.s., anthrone CH_2), 6.10(3H,s,Ar-OMe); 7.15(2H,t.J=8,α- CH_2);7.63(3H,br.s.,Ar-Me); 8.03(2H,t.J=8, β - CH_2); 8.30(6H,s, γ - Me's).

M.S. : M^+ (apparent) at $^m/e$ = 338(0.21); also 323(0.08),295(0.24), 284(0.24), 283(1,00), 253(0.18),225(0.11),165(0.10),131(0.13).

Accurate M^+(apparent) at $^m/e$ = 338.1512; $C_{21}H_{22}O_4$ requires 338.1517.

$C_{21}H_{23}O_4Cl$ requires m.w. = 374.5.

Analysis: Found C: 67.10, H: 6.36, Cl.: 9.40;

$C_{21}H_{23}O_4Cl$ requires C: 67.29, H: 6.17, Cl :9.48.

The chloro-derivative (166, 20mg.)in acetone (20ml.) at 0°C was treated with Jones reagent (5mls.), with stirring, for 5 minutes. Methanol (25 ml.) was added, the mixture extracted with ethyl acetate (25 mls.), the organic layer washed with brine to neutrality and dried, giving an orange solid (70mg.). Recrystallisation (ethyl acetate) gave a product (8mg., 37%) identical with the product (166) from the action of hydrochloric acid - acetic acid on 2-(γ,γ - dimethylallyl)-physcion (73).

b) Reaction with thionyl chloride and pyridine

Asperflaschin (159,10mg.) was added to a mixture of pyridine (1ml.) and freshly distilled thionyl chloride (0.1 ml.) and the solution allowed to stand at 0°C overnight. TLC indicated that no reaction had taken place.

c) Reaction with potassium bisulphate

An intimate mixture of asperflaschin (159,20mg.) and potassium bisulphate(13.6mg.)were heated at about 150°C for 25 minutes. Extraction of the resulting solid with ethyl acetate (2x20mls.) and evaporation of solvents gave a dark green solid (16.4 mg.). PLC (50% chloroform-light petroleum) gave a yellow band (R_f 0.38) as main product (4.4 mg.) which stained pink-orange with Ce^{4+}. Because of the low yield, this approach was not pursued further.

d) Reaction with N,N'-dicyclohexylcarbodiimide

Asperflaschin (159,312 mg.) was refuluxed with N,N'- dicyclohexylcarbodiimide (D.C.C., 2.35g., 13 equiv.) in dry toluene (100mls.), under nitrogen, for 24 hours. Ethyl acetate (100 mls.) was added and the solution washed with a 50/50 (v/v) mixture of brine and 10% acetic acid solution (100 mls. of mixture x 10). A white precipitate which formed (dicyclohexylurea) was filtered off from time to time. The

filtrate was washed with brine to neutrality, dried and the solvents evaporated, giving a brown oil (0.797 g.). PLC and extraction of a pale yellow band (R_f 0.61) gave a yellow solid (31 mg., 11%) probably consisting mainly of the 10,10' dimer of 2-(γ,γ - dimethylallyl)-physcion anthrone (140). The sparing solubility of this compound in $CHCl_3$ contrasted with that of 2-(γ,γ - dimethylallyl)-physcion anthrone(140, fairly soluble) and of asperflaschin (159, readily soluble).

T.L.C.: R_f 0.61(50% chloroform-light petroleum); Ce^{4+} scarlet.

I.R. ν_{max} (KBr. disc)cm^{-1}: 2968(w), 2920(w),2850(w),1633(m), 1619(s), 1596(s), 1563(w).

U.V. λ_{max} ($CHCl_3$) nm.: 278(4.19), 360(4.37).

N.M.R. (60 MHz) τ ($CDCl_3$): -2.65(1H,s,-OH); -2.37(1H,s,-OH); -2.33(1H,s,-OH); -1.97(1H,s,-OH); 3.33(2H,br.s., H-7,7'); 3.48, 3.58(2H,H-5,5'); 4.48(2H,br.s., H-4,4'); ca.4.73 (2H,m.,β-H's); 5.77(2H,br.s., anthrone CH_2); 6.06 (3H,s,Ar.OMe) 6.30(3H,s,Ar-OMe); ca.6.65 (4H,m, α-H's); 7.57(3H,s,Ar-CH_3); 7.62(3H,s,Ar - CH_3); 8.18(6H,br.s.,γ-Me's); (6H,br.s., γ-Me's);

M.S. M^+ (apparent) at $^m/_e$ = 338(0.53); also 323(0.30), 296(0.20), 295(1.00), 283(0.80).

Accurate M^+(apparent) at $^m/_e$ = 338.1509; $C_{21}H_{22}O_4$ requires 338.1518.

$C_{42}H_{42}O_8$ requires m.w. = 674.

e) Reaction with collidine (Formation of 2- (γ,γ-dimethylallyl)-physcion anthrone

Asperflaschin (159, 43mg.) was refluxed with collidine (4ml.) under nitrogen for 20 minutes. Ethyl acetate (15mls.) was added and the mixture washed with dilute hydrochloric acid (12mls.). The organic layer was washed with bfine to neutrality, dried and the solvents evaporated, giving a fawn-coloured solid (46 mg.). Recrystallisation (ethyl acetate) gave 2-(γ,γ- dimethylallyl)

- physcion anthrone (140, 31.5 mg., 77%) as off-yellow prisms, m.p. 187 - 9°C.

T.L.C.: R_f 0.63 (50% chloroform-light petroleum); Ce^{4+} red.

I.R. ν_{MAX}(KBr. disc)cm.$^{-1}$: 2955(w), 2915(w), 2850(w), 1636(m), 1618(s), 1595(s), 1554(m).

U.V. λ_{MAX}(MeOH)nm. : 226(4.41), ca.232(sh., 4.38),273.5(3.93), ca. 284.5 (3.79), ca. 307(3.79), 358(4.15).

(MeOH-NaOH)nm.: 217.5(4.74), ca. 234(sh.,4.41),268(4.27),312.5(3.83), 365(3.94), 384(3.98), 440(3.62), 522(3.60). Did not revert to original spectrum on neutralisation.

N.M.R. (60MHz)τ($CDCl_3$): - 2.65(1H,s,-OH); -2.35(1H,s,-OH); 3.36(2H,br.s., H-5,7); 3.63(1H,s,H - 4); ca. 4.80(1H,m,β - H); 5.85(2H,s, anthrone CH_2), 6.13(3H,s,Ar-OMe); 6.67(2H,d,J = 8, α- CH_2); 7.65(3H,br.s., Ar - CH_3); 8.20(3H,br.s., cis γ-Me); 8.32(3H,br.s., trans γ - Me).

M.S. : M^+ at $^m/_e$ = 338(0.53); also 323(0.30), 296(0.20),295(1.00), 283(0.80). $C_{21}H_{22}O_4$ requires m.w. = 338.

Analysis: Found C ; 74.25, H: 6.55; $C_{21}H_{22}O_4$ requires C:74.54, H:6.55.

The action of sodium hydroxide on asperflaschin

Asperflaschin(159,40mg.) was allowed to stand in 1M sodium hydroxide (40mls.) for 4 days. The mixture was brought to neutrality with dilute hydrochloric acid and extracted with ethyl acetate (2 x 20 ml.). The orgamic layer was washed with brine (2 x 20 mls.), dried and the solvents evaporated, giving a brown oil (63mg.). PLC (eluting twice with 50% chloroform - light petroleum) gave starting material (159,30mg.) and 2-(γ, γ - dimethylallyl)- physcion (73,10mg., quantitative conversion based on recovered unreacted starting material) identified by comparison (spectral data) with authentic material.

Attempted synthesis of 2- (γ, γ - dimethylallyl)-emodin anthrone(168)

a) Attempted prenylations of emodin anthrone

i) Using dimethylallyl bromide and silver oxide.

Emodin anthrone (103, 50mg.) in dry dioxan (50mls.) was stirred overnight with freshly prepared silver oxide (ca. 70 mg.) and freshly distilled dimethylallyl bromide (2drops). Ether(30ml.) was added and the reaction mixture washed with water (3 x 30 mls.), the organic layers washed with brine, dried, giving a brown resin (81 mg.). PLC (50% chloroform- light petroleum) gave only a complex mixture of products from which no pure product could be obtained.

When pyridine was used as base, a black residue was the only product, no reaction being detectable by TLC.

ii) Using 2- methyl-3-buten-2-ol and formic acid

To a solution of emodin anthrone (103, 20mg.) in diglyme (2ml.) was added aqueous formic acid (1 drop). The mixture was heated to 80°C and 2-methyl-3-buten-2-ol (3.36 mg., 2 eq.) was added. The mixture was allowed to stand at room temperature for 48 hours, ether (30ml.) added and the solution washed with water (4x20mls.), dried and the solvents evaporated, giving a brown oil (81mg.) from which no pure compound was isolable by PLC.

iii) Using 3-chloro-3-methyl-but-1-yne.

Emodin anthrone (103, 50 mg.), potassium carbonate(50mg.), potassium iodide (8.8 mg.) and aqueous acetone (10 ml.) were stirred under nitrogen for one hour at room temperature. Freshly distilled 3-chloro-3-methyl-but-1-yne (1drop, prepared by the method of Hennion and Boiselle) was added, and the mixture refluxed for 6 hours, when second aliquots of potassium carbonate (50mg.) and 3-chloro-3-methyl-but-1-yne (1 drop) were added. The mixture was refluxed for a further 24 hours,

the solvents evaporated and ethyl acetate added. This solution was washed with water (2 x 20 mls.), brine (25 mls.) and dried, giving a purple oil (80mg.). No pure compound could be isolated from the mixture by PLC although spectral data (n.m.r., i.r.) indicated multiple alkylation of the substrate.

N.M.R. : complex mass of signals which was interpreted as indicating the presence of several alkyl (dimethylpropargyl) groups; also no anthrone signals.

M.S. : M^+ at $^m/_e$ = 388(0.65); also ions at 373(0.41),321(1.00), 306(0.15), 293(0.22), 265(0.36),255(0.14),239(0.16).

Also, no single product could be obtained when the reaction was repeated at room temperature.

b) From asperflaschin with lithium iodide in collidine

To a solution of fresh lithium iodide (large excess, prepared by heating lithium iodide at ca. 240°C under vacuum for 1 hour and allowing to cool under vacuum) in refluxing collidine (5ml.) was added a solution of asperflaschin (159,39mg.) in collidine (4ml.). The mixture was stirred and refluxed under nitrogen overnight. Ethyl acetate (25 ml.) was added and the mixture washed with dilute hydrochloric acid until collidine could no longer be detected, washed with brine to neutrality and dried, giving a dark brown gum (32 mg.) from which no pure compound could be isolated by PLC.

c) From asperflaschin with hydriodic acid and acetic acid (Formation of the dihydropyran,173)

Asperflaschin (159, 50 mg.) was refluxed under nitrogen with hydriodic acid (45% v/v, 2.5ml.) in glacial acetic acid (6.03 ml.) for 3 hours. On cooling, a precipate formed which was removed, washed with water and dried. The mother liquors were diluted with water (3 - 4 volumes) and further material removed, washed and dried. Recrystallisation (ethyl acetate)

gave the dihydropyran derivative (173, 29mg., 44%) as a fawn-coloured amorphous solid, m.p. 187 - 9°C.

T.L.C.: R_f 0.73(50% chloroform-light petroleum):Ce^{4+}: yellow.

I.R. ν_{MAX} (KBr. disc)cm^{-1}.:2975(w), 2940(w), 2915(w),1621(s), 1596(s), 1567(m).

U.V. λ_{MAX} (MeOH)nm.: 219.5(4.45),ca.224(sh.,4.42),ca.251(inf.,3.85) 263(3.84), 273(3.96), ca. 323(sh.,4.05), 357(4.30).

(MeOH-NaOH)nm.: 214(5.07),ca.234(sh.,4.40),265(4.33),ca.301(inf.,3.98), 366(3.96), 385(4.06),510(4.13). Did not revert to original spectrum on neutralisation.

N.M.R. (60MHz)τ($CDCl_3$);-2.92(1H,s,-OH); -2.40(1H,s,-OH); 3.35(2H,br.s., H- 2,4); 3.68(1H,br.s.,H-5);5.85(2H,br.s., anthrone CH_2) 7.27(2H,t.J=6, α -CH_2); 7.65(3H,s,Ar-Me); 8.17(2H,t.J=6, β - CH_2); 8.65(6H,s, γ - Me's).

M.S. : M^+ at $^m/e$ = 324(0.53); also 309(0.11), 281(0.37), 269(1.00), 268(0.37), 240(0.08), 164(0.11).

$C_{20}H_{20}O_4$ requires m.w. = 324.

Analysis: Found C: 73.97, H: 6.30; $C_{20}H_{20}O_4$ C: 74.06, H: 6.21.

Physcion - dimethylallyl emodin - 10,10'- bianthrone (174)

This compound was isolated by column chromatography of asperflaschin-rich fractions of an earlier column, as described earlier, as a grey-green amorphous solid.

T.L.C. R_f 0.12 (50% chloroform-light petroleum); Ce^{4+}: dull green.

I.R. ν_{MAX} (KBr disc) cm^{-1} : 3400(w), 1630(s),1618(s), 1597(s), 1585(s).

U.V. λ_{MAX} ($CHCl_3$) nm.: 279(4.17), 353(4.26).

N.M.R. (60MHz)τ($CDCl_3$): -2.47($\frac{1}{2}$H,s,-OH); -2.43($\frac{1}{2}$H,s,-OH); -2.13($\frac{1}{2}$H,s,-OH); -2.06($\frac{1}{2}$H,s,-OH);-1.83(1H,s,-OH);-1.78(1H,s,-OH); 3.35(2H,br.s.,H-7,7'); 3.66(1H,br.s.,H-2'); 3.87 - 4.18(4H,m,H-4,4'5,5'); 4.73(1H,m, β -H); 6.17(^{3}/2H,s, Ar-OMe);6.23(^{3}/2H,s, Ar-OMe); 6.65(2H,d.J = 6, α - CH_2); 7.70(^{6}/2H, br.s., Ar-CH_3); 7.77(^{6}/2H,br.s.,

Ar - CH_3); 8.18(3H,s, γ - Me); 8.25 (3H,s, γ- Me).

M.S.: M^+(apparent) at $^m/_e$ = 324(0.32); also 307(0.33), 281(0.25), 270(1.00), 269(0.66), 255(0.25), 240(0.18), 227(0.50).

Feeding of labelled precursors to cultures of A. flaschentraegeri (Biosynthesis of asperflaschin, 159)

a) Preliminary time study with ^{14}C- labelled precursors.

After removal of the physcion anthrone (139) from the mycelial extracts of those cultures (2 Roux bottles per experiment) which had been incubated for various times (see first group of experiments in the time - study described earlier, chapter 3), inactive asperflaschin (159, 20 mg.) was, in each case, added to the remaining extract and separation of metabolites achieved by PLC (eluting twice with 50% chloroform-light petroleum). The band containing asperflaschin was removed, extracted and weighed after evaporation of solvents. The entire band was dissolved in a mixture of toluene (250 mls.) and D.M.F. (10mls.) and 1 ml. of this solution was then removed and counted.

A radiotracer experiment ([1 - ^{14}C] - sodium acetate, 0.1015 mCi) was carried out in parallel with the ^{13}C - labelled (4 - 7)day experiment, described in chapter 3, leading to the elucidation of the biosynthesis of physcion anthrone (139). In the ^{14}C-precursor experiment, inactive sodium acetate (300mg.) was added to the radioactive precursor solution and the mixture administered in 6 pulses to surface cultures of A.flaschentraegeri (2 bottles).

After removal of labelled physcion anthrone in the usual way, the remaining mycelial extract was chromatographed, asperflaschin isolated and solutions prepared for counting, as above.

Incorporation of $[1-^{14}C]$ - sodium acetate into asperflaschin (159).

Feeding Period (days)	Total Weight of Mycelial Extract (mg.) Chromatographed	Weight Isolated as Asperflaschin Band (mg.)	Sp. Activity (dpm/mg.x 10^4)	% Incorp.
3-5 (single pulse)	131	58	1.58	0.40
3-6 (" ")	131	80	0.95	0.33
3-7 (" ")	194	81	0.77	0.30
4-6 (" ")	190	66	2.34	0.69
4-7 (" ")	223	66	1.46	0.42
5-7 (" ")	219	48	3.50	0.74
4-7 (six pulses)	460	89	2.41	0.95

The aim of this latter experiment was to monitor the effect on the incorporation rate of the presence of large amounts of acetate, as is the case in ^{13}C - labelled precursor experiments. The results from these experiments are summarised opposite.

b) Experiments with ^{13}C- labelled precursors.

A solution of $[1,2\ ^{13}C]$ - sodium acetate (375 mg.) and unlabelled sodium acetate (750 mg.), in distilled water (60 mls.) was divided into 6 equal portions, sterilised and added to 4 - day old surface cultures of A. flaschentraegeri (10 bottles), in 6 pulses, as described earlier (chapter 2). The cultures were harvested on the seventh day of growth and the broth and mycelium separated. Extraction of the mycelium with light petroleum (b.p. 100 - 120°) in the usual way afforded physcion anthrone (139,397mg, 200 $mg.l.^{-1}$).

The mother liquors were combined with ethyl acetate and chloroform (Soxhlet) extracts of the dried mycelium. Repeated chromatography and crystallisation (ethyl acetate) afforded labelled asperflaschin (159, 76mg., 38 $mg.l.^{-1}$) The ^{13}C- n.m.r. spectrum of this material was obtained in $CDCl_3$ (Table 4.8).

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