MYCOPHENOLIC ACID AND OTHER METABOLITES OF *PENICILLIUM BREVICOMPACTUM*

A THESIS PRESENTED BY

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TO THE UNIVERSITY OF GLASGOW FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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SUMMARY

The major part of this thesis describes studies on the biosynthesis of the benzoylated drimane sesquiterpene pebrolide and on mycophenolic acid.

The location of tritium in pebrolide biosynthesised from [2-T, 2-^{14}C] mevalonate was established by degradation and in particular the 4α-oxymethylene group was shown to be derived from the C_2 methylene group of mevalonate, in agreement with the specificity of cyclisation found in di- and tri- terpene biosynthesis.

A number of degradative sequences for the determining of the level of tritium incorporation into different parts of mycophenolic acid were studied. Application of one of these to material biosynthesised from [2-T, 2-^{14}C] sodium acetate showed that the incorporation of acetate into the phthalide methylene group was in accord with the intermediacy of a tetraketide precursor. A farnesyl analogue of mycophenolic acid, its methyl ether (which were isolated from cultures of P. stoloniferum) and desmethylmycophenolic acid were tested (by radiotracer methods) as precursors, and the final steps in the biosynthesis of mycophenolic acid elucidated.

The stereochemistry of a dihydrobenzofuran metabolite of P. brevicompactum, related to mycophenolic acid, has been established by synthesis from mycophenolic acid.

A minor metabolite (brevigellin) of P. brevicompactum was isolated and shown by chemical and spectroscopic means to be a cyclodepsipeptide containing a previously unreported amino acid residue.

Contents

Fungi, together with algae and bacteria, are traditionally classified as members of the plant Sub-kingdom Thallophyta which comprises "plants" that are without functional roots, stems, leaves or flowers, do not form embryos (as in seeds) and may consist of only one cell or of an aggregation of cells. In view of the dissimilarities to the rest of the plant kingdom the classification of the non-photosynthetic members of this group as plants is somewhat dubious, and some authorities have considered the fungi as neither plant nor animal but belonging to a separate kingdom.

The classification of the fungi rests upon the sexual reproductive mechanism exhibited by the organism and on the nature of the mycelium (or thallus)[1,2]. The most primitive class is the Phycomycetes, the simplest of which are unicellular and often aquatic. Some species do form extensive mycelia, such as the water moulds of the order Saprolegniales and the common black bread mould Mucor. Rhizophus nigricans. The Ascomycetes derive their name from the sac-like vessel (asci) in which sexual spores are formed. This class includes the the yeasts and the filamentous fungi typified by the genera Aspergillus and Penicillia. The Basidiomycetes are the highest form of fungi and are familiar to the layman as mushrooms, toadstools and the smuts and rusts of cereals. In contrast to the previous classes Basidiomycetes rarely form asexual spores.

A fourth class, the Fungi Imperfecti, is artificial and comprises those species in which a sexual (or perfect) stage has not been

observed. Many of these fungi are known to be the conidial stages of perfect fungi (generally the Ascomycetes). For example, Fusarium moliforme is the conidial stage of the gibberellin producing Ascomycete Gibberella fujikuroi. This class contains the carniverous fungi such as the nematode predator Dactylaria gracilis[3] and several pathogenic species such as Candida albicans which is responsible for pulmonary candidiasis in man and animals, and Epidermophyton floccosum which causes both tinea (ring worm) and athlete's foot.

The lichens are symbiotic associations of fungi (Ascomycetes or Basidiomycetes) with unicellular algae. The alga, being photosynthetic, provides organic nutrients for the fungus (mycobiot) which, in turn, provides mechanical support and minerals for the alga (phycobiot). In a number of cases the component organisms have been cultivated separately under laboratory conditions.

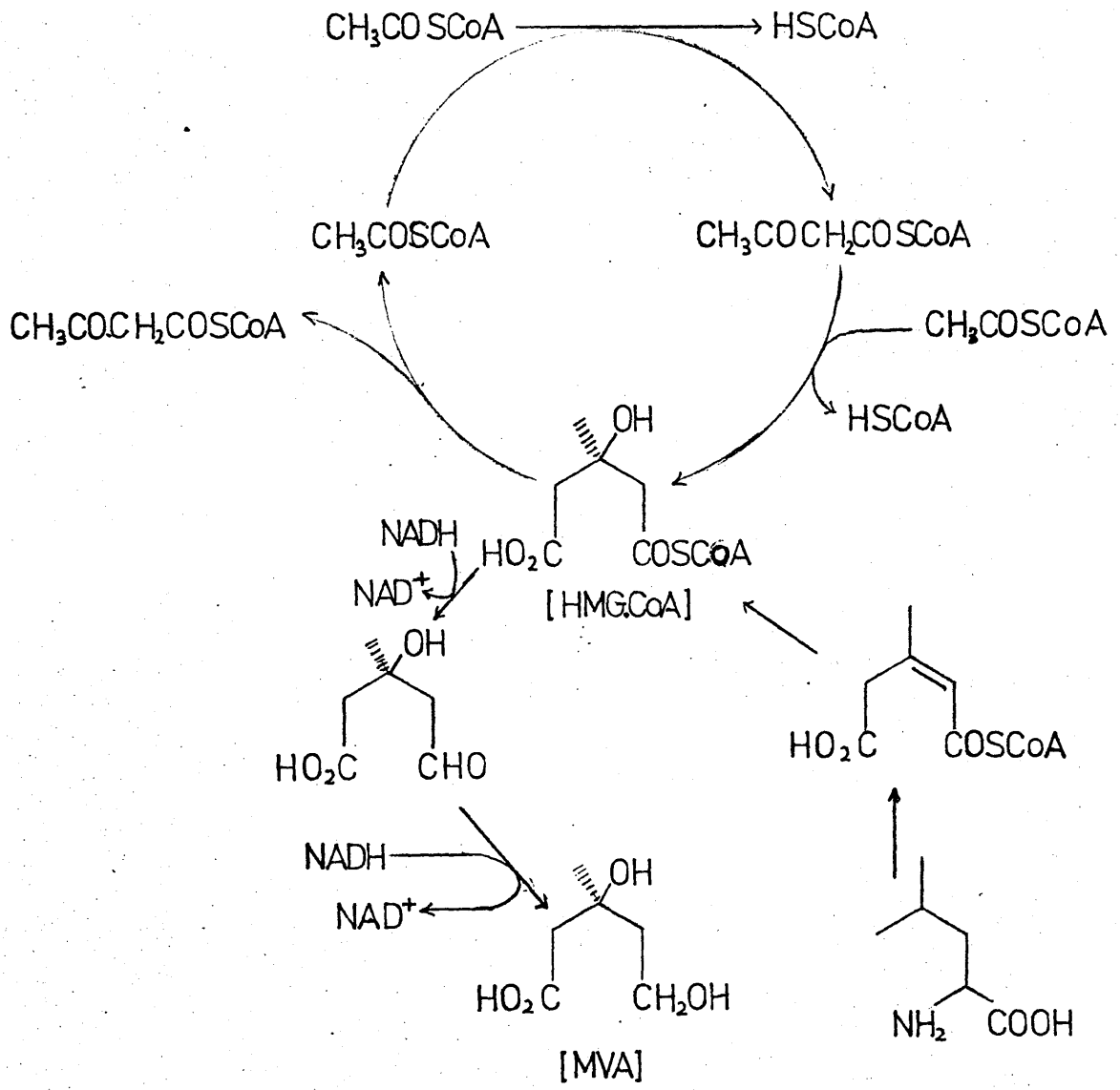
In general the chemical processes essential to the support of life are common to all organisms, these processes provide the synthetic intermediates and the key macromolecules such as the proteins and DNA and also the energy necessary to support metabolism. In contrast to these primary metabolic processes the fungi, in common with the higher plants and bacteria, possess a secondary synthetic form of metabolism the products of which are species and sometimes strain dependent, but play no obvious role in the natural economy of the organism. The diversity of chemical structure displayed by the secondary metabolites of fungi led Raistrick[4] to term the phenomena 'polychemism'. Although in a number of cases specific function has

been tentatively ascribed to a number of these metabolites[53], in general they have generated interest not through their function in the producing organism but rather by the effect which some of these metabolites have on alien systems. The discovery of the effectiveness of penicillin, produced by the mould Penicillium chrysogenum, in treating bacterial infections in man gave tremendous impetus to the study of fungal secondary metabolites, leading to the isolation of many other antibiotics of economic importance.

While a number of secondary metabolites are structurally similar to primary metabolites, and are presumably formed by modification of the processes producing these, most of these compounds possess structural features unknown in primary metabolites and consequently the biosynthetic steps leading to their formation are of intrinsic interest. The main body of this thesis describes an attempt to elucidate the sequences involved in the biosynthesis of two such metabolites of the mould Penicillium brevicompactum (Fungi Imperfecti).

CHAPTER 1

Fig. 1:1 - The biosynthesis of mevalonic acid.

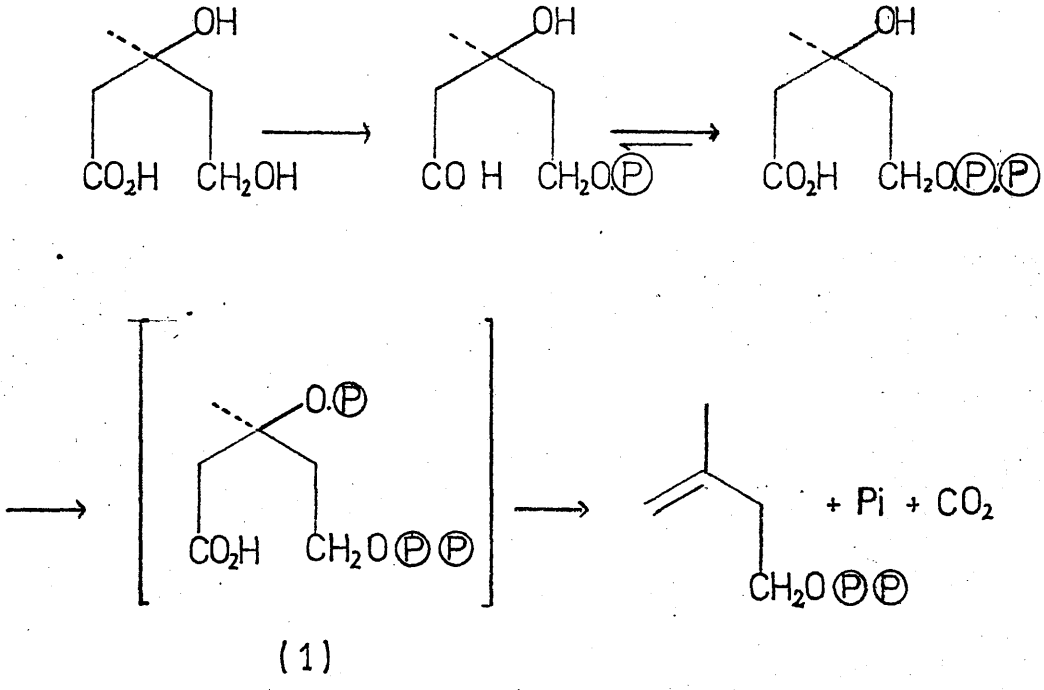


The plant terpenes are probably the largest known class of naturally occurring compounds. Despite their structural and functional diversity, a certain regularity of structural features was noted by early workers and this was explained as resulting from a common derivation involving polymerisation of a 'C_5 nucleus'. These ideas were rationalised by Ruzicka[5] in his "Biogenetic Isoprene Rule" which postulated that terpene structures could be derived from 'head-to-tail' linkage of 'active isoprene'.

Although Bloch[6] had demonstrated that the carbon skeleton of cholesterol was totally derived from acetate, the origin of the C_5 unit was largely a matter of speculation until Folker et al.[7] showed that mevalonic acid was a better precursor of cholesterol. The stages of mevalonic acid (MVA) biosynthesis from acetate have been demonstrated with purified enzyme systems[9], and are outlined in Fig. 1:1. Alternative pathways for the formation of mevalonic acid have been demonstrated[10]. Certain plants and microorganisms are capable of utilising leucine as a precursor[11] (Fig. 1:1).

The phosphorylation of L-mevalonic acid to 5-phosphomevalonic acid is irreversible and is virtually the only specific enzymatic reaction of mevalonic acid. A second phosphorylation gives 5-pyrophosphomevalonic acid[12], which reacts with a molecule of ATP to give ADP, inorganic phosphate and isopentenyl pyrophosphate (IpPP) (Fig. 1:2). Experiments with [3-^{18}O] mevalonic acid[13] have shown that the labelled oxygen is located in the inorganic phosphate formed, which suggests the intermediacy of (1). It has been shown[9] that

Fig. 1:2 - Formation of isopentenylpyrophosphate.



(1)

FIg. 1:3 - Isomerism of isopentenylpyrophosphate.

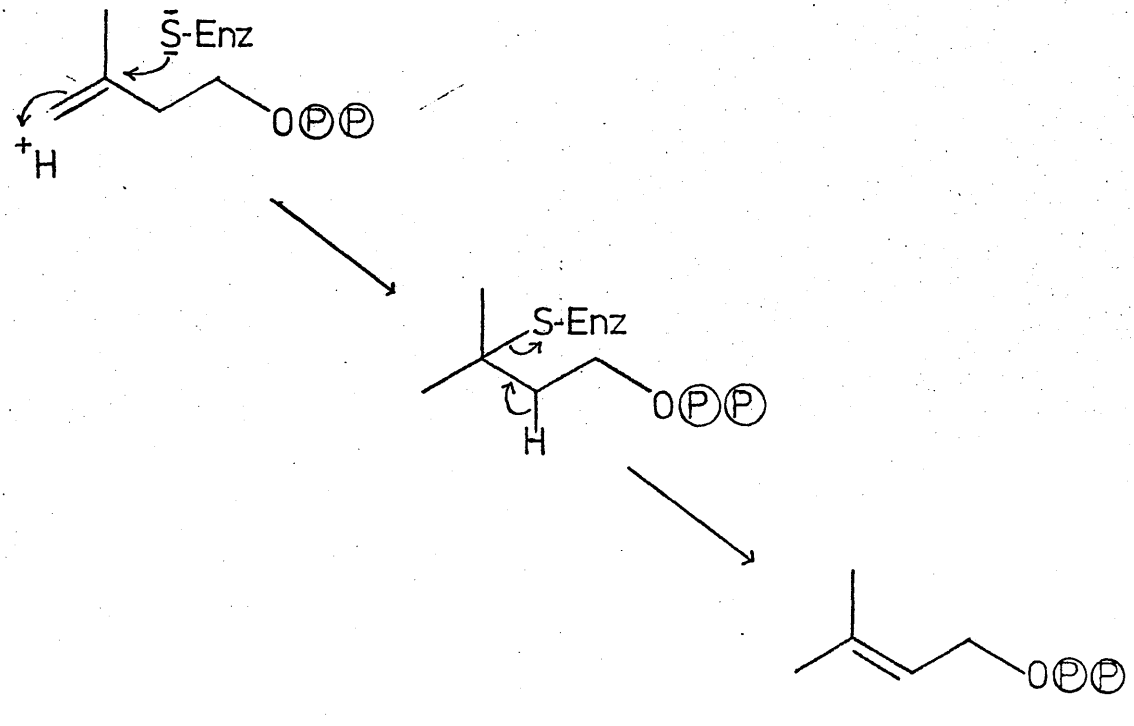


Fig. 1:4 - Biosynthesis of polyisoprenoids.

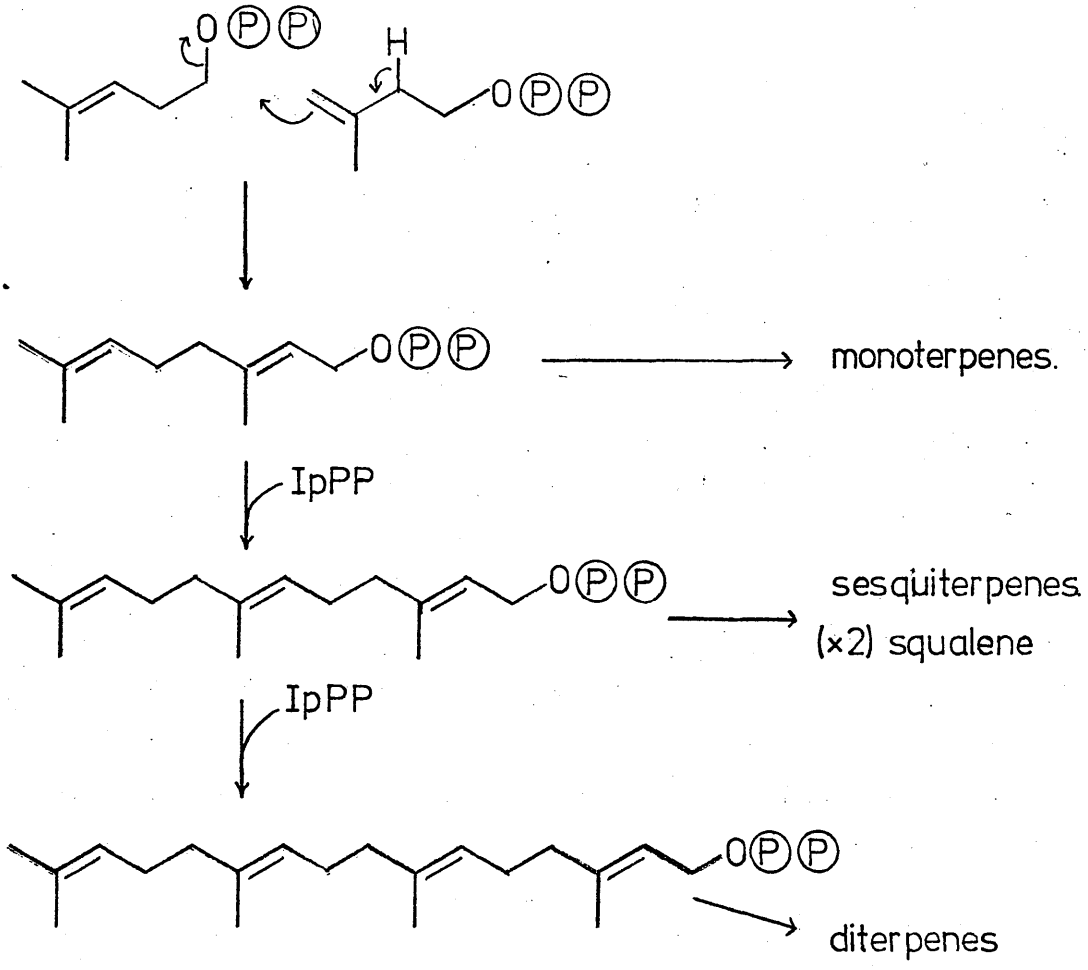
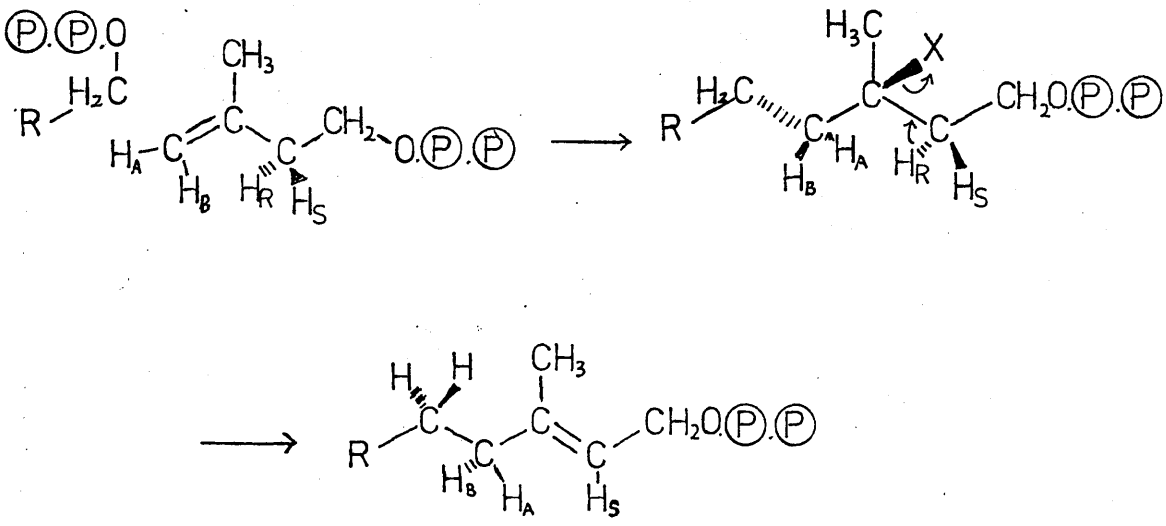


Fig. 1:5

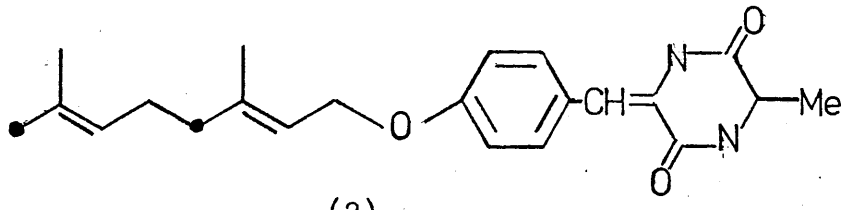


the elimination of inorganic phosphate from this molecule proceeds with trans stereochemistry. Isopentenyl pyrophosphate, which can be regarded as the formal 'isoprene unit' required by Ruzicka's hypothesis is converted enzymatically[8, 14] by a prototropic shift[16] to dimethylallyl pyrophosphate (DmPP) (Fig. 1:3).

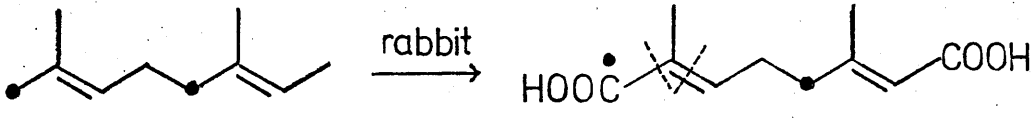
The coupling of dimethylallyl pyrophosphate with one molecule of isopentenyl pyrophosphate to yield geranyl pyrophosphate and the successive addition of further molecules of isopentenyl pyrophosphate to give farnesyl and geranylgeranyl pyrophosphates (Fig. 1:4) have been demonstrated with enzyme systems isolated from both plant and animal sources. Popjak[15] has isolated a single enzyme capable of synthesising farnesyl pyrophosphate.

The stereochemistry of the coupling process has been the subject of extensive studies by Cornforth and Popjak[16] using substrates asymmetrically labelled with hydrogen isotopes. They have proposed that the reaction proceeds in two distinct steps (Fig. 1:5). The first stage is the trans addition of the allylic group and of a nucleophilic group X to the double bond of isopentenyl pyrophosphate; and the second a trans elimination of X and a proton (H_R) from the intermediate. The identity of the group X is in some doubt. Johnson[17] has suggested that it may be regarded as an oxygen atom of the pyrophosphate leaving group.

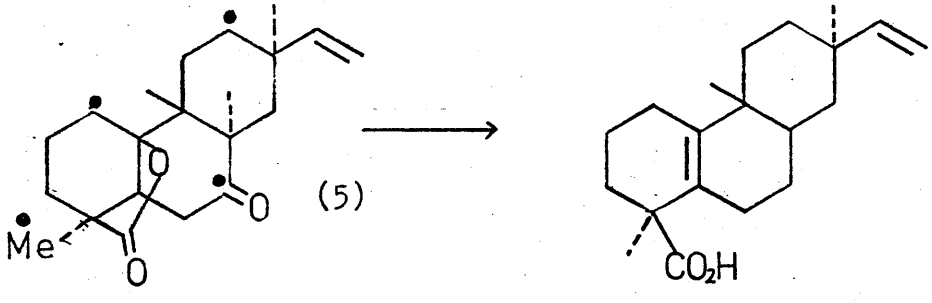
As a consequence of the stereospecificity of the isomerisation of isopentenyl pyrophsophate the C_2 of mevalonic acid becomes the trans methyl of the isopropylidene group of geranyl pyrophosphate.



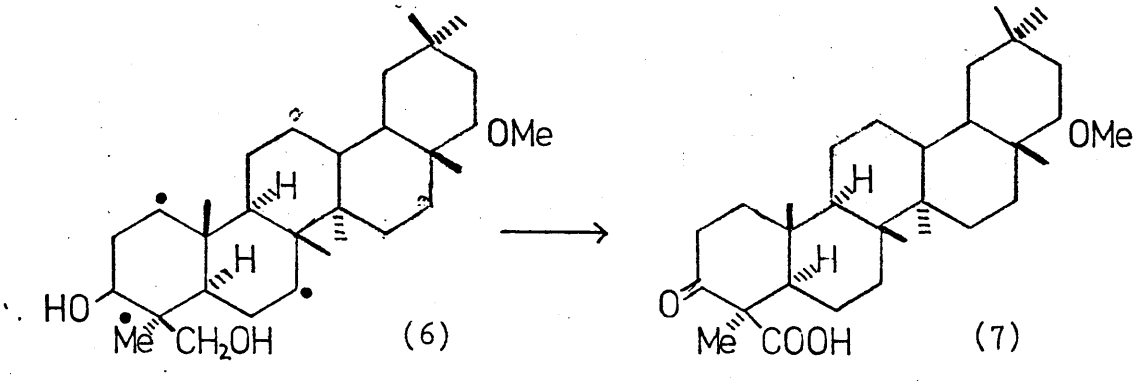
(2)



(3) (4)



(5)



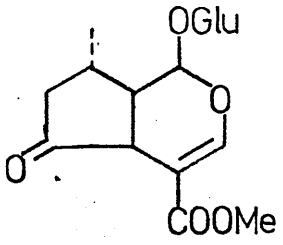
(6) (7)

This retention of stereochemical individuality has been demonstrated by Birch in the case of mycelianamide (2)[18] labelled with [2-^{14}C] mevalonate. Cleavage with sodium in liquid ammonia gave the hydrocarbon (3) which was fed to a rabbit, and recovered from its urine as the acid (4). Ozonolysis of the acid gave essentially unlabelled acetaldehyde.

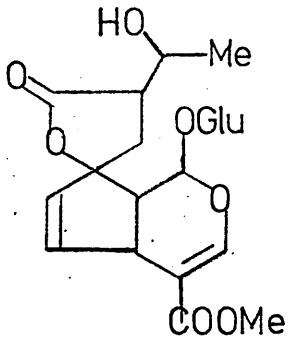
The cyclic terpenes can be considered to arise from cyclisation of appropriate acyclic terpenoid species; thus cyclisation of farnesyl pyrophosphate (three C_5 units), geranylgeranyl pyrophosphate (four C_5 units) and squalene (two farnesyl units linked[19] head-to-head) give rise to the sesqui-, di- and tri- terpenes respectively. The mechanisms which have been proposed for the cyclisation processes[16, 20] demand the retention of stereochemical individuality of the angular methyl groups of the cyclised terpenes. Proof of this has been presented in a number of cases including that of the fungal diterpene rosenonolactone (5)[21]. Wolf-Kishner reduction of the keto group of rosenonolactone labelled with [2-^{14}C] mevalonate, was accompanied with opening of the lactone ring. The resulting unsaturated acid was decarboxylated to give CO_2 which contained no ^{14}C.

In the triterpene series, Arigoni[21] has shown that there is also retention of stereochemical identity. The plant triterpene soyasapogenol-D (6) was labelled with [2-^{14}C] mevalonate and oxidised to the corresponding keto acid (7), decarboxylation of which gave inactive CO_2.

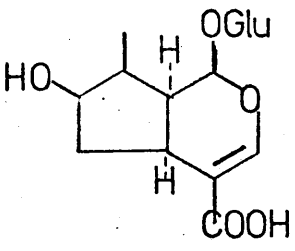
In contrast, labelling studies on the biosynthesis of the



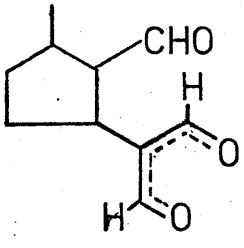
(8)



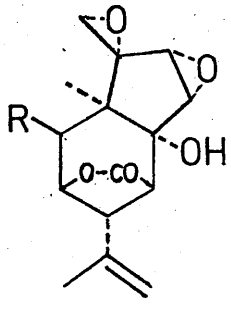
(9)



(10)

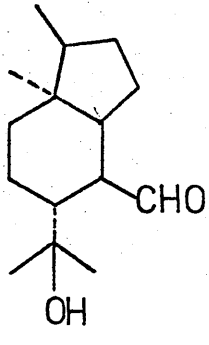


(11)



(13) R = H

(12) R = OH

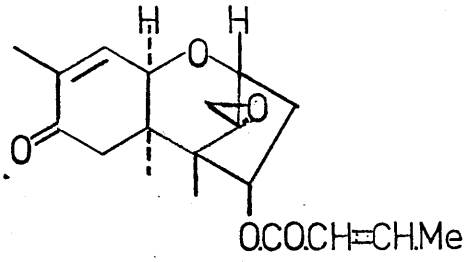


(14)

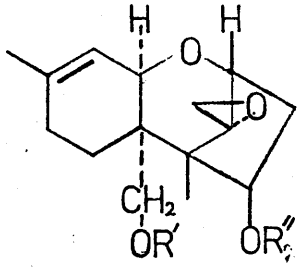
monoterpene plant glucosides verbenalin (8)[22], plumieride (9)[23] and loganic acid (10)[24] have shown that in these cases the identity of the gem methyl groups is lost, suggesting the intermediacy of a species (11) in which the isopropylidene carbons are indistinguishable.

Randomisation has also been reported in the biosynthesis of the sesquiterpenes tutin (12)[25, 26] and coriamyrtin (13)[25]. In experiments with [2-^{14}C] mevalonate the methyl and methylene of the isopropylidene group are equally labelled. If the methyl groups in an intermediate were part of an isopropyl group, they would almost certainly be diasteriotopic (chemically and biologically non-equivalent). Loss of biosynthetic identity must therefore reflect the occurrence of at least one non-specific process in the formation of these compounds, e.g. the elimination of water from an intermediate type (14).

Fig. 1:6 Fungal sesqiterpenes.



tricothecin

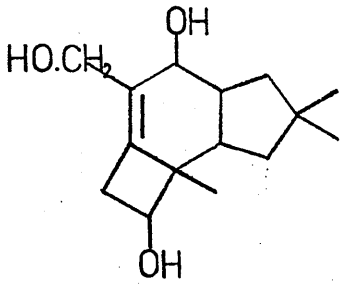


Me

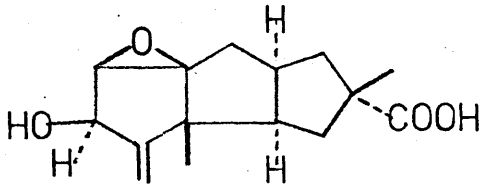
R',R'' = CO.CH=C.CH2CH2OCH-CH(OH)Me

—CO.CH=CH.CH=CH

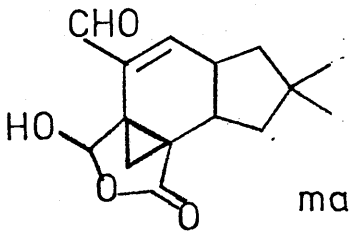
roridin E



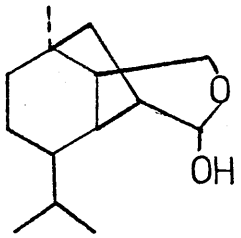
illudol



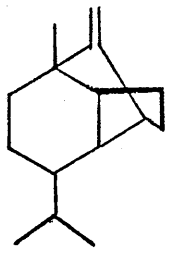
hirsutic acid



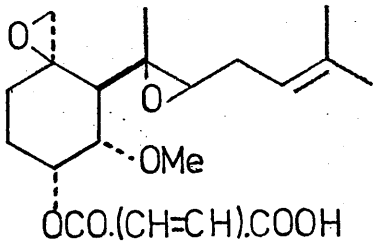
marasmic acid



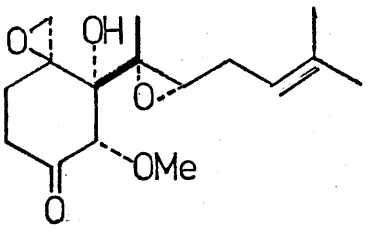
prehelminthosporol



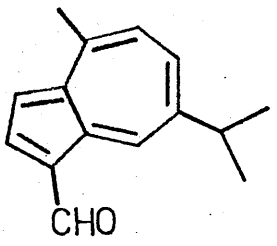
sativene



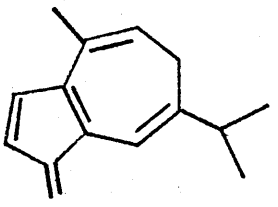
fumagillin



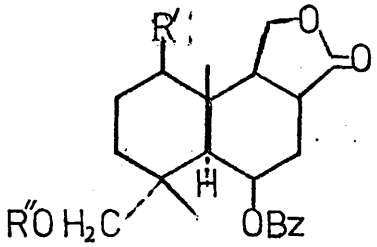
ovalicin



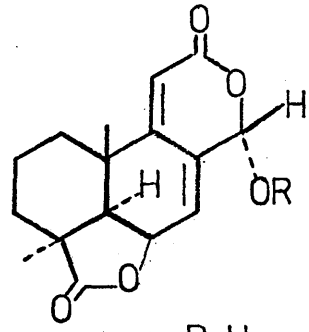
lactaroviolin



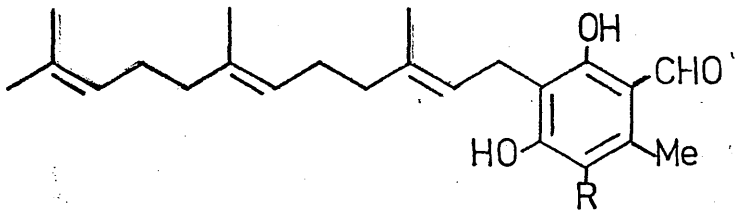
lactarofulvene



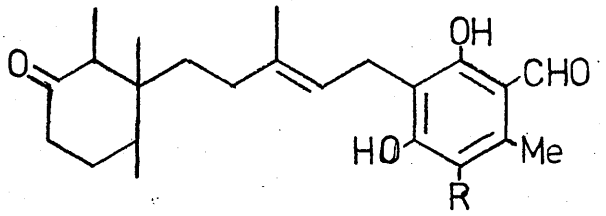
(15) R′=OH, R″=Ac
(16) R′=OH, R″=H
(17) R′=H , R″=Ac



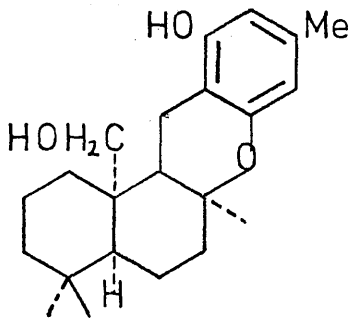
(18) R=H
(19) R=Me



(20) R=H
(21) R=Cl



(22) R=H
(23) R=Cl



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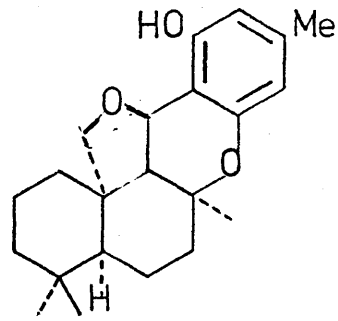


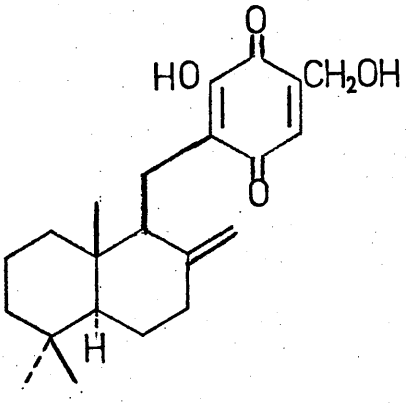
(25)

Fungal sesquiterpenes

In contrast to the large number of sesquiterpenes isolated from higher plants, the fungal sesquiterpenes form a relatively small class, few of which have skeletons similar to those found in plants. The majority of fungal sesquiterpenes can be classified on biogenetic grounds into five distinct groups; the tricothecanes, the "protoilludanes", the helminthosporal group, the fungallin group and the azulenes. (Fig. 1:6). The isoprenoid origin of each of the first four groups has been demonstrated and possible biosynthetic routes from farnesyl pyrophosphate have been proposed[27, 28]. The azulenes appear to be derived from farnesyl pyrophosphate without rearrangement of the intermediates.

The sesquiterpene benzoates pebrolide (15), desacetylpebrolide (16) and deoxypebrolide (17)[29] are the only drimane sesquiterpenes to have been isolated from a fungus. The Acrostalogmus sp. antibiotics (18) and (19)[30] are probably degraded diterpenes but could be formed by introduction of a one carbon unit into a drimane skeleton.

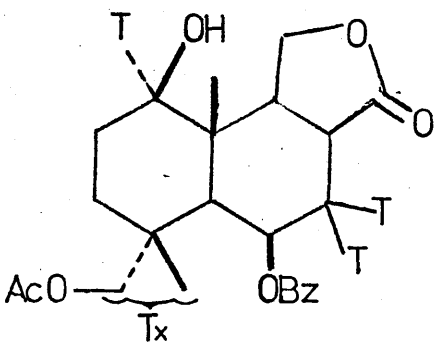
A quite separate group of sesquiterpenes are those derived by alkylation of a polyketide derived nucleus with a farnesyl unit. The occurrence of cyclic and acyclic metabolites in the same organisms suggests that cyclisations occur after introduction of the side chain. For example, the acyclic antibiotics (20) and (21), and the cyclised metabolites (22) and (23) are produced by the same strain of Fusarium sp.[31]. Siccanochromene E (24)[32] and its co-metabolite siccanin (25)[33] exhibit an unusual cis ring fusion. Tauranin (26)[34] has a drimane



(26)

skeleton which has the trans configuration analogous to that of pebrolide (15).

DISCUSSION



(15a)

Radiotracer studies in the biosynthesis of pebrolide

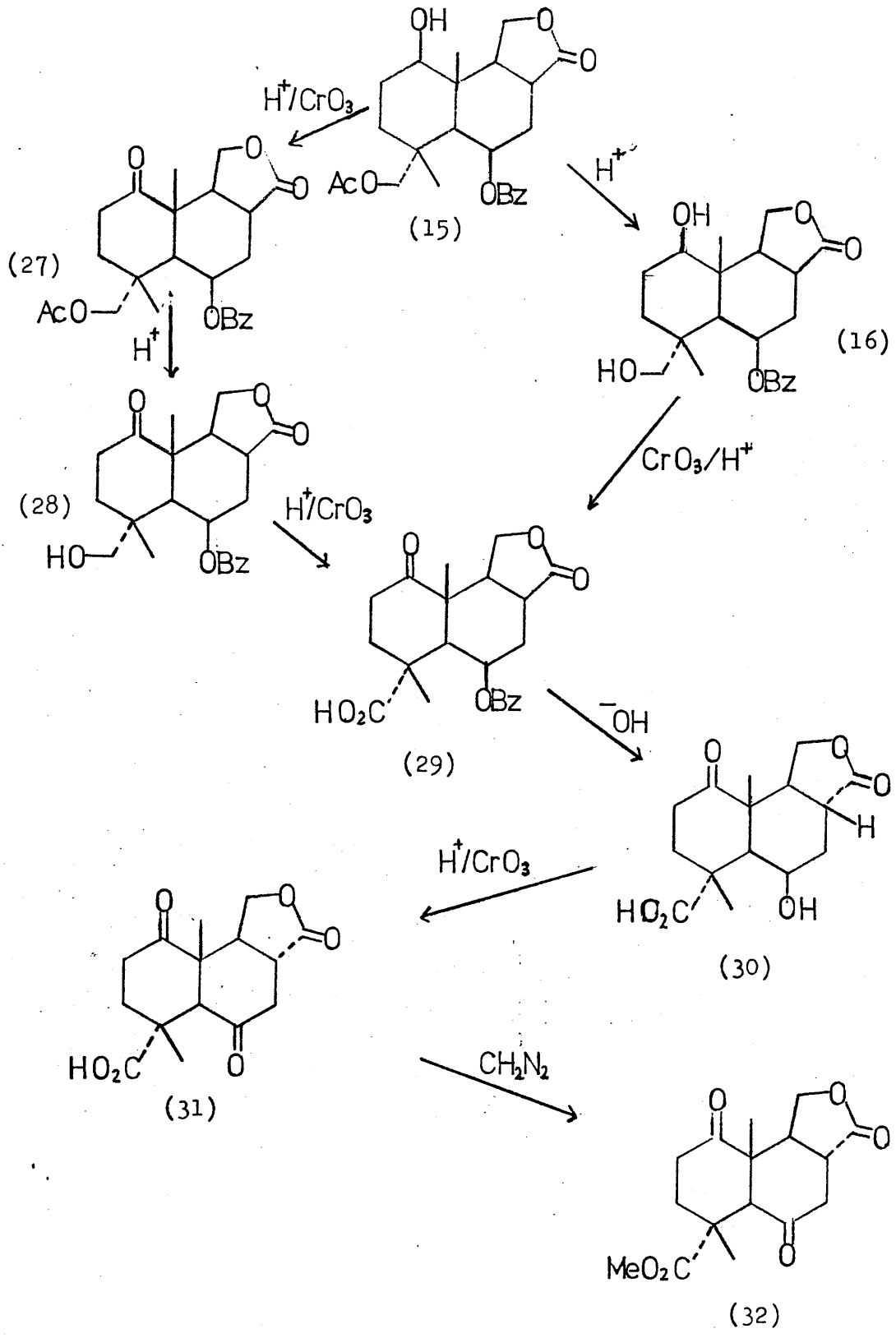
Pebrolide (15) and the closely related metabolites desacetylpebrolide (16) and 1-deoxypebrolide (17) were isolated from the fungus Penicillium brevicompactum and their structures determined by a combination of degradative chemistry and X-ray analysis. The terpenoid origin of pebrolide was demonstrated by the incorporation of [2-^{14}C] mevalonate[29].

The principal aim of the following experiments was to establish whether the biosynthetic individuality of the gem dimethyl of the precursor are preserved in the same way in the biosynthesis of this sesquiterpene, as in the formation of di- and tri- terpenes. The structure of pebrolide offers unique advantage in that the 4α- and 4β- carbon atoms are chemically distinct.

Pebrolide derived from [2-T] mevalonate might be expected to have the labelling pattern shown in (15a). If the 4β-methyl group is derived from the C_2 of mevalonate then 2 Tritium atoms will be incorporated in this group, but if the 4α-acetoxymethyl group is derived from C_2 then only $1\frac{1}{3}$ tritium atoms (the statistical residue after oxidation of a doubly tritiated methyl) will be present. Complete randomisation of label due to a symmetrical intermediate would lead to 1 tritium atom being located in the 4β-methyl group, and $\frac{2}{3}$ of a tritium atom in the 4α-acetoxymethyl group.

A degradative sequence suitable for determining the distribution of tritium in pebrolide biosynthesised from [2-T] mevalonate had previously been investigated using inactive material by C. H.

Fig. 1:7 - Degradation of pebrolide (15).



Cadzadilla[29], in this laboratory as summarised in Fig. 1:7. The labelled compounds derived from pebrolide biosynthesised from [2-^{14}C] mevalonate were identified by comparison by thin layer chromatography and mixed melting point with authentic samples.

A preliminary study of sesquiterpene production by P. brevicompactum in surface culture, by analytical t.l.c. of the culture filtrate extracts, had shown that pebrolide was produced from the fourth day onwards, whilst significant quantities of desacetylpebrolide were not produced until after the eighth day. However another metabolite, the depsipeptide, brevigellin (208), traces of which prevent the crystallisation of pebrolide and which is not easily separated from pebrolide by preparative t.l.c., is produced from the seventh day onwards. For this reason the best time for feeding was thought to be between the fourth and sixth day of growth.

The first of the two feeding experiments described was carried out by C. H. Cadzilla[29], whose results are reproduced in Table 1:1.

An aqueous solution of DL-[2-^{14}C] and [2-T] mevalonic acid lactone (0.05 and 2.0 mCi. respectively) was fed to a four day old surface culture of P. brevicompactum which was then harvested after a further two days growth. The metabolites present in the culture filtrate were isolated using the charcoal/acetone method. Inactive pebrolide was added to the extract, reisolated by crystallisation and purified by repeated crystallisation. However, t.l.c. radioscan of the labelled pebrolide showed that even after several crystallisations traces of a slightly less polar metabolite of much

Table 1:1 Degradation of [^{14}C,T] pebrolide from experiment I

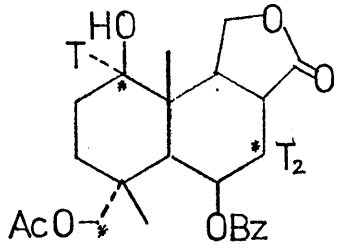
Compound	rel. molar activity (dpm./mM.) ^{14}C	T	%T based on pebrolide	T/^{14}C atomic
pebrolide (15a)	3.2×10^4	8.7×10^5	100	4.35/3 (4.47/3)*
(27a)	3.18×10^4	7.15×10^5	82	3.36/3 (3.78/3)*
(29a)	--	--	--	(1.76/3)*

( )* denotes results obtained by C. Hernandez Cadzadilla[29]

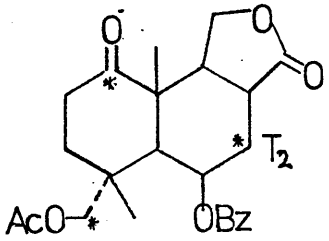
higher specific activity were present. On oxidation with Jones' reagent the contaminant was converted to a product (or products) slightly less polar than pebrolide ketone (27).

Samples of pebrolide and the ketone were purified by repeated crystallisation until the contaminants could no longer be detected by t.l.c. radioscan. The total activity of the reisolated pebrolide was 0.001 μCi. with respect to ^{14}C, which represents an incorporation of 0.014% based on L-[2-^{14}C] mevalonate.

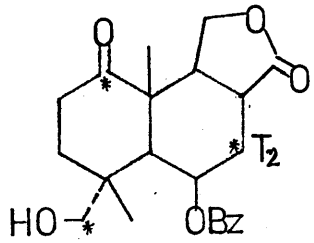
The polarity of the contaminant mentioned above and that of its oxidation product, coupled with the high specific activity observed suggested that it may have been a sterol or a closely related mixture of sterols. Analytical t.l.c. examination of the mycelial extracts of P. brevicompactum showed that ergosterol (the only sterol produced in significant quantities by this fungus) is produced from the fifth day of growth onwards. In order to reduce contamination from this source it was thought that the cultures should be harvested at an earlier stage of growth. Consequently, in a second experiment cultures of P. brevicompactum were fed with DL-[2-^{14}C] and [2-T] mevalonic acid lactone (0.1 and 4 mCi. respectively) on the third day of growth and harvested on the fourth day. Inactive pebrolide and desacetylpebrolide were added to the extract of the culture filtrate and were reisolated by a combination of column and preparative layer chromatography. Samples of both were crystallised until constant activity was achieved. The incorporation of [2-^{14}C] mevalonate into pebrolide was 0.002% (based on L-[2-^{14}C] mevalonate) and that into desacetylpebrolide was 0.0004%.



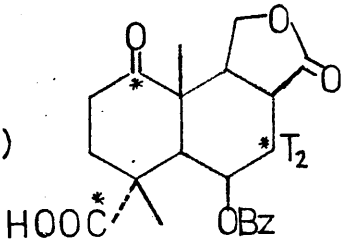
(15a)



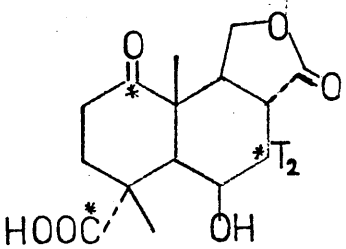
(27a)



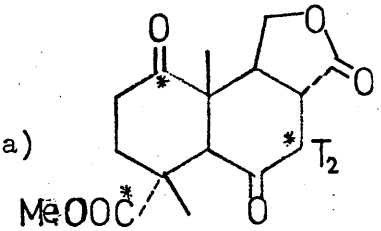
(28a)



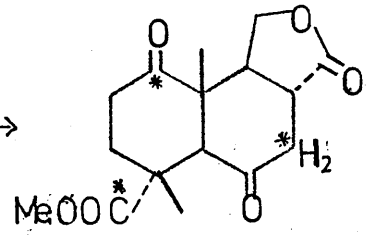
(29a)



(30a)



(32a)



(32b)

Table 1:2 Degradation of [^{14}C,T] pebrolide from experiment II

Compound	rel. molar activity (dpm./mM.) ^{14}C	T	%T based on pebrolide	T/^{14}C (atomic)
pebrolide (15a)	2.50×10^4	3.87×10^5	100	4.36/3
(29a)	1.51×10^4	1.75×10^5	45	1.98/3
(32a)	1.50×10^4	1.62×10^5	41.6	1.87/3
(32b)	1.49×10^4	1.0×10^4	2.6	0.12/3

Table 1:3

Position	%T	
	exptl.	calc.
1	18	23
4α-methylene and CH_3CO-	37	31
2,6,8	3.4	0
7	39	46

Pebrolide from each of the feeding experiments was degraded by the scheme outlined in Fig. 1:7; the results are summarised in Tables 1:1, 1:2, and 1:3.

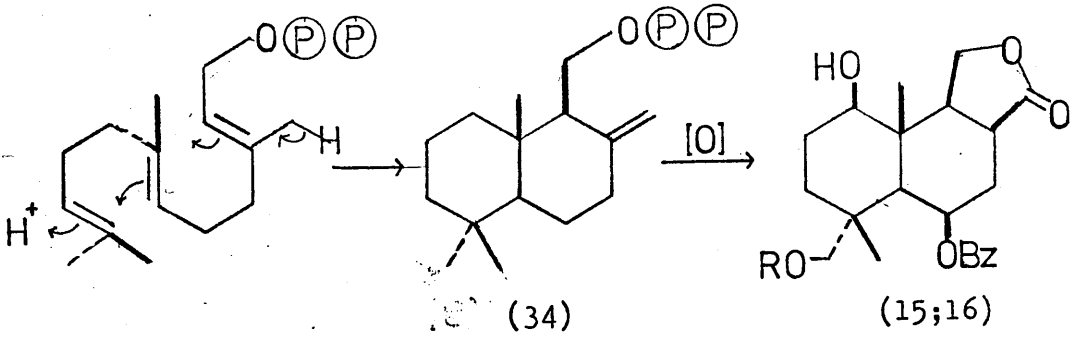
The atomic ($T/^{14}C$) ratio of pebrolide from both feedings is in accord with the predicted ratio (4.33/3) in the case where the 4α-methylene is stereoselectively derived from the C_2 of mevalonate.

Oxidation of pebrolide to pebrolide ketone with Jones' reagent results in the loss of 18% of the tritium activity, which confirms the incorporation of one tritium atom at C_1. The atomic ratio of the ketone (3.36/3) is in good agreement with a predicted ratio of 3.33/3.

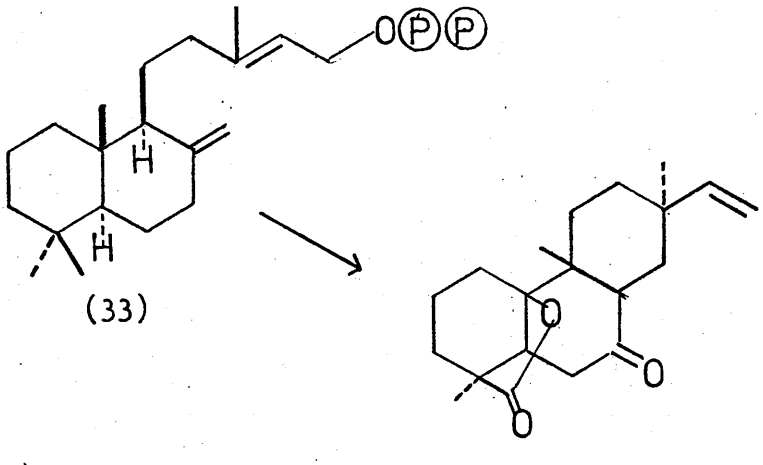
Selective hydrolysis of pebrolide ketone (27a) with dilute acid and oxidation of the alcohol (28a) with Jones' reagent gave the keto acid (29a), which retained only 45% of the tritium activity of pebrolide. The difference in the atomic ratios found for the keto acid (1.98/3) and the ketone (27a) is almost exactly that predicted.

Basic hydrolysis of the benzoate, which was accompanied by epimerisation of the lactone, gave the keto hydroxy acid (30a) which was oxidised to the diketo acid (31a) with Jones' reagent, and this was esterified with diazomethane to give the methyl ester (32a). Comparison of the activity of tritium in the keto acid (29a) and the methyl ester (32a) shows that only a small fraction of the total tritium has been lost (3.4% of the total in pebrolide). This activity corresponds to incorporation in the positions 2, 6 and 8 of the pebrolide skeleton.

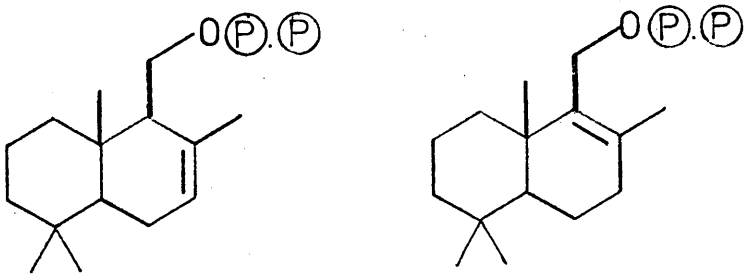
Fig. 1:8 - Proposed biogenesis of pebrolide.



(34) (15;16)



(33) (5)



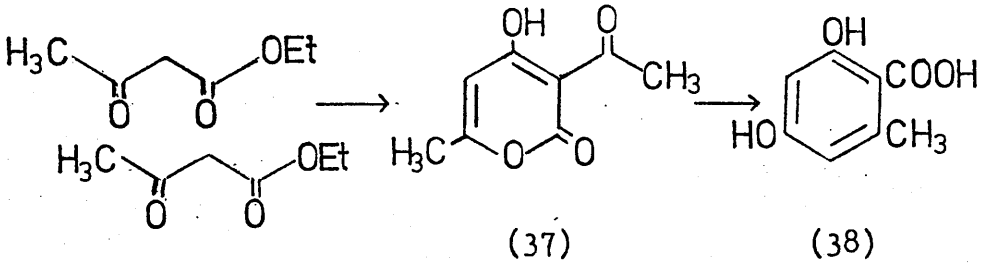
(35) (36)

Exchange of the tritium at positions 7 and 5 with refluxing dilute aqueous sodium hydroxide was accompanied by hydrolysis of the ester (32a) to the corresponding acid (31b), which was re-esterified with ethereal diazomethane prior to counting. The tritium content of the product was only 2.5% of that of the ester prior to treatment with base, indicating that the remaining two tritium atoms had been incorporated at the 7 position as predicted.

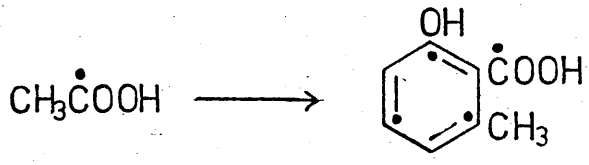
The results indicate that in the biosynthesis of pebrolide the α-carbon atom at C_4 is directly derived from the C_2 of mevalonate, and that the stereoselectivity of ring closure is identical to that observed in the di- and tri- terpenoids.

A possible biosynthetic route from farnesyl pyrophosphate is outlined in Fig. 1:8. By analogy with the pyrophosphate (33) which has been shown to be an intermediate[35] in the biosynthesis of rosenonolactone (5) by Trichothecium roseum, the drimenyl pyrophosphate (34) could be an intermediate in the biosynthesis of pebrolide. However, the intermediacy of either of the isomeric pyrophosphates (35) and (36) is also possible. The location of 2 tritium atoms at C_7 of pebrolide derived from [2-T] mevalonate (Table 1:3) would be impossible if the intermediate (35) were involved. Experiments with [4-T] mevalonate would be required to show whether (36) is a possible intermediate.

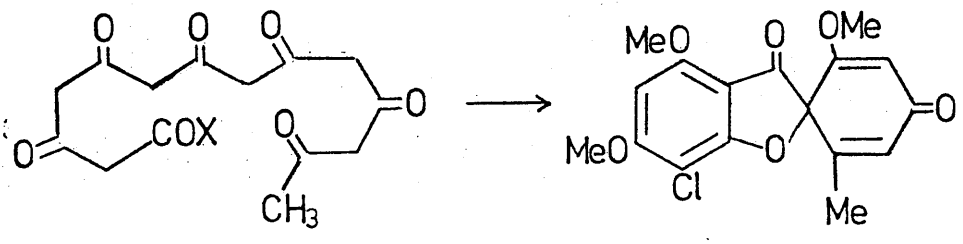
CHAPTER 2


H3C
OEt
O
O
H3C
OEt
O
O
OH
O
CH3
H3C
O
O
OH
COOH
HO
CH3

(37) (38)


CH3COOH
OH
COOH
CH3

(39)


O
O
O
O
O
COX
O
CH3
MeO
O
OMe
O
MeO
O
Cl
Me

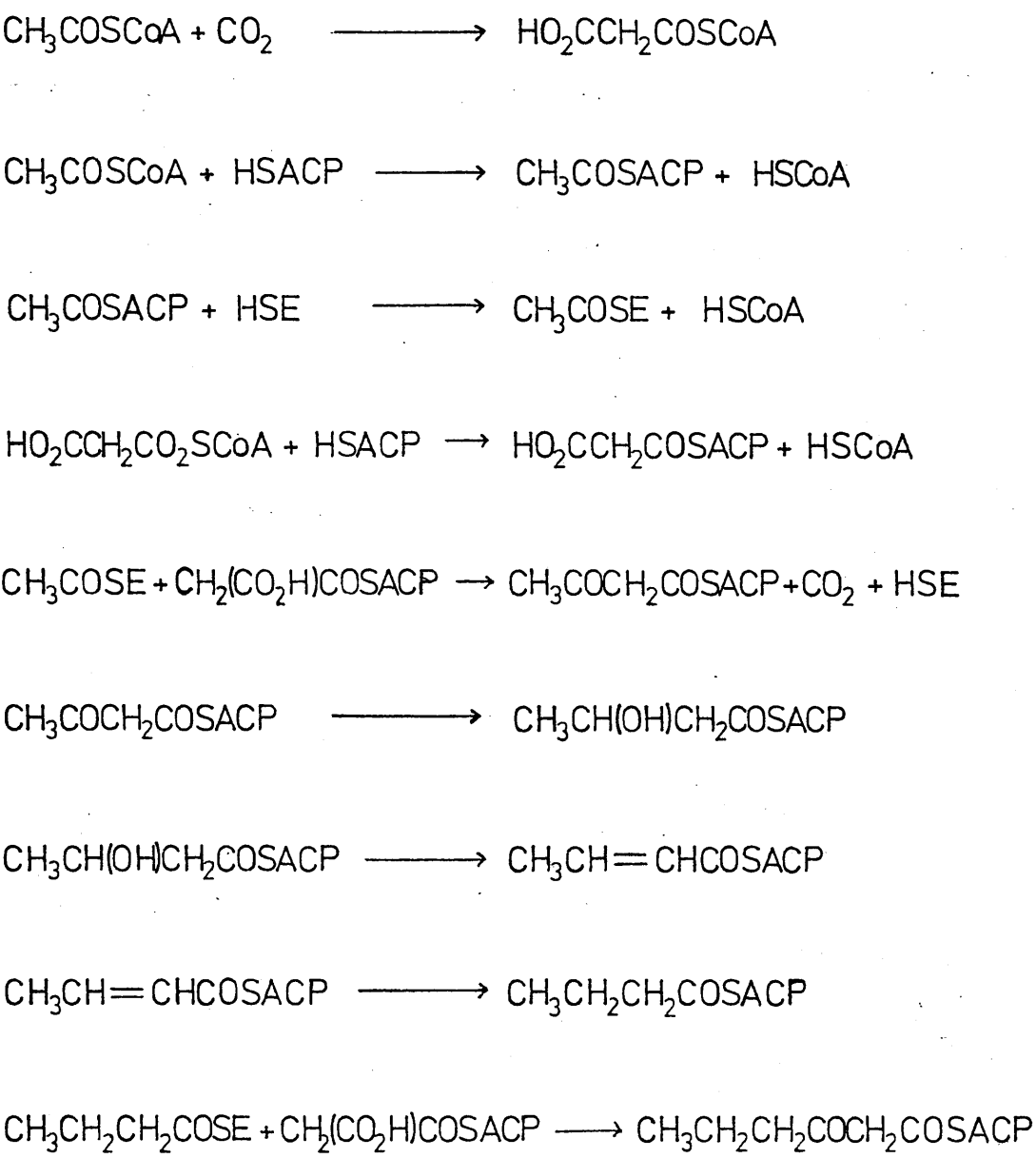
(40) (41)

Introduction

The implication of acetate in biosynthesis was originally proposed by Collie[36] (1907), whose synthesis of orsellinic acid (38) from the dehydracetic acid (37) which he obtained from condensation of two molecules of ethyl acetoacetate prompted him to suggest that polymers of acetate (or rather ketene (CH_2=C=O)) might be involved in the biosynthesis of this and other naturally occurring aromatic compounds. This concept lay dormant until re-examined by Birch[37] (1953) who used it to rationalize the oxygenation patterns observed in a large number of fungal metabolites, by postulating the intermediacy of a poly-β-keto-methylenic acid, $CH_3(CO.CH_2)_nCO_2H$, in their biosynthesis. The first experimental verification[38] of this was given by the specific incorporation of [1-^{14}C] acetate into 6-methylsalicylic acid (39) by cultures of Penicillium griseofulvum. Griseofulvin (41), also produced by P. griseofulvum, can also be derived 'on paper'[37] from a polyketide precursor (40) and the labelling pattern of this compound derived from [1-^{14}C] acetate[39] has been shown to be consistent with an intermediate of this type.

As will be discussed later, there is a close relationship between polyketide biosynthesis and the biosynthesis of fatty acids, the mechanism of which has been studied in detail and is outlined in Fig. 2:1[40]. An acetyl residue is transferred from coenzyme A, first to acyl carrier protein (ACP) and then to an enzyme (ESH). The malonate moeity of malonyl coenzyme A, formed by the carboxylation of acetyl coenzyme A, is similarly transferred to ACP and condensation

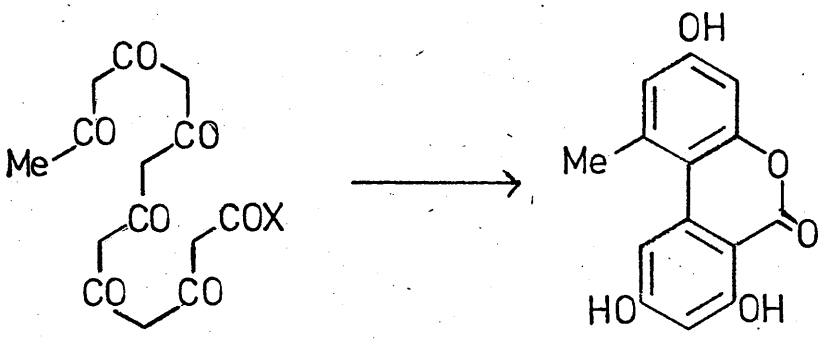
Fig. 2:1 - Fatty acid biosynthesis.



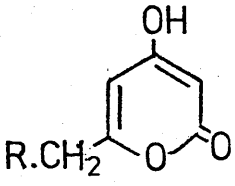
occurs with the enzyme bound acetyl unit giving acetoacetyl ACP with the elimination of CO_2. Successive reduction, dehydration and reduction give butyryl ACP which is transferred to an enzyme and condensed with a further malonyl ACP unit to give a C_6 intermediate bound to ACP. The process is continued until the desired length of chain is formed when the free acid or its coenzyme A derivative is liberated from the enzyme complex.

The process of polyketide biosynthesis has not been the subject of such an extensive study, but the body of evidence which has been accumulated points to a close similarity. Firstly, experiments with whole cells[41] and partially purified enzyme systems[42,43,44] have shown that the precursors of the polyketide chain are malonyl and acetyl coenzyme A derivatives. Secondly, the physical properties of the "polyketide synthetase" enzymes[45,48] are similar to those of the fatty acid synthetase enzyme complexes, and thirdly, the distribution of radioactivity in fatty acids and 6-methylsalicylic acid derived from 1-^{14}C and 2-T acetate in cultures of P. griseofulvum[46] and P. urticae[47] have been shown to be consistent with their formation by a similar condensation process. A further similarity is the absence of free intermediates between acetate and the fully assembled and stabilised polyketides.

The biosynthetic steps involved in polyketide formation can be envisaged as follows; the initial condensation between protein bound acetate and malonate units give rise to a protein bound C_4 β-keto acyl unit. The further extensions of this unit include



(42)



R = H
R = Ac

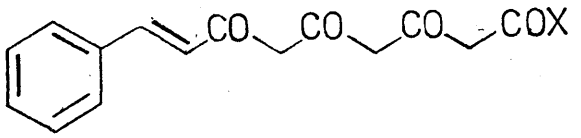
(43)

poly-β-keto intermediates which are bound to the enzyme surface, possibly by hydrogen bonding, and release from the enzyme surface occurs after aromatisation. Evidence for the intermediacy of protein bound poly-β-keto compounds has been presented by Gatenbeck[45] who has shown that the alternariol (42) synthesising enzyme system is more strongly inhibited by the β-keto compounds, acetylacetone and ethyl acetoacetate than by the β,δ -diketo compound acetonylacetone.

In the absence of NADPH, purified fatty acid synthetase[49] produces the tri- and tetra- acetic acid lactones (43) which can be considered as stabilized polyketide chains. Such pyrones do not themselves serve as intermediates[50,51] in the biosynthesis of other polyketides in vivo (although their conversion in vitro to a number of naturally occurring structural types of polyketides has been demonstrated[43,52]) indicating that "polyketide synthetase complex" in contrast to fatty acid synthetase, must be able to stabilize the reactive poly-β-ketone intermediates. It has been suggested that metal chelation may play a part in the stabilization of these intermediates and the control of their specific cyclisations[53].

The fact that only the first two carbon atoms of a polyketide chain are directly derived from acetate suggests that acetate should be incorporated into this position more efficiently than malonate. This starter effect has been demonstrated in a number of experiments both with enzyme systems[42,43] and whole cells[46,47].

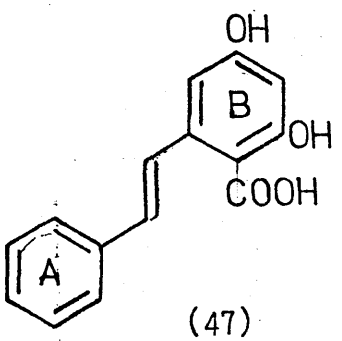
Acyl groups other than acetyl are also known to initiate polyketide chain formation. Addition of three malonyl units to a cinnamoyl



(44)

Aldol

Claisen



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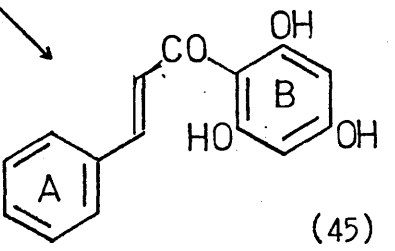

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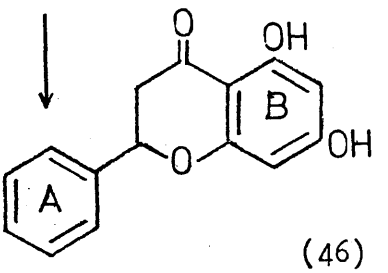
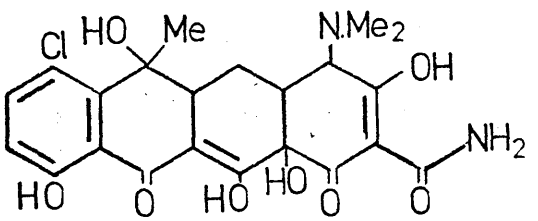
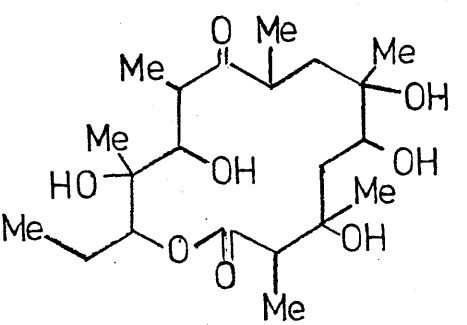


(46)



(48) 7-Chlorotetracycline



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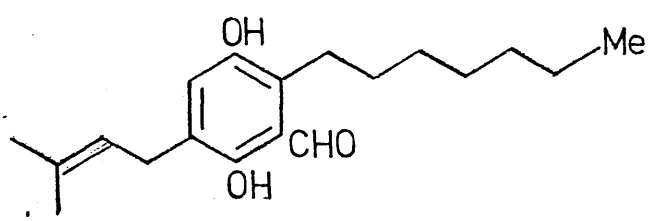

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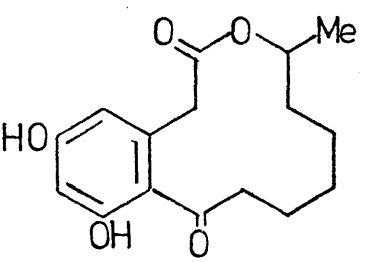

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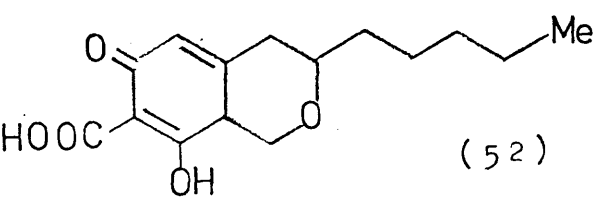
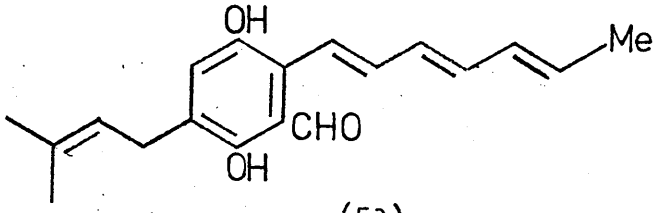


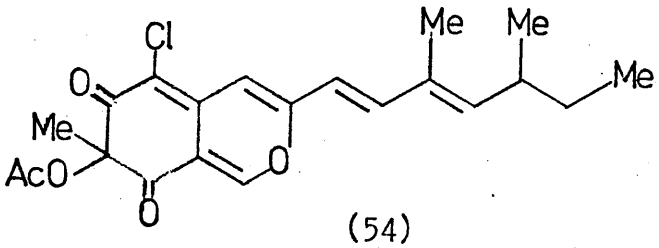
(52)

starter group could produce a polyketide (44), which would give chalcone (45) and compounds of the flavanoid series such as flavanone (46) by Claisen-type condensation, or compounds of the stilbene series such as pinosylvic acid (47) by aldol-type cyclisation. Labelling experiments support these schemes[54]; although the cinnamoyl group (ring A) is derived from shikimic acid, ring B is derived from acetate. In the biosynthesis of the tetracyclines (48) by actinomycetes of Streptomyces spp. the 'starter unit' is probably malonamoyl coenzyme A[55]. In actinomycetes propionate can function both as a 'starter' and (through methylmalonyl coenzyme A) as a chain extender unit, as in the biosynthesis of the macrolide antibiotic erythronolide (49), which is wholly derived from propionate[56]. In fungi, however, intervention of units other than acetyl is rare although an ambiguity arises in the case of compounds which possess saturated straight chain residues, such as flavoglaucin (50) and curvularin (51), in which the incorporation of a preformed fatty acid as 'starter' can be envisaged. It has been shown, however, that hexanoate was not specifically incorporated into pulvilloric acid (52).

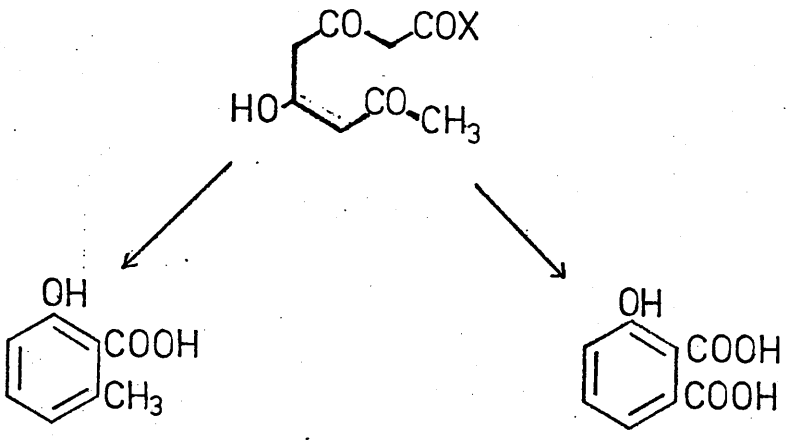
Reduction of carbonyl groups and dehydration without a final reduction step (cf. fatty acid biosynthesis) would account for the unsaturated side chains of auroglaucin (53) and sclerotiorin (54) and also for the 'missing oxygen atoms' of a large number of polyketides, e.g. 6-methylsalicylic acid (39) and 3-hydroxyphthalic acid (55) which can be formally derived from a reduced orsellinic acid-type precursor.


OH
Me
CHO
OH

(53)

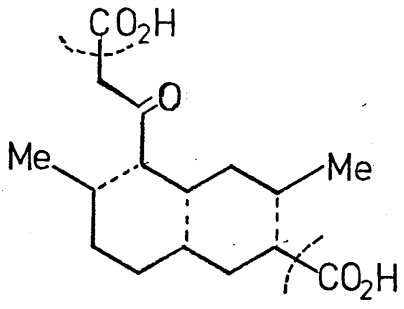

Cl
Me
Me
Me
O
Me
AcO
O
O

(54)

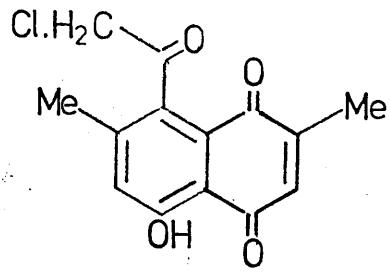

CO
COX
HO
CO
CH3
OH
COOH
CH3
OH
COOH
COOH

(39)

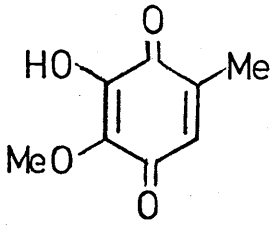
(55)



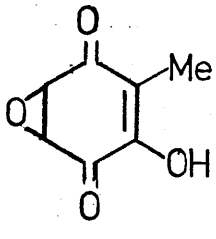
(56)



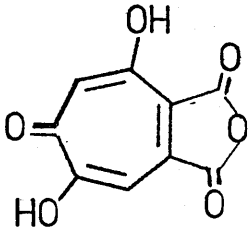
(57)



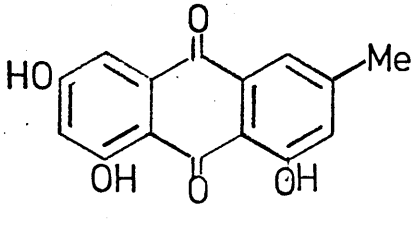
(58)



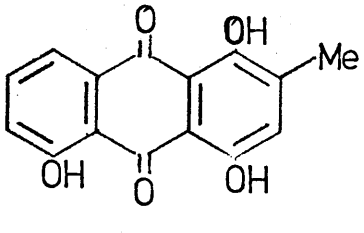
(59)



(60)



(61)



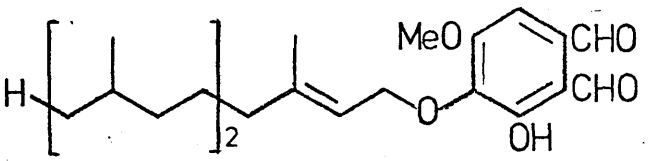
(62)

A small class of metabolites are known to be derived from the condensation of separate polyketide chains. Mollisin (57) a metabolite of Mollisia caesia has been shown to arise wholly from acetate[58,59] which is consistent with formation from two tetraketide chains (56) with subsequent decarboxylation.

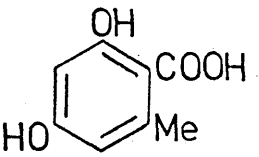
Decarboxylation is a common feature of polyketide biosynthesis; fumigatin (58) has been shown to be derived from orsellinic acid[60] and terreic acid (59) from 6-methylsalicylic acid[61], indicating that decarboxylation occurs after aromatisation of a polyketide precursor. In certain cases the enzymes which catalyse the decarboxylation of polyketide substrates have been isolated and characterised, e.g. orsellinic acid[62], 6-methylsalicylic acid[63], and stipitatonic acid (60)[64] decarboxylases have been reported.

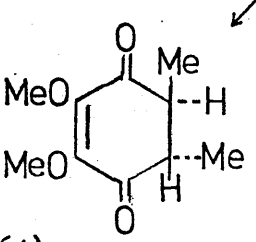
The biosyntheses of fumigatin (58) and terreic acid (59) also involve the introduction of 'extra' oxygen atoms, a process which occurs late in a biosynthetic sequence, often after stabilization of the polyketide, and is catalysed by relatively non-specific enzymes. The biosynthesis of many of the anthraquinones, e.g. emodin (61) and islandicin (62), involve both decarboxylation and the introduction of oxygen, although it is not known in which order these occur.

In general O-alkylation involves methionine (as its S-adenosyl derivative) as a methyl donor[65] or the prenylpyrophosphates as donors of terpenyl units[66], and is a terminal step in biosynthesis. Asperugin (63), a metabolite of Aspergillus rugulosus[67], contains both types of O-alkyl substituent.

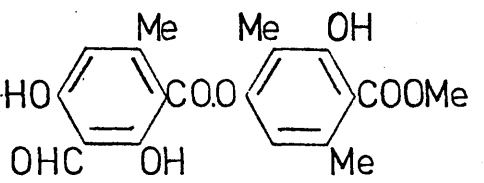


(63)

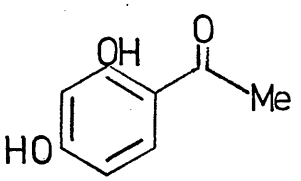




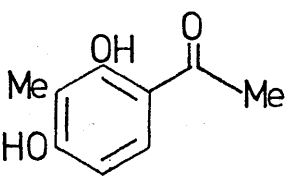
(64)



(65)



(66)



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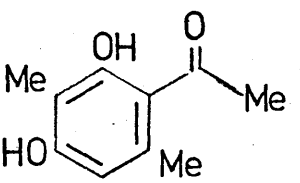

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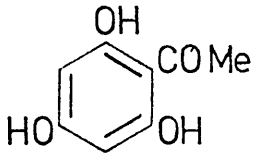

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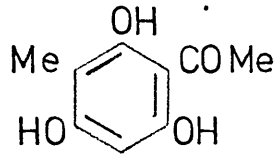

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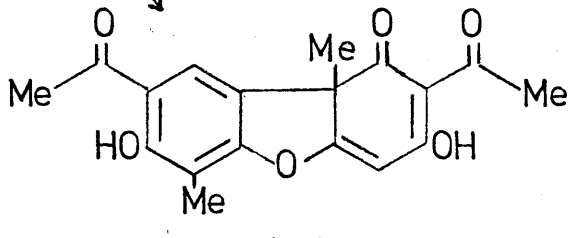

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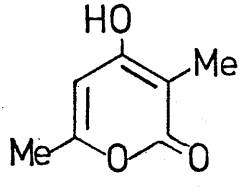
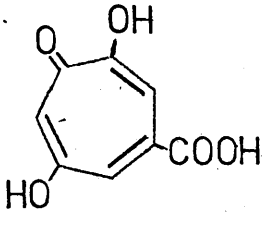
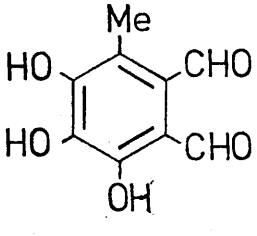


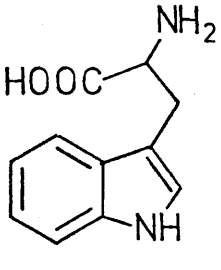
(72)



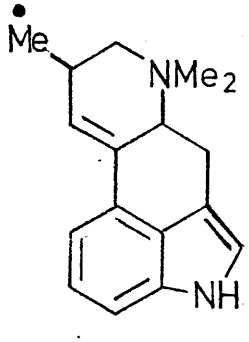
(73)



(64A)



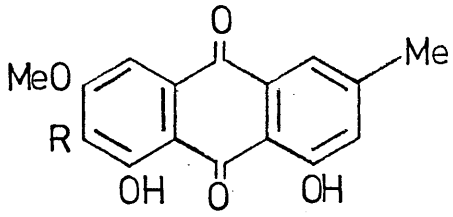
$[2\text{-}^{14}C]MVA$



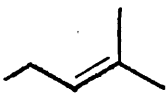
(74)

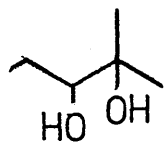
Methyl transfer from S-adenosyl methionine is also responsible for the 'extra' C-methyl groups of many polyketides[65,68]. C-methylation was thought earlier to occur at a post-aromatic stage, but the body of evidence now available; vis. the fact that orsellinic acid is not incorporated into gliorosein(64)[69], and atranorin (65)[70], that the ketones (66, 67) are not incorporated into clavatol (68)[71], and that although methylphloroacetophenone (69) is a precursor of usnic acid (71) the desmethyl compound (70) is not[72]; suggests that it occurs at some pre-aromatic stage. The isolation of methyl triacetic acid lactone (72) as a by-product of stipitatic acid (73) biosynthesis in P. stipitatum[73] has been interpreted as evidence that C-methylation precedes the introduction of the final malonyl unit to the growing polyketide chain, but the reported catalysis of the methylation of a protein bound substrate, believed to be tetraacetic acid, by extracts of Aspergillus flavipes[74] (which produces flavipin (64A)), indicates that methylation in this case occurs after chain formation. The mechanism and timing of C-methylation requires detailed study with pure enzymes before any conclusions can be reached.

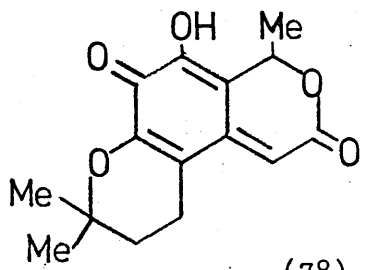
On the other hand C-alkylation by prenyl units probably does occur at a post aromatic stage. The prenylation of p-hydroxybenzoic acid (derived from shikimate) in bacterial systems has been demonstrated[75] and the formation of the ergoline base agroclavine (74) by Claviceps purpurea shown to involve alkylation of tryptophan with a mevalonate derived C_5 unit[76]. The recent isolation of the prenylanthraquinones (76) and (77) from a physcion (75) producing strain of Aspergillus



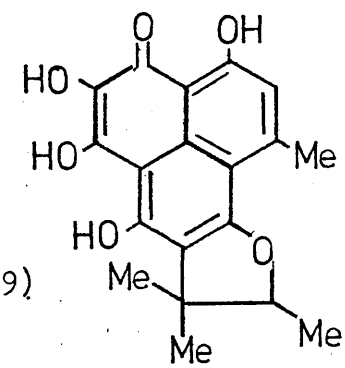
(75) R = -H

(76) R = 

(77) R = 

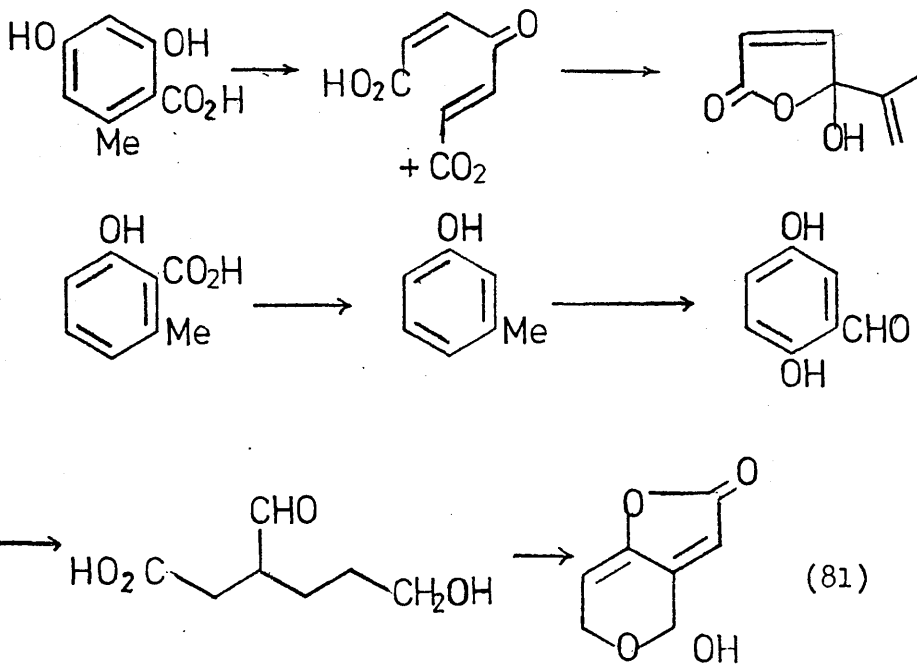


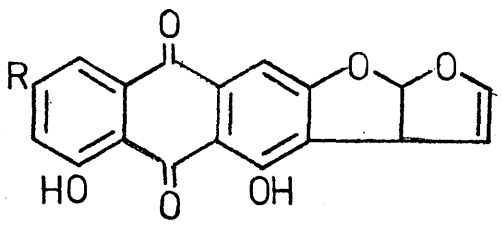
(78)



(79)

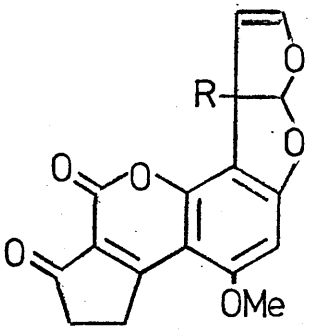
Fig. 2:2 The biosynthesis of penicillic acid and patulin.





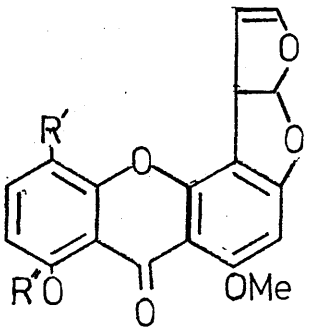
(82)

R = OH versicolorin A.
= H deoxyversicolorin A.



(83)

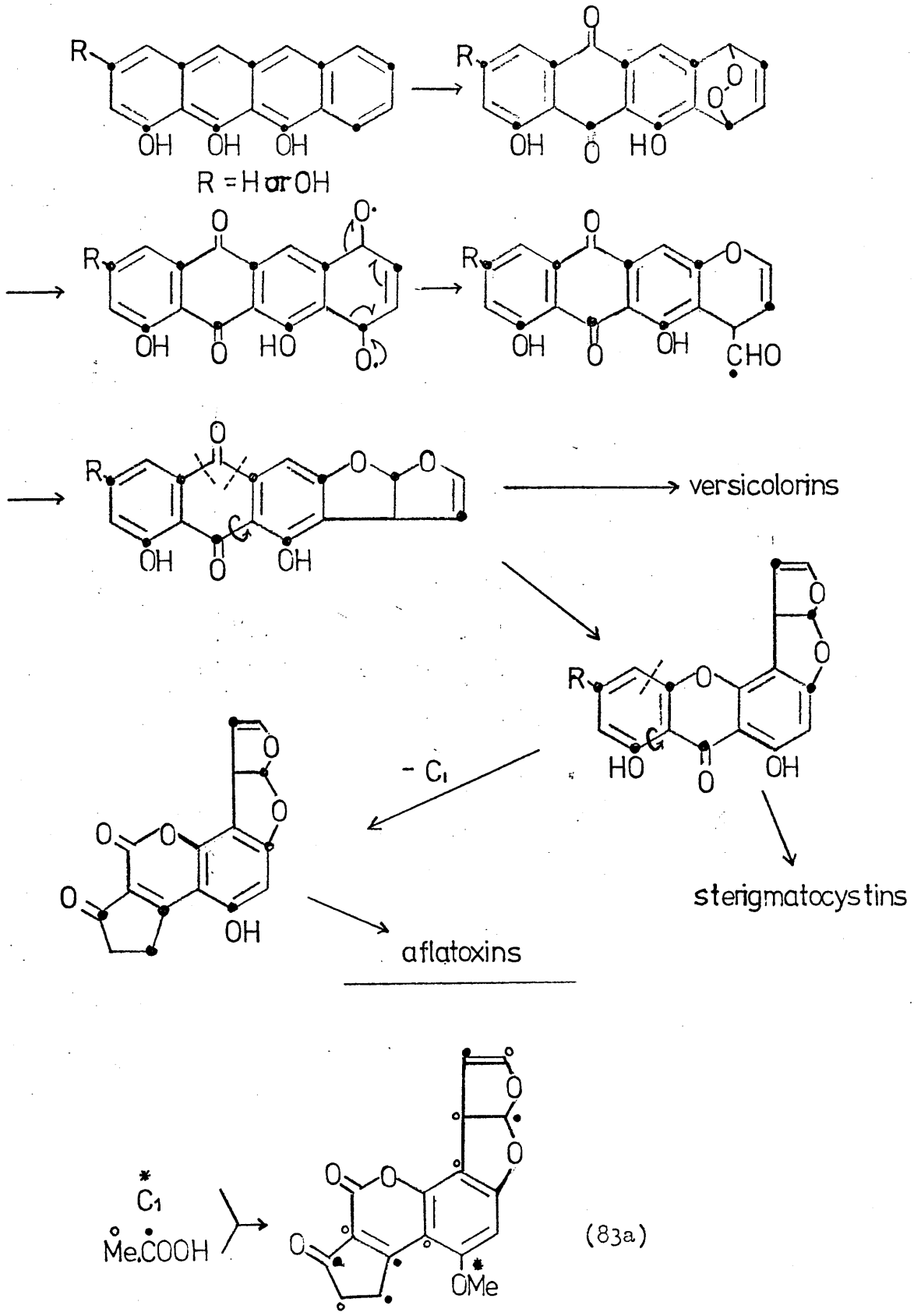
R = H aflatoxin B_1.
= OH aflatoxin M_1.



(84)

R" = R' = H
sterigmatocystin.

Fig. 2:3 - Proposed biosynthesis of the bisfurans.



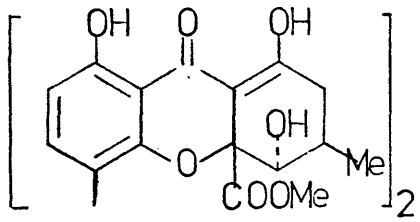
(83a)

flashentraegeri[77] suggests that the same order applies in polyketide prenylation. Auroglaucin (53)[78] and flavoglaucin (50) from Aspergillus glaucus[79] are derived by introduction of an isopentenyl unit derived from mevalonate, in fuscin (78)[78] and atrovenetin (79)[80] cyclisation of the terpenyl side chain has occurred. Metabolites formally derived by alkylation of a tetraketide nucleus with a farnesyl unit have been described previously (p. 8).

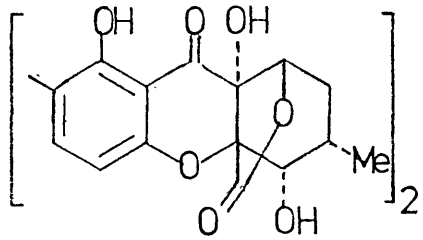
The biosynthesis of a number of acetate derived fungal metabolites involves rearrangement of the carbon skeletons of the initial polyketide precursors. The sequences involved in the formation of penicillic acid (80) from orsellinic acid[81], and patulin (81) from 6-methylsalicylic acid[82,83] are shown in Fig. 2:2.

The origin of the bisfurano-metabolites of Aspergillus versicolor and A. flavus (e.g. the versicolorins (82) and aflatoxins (83))[84] is not immediately obvious from inspection of their structures. The specific incorporations of [1-^{14}C] and [2-^{14}C] acetate and [$^{14}CH_3$] methionine into aflatoxin B_1 (83a) have been determined by Buchi and co-workers[85], who proposed the biosynthetic sequence (Fig. 2:3) as a possible route from a nonaketide precursor. Tanabe et al.[86] have examined the incorporation of ^{13}C labelled precursors into the related metabolite sterigmatocystin (84, Fig. 2:3) and shown that these are consistent with derivation from a nonaketide, rather than from two acetate-derived chains as previously proposed[87].

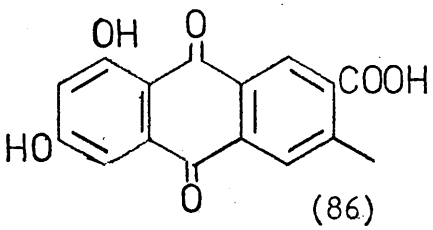
The co-occurrence of the ergochromes (85) and the anthraquinones (86) and (87) in cultures of Claviceps purpurea suggested that the



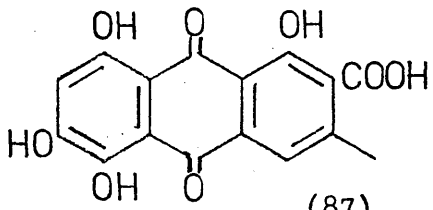
ergochrome AA (85)



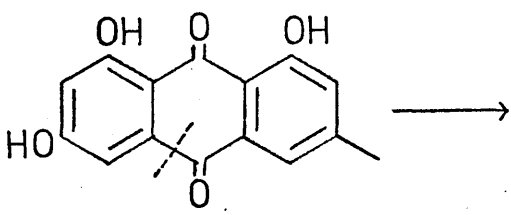
ergochrome CC



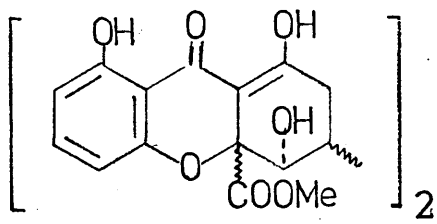
(86)

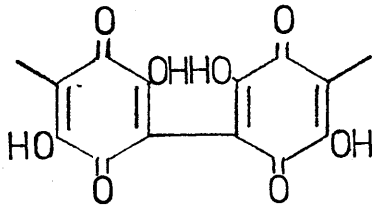


(87)



(88)





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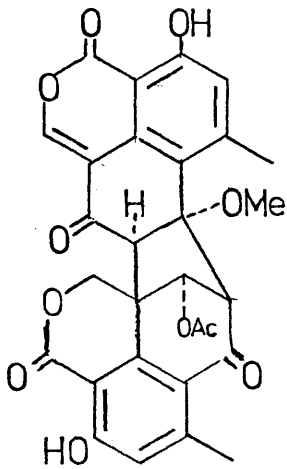

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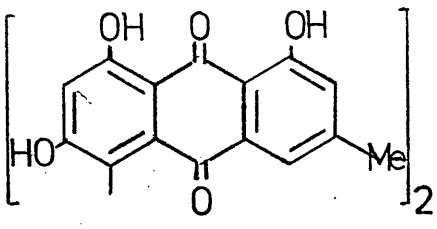

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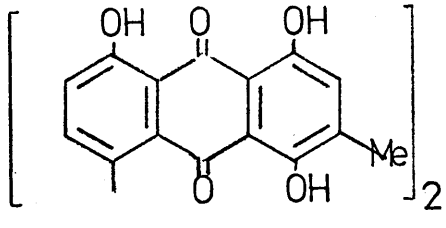

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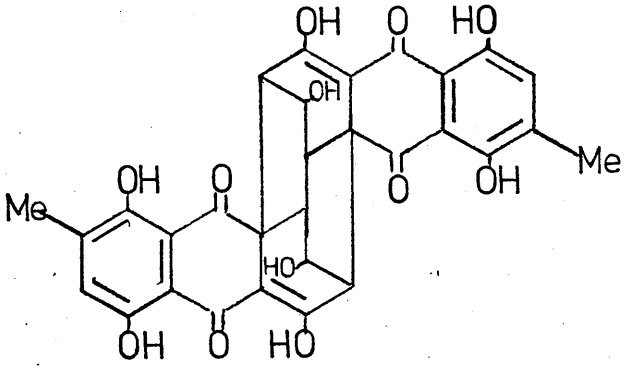

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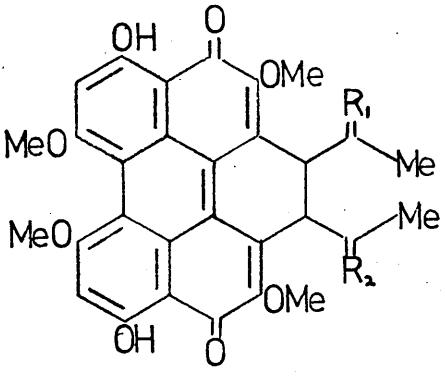


$R_1 = R_2 = O.$

$R_1 = R_2 = OH, H.$

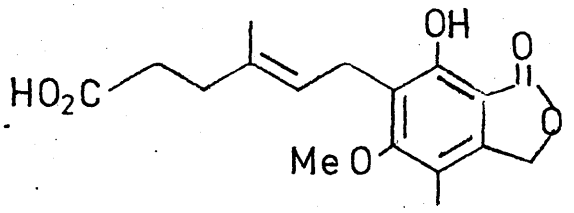
(94)

former might be formed by oxidative cleavage of an anthraquinone precursor. This has been verified by Franck et al.[88] who have shown that emodin (89) is a precursor of these compounds.

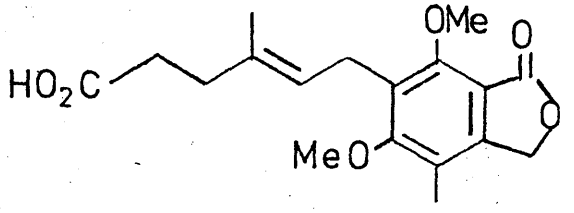
The dimeric nature of the ergochromes introduces a further common feature of fungal metabolism, phenol-oxidative coupling[89], a biosynthetic process exhibited by a large number of plant systems. Both inter- and intra- molecular couplings are known. The coupling of orsellinic acid residues in the biosynthesis of oosporein (89)[90] and of methylphloroacetophenone (69) in the formation of usnic acid (71)[91] have been demonstrated, and the formation of an ether linkage by this process in the biosynthesis of griseofulvin (41) has been suggested[92]. Duclauxin (90)[93] is presumably formed by coupling of the corresponding monomer. Although the dimeric anthraquinones of Penicillium islandicum[94], e.g. skyrin (91) and iridoskyrin (92), and their monomers occur as co-metabolites, the monomers are not incorporated into the dimeric compounds[95], suggesting that coupling occurs at a stage prior to quinone formation possibly at the level of the intermediate anthrones. Intramolecular repetition of the coupling process could lead to the novel structures of luteoskyrin (93)[94] and the elsinochromes (94)[95].

DISCUSSION

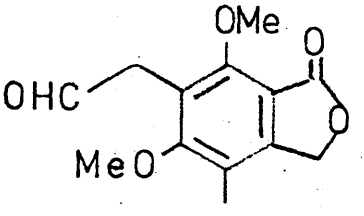
PART 1



(95)



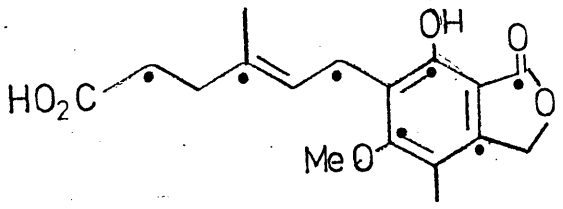
(96)



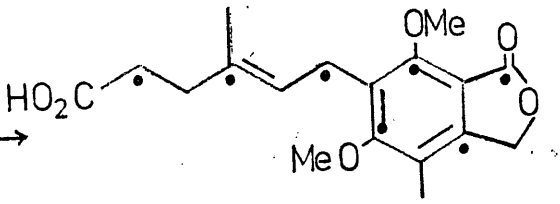
(97)

Mycophenolic acid (95) holds a position unique in the history of the study of fungal metabolites. It was named by Alsberg and Black (1915) who isolated it from cultures of Penicillium stoloniferum[97], although Gosio (1896)[98] had described the isolation of a crystalline antibiotic substance from cultures of "Penicillium glaucum" which was probably identical. Raistrick and co-workers isolated mycophenolic acid, together with other phenolic compounds, from twelve different strains of the Penicillium brevicompactum series[99] (to which P. stoloniferum belongs) and advanced the structure (95) on the basis of extensive chemical degradation[100,102]. This structure has been confirmed by the synthesis of the ozonolysis product (97)[103] of the methyl ether (96) and more recently by total synthesis of mycophenolic acid[104,105].

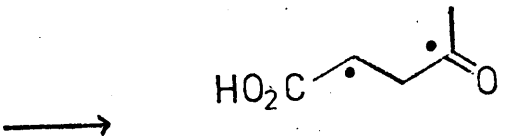
Early investigations into the antifungal and antibacterial properties of mycophenolic acid showed that the metabolite suppressed the growth of certain pathogenic fungi and Gram positive bacteria, but the activity/toxicity ratio was considered too low to permit its use as a therapeutic agent[98,106]. Recently, however, interest in mycophenolic acid and its derivatives has been revived with the discovery that the free acid has marked antiviral[107,108], immunosuppressive[109] and antitumor[107,110,111] activity in vivo. The antimitotic and anti-cancer properties of mycophenolic acid have been ascribed to its inhibitory action on inosinic acid dehydrogenase which results in a suppression of quanine and xanthine nucleotide biosynthesis[110,113]. In contrast to earlier reports, it has also been shown to inhibit the growth of certain pathogenic fungi in very



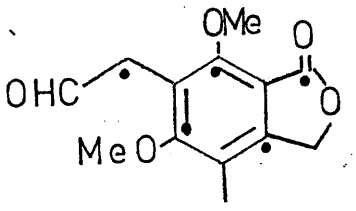
(95a)



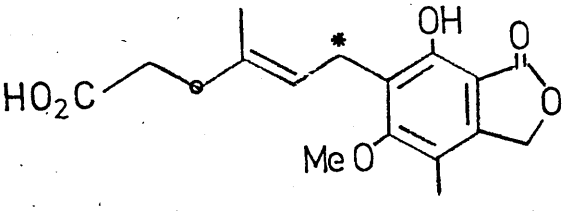
(96a)



(98a)

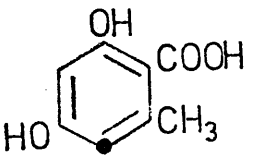


(97a)

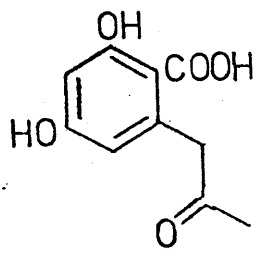


[2-^{14}C]MVA - °
[4-^{14}C]MVA - *

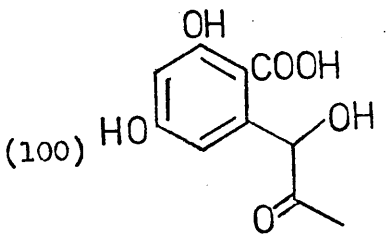
(95b)



(38a)



(99)



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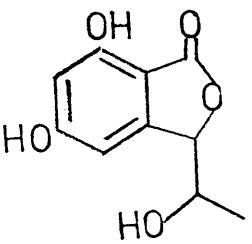
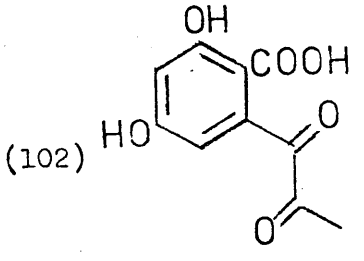


(101)



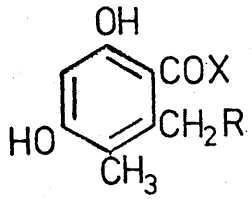
(102)

low concentration[112].

The polyketide origin of the aromatic nucleus of mycophenolic acid was first suggested by Birch and co-workers[114] who showed that label from [1-^{14}C] acetate was incorporated into both the side chain and the nucleus (95a). Ozonolysis of the labelled O-methylmycophenolic acid (96a) afforded the aldehyde (97a) and laevulinic acid (98a). Extensive degradation of the laevulinic acid, which possessed 2/7 of the activity of the mycophenolic acid, showed a labelling pattern consistent with terpenoid origin of the side chain. This was confirmed by the incorporation of [2-^{14}C] and [4-^{14}C] mevalonate into mycophenolic acid (95b)[114,115]. Feeding experiments with [$^{14}CH_3$] and [CD_3] methionine have shown that the methoxyl and aromatic methyl groups are derived from the C_1 pool[65,116].

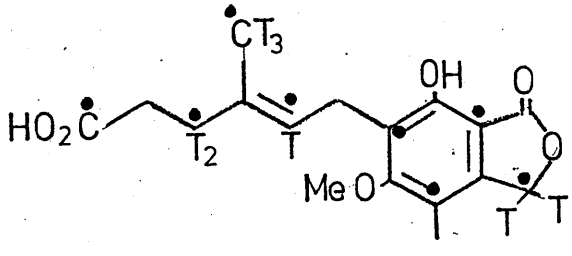
Although the above experiments had given an indication of the primary precursors involved, nothing was known of the biosynthetic sequence of their assembly. Birch[115] has claimed* the incorporation of [5-^{14}C] orsellinic acid (38a) but the location of label in the mycophenolic acid formed is consistent with breakdown of the orsellinic acid to acetate prior to incorporation rather than incorporation of the acid as an intact unit. It has been suggested[115], in view of the co-occurrence of the pentaketides (99), (100), (101) and (102) in P. brevicompactum[99,117,29], that a compound of this type could

*It has recently been reported that [1-^{14}C] orsellinic acid is not incorporated into mycophenolic acid[124].



R = H or $COCH_3$

(103)



(95a)

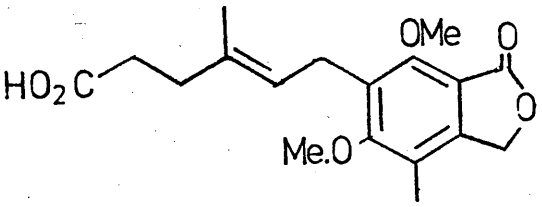
be a precursor, a C_2 unit being removed at some later stage in biosynthesis. Another possibility, by analogy with the sequence found in the biosynthesis of other C-methylated polyketides (p.20) is that introduction of the C-methyl group precedes aromatisation of the nucleus, and that the first free intermediate is of the type (103). It has been claimed[118], without experimental details however, that a "starter effect" has been detected in feeding experiments with malonyl CoA which would imply that the aromatic precursor is a tetraketide. It was proposed to investigate whether or not a starter effect is observed using a double labelling technique.

[2-^{14}C, 2-T] Acetate should be incorporated both into the side chain and the nucleus of mycophenolic acid (95c). Having established from the ^{14}C activities of suitable fragments the relative incorporations into the nucleus and side chain, the tritium content of the phthalide methylene group could be predicted, assuming uniform incorporation of acetate into the nucleus. A substantially higher value than predicted would indicate a starter effect, and hence a tetraketide precursor, while a lower value would indicate the intermediacy of a precursor more highly oxygenated at this position.

In an analogous experiment with [2-^{14}C, 2-T] malonate a certain proportion of the total tritium content would again be predicted to lie in the phthalide methylene group, assuming uniform incorporation of malonate into the nucleus, as would be expected for a pentaketide precursor. A lower proportion again would indicate a starter effect.

In order to assess the incorporations involved, a degradative

scheme was required which enabled the incorporation of tritium in the phthalide methylene group, the incorporation of ^{14}C in the nucleus and the incorporation of tritium and ^{14}C in the side chain to be determined independently. The following section describes the investigation of degradative sequences which were carried out using inactive material.



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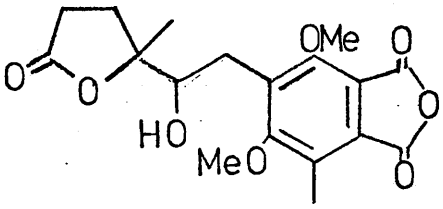
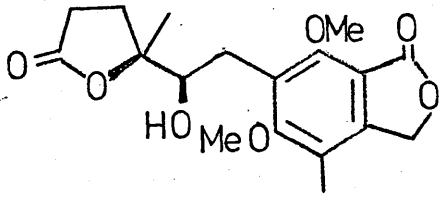


(104)



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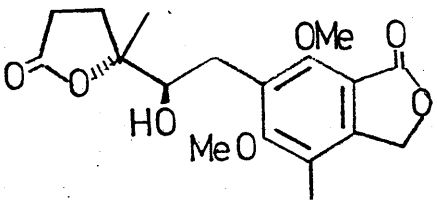

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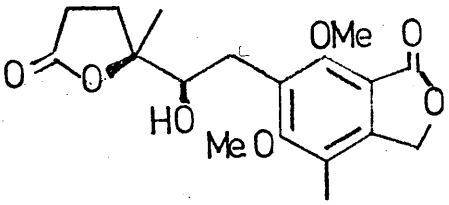


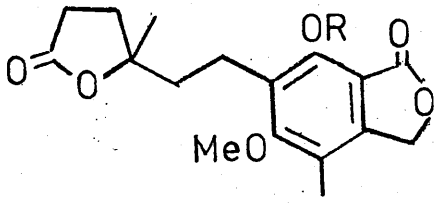
(107)

Degradations of Mycophenolic acid

(i) The specific oxidation of the phthalide methylene group

The oxidation of O-methylmycophenolic acid (96) with refluxing alkaline potassium permanganate was reported by Raistrick[101] to give the anhydride (104) in high yield, and this structure was later confirmed spectroscopically by Birch[116]. Oxidation of (96) under the conditions described by these authors afforded a mixture of compounds, preparative t.l.c. of which gave one major crystalline product m. 150-151°C. (14%). Although the melting point of this compound was similar to that reported for the anhydride (104) (153°C)[101], elemental analysis and mass spectrometry were consistent with a molecular formula $C_{18}H_{22}O_7$ rather than that recorded for the anhydride ($C_{18}H_{20}O_8$). Absorption bands at 3480 and 1755 $cm.^{-1}$ in the i.r. confirmed the presence of hydroxyl and γ-lactone functions but the bands characteristic of a phthalic anhydride were absent. The presence of a 2H singlet at 4.85τ in the n.m.r. showed that the phthalide methylene group was intact. The n.m.r. spectrum was in accord with the hydroxylactone structure (105) and comparison with the spectra of the available diasteriomeric hydroxylactones (106) and (107)[119] showed that the product had the erythro configuration.

The oxidation was repeated using a much larger excess of the permanganate reagent, affording a mixture of compounds from which the anhydride (104) could be separated by crystallisation from ether (13%). The melting point of this compound was depressed on admixture



(108) R = H

(109) R = Me

with a sample of (105) but the compounds could not be distinguished by analytical t.l.c. The i.r. (KBr) of the anhydride (104) showed bands at 3560, 3460, 1840 and 1770 $cm.^{-1}$ in agreement with the values reported[116]. Confirmatory evidence for this structure was provided by the absence in the n.m.r. of the resonance characteristic of a phthalide methylene group and the low value of the aryl methyl group resonance (Table 2:1) which suggests that it is deshielded by an ortho carbonyl group.

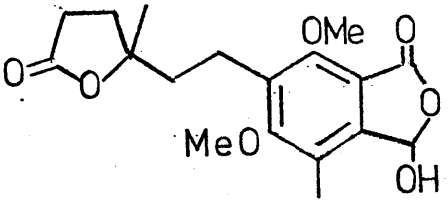
Subsequent attempts to increase the yield of the anhydride by varying the conditions of the reaction afforded mixtures of the hydroxylactone (105), the anhydride (104) and several more polar products, from which the anhydride could not be readily separated. In order to avoid possible side reactions involved in oxidation the side chain the oxidation was repeated using the methyl ether of the lactone (108).

The lactone (108), prepared in almost quantitative yield by treatment of mycophenolic acid with trifluoroacetic acid[29] or boron trifluoride etherate, was methylated with diazomethane to give the corresponding methyl ether (109, 96%). The failure of this compound to give a stain with ferric chloride (t.l.c.) and the presence in its n.m.r. spectrum of an additional 3H singlet at 5.90 τ are consistent with this structure.

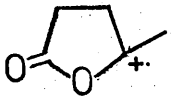
Treatment of the lactone (109) with an excess of alkaline permanganate, under the conditions which had been found most effective in the oxidation of O-methylmycophenolic acid (96), yielded, in addition to starting material, a mixture of polar compounds. These

Table 2:1 Chemical shifts (τ) of protons in the oxidation products of (96) and (109)

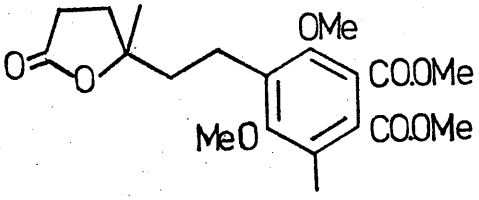
Compound	Ar.$\underline{CH_2}$O-	Ar.$\underline{CH}$(OH)O-	Ar$\underline{Me}$	Ar(O$\underline{Me}$)$_2$
(105)	4.85	--	7.80	5.92; 6.16
(104)	--	--	7.45	5.82; 6.16
(110)	--	3.52	7.68	5.92; 6.19
(112)	--	--	7.66	6.03; 6.19



(110)



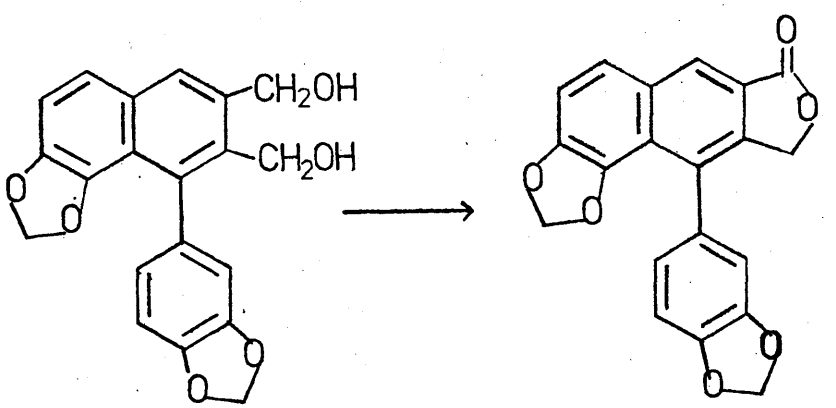
(111)



(112)

were treated with ethereal diazomethane and the two major products isolated by preparative t.l.c. The oily major product (20%), the psuedo acid (110), showed hydroxyl absorption at 3400 $cm.^{-1}$ in the i.r. but lacked the bands characteristic of an anhydride. Also, in place of the resonance characteristic of a phthalide methylene group, the n.m.r. showed a broad 1H singlet at 3.52τ which sharpened on treatment with D_2O. The low frequency of this proton can be attributed to the deshielding of a benzylic proton by the two oxygen functions. The aryl methyl group resonated at a slightly lower value than that of the lactone (109) in keeping with lone pair deshielding by an ortho carbinol substituent. Evidence that the remainder of the molecule was unchanged was provided by the mass spectrum in which the base peak ($^m/e$ 99) corresponds to the fragment (111), and by the upper region of the n.m.r. spectrum which is almost identical to that of the starting material (109).

The second product, the dimethyl phthalate (112), was isolated in 5% yield as a colourless oil. In the i.r. (CCl_4) bands at 1780 and 1741 $cm.^{-1}$ showed the presence of both γ-lactone and ester groups, while the presence of two carbomethoxyl groups was shown by two additional 3H singlets at 6.03τ and 6.11τ in the n.m.r. The aryl methyl group is apparently deshielded to a smaller extent in this compound than the corresponding group in the anhydride (104) (Table 2:1) presumably since the ortho carbomethoxyl group is free to rotate out of the plane of the aromatic ring. Attempts to improve the yield of the phthalate (112) at the expense of the pseudo acid (110) by increasing the concentration of the reagent and prolonging the reaction



(113)

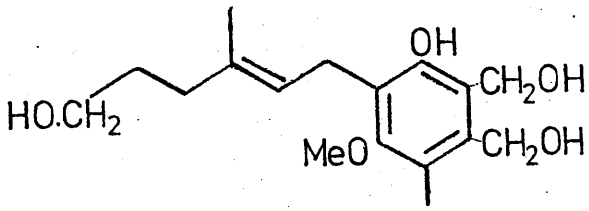
(114)

time were unsuccessful.

The difficulty experienced, as above, in converting phthalides to phthalic acid derivatives can be attributed partly to the difficulty of opening the phthalide ring and partly to steric limitations imposed on the approach of an oxidising agent by the bulk of the groups ortho to the hydroxymethyl group in the ring opened compound. It was thought that if the phthalide system were reduced to the corresponding 1,4 diol, the hydroxymethyl group ortho to the aryl methyl group might be more accessible to oxidising agents than in the above system.

The oxidation of 1,4 diols has been reported to yield the corresponding γ-lactones in high yield, except in cases where the configuration prohibits cyclisation[123]. In the case of a phthalol no such restriction exists and the product is the corresponding phthalide. In non-symmetric systems where there is no difference in the bulk of the substituents ortho to the hydroxymethyl groups, or in the activation of these groups, a 1:1 mixture of the isomeric phthalides would be produced. Otherwise it would be expected that the major product would be that in which oxidation of the least hindered alcohol has occurred. An extreme example of this has been reported in the oxidation of the phthalol (113) with silver carbonate/celite which yielded helioxanthin (114) exclusively, due to the restriction to the approach of the reagent to the hydroxymethyl group ortho to the bulky methylenedioxyphenyl substituent[120].

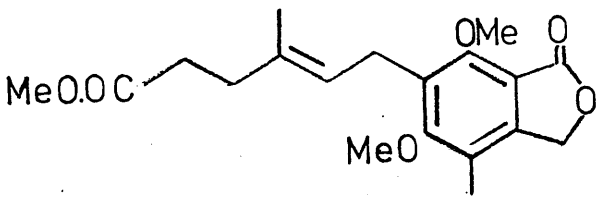
If mycophenolic acid or a suitable derivative could be efficiently reduced to a phthalol, it was thought that subsequent oxidation might afford the 3,5 dioxygenated phthalide in workable overall yield.



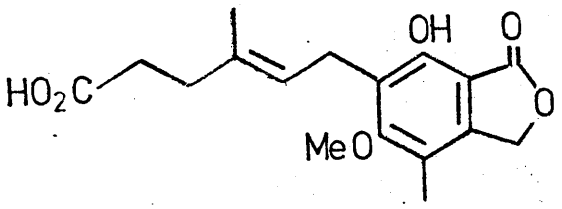
(115)

Preliminary attempts to reduce mycophenolic acid with lithium aluminium hydride in ether afforded a mixture of compounds, none of which was produced in sufficient yield to merit further investigation. However, when the reduction was carried out in tetrahydrofuran, analytical t.l.c. showed the presence of one major phenolic product (21%) which was more polar than mycophenolic acid. Purification of this compound by preparative t.l.c. was complicated by the difficulty of recovery from silica gel. The n.m.r. spectrum showed a 4H multiplet at 5.4τ and a 2H multiplet at 6.6τ which could be ascribed to the presence of two aryl and one alkyl hydroxymethyl groups. The phenolic nature of the compound was shown by a positive ferric chloride reaction (t.l.c.) and the presence of the double bond was suggested by a 1H multiplet at 4.8τ in the n.m.r. Integration of the upper region of the spectrum before and after treatment with D_2O indicated the presence of three hydroxylic protons. Intense absorption at 3500 $cm.^{-1}$ in the i.r. (liquid film) confirmed the polyol nature of the compound, absorption corresponding to carbonyl functions being absent. On the basis of the above the product can be formulated as the phenolic triol (115). In the light of the following work no attempt was made to pursue the investigation of this compound.

Although the successful reduction of phenolic phthalides with metal hydrides have been reported[121], most of the examples in which high yields have been obtained, are of mono- or di- substituted phthalides. Poor yields can be expected where the phenolate complex is sparingly soluble under the reaction conditions. For this reason it was thought that improved yields might be obtained if the reduction



(117)



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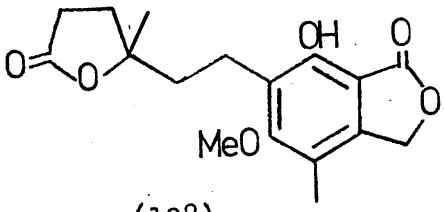

(108)

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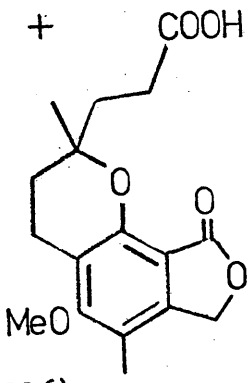
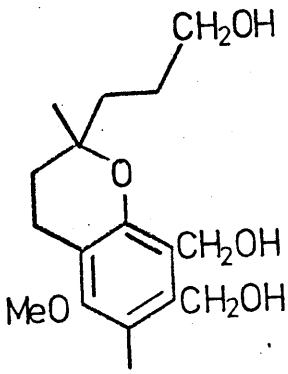


(116)

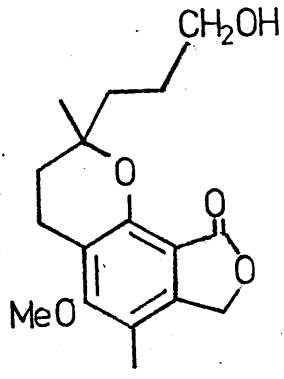


(118)

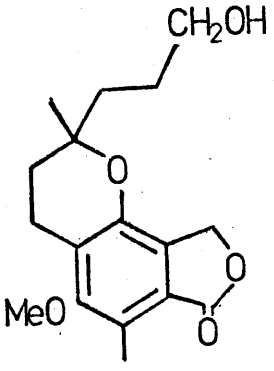
were carried out on derivatives of mycophenolic acid in which the phenolic group was protected. Mycochromanic acid (116) and methyl O-methylmycophenolate (117) were chosen as starting materials in view of the high yields in which they can be prepared from mycophenolic acid.

The conversion of mycophenolic acid to mycochromanic acid in a refluxing solution of acetic acid and sulphuric acid has been reported to proceed in high yield, but the exact proportions of the acids were not recorded[29]. In order to find the optimum conditions a series of small scale reactions was carried out using varying proportions of sulphuric acid and acetic acid as solvents, and these were monitored by analytical t.l.c. In most cases both mycochromanic acid (116) and the lactone (108) were formed, but the optimum yield of the former was achieved where 3% sulphuric acid in glacial acetic acid was used. On a preparative scale this procedure afforded mycochromanic acid (90%) and the lactone (108, 5%), the yields of which were not noticeably affected by prolonged reaction. However the lactone (108) could be converted to mycochromanic acid (in 50% yield) under the same conditions, perhaps suggesting that there is a degree of equilibrium between the two isomers.

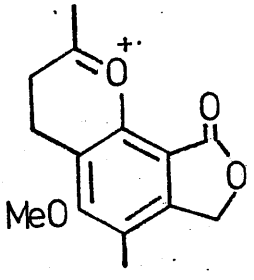
Reduction of mycochromanic acid with lithium aluminium hydride in refluxing tetrahydrofuran afforded the triol (118), a viscous oil, as the sole product (98%). This showed a strong peak at 3500 $cm.^{-1}$ in the i.r. (liquid film) suggesting the presence of hydroxyl groups and lacked significant absorption in the carbonyl region. In the n.m.r. spectrum a 4H multiplet at 5.39τ and a 2H multiplet between



(119)



(120)



(121)

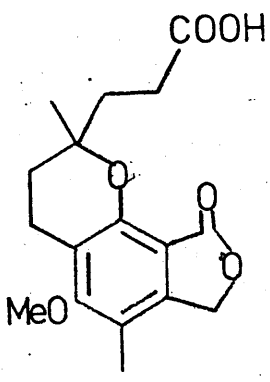
7.2 and 7.5τ suggested the presence of two aryl and one alkyl hydroxymethyl groups.

The n.m.r. spectrum of the corresponding triacetate (i.r. bands at 1747 and 1241 $cm.^{-1}$) showed the expected singlets at 7.97τ(3H) and 7.98τ(6H), the protons geminal to the acetoxyl groups appearing as 2H singlets at 4.80 and 4.81τ and as a 2H doublet (J = 5.5 Hz.) at 5.92τ.

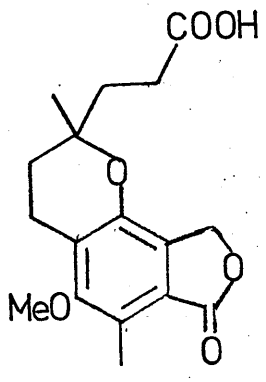
The triol (118) was oxidised by stirring with "active" manganese dioxide in acetone under nitrogen for 48 hr., to give a mixture of the two isomeric alcohols (119, 20%) and 120, 30%) which were separated by preparative t.l.c. The minor product (119) was crystalline, but the major product (120) was isolated as a colourless oil. Both products showed bands in the i.r. ($CHCl_3$) corresponding to hydroxyl and phthalide carbonyl groups (3620 and 1755 $cm.^{-1}$; 3525 and 1760 $cm.^{-1}$). The mass spectra of each of these compounds showed a molecular ion at $^{m}/_{e}$ 306, an ion at $^{m}/_{e}$ 288, corresponding to loss of water, and an abundant (98% and 67%) ion at $^{m}/_{e}$ 247. An ion of the latter $^{m}/_{e}$ is found in the spectra of mycochromanic acid and its methyl ester, probably due to the fragment (121).

In the n.m.r. spectrum of the alcohol (119) the chemical shift of the aryl methyl group (7.83τ) is close to that found in the spectra of mycochromanic acid and its methyl ester, confirming assignment as the 2,4 dioxygenated isomer, while in the spectrum of (120), the aryl methyl resonance appears as 7.50τ due to the deshielding effect of the ortho carbonyl group.

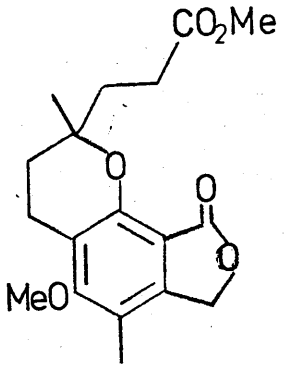
Oxidation of the triol (118) with silver carbonate/celite in



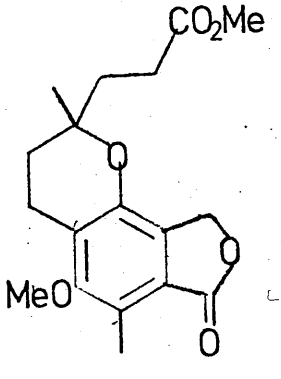
(122)



(124)



(123)

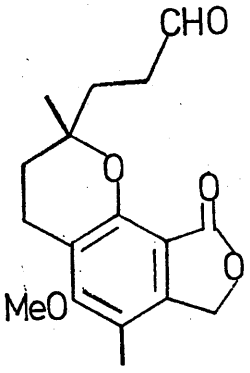


(125)

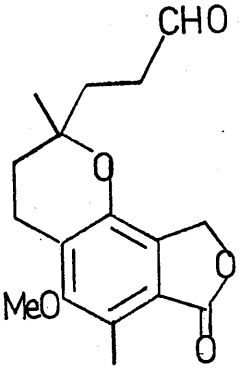
refluxing benzene afforded a mixture of the alcohols (119) and (120) in lower overall yield. (16% and 24% respectively.) However the specificity of oxidation with this reagent appears identical to that of manganese dioxide, i.e. $^{(120)}/_{(119)} = {}^{3}/_{2}$.

Treatment of an acetone solution of the triol (118) with an excess of Jones' reagent gave a mixture of two polar compounds, presumably the acids (122) and (124), which could not be completely separated by analytical t.l.c. The corresponding methyl esters formed by treatment of the mixture with ethereal diazomethane were, however, separable and were obtained as oils (123, 20% and 125, 19%) by preparative t.l.c. The presence of a carbomethoxyl function was indicated in each case by a band at 1740 $cm.^{-1}$ in the i.r. and a 3H singlet at 6.35τ in the n.m.r. The remainder of the n.m.r. spectra were similar in appearance to that of mycochromanic acid with the exception of the aryl methyl group in the spectrum of the 3,5 dioxygenated phthalide (125) which appeared at lower field (7.46τ). The 2,4 dioxygenated isomer (123) was identical (t.l.c., i.r., n.m.r.) with a sample prepared by treatment of mycochromanic acid with diazomethane.

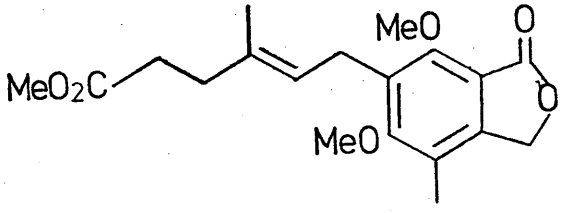
The course of this oxidation, with Jones' reagent at $0^{\circ}C$, was followed by t.l.c. examination of aliquots withdrawn at 30 sec. intervals, the reaction being quenched in each case by addition of ice water. Four intermediates were detected in this way. Two compounds corresponding in Rf. values and staining properties with the alcohols (119) and (120) were formed almost immediately together with small quantities of two less polar compounds. On a preparative scale work



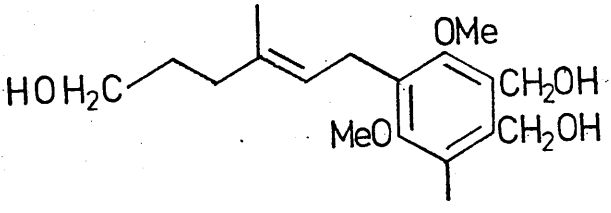
(126)



(127)



(117)

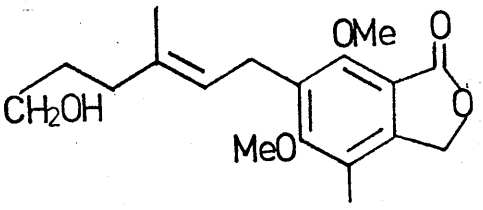


(128)

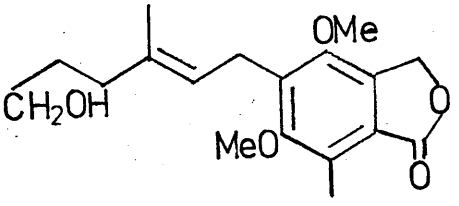
up after oxidation for 30 sec. afforded the alcohols (119, 9%) and (120, 12%) together with two compounds whose i.r. spectra were very similar. Neither showed significant absorption in the hydroxyl region but both showed a maximum at 1726 $cm.^{-1}$ which, together with weak maxima at 2720 $cm.^{-1}$ and 2715 $cm.^{-1}$ respectively, suggested the presence of a formyl grouping. The compounds are probably the aldehydes (126) and (127) produced by oxidation of the corresponding primary alcohols. From the relationship found between the phthalide carbonyl stretching frequencies of the carbomethoxy compounds (123) and (125) the values in this case, 1770 $cm.^{-1}$ and 1760 $cm.^{-1}$ allow tentative assignment of structures (126) and (127), respectively, to the more and less polar compounds.

In the above oxidations the desired selectivity was achieved with manganese dioxide (and silver carbonate/celite) but the low overall yield of the alcohol (120) and the fact that neither this compound nor the triol (118) could be obtained in a crystalline form are weak points in this particular degradative scheme. Preliminary attempts to obtain crystalline derivatives of these compounds were unsuccessful and in view of the work described subsequently these were not further investigated.

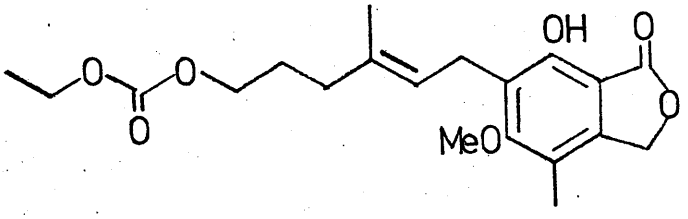
Reduction of methyl O-methylmycophenolate (117) with lithium aluminium hydride in tetrahydrofuran afforded the crystalline triol (128) in 72% yield. Again the absence of absorption in the carbonyl region and a strong peak at 3500 $cm.^{-1}$ in the i.r. indicated that complete reduction of the carbonyl functions to the corresponding alcohols had been accomplished. This was confirmed by the appearance



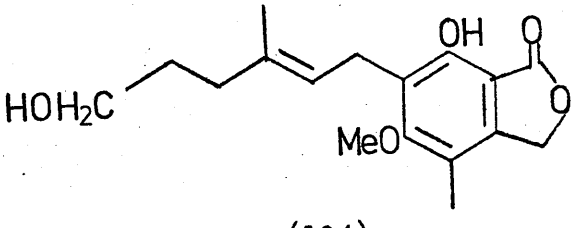
(131)



(132)



(133)



(134)

in the n.m.r. of two 2H singlets at 5.30 and 5.33τ indicative of the presence of two aryl hydroxymethyl groups and a 2H triplet (J = 6 Hz.) at 6.53τ corresponding to the presence of a terminal hydroxymethyl grouping in the side chain. In agreement with the structure (118) the n.m.r. spectrum showed a 1H triplet (J = 7 Hz.) corresponding to a single olefinic proton.

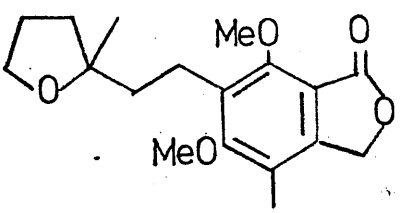
Oxidation of the triol (128) with freshly prepared "active" manganese dioxide in chloroform gave a mixture of two compounds which could not however be separated by preparative t.l.c. In the i.r. (liquid film) of the mixture a broad peak at 1750 $cm.^{-1}$ suggested that oxidation of the phthalol system to the corresponding phthalides had occurred, but the presence of a peak at 3450 $cm.^{-1}$ indicated that the aliphatic primary alcohol was intact. It could be inferred from the appearance of the n.m.r. spectrum of the mixture that the two products were the isomeric phthalides (131) and (132).

In order to establish whether the phthalide (131) was indeed a product of the above oxidation an unambiguous synthesis of this compound was carried out as follows. The mixed anhydride prepared from mycophenolic acid and ethyl chloroformate was reduced with sodium borohydride in aqueous tetrahydrofuran affording the carbonate (133) in high yield. Hydrolysis of the carbonate with aqueous sodium hydroxide gave the alcohol (134). The presence of a terminal hydroxymethyl group in the side chain of this compound was reflected by the presence in the n.m.r. of a 2H triplet (J = 6 Hz.) at 6.35τ

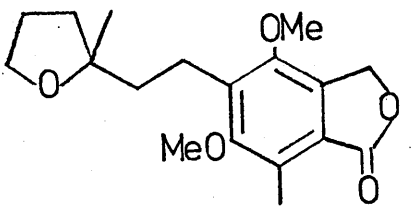
and by a single carbonyl band in the i.r. ($CHCl_3$) at 1742 $cm.^{-1}$, characteristic of a phthalide carbonyl group ortho to a free phenolic hydroxyl group (cf. 1-hydroxyphthalide[122] ν_{CO} ($CHCl_3$): 1738 $cm.^{-1}$). Methylation of the alcohol (134) with ethereal diazomethane yielded the methyl ether (131).

Comparison of the n.m.r. spectrum of (131) with that of the mixture obtained by oxidation of the triol (128) showed that this compound was the minor component. On the basis of its lower aryl methyl group resonance (7.47τ) the other component can be assigned the structure (132). Integration of the singlets at 7.81τ and 7.47τ (the aryl methyl group resonances of (131) and (132) respectively) showed that the selectivity of oxidation was greater in this case $(132)/_{(131)} = 2/_1$ than in the oxidation of the triol (118). This might be predicted on the basis of the greater steric bulk of a methoxyl substituent as compared with that of the ring residue in the corresponding position in the triol (118).

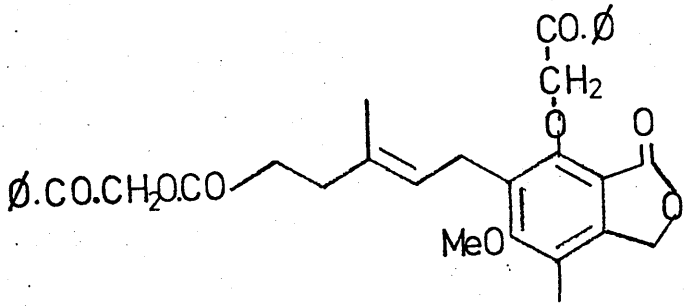
An effort was made to separate these compounds via their 6′-acetoxy derivatives, but these were even less distinguishable by analytical t.l.c. An attempt to selectively remove the methoxyl group ortho to the phthalide carbonyl group in (131) with "magnesium iodide etherate" gave a mixture of two less polar compounds, which were indistinguishable by t.l.c. The i.r. spectrum (liquid film) of the mixture showed no absorption in the region 3000 - 3600 $cm.^{-1}$ indicating that the hydroxyl group was no longer present, while in the n.m.r. the absence of olefinic resonances and the high value (8.66τ) of the alkyl methyl group resonance (c.f. the spectrum of



(129)



(130)



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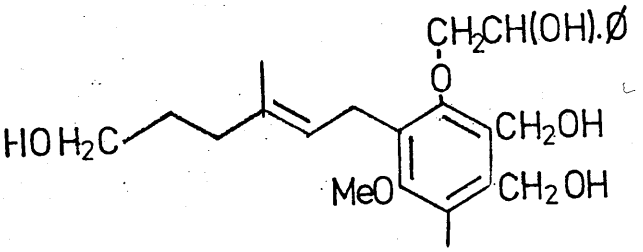
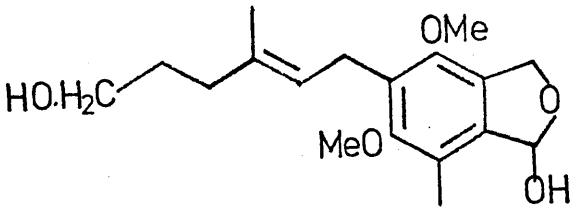


(136)

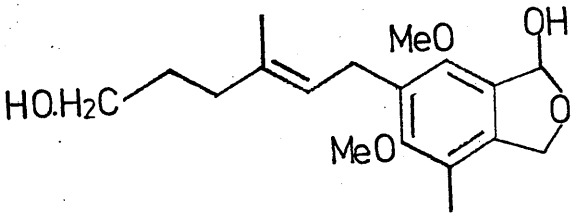
the lactone (108) in which the C-Me group resonates at 8.57) indicated that cyclisation to the corresponding tetrahydrofurans (129, 130) had occurred. This cyclisation is analogous to the lactonisation of mycophenolic acid in the presence of Lewis acids described earlier.

Since the selectivity in the oxidation of the triol (128) was greater than that of the triol (118), attempts were made to prepare the tetrahydropyranyl, methoxy methyl and ^{t}butyl ethers of mycophenolic acid by recognised procedures. In each case very low yields of the desired product was obtained, which is understandable in view of the lowered reactivity of the phenolic hydroxyl due to hydrogen bonding with the adjacent carbonyl group and the steric limitations imposed by the bulk of the ortho alkyl substituent. It was possible to prepare the di-p-bromophenacyl derivative (135) in high yield, however, by reaction of the dipotassium salt of mycophenolic acid with p-bromophenacylbromide in refluxing acetone, but the low yield of the tetrol (136) obtained on reduction made this route impractical.

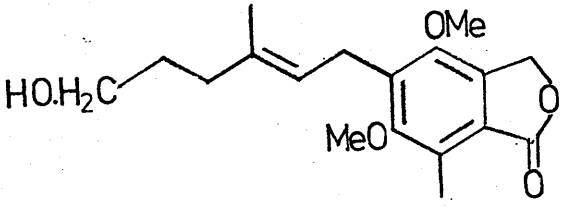
Reinvestigation of the oxidation of the triol (128) using "active" manganese dioxide which had been stored in the laboratory for twelve months, showed that oxidation with this reagent proceeded much more slowly than that with freshly prepared material. Complete oxidation over a period of three days gave a 9:1 mixture of (132) and (131) in an overall yield of 95% but the major product could still not separated from its isomer. In the course of monitoring this reaction by analytical t.l.c. the intermediacy of a compound slightly less polar than the starting material was observed. When the oxidation was stopped after 5 hr. (at which time none of the starting material



(137)



(138)



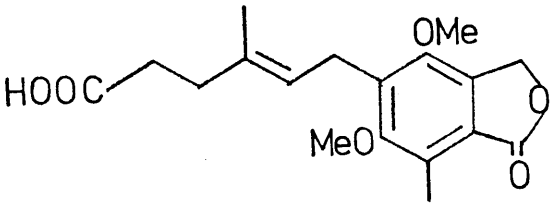
(132)

was left) separation of the mixture by preparative t.l.c. afforded a mixture of the phthalides (131) and (132) (5%) and the more polar product, which was shown to be the hemiacetal (137, 82%).

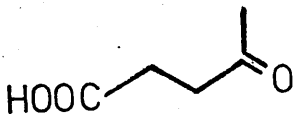
The presence of the hemiacetal function was evident from the n.m.r. spectrum in which the methine proton appeared as a 1H singlet at 3.6τ whose broadening was shown to be due to coupling to the ortho oxymethylene protons (a broad 2H singlet at 4.8τ) and also to a hydroxyl proton (an exchangeable signal at ca. 8τ). The position of the hydroxyl group was also reflected in the low value of the aryl methyl resonance (7.72τ) and the similar chemical shifts of the methoxyl groups (6.24τ and 6.28τ). The remainder of the n.m.r. spectrum was virtually identical to that of the phthalide (131).

Since the n.m.r. spectrum of the mixture of phthalides formed in the foregoing reaction showed that these were produced in the ratio $^{(132)}/_{(131)} = {}^{1}/_{5}$, and since only the hemiacetal (137) was detected, oxidation of the isomeric hemiacetal (138) is evidently much faster, perhaps because the activity of the hemiacetal hydroxyl group is enhanced by hydrogen bonding with the ortho methoxyl group.

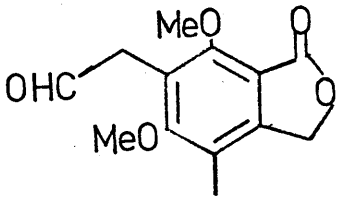
The hemiacetal (137) was a fairly unstable compound, decomposing under acidic and basic conditions. However, it was smoothly oxidised to the phthalide (132, 95%) by prolonged treatment with manganese dioxide. The identity of this compound was evident from the presence in the i.r. ($CHCl_3$) of a band at 1760 $cm.^{-1}$ characteristic of the phthalide carbonyl group, and the low value of the aryl methyl group resonance (7.47τ) in the n.m.r. The phthalide (132) could not, however, be obtained in a crystalline form. Oxidation of this compound with



(139)



(98)



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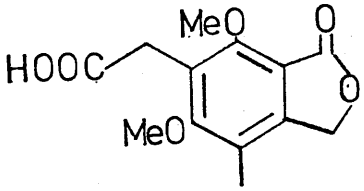

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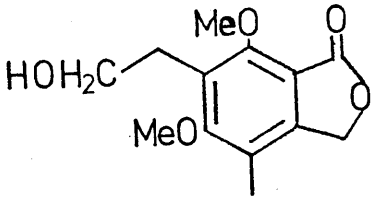

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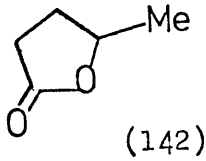


(142)

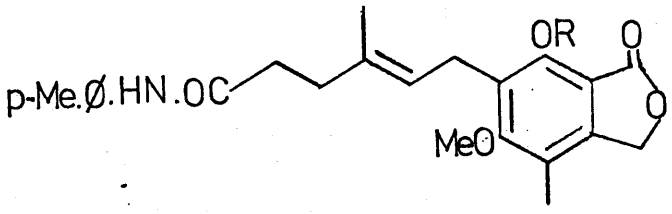
Jones' reagent, however, afforded the corresponding crystalline acid (139) in high yield.

In view of the high overall yield of the acid (139) from the triol (128) (47%) and the crystallinity of both these compounds, this degradative scheme was subsequently used for the estimation of the amount of tritium located in the phthalide methylene group.

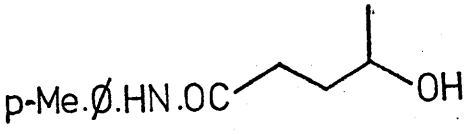
(ii) Cleavage of the terpenoid side chain

The ozonolysis of O-methylmycophenolic acid in chloroform at room temperature and subsequent degradation of the ozonide with water, was reported by Raistrick et al.[102] to yield the aldehyde (97) and laevulinic acid (98) in high yield. In our hands, however, this procedure gave a mixture of the aldehyde (97) and the corresponding acid (140) in low yield, together with trace quantities of laevulinic acid, which was trapped as its 2,4 dinitrophenylhydrazone. No significant improvement was observed when the ozonolysis was carried out at lower temperatures. However, the ozonolysis of methyl O-methylmycophenolate (117) in ethyl acetate at -80°C and reduction of the ozonide with sodium borohydride in aqueous methanol afforded the alcohol (141) in fair yield. The other expected product, methyl 4-hydroxypentanoate, could not be detected by analytical t.l.c. In view of the large excess of sodium borohydride employed it is possible that reduction to pentan-1,4-diol or hydrolysis and cyclisation to the volatile γ-lactone (142) had occurred.

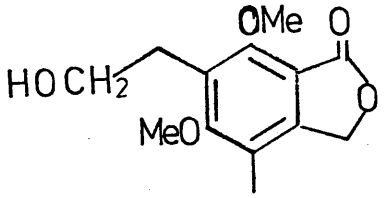
In order that the side chain fragment could be isolated, mycophenolic acid was converted to its p-toluidide (143, 90%) and methylated with



(143) R = H
(144) R = Me



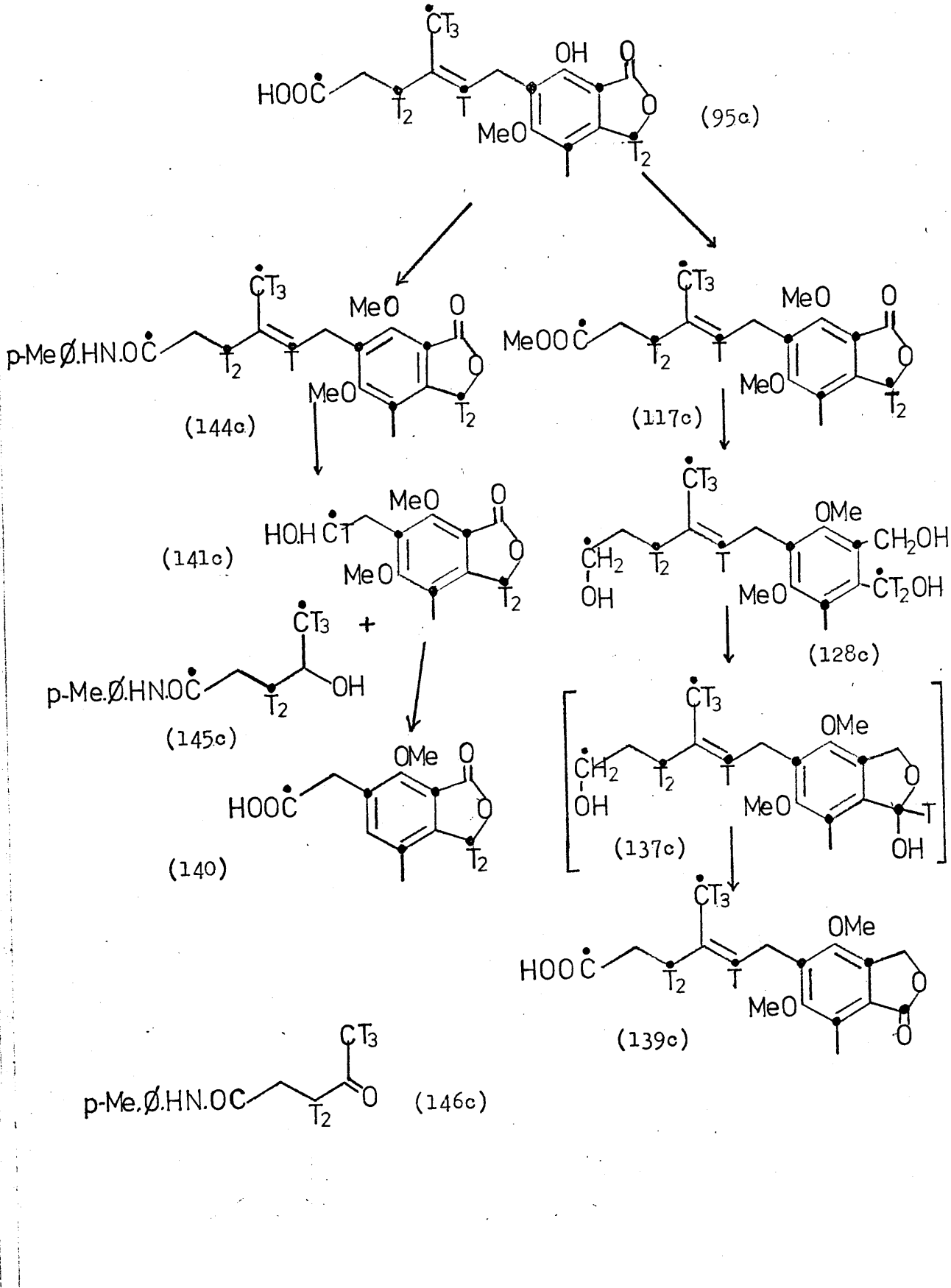
(145)



(141)

ethereal diazomethane yielding the corresponding methyl ether (144) in quantitative yield. Ozonolysis of this compound and subsequent reduction of the ozonide (as above) yielded the alcohol (141) and the amide (145) both in 30% yield. These compounds were easily separated by preparative t.l.c. and subsequent crystallisation. Oxidation of the alcohol (141) with an excess of Jones' reagent afforded the acid (140) in 70% yield.

Fig. 2:4 - Degradation of labelled mycophenolic acid (95c).



The incorporation of [2-T, 2-^{14}C] acetate into mycophenolic acid

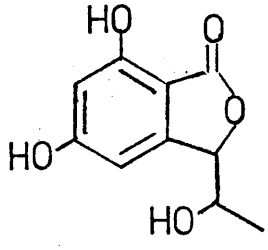
A strain of P. stoloniferum which was an efficient producer of mycophenolic acid was used in biosynthetic studies in preference to the strain of P. brevicompactum used for production of various other metabolites. Production of mycophenolic acid in shake flask cultures (in which there is a higher metabolite turnover than in surface cultures) was found to progress almost linearly from the third day of growth onwards (see Part II).

[2-T, 2-^{14}C] Sodium acetate (isotopic ratio $^{T}/_{14_C}$ (dpm) = $^{178}/_{1}$), was fed to two shake flask cultures late on the second day of growth and the cultures harvested on the fifth day. The crude acidic fraction of the material extracted with ethyl acetate was diluted with inactive mycophenolic acid and the labelled mycophenolic acid isolated and purified by repeated crystallisations. Difficulties were encountered in obtaining radiochemically pure material due to the presence of a small quantity of a highly active compound which was identified as orsellinic acid. However mycophenolic acid could be freed from this impurity by crystallisation after further dilution. Crystallisation to constant activity finally gave material with an isotopic ratio $^{T}/_{14_C}$ (dpm) of $^{55.6}/_{1}$ containing 0.34% of the total tritium, and 1.05% of the total ^{14}C of the fed acetate.

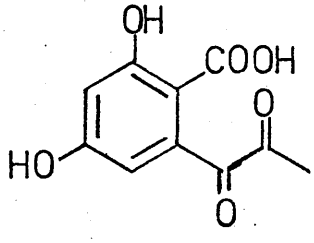
The degradations carried out on the labelled metabolite are summarised in Fig. 2:4 and the relative molar activities of the degradation products in Tables 2:2 and 2:3.

Ozonolysis and reduction of the ozonide of the p-toluidide methyl ether (144c) gave the alcohol (141c) which contained 59% of the total ^{14}C of mycophenolic acid, and the amide (145c) containing 36% of the total ^{14}C. Since the three labelled carbon atoms of the amide must be derived *via* mevalonate labelled in the 2,3 and 4 positions, the hydroxymethyl group of the alcohol (141c) must contain about 12% of the total ^{14}C activity. Hence the total incorporation of acetate into the isoprenoid moeity is almost identical (48%) with that into the polyketide nucleus (47%). This result is in agreement with that found by Birch *et al.*[114] in their experiments with [1-^{14}C] acetate. However, in a separate series of experiments the incorporation of [2-^{14}C] acetate into mycophenolic acid was reported to give preferential labelling of the terpenoid side chain[115].

The $^{T}/_{14_C}$ (dpm) ratio found for the amide (145c) ($^{73}/_{1}$) is lower than expected (5 tritium atoms derived from [2-T] acetate should give rise to a ratio of $\sim^{100}/_{1}$) but can be explained by the partial equilibration of the ketone (146c) during reduction with sodium borohydride. Oxidation of the alcohol (141c) to the corresponding acid resulted in the loss of 12% of the total tritium content, i.e. a drop in atomic ratio ($^{T}/_{14_C}$) from $^{3.4}/_{5}$ to $^{2.6}/_{5}$, corresponding to the loss of one tritium atom. This indicates that 32% of the total tritium content is located in the nucleus (a ratio of $^{2.6}/_{4}$). Transformation of mycophenolic acid to the acid (139c) was effected with loss of 28% of the total tritium activity indicating that all (or almost all) of the tritium incorporated into the aromatic nucleus is located in the phthalide methylene group. The $^{T}/_{14_C}$ (atomic)



(101)



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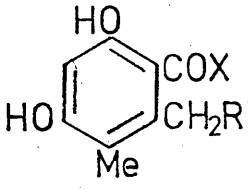

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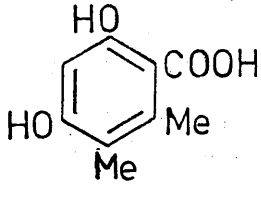


(147)

ratio of the polyketide derived moeity ($^{2.4}/_{4}$) is incompatible with the intermediacy of oxygenated compounds such as (101) and (102). While this result does not directly distinguish between a pentaketide intermediate (103, X = $-CO.CH_3$) and a tetraketide intermediate (103, X = H), the fact that the ratio is substantially higher than would be predicted for equal incorporation into each of the "acetate units" in the polyketide chain seems to be consistent with a starter effect. (ca. 130%). In view of the strong evidence which this provides for the implication of a tetraketide precursor the proposed experiment (p. 25) with [2-T, 2-^{14}C] malonate was not carried out. It has been shown subsequently that [1-^{14}C] 5-methylorsellinic acid (147) is specifically incorporated into mycophenolic acid[124, 126], and in the light of the above result this compound is probably the first aromatic precursor of the nucleus.

Table 2:2 [^{14}C,T] Mycophenolic acid from [2-^{14}C] acetate

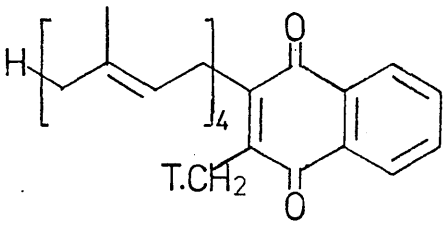
Compound	Dilution %	Wt. (mg.)	dpm. ^{14}C	dpm. T	dpm./mg. ^{14}C	dpm./mg. T	dpm./mg. (for a dilution of 1%) ^{14}C	dpm./mg. (for a dilution of 1%) T	dpm./mM. (for dilution of 1%) ^{14}C ($\times10^{-5}$)	dpm./mM. (for dilution of 1%) T ($\times10^{-5}$)	Ratio T/^{14}C dpm.	Ratio T/^{14}C atomic	^{14}C % of total	T % of total
mycophenolic acid (95c)	3.500	2.65 (x2)	8802	499161	3320	188840	391	22200	1.25	71.0	56.5		100%	100%
		0.90 (x3)	2955	158160	3280	176000	386	20700	1.24	66.3	53.7			
		1.10 (x4)	3622	199100	3310	181000	390	21300	1.25	68.2	56.5			
		0.85 (x5)	2818	156704	3320	184050	381	21620	1.25	69.7	55.5			
	0.760	2.02 (x2)	595	33875	295	16800	388	22100	1.24	70.7	57.0		100%	100%
		2.20 (x3)	683	37446	310	17000	408	22400	1.30	71.7	55.0			
		1.95 (x4)	574	32473	295	16700	388	21960	1.24	70.3	56.8			
		2.38 (x5)	717	38673	307	16250	395	21400	1.27	68.5	53.2			
	0.590	2.13 (x2)	505	27700	235	13150	398	22800	1.27	73.0	56.0		100%	100%
		4.28 (x3)	683	37508	228	12550	386	21300	1.24	68.2	55.0			
		1.83 (x4)	421	23055	230	12650	390	21500	1.25	68.8	55.0			
									1.25	69.5	55.6	0.936		

Table 2:3 Degradation of [^{14}C,T]mycophenolic acid

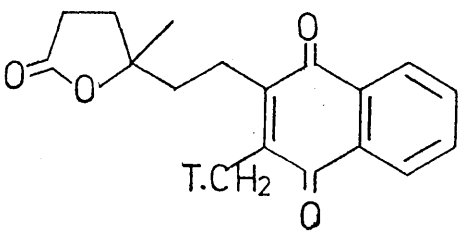
Compound	Dilution %	Wt. (mg.)	dpm. ^{14}C	dpm. T	dpm./mg. ^{14}C	dpm./mg. T	dpm./mg. (for a dilution of 1%) ^{14}C	dpm./mg. (for a dilution of 1%) T	dpm./mM. (for a dilution of 1%) ^{14}C ($\times 10^{-5}$)	dpm./mM. (for a dilution of 1%) T ($\times 10^{-5}$)	Ratio $^{T}/_{^{14}C}$ dpm.	Ratio $^{T}/_{^{14}C}$ atomic	^{14}C %	T %
(128c)	0.7600	1.31 (x2)	364	20740	278	15800	366	20800	1.19	67¼	57.0		97%	96%
		0.67 (x3)	192	20685	286	15950	376	21000	1.22	68.0	55.5			
	1.325	2.59 (x2)	1283	68925	495	26600	376	20200	1.22	65.5	53.6			
		2.80 (x3)	2378	74802	492	26800	373	20300	1.21	65.8	54.4			
									1.21	66.6	55.1	0.930		
(139c)	1.325	0.68 (x2)	323	13450	477	19900	362	15100	1.21	50.5	41.5		98%	72%
		0.37 (x3)	182	7260	489	19650	370	14900	1.23	49.8	40.0			
									1.22	50.2	40.8	0.685		
(141c)	0.590	1.57 (x3)	250	11024	159	7030	270	11900	0.67	30.0	44.0		59%	44%
		1.35 (x4)	215	9344	159	6920	270	11700	0.67	29.5	43.5			
		0.75 (x5)	126	5220	168	7000	285	11900	0.72	30.0	41.7			
									0.74	29.8	40.3	0.684		
(140c)	0.590	2.14 (x2)	280	8749	131	4050	222	6860	0.59	18.3	30.8		58%	32%
		1.98 (x3)	303	9335	152	5000	258	8500	0.72	22.6	32.8			
		0.66 (x4)	107	3208	163	5000	276	8500	0.74	22.6	30.6			
									0.73	22.6	31.0	0.520		
(145c)	0.590	1.50 (x3)	193	14350	129	9660	219	16400	0.45	33.0	75.0		36%	46%
		1.11 (x4)	138	9841	124	8850	211	15000	0.43	31.0	71.4			
		2.09 (x5)	281	21031	134	9660	227	16400	0.47	33.0	72.0			
									0.45	32.0	72.7	1.23		

DISCUSSION

PART 2



(148)



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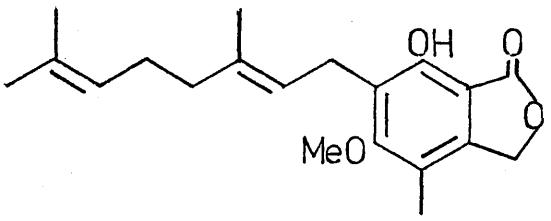

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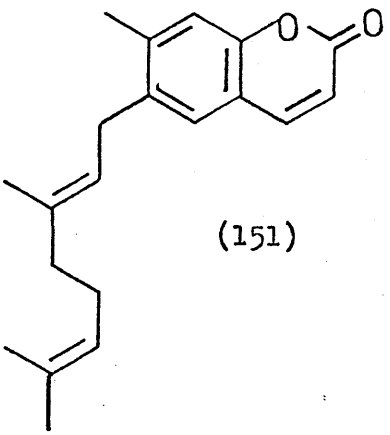

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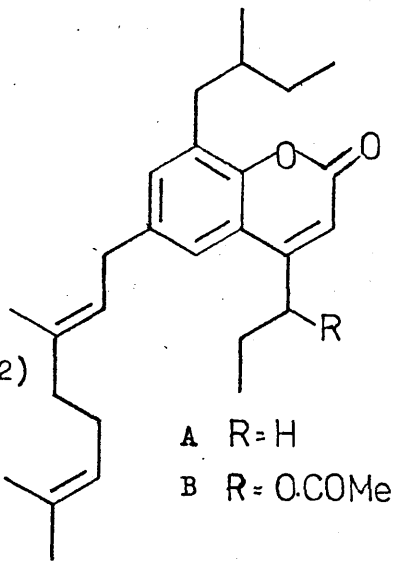
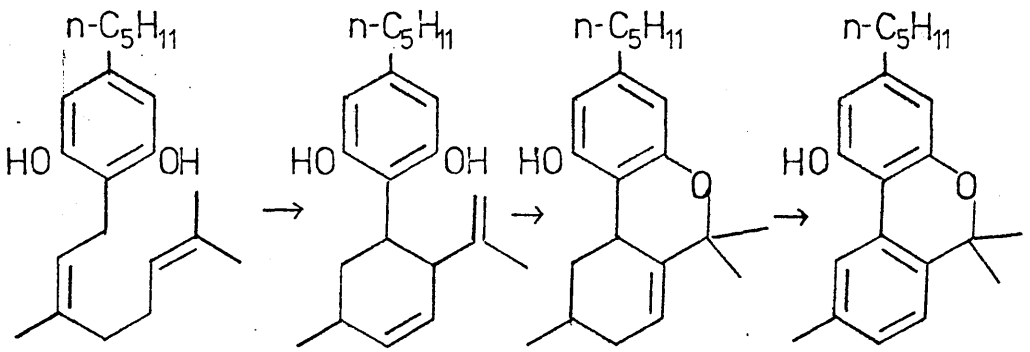


(152)

A R = H

B R = O.COMe

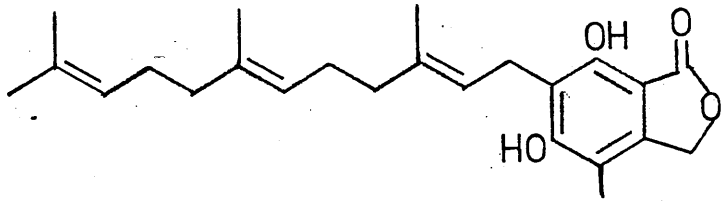


(153) (154) (155) (156)

The stage of biosynthesis at which the terpenoid side chain is attached to the nucleus, by analogy with the biosynthesis of Coenzyme Q* and the prenyl alkaloids (see p. 20) probably occurs after aromatisation, although it may or may not precede O-methylation or oxidation of the starter methyl group. The identity of the alkylating species is also in doubt, since the possibility of alkylation by a preformed C_7 unit cannot be entirely ignored, although by analogy with the biosynthetic relationship elucidated between the prenyl coumarins and the co-occurring furans[127,128], alkylation by a geranyl or higher prenyl unit and subsequent oxidative cleavage is more probable. The metabolic oxidative degradation sequence established for the menaquinone (148) giving the lactone (149) is analogous[129]. The isolation of [2-^{14}C] acetone and [4'-^{14}C] mycophenolic acid of virtually the same specific activities from a culture of P. brevicompactum fed with [2-^{14}C] mevalonic acid[130] implicates the latter hypothesis and has been generally accepted as an indication of the intermediacy of a geranyl precursor (150) although it does not exclude the possibility of more extensive oxidation of a longer side chain.

C-geranyl compounds are known in higher plants, notably the prenyl coumarins ostruthin (151)[131] and surangin A and B (152)[132], and the Cannabis phenols (154), (155) and (156) (which probably arise by cyclisation of the simple derivative (153))[133], but their occurrence

*Coenzyme Q9 was also isolated from the mycelium of P. brevicompactum (p. 72).



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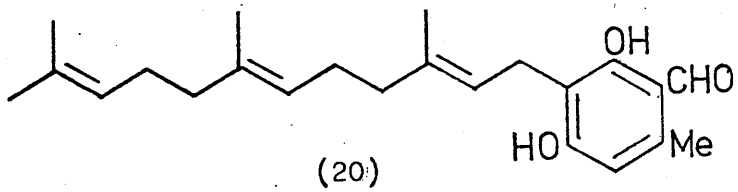

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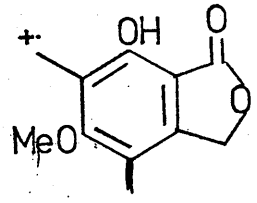


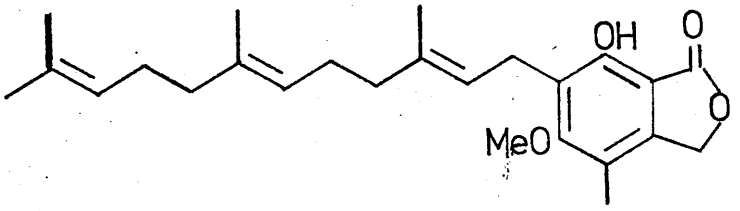
(158)

in fungi has not been reported*. Consequently the postulate of an intermediate with a geranyl side chain seems not entirely convincing. The isolation of the farnesyl compound (157) as a cometabolite of mycophenolic acid from the mycelium of submerged cultures of a strain of P. stoloniferum suggested that this compound might be a precursor of mycophenolic acid.

The structure (157) of the above metabolite was assigned by comparison of its n.m.r. spectrum with those of mycophenolic acid and the antibiotic (20)[31], and the all trans nature of the side chain inferred by comparison with the spectra of the isomeric methyl farnesoates[135] and farnesols[136]. The all trans structure (157) has subsequently been confirmed by synthesis[137].

T.l.c. examination of the light petroleum extract of the mycelia of these cultures showed the presence of another metabolite slightly less polar than the farnesyl compound (157) and present in smaller quantities but giving the same blue colouration with ferric chloride (t.l.c.) as the farnesyl compound (157) and mycophenolic acid. Fractionation of the extract by column chromatography and subsequent purification by preparative t.l.c. afforded the pure metabolite as a colourless oil. The molecular formula $C_{25}H_{34}O_4$ was assigned by high resolution mass spectroscopy. The base peak of the mass spectrum, corresponding to the fragment $(C_{11}H_{11}O_4)^+$ was indicative of the ion (158)

*Monoterpenes as a class are not nearly as common in fungal systems as in the higher plants. The only authentic examples known to the author are the geranyl side chain of mycelianamide (2) and the production of geraniol, linalool, citranellol and neral by the filamentous fungus Ceratocystis variospora[134].



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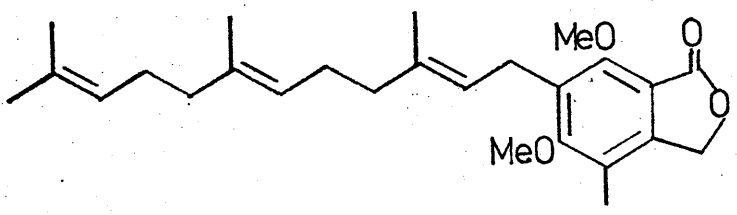


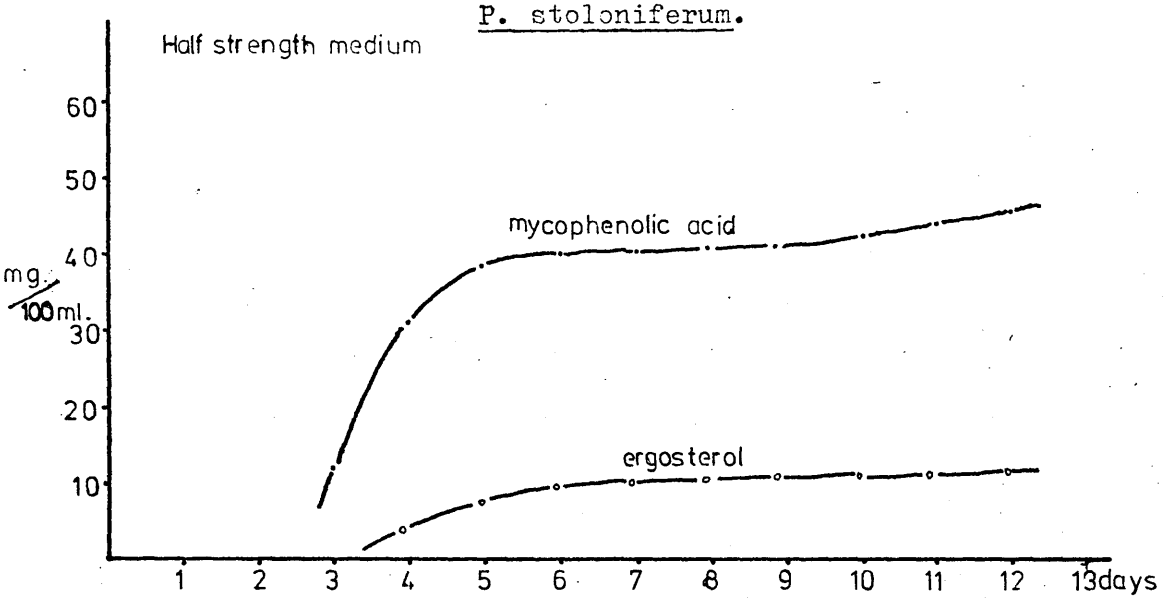
(160)

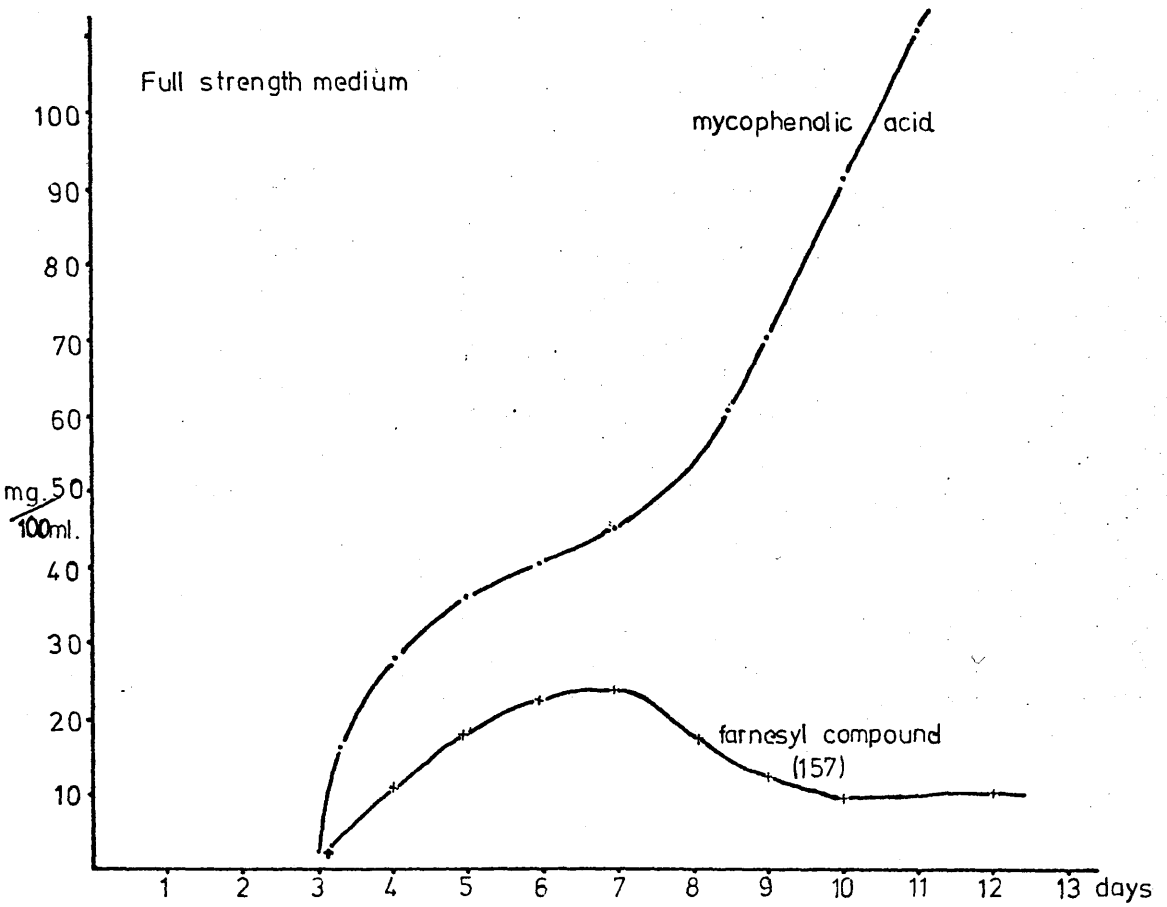
also found in the spectra of mycophenolic acid and its esters, and a fairly abundant ion $(C_{14}H_{23})^+$ (33%), were consistent with formulation as a farnesyl derivative. The overall similarity of the n.m.r. spectrum to that of the farnesyl compound (157), with the exception of an additional 3H singlet at 6.29τ was consistent with the structure (159). Unambiguous assignment of this structure was obtained by synthesis from the farnesyl compound (157), methylation of which with methyl iodide in the presence of anhydrous potassium carbonate afforded the corresponding O-methyl and O,O′-dimethylfarnesyl derivatives. The O-methylfarnesyl compound (159) was identical to the above metabolite (t.l.c., i.r., n.m.r.), while in the n.m.r. spectrum of the O,O′-dimethylfarnesyl compound (160) the lower chemical shift of the second methoxyl group protons (5.95τ) was consistent with a position ortho to the phthalide carbonyl group.

The presence of the metabolites (157) and (159) in cultures which afford mycophenolic acid is in itself consistent with a biosynthetic sequence involving prenylation, O-methylation and oxidative cleavage of the farnesyl side chain. In order to carry out the labelling experiments necessary to test this hypothetical scheme the growth of the mould under conditions more amenable to small scale experimentation had to be ascertained. Shake flask cultures were preferred to surface cultures since the former was taken to be nearer in type of growth to submerged cultures and to involve a faster metabolic turnover.

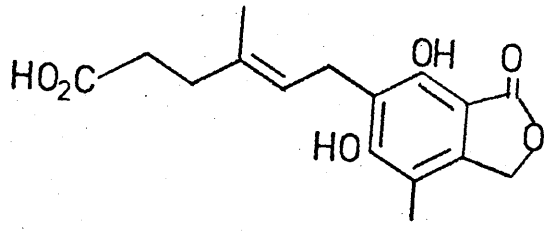
Analysis (by analytical t.l.c. and G.L.C. after silylation) of the extracts of shake flask cultures of P. stoloniferum grown on the

Fig. 2:8 - Production of metabolites by shake flask cultures of P. stoloniferum.





same medium as used in the submerged culture experiments (half strength medium) showed that neither of the farnesyl metabolites, (157) and (159), were produced in detectable quantities. These compounds were produced in substantial quantities however, when a medium of twice that concentration (full strength medium) was used. The metabolite concentrations vs. growth relationships for both media are reproduced graphically in Fig. 2:8.



(161)

The incorporation of labelled precursors into mycophenolic acid

An aqueous solution of DL-[$^{14}CH_3$, CT_3] methionine (50 μCi. and 1 mCi. respectively) was fed to a two day culture of P. stoloniferum grown on half strength medium and the culture harvested after a further two days. The labelled mycophenolic acid isolated from extracts of the culture possessed 26.6% of the total fed activity and had an isotopic ratio (atomic) of approximately 1, indicating that no exchange of tritium had taken place. This result is consistent with those of Lederer et al.[116] who showed that the CD_3 moeity of [CD_3] methionine was incorporated intact. Demethylation of the labelled mycophenolic acid using lithium iodide in collidine afforded O-desmethylmycophenolic acid (161) with half of the specific activity and an identical isotope ratio. Since C- and O- methylations would be expected to occur at different stages in the biosynthesis, this indicates that biosynthesis in this growth medium is extremely rapid. Labelled farnesyl compound (157) could not be detected by t.l.c. radioscan of the mycelial extracts.

Feeding of [$^{14}CH_3$] methionine (10 μCi.) to a culture grown on full strength medium, over the same period of time, led to the isolation of labelled mycophenolic acid (3.08×10^7 dpm./mM), farnesyl compound (157) (2.38×10^7 dpm./mM) and O-methylfarnesyl compound (159) (5.40×10^7 dpm./mM). The higher specific activities of the farnesyl derivatives (relative to the number of methionine derived carbon atoms) is consistent with a precursor relationship.

Reintroduction of the ^{14}C labelled farnesyl compound (0.297 μCi.) from the above culture into a two day old culture grown on full strength

Table 2:4 Incorporation of labelled precursors into mycophenolic acid

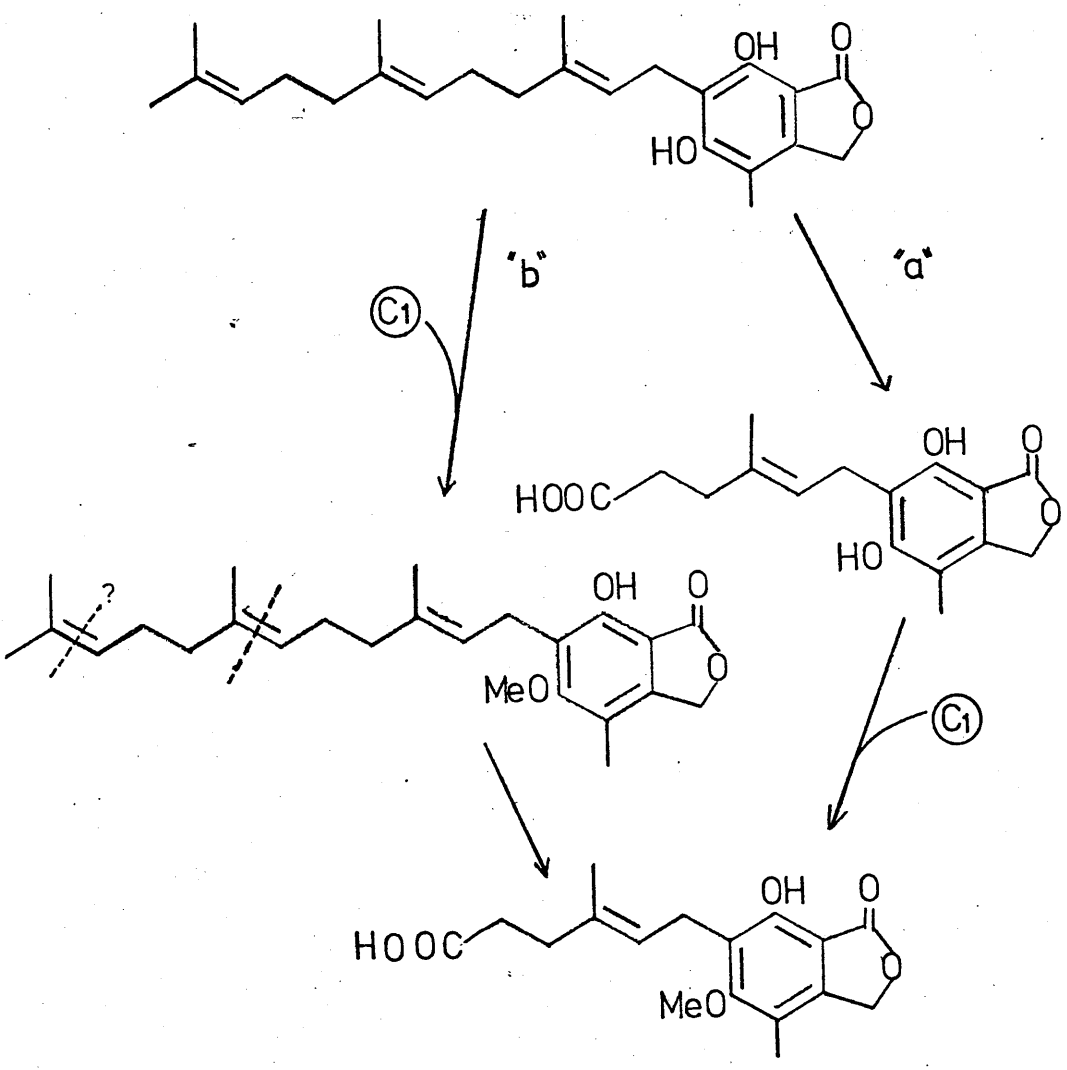
Precursor	Culture medium	Incorporation %	Molar incorporation %
Farnesyl compound (157)	full strength	7.5	9.0
O-methylfarnesyl compound (159)	full strength	11.8	14.7
	half strength	20.2	25.0
O-desmethylmycophenolic acid (161)	full strength	0.8	0.8

medium gave, after 24 hr., mycophenolic acid containing 4.5% of the fed label. However t.l.c. radioscan of the mycelial and culture filtrate neutral extracts showed that not all of the labelled farnesyl compound had been utilised and this was reisolated by preparative t.l.c. and introduced to a three day culture grown on the same medium. After a further four days, when t.l.c. radioscan of the culture filtrate showed that the farnesyl compound was essentially unlabelled, the contents of the flask were extracted giving mycophenolic acid containing 3% of the total administered label. On the basis of the total reisolated activity (other than that located in mycophenolic acid) the overall incorporation of the farnesyl compound (157) into mycophenolic acid was 7.5% (a molar incorporation of 9.0%).

The implication of the farnesyl compound (157) as a precursor means that O-methylation and cleavage of this side chain must occur after prenylation of the nucleus, but the order in which these processes occur is open to question. The presence of the O-methylfarnesyl compound (159) as a metabolite does suggest that methylation precedes cleavage but is not proof of this since methylation of the farnesyl compound is possibly a side reaction. Dilution of the acidic extracts of the above culture with inactive O-desmethylmycophenolic acid (161) and reisolation afforded material containing 0.05% of the administered label, indicating that desmethylmycophenolic acid might be a high activity precursor present in very small amounts.

In order to test this, desmethylmycophenolic acid prepared by demethylation of mycophenolic acid derived from [$^{14}CH_3$, CT_3] methionine (above) was pulse fed as its sodium salt (0.347 μCi.) to a two day

Fig. 2:5.



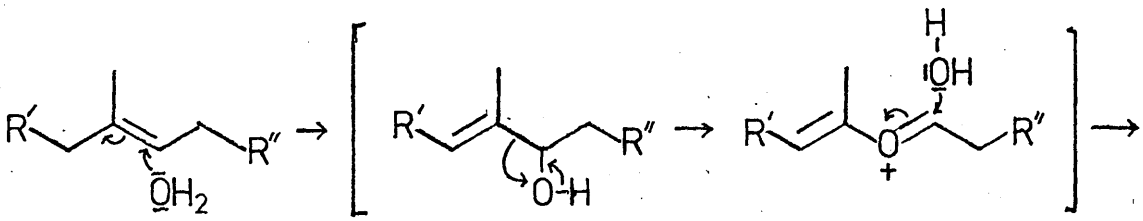
culture grown on full strength medium over 24 hr. Mycophenolic acid isolated from this culture after a further three days growth possessed only 0.8% of the administered label, showing that O-desmethylmycophenolic acid is not such an efficient precursor as the farnesyl compound.

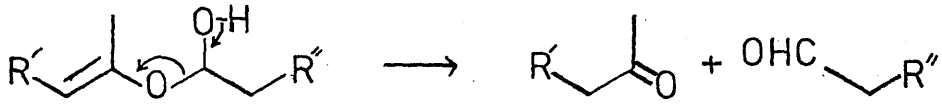
The yields of the O-methylfarnesyl compound (159) from shake flask cultures were too low to permit use of biological labelling as a method for preparation of radioactive material for precursor studies, but material of high specific activity was prepared by methylation of inactive farnesyl compound with [^{14}C] methyl iodide (in 50% radiochemical yield). Methoxyl labelled O-methylfarnesyl compound (2.16 μCi.) prepared in this way was fed to a two day old culture of P. stoloniferum cultivated on full strength medium. Harvesting the culture after a further three days gave mycophenolic acid containing 11.8% of the introduced label (14.5% molar incorporation). The higher incorporation of the O-methylfarnesyl compound (159) into mycophenolic acid than that of the farnesyl compound (157) is indicative of a biosynthetic sequence in which oxidative cleavage of the side chain is the last step.

Feeding of the labelled O-methylfarnesyl compound (159) (1.81 μCi.) to cultures grown on half strength medium, over the same period of growth as above, led to the isolation of mycophenolic acid with a higher activity (25% molar incorporation). In view of the higher rate of production of mycophenolic acid in cultures grown on this medium, a higher incorporation of precursor is not unexpected.

The above results are clearly consistent with a major pathway

Fig. 2:6 - Possible mechanisms for cleavage of the farnesyl side chain.





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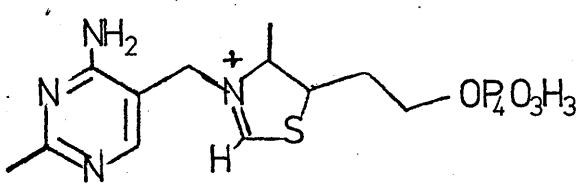

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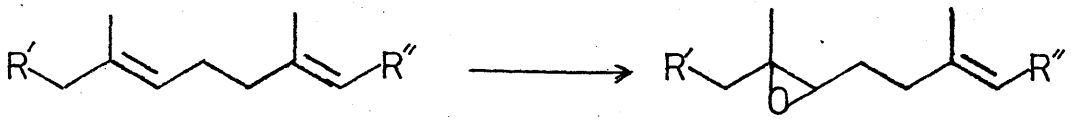
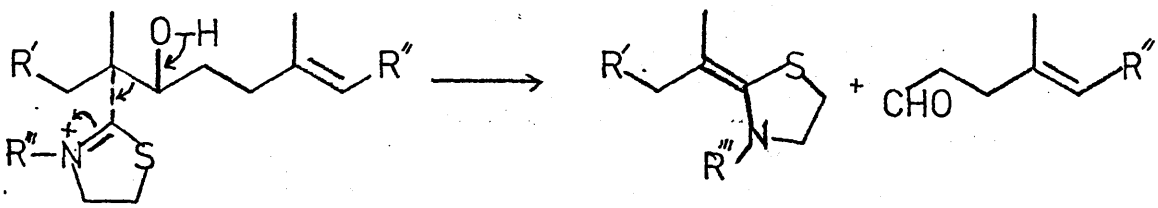
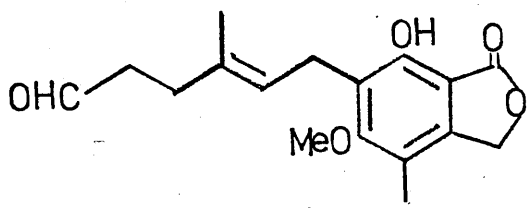




from the farnesyl compound (157) via the O-methylfarnesyl compound (159) (Fig. 2:5'a'), although the low incorporation of the farnesyl compound (157) into O-desmethylmycophenolic acid (161) and the incorporation of this compound into mycophenolic acid (Fig. 2:5'b') affords some evidence for the operation of two alternate pathways. The utilisation of added O-desmethylmycophenolic acid (161) as a precursor, relative to that of the O-methylfarnesyl compound (159), may however be due to a permeability effect and is possibly not entirely representative of the process in vivo.

Various possibilities exist for the mechanism of double bond cleavage, the intermediacy of an epoxide seems most likely although hydroperoxidation and subsequent biological Baeyer-Villiger type cleavage[138] to the corresponding hemiacetal (162) is possible (Fig. 2:6). Cleavage of an epoxide, in the absence of neighbouring group participation probably involves thiamine pyrophosphate (TH) (163) as a cofactor. Addition of TH across the epoxide followed by retro-aldol fission of the C-C bond would afford the aldehyde (164) (Fig. 2:6). Since the aldehyde of mycophenolic acid is oxidised to the acid on exposure to air, it would not be detected as an intermediate.

It is unknown whether the terminal double bond of the farnesyl side chain is also cleaved, and if so, if it is cleaved before, at the same time as, or after the cleavage of the central double bond. The isolation of [2-^{14}C] acetone by Birch discussed previously (p. 45) does however indicate that this cleavage occurs at the same time or earlier than the central bond cleavage. If the oxidative loss of acetone is the first step then the acid (165) would presumably



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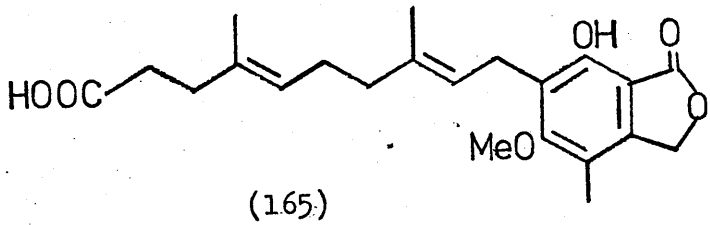

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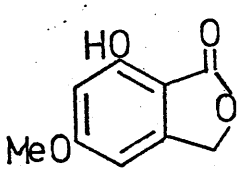

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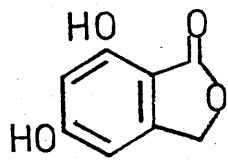
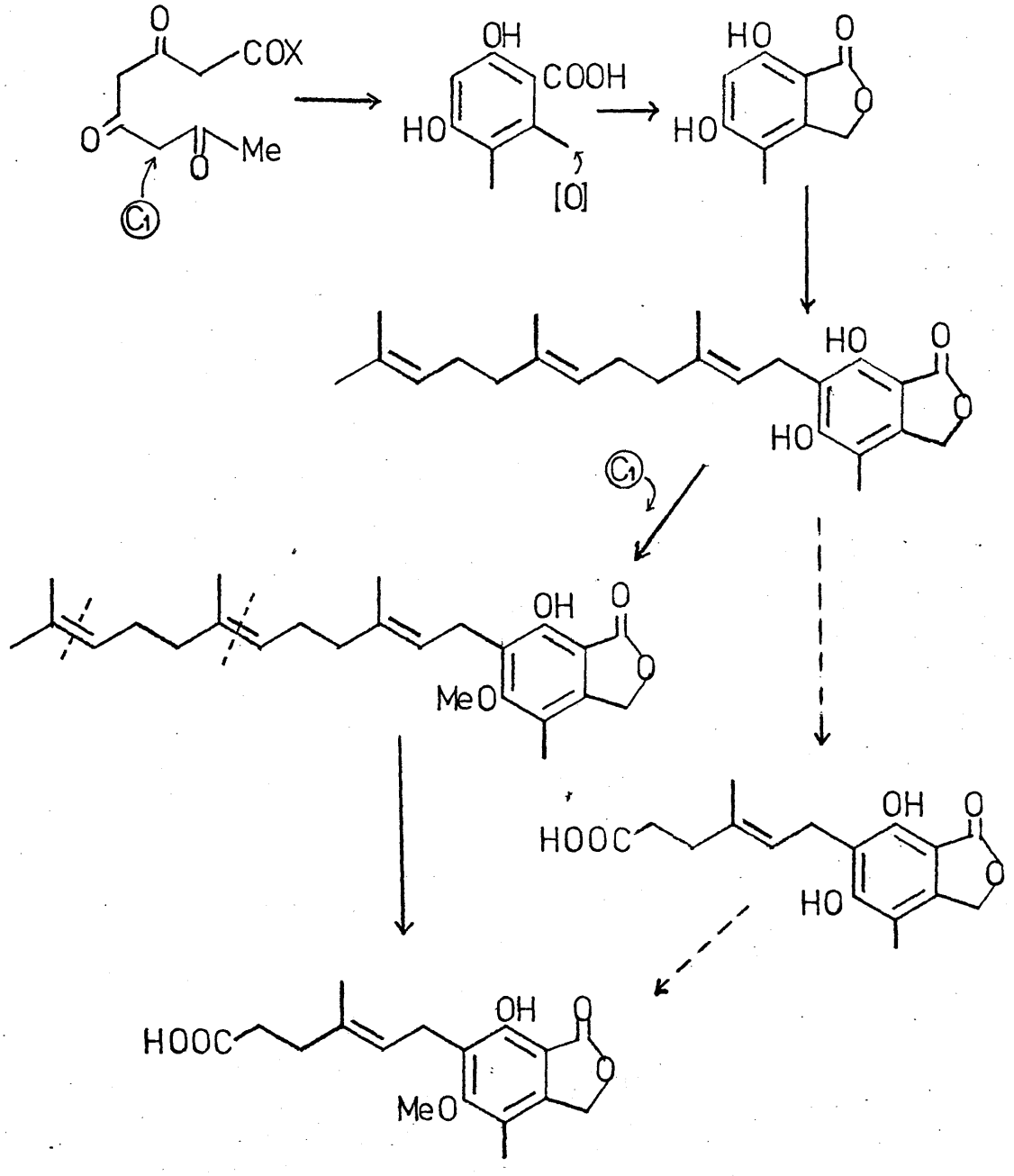


(167)

Fig. 2:7 - Proposed biosynthetic sequence for mycophenolic acid.

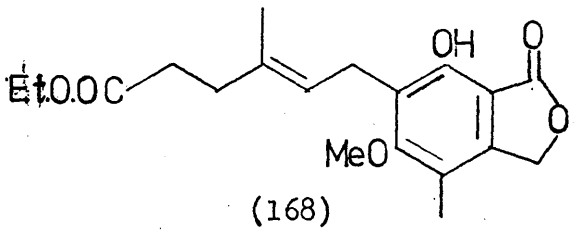


be an intermediate precursor. This compound has not been detected in P. stoloniferum or P. brevicompactum cultures, but modification of a recent synthetic method[139] may enable it to be synthesised and tested as a precursor.

Attempts to trap [3-^{14}C] laevulinic acid from shake flask fermentations fed with [2-^{14}C] mevalonate were inconclusive. Although t.l.c. radioscan of the ethereal extracts of the culture filtrate showed the presence of a highly active compound similar in Rf and staining properties to laevulinic acid, attempts to trap this as its p-bromophenacyl ester gave material of very low activity. (1.2×10^{-6}% incorporation based on L-[2-^{14}C] mevalonate.)

Subsequent to the work described above, extensive studies on the biosynthesis of mycophenolic acid have been reported by Canonica et al.[125,126] and Money et al.[124]. Both groups have shown that 5-methylorsellinic acid (147) is a specific precursor of mycophenolic acid in P. brevicompactum. A molar incorporation of 54.8% has been reported[126]. Canonica et al.[125] have shown that whereas 2-hydroxy-4-methoxy-5-methylphthalide (166) is not incorporated, the corresponding O-desmethyl compound (167) is incorporated to an extent of 97% (molar), indicating that C-prenylation occurs after oxidation of 5-methylorsellinic acid to the phthalide (167), but prior to O-methylation. These workers also succeeded in trapping the farnesyl compound (157)[126] by feeding large quantities of the phthalide (167) to cultures of P. brevicompactum and have shown that this compound is a precursor. The biogenesis of mycophenolic acid can be envisaged as outlined in Fig. 2: 7.

CHAPTER 3



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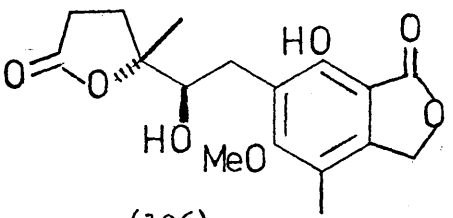

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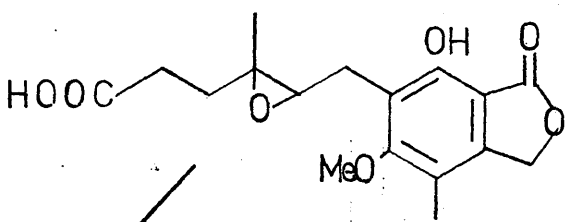

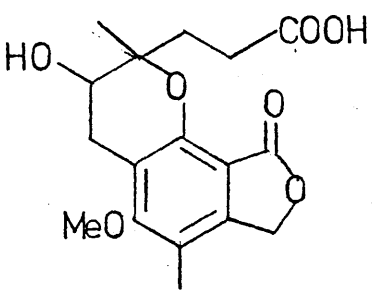

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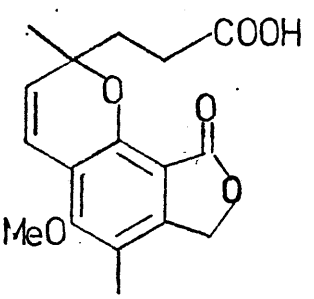

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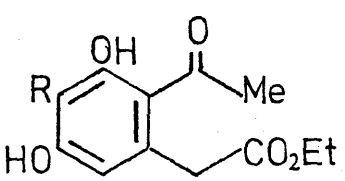
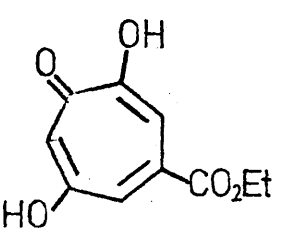


(175) R = H

(176) R = OMe



(177)

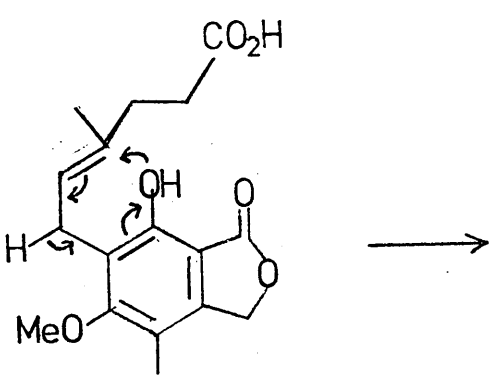
A feature of the metabolism of the strain of Penicillium brevicompactum described previously is the occurrence of a number of metabolites related to mycophenolic acid. Ethyl mycophenolate (168)[119] is the only simple ester* of mycophenolic acid reported from this strain although methyl mycophenolate was isolated from submerged cultures of P. stoloniferum during the work described in the previous chapter. The remainder of these co-metabolites appear to be formed in vivo by oxidation of the 2'3' double bond of the terpenoid side chain. It was thought that study of these compounds might provide some insight into the mechanism of side chain cleavage involved in the biosynthesis of mycophenolic acid.

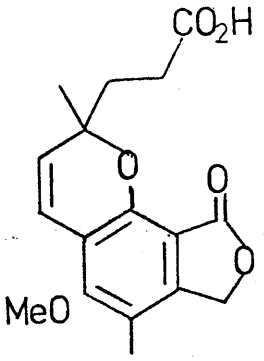
The hydroxylactone (106)[119] was assigned threo-stereochemistry by comparison with the synthetic erythro- and threo- diasteriomers prepared respectively by osmylation and peracid oxidation of the 2'3' double bond of mycophenolic acid, and can be considered to be formed in vivo by epoxidation and subsequent cyclisation of the acidic side chain of mycophenolic acid.

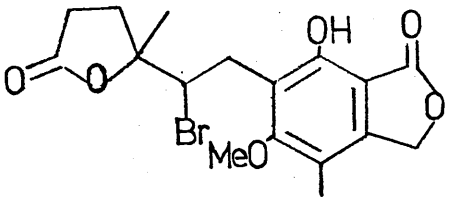
Mycochromenic acid (170)[119] could be derived by the attack of the phenolate anion upon the 2'3' epoxide of mycophenolic acid followed by dehydration of the resultant hydroxypyran (169), or alternatively by a direct oxidation process (Fig. 3:1). In favour of the latter pathway mycochromenic acid (170) has been prepared from mycophenolic

*Ethyl esters are comparatively rare in nature, a number of examples have however been isolated from fungi, e.g. ethyl curvulinate (175)[140], ethyl curvulate (176)[141], ethyl stipitatate (177)[142], and ethyl acetate[4].

Fig. 3:1.







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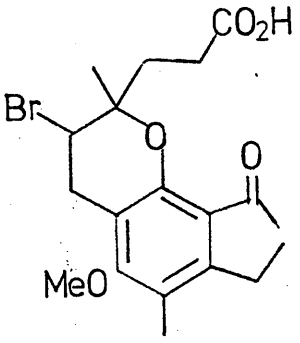

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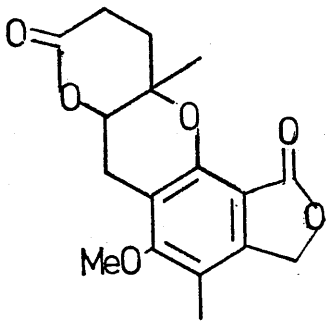

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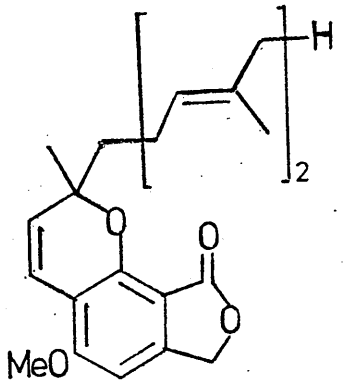

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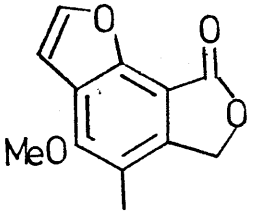
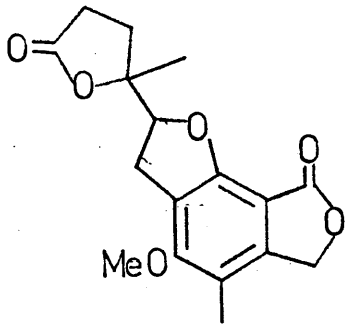


(178)

acid by the action of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)[119], and independently by the action of alkaline potassium ferricyanide[143]. It could not be obtained by the action of base on the hydroxylactone (106)[119]. However, formation from the epoxide of mycophenolic acid (or a related compound) cannot be ruled out since mycochromenic acid (170) has been prepared by treatment of the bromolactone (171) with refluxing pyridine and by the action of aqueous base on the bromopyran (172), which can be prepared by treatment of the bromolactone (171) with concentrated acid, and the δ-lactone (173) has been prepared by treatment of the hydroxylactone (106) with concentrated acid[143]. It may also be significant that the hydroxylactone (106) and mycochromenic acid (170) are not only co-metabolites of P. brevicompactum but are both produced by cultures Helminthosporium bicolor fed with mycophenolic acid[143]. It is also possible that the biosynthesis of mycochromenic acid (170) may involve the O-methylfarnesyl compound (159) and that the immediate precursor is in fact the chromene (174).

Mycofuranolide (178) has been isolated from a number of fermentations of P. brevicompactum and the structure confirmed by synthesis from mycophenolic acid[29], but whether biosynthesis of this compound involves direct formation from the O-methylfarnesyl compound (159) or formation via mycophenolic acid is uncertain. The growth of the mould in the presence of radiolabelled mycophenolic acid and the O-methylfarnesyl compound (159) would be required to clarify the biogenesis of this compound and of mycochromenic acid (170).

Another metabolite, related to mycophenolic acid was detected



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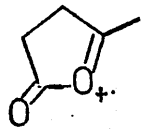
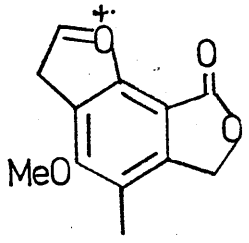


(180)



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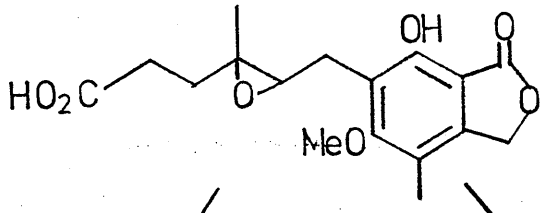
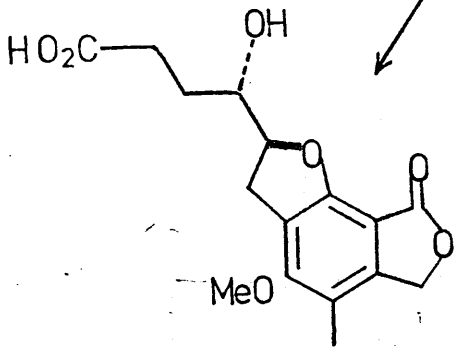
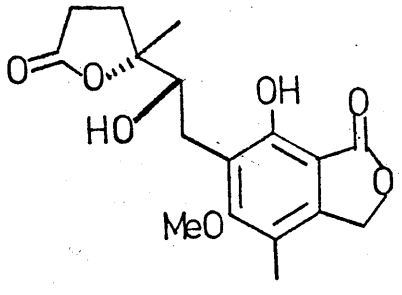






(106)

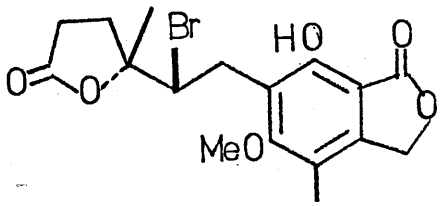
(182)

(183)

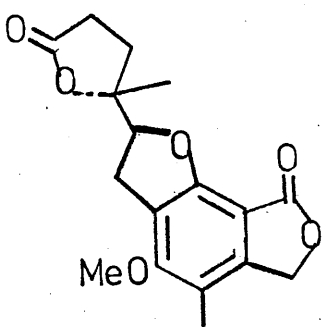
in trace amounts in several fermentations of P. brevicompactum by C. Hernandez Calzadilla[29] who managed to obtain sufficient quantities for examination. The compound was non-phenolic, as indicated by its failure to give a colouration with ferric chloride (t.l.c.) and the lack of change in the u.v. spectrum with base. The presence of the substituted phthalide nucleus of mycophenolic acid was evident from the n.m.r. spectrum which was very similar to that of the hydroxylactone (106). However the absence of hydroxyl absorption in the i.r. suggested the dihydrobenzofuran structure (179). This was substantiated by the appearance, in the mass spectrum, of ions at $^{m}/_{e}$ 99 (100%) and 219 (98%), which can be assigned to the fragments (180) and (181) respectively.

Biogenetically a compound of this gross structure could arise by intramolecular attack of the phenolate anion on the 2′ position of a 2′3′ epoxy intermediate, followed by lactone formation giving a product with threo-stereochemistry (182), or alternatively from the threo-hydroxylactone (106) by Sn_2 elimination of the 2′ hydroxyl group by a phenolate anion to give the erythro-dihydrobenzofuran (183). In order to determine the stereochemistry of the naturally occurring dihydrobenzofuran, attempts were made to prepare the two possible diasteriomers.

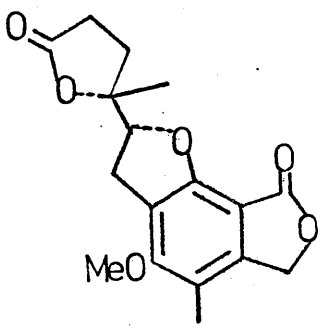
Treatment of mycophenolic acid with bromine in chloroform gave the bromolactone (171) in high yield[143]. Since this compound could also be prepared, albeit in slightly reduced yield, by the reactions of mycophenolic acid with N-Bromosuccinamide, N-bromoacetamide and



(171)



(182)



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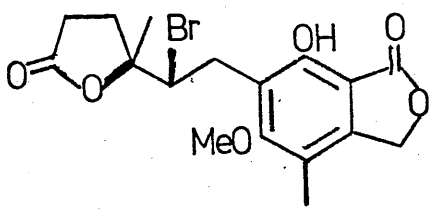
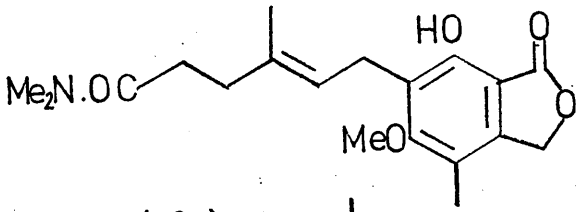


(184)

dibromo-dimethyl hydantoin it is presumably formed by backside attack by the carboxylate grouping on the bromonium ion formed from the 2′3′ double bond. This has been reported as a general reaction of 3,4 and 4,5 unsaturated acyclic acids[144]. The bromolactone (171) would therefore be expected to have threo-stereochemistry. Internal Sn_2 displacement of the 2′ bromine atom by a phenolate anion would give the erythro-dihydrobenzofuran (183). Treatment of the bromolactone (171) in methanol with dilute aqueous sodium hydroxide afforded the desired product in good yield.

The naturally occurring material and the synthetic erythro-diasteriomer (183) were identical by t.l.c. and mixed m.pt., but the G.L.C. of the former showed the presence of a small amount (~5%) of impurity with a shorter retention time. This was borne out by the slightly lower melting point of the sample (197-200°C) cf. that of the synthetic material (200-201°C.). The n.m.r. spectrum of a sample of the metabolite was essentially the same as that of the synthetic erythro-dihydrobenzofuran (183).

It was hoped that the concerted lactonisation which occurs in the bromination of mycophenolic acid might be avoided by protection of the carbonyl group as a dimethylamide and that the resultant trans 2′3′ dibromo derivative might be hydrolysed to give the erythro-bromolactone (184) which could (presumably) be converted to the desired threo-dihydrobenzofuran (182) by treatment with base. Bromination of N,N dimethylmycophenolamide (185) afforded a crystalline compound much more polar (t.l.c.) than mycophenolic acid. This was shown to be phenolic by its blue stain (characteristic of mycophenolic



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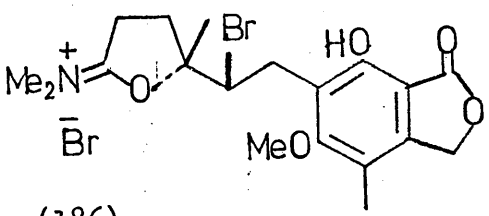

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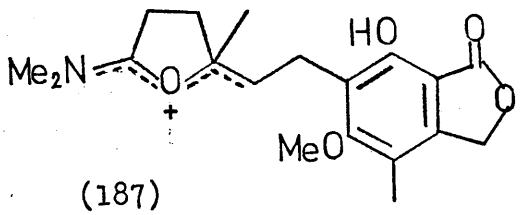

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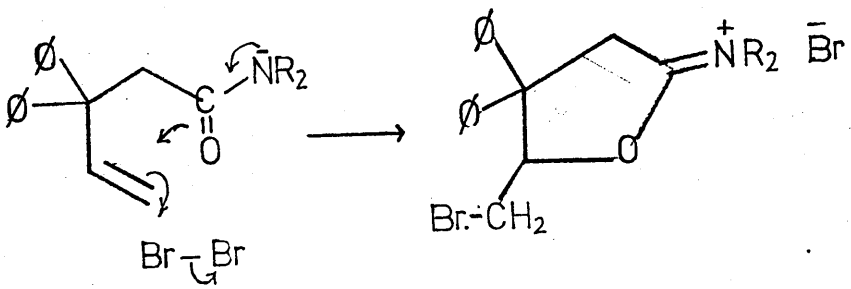


(188) (189)

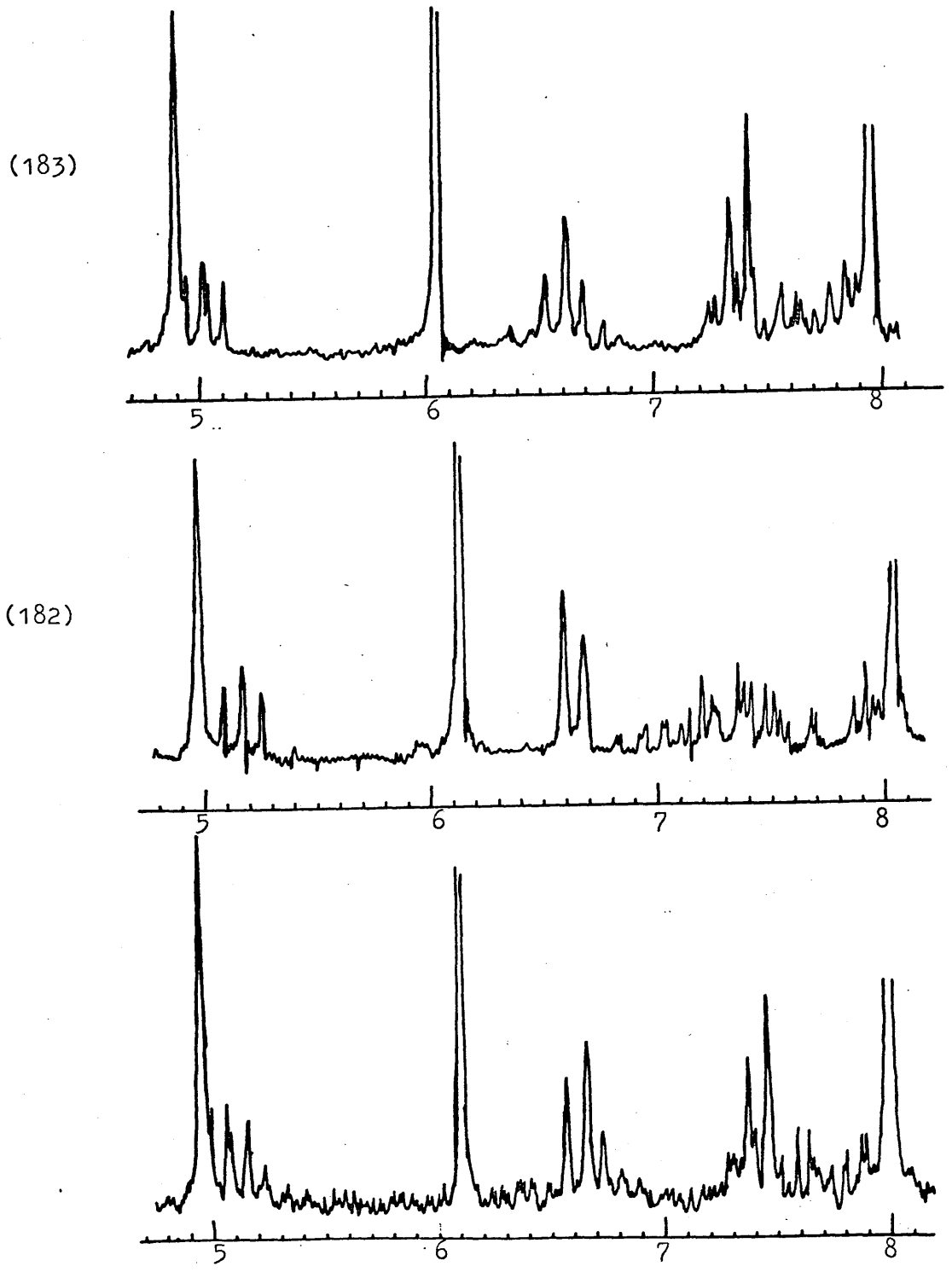
acid derivatives) with ferric chloride (t.l.c.) and by the presence of a band at 3500 $cm.^{-1}$ in the i.r. In the n.m.r. the 3H singlets corresponding to the amide methyl groups appeared at much lower field (6.45 and 6.66τ) than in the spectrum of the starting material (7.0 and 7.1τ) suggesting the formation of an immonium salt. A 1H triplet (J = 7 Hz.) at 5.20τ indicated the presence of a proton geminal to a bromine substituent in the 2′ position. These data can be accommodated by formulation as the cyclic immonium bromide (186). The highest ion in the mass spectrum of this compound appeared at $^{m}/_{e}$ 347 corresponding to loss of bromine from the immonium cation to give the fragment (187).

Hydrolysis of the immonium bromide (186) with water at room temperature afforded the threo-bromolactone (171) in good yield. Analogous cyclisations of the amides of 2,2 diphenyl-4-pentanoic acid (188) to the immonium bromides (189) have been reported, although in these cases the stability of the salts of tertiary amides to hydrolysis was found to be greater[145].

Derivatives of the carboxylic acid group were equally ineffective in preventing carboxylate participation in peracid reactions. Thus peracid oxidation of N,N diethylmycophenolamide (190) gave the hydroxylactone (106) as the sole product; peracid oxidation of ethyl mycophenolate (168) gave in addition to the hydroxylactone (106) trace amounts of the erythro-dihydrobenzofuran (183).

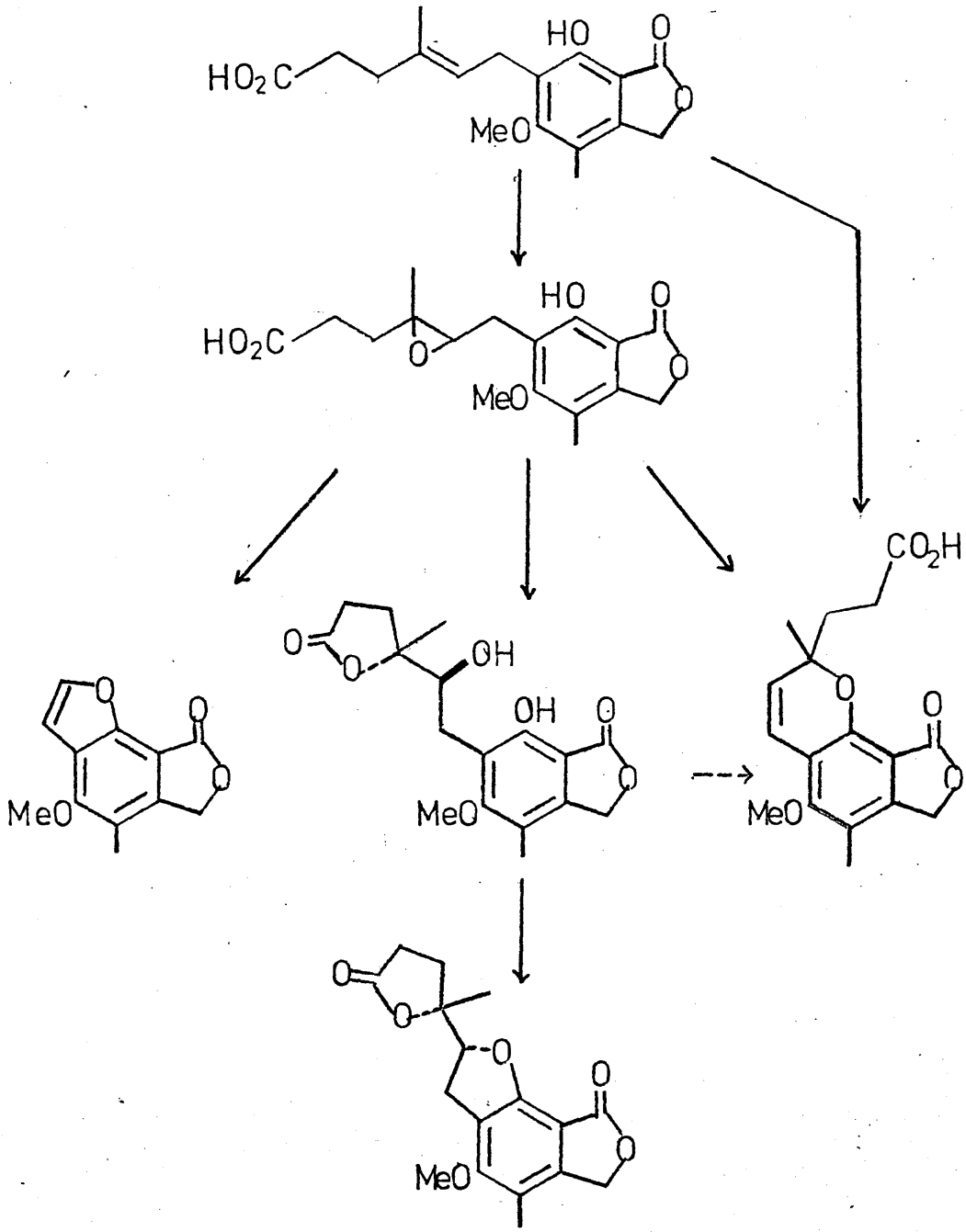
At this point we received from Dr. D. F. Jones a sample of the threo-dihydrobenzofuran (182) which had been obtained by treatment of the bromolactone (171) with lithium chloride in refluxing

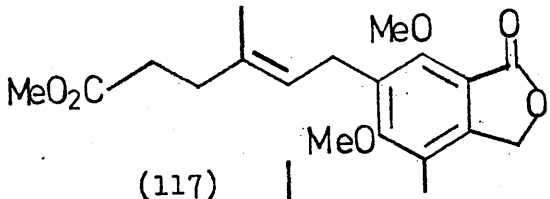
Fig. 3:2 NMR comparison of the diasteriomeric dihydrobenzofurans



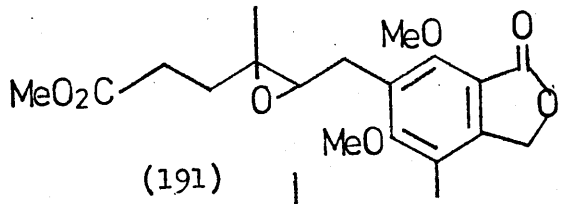
Metabolite isolated from P. brevicompactum

Fig. 3:3 - Possible interrelationship between P. brevicompactum metabolites.





(117)



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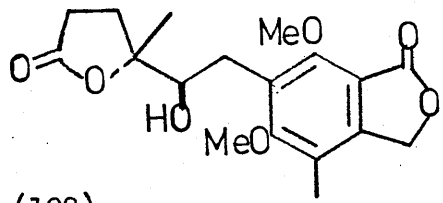


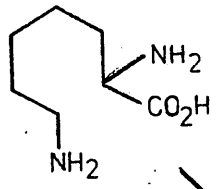
(192)

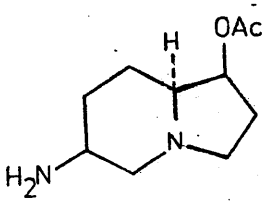
dimethylformamide, and also as a minor product in the synthesis of mycochromenic acid (170) from the bromolactone (171)[143]. The diasteriomeric dihydrobenzofurans could be distinguished by t.l.c., G.L.C., i.r. and n.m.r. (Fig. 3:2), but the presence of the threo-isomer could not be detected in the material isolated from P. brevicompactum. Consequently the only natural stereoisomer is the erythro-dihydrobenzofuran (183).

A tentative biosynthetic scheme for the formation of the oxygenated metabolites related to mycophenolic acid in P. brevicompactum is outlined in Fig. 3:3.

The instability of the 2′3′ epoxy derivatives of the esters and amides of mycophenolic acid can be attributed to the presence of a neighbouring free phenolic hydroxyl group in the molecule which is capable of protonating the epoxide, creating an electrophilic centre at the 3′ position. In contrast the peracid oxidation of methyl O-methylmycophenolic acid (117) afforded the epoxide (191) in good yield. The product was fairly stable at room temperature but was converted quantitatively to the threo-hydroxylactone (192) on treatment with trifluoroacetic acid.

CHAPTER 4





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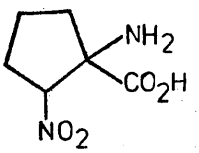

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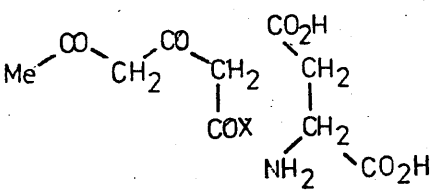
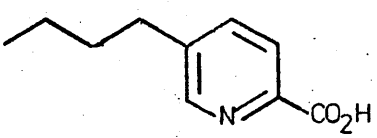
$NO_2CH_2CH_2CO_2H$

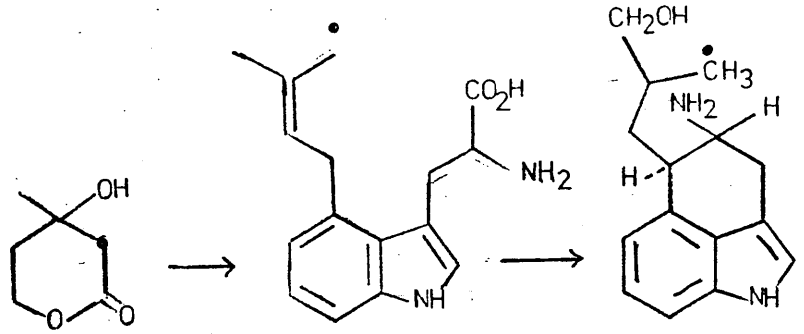
(195)



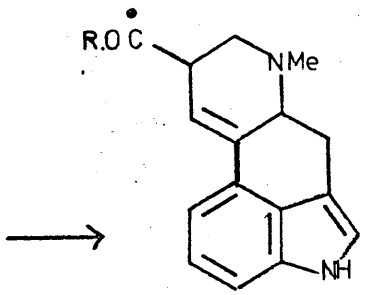


(196)

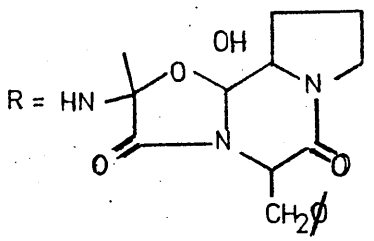
Fig. 4:1



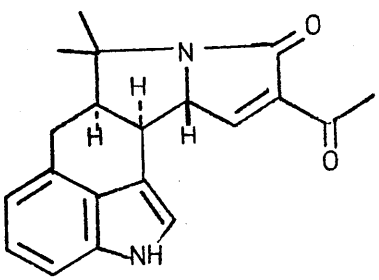
[2-^{14}C]MVA



R = OH lysergic acid.



ergotamine



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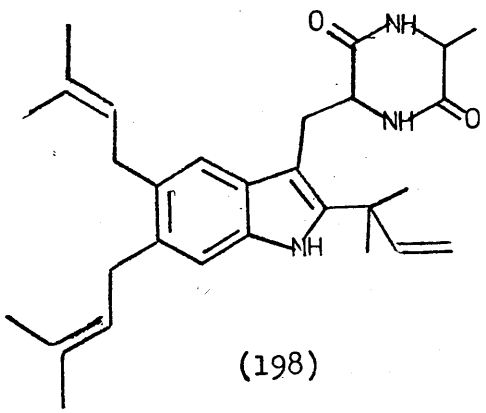

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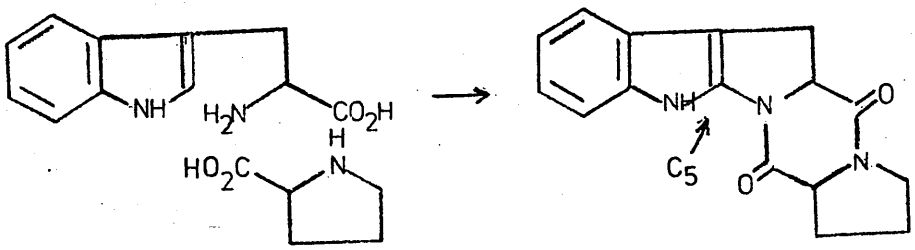
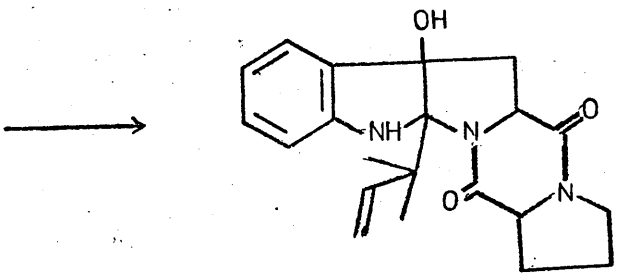
Fig. 4:2.





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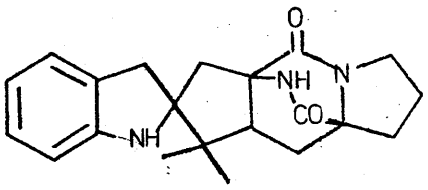


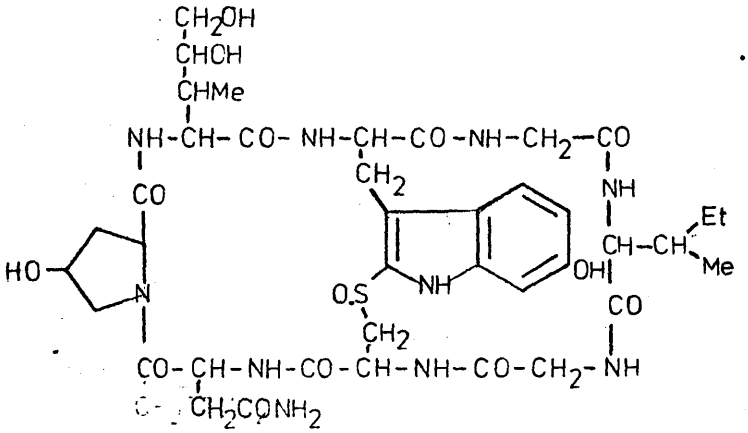
(200)

Introduction

A large number of fungal metabolites can be classified biosynthetically as being derived from amino acids. Many of these compounds are simple derivatives of the common amino acids found in both animal and plant systems. Slaframine (193)[151] and 1-amino-2-nitrocyclopentane carboxylic acid (194)[152] are derived from lysine, and β-nitropropionic acid (195) which has been isolated from a number of Aspergillus spp.[153] has been shown to be derived from aspartic acid[154]. Fusaric acid (196) a metabolite of several Fusarium spp. has been shown to arise from the condensation of a triketide unit with aspartic acid[155].

The prenylation of tryptophan with a C_5 unit derived from mevalonic acid is the first stage in the biosynthesis of the ergot alkaloids of Claviceps spp. (Fig. 4:1)[156]. The 4-dimethylallyl-tryptophan moeity is also present in cyclopiazonic acid (197) where it has undergone (formal) condensation with an acetoacetyl unit[157]. The substitution of tryptophan by isoprene units also occurs in the formation of echinulin (198)[158] a metabolite of Aspergillus spp. in which the C_3 unit of the diketopiperazine ring is derived from alanine[159]. Brevianamide E (199) isolated from P. brevicompactum can be formally derived from tryptophan, proline and mevalonate (Fig. 4:2), and brevianamide A (200) may be a rearrangement product[161]. In gliotoxin (201)[162] and sporidesmin (202)[163] the diketopiperazine ring is bridged by sulphur.

Cyclic peptides and cyclodepsipeptides have also been isolated



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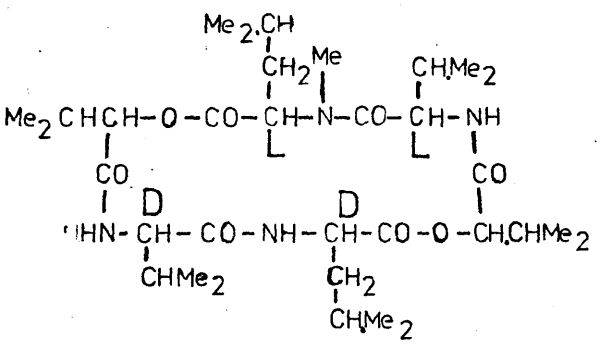

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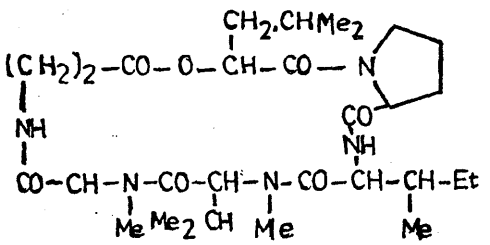


(207)

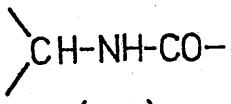
from fungi, e.g. islanditoxin (203) the toxic principle of P. islandicum[164], maliformin A (204) from several Aspergillus spp.[165] the toxins of Amanita spp.[166] (e.g. α-Amanitin (205)), the sporodesmolides (e.g. sporodesmolide I (206) from Sporodesmium bakeri[167] and the insecticide destructuxin B (207) from Oospora destructor[168]. In common with the large number of peptide metabolites isolated from other microbial sources, especially the Streptomycetes and Bacilli, fungal peptides possess certain features which are rare in proteins. Many contain proline, N-methylated amino acids and imino acids, which may prohibit helix formation and hence favour cyclisation, while arginine, histidine and methionine which are commonly found in proteins are rare. The occurrence of both D- and L- forms of certain amino acids is common in a number of cases being found in the same molecule[169].

Some bacterial cyclic peptides are known to function as metal transport agents, and their role in sporulation has been discussed[170]. It has also been suggested that these compounds may be "fossils" of an earlier less specific metabolic process[169].

Brevigellin

Brevigellin (208) was isolated from the neutral extract of the culture filtrate of P. brevicompactum by column chromatography and subsequently purified by preparative t.l.c. The pure metabolite was a colourless hydroscopic glass (m. 209-212°C.) which could not be obtained in a crystalline form; addition of light petroleum to a chloroform solution of the metabolite resulted in the formation of a gel. The molecular formula $C_{31}H_{41}N_5O_7$ was assigned on the basis of high resolution mass spectroscopy (Table 4:1). Bands at 3400, 1658 (ε = 836), and 1500 $cm.^{-1}$ in the i.r. ($CHCl_3$) suggested the presence of at least one secondary amide group which was supported by the presence of a low intensity peak at 3061 $cm.^{-1}$ in the solid state spectrum (KBr.)[171]. A band at 1758 $cm.^{-1}$ (ε = 236) could be assigned to an ester grouping. A sharp peak at 1600 $cm.^{-1}$ indicated the presence of an aromatic ring, which was confirmed by the n.m.r., in which the pattern characteristic of a benzoyl group was evident, and by the presence of an ion $^{m}/_{e}$ 105.0345 (73.9%) in the mass spectrum, corresponding to the fragment $(C_7H_5O)^+$. The presence of a minor ion $^{m}/_{e}$ 121.0531 (5.5%) corresponding to $(C_7H_7NO)^+$ suggested that the benzoyl fragment resulted from fragmentation of a benzamide grouping.

Only one exchangeable proton (a 1H doublet, J = 9 Hz., at 3.3τ) was observed in the n.m.r. spectrum, exchange of which did not take place on shaking with D_2O but did in the presence of a trace of trifluoroacetic acid indicating that this was an amide proton. On exchange of this proton a 1H doublet (J = 9 Hz.) at 4.91τ was reduced

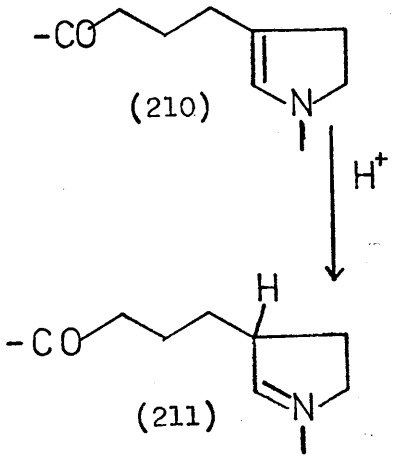


(209)

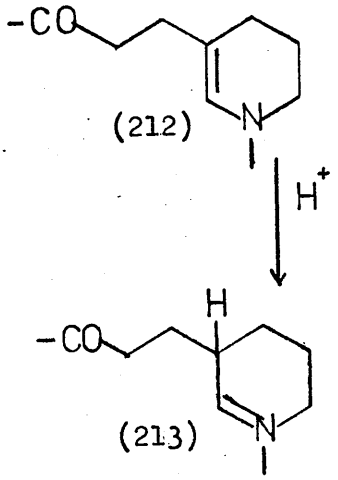
to a broad singlet indicating the presence of the system (209) in which the methine proton is not further coupled.

The above data suggested that brevigellin might have a 'peptide' type structure and this was also indicated by a characteristic yellow stain with a blue halo, which it gave on t.l.c. when sprayed with sodium hypochlorite and then potassium iodide/O-toluidide reagent[174]. In keeping with this hydrolysis with 2.5N aqueous hydrochloric acid afforded a mixture of four polar ninhydrin staining compounds, which could be separated by analytical paper chromatography. The three most polar of these compounds gave colourations with ninhydrin in the cold (indicative of an α-amino acid) and were identified as threonine, α-alanine and proline on the basis of their Rf. values and staining properties with ninhydrin and isatin. Comparison with authentic samples of these amino acids, by paper chromatography confirmed these assignments. The least polar of the four ninhydrin positive products (Rf. 0.42), gave a lilac stain with ninhydrin on heating and a green-blue stain with isatin. Chromatographic comparison with β- and γ- amino butyric acids, which have almost identical Rf. values in the solvent system used, suggested that the product was β-aminobutyric acid.

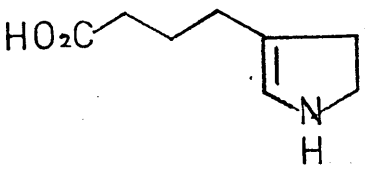
Amino acid analysis of the hydrolysis mixture confirmed the presence of threonine, α-alanine and proline and showed the presence of an additional compound which had a longer retention time than α-alanine. Under the conditions of analysis β-aminobutyric acid is not observed, which suggested the presence of another nitrogen containing compound in the mixture. When brevigellin was hydrolysed



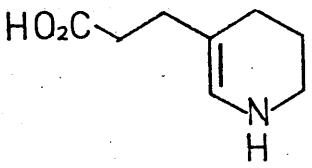
(210) (211)



(212) (213)

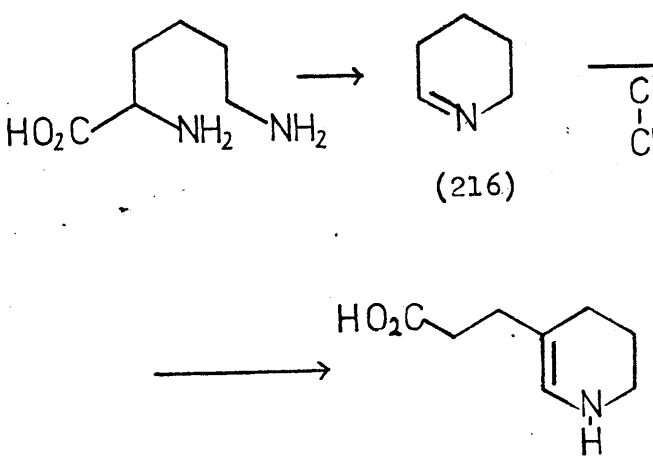


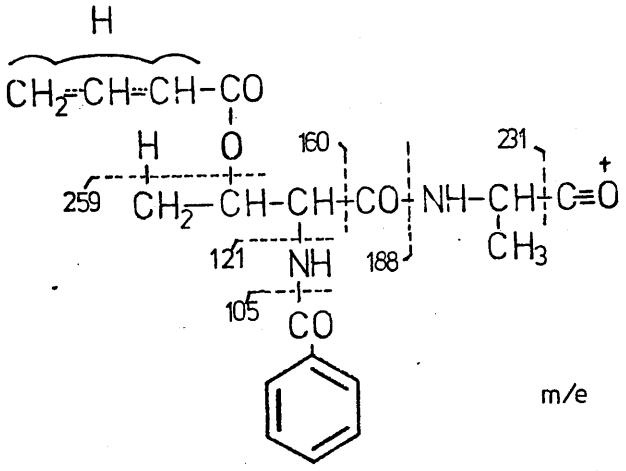
(214)



(215)

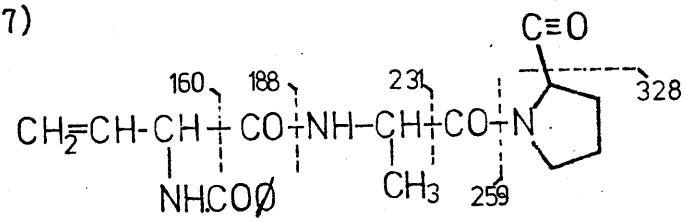
Fig. 4:3.





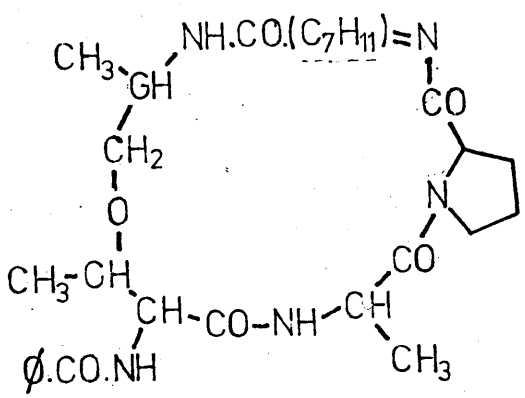
m/e 345.1446

(217)



(218)

m/e 356.1600



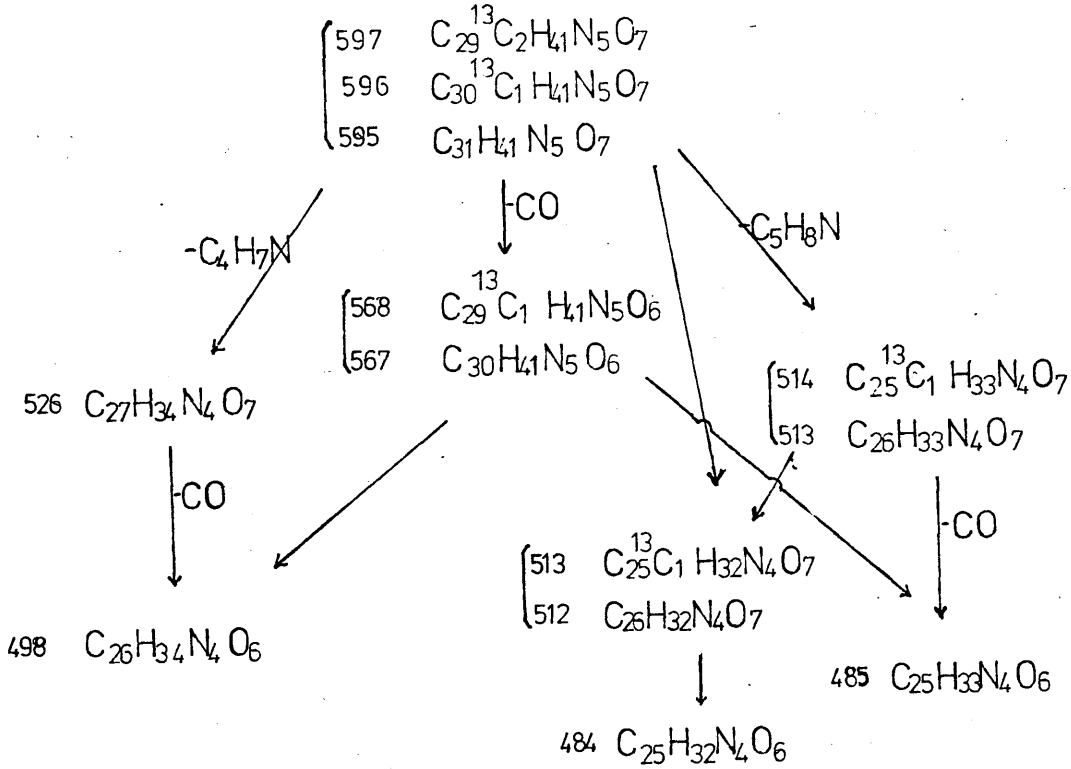
(219)

under more forceful conditions, however, (6N HCl, 12 hr. at 120° in a sealed tube) this compound was not observed indicating that it is not especially stable under acid conditions.

The presence of the four identified amino acid residues in the molecule accounts for some additional features of the n.m.r. spectrum. The 9H multiplet at 8.71τ can now be assigned to the protons of the methyl groups of threonine, alanine and β-aminobutrate, while a 1H quartet (J = 6 Hz.) at 4.64τ, which collapses to a broad singlet on irradiation of the 8.71τ multiplet, can be assigned to the β-methine proton of threonine.

Subtraction of the four identified residues from the molecular formula ($C_{31}H_{41}N_5O_7$) leaves the residue $C_8H_{11}NO$ to be accounted for. The lack of free carboxyl or amino groups in the molecule was evident from its non-polar nature and the absence of the characteristic absorption bands in the i.r., suggesting that the $C_8H_{11}NO$ moeity is part of a ring residue. In the n.m.r. eight of the protons of this unit are observed at 8.25τ typical of C-$\underline{CH_2}$-C, and a further 2H appear at 6.31τ indicating the presence of a C-$\underline{CH_2}$-N grouping. The remaining proton appears 2.2τ in $CDCl_3$, and is obscured by the resonances of the ortho-protons of the benzoyl group. When the spectrum was recorded in 5% trifluoroacetic acid/$CDCl_3$ however, the resonance of this proton appeared as a doublet (J = 9 Hz.) at 1.75τ suggesting that it was an olefinic proton α- to nitrogen. In addition, integration of the multiplet between 5.2 and 6.0τ in this spectrum indicated the presence of an additional proton, irradiation of which (ca. 5.4τ) resulted in the collapse of the 1.75τ doublet to a broad singlet.

Fig. 4:4 - Fragmentation of brevigellin (208)



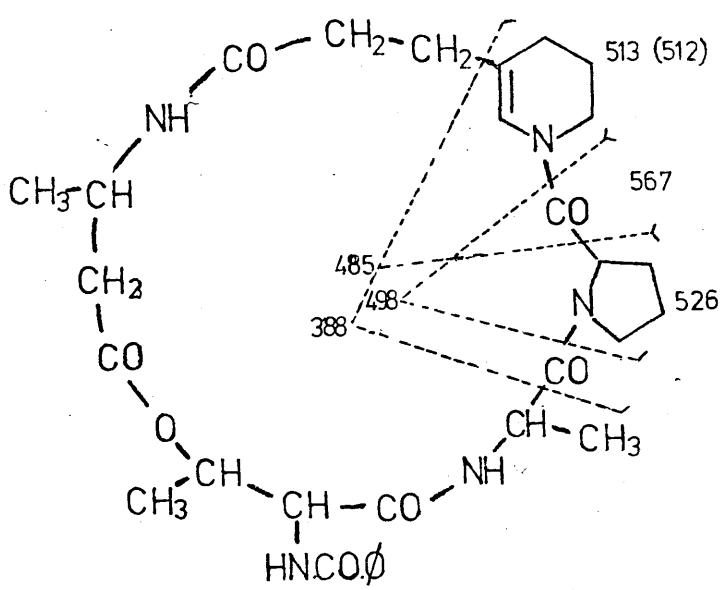


Table 4:1 The Mass Spectrum of Brevigellin (208)

m/e	rel. abundance %	assignment		m/e	rel. abundance %	assignment
597.3090	4.1	$C_{29}{}^{13}C_2H_{41}N_5O_7$	$(M+2)^+$	356.1600	9.5	$C_{19}H_{22}N_3O_4$
596.3038	19.0	$C_{30}{}^{13}C\,H_{41}N_5O_7$	$(M+1)^+$	345.1446	30.0	$C_{18}H_{21}N_2O_5$
595.3013	48.2	$C_{31}H_{41}N_5O_7$	M^+	328.1665	0.8	$C_{18}H_{22}N_3O_3$
567.3051	3.2	$C_{30}H_{41}N_5O_6$		259.1082	59.0	$C_{14}H_{15}N_2O_3$
526.2427	0.6	$C_{27}H_{34}N_4O_7$		231.1127	15.5	$C_{13}H_{15}N_2O_2$
513.2318	4.2	$C_{26}H_{33}N_4O_7$		208.1216	11.8	--
512.2249	6.8	$C_{26}H_{32}N_4O_7$		188.0704	14.5	$C_{11}H_{10}N\,O_2$
498.2460	0.8	$C_{26}H_{34}N_4O_6$		168.0896	14.8	--
485.2394	0.8	$C_{25}H_{33}N_4O_6$		167.088	12.9	--
484.2335	2.6	$C_{25}H_{32}N_4O_6$		160.0753	35.3	$C_{10}H_{10}N\,O$
468.2360	1.3	$C_{25}H_{32}N_4O_5$		121.0531	5.5	C_7H_7NO
439.2337	1.0	$C_{24}H_{31}N_4O_4$		106.0379	10.0	$C_6{}^{13}CH_5O$
388.1872	1.0	$C_{20}H_{26}N_3O_5$		105.0345	73.9	C_7H_5O

These features can be accommodated in the partial structures (210) and (212), protonation of which would give the salts (211) and (213) Although neither of the amino acids (214) or (215) have been reported the latter could be derived biogenetically by condensation of lysine derived piperideine (216) with β-alanine imine or oxaloacetic acid (Fig. 4:3).

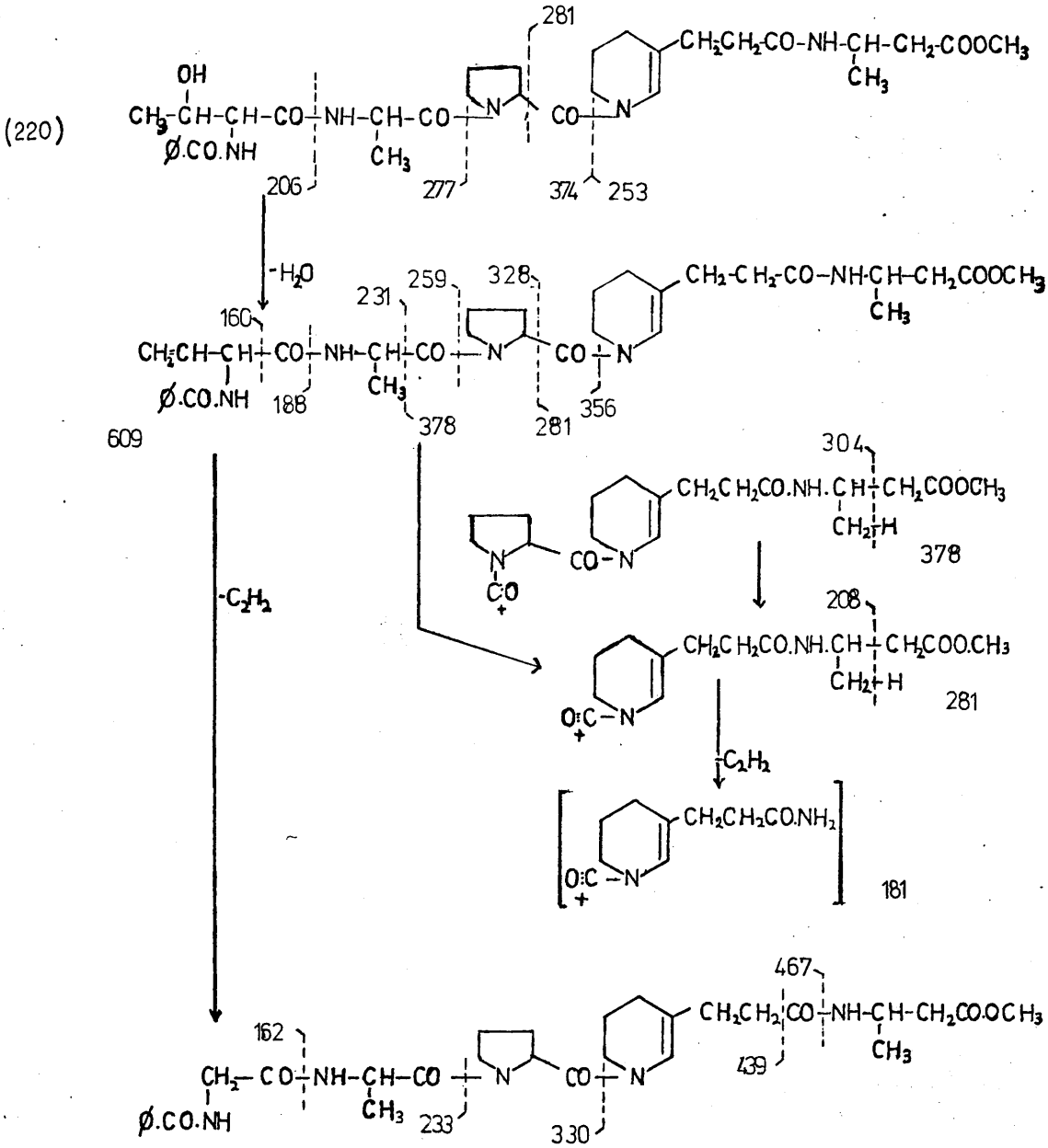
The order in which the amino acid residues are coupled in the molecule was elucidated from the mass spectrum (Table 4:1). An ion at $^{m}/_{e}$ 345.1446 ($C_{18}H_{21}N_2O_5$, 30%) could be assigned to the fragment (217) in view of the presence of ions at $^{m}/_{e}$ 259, 231, 188, 121 and 105 which correspond to sequential fragmentation of this ion. The presence of an ion $^{m}/_{e}$ 356.1600 ($C_{19}H_{22}N_3O_4$, 10%) could be ascribed to the fragment (218) loss of CO from which would give the ion $^{m}/_{e}$ 328.1646 ($C_{18}H_{22}N_3O_3$, 0.75%) and subsequent fragmentation of this ion could also give rise to the fragments 259, 231, 188, 160, 121 and 105. From the order of the amino acid residues indicated by these ions the partial structure (219) can be written. The upper region of the spectrum is characterised by low intensity ions (Table 4:1) which can be assigned to the fragmentations proposed in Fig. 4:4. The observed losses of 82 (C_5H_8N), 83 (C_5H_9N), 110 (C_6H_8NO), 207 ($C_{10}H_{15}N_2O$) and 207 ($C_{11}H_{15}N_2O_2$) favour the structure (215) proposed for the fifth amino acid rather than (214).

Specific hydrolysis of the ester linkage was achieved using 0.5N methanolic potassium hydroxide yielding a single polar product, which gave no stain with ninhydrin indicating the absence of a free amino group. Esterification with ethereal diazomethane afforded

Table 4:2 The mass spectrum of the methyl ester (220)

m/e	rel. abundance %	assignment	m/e	rel. abundance %	assignment
609.3143	0.5	$C_{32}H_{43}N_5O_7$	253.1520	18.8	$C_{13}H_{21}N_2O_3$
467.2272	3.9	$C_{25}H_{31}N_4O_5$	233.0928	11.9	$C_{12}H_{13}N_2O_3$
457.2438	8.8	--	231.1131	9.2	$C_{13}H_{15}N_2O_2$
441.2126	10.6	--	208.1205	11.8	$C_{11}H_{16}N_2O_2$
440.2097	15.9	--	207.1129	6.9	$C_{11}H_{15}N_2O_2$
439.2318	5.3	$C_{24}H_{31}N_4O_4$	206.0867	9.4	$C_{11}H_{12}NO_3$
413.2174	16.9	$C_{22}H_{29}N_4O_4$	188.0706	9.7	$C_{11}H_{10}NO_2$
378.2017	10.5	$C_{19}H_{28}N_3O_5$	181.1309	31.5	$C_9H_{13}N_2O_2$
374.1713	41.5	$C_{19}H_{24}N_3O_5$	167.0854	14.5	--
356.1605	21.9	$C_{19}H_{22}N_3O_4$	165.0787	7.8	$C_9H_{11}NO_2$
331.1473	14.7	$C_{16}{}^{13}CH_{20}N_3O_4$	162.0552	14.4	$C_9H_8NO_2$
330.1443	65.8	$C_{17}H_{20}N_3O_4$	160.0757	7.2	$C_{10}H_{10}NO$
304.1673	1.1	$C_{16}H_{22}N_3O_3$	144.1021	12.2	--
328.1646	0.6	$C_{18}H_{22}N_3O_3$	142.0867	16.8	--
281.1514	11.7	$C_{14}H_{21}N_2O_4$	121.0535	2.7	C_7H_7NO
277.1206	9.4	$C_{14}H_{27}N_2O_4$	106.0370	10	$C_6{}^{13}C\,H_5O$
259.1082	46.7	$C_{14}H_{15}N_2O_3$	105.0323	100	C_7H_5O

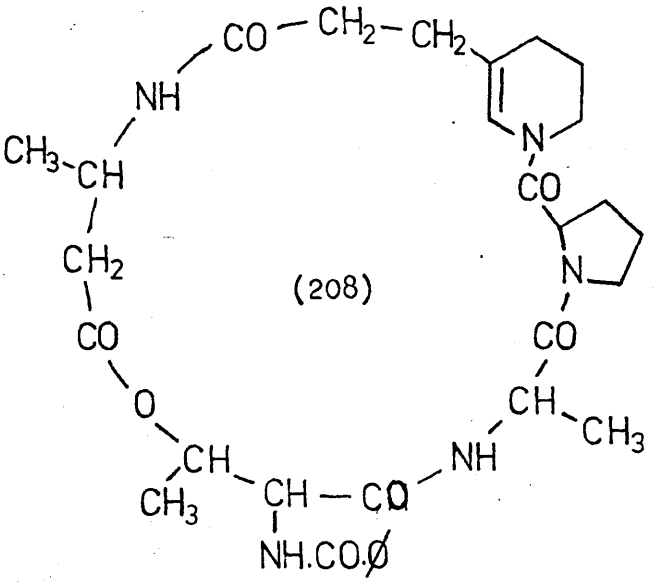
Fig. 4:5 - Mass spectral fragmentation of the methyl ester (220).



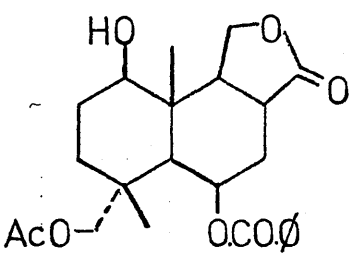
a product slightly less polar than brevigellin. The highest ion in the mass spectrum of this compound appeared at $^{m}/_{e}$ 609.3143 ($C_{32}H_{42}N_5O_7$, 0.5%) corresponding to loss of water from the structure (220). However the presence of the ions $^{m}/_{e}$ 374.1713 ($C_{19}H_{24}N_3O_5$, 41.5%) 277.1206 ($C_{14}H_{21}N_2O_4$, 9.5%) and 206.0867 ($C_{11}H_{12}NO_3$, 9.4%) suggests that the structure of the hydrolysis/methylation product is in fact (220) and that loss of water occurs in the mass spectrometer. A plausible fragmentation scheme for this molecule is outlined in Fig. 4:5.

In view of the extremely low yields of brevigellin from P. brevicompactum (ca. 0.2 mg./l. of culture filtrate after 20 days) insufficient material was available to obtain samples of the constituent amino acids for specific rotation measurements. However the almost negligible coupling (< 1 Hz.) between the adjacent methine protons of the threonine residue (4.64 and 4.91τ) indicates that these lie at almost 90° to one another, a configuration which can be accommodated in a structure where the β-aminobutyrate carbonyl forms a hydrogen bond with the nitrogen atom of alanine residue. This is shown in Fig. 4:6, in which a hydrogen bond between the carbonyl of proline and the nitrogen atom of β-aminobutyrate has also been constructed.

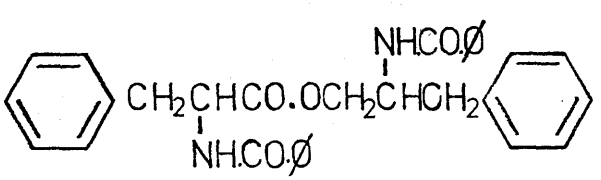
An unusual feature in the structure of brevigellin (208) is the presence of an N-benzoyl substituent; benzoyl groups, which are presumably derived via phenylalanine, are uncommon in microbial metabolism, although pebrolide (15) (Chapter I) and candipolin (221)[172] from P. canadense both contain benzoyl substituents. Benzoyl



(208)

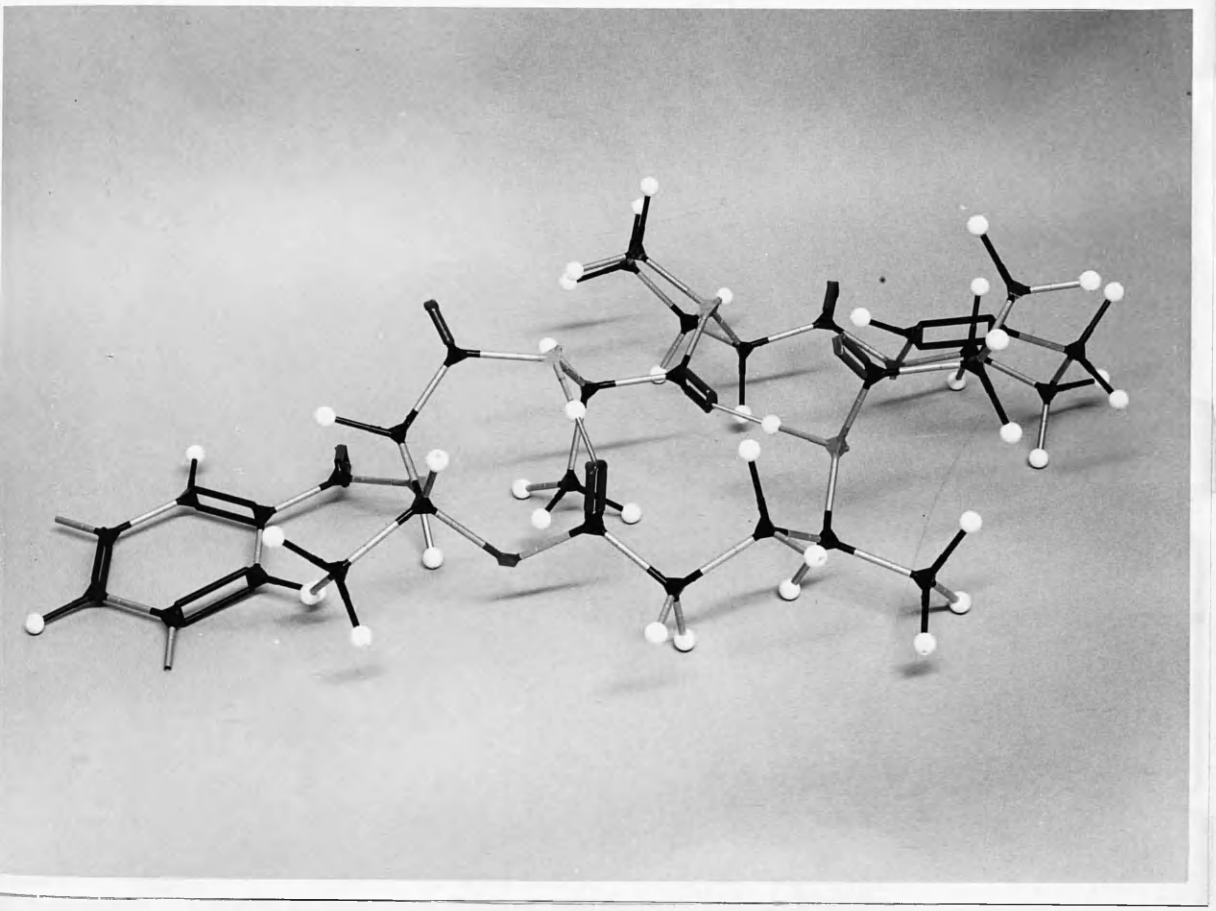


(15)



(221)

esterases[173] have been isolated from a number of fungi, e.g. Aspergillus terricola and P. dahlea which suggests that this grouping may be a more common feature in metabolism than previously supposed.



EXPERIMENTAL

General - Instrumental

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected, boiling points are uncorrected. Ultraviolet spectra were obtained on a Unicam SP 800 recording spectrophotometer. Infra red spectra were measured with a Unicam SP 200, and Perkin Elmer 225 and 257, spectrometers. Nuclear magnetic resonance spectra were recorded with Varian HA-60 and HA-100 spectrometers. Unless otherwise stated all values quoted were recorded in deuterochloroform with tetramethyl silane as internal standard. Routine mass spectra were obtained with an A.E.I. M.S.12 mass spectrometer and exact mass determinations on an A.E.I. M.S.9 mass spectrometer.

Thin layer chromatography (t.l.c.)

Rf. values were determined from elution on 0.1 mm. layers of Kieselgel GF $_{254}$ the compounds being located under ultra violet light, with iodine and 3% methanolic ferric chloride spray ($FeCl_3$), and standardized by comparison with the Rf. values of sudan yellow and p-amino azobenzene.

	p-amino azobenzene	sudan yellow
chloroform	0.65	0.30
5% methanol/chloroform	0.70	0.50

Values quoted for other solvent systems refer to analytical t.l.c. (below) and are approximate.

Analytical t.l.c. was performed on 0.25 mm. layers of Kieselgel G, and the compounds located by spraying with 3% methanolic ferric chloride ($FeCl_3$) or 1% ceric ammonium nitrate in 10% sulphuric acid (Ce^{4+}). The colours observed with the above reagents are abbreviated as follows: gr. (grey), y. (yellow), bn. (brown), bk. (black), gn. (green), r. (red) and pur. (purple).

Radioactive compounds were located on thin layer chromatograms by scanning with either a Packard or Texas Instruments' radioscanner.

Counting of radioactive materials

Radioactive assays were carried out using a Phillips Liquid scintillation counter. In experiments with doubly labelled precursors the external channel ratio method was used invariably.

Solid samples were weighed on metal foil, transferred to Packard scintillation vials and dissolved in toluene scintillation solution (15 ml.) 2,5-diphenyloxazole (6 g.); 1,4-bis-2 (4 methyl-5-phenyl oxazalyl) benzene (0.1 g.); toluene (1 l.) . Liquid samples were weighed directly into the vials. Aqueous solutions were counted in toluene scintillator (13.5 ml.). Bio-solv. BBS-2 solution (1 ml.) made up to 15 ml. with distilled water. Samples were counted for sufficient time to achieve a counting error of less than $\pm 3\%$.

General

Acetone was dried over anhydrous magnesium sulphate, and

tetrahydrofuran dried over metallic sodium and redistilled from lithium aluminium hydride prior to use.

Diazomethane was prepared by the method of Moore and Reed[146] from bis (N-methyl-N-nitroso)-terephthalimide. Jones' reagent was prepared by the procedure of E. R. H. Jones et al.[147] and Fetizon's reagent by the procedure of Fetizon and Golfier[148]. "Active" manganese dioxide was prepared by the method of Attenburrow et al.[149] "Magnesium iodide etherate" was prepared, immediately prior to use, as described by Attenburrow et al.[150] All organic extracts were routinely washed with water and dried over anhydrous magnesium sulphate. Solvents were removed using a rotary film evaporator. Light petroleum unless otherwise stated refers to a light petroleum fraction b.pt. 40-60°C.

The following abbreviations are used in reporting spectral data: s., singlet; d., doublet; t., triplet; q., quartet; m., multiplet; br., broad; obs., pattern obscured by other resonances.

In the following typical description of n.m.r. data

5.6 (2H, q, J = 8Hz., irr. 8.6 → s, $-O\underline{CH_2}-CH_3$) irradiation at 8.62τ has resulted in the collapse of the 2H quartet at 5.6τ.

Table I Composition of Czapek Dox medium (g./l.)

sodium nitrate	2
dipotassium hydrogen phosphate	1
potassium chloride	0.5
magnesium sulphate	0.5
ferric sulphate	0.01
glucose	50
corn steep liquor	10

The growth and extraction of surface cultures of P. brevicompactum

The strain of Penicillium brevicompactum described was originally supplied by the Commonwealth Mycological Institute (No. 49162) and has been cultured in the Glasgow University Joint Mycology Laboratories since 1964.

Typically a spore suspension of this strain was used to inoculate 100 Roux bottles, each of which contained 200 ml. of sterile Czapek Dox medium (Table I). The cultures were allowed to grow undisturbed at 25°C and 70% relative humidity, under artificial lighting. After the prescribed period of growth the mycelial mats were physically separated from the culture filtrate, dried, pulverised and extracted in a Soxhlet apparatus with acetone for 24 hr. The culture filtrate (20 l.) was stirred with charcoal (200 g.) for 2 hr., the charcoal removed by filtration and extracted in a Soxhlet apparatus with acetone for 24 hr.

Separation of the metabolites present in the culture filtrate extract

A typical procedure for the extract of an 18 day culture was as follows. The acetone extract was dried and the solvent evaporated to give a brown gum (1.8 g.). This was partitioned between chloroform and water. The chloroform solution was extracted with saturated aqueous sodium bicarbonate. Neutralisation of the bicarbonate extract with dilute hydrochloric acid gave mycophenolic acid (0.4 g.), which was purified by crystallisation from aqueous methanol.

The chloroform solution was dried and the solvent evaporated

Table II Separation of the neutral fraction of P. brevicompactum culture filtrate by column chromatography on silica gel

Eluant		Yield (mg.)
benzene	unidentified mixture	100
10% chloroform/benzene	acetyl tri^{n}butyl citrate	20
	deoxypebrolide	4
10-90% chloroform/ benzene	ethyl mycophenolate	2
	(erythro)-hydroxylactone	5
	mycophenolic acid	30
chloroform	pebrolide	30
2% methanol/chloroform	brevigellin	4
5% methanol/chloroform	desacetylpebrolide	45

Table III Separation of the mycelial extract of P. brevicompactum by column chromatography on silicic acid

Eluant		Yield (mg.)
50% light petroleum (60-80°)/benzene	Coenzyme Q9	1
	unidentified mixture	10
benzene	n-butyl phthalate	250
10% chloroform/benzene	tetracosanoic acid	2
10-90% chloroform/benzene	"fatty acids"	500
	ergosterol	20
-chloroform	mycophenolic acid	30

to give a brown gum (1 g.). This was chromatographed on a column of silica gel (40 g.) (Table II).

Mycophenolic acid (95), ethyl mycophenolate (168), the (erthro-) hydroxylactone (106), pebrolide (15), deoxypebrolide (17), desacetyl-pebrolide (16) and acetyl tri-^{n}butyl citrate were identified by comparison (t.l.c., mixed m.pt., i.r.) with authentic samples isolated and characterised by previous workers in this laboratory.

Separation of metabolites present in the mycelial extracts

The acetone extract of the mycelium (18 day culture) was dried, and the solvent evaporated to leave a brown oil (2 g.). This was chromatographed on a column of silicic acid (90 g.) (table III).

Coenzyme Q9(222)

P.l.c. of the crude fractions and subsequent crystallisation from methanol gave orange prisms, m. 41°C (lit. 42-43.5°C).

T.l.c.: benzene, Rf. 0.5, bn.-bk. (Ce^{4+}).

I.R.νmax. (CCl_4): 2920, 2870, 1665, 1651, 1614, 1451, 1384, 1267 cm.$^{-1}$.

UV.λmax. (EtOH): 210 (ε=51000), 270 (ε=36200) nm.

NMR. (100 Mc/s.) τ $CDCl_3$: 4.90 (4H, m., = CH.CH_2-), 6.08 (3H, s., MeO-), 6.09 (3H, s., MeO-), 6.88 (2H, d., J = 6.5Hz., $ArCH_2CH$ =), 8.07 (3H, s., Ar.Me), 8.32, 8.38, 8.46 (27H, 3s., -CH_2CH_2- and C-Me).

MS. m/e (rel. abundance): 806 (30, M^+), 235 (100), 197 (67), 69 (68).

Tetracosanoic acid

This compound crystallised from fractions eluted with 10% chloroform/benzene. Crystallisation from methanol gave colourless prisms, m. 82-86°C. (lit. 88°C.), identical (t.l.c., mixed m.pt., i.r., m.s.) with an authentic sample.

EXPERIMENTAL

CHAPTER 1

Pebrolide (15) and desacetylpebrolide (16) were isolated from the neutral fraction of the culture filtrate extract of P. brevicompactum as described in the previous section (Table II).

Pebrolide (15) crystallised as colourless needles, m. 167-170°C., from chloroform/light petroleum.

T.l.c.: 10% methanol/chloroform, Rf. 0.6: bn. (Ce^{4+}).

Desacetylpebrolide (16) crystallised as colourless needles, m. 252-255°C., from chloroform/light petroleum.

T.l.c.: 10% methanol/chloroform, Rf. 0.4; bn.-bk. (Ce^{4+}).

The following transformations were carried out in order to obtain sufficient quantities of the degradation products as were required for radiotracer studies. The identity of these compounds was established by comparison (t.l.c., mixed m.pt., i.r.) with authentic samples. Rf. values were determined using 10% methanol/chloroform as eluant.

Desacetylpebrolide (16)

To a solution of pebrolide (15) (105 mg.) in acetone (10 ml.), 6N sulphuric acid (2 ml.) was added and the solution left at room temperature for 6 hr. Extraction with chloroform (20 ml.) and subsequent evaporation of the solvent afforded desacetylpebrolide (16)

(70 mg., 70%) which crystallised from chloroform/light petroleum as colourless needles, m. 252-255°C.(Rf. 0.4).

Pebrolide ketone (27)

Pebrolide (15) (20 mg.) in acetone (5 ml.) at 0°C was treated with Jones' reagent (0.1 ml.) for 10 min., the solution diluted with ice water (5 ml.) and extracted with chloroform (10 ml.). Evaporation of the solvent gave the ketone (27) (15 mg., 75%) which crystallised from diethyl ether as colourless needles, m. 187-190°C. (Rf. 0.8).

Keto acid (29)

Desacetylpebrolide (16) (50 mg.) was oxidised with Jones' reagent using a procedure similar to that used in the previous reaction. Evaporation of the solvent yielded the keto acid (29) (35 mg., 65%) which crystallised as colourless needles, m. 123-125°C. from chloroform/light petroleum (Rf. 0.45).

Diketo methyl ester (32)

The keto acid (29) (35 mg.) was dissolved in 4N sodium hydroxide (5 ml.) and the solution refluxed for 4 hr., acidified with dilute hydrochloric acid and extracted with chloroform (10 ml.). Evaporation of the solvent gave the alcohol (30) which, without purification, was dissolved in acetone (5 ml.) and treated with Jones' reagent (0.1 ml.) at 0°C. for 10 min. Ice water (5 ml.) was added and the mixture extracted with chloroform (10 ml.). Evaporation of the solvent gave the crude diketo acid (31) (16 mg.). The crude acid was dissolved in methanol (2 ml.) and treated with an excess of ethereal diazomethane

for 5 min. Filtration and evaporation of the solvent gave an oil, from which the diketo methyl ester (32) crystallised on standing. The crystals were washed with light petroleum to remove traces of methyl benzoate. Recrystallisation from chloroform/light petroleum gave the diketo methyl ester (32) (10 mg., 30%) as colourless needles, m. 182-186°C. (Rf. 0.7).

Table 1:I Degradation of [^{14}C,T] pebrolide (I)

Compound	wt./mg.	dpm. ^{14}C	dpm. T	T/^{14}C (dpm.)
pebrolide (15a)	1.60 (x6)	113	3340	29
	1.55 (x7)	107	3060	
(27a)	2.10 (x2)	150	3580	22.5
	2.20 (x3)	158	3610	

Table 1:II

Compound	wt./mg.	dpm. ^{14}C	dpm. T	T/^{14}C (dpm.)
pebrolide (15a)	1.09 (x6)	34.0	914	26.5
	0.67 (x7)	21.1	552	
desacetylpebrolide (16a)	1.22 (x6)	7.9	190	24.2

Table 1:III Degradation of [^{14}C,T] pebrolide (II)

Compound	wt./mg.	dpm. ^{14}C	dpm. T	T/^{14}C (dpm.)
(29a)	1.99 (x2)	75.0	907	11.5
	2.00 (x3)	74.3	825	
(32a)	0.62 (x2)	30.0	330	10.8
	0.64 (x3)	31.1	324	
(32b)	0.70 (x2)	34.4	6.38	--
	0.71 (x3)	33.4	8.08	

Feeding of labelled precursors to cultures of P. brevicompactum (I)

DL-[2-^{14}C] mevalonic acid lactone (0.05 mCi; 5.03 mCi/mM) and DL-[2-T] mevalonic acid lactone (2 mCi; 90 mCi/mM) were dissolved in sterile water (11 ml.). 10 ml. of this solution was added to the culture filtrates of a 4 day old surface culture of P. brevicompactum (5 Roux bottles). The cultures were harvested after a further 48 hr.

Isolation of [^{14}C,T] pebrolide

The culture filtrate and mycelial washings (1 l.) from the above, were stirred with charcoal (10 g.) for 2 hr. and the charcoal subsequently extracted in a soxhlet apparatus with acetone. The acetone extract was partitioned between chloroform and water, and the chloroform extract washed with saturated aqueous sodium bicarbonate. To the dried chloroform extract, inactive pebrolide (187 mg.) was added. After precipitation with light petroleum, crystallisation gave pebrolide (150 mg.), homogeous by t.l.c. Seven successive crystallisations yielded material of constant activity (Table 1:I).

Degradation of [^{14}C,T] pebrolide

[^{14}C,T] Pebrolide ketone

[^{14}C,T] pebrolide (100 mg.) was oxidised to pebrolide ketone as described above. Two successive crystallisations gave chemically pure (t.l.c., mixed m.pt., i.r., identical with an authentic sample) pebrolide ketone (50 mg.). Repeated preparative t.l.c. on HF_{254}

silica, using chloroform as eluant, followed by further crystallisation gave material of constant activity (Table 1:I)

Feeding of labelled precursors to cultures of P. brevicompactum (II)

DL-[2-^{14}C] mevalonic acid lactone (0.1 mCi.; 99 mCi./mM) and DL-[2-T] mevalonic acid lactone (4 mCi./mM) were dissolved in sterile water (25 ml.). 24 ml. of this solution was added to the culture filtrates of a 3 day old surface culture of P. brevicompactum (11 Roux bottles). The cultures were harvested after a further 20 hr.

Determination of total activity of precursor added

0.25 ml. of the precursor solution (containing 1/100 of the total activity) was diluted to 100 ml. with distilled water. 0.2 ml. aliquots of this solution were counted in biosolve/toluene scintillator. Total activity fed (24/25 of precursor solution): 0.090 mCi. ^{14}C; 3.15 mCi. T; T/^{14}C = 36.0.

Isolation of [^{14}C,T] pebrolide and [^{14}C,T] desacetylpebrolide

The procedure followed was essentially that of the previous experiment. To the dried chloroform extract, inactive pebrolide (205 mg.) and desacetylpebrolide (140 mg.) were added and the mixture chromatographed on a column of silica gel (40 g.). Fractions eluted with chloroform contained crude pebrolide (260 mg.). Fractions eluted with 5% methanol/chloroform contained crude desacetylpebrolide (150 mg.)

[^{14}C,T] Pebrolide (t.l.c., mixed m.pt., i.r. identical to an authentic sample) was isolated from the crude column fractions by preparative t.l.c. on HF_{254} silica, using 5% methanol/chloroform

as eluant, and subsequent crystallisation. Six successive crystallisations yielded material of constant activity (Table 1:II).

The crude desacetylpebrolide was purified by crystallisation from chloroform/light petrol giving [^{14}C,T] desacetylpebrolide (t.l.c., mixed m.pt., i.r. identical to an authentic sample). Five successive crystallisations gave material of constant activity (Table 1:II).

Degradation of [^{14}C,T] pebrolide

[^{14}C,T] Pebrolide (120 mg.) was hydrolysed to (28a) (83 mg.) and (28a) oxidised to (29a) (69 mg.). Basic hydrolysis of (29a) (60 mg.) afforded (30a) (25 mg.) which was oxidised to (31a) (16 mg.). Methylation of (31a) afforded (32a) (15 mg.).

The diketo methyl ester (32a) (9 mg.) was refluxed with 5N sodium hydroxide (5 ml.) for 4 hr., the solution neutralised with dilute hydrochloric acid and extracted with chloroform (5 ml.). Evaporation of the solvent afforded the diketo acid (31b) which was esterified with ethereal diazomethane in methanol giving the diketo methyl ester (32b) (4.5 mg.)

The above compounds were purified to constant activity by successive crystallisation (Table 1:III).

EXPERIMENTAL

CHAPTER 2:1

Methyl O-methylmycophenolate (117)

Mycophenolic acid (200 mg.) in methanol (10 ml.) was treated with an excess of ethereal diazomethane and the solution left overnight at room temperature. Filtration and evaporation of the solvent afforded methyl O-methylmycophenolate (117) (216 mg., 99%) which crystallised from diethyl ether/light petroleum as colourless needles, m. 57-58°C (lit.[100] 58°C), identical (t.l.c., mixed m.pt., i.r.) with an authentic sample.

O-Methylmycophenolic acid (96)

A solution of methyl O-methylmycophenolate (117) (170 mg.) in methanol (2 ml.) was refluxed with 2N sodium hydroxide (20 ml.) for 15 hr. After acidification with dilute aqueous hydrochloric acid, extraction with chloroform (30 ml.) gave O-methylmycophenolic acid (96) (120 mg., 75%) which crystallised from ethyl acetate/light petroleum as colourless needles, m. 109-112°C (lit. 112°C), identical (t.l.c., mixed m.pt., i.r.) with an authentic sample.

T.l.c.: 5% methanol/chloroform, Rf. 0.38, gr. (Ce^{4+}).

(erythro-) 5,7-Dimethoxy-6 2-hydroxy-2(methyl-5-oxotetrahydro-2-furyl) ethyl -4-methylphthalan-1-one (105)

To a solution of O-methylmycophenolic acid (96) (100 mg.) in 2N aqueous potassium hydroxide at 100°C, 0.5 ml. aliquots of a solution of potassium permanganate (1 g.) in 0.45N aqueous potassium hydroxide (20 ml.) were added at 5 min. intervals until the purple colour persisted (approximately 6 ml. added). The solution was filtered, reduced to 20 ml., acidified to pH 2 with dilute aqueous hydrochloric

acid, and subjected to constant extraction with ether overnight. Evaporation of the ether and subsequent p.l.c. of the residue on HF_{254} silica, using 5% methanol/chloroform as eluant, gave the dimethoxy-hydroxylactone (105) (15 mg., 14%) which crystallised from chloroform/light petroleum as colourless needles, m. 150-151°C. (mixed m.pt. with the (threo-)dimethoxy-hydroxylactone (192) - 130-135°C.)

T.l.c.: chloroform, Rf. 0.56; bn. (Ce^{4+}).

Analysis: Found C: 61.58%, H: 6.19%.

$C_{18}H_{22}O_7$ requires C: 81.71%, H: 6.33%.

I.R. ν max. (KBr): 3480, (ν_{OH}), 2980, 2940, 2870, 1755 (ν_{CO}), 1604, 1477, 1203, 1130, 1075, 943, 870, 789 $cm.^{-1}$.

UV. λ max. (EtOH): 226 (ε=20000), 251 (ε=9930), 296 (ε=3180) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.85 (2H, 1., $ArCH_2O$-), 5.92 (3H, s., MeO-), 6.16 (3H, s., MeO-), 6.36 (1H, m., -CH(OH)-), 6.9-7.5 (4H, m.), 7.15 (1H, exchangeable with D_2O), 7.6-8.0 (2H, m.), 7.80 (3H, s., ArMe), 8.49 (3H, s., C-Me)

MS. M/e (rel. abundance): 350 (M) (13), 251 (100), 221 (68), 99 (40).

(erythro-) 5,7-Dimethoxy-6 2-hydroxy-2(methyl-5-oxotetrahydro-2-furyl) ethyl -4-methylphthalic anhydride (104)[102]

O-Methylmycophenolic acid (96) (70 mg.) in 2N aqueous potassium hydroxide (2 ml.) was treated at reflux with an alkaline solution of potassium permanganate as before. The solution was refluxed for 2 hr., filtered, made acid with dilute aqueous hydrochloric acid and

subjected to constant extraction with ether overnight. Evaporation of the solvent gave a yellow oil. After drying in a desiccator over potassium hydroxide, the oil was treated with a small volume of ether to give the dimethoxyphthalic anhydride (104), as colourless crystals, which crystallised from chloroform/light petroleum as prisms, m. 149-151°C. (10 mg., 13%).

T.l.c.: chloroform, Rf. 0.55, y.-bn. (Ce^{4+}).

Analysis: Found C: 59.37%, H: 5.48%

Calculated for $C_{18}H_{20}O_8$ C: 59.34%, H: 5.53%.

I.R. ν max. (KBr): 3560, 3460, 2985, 2958, 2860, 1840 (ν_{CO} anhydride), 1770 (ν_{CO} γ-lactone), 1616, 1588, 1471, 1220, 978, 752, 639 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 5.83 (3H, s., MeO-), 6.16 (3H, s., MeO-), 6.35 (1H, m., -CH(OH)-), 6.8-7.4 (5H, m., 1H exchangeable with D_2O), 7.45 (3H, s., ArMe), 7.5-8.0 (2H, m.), 8.52 (3H, s., C-Me).

MS. m/e (rel. abundance): 364 (M^+) (6), 346 (5), 266 (99), 99 (100).

Lactonisation of mycophenolic acid

(a) A solution of mycophenolic acid (100 mg.) in trifluoroacetic acid (5 ml.) was left at room temperature for 1 hr. Evaporation gave the lactone (108) (95 mg., 95%) which crystallised from chloroform/light petroleum as colourless needles, m. 161-162°C. Identical (t.l.c., mixed m.pt., i.r.) to an authentic sample.

(b) Mycophenolic acid (100 mg.) in chloroform (5 ml.) was treated with 3 drops of borontrifluoride etherate solution and the solvents

evaporated almost immediately giving the lactone (108) (98 mg., 98%) identical (t.l.c., mixed m.pt., i.r.) to an authentic sample.

5,7-Dimethoxy-4-methyl-6 2(2-methyl-5-oxotetrahydro-2-furyl)ethyl phthalan-1-one (109)

The lactone (108) (50 mg.) in ether (5 ml.) was treated with an excess of ethereal diazomethane and the solution left overnight at room temperature. Filtration and evaporation of the solvent gave a colourless oil, b.pt. 150°C/0.003 mm. (50 mg., 96%). The distillate crystallised from chloroform/light petroleum giving the dimethoxylactone (109) as needles, m. 72-73°C.

T.l.c.: chloroform, Rf. 0.60, y. (Ce^{4+}).

Analysis: Found C: 64.71%, H: 6.48%

$C_{19}H_{22}O_6$ requires C: 64.66%, H: 6.63%

I.R. νmax. (film): 2980, 1759, 1605, 1140, 768 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.86 (2H, s., $ArCH_2O$-), 5.90 (3H, s., MeO-), 6.16 (3H, s., MeO-), 7.0-7.5 (4H, m.), 7.80 (3H, s., ArMe), 7.7-8.3 (4H, m.), 8.50 (3H, s., C-Me).

MS. m/e (rel. abundance): 334 (M^+) (48), 221 (100), 220 (99), 99 (42).

Oxidation products of the dimethoxylactone (109) 3-Hydroxy-5,7-dimethoxy-4-methyl-6 2(2-methyl-5-oxotetrahydro-2-furyl)ethyl phthalan-1-one (110) and 1,2-Carbomethoxy-3,5-dimethoxy-6-methyl-4 2(2-methyl-5-oxotetrahydro-2-furyl)ethyl - benzene (112)

A refluxing solution of the dimethoxylactone (109) (200 mg.) in aqueous potassium hydroxide (4 ml.) was treated with alkaline

potassium permanganate solution and extracted as before. The products, in methanol (5 ml.), were treated with an excess of ethereal diazomethane for 30 min. The solution was filtered and the solvent evaporated. P.l.c. of the residue on HF_{254} silica, using 10% methanol/chloroform as eluant, gave starting material (90 mg.), the pseudo acid (110) (30 mg., 29%) and the dimethyl phthalate (112) (6 mg., 5%).

The pseudo acid (110) was obtained as a yellow oil which decomposed slightly on distillation (b.pt. 170°C/0.01 mm.).

T.l.c.: 10% methanol/chloroform, Rf. 0.7; gr. (Ce^{4+}).

I.R. νmax.($CHCl_3$): 3400 (br., ν_{OH}), 3022 (ν_{OH}), 2981, 2950, 1764 (ν_{CO}), 1600 $cm.^{-1}$.

νmax.(film): 2450 (br., ν_{OH}), 1760 (ν_{CO}), 1603, 1478, 1390, 1312, 1150, 970, 777 $cm.^{-1}$.

NMR.(60 Mc/s.) τ $CDCl_3$: 3.52 (1H, s., Ar<u>CH</u>(OH)O-), 5.92 (3H, s., <u>Me</u>O-), 6.19 (3H, s., <u>Me</u>O-), 7.0-7.5 (5H, m., 1H exchangeable with D_2O), 7.68 (3H, s., Ar<u>Me</u>), 7.83-8.3 (4H, m), 8.52 (3H, s., C-<u>Me</u>).

MS. m/e (rel. abundance): 350 (M^+) (23), 332 (100), 235 (26), 149 (40), 99 (40).

The dimethyl phthalate (112) was isolated as a viscous oil.

T.l.c.: chloroform, Rf. 0.65, bn. (Ce^{4+}).

I.R. ν max. (CCl_4): 2955, 1780 (ν_{CO} γ-lactone), 1741 (ν_{CO} phthalate), 1566, 1327, 1224 $cm.^{-1}$.

NMR. (60 Mc/s.) $\tau CDCl_3$: 6.03 (6H, s., 2MeO-), 6.11 (3H, s., MeO-), 6.19 (3H, s., MeO-), 7.0-7.5 (4H, m.), 7.66 (3H, s., ArMe), 7.7-8.1 (4H, m.), 8.45 (3H, s., C-Me).

1,2-Dihydroxymethyl-3-hydroxy-4(6-hydroxy-3-methylhex-2-enyl)-5-methoxy-6-methylbenzene (115)

A solution of mycophenolic acid (95 mg.) in dry tetrahydrofuran (5 ml.) was slowly added to a stirred suspension of lithium aluminium hydride (65 mg.) in dry tetrahydrofuran (5 ml.) and the solution refluxed for 3 hr. To the cooled reaction mixture saturated aqueous sodium sulphate (2 ml.) was added and the solution stirred for a further 30 min. The reaction mixture was diluted with water (5 ml.) and extracted with ethyl acetate (2 x 10 ml.). Evaporation of the solvent and p.l.c. of the residue on HF_{254} silica, using 10% methanol/chloroform as eluant, gave the triol (115) (20 mg., 21%) as a yellow gum.

T.l.c.: 10% methanol/chloroform, Rf. 0.4; bl. ($FeCl_3$), bn. (Ce^{4+}).

I.R. νmax. (film): 3500 (br., ν_{OH}), 1605, 1475, 1100, 975, 720 $cm.^{-1}$.

NMR. (60 Mc/s.) $\tau CDCl_3$: 4.80 (1H, m., =CHCH$_2$-), 5.4 (4H, m., ArCH$_2$OH), 6.35 (3H, s., MeO-), 6.55 (4H, m., ArCH$_2$CH = -CH$_2$OH), 7.76 (3H, s., ArMe), 7.5-8.5 (7H, m., 3H exchangeable with D_2O), 8.22 (3H, br., s., C-Me).

Mycochromanic acid (116)

(a) A solution of mycophenolic acid (430 mg.) in 3% sulphuric acid/glacial acetic acid was refluxed for 4 hr. The cooled solution was

extracted with chloroform (20 ml.). Evaporation of the solvent and crystallisation of the residue from chloroform/light petroleum afforded mycochromanic acid (116) (380 mg., 90%) as colourless needles, m. 172-175°C, identical (t.l.c., mixed m.pt., i.r.) with an authentic sample.

Addition of light petroleum to the mother liquor of the foregoing crystallisation gave the lactone (108) (20 mg., 5%) as colourless needles, m. 161-162°C, identical with an authentic sample.

(b) A solution of the lactone (108) (60 mg.) in 3% sulphuric acid/glacial acetic acid was refluxed for 12 hr. Extraction with chloroform (10 ml.), evaporation of the solvent and subsequent p.l.c. on HF_{254} silica, using 5% methanol/chloroform as eluant, gave starting material (25 mg.) and mycochromanic acid (116) (30 mg., 50%).

2,3-Dihydroxymethyl-8(3-hydroxypropyl)-5-methoxy-4,8-dimethyldihydro 1 benzopyran (118)

A suspension of lithium aluminium hydride (100 mg.) in dry tetrahydrofuran (10 ml.) was slowly added to a stirred solution of mycochromanic acid (200 mg.) in dry tetrahydrofuran and the solution refluxed for 1 hr. To the cooled reaction mixture saturated aqueous sodium sulphate (2 ml.) was added and the solution stirred for a further 30 min. The reaction mixture was diluted with water (10 ml.) and extracted with ethyl acetate (20 ml. x 2). Evaporation of the solvent gave the triol (118) (200 mg., 98%) as a colourless oil, which decomposed on attempted vacuum distillation.

T.l.c.: 5% methanol/chloroform, Rf. 0.45; r.-pur. (Ce^{4+}).

I.R.νmax. (film): 3500 (br.ν_{OH}), 3000, 1630, 1580, 1450, 1320, 1110, 1000, 770 $cm.^{-1}$.

NMR. (60 Mc/s.)$\tau$$CDCl_3$: 5.39 (4H, m., 2Ar$\underline{CH_2}$OH), 6.36 (3H, s., MeO-), 6.50 (2H, m., $CH_2\underline{CH_2}$OH), 7.2-7.5 (2H, m., Ar$\underline{CH_2}CH_2$-), 7.60 (3H, s., exchangeable with D_2O), 7.75 (3H, s., Ar$\underline{Me}$), 8.2 (6H, m.), 8.90 (3H, s., C-$\underline{Me}$).

The triol (118) (50 mg.) was dissolved in pyridine (1 ml.), acetic anhydride (0.5 ml.) and allowed to stand overnight at room temperature. Evaporation of the reagents afforded the triacetate (118A) (60 mg.), b.pt. 190°C/0.03 mm.

T.l.c.: chloroform, Rf. 0.8; bn. (Ce^{4+}).

Analysis: Found C: 63.83%, H: 7.53%,

$C_{23}H_{32}O_8$ requires C: 63.30%, H: 7.40%.

I.R.ν max. (CCl_4): 3385, 3354, 1747, 1604, 1590, 1384, 1366, 1331, 1241, 1118, 1028 $cm.^{-1}$.

ν max. (KBr): 3318, 3282, 1744, 1603, 1590, 1390, 1370, 1334, 1250, 1119, 1031 $cm.^{-1}$.

NMR. (60 Mc/s.)$\tau$$CDCl_3$: 4.80 (2H, s., Ar$CH_2$OH), 4.81 (2H, s., Ar$\underline{CH_2}$OH), 5.92 (2H, t., J = 5.5 Hz., -$CH_2\underline{CH_2}$OH), 6.30 (3H, s., MeO-), 7.21 (2H, t., J = 6.5 Hz., Ar$\underline{CH_2}CH_2$-), 7.76 (3H, s., Ar$\underline{Me}$), 7.97 (3H, s., $\underline{Me}$.CO.O-), 7.98 (6H, s., 2$\underline{Me}$.CO.O-), 8.0-8.4 (4H, m.), 8.73 (3H, s., C-$\underline{Me}$).

MS. m/e (rel. abundance): 436 (M^+) (67), 408 (17), 376 (73), 348 (87), 334 (46), 316 (69), 235 (67), 215 (100), 193 (95).

m*: 324 (436→376), 296.5 (376→335), 265.6 (376→316), 146.5 (316→215).

Oxidation of the triol (118)

(a) With "active" manganese dioxide

The triol (118) (160 mg.) in dry acetone was stirred with powdered manganese dioxide (1.6 g.) for 48 hr. under nitrogen. The mixture was filtered and the solvent evaporated leaving an oil (90 mg.). Multiple p.l.c. on HF_{254} silica, using 5% methanol/chloroform (x1) and chloroform (x3) as eluants, afforded the isomeric alcohols (119) and (120).

2(3-hydroxypropyl)-5-methoxy-2,6-dimethyldihydro 1 benzopyran-9 7H - one (119)

(33 mg., 20%) crystallised from chloroform/light petroleum as colourless prisms, m. 148-150°C.

T.l.c.: chloroform, Rf. 0.4; bn. (Ce^{4+}).

Analysis: Found C: 66.84%, H: 7.35%,

$C_{17}H_{22}O_5$ requires C: 66.65%, H: 7.24%.

I.R. ν max. ($CHCl_3$): 3525 (ν_{OH}), 2935, 2875, 1760 (ν_{CO} phthalide), 1607 $cm.^{-1}$.

ν max. (KBr): 3425 (ν_{OH}), 2925, 2860, 1760 (ν_{CO} phthalide), 1604, 1325, 1133, 1104, 1032, 787 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.93 (2H, s., $Ar\underline{CH_2}O$-), 6.18 (3H, s., $\underline{Me}O$-), 6.25 (2H, m., $-CH_2\underline{CH_2}OH$), 7.21 (2H, t., J = 6Hz., $Ar\underline{CH_2}CH_2$-), 7.83 (3H, s., $Ar\underline{Me}$), 7.95 (1H, exchangeable with D_2O), 8.21 (4H, br., s.), 8.0-8.4 (2H, obs.), 8.65 (3H, s., C-$\underline{Me}$).

MS. m/e (rel. abundance): 306 (M^+) (37), 288 (10), 247 (98), 207 (100), 159 (98), 85 (97),

m*: 271 (306→288), 122.5 (207→159).

2(3-hydroxypropyl)-5-methoxy-2,6-dimethyldihydro 1 benzopyran-7 9H - one (120)

(48 mg., 30%) a colourless oil, b.pt. 160°C/0.003 mm.

T.l.c.: chloroform, Rf. 0.48; bn. (Ce^{4+}).

Analysis: Found C: 66.40%, H: 7.16%,

$C_{17}H_{22}O_5$ requires C: 66.65%, H: 7.24%.

I.R. ν max. ($CHCl_3$): 3620 (ν_{OH}), 2950, 2880, 1755 (ν_{CO} phthalide), 1618 $cm.^{-1}$.

νmax. (KBr): 3430 (ν_{OH}), 2935, 2864, 1755 (ν_{CO} phthalide), 1620, 1477, 1380, 1329, 1116, 1030, 782 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.95 (2H, s., $Ar\underline{CH_2}O$-), 6.25 (3H, s., $\underline{Me}O$-), 6.38 (2H, m., $-CH_2\underline{CH_2}OH$), 7.20 (2H, t., J = 6Hz., $Ar\underline{CH_2}CH_2$-), 7.50 (3H, s., $Ar\underline{Me}$), 8.19 (2H, t., J = 6Hz., $Ar\underline{CH_2}CH_2$-), 8.3 (1H, exchangeable with D O), 8.32 (4H, br., s., $-\underline{CH_2}\underline{CH_2}CH_2OH$), 8.68 (3H, s., C-$\underline{Me}$).

MS. m/e (rel. abundance): 306 (M^+) (83), 247 (67), 207 (93), 177 (67), 85 (100).

(b) With silver carbonate/celite (Fetizon's reagent)

The triol (118) (55 mg.) and silver carbonate/celite (1.1/ 1) (60 mg.) were weighed into a dry flask and benzene (10 ml.) was distilled (from sodium) into the flask. The mixture was stirred and refluxed for 20 hr. The solvent was evaporated and the residue extracted in a Soxhlet apparatus using ethyl acetate (50 ml.) for 12 hr. Evaporation of the ethyl acetate and multiple p.l.c. of the residue, as above, gave the isomeric alcohols (119) (8 mg., 16%) and (120) (12 mg., 24%). The products were identified by t.l.c. and i.r. comparison with samples from the previous oxidation.

(c) With chromium trioxide (sulphuric acid (Jones' reagent)

(i) A solution of the triol (118) (140 mg.) in acetone (10 ml.) was treated with Jones' reagent (0.2 ml.) for 5 min., diluted with ice water (5 ml.) and extracted with ethyl acetate (20 ml.). The extract was dissolved in methanol (5 ml.) and treated briefly with ethereal diazomethane. Filtration, evaporation of the solvent and multiple p.l.c. of the residue on HF_{254} silica, using 2% methanol chloroform as eluant, afforded methyl mycochromanate (123) (28 mg., 19%) identical (t.l.c., i.r., n.m.r.) with an authentic sample, and the isomeric ester, 2(2-carbomethoxyethyl)-5-methoxy-2,6-dimethyldihydro 1 benzopyran-7 9H -one (125) (30 mg., 20%) as a colourless oil, b.pt. 160°C/0.005 mm.

T.l.c.: chloroform, Rf. 0.50, bn. (Ce^{4+}).

Analysis: Found C: 64.41%, H: 6.61%,

$C_{18}H_{22}O_6$ requires C: 64.66%, H: 6.36%.

I.R. ν max. (CCl_4): 2940, 1764 (ν_{CO} phthalide), 1742 (ν_{CO} ester), 1615, 1323, 1112, 1030, 1015 $cm.^{-1}$.

ν max. (KBr): 2930, 1760 (ν_{CO} phthalide), 1740 (Sh. ν_{CO} ester), 1618, 1472, 1325, 1112, 1024, 993, 780 $cm.^{-1}$.

NMR. (60 Mc/s.) $\tau CDCl_3$: 4.92 (2H, s., $ArCH_2O$-), 6.21 (3H, s., MeO-), 6.33 (3H, s., MeO.CO-), 7.14 (2H, t., J = 7.5 Hz., $ArCH_2CH$), 7.48 (3H, s., ArMe), 7.4-7.7 (2H, m.), 7.8-8.1 (2H, m.), 8.16 (2H, t., J = 7.5 Hz., $ArCH_2CH_2$-), 8.7 (3H, s., C-Me).

MS. m/e (rel. abundance): 334 (M^+) (100), 274 (59), 208 (82), 207 (56), 177 (47), 149 (43), 95 (35).

(ii) A solution of the triol (118) (137 mg.) in acetone at 0°C was treated with Jones' reagent (0.1 ml.) for 30 sec., diluted with ice water and extracted as before. Evaporation of the solvent and p.l.c. of the residue on HF_{254} silica, using 2% methanol/chloroform as eluant, afforded starting material (10 mg.), the isomeric alcohols (120) (14 mg., 12%) and (119) (11 mg., 9%) and two less polar compounds (126) (3 mg.) and (127) (4 mg.).

(126) T.l.c.: chloroform, Rf. 0.60; m. (Ce^{4+}).

I.R. ν max. (CCl_4): 2940, 2715 (ν_{CH} aldehyde), 1760 (ν_{CO} phthalide), 1726 (ν_{CO} aldehyde), 1610, 1470 $cm.^{-1}$.

(127) T.l.c.: chloroform, Rf. 0.65; bn. (Ce^{4+}).

I.R. ν max. (CCl_4): 2950, 2720 (ν_{CH} aldehyde), 1770 (ν_{CO} phthalide), 1726 (ν_{CO} aldehyde), 1610, 1473 $cm.^{-1}$.

Methyl mycochromanate (123)

Mycochromanic acid (116) (24 mg.) in methanol (5 ml.) was treated with an excess of ethereal diazomethane for 5 min., the solution filtered and the solvent evaporated to give methyl mychromanate (123) (26 mg.), a colourless oil, b.pt. 165°C/0.004 mm.

T.l.c.: chloroform, Rf. 0.45; bn. (Ce^{4+}).

Analysis: Found C: 64.40%, H: 6.57%,

$C_{18}H_{22}O_6$ requires C: 64.66%, H: 6.63%.

I.R. ν max. (CCl_4): 2985, 2955, 2880, 1774 (ν_{CO} phthalide), 1740 (ν_{CO} ester), 1608, 1473, 1335, 1327, 1302, 1036, 872 $cm.^{-1}$.

ν max. (KBr): 2950, 1763 (ν_{CO} phthalide), 1738 (ν_{CO} ester), 1606, 1334, 1323, 1300, 1032, 870, 787 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.95 (2H, s., $ArCH_2O$-), 6.20 (3H, s., MeO-), 6.35 (3H, s., MeO-), 7.20 (2H, t., J = 7.5 Hz., $ArCH_2CH_2$-), 7.1-7.6 (2H, m.), 7.9-8.3 (2H, m.), 7.88 (3H, s., ArMe), 8.16 (2H, t., J = 7.5 Hz., $ArCH_2CH_2$-), 8.66 (3H, s., C-Me).

MS. m/e (rel. abundance): 334 (M^+) (58), 316 (49), 247 (100), 207 (98.5), 159 (61).

m*: 299 (334→316).

1,2 Dihydroxymethyl-3,5-dimethoxy-4(6-hydroxy-3-methylhex-2-enyl)-6-methylbenzene (128)

To a stirred solution of methyl O-methylmycophenolate (117) (570 mg.) in dry tetrahydrofuran (15 ml.) at room temperature, a suspension of lithium aluminium hydride (153 mg.) in dry tetrahydrofuran (15 ml.) was added over 15 min. The mixture was stirred for 2 hr., saturated aqueous sodium sulphate (5 ml.) added and stirred for a further 30 min. The solvent was partially evaporated and the mixture extracted with ethyl acetate (2 x 20 ml.). Evaporation of the solvent gave the triol (128) (370 mg., 72%) which crystallised as colourless prisms, m. 91°C, from chloroform/light petrol.

T.l.c.: 5% methanol/chloroform, Rf. 0.45, r.-bn. (Ce^{4+}).

Analysis: Found C: 65.89%, H: 8.67%,

$C_{17}H_{26}O_5$ requires C: 65.78%, H: 8.44%.

I.R. ν max. ($CHCl_3$): 3610, 1480 (ν_{OH}, br.), 2940, 1572 $cm.^{-1}$.

ν max. (KBr): 3300 (ν_{OH}, br.), 2940, 2903, 1579, 1410, 1101, 974, 722 $cm.^{-1}$.

UV. λ max. (EtOH): 260 (ε=104), 275 (ε=4140), 283 (ε=4660) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.82 (1H, t., J = 7 Hz., =C<u>H</u>.CH_2-), 5.30 (2H, s., Ar<u>CH_2</u>OH), 5.33 (2H, s., Ar<u>CH_2</u>OH), 6.27 (3H, s., <u>Me</u>O-), 6.34 (3H, s., <u>Me</u>O-), 6.3-6.8 (3H, obs., exchangeable with D_2O), 6.53 (2H, t., J = 6 Hz., -CH_2<u>CH_2</u>OH), 6.65 (2H, t., J = 7 Hz., Ar<u>CH_2</u>CH=), 7.69 (3H, s., Ar<u>Me</u>), 7.8-8.5 (4H, m., -<u>CH_2</u><u>CH_2</u>-), 8.21 (3H, s., C-<u>Me</u>)

MS. m/e (rel. abundance): 324 (M^+), 306 (80), 288 (62), 275 (33), 247 (58), 207 (83), 193 (58), 85 (100).

m*: 271 (306→288).

Oxidation of the triol (128)

(a) With "active" manganese dioxide

The triol (128) (60 mg.) in dry chloroform (20 ml.) was stirred with powdered manganese dioxide (600 mg.) under nitrogen for 24 hr. Filtration and evaporation of the solvent gave an oily mixture of the isomeric alcohols, 5,7-dimethoxy-6(6-hydroxy-3-methylhex-2-enyl)-4-methylphthalan-1-one (131) (10 mg.) and 4,6-dimethoxy-6(6-hydroxy-3-methylhex-2-enyl)-4-methylphthalan-1-one (132) (20 mg.) which could not be separated by p.l.c.

Conversion to the corresponding 6′-acetoxy derivatives was accomplished using a procedure similar to that used in the acetylation of the triol (118). The 6′-acetoxy derivatives could not be distinguished from each other on t.l.c.

Attempted selective demethylation of the alcohol (131)

The above mixture of alcohols (27 mg.) in refluxing dry benzene (10 ml.) was treated with "magnesium iodide etherate" reagent (0.2 ml.) and the solution stirred and refluxed for 3 hr. under nitrogen. The cooled solution was washed with 5N hydrochloric acid (10 ml.), water (10 ml.) added, and extracted with chloroform (10 ml.). Evaporation of the solvent and p.l.c. of the residue on HF_{254} silica, using 2% methanol/chloroform as eluant, gave an oil (15 mg.),

apparently homogeneous by t.l.c. but consisting of a mixture of the isomeric tetrahydrofurans (129) and (130).

T.l.c.: chloroform, Rf. 0.6; bn. (Ce^{4+}).

I.R. ν max. (film): 3500, 3000, 1745 (ν_{CO} phthalides), 1610, 1475, 1050, 770 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.68 (2H, s., $ArCH_2O$-), 4.86 (1H, s., $ArCH_2O$-), 9.51 ($1\frac{1}{2}$H, s., MeO-), 6.08 (3H, s., MeO-), 6.16 ($1\frac{1}{2}$H, s., MeO-), 6.22 (3H, s., MeO-), 7.0-7.5 (6H, m.), 7.42 (3H, s., ArMe), 7.80 ($1\frac{1}{2}$H, s., ArMe), 7.8-8.6 (9H, m.), 8.66 ($4\frac{1}{2}$H, s., C-Me).

(b) With "aged" manganese dioxide 4,6 dimethoxy-1-hydroxy-5(6-hydroxy-3-methylhex-2-enyl)-3-methylphthalan (137)

"Active" manganese dioxide prepared by the method of Attenburrow et al.,[149] and stored at room temperature and humidity for 12 months was used in the following experiments.

The triol (128) (97 mg.) in dry chloroform was stirred at room temperature under nitrogen with powdered manganese dioxide (1 g.) until t.l.c. of the mixture indicated that no starting material remained (5 hr.). P.l.c. of the product on HF_{254} silica, using 2% methanol/chloroform as eluant, gave a mixture of the isomeric alcohols (131) and (132) (10 mg.) and the hemiacetal (137) (70 mg., 82%) as an unstable colourless oil.

T.l.c.: 5% methanol/chloroform, Rf. 0.5; bn. (Ce^{4+}).

I.R. ν max. ($CHCl_3$): 3500 (br. ν_{OH}), 2835, 1655, 1600 $cm.^{-1}$.

NMR. (60 Mc/s.) $\tau CDCl_3$: 3.6 (1H, br. s., irr. 4.8→s., Ar<u>CH</u>(OH)O-), 4.75 (1H, m. = <u>CH</u>CH$_2$-), 4.8 (2H, br.,s., irr. 3.6→s., Ar<u>CH$_2$</u>O), 6.24 (3H, s., <u>Me</u>O-), 6.28 (3H, s., <u>Me</u>O-), 6.28 (3H, s., <u>Me</u>O-), 6.45 (2H, t., J = 6.5 Hz., -CH$_2$<u>CH$_2$</u>OH), 6.65 (2H, d., J = 7.5 Hz., Ar<u>CH$_2$</u>CH=), 7.72 (3H, s., Ar<u>Me</u>), 7.6-8.4 (6H, m., 2H exchangeable with D_2O), 8.22 (3H, br., s., C-<u>Me</u>).

<u>5(5-carboxy-3-methylpent-2-enyl)-4,6-dimethoxy-7-methylphthalan-1-one (139)</u>

The hemiacetal (137) (70 mg.) was dissolved in dry chloroform (15 ml.) and stirred with powdered manganese dioxide (700 mg.) under nitrogen for 12 hr. Filtration and evaporation of the solvent afforded <u>the alcohol (132)</u> (65 mg., 95%) as an oil.

I.R. ν max. ($CHCl_3$): 3500 (br., ν_{OH}), 2940, 1760 (ν_{CO} phthalide), 1600 cm.$^{-1}$.

NMR. (60 Mc/s.) $\tau CDCl_3$: 4.60 (2H, s., Ar<u>CH$_2$</u>O-), 4.81 (1H, m., = <u>CH</u>.CH$_2$-), 6.14 (3H, s., <u>Me</u>O-), 6.25 (3H, s., <u>Me</u>O-), 6.41 (2H, t., J = 6.5 Hz., -CH$_2$<u>CH$_2$</u>OH), 6.58 (2H, d., J = 6.5 Hz., Ar<u>CH$_2$</u>CH=), 7.47 (3H, s., Ar<u>Me</u>), 7.7-8.6 (5H, m., 1H exchangeable with D_2O), 8.20 (3H, br., s., C-<u>Me</u>).

The alcohol (132) (65 mg.) in ice cold acetone (2 ml.) was treated with Jones' reagent (0.2 ml.) for 15 min. Ice water (2 ml.) was added and the solution extracted with chloroform (10 ml.). The extract was washed with water (5 ml.) and extracted with saturated aqueous sodium bicarbonate (10 ml.). The bicarbonate extract was made acid

with dilute hydrochloric acid and extracted with ethyl acetate (10 ml.). Evaporation of the solvent gave the acid (139) (55 mg., 83%) which crystallised as colourless needles, m. 107-110°C, from chloroform/light petroleum. (mixed m.pt. with O-methylmycophenolic acid (96) 91-96°C).

T.l.c.: 5% methanol/chloroform, Rf. 0.4; gr. (Ce^{4+}).

Analysis: Found C: 64.42%, H: 6.78%,

$C_{18}H_{22}O_6$ requires C: 64.66%, H: 6.63%

I.R. ν max. ($CHCl_3$): 3520 (br.), 2940, 1760 (ν_{CO} phthalide), 1711 (ν_{CO} acid), 1595 $cm.^{-1}$.

ν max. (KBr): 3200-2850 (br.), 1754 (ν_{CO} phthalide), 1695 (ν_{CO} acid), 1595, 1459, 1426, 1295, 1116, 1032 $cm.^{-1}$.

UV. λ max. (EtOH): 210 (ε=16000), 250 (ε=10900), 295 (ε=5450) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 0.5 (1H, br., exchangeable with D_2O, -COOH), 4.96 (2H, s., $ArCH_2O$-), 4.88 (1H, obs., =CH.CH_2-), 6.15 (3H, s., MeO-), 6.28 (3H, s., MeO-), 6.58 (2H, d., J = 7 Hz., $ArCH_2CH$=), 7.44 (3H, s., ArMe), 7.64 (4H, br., s., $-CH_2CH_2-$), 8.20 (3H, s., C-Me).

7-Hydroxy-6(6-hydroxy-3-methylhex-2-enyl)-5-methoxy-4-methylphthalan-1-one (134)

Ethyl chloroformate (0.88 ml.) in tetrahydrofuran (2.5 ml.) was added to a solution of mycophenolic acid (1.46 g.) and triethylamine (1.28 ml.) in tetrahydrofuran (30 ml.) at -10°C. The mixture was stirred for 1 hr., filtered and the filtrate added over 30 min. to a stirred solution of sodium borohydride (1.05 g.) in

water (10 ml.) at 10-15°C. The mixture was stirred at room temperature for 18 hr., acidified with 3N hydrochloric acid and extracted with ethyl acetate (30 ml.). Evaporation of the solvent gave the carbonate (133) (1.68 g., 97%) a colourless oil, b.pt. 180°C/ 0.005 mm.

T.l.c.: chloroform, Rf. 0.47; bl. ($FeCl_3$), bn. (Ce^{4+}).

Analysis: Found C: 63.50%, H: 7.08%,

$C_{20}H_{26}O_7$ requires C: 63.48%, H: 6.93%.

I.R. ν max. (film): 3550 (ν_{OH} phenol), 3000, 1760 (ν_{CO} phthalide) 1725 (ν_{CO} carbonate), 1620, 1245, 1218, 1048, 775 $cm.^{-1}$.

UV. λ max. (EtOH): 222 (ε=13580), 247 (ε=7830), 291 (ε=1960), 308 (ε=1730) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.82 (2H, s., $Ar\underline{CH_2}O$-), 4.83 (1H, t., J = 6.5 Hz., $=\underline{CH}.CH_2$-), 5.62 (2H, q., J = 8 Hz., $CH_3\underline{CH_2}O$-), 6.16 (3H, s., $\underline{Me}O$-), 6.45 (2H, t., J = 6 Hz., $-CH_2\underline{CH_2}OCO-$), 6.60 (2H, t., J = 6.5 Hz., $Ar\underline{CH_2}CH=$), 7.76 (3H, s., $Ar\underline{Me}$), 7.8-8.4 (4H, m., $-\underline{CH_2}\underline{CH_2}CH_2O$-), 8.23 (3H, s., $C-\underline{Me}$), 8.60 (3H, t., J = 8 Hz., $\underline{CH_3}CH_2O$-),

MS. m/e (rel. abundance): 379 (M + 1) (0.5), 306 (35), 288 (31), 247 (98.5), 229 (46), 207 (100), 159 (57), 85 (93).

The carbonate (133) was heated with 3N aqueous sodium hydroxide (20 ml.) at 110°C for 3 hr., the solution made acid with dilute hydrochloric acid and extracted with ethyl acetate (60 ml.). The extract was washed with saturated sodium bicarbonate (10 ml.) and water (10 ml.) and dried. Evaporation of the solvent afforded the alcohol (134) (970 mg., 70%) which crystallised as colourless needles,

m. 105-106°C.(ref. 106-107°C.) from ethyl acetate /light petroleum (60-80°C.).

T.l.c.: chloroform, Rf. 0.45; bl. ($FeCl_3$), bn. (Ce^{4+}).

Analysis: Found C: 66.50%, H: 7.19%,

Calculated for $C_{17}H_{22}O_5$ C: 66.65%, H: 7.21%.

I.R. νmax. ($CHCl_3$): 3630 (br., ν_{OH} phenol), 3100 (br.ν_{OH} alcohol), 3016, 2945, 1742 (ν_{CO} phthalide), 1631, 1493 cm.$^{-1}$.

νmax. (KBr): 3436 (ν_{OH} phenol), 3100 (br.ν_{OH} alcohol), 2940, 1735 (ν_{CO} phthalide), 1622, 1614, 1459, 1089, 1003 cm.$^{-1}$.

NMR. (60 Mc/s.) $\tau CDCl_3$: 2.22 (1H, s., exchangeable with D_2O, ArO<u>H</u>), 4.68 (1H, t., J = 7.5 Hz., = <u>CH</u>.CH_2-), 4.73 (2H, s., Ar<u>CH_2</u>O-), 6.15 (3H, s., <u>Me</u>O-), 6.35 (2H, t., J = 6 Hz., -CH_2<u>CH_2</u>OH), 6.54 (2H, d., J = 7.5 Hz., Ar<u>CH_2</u>CH=), 7.79 (3H, s., Ar<u>Me</u>), 7.8-8.4 (4H, m., -<u>$CH_2$$CH_2$</u>-), 8.16 (3H, s., C-Me), 8.38 (1H, s., exchangeable with D_2O, -O<u>H</u>).

MS. m/e (rel. abundance): 306 (M^+) (27), 288 (20), 247 (87), 229 (28), 207 (91), 159 (45), 85 (100).

m*: 271 (306→288), 253 (288→270), 122 (207→159).

<u>5,7-Dimethoxy-6(6-hydroxy-3-methylhex-2-enyl)-4-methylphthalan-1-one (131)</u>

The alcohol (134) (60 mg.) in methanol (10 ml.) was treated with an excess of ethereal diazomethane overnight. Filtration and evaporation of the solvent gave <u>the dimethoxy alcohol (131)</u> (62 mg.) as a colourless oil, b.pt. 165°C/0.005 mm.

T.l.c.: 2% methanol/chloroform, Rf. 0.4; bn. (Ce^{4+}).

Analysis: Found C: 67.33%, H: 7.46%,

$C_{18}H_{24}O_5$ requires C: 67.48%, H: 7.55%.

I.R. νmax (KBr): 3400 (ν_{OH}), 2930, 1760 (ν_{CO} phthalide), 1600, 1473, 1359, 1316, 1128, 1033, 994, 966, 914 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.82 (1H, t., J = 8 Hz., = CH.CH_2-), 4.89 (2H, s., ArCH_2O-), 5.96 (3H, s., MeO-), 6.21 (3H, s., MeO-), 6.41 (2H, t., 6.5 Hz., -CH_2CH_2OH), 6.62 (2H, s., J = 8 Hz., ArCH_2CH =), 7.81 (3H, s., ArMe), 7.8-8.5 (5H, m., 1H exchangeable with D_2O), 8.20 (3H, s., C-Me).

MS. m/e (rel. abundance): 320 (M^+) (58), 261 (40), 221 (73), 207 (60), 85 (100).

2,4-Dimethoxy-3(2-hydroxyethyl)-5-methylphthalan-1-one (141)

Methyl O-methylmycophenolate (117) (115 mg.) in ethyl acetate (10 ml.) was treated at -80°C. with a stream of ozone (2 mg./min.) for 30 min. The solution was brought to room temperature, the solvent evaporated and the oily residue in methanol (2 ml.) added to a solution of sodium borohydride in methanol (3 ml.)/water (5 ml.). The mixture was stirred for 2 hr. at room temperature and extracted with ethyl acetate (20 ml.). Evaporation of the solvent afforded the alcohol (141) (40 mg., 45%) which crystallised from chloroform/light petroleum as colourless needles, m. 98-100°C.

T.l.c.: 5% methanol/chloroform, Rf. 0.65; bn. (Ce^{4+}).

Analysis: Found C: 61.99%, H: 6.41%,

$C_{13}H_{16}O_5$ requires C: 61.90%, H: 6.39%.

I.R. νmax. ($CHCl_3$): 1754, 1597 $cm.^{-1}$.

νmax. (KBr): 3420 (ν_{OH} alcohol), 2935, 1761, 1738, 1590, 1466, 1354, 1301, 1124, 1100, 954 $cm.^{-1}$.

UV. λmax (MeOH): 217, 249 (ε=5500), 295 (ε=1520) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.89 (2H, s., $Ar\underline{CH_2}O$-), 5.92 (3H, s., $\underline{Me}O$-), 6.20 (3H, s., $\underline{Me}O$-), 6.25 (2H, obs., -$\underline{CH_2}OH$), 7.01 (2H, t., J = 6.5 Hz., $Ar\underline{CH_2}\underline{CH_2}$-), 7.81 (3H, s., $Ar\underline{Me}$), 8.0 (1H, br., s., exchangeable with D_2O).

MS. m/e (rel. abundance): 252 (M^+) (60), 234 (26), 221 (100), 220 (57), 191 (80), 163 (59), 161 (57), 133 (74), 91 (51), 77 (44).

m*: 217.5 (252→234), 192 (252→221), 165 (221→191), 139 191→163), 136 (191→161), 110 (161→133).

3-Carboxymethyl-2,4-dimethoxy-5-methylphthalan-1-one (140)

The alcohol (141) (17 mg.) in acetone (1 ml.) at 5°C, was treated with Jones' reagent (0.05 ml.) for 10 min. The solution was diluted with ice water (3 ml.) and extracted with chloroform (3 ml.). The chloroform solution was extracted with saturated aqueous sodium bicarbonate (5 ml.). Neutralisation of the aqueous fraction with dilute hydrochloric acid, extraction with ethyl acetate (5 ml.) and evaporation of the solvent afforded the acid (140) (12 mg., 70%), which crystallised from chloroform/light petroleum as colourless prisms, m. 148-150°C.

T.l.c.: 5% methanol/chloroform, Rf. 0.15; bn. (Ce^{4+}).

Analysis: Found C: 58.30%, H: 5.21%,

$C_{13}H_{14}O_6$ requires CL 58.63%, H: 5.30%.

I.R. ν max. ($CHCl_3$): 1759 (ν_{CO} phthalide), 1710 (ν_{CO} acid), 1601 cm.$^{-1}$.

ν max. (KBr): 3000 (br.), 2940, 1752 (ν_{CO} phthalide), 1699 (ν_{CO} acid), 1599, 1355, 1297, 1228, 1200, 1129, 1100, 1070, 1035, 989, 965 cm.$^{-1}$.

UV. λ max. (EtOH): 217 (ε=26000), 250 (ε=5360), 295 (ε=1515) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 3.0 (1H, br., exchangeable qith D_2O), 4.86 (2H, s., Ar$\underline{CH_2}$O-), 5.91 (3H, s., $\underline{Me}$O-), 6.21 (3H, s., $\underline{Me}$O-), 6.24 (2H, s., Ar$\underline{CH_2}$CO-), 7.82 (3H, s., Ar$\underline{Me}$).

MS. m/e (rel. abundance): 266 (M^+) (92), 248 (77), 221 (63), 207 (100), 163 (59), 161 (57), 133 (74), 91 (51), 77 (44).

m*: 231.5 (266 248), 165 (221 191), 139 (191 163), 136 (191 161), 110 (161 133).

Note: treatment of the ozonide of methyl O-methylmycophenolate (117) (previous reaction) in tetrahydrofuran with water afforded the acid (140) (30%) identical (t.l.c., mixed m.pt., i.r.) with a sample obtained above.

Mycophenolic acid p-toluidide (143)

To a stirred suspension of mycophenolic acid (520 mg.) in dry benzene (50 ml.) an excess of oxalyl chloride (1 ml.) was added and the mixture stirred for 2 hr. at room temperature. The solvent and reagent were evaporated and the residue dissolved in dry benzene (30 ml.). To this a solution of p-toluidine (230 mg.) in benzene (20 ml.

was added and the solution stirred at reflux for 2 hr. The cooled reaction mixture was washed with water (10 ml.), dilute hydrochloric acid (5 ml.) and saturated aqueous sodium bicarbonate (5 ml.). Evaporation of the solvent afforded mycophenolic acid p-toluidide (143) (590 mg., 90%) which crystallised from aqueous ethanol as colourless prisms, m. 103-104°C.

T.l.c.: chloroform, Rf. 0.45, r.-bn. (Ce^{4+}).

Analysis: Found C: 70.23%, H: 6.55%, N: 3.46%,

$C_{24}H_{27}NO_5$ requires C: 70.40%, H: 6.65%, N: 3.42%.

I.R. ν max. (nujol): 3450 (ν_{OH}), 3360 (ν_{NH}), 1752 (ν_{CO} phthalide), 1660 (ν_{CO} amide), 1618, 1600, 1535 $cm.^{-1}$.

UV. λ max. (EtOH): 217 (ε = 33000), 250 (ε = 17400), 306 (ε = 4500) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 2.5 (1H, br., exchangeable with D_2O, ArO<u>H</u>), 2.60 (1H, br., s., exchangeable with D_2O/TFA, -CO<u>NH</u>-), 2.86 (4H, ABA' B' system, J ≈ 3 Hz., Ar<u>H</u>), 4.70 (1H, t., J = 7 Hz., = <u>CH</u>CH -), 4.89 (2H, s., Ar<u>CH_2</u>O-), 6.82 (3H, s., <u>Me</u>O-), 6.61 (2H, d., J = 7 Hz., Ar<u>CH_2</u>CH=), 7.60 (4H, s., -<u>CH_2CH_2</u>-), 7.72 (3H, s., ArMe), 7.95 (3H, s., Ar<u>Me</u>), 8.18 (3H, s., C-<u>Me</u>).

<u>O-Methylmycophenolic acid p-toluidide (144)</u>

Mycophenolic acid p-toluidide (180 mg.) in toluene (10 ml.) was treated with an excess of ethereal diazomethane and allowed to stand overnight at room temperature. Filtration and evaporation of the solvent afforded the methyl ether (144) as a yellow oil (200 mg.) homogeneous by t.l.c.

T.l.c.: chloroform, Rf. 0.50·,r.bn. (Ce^{4+}).

I.R. ν max. (film): 3390 (ν_{NH}), 1850 (ν_{CO} phthalide), 1659 (ν_{CO} amide), 1600, 1535, 1315, 1132, 770 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 2.63 (1H, br., s., exchangeable with D_2O/TFA, -CONH-), 2.88 (4H, ABA'B' system, J ≈3 Hz., ArH), 4.78 (1H, t., J = 7 Hz., = CH.CH_2-), 4.95 (2H, s., $ArCH_2O$-), 5.95)3H, s., MeO-), 6.26 (3H, s., MeO-), 6.61 (2H, d., J = 7 Hz., $ArCH_2CH$=), 6.60 (4H, s., -CH_2CH_2-), 7.72 (3H, s., ArMe), 7.89 (3H, s., ArMe), 8.17 (3H, s., C-Me).

Ozonolysis of O-methylmycophenolic acid p-toluidide (144)

O-Methylmycophenolic acid p-toluidide (180 mg.) was ozonised in ethyl acetate and the ozonide reduced with sodium borohydride (50 mg.) using the procedure described for the ozonolysis of methyl O-methylmycophenolate (p. 100).

The products were separated by p.l.c. on HF_{254} silica, using 5% methanol/chloroform as eluant, giving the alcohol (141) (30 mg., 30%), identical (t.l.c., mixed m.pt., i.r.) with an authentic sample, and

4-hydroxypentanoic acid p-toluidide (145) (25 mg.) 30%) which crystallised from chloroform/light petroleum as colourless prisms, m. 114-115°C.

T.l.c.: 5% methanol/chloroform, Rf.04; r.-bn. (Ce^{4+}).

Analysis: Found C: 69.38%, H: 8.04%, N: 6.85%,

$C_{12}H_{17}NO_2$ requires C: 69.54%, H: 8.27, N: 6.76%.

I.R. ν max. ($CHCl_3$): 3435, 2960, 1623, 1598, 1510 $cm.^{-1}$.

ν max. (KBr): 3330 (br. ν_{OH}), 3250, 3180, 3120, 2065, 2960, 2920, 1670 (ν_{CO} amide), 1608, 1550, 1511, 1310, 1128, 816 cm.$^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 1.9 (1H, br., s., exchangeable with D_2O/TFA, -CONH-), 2.80 (4H, ABA'B' system, J≃3 Hz., ArH), 6.15 (1H, m., -CH(OH)-), 6.79 (1H, br., s., exchangeable with D_2O), 7.57 (2H, t., J = 6.5 Hz., $-CH_2CO-$), 7.70 (3H, s., ArMe), 8.0-8.4 (2H, m., $-CH_2-$), 8.80 (3H, d., J = 6.5 Hz., $CH_3CH(OH)-$).

Feeding of labelled sodium acetate to shake flask cultures of P. stoloniferum

[2-^{14}C] sodium acetate (0.5 mCi., 55 mCi./mM.) and [2-T] sodium acetate (25 mCi., 500 mCi./mM.) were dissolved in water (10 ml.) and the solution sterilised (15 min. with steam at 240°C and 15 p.s.i.). 9 ml. of this solution was added to a shake flask culture of P. stoloniferum (2 day old, 2 shake flasks each containing 100 ml. of culture filtrate) growing on full strength medium. The cultures were harvested on the fifth day of growth.

Determination of total activity of precursor added

0.05 ml. of the precursor solution (containing 1/200 of the total activity) was diluted to 20 ml. with distilled water. 0.1 ml. aliquots of this solution were counted in toluene/biosolv. scintillator solution. Total activity fed (9/10 of precursor solution)

37.25×10^6 dpm. ^{14}C; 64.4×10^8 dpm.T; T/^{14}C (dpm): 178/1.

Isolation of [^{14}C,T] mycophenolic acid

The mycelial tissue was removed by filtration, dried, and extracted with ethyl acetate (100 ml.) for 6 hr. in a Soxhlet apparatus. The culture filtrate (200 ml.) was extracted with ethyl acetate (100 ml.) at natural pH, acidified with concentrated hydrochloric acid and again extracted with ethyl acetate (100 ml.). The combined ethyl acetate solutions were extracted with saturated sodium bicarbonate, and the crude acidic metabolites (150 mg.) recovered, after acidification with concentrated hydrochloric acid, by extraction

with ethyl acetate. To this, inactive mycophenolic acid (815 mg.) was added and reisolated by crystallisation from methanol/water, giving chemically pure (t.l.c., mixed m.pt.) mycophenolic acid after 3 crystallisations (810 mg.). Further crystallisation gave material by constant activity (Table 2:2).

Hence the activity of undiluted mycophenolic acid: 3.9×10^4 dpm ^{14}C/mg., 217×10^4 dpm T/mg., ($T/^{14}C$ (dpm): 55.6) and incorporation (based on [2-^{14}C] acetate) 1.05%.

Degredation of [^{14}C,T] mycophenolic acid

The following degredations were carried out on [^{14}C,T] mycophenolic acid obtained as above, further diluted with inactive mycophenolic acid. Experimental details are described in the previous section.

(a) [^{14}C,T] Mycophenolic acid (220 mg., % dilution 0.59) was converted to the p-toluidide (143c) (230 mg.) which afforded (144c) on methylation. Ozonolysis followed by reduction gave (141c) (60 mg.) and (145c) (65 mg.). The alcohol (141c) (50 mg.) was oxidised to the corresponding acid (140c) (40 mg.)

(b) [^{14}C,T] Mycophenolic acid (200 mg., % dilution 1.325) was converted to (117c) on methylation. (117c) (205 mg.) was reduced to the triol (128c) (140 mg.) with lithium aluminium hydride and the triol (128c) (100 mg.) oxidised to the hemiacetal (137c) (80 mg.) which was oxidised to the acid (139c) (50 mg.).

The above compounds were purified to constant activity by successive crystallisation (Table 2:3).

Orsellinic acid (38)

P.l.c. of the acidic fraction of the culture filtrate extract of P. stoloniferum shake flask cultures afforded the crude metabolite which was purified by crystallisation from ethyl acetate/light petroleum giving colourless needles, m. 162-166°C. (lit. 176°C.- monohydrate).

T.l.c.: ethyl acetate/benzene/formic acid (33/66/1), Rf. 0.5, r. bn., ($FeCl_3$), bn. (Ce^{4+}).

I.R. ν max. (KBr): 3370,(ν_{OH} phenol), 3000 (br.), 1627 (ν_{CO}), 1595 (sh.), 1450, 1358, 1259, 1176 $cm.^{-1}$.

UV. λ max. (EtOH): 223 (ε = 20,000), 255 (ε = 13,000), 300 (ε = 5,000) nm.[175]

NMR. (60 Mc/s.) τ d_6 acetone: 2.7-4.0 (3H, br., exchangeable with D_2O), 3.65 (2H, m., Ar<u>H</u>), 7.5 (3H, s., Ar<u>Me</u>).

MS. m/e (rel. abundance): 152 (M^+) (46), 134 (100), 108 (60), 106 (77), 93 (40), 78 (40).

EXPERIMENTAL

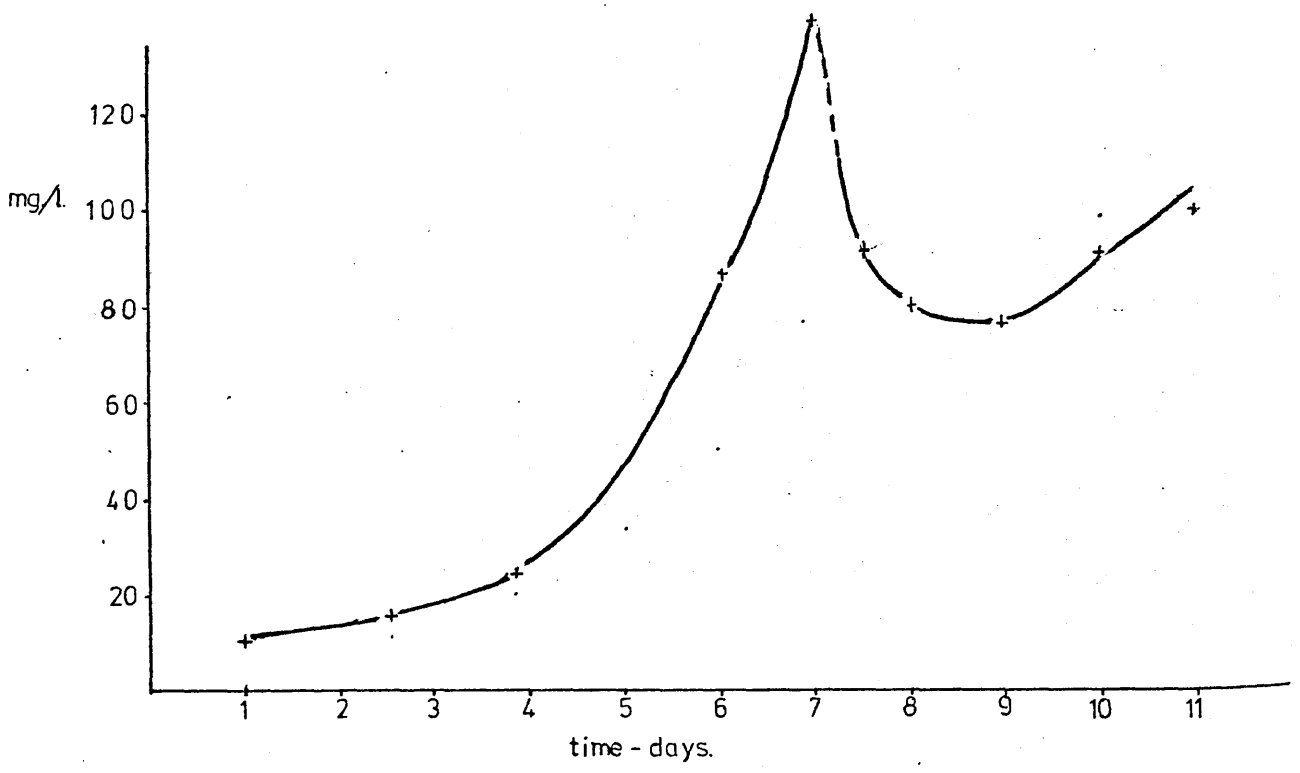
CHAPTER 2:2

Table 2:I Composition of culture media (g./l.)

Component	Full strength medium	Half strength medium
Dextrose monohydrate	120	60
Ammonium nitrate	4.6	2.3
Potassium hydrogen phosphate	5.0	2.5
Magnesium sulphate (hydrate)	1.0	0.5
Yeast extract	1.0	0.5
*Minor element concentrate	2 ml.	1 ml.

*Minor element concentrate: ferric sulphate (1 g.), zinc sulphate (1 g.), potassium molybdate (0.2 g.), copper sulphate (0.15 g.), manganese sulphate (0.1 g.)/litre.

Fig. 2:I Production of the farnesyl compound (157) by P. stoloniferum in deep culture (half strength medium)



Growth of P. stoloniferum in deep culture

The strain of Penicillium stoloniferum (P. brevicompactum group) used was a mutant developed by Imperial Chemical Industries Pharmaceutical Division (I.C.I. culture collection No. 4408).

Spores of this fungus were used to inoculate 80 l. batches of sterile half-strength medium (Table 2:I). The cultures were grown at 25°C with constant stirring and aeration in stainless steel deep culture vessels.

The concentration of the farnesyl compound (157) with respect to growth was estimated as follows. From a continuous culture, 250 ml. aliquots were taken at regular intervals (24 hr.) over a period of 14 days. Each of the samples was treated as follows. The mycelium was removed by filtration, dried and extracted with ethyl acetate in a Soxhlet apparatus for 12 hr. The extract was evaporated to 5 ml., and 25 ml. of this was treated with 0.5 ml. of (bis-)trimethyl silyl acetamide (BSA) for 20 min. at room temperature. 5 l. of this were injected into a 2% E 301 G4C column at 250°C and the intensity of the resulting peak (RT: 8.5 min.), corresponding to the silylated farnesyl compound (157), compared with that produced by 5 µl. of a standard solution.

The variation of concentration with growth is shown graphically in Fig. 2:I.

Table 2:II Separation of the mycelial extract of P. stoloniferum by column chromatography on silica gel

Eluant		Yield (mg.)
benzene	Unidentified mixture	500
5-80% chloroform/ benzene	O-methylfarnesyl compound (159)	300
80% chloroform/ benzene-chloroform	farnesyl compound (157)	500
chloroform	methyl mycophenolate	20
	ergosterol	50

Extraction of P. stoloniferum mycelium

The mycelium of a 7 day culture was removed by filtration, washed with ethyl acetate, dried and extracted with light petroleum (60-80°C.) for 12 hr. in a Soxhlet apparatus. Evaporation of the solvent gave 9.7 g. of a brown oil. This was chromatographed on a column of silica gel (320 g.) (Table 2:II)

The farnesyl compound (157)

P.l.c. of the appropriate column fractions (Table 2:II) on HF_{254} silica, using chloroform as eluant, and successive crystallisation from methanol and diethyl ether/light petroleum gave colourless needles, m. 85-87°C. The melting point was unchanged on sublimation (140°C/0.01 mm.) followed by crystallisation from diethyl ether/light petroleum. The identity of this compound was confirmed by comparison (t.l.c., mixed m.pt., i.r., n.m.r.) with authentic samples.

T.l.c.: chloroform, Rf. 0.60; bl. ($FeCl_3$), r.-bn. (Ce^{4+}).

I.R. ν max. (CCl_4): 2420 (ν_{OH}), 2920, 1738 (ν_{CO} phthalide), 1635, 1625, 1449, 1358, 1307, 1147, 1091, 1060 $cm.^{-1}$.

NMR. (100 Mc/s.) τ $CDCl_3$: 2.32 (1H, br. s., exchangeable with D_2O), 3.80 (1H, br. s., exchangeable with D_2O), 4.82 (1H, t., J = 7 Hz., $=\underline{CH}CH_2Ar$), 4.8-5.1 (2H, m., $-CH_2\underline{CH}=$), 4.89 (2H, s., $Ar\underline{CH_2}O-$), 6.58 (2H, d., J = 7 Hz., $Ar\underline{CH_2}CH=$), 7.8-8.1 (8H, m., $-\underline{CH_2}\underline{CH_2}-$), 8.18 (3H, s., $=C(\underline{CH_3})-$), 8.36 (3H, s., $=C(\underline{CH_3})-$), 8.44 (6H, s., $=C(\underline{CH_3})_2$).

The O-methylfarnesyl compound (159)

(a) P.l.c. of the appropriate column fractions (Table 2:II) on HF_{254} silica, using chloroform as eluant, gave the O-methylfarnesyl compound (157) as a colourless oil.

T.l.c.: chloroform, Rf. 0.45; bl. ($FeCl_3$), r.-bn. (Ce^{4+}).

MS. gives M^+ at 398.2467, $C_{25}H_{34}O_4$ requires 398.2457.

I.R. ν max. (CCl_4): 3450 (ν_{OH}), 2920, 1750 (ν_{CO} phthalide), 1630, 1455, 1370, 1240 $cm.^{-1}$.

UV. λ max. (EtOH): 252 (ε=14800), 360 (ε=8000) nm.

NMR. (100 Mc/s.) $\tau CDCl_3$: 2.0-3.0 (1H, br., exchangeable with D_2O), 4.84 (2H, s., $ArCH_2O$-), 4.75-5.05 (3H, m., = $\underline{CH}CH_2$-), 6.29 (3H, s., $\underline{Me}O$-), 6.64 (2H, d., J = 7 Hz., $Ar\underline{CH_2}CH$=), 7.90 (3H, s., $Ar\underline{Me}$), 8.0-8.1 (8H, m., -$\underline{CH_2CH_2}$-), 8.22 (3H, s., = $C(\underline{CH_3})$-), 8.36 (3H, s.,=$C(\underline{CH_3})$-), 8.45 (6H, s., = $C(\underline{CH_3})_2$).

MS. m/e (rel. abundance): 398.2467 $(C_{25}H_{34}O_4)^+$ (6), 329.1732 $(C_{20}H_{25}O_4)^+$ (5), 261.1124 $(C_{15}H_{17}O_4)^+$ (39), 207.0636 $(C_{11}H_{11}O_4)^+$ (100), 191.1716 $(C_{14}H_{23})^+$ (33).

(b) The farnesyl compound (157) (65 mg.) in dry acetone (5 ml.) was stirred and refluxed with anhydrous potassium carbonate (28 mg.). A solution of methyl iodide (34 mg.) in dry acetone (5 ml.) was added to the mixture in 0.5 ml. aliquots at 0.5 hr. intervals. After 4 hr. the solvent was evaporated and the residue partitioned between ethyl acetate (10 ml.) and water (5 ml.). Evaporation of the ethyl acetate and p.l.c. of the residue on HF_{254} using multiple elution with

benzene as eluant, gave starting material (5 mg.), the 0,0′-dimethylfarnesyl compound (160) (20 mg., 31%), identical (t.l.c., i.r., n.m.r.) to material isolated in (a) above, and the O-methylfarnesyl compound (159) (15 mg., 24%) as colourless oils.

The 0,0′-dimethylfarnesyl compound (160)

Prepared as described in (a) above, as a colourless oil, b.pt. 175°C/0.05 mm.

T.l.c.: chloroform, Rf. 0.50; r.-pur. (Ce^{4+}).

MS. gives M^+ at 412.2610, $C_{26}H_{36}O_4$ requires 412.2613.

I.R. ν max. (film): 2920, 1760 (ν_{CO} phthalide), 1603, 1478, 1315, 1130, 1045, 979 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.75-5.15 (3H, m., $=CHCH_2-$), 4.91 (2H, s., $ArCH_2O-$), 5.95 (3H, s., $MeO-$), 6.22 (3H, s., $MeO-$), 6.60 (2H, d., J = 6.5 Hz., $ArCH_2CH=$), 7.81 (3H, s., $ArMe$), 7.9-8.15 (8H, m., $-CH_2CH_2-$), 8.22 (3H, s., $=C(CH_3)-$), 8.34 (3H, s., $=C(CH_3)-$), 8.42 (6H, s., $=C(CH_3)_2$).

MS. m/e (rel. abundance): 412.2610 $(C_{26}H_{36}O_4)^+$ (8), 343.1959 $(C_{21}H_{27}O_4)^+$ (2), 275.1288 $(C_{16}H_{19}O_4)^+$ (86), 221.0814 $(C_{12}H_{13}O_4)^+$ (100), 191.1807 $(C_{14}H_{23})^+$ (13).

Demethylation of the 0,0′-dimethylfarnesyl compound (160) with "magnesium iodide etherate"

The 0,0′-dimethylfarnesyl compound (160) (15 mg.) in dry refluxing benzene (10 ml.) was treated, under nitrogen, with freshly prepared "magnesium iodide etherate" reagent (70 µl.). The solution was

stirred and refluxed for 3 hr., cooled, washed with 5N hydrochloric acid (10 ml.), water (5 ml.) added and the solution extracted with chloroform (10 ml.). Evaporation of the solvents and p.l.c. of the residue on HF_{254} silica, using 12% ethyl acetate/light petroleum as eluant, gave a mixture of compounds apparently homogeneous by t.l.c.

T.l.c.: chloroform; Rf. 0.45: bl. ($FeCl_3$), r.-bn. (Ce^{4+}).

I.R. ν max. (CCl_4): 3450 (ν_{OH}), 2920, 1750 (ν_{CO} phthalide), 1630, 1456, 1775, 1240 $cm.^{-1}$.

NMR. (100 Mc/s.) τ $CDCl_3$): 2.35 (1H, br., s., exchangeable with D_2O, Ar<u>OH</u>), 4.95 (2H, s., Ar<u>CH_2</u>O-), 6.30 (3H, 3s., <u>Me</u>O-), 7.2-7.6 (2H, m.), 7.94 (3H, s., Ar<u>Me</u>), 7.7-8.9 (14H, m.), 9.05 (12H, m., 4<u>CH_3</u>).

<u>Methyl mycophenolate</u>

(a) Mycophenolic acid (80 mg.) in 3% methanolic hydrogen chloride (10 ml.) was left at room temperature for 3 days, the solvent evaporated and the residue crystallised from chloroform/light petroleum as colourless prisms, m. 102-104°C.

T.l.c.: chloroform, Rf. 0.66; bl. ($FeCl_3$), bn. (Ce^{4+}).

Analysis: Found C: 64.19%, H: 6.35%,

$C_{18}H_{22}O_6$ requires C: 64.66%, H: 6.63%.

I.R. ν max. (nujol): 3475, 1720, 1620, 1170, 1075 $cm.^{-1}$.

UV. λ max. (EtOH): 220 (ε=36000), 250 (ε=10000), 300 (ε= 4600) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 2.32 (1H, s., exchangeable with D_2O), 4.78 (1H, t., J = 7 Hz., = <u>CH</u>CH_2-), 4.81 (2H, s., Ar<u>CH_2</u>O-), 6.21 (3H, s., <u>Me</u>O-), 6.38 (3H, s., <u>Me</u>OCO-), 6.63 (2H, d.,

J = 7 Hz., Ar$\underline{CH_2}$CH=), 7.65 (4H, br. s., -$\underline{CH_2CH_2}$-), 7.85 (3H, s., Ar$\underline{Me}$), 8.20 (3H, s., C-$\underline{Me}$).

(b) P.l.c. of the appropriate column fractions (Table 2:II) on HF_{254} silica, using chloroform as eluant, afforded methyl mycophenolate ( ) which was identified by comparison (t.l.c., mixed m.pt., i.r.) with material from (a).

Ergosterol

P.l.c. of the appropriate column fractions (Table 2:II) on HF_{254} silica, using chloroform as eluant, and crystallisation from ethanol, gave ergosterol as colourless needles, m. 160-164°C, identical (t.l.c., mixed m.pt., u.v.) with an authentic sample.

O-Desmethylmycophenolic acid (161)

Mycophenolic acid (29 mg.) in collidine (2 ml.) was added to a stirred solution of lithium iodide (dried at 300°C. under nitrogen for 1 hr.) (200 mg.) in refluxing collidine (6 ml.). The solution was stirred and refluxed for 7.5 hr., allowed to cool and dilute hydrochloric acid (10 ml.) added. Extraction with ethyl acetate, evaporation of the solvent and p.l.c. of the residue on GF_{254} silica, using benzene/ethyl acetate/formic acid as eluant, afforded starting material (10 mg.) and O-desmethylmycophenolic acid (161) (5 mg., 28%) which crystallised from acetone/light petroleum.as colourless prisms, m. 147-149°C., identical (t.l.c., mixed m.pt., i.r.) with an authentic sample.

Table 2:IIA Retention times (R_T) of P. stoloniferum metabolite TMS derivatives

Metabolite	R_T (min.) 2% E301	1% XE60
mycophenolic acid	3.2	4.5
O-methylfarnesyl compound (159)	6.8	10.5
farnesyl compound (157)	8.5	11.2
ergosterol	8.6	11.5
O-desmethylmycophenolic acid (161)	2.0	--

Growth of P. stoloniferum in shake flask cultures

250 ml. shake flasks containing 100 ml. of sterile medium were inoculated with spores of P. stoloniferum. The flasks were shaken continuously on a Gallenkamp rotary shaker (200 rev./min.) at 25°C under continuous illumination from fluorescent tubes.

The variation in metabolite concentration with growth for different medium strengths was determined as follows:

Two series of shake flasks containing full strength and half strength media respectively (Table 2:I) were inoculated and grown as described above. At regular intervals (24 hr.) two flasks of each series were removed and extracted in identical fashion.

The mycelium was removed by filtration, dried and extracted with ethyl acetate (25 ml.) in a Soxhlet apparatus for 12 hr. The culture filtrate was extracted with ethyl acetate (25 ml.), acidified with concentrated hydrochloric acid to pH 2, and again extracted with ethyl acetate (25 ml.). The extracts were combined, dried and reduced to 5 ml., and 2.5 ml. of this solution was silylated with 0.5 ml. of BSA for 20 min. at room temperature. 5 l. of this were injected into a 2% E301 GLC column at 250°C. and the intensity of the resulting peaks corresponding to the silylated metabolites, compared with those produced by standard solutions (Table 2A). This procedure was repeated on a 1% E60 GLC column at 240°C.

The variations of metabolite concentrations with growth are shown in Fig. 2:8.

FEEDING OF LABELLED PRECURSORS TO SHAKE FLASK CULTURES OF P. STOLONIFERUM

General

In the following experiments solutions of the appropriate precursors were sterilized by passage through a sterile Sykes' filter, added to shake flask cultures between the second and third day of growth and the cultures harvested between the fourth and seventh day.

The mycelium was removed by filtration, dried and extracted with ethyl acetate in a Soxhlet apparatus for 24 hr. The culture filtrate was extracted with ethyl acetate at natural pH, acidified to pH 2 with concentrated hydrochloric acid, and again extracted with ethyl acetate. The combined extracts were separated into acidic and neutral fractions as described earlier.

The farnesyl compound (157), its O-methyl ether (159) and ergosterol were isolated by p.l.c. of the mycelial and culture filtrate neutral extracts. Mycophenolic acid was isolated from the culture filtrate acidic fraction.

Total activities of aqueous precursor solutions were determined by counting diluted aliquots in toluene/biosolv. scintillator solution. The purity of sufficiently radioactive compounds ($\geqslant 10^3$ dpm/mg.) was determined by TLC radioscan, prior to determination of activity by dilution with inactive material and crystallisation to constant activity.

Table 2:III Feeding of DL-[^{14}C,T] -methionine to half strength cultures of P. stoloniferum

Compound	dilution %	Wt./mg.	dpm. ^{14}C	dpm. T	dpm./mg. ^{14}C	dpm./mg. T	^{14}C (undiluted)	T (undiluted)	T/^{14}C ratio dpm.	T/^{14}C ratio atomic	Estimated total wt. from culture (mg.)	total activity dpm. ^{14}C	total activity dpm. T	Incorporation %
Mycophenolic acid	7.95	1.06 (x1)	34976	743560	33050	702500	4.16 x 10^5	8.83 x 10^6	21.0	1.05	64	2.66×10^7	5.98×10^8	23.6
		0.72 (x2)	23892	534381	33200	743500	4.17 x 10^5	9.34 x 10^6	22.4	1.12				
		0.74 (x3)	24439	550213	32950	742500	4.15 x 10^5	9.36 x 10^6	22.4	1.12				
Ergosterol	5.4	0.17 (x1)	2033	38687	11970	227900	2.22 x 10^5	4.21 x 10^6	19	0.96	10	1.3×10^6	24.6×10^7	1.26
		0.20 (x2)	2420	45602	12100	228000	2.24 x 10^5	4.23 x 10^6	18.9	0.95				
Unidentified metabolite	100	0.5		9725	220110	19450	440220	194 x 10^5	440220	226	1.15	5	1×10^5	2.2×10^6	0.078

Feeding of labelled DL-methionine to P. stoloniferum

I Using half strength medium

11.7 ml. of a solution, of DL-[$^{14}CH_3$] methionine (50 Ci; 60 mCi./mM) and DL-[CT_3]-methionine (1.0 mCi.; 100 mCi./mM) in water (12 ml.), were added to 2 shake flask cultures (200 ml. of medium) on the third day of growth. The cultures were harvested on the fifth day. (Total fed activity: 1.129 x 10^8 dpm ^{14}C, 22.4 x 10^8 dpmT; T/^{14}C (dpm): 19.8)

[^{14}C,T] Mycophenolic acid (65 mg.) was isolated by crystallisation of the residue of the culture filtrate acidic fraction. [^{14}C,T] Ergosterol (6 mg.) was isolated by p.l.c. of the mycelial extract and subsequent crystallisation (Table 2:III).

P.l.c. of the mycelial extract on GF_{254} silica, using chloroform as eluant, gave an unidentified sterol ester (5 mg.) as a colourless oil.

T.l.c.: chloroform, Rf. 0.90; bk. (chromic acid), bk. (Ce^{4+}).

GLC.: 2% E301 (250°), R.T.: 1.2 min.

I.R. ν max. (CCl_4): 2920, 1740, 1365, 1240, 1045, 645 $cm.^{-1}$.

UV. λ max. (MeOH): 210, 175nm .

II Using full strength medium

12.5 ml. of a solution, of DL-[$^{14}CH_3$]-methionine (10 Ci; 60 mCi./mM) in water (13 ml.), were added to one shake flask culture (100 ml. of medium) on the third day of growth. The culture was harvested on the fifth day. (Total fed activity 2.06 x 10^7 dpm. ^{14}C.)

Table 2:IV Feeding of DL-[^{14}C,T]methionine to full strength cultures of P. stoloniferum

Compound	dilution %	Wt./mg.	dpm. ^{14}C	dpm./mg. ^{14}C	dpm./mg. (undiluted) ^{14}C	Estimated total wt. from culture (mg.)	total activity dpm. ^{14}C	Incorporation %
Mycophenolic acid	11.36	1.92 (x1)	21636	11300	9.96×10^4	36	3.46×10^6	26
		0.4 (x2)	4396	10956	9.66×10^4			
		0.4 (x3)	4350	10875	9.55×10^4			
Farnesyl compound	4.6	0.6 (x2)	2937	2820	6.18×10^4	20	1.24×10^6	6.0
		2.24 (x3)	4087	1823	6.38×10^4			
	2.9	1.12 (x4)	1938	1730	6.06×10^4			
Farnesyl compound monomethyl ether	3.76	0.7 (x1)	3681	5250	1.40×10^5	0.5	7.0×10^4	0.34
		1.05 (x2)	5571	5000	1.33×10^5			

Table 2:V Mycophenolic acid from cultures fed with ^{14}C farnesyl compound (157)

Compound	dilution %	Wt./mg.	dpm. ^{14}C	dpm./mg. ^{14}C	dpm./mg. (undiluted)	Estimated total wt. from culture	total activity dpm. ^{14}C	Incorporation % (Molar %)
Mycophenolic acid (3 day)	1	0.9 (x2)	716	795	795	Isolated by dilution with 0.5 mg. cold material	2.8×10^4	4.5% (5.4%)
		0.6 (x3)	486	806	806			
Mycophenolic acid (7 day)	1	1.92 (x2)	1380	720	733	23 mg.	1.69×10^4	7.5% (9.0%)
		1.85 (x2)	1364	738				
		2.68 (x3)	2036	760				
		1.07 (x3)	766	716				
Farnesyl compound	0.56	0.3 (x2)	77	258	4608	Isolated by dilution with 9.8 mg. cold material	4.5×10^4	
Desmethyl mycophenolic acid	1	2.03 (x2)	27.4	13.45	12.8	Isolated by dilution with 30 mg. cold material	384	0.065% (0.082%)
		2.15 (x3)	26.2	12.13				

[^{14}C] Mycophenolic acid (20 mg.) was isolated by p.l.c. of the culture filtrate acidic fraction and subsequent crystallisation. The [^{14}C] farnesyl compound (157) (12.6 mg.) and the [^{14}C] O-methylfarnesyl compound (2 mg.) were isolated by p.l.c. of the mycelial and culture filtrate neutral extracts and purified by crystallisation and p.l.c. respectively (Table 2:III).

Feeding of [^{14}C] farnesyl compound (157) to cultures of P. stoloniferum grown on full strength medium

A solution of [^{14}C] farnesyl compound (157) (10.60 mg., 6.6 x 10^5 dpm) in acetone (0.5 ml.) was added to one shake flask culture (50 ml. of medium) on the second day of growth. The culture stopped growing between the second and third day, and was harvested on the latter. Inactive mycophenolic acid (3.5 mg.) was added to the culture and reisolated as described above (Table 2:IV).

The remaining [^{14}C] farnesyl compound was isolated by p.l.c. of the appropriate extracts, dissolved in acetone (0.1 ml.) and added to one shake flask culture (50 ml. of medium) on the third day of growth and the culture harvested on the seventh day. [^{14}C] Mycophenolic acid (20 mg.) was isolated by crystallisation of the culture filtrate acidic fraction. To the remainder of this fraction inactive O-desmethylmycophenolic acid (161) (30 mg.) was added and reisolated by p.l.c. on HF_{254} silica, using benzene/ethyl acetate/formic acid (66/33/1) as eluant, and crystallisation from acetone/light petroleum. Inactive farnesyl compound (157) (9.8 mg.) was added to the mycelial and culture filtrate neutral extracts and reisolated by p.l.c. and

Table 2:VI O-desmethylmycophenolic acid from [$^{14}CT_3$] mycophenolic acid

Wt./mg.	dilution %	dpm. ^{14}C	dpm. T	dpm./mg. ^{14}C	dpm./mg. T	dpm./mg. (undiluted) 14C	dpm./mg. (undiluted) T	T/14 dpm.	T/14 atomic
2.02 (x1)		15451	282107	7650	13940				
1.85 (x2)	3.75	14010	253115	7570	13860				
1.20 (x3)		9208	167047	7600	13900				
				76100	13950	2.03×10^5	37.2×10^5	18.3	0.93

subsequent crystallisation (Table 2:V). The mycelial tissue was finally extracted with ethyl acetate for 3 days in a Soxhlet apparatus. The resulting material (10 mg.) was found to have an activity of 2650 dpm/mg.

[^{14}C,T] O-Desmethylmycophenolic acid (161)

[^{14}C,T] Mycophenolic acid (29 mg., 4.95 x 10^5 dpm/mg. ^{14}C; 8.50 x 10^6 dpm/mg. T) isolated from P. stoloniferum fed with $^{14}CT_3$ methionine (p. 117) was demethylated by the procedure outlined previously (p. 114) giving ^{14}C,T O-desmethylmycophenolic acid (161) (5 mg., 18%) identical (t.l.c., mixed m.pt., GLC (TMS derivative)) to an authentic sample. The specific activity was determined by dilution (Table 2:VI).

Feeding of [^{14}C,T] O-desmethylmycophenolic acid to cultures of P. stoloniferum grown on full strength medium.

A solution of [^{14}C,T] O-desmethylmycophenolic acid (3.80 mg.) in 3N sodium hydroxide (0.5 ml.) was diluted with distilled water and neutralised with 3N hydrochloric acid (Total volume 7.0 ml.). Aliquots of the sterilised solution were added to a shake flask culture (100 ml. of medium) at the following times: 30 hr. (3 ml.), 43 hr. (2 ml.), 51 hr. (1.8 ml.). The culture was harvested after 100 hr. (Total fed activity 7.45 x 10^5 dpm ^{14}C, 14.2 x 10^6 dpm T, T/^{14}C (dpm): 19/1).

[^{14}C,T] Mycophenolic acid (11 mg.) was isolated from the culture filtrate acidic fraction by p.l.c. on GF_{254} silica, using benzene/ethyl acetate/formic acid (66/33/1) as eluant, and subsequent

Table 2:VII Mycophenolic acid from culture fed with [^{14}C,T] O-desmethylmycophenolic acid

wt./mg.	dpm. ^{14}C	dpm. T	dpm./mg. ^{14}C	dpm./mg. T	Estimated total weight from culture	dpm. (total) ^{14}C	dpm. (total) T	ratio ($^{T}/_{14C}$) dpm.	ratio ($^{T}/_{14C}$) atomic	Incorporation %
1.02 (x1)	200	4286	197	4202				21.20		
1.40 (x2)	277	5926	198	4240	30 mg.			21.40		
1.10((x3)	215	4582	196	4160				21.20		
			197	4208		4.91×10^3	1.26×10^5	21.27	1.07	0.8%

Table 2:IIX Dilution of synthetic [methyl-^{14}C] O-methylfarnesyl compound

Dilution %	wt.	purification (PLC)	dpm.	dpm./mg.	dpm./mg. (undiluted)
1.135	2.13	x1	36535	1.72×10^4	
	3.75	x2	64950	1.73×10^4	
	0.44	x3	7610	1.73×10^4	
	2.44	x4	41000	1.71×10^4	
				1.725×10^4	1.52×10^6

crystallisation (Table 2:VII).

[methyl-^{14}C] O-Methylfarnesyl compound (159)

[^{14}C] Methyl iodide (0.24 mg., 100 Ci.) was diluted with inactive methyl iodide (34 mg.) in dry acetone (5 ml.). 1 ml. aliquots of this solution were added at 30 min. intervals to a stirred refluxing solution of the farnesyl compound (157) (63 mg.) and anhydrous potassium carbonate (31 mg.) in dry acetone (10 ml.). A dry ice condenser was used to prevent loss of the methyl iodide. The reflux was continued for 1½ hr. after all the methyl iodide solution had been added. The solvent was evaporated and the residue partitioned between water (1 ml.) and chloroform (5 ml.). Evaporation of the chloroform fraction and p.l.c. of the residue on HF_{254} silica, using chloroform as eluant, gave starting material (23 mg.) and [methyl-^{14}C] O-methylfarnesyl compound (157) (22 mg., 53%) identical (t.l.c., u.v., n.m.r.) with an authentic sample. The specific activity was established by dilution (Table 2:IIX).

Feeding of [methyl-^{14}C] O-methylfarnesyl compound (157) to cultures of P. stoloniferum

I Using half strength medium

[methyl-^{14}C] O-Methylfarnesyl compound (3.16 mg., 4.80×10^6 dpm) in acetone (0.1 ml.) was added to one shake flask culture (100 ml. of medium) on the second day of growth. The culture was harvested on the fifth day.

[^{14}C] Mycophenolic acid was isolated by crystallisation from

Table 2:IX Mycophenolic acid from half strength cultures fed with [methyl-^{14}C]

O-methylfarnesyl compound (159)

Wt (mg.)	dpm. ^{14}C	dpm./mg. ^{14}C	Estimated total wt. from culture	total activity	% incorp.	% molar incorp.
2.10 (x1)	19700	1.97×10^4				
1.41 (x2)	20500	2.05×10^4	40 mg.	8.2×10^5	20.2	25
0.80 (x3)	2100	2.10×10^4				
		2.04×10^4				

Table 2: X Mycophenolic acid from full strength cultures fed with [methyl-^{14}C]

O-methylfarnesyl compound (159)

Wt. (mg.)	dpm. ^{14}C	dpm./mg. ^{14}C	Estimated total wt. from culture	total activity	% incorp.	% molar incorp.
0.58 (x1)	8965	1.55×10^4				
0.59 (x2)	9674	1.64×10^4	36 mg.	5.65×10^5	11.8	14.7
0.33 (x3)	5065	1.54×10^4				
		1.57×10^4				

the residue of the culture filtrate acidic fraction (Table 2:IX).

II Using full strength medium

[methyl-^{14}C] O-Methylfarnesyl compound (2.70 mg., 4.03 x 10^6 dpm) in acetone (0.1 ml.) was added to one shake flask culture (100 ml. of medium on the second day of growth. The culture was harvested on the fifth day.

[^{14}C] Mycophenolic acid was isolated by p.l.c. of the residue from the culture filtrate acidic fraction on HF_{254} silica, using benzene/ethyl acetate/formic acid (66/33/1) as eluant, and subsequent crystallisation (Table 2:X).

Feeding of [2-^{14}C] mevalonic acid lactone to cultures of P. stoloniferum grown on full strength medium

9.5 ml. of a solution of [2-^{14}C] mevalonic acid lactone (50 Ci.) in distilled water (10 ml.) was added to one shake flask culture (100 ml. of medium) on the second day of growth. The culture was harvested on the fourth day.

The mycelial tissue was removed by filtration and the culture filtrate concentrated to 50 ml. in vacuo on a rotary film evaporator. The filtrate was passed through an Amberlite IR-120 (Na^+) column, the pH adjusted to 7 and extracted with ethyl acetate (50 ml.). The aqueous layer was acidified to pH 2 with dilute hydrochloric acid and subjected to constant extraction with ether overnight. The ether extract was evaporated to dryness and the residue p.l.c'd on HF_{254} silica, using pyridine/light petroleum (60-80°) as eluant.

The band corresponding in Rf. to laevulinic acid was removed and extracted with water (5 ml.). The pH of the aqueous extract was adjusted to 8 with dilute sodium hydroxide, 5N hydrochloric acid (2 drops) and p-bromophenacyl bromide (500 mg.) in ethanol (5 ml.) were added and the solution refluxed for 2 hr. The cooled mixture was extracted with chloroform (20 ml.) and to the extract p-bromophenacyl laevulinate (38 mg.) was added. Evaporation of the solvent and p.l.c. of the residue on HF_{254} silica, using chloroform as eluant, gave [^{14}C] p-bromophenacyl laevulinate (25 mg.). Successive crystallisation from diethyl ether/light petroleum gave material of very low constant activity. Total activity (for 38 mg.) = 65 dpm ^{14}C. .

EXPERIMENTAL

CHAPTER 3

(threo-)6 2-Bromo-2(methyl-5-oxotetrahydro-2-furyl)ethyl -5-methoxy-4-methylphthalan-1-one (171)

To mycophenolic acid (220 mg.) in chloroform (12 ml.) a solution of bromine in chloroform was added until the colour persisted. The mixture was extracted with aqueous sodium metabisulphite, saturated aqueous sodium bicarbonate and brine. Evaporation of the solvent afforded the bromolactone (171) (200 mg., 73%) which crystallised from chloroform/light petroleum as colourless prisms, m. 180-182°C.

T.l.c.: chloroform, Rf. 0.58; bl. ($FeCl_3$), gn. (Ce^{4+}).

Analysis: Found C: 50.95%, H: 4.72%,

$C_{17}H_{19}O_6Br$ requires C: 51.20%, H: 4.76%.

I.R. ν max. ($CHCl_3$): 3418 (ν_{OH}), 2940, 1786 (ν_{CO} phthalide), 1746 (ν_{CO} lactone), 1630 $cm.^{-1}$.

ν max. (KBr): 3425 (ν_{OH}), 2970, 1763 (ν_{CO} phthalide), 1722 (ν_{CO} lactone), 1618, 1138, 1068, 624, 609 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 2.15 (1H, s., exchangeable with D_2O), 4.74 (2H, s., $ArCH_2O$-), 5.41 (1H, t., J = 8 Hz., -CHBr-), 6.18 (3H, s., MeO-), 6.68 (2H, d., J = 8 Hz., $ArCH_2CHBr$-), 7.2-7.8 (4H, m., $-CH_2CH_2-$), 7.83 (3H, s., ArMe), 8.33 (3H, s., C-Me).

MS. m/e (rel. abundance): 400 (M^+, ^{81}Br) (2.6), 399 (^{81}Br) (5.2), 382 (^{81}Br) (2.5), 319 (31), 318 (33), 301 (26), 220 (99), 219 (99), 207 (44), 205 (38), 109.5 (1.7), 99 (99), 44 (100).

(erythro-)4-Methoxy-5-methyl-2(2-methyl-5-oxotetrahydro-2-furyl)-2,3-dihydrobenzo 2,1-6:3-4-C -8(6H)-one (183)

To the bromolactone (171) (100 mg.) in methanol, 3N aqueous sodium hydroxide was added to pH 11. The solution was immediately neutralised with dilute hydrochloric acid and extracted with chloroform (10 ml.) Evaporation of the solvent gave the dihydrobenzofuran lactone (183) (55 mg., 60%) which crystallised from chloroform/light petroleum as colourless needles, m. 201-202°C. (lit. 201-202°C.), identical (t.l.c., mixed m.pt., i.r., n.m.r.) with an authentic sample.

T.l.c.: chloroform, Rf. 0.56; bn. (Ce^{4+}).

GLC: OV17 (255°C.), RT. 8.5 min.

I.R. ν max. ($CHCl_3$): 3006, 2935, 1763 (ν_{CO} phthalide), 1751 (shoulder, ν_{CO} γ-lactone), 1637, 1613 $cm.^{-1}$.

UV. λ max. (EtOH): 252 (ε=5900), 305 (ε=3650) nm.

NMR. (100 Mc/s.) τ $CDCl_3$: 4.90 (2H, s., ($Ar\underline{CH_2}O$-), 5.02 (1H, q., $J_{AX} + J_{BX}$ = 8.5 Hz., irr. 6.62 s., $\underline{CH}(O)CH_2$-), 6.06 (3H, s., $\underline{Me}O$-), 6.62 (2H, octet, J_{AX} = 5 Hz., J_{BX} = 3.5 Hz., J_{AB} = 8 Hz., irr. 5.02 s., -$\underline{CH_2}CH(O)$-), 7.2-9.7 (4H, m., -$\underline{CH_2CH_2}$-), 7.96 (3H, s., Ar$\underline{Me}$), 8.58 (3H, s., C-$\underline{Me}$).

MS. m/e (rel. abundance): 318 (M^+) (13.5), 317 (65), 220 (98), 219 (98), 205 (63), 175 (42), 99 (100).

N,N-Dimethylmycophenolamide (185)

Mycophenolic acid (450 mg.) was converted to the dimethylamide (185) (375 mg., 77%) using a procedure similar to that used in the

preparation of mycophenolic acid p-toluidide (143) (p. 102). The dimethylamide (185) crystallised from benzene/light petroleum as colourless needles, m. 86°C.

T.l.c.: chloroform, Rf. 0.50; bl. ($FeCl_3$), bn. (Ce^{4+}).

Analysis: Found C: 65.38%, H: 7.12%, N: 3.93%,

$C_{19}H_{25}NO_5$ requires C: 65.60%, H: 7.20%, N: 4.04%.

I.R. ν max. ($CHCl_3$): 3450 (ν_{OH}), 1735 (br., ν_{CO} γ lactone, phthalide), 1632 $cm.^{-1}$.

ν max. (KBr): 3420-3000 (br. ν_{OH}), 1753, 1620, 1597 $cm.^{-1}$.

UV. λ max. ($CHCl_3$): 250 (ε 5050), 305 (ε 3800) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 2.85 (1H, br. s., exchangeable with D_2O), 4.74 (1H, t., J = H Hz., = $\underline{CH}CH_2$-), 4.80 (2H, s., Ar$\underline{CH_2}$O-), 6.20 (3H, s., $\underline{Me}$O-), 6.60 (2H, d., J = Hz., Ar$\underline{CH_2}$CH=), 7.0 (3H, s., N.$\underline{Me}$), 7.1 (3H, s., N.$\underline{Me}$), 7.63 (4H, s., -$\underline{CH_2CH_2}$-), 7.82 (3H, s., Ar$\underline{Me}$), 8.18 (3H, s., C-$\underline{Me}$).

MS. m/e (rel. abundance): 348 (M^+) (13), 347 (M^+) (57), 261 (34.5), 207 (34.5), 173.5 (M^{2+}) (2.8), 159 (34.5), 140 (63), 87 (100), 72 (97).

N,N-Diethylmycophenolamide (190) was prepared by a similar procedure. Crystallisation from benzene/light petroleum gave colourless needles, m. 94.5-96°C.

T.l.c.: chloroform, Rf. 0.52; bl. ($FeCl_3$), bn. (Ce^{4+}).

Analysis: Found C: 67.20%, H: 7.80%, N: 3.0%,

$C_{21}H_{29}NO_5$ requires C: 67.24%, H: 7.74%, N: 3.7%

I.R. ν max. (CCl_4): 3450 (ν_{OH}), 1750 (br., ν_{CO} γ-lactone, phthalide), 1650, 1440, 1407, 1420 $cm.^{-1}$.

UV. λ max ($CHCl_3$): 250 (ε=5000), 305 (ε=3800) nm.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.75 (1H, br. s., exchangeable with D_2O), 4.75 (1H, m., = $\underline{CH}CH_2$-), 4.80 (2H, s., $ArCH_2O$-), 6.20 (3H, s., MeO-), 6.5-6.9 (6H, m., $-N(CH_2CH_3)_2$ $ArCH_2CH$=), 7.7 (4H, s., $-CH_2CH_2$-), 7.90 (3H, s., ArMe), 8.20 (3H, s., C-Me), 8.9 (6H, 2t. superimposed, $-N(CH_2CH_3)_2$).

MS. m/e (rel. abundance): 376 (M+1) (2), 375 (M^+) (10), 207 (19.5), 187.5 (M^{2+}) (2.3), 168 (37), 159 (24), 155 (11.4), 115 (100), 100 (97).

(threo-)6 2-Bromo-2(methyl-5-dimethylimmonium-tetrahydro-2-furyl) ethyl -7-hydroxy-5-methoxy-4-methylphthalon-1-one bromide (186)

N,N dimethylmycophenolamide (160 mg.) in chloroform (20 ml.) at 0°C, was treated with a solution of bromine in chloroform until the colour persisted. After evaporation of the excess bromine and the solvent, crystallisation from chloroform/ethyl acetate afforded the immonium salt (186) (238 mg., 94%) as colourless prisms, m. 129-134°C.

T.l.c.: 10% methanol/chloroform, Rf. 0.05; bl. ($FeCl_3$), bn. (Ce^{4+}).

I.R. νmax. ($CHCl_3$): 3430 (ν_{OH} phenol), 2940, 1740 (ν_{CO} phthalide), 1700, 1630, 1600 $cm.^{-1}$.

νmax. (KBr): 3415, 2940, 1730, 1703, 1623, 580, 540 (ν_C - Br) cm^{-1}.

NMR. (60 Mc/s.) $\tau CDCl_3$: 4.84 (2H, s., $ArCH_2O$-), 5.20 (1H, t., -CHBr-), 6.11 (3H, s., MeO-), 6.45 (3H, s., $= \overset{+}{N}CH_3$), 6.66 (3H, s., $= \overset{+}{N}CH_3$), 6.86 (2H, d., J = 7 Hz., $ArCH_2CHBr$-), 6.0-6.5 (2H, m.), 7.3 (2H, m.), 7.83 (3H, s., ArMe), 8.02 (3H, s., C-Me).

MS. m/e (rel. abundance): 347 (M-Br) (15), 346 (M-HBr) (14), 345 (28), 273 (24), 259 (37), 245 (98), 207 (23), 159 (23), 140 (39), 138 (42), 87 (99), 72 (100).

Hydrolysis of the immonium bromide (186)

The immonium salt (186) (30 mg.) in benzene (5 ml.) was stirred with 2 drops of water for 2 hr. at room temperature. Evaporation of the solvent afforded the(threo-)bromolactone (171) (25 mg., 90%) which crystallised from chloroform/light petroleum as colourless needles, m. 180-182°C., identical (t.l.c., mixed m.pt., i.r.) to an authentic sample.

(threo-)7-hydroxy-6 2-hydroxy-2(2-methyl-5-oxotetrahydro-2-furyl) ethyl -5-methoxy-4-methylphthalan-1-one (106)

(a) From mycophenolic acid

m-Chloroperbenzoic acid (150 mg.) in chloroform (10 ml.) was added to a solution of mycophenolic acid (150 mg.) in chloroform and the solution left overnight at room temperature. Extraction with saturated aqueous sodium carbonate (10 ml.) and evaporation of the solvent afforded the crude hydroxylactone (70 mg.). Crystallisation from aqueous acetic acid gave pure (threo-)hydroxy-lactone (106) (50 mg., 32%) as colourless needles, m. 215-218°C.,

identical (t.l.c., mixed m.pt., i.r.) with an authentic sample.

(b) From ethyl mycophenolate (168)

m-Chloroperbenzoic acid (50 mg.) in chloroform (5 ml.) was added to a solution of ethyl mycophenolate (168) (50 mg.) in chloroform (5 ml.) and the solution left for 3 days at room temperature. Extraction with saturated aqueous sodium carbonate (5 ml.), evaporation of the solvent and p.l.c. of the residue on HF_{254} silica, using 10% methanol/chloroform as eluant, gave the (threo-)hydroxylactone (106) (10 mg., 20%), identical (t.l.c., mixed m.pt., i.r.) with an authentic sample, and the (erythro-)dihydrobenzofuran (183) (5 mg., 11%), identical (t.l.c., mixed m.pt., i.r.) with an authentic sample.

(c) From N,N-diethylmycophenolamide (190)

m-Chloroperbenzoic acid (50 mg.) in chloroform (5 ml.) was added to a solution of the amide (190) (50 mg.) in chloroform (5 ml.) and the solution left at room temperature overnight. Extraction with saturated aqueous sodium carbonate (10 ml.) followed by evaporation of the solvent afforded the (threo-)hydroxylactone (106) (40 mg., 90%) which crystallised from chloroform/light petroleum as colourless needles, m. 216-218°C., identical (t.l.c., mixed m.pt., i.r.) to an authentic sample.

(threo-)Dihydrobenzofuran (182)

The following data were obtained from a sample gifted by Dr. D. Jones (I.C.I. Pharmaceuticals), m. 252-253°C.

T.l.c.: chloroform, Rf. 0.57; bn. (Ce^{4+}).

GLC: OV17 column (255°C.) RT: 6.5 min.

I.R. νmax (nujol): 1760 (ν_{CO} phthalide, γ-lactone), 1630, 1608, 1160, 991, 950, 784 $cm.^{-1}$.

NMR. (100 Mc/s.) τ $CDCl_3$: 4.97 (2H, s., $ArCH_2O$-), 5.16 (1H, q., $J_{AX} + J_{BX}$ = 9 Hz., -CH(O)CH_2-), 6.12 (3H, s., MeO-), 6.64 (2H, br. d., $ArCH_2CH(O)$-), 7.0-8.0 (4H, m., $-CH_2CH_2-$), 8.03 (3H, s., $ArCH_3$), 8.56 (3H, s., C-Me).

Methyl O-methylmycophenolate epoxide (191)

Methyl O-methylmycophenolate (117) (100 mg.) in chloroform (10 ml.) was added to a solution of m-chloroperbenzoic acid (100 mg.) in chloroform (5 ml.) and the solution left overnight at room temperature. Extraction with saturated aqueous sodium bicarbonate (10 ml.), evaporation of the solvent and p.l.c. of the residue on HF_{254} silica, using chloroform as eluant, gave starting material (10 mg.) and the more polar epoxide (191) (75 mg., 80%), as a colourless oil.

T.l.c.: chloroform, Rf. 0.72; y.-bn. (Ce^{4+}).

I.R. ν max. (CCl_4): 2990, 2940, 2870, 1768 (ν_{CO} phthalide), 1741 (ν_{CO} ester), 1355, 1128 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.86 (2H, s., $ArCH_2O$-), 5.90 (3H, s., MeO-), 6.19 (3H, s., MeO-), 6.38 (3H, s., MeO.CO), 7.06 (3H, br. s., $ArCH_2CH$-), 7.4-7.8 (2H, m.), 7.81 (3H, s., ArMe), 7.9-8.3 (2H, m.), 8.59 (3H, s., C-Me).

MS. m/e (rel. abundance): 364 (M^+) (8), 333 (25), 235 (100), 221 (48), 143 (23), 115 (28), 99 (28).

(threo-)6 2-Hydroxy-2(2-methyl-5-oxotetrahydro-2-furyl)ethyl -5,7-dimethoxy-4-methylphthalan-1-one (192)

A solution of the epoxide (191) (45 mg.) in trifluoroacetic acid (5 ml.) was left at room temperature for 2 hr. Evaporation of the acid afforded the dimethoxy-hydroxylactone (192) (42 mg., 95%) which crystallised from chloroform/light petroleum as colourless needles, m. 158-159°C. (mixed m.pt. with the (erythro-)dimethoxy-hydroxylactone (105) - 130-135°C.)

T.l.c.: chloroform, Rf. 0.53; bn. (Ce^{4+}).

Analysis: Found C: 61.68%, H: 6.01,

$C_{18}H_{22}O_7$ requires C: 61.71%, H: 6.33%.

I.R. ν max. ($CHCl_3$): 3500 (br. ν_{OH}), 2940, 1764 (ν_{CO} phthalide, γ-lactone), 1604 $cm.^{-1}$.

ν max. (KBr): 3450 (br.), 2950, 1760, 1597, 1475, 1355, 1300, 1201, 1124 $cm.^{-1}$.

NMR. (60 Mc/s.) τ $CDCl_3$: 4.95 (2H, s., $ArCH_2O$-), 5.98 (3H, s., MeO-), 6.22 (3H, s., MeO-), 6.2 (1H, obs., -CH(OH)-), 7.2 (1H, s., exchangeable with D_2O), 7.1-8.2 (6H, m.), 7.84 (3H, s., ArMe), 8.54 (3H, s., C-Me).

EXPERIMENTAL

CHAPTER 4

General

Paper chromatography was carried out on Whatman's No. 1 grade paper using n-butanol/acetic acid/water (12/3/5) as eluant. The chromatograms were developed with either ninhydrin (0.5% indane-trione in n-butanol) or isatin (0.2% w/v in acetone).
Amino acid analyses were carried out on a Beckmann Unichrom autoanalyser, using a 69 x 0.9 cm. column, resin type PA-28, and eluting with 0.20 N citrate buffer at 50 ml./hr.

Brevigellin

This compound was isolated from the neutral extracts of the culture filtrate of P. brevicompactum as described earlier (p. 72). Purification by p.l.c. afforded the metabolite as a colourless glass, m. 209-212°C.

T.l.c.: 10% methanol/chloroform, Rf. 0.6, y. (I_2); l.bn., blue fluorescence at 354 nm. (Ce^{4+}).

Analysis: Found C: 59.8%, H: 6.8%, N: 10.2%

$C_{31}H_{41}N_5O_7 \cdot 1\frac{1}{2}H_2O$ requires C: 59.7%, H: 7.05%, N: 11.2%.

MS. gives M^+ at 595.30132, $C_{31}H_{41}N_5O_7$ requires 595.30058.

I.R. νmax. ($CHCl_3$): 3400, 2295, 2920, 2845, 1758 (ε = 236, ν_{CO} ester), 1658 (ε = 836, amide I), 1600, 1500 $cm.^{-1}$.

νmax. (KBr.): 3422, 3295 (br., ν_{NH}); 3016 (ν_{NH}), 2943, 2870, 1758 (ν_{CO} ester), 1660, 1634 (amide I), 1519 (amide II), 1490, 1265, 1240, 1175, 755, 720 $cm.^{-1}$.

UV. λmax. (EtOH): 230 (ε = 10000) nm.

NMR. (100 Mc/s.) τ $CDCl_3$: 2.2 (3H, m., O-H of Ar.CO-, =CH-N.CO-), 2.5 (3H, m.; m-, p- H of Ar.CO-), 2.86 (1H, d., J = 9 Hz., irr. 4.91→s., -CO.NH.CH-), 4.64 (1H, q., J = 6 Hz., irr. 8.71→s., -CH-CH_3), 4.91 (1H, d., J = 9 Hz., irr. 2.86→s., -CH-NH), 5.3-6.0 (5H, br., m., -CO.CHN-, -NH.CO-), 6.31 (4H, br., m., irr. 8.25→sharpens, -CH_2-N), 8.25 (14H, br., m., irr. 6.31→sharpens, -CH_2-), 8.71 (9H, m., irr. 4.64→sharpens, irr. 5.54→sharpens, C-CH_3).

NMR. (100 Mc/s.) τ 5% TFA/$CDCl_3$: 1.75 (1H, d., J = 9 Hz., irr. 5.4 ⟶ s., -CH = N.CO-), 2.2 (2H, m., O-H of Ar.CO-), 2.5 (3H, m., m-, p- H of Ar.CO-), 2.7 (1H, d., J = 9 Hz., exchanged with D_2O, -NH-CH-), 4.62 (1H, q., J - 6 Hz., -CH-CH_3), 4.91 (1H, d., J = 9 Hz.,→ s. on exchange with D_2O, -CH-NH-), 5.2-6.0 (6H, m.,), 6.3 (4H, br., m., -CH_2-N), 8.25 (14H, br., m., -CH_2-), 8.71 (9H, m., C-CH_3).

MS. (see text p. 65)

Acid hydrolysis of brevigellin (208)

(a) Brevigellin (8.03 mg.) was refluxed in 2.5N hydrochloric acid (1 ml.) for 6 hr., the cooled solution neutralised with aqueous ammonium hydroxide and evaporated to dryness. The hydrolysis products were identified by comparison with samples of the appropriate amino acids by paper chromatography (Table 4:I).

Amino acid analysis R_T: 86 min. (threonine, 24%), 116 min. (proline, 25%), 150 min. (alanine, 25%), 202 min. (unidentified component, 20%).

Table 4:I Separation of brevigellin hydrolysis products by paper chromatography

Component	Rf.*	Ninhydrin	Isatin
Threonine	0.25	red-brown	red-brown
Alanine	0.31	purple	--
Proline	0.34	yellow	blue
β-aminobutyrate	0.42	lilac	green

*Rf. values were standardised by comparison with glycine (0.23) and phenylalanine (0.60).

(b) Brevigellin (1.02 mg.) in 6N hydrochloric acid (0.5 ml.) was heated at 120°C in a sealed tube for 12 hr. and the hydrolysis products directly analysed.

Amino acid analysis, R_T: 85 min. (threonine, 31%), 118 min. (proline, 34%), 150 min. (alanine, 33%).

Alkaline hydrolysis of brevigellin (208)

Brevigellin (5 mg.) was treated with 0.5N methanolic potassium hydroxide (1 ml.) for 6 hr. and the solution carefully neutralised with 0.5 N hydrochloric acid. Extraction with chloroform (5 ml.) and subsequent evaporation of the solvent afforded a colourless residue (2 mg.) which was dissolved in methanol (1 ml.) and treated with an excess of ethereal diazomethane for 5 min. Evaporation of the solvent gave the methyl ester (**220**, 1 mg.).

T.l.c.: 10% methanol/chloroform, Rf. 0.7, y. (I_2), l. bn., (Ce^{4+}).

MS. (see text p. 66).

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