F U N G A L T E R P E N O I D S

A THESIS PRESENTED BY

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TO THE UNIVERSITY OF GLASGOW FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

THE CHEMISTRY DEPARTMENT

DECEMBER 1968

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

My thanks are also due to Dr. G. Ferguson for his assistance in the X-ray crystallographic analysis.

I am indebted to the P.I.O. for maintenance during one year and to Professor R.A. Raphael, F.R.S., for providing the opportunity and facilities for performing the work described here.

For their assistance and advice, my thanks are due to Mr. J.M.L. Cameron, B.Sc. and his staff (micro-analyses), to Mrs. F. Lawrie and technical staff (infra red spectra), to Dr. J.S. Roberts (mass spectra) to Mr. J. Gall (nuclear magnetic resonance spectra), to Miss M. McKenzie and Mrs. J. MacIntosh (preparation of fungal extracts), to the X-ray technicians, to Miss M.H. Linton (manuscript typing) and finally to my many laboratory colleagues.

SUMMARY

Studies of the terpenoid constituents of the fungus Penicillium brevicompactum have established the presence of two new compounds(a furan and a dihydrofuran) with the same nucleus as mycophenolic acid but with modified terpenoid side chains. Elucidation of the structure of these has been assisted by various transformations involving the side chain.

The structure of mycochromenic acid,a further compound of this type which was previously isolated from P.brevicompactum has been confirmed by a synthesis which establishes a new route to chromenes.

From the same fungus three related sesquiterpene benzoates (pebrolide,deoxypebrolide and desacetylpebrolide) were isolated and their structures elucidated by chemical studies and by an X-ray analysis of bromoacetylpebrolide. NMR studies of pebrolide and its derivatives and the X-ray data showed that the preferred conformation in solution and the conformation in the crystal are very similar.

A sequence for degradation of biosynthetically labelled pebrolide was established. This was applied to material derived from doubly labelled $2^{14}C/2^{3}H$ mevalonate and the presence of label at C-1 and at the 4α carbon established.

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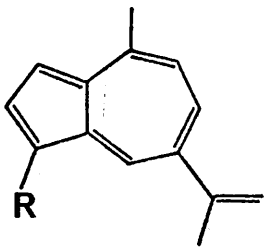
GENERAL INTRODUCTION



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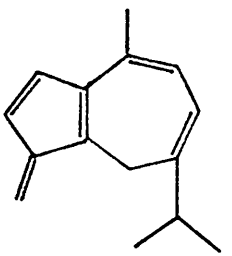

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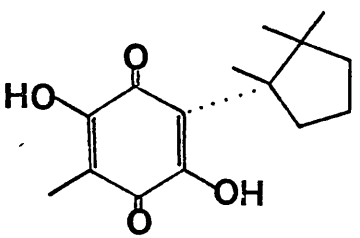

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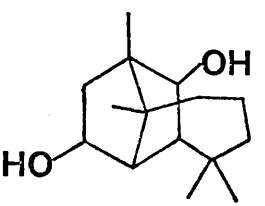

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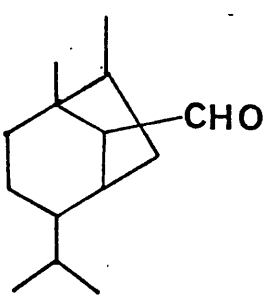

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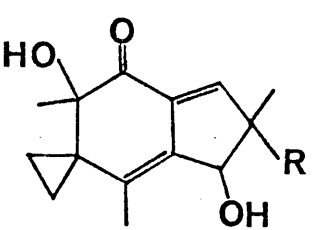
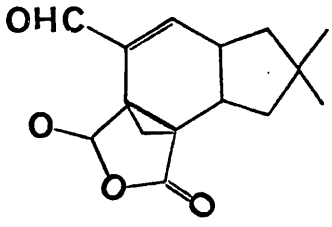


7

GENERAL INTRODUCTION

The discovery of the effectiveness of the mould product, penicillin, in treating many bacterial infections in man, gave tremendous impetus to the isolation and screening of micro-organisms and their metabolites for antibiotics.

The variety of mould metabolites is very large and a considerable number of them possess terpenoid structure. The last few years have witnessed many notable advances in the knowledge of pharmacological and physiological properties of the terpenoid group. In the case of the sesquiterpenes, those produced by fungi show a particularly wide variety and novelty of structure, relatively few members having skeletons similar to those found in plants. In the azulenes, from Lactarius deliciosus, lactaroviolin (1)[1] , lactarazulene (2)[1] , lactarofulvene (3)[2] , in the benzoquinone helicobasidin (4)[3] , and in culmorin (5)[4] , the head-to-tail C_5 units are easily visible, whereas, in helminthosporal (6)[5] , the illudins (7)[6], marasmic acid (8)[7] , and hirsutic acid (9)[8] , a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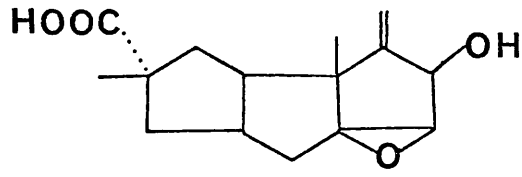
O
O
O

8


HOOC
OH
O

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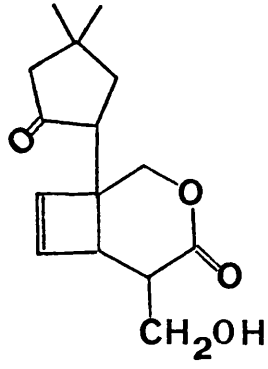
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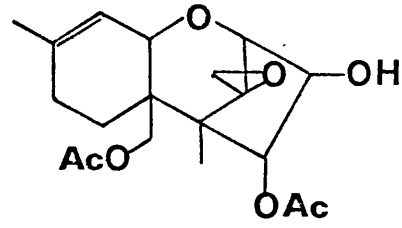
O
O
CH2OH

10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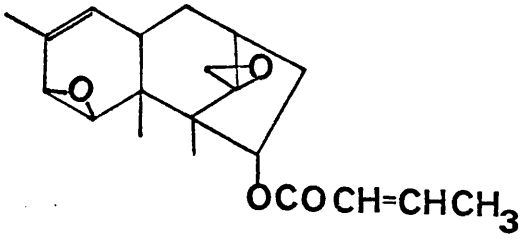
O
OH
AcO
OAc

11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O
OCOCH=CHCH3

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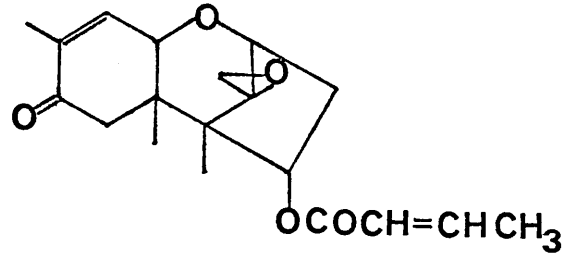
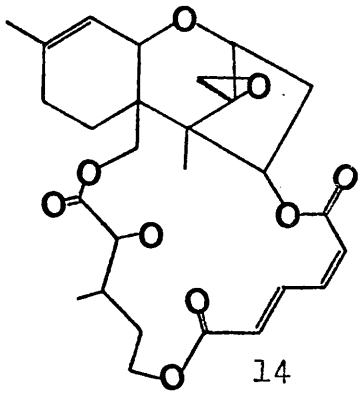

O
O
O
OCOCH=CHCH3

13



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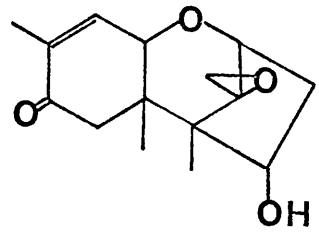

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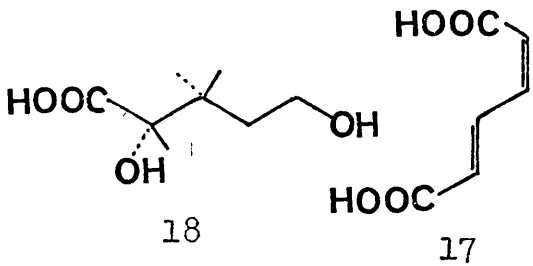
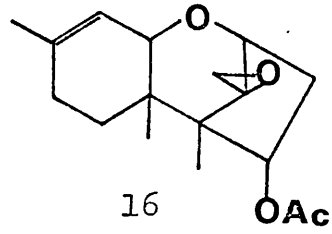


18 17



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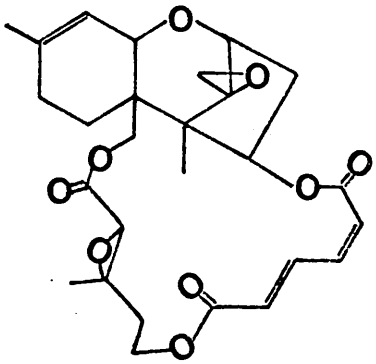

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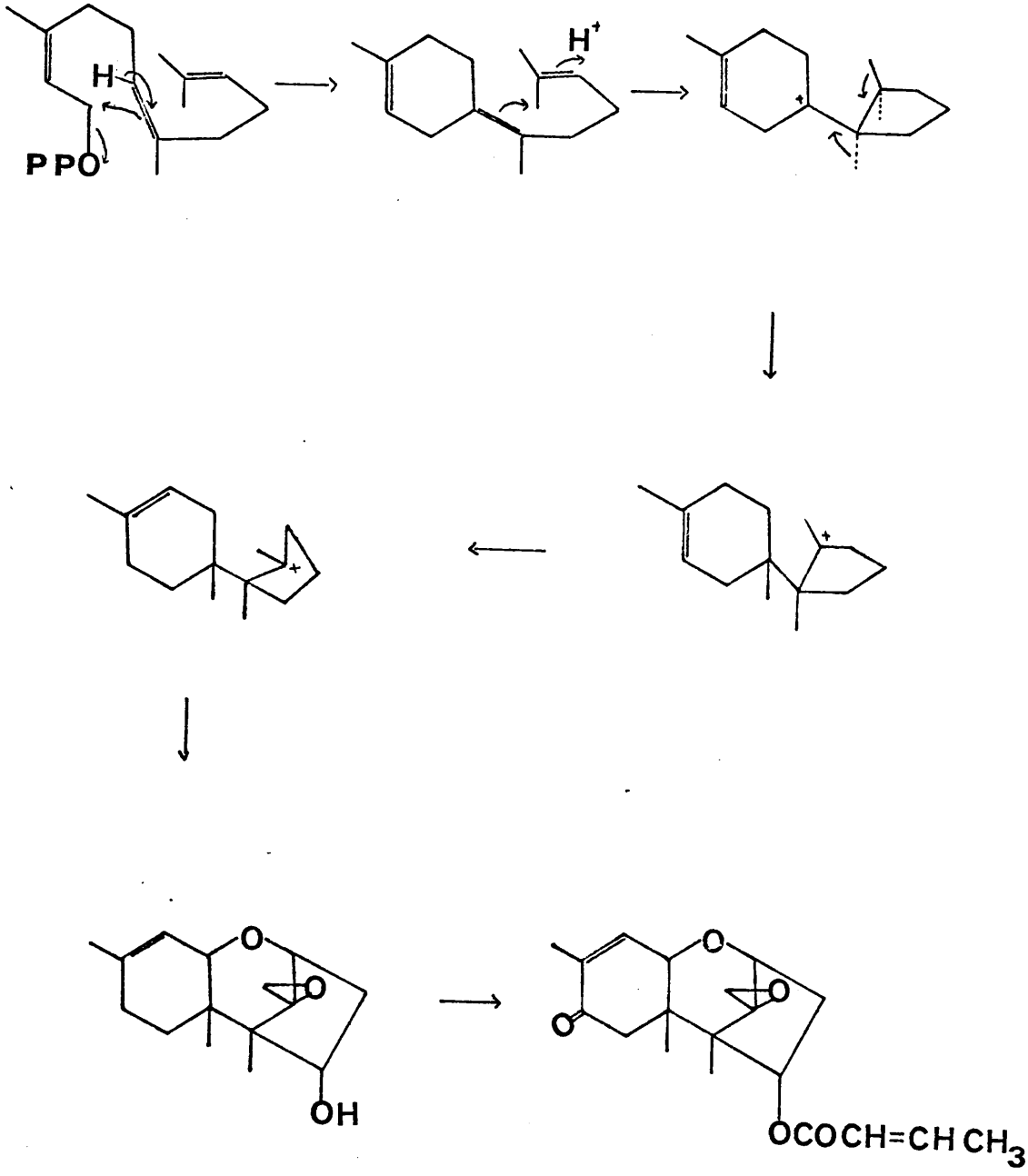
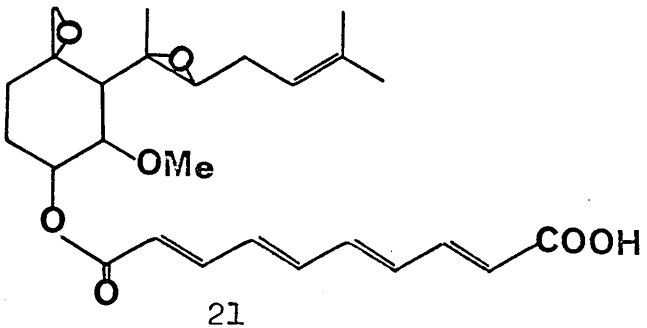

H
PPO
H+
O
O
OH
O
O
O
OCOCH=CH CH3

more complex pattern is observed.

Fomannosin (10)[9] , is the first example of a sesquiterpene containing the cyclobutene moiety. An expanding group of sesquiterpenes contain an oxabicyclo-(3,2,1)-octane system including diacetoxyscirpenol (11)[10] , crotocin (12)[11] , trichothecin (13)[12] , the verrucarins e.g. verrucarin A (14)[13] , the roridins e.g. roridin C (15)[12] , trichodermin (16)[14] . The conjugate acid in trichothecin and crotocin is crotonic acid, probably arising from condensation of two acetate units. The macro-ring of verrucarin A is formed by the cis,trans muconic acid (17) , and an isomer of mevalonic acid (18) . Verrucarin B (19)[15] contains an epoxy acid related to this mevalonate isomer.

Trichothecin appears to be derived from farnesyl pyrophosphate by a pathway involving oxidations and alkyl shifts as shown in the figure (20)[16] . The unusual primary epoxide grouping present in these antibiotics is also present in the unrelated antiamoebic antibiotic, fumagillin (21)[17] , from Aspergillus fumigatus, which possesses an isopentenyl



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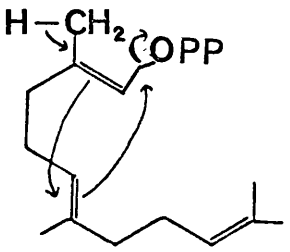

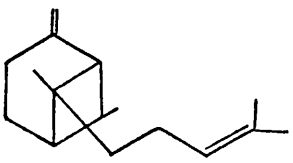

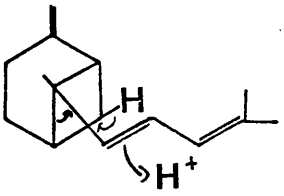

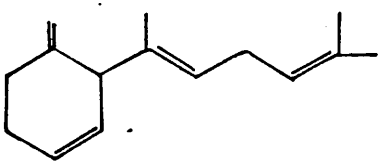

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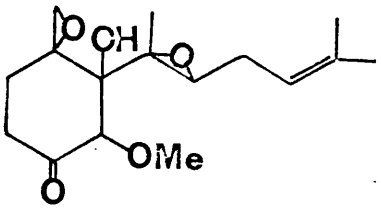

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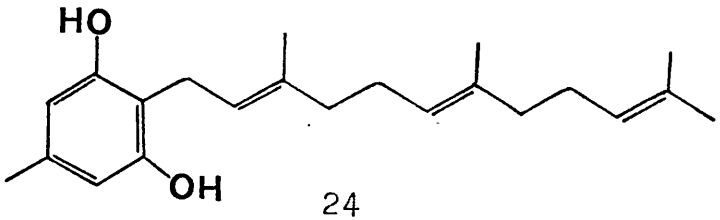

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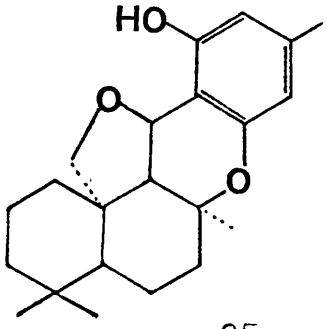

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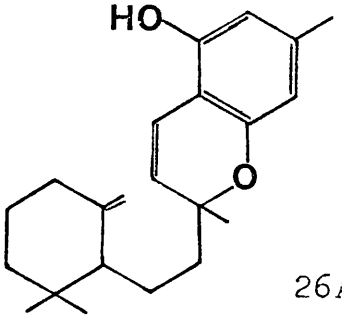

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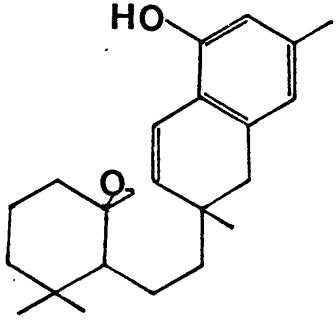

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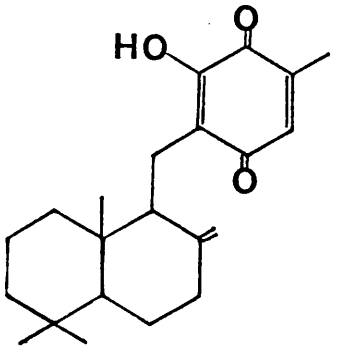
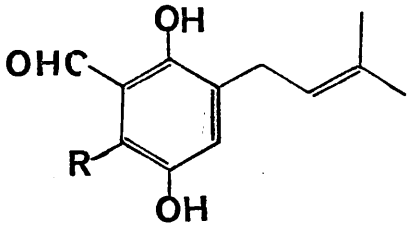


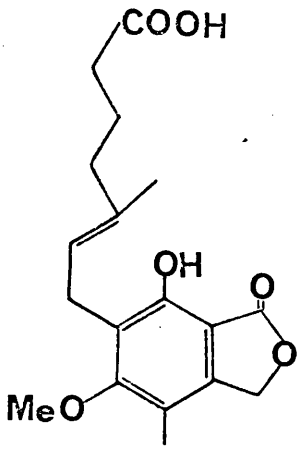
27

trimer nucleus and a decatetraendioic acid side chain apparently derived from the normal C_{10} fatty acid. A possible biosynthesis is indicated in scheme (22)[16]. The antibiotic ovalicin (23)[18], recently isolated from Pseudeurotium ovalis is closely related.

One separate group of terpenoids is that involving alkylation of an otherwise derived nucleus with isoprenoid units. While this type of compound is very common in the higher organisms, in micro-organisms it is relatively rare. Grifolin (24)[19], is derived by alkylation of orcinol with trans trans farnesyl pyrophosphate. Siccanin (25)[20] is a related antifungal antibiotic in which cyclisation has occurred. Siccanin (25) has a sesquiterpene portion (drimane skeleton) attached to the orcinol ring. From the same fungus chromenes have recently been isolated named siccanochromenes A and B (26)[21] in which partial cyclisation has occurred. Tauranin (27)[22] also possesses a non isoprenoid side chain derived from orcinol. Other compounds in the same group include auroglaucin (28)[23] and flavoglaucin (29)[24] with an isopentenyl substituent, mycophenolic acid (30)[25] with



28 $(CH=CH)_3CH_3$
29 $(CH_2)_6CH_3$



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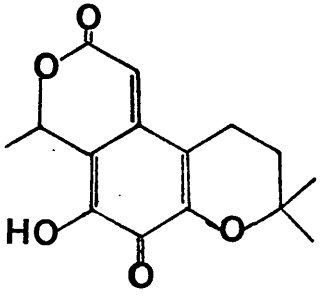

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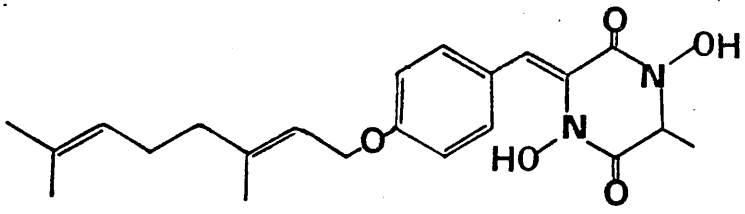

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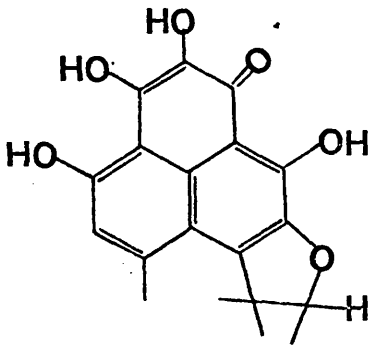

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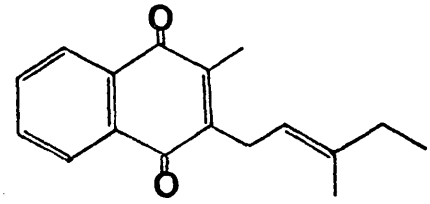

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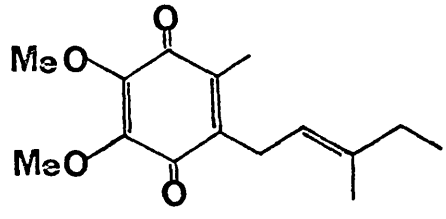

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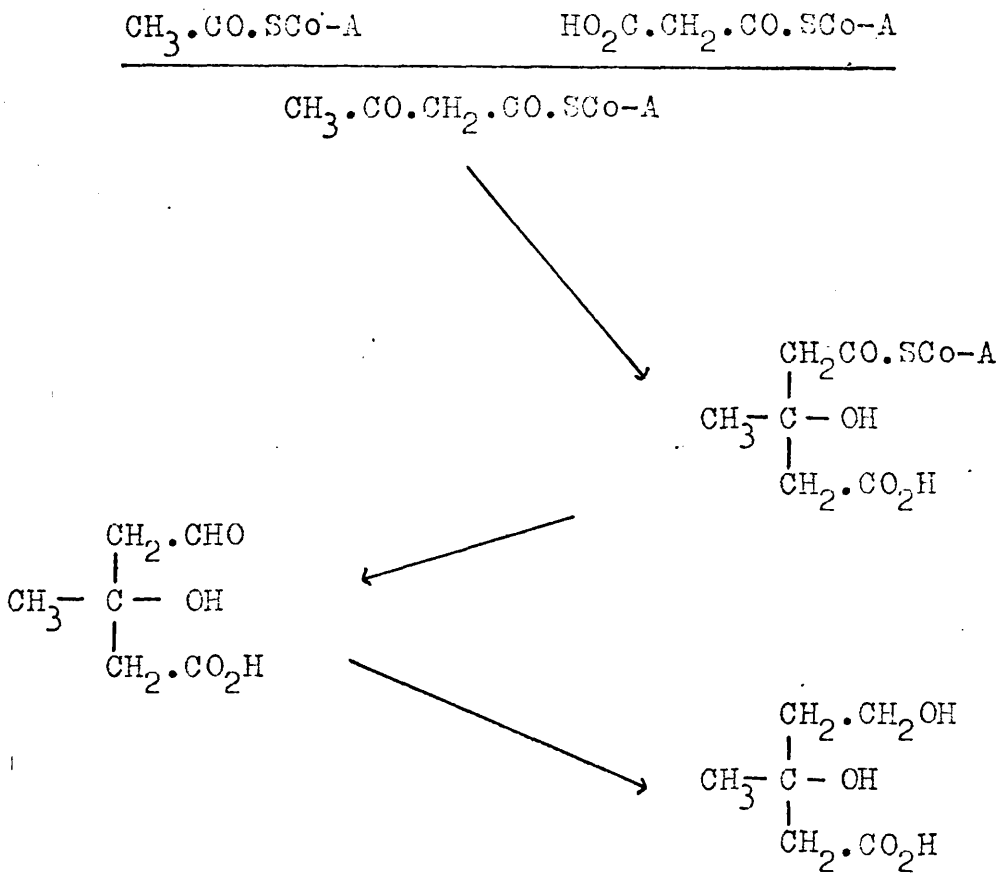

CH3.CO.SCo-A
HO2C.CH2.CO.SCo-A
CH3.CO.CH2.CO.SCo-A
CH2CO.SCo-A
CH3 C OH
CH2.CO2H
CH2.CHO
CH3 C OH
CH2.CO2H
CH2.CH2OH
CH3 C OH
CH2.CO2H

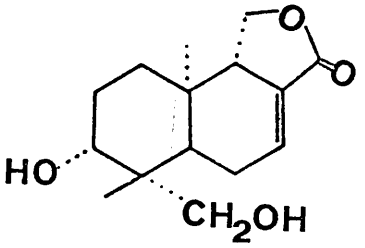
a degraded geranyl side chain (oxidative loss of acetone), fuscin (31)[26] , mycelianamide (32)[27] , with a terpenoid ether grouping, atrovenetin (33)[28] , and quinone coenzymes (vitamin K (34)[29] , coenzyme Q (35)[30].). Benzoquinones with side chains containing six to nine isoprenoid units are generally found in fungi, bacteria and plants, whereas those containing nine to ten units are chiefly found in animal tissues.

The fungal metabolite mycophenolic acid was one of the first secondary metabolites in which the terpenoid portion was shown to be derived from mevalonic acid [31] . The accepted sequence of reactions from acetyl Co A up to mevalonic acid is based on work in mammalian systems and is shown in scheme (36) . The acetyl Co. A is carboxylated to give malonyl Co A which is then decarboxylatively coupled to a second molecule of acetyl Co A to yield acetoacetyl Co A. Claisen condensation of this species with a third molecule of acetyl Co A leads to β - hydroxy β - methyl glutaryl Co A, The evidence suggests that up to this point the reactions are reversible, but that the reduction of mevalonic acid is effectively irreversible and that this is one of the points at which terpene synthesis may be

controlled [32].

It has been proposed[33] that the first three steps may involve enzyme - bound species rather than the free Co A esters and that the final reduction to mevalonic acid releases it from the enzyme surface. The focal point of sesquiterpene biogenesis is the naturally occurring compound farnesol whose formation from acetyl Co A via mevalonic acid has found experimental verification.[34] Lynen[35] and his collaborators identified farnesyl pyrophosphate as a precursor of squalene in yeast and it was later shown that farnesyl pyrophosphate was preceded in biosynthetic sequence by geranyl pyrophosphate. The first step in the conversion of isopentenyl pyrophosphate into farnesyl pyrophosphate was the enzymic isomerisation of isopentenyl pyrophosphate to dimethylallyl pyrophosphate, which was then converted into geranyl pyrophosphate and the latter, in turn, to trans,trans farnesyl pyrophosphate. This pathway has been shown to operate in mammalian liver by Popjak et.al.[36], who have also established the trans,trans structure for farnesyl pyrophosphate.

Various cyclisations of farnesyl pyrophosphate give



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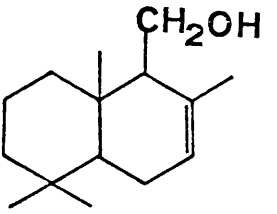

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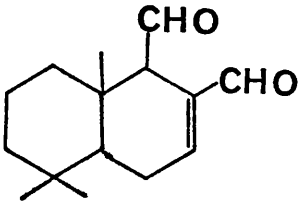

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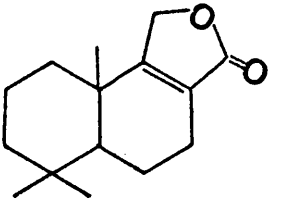

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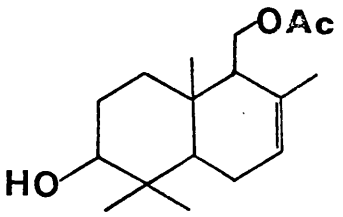

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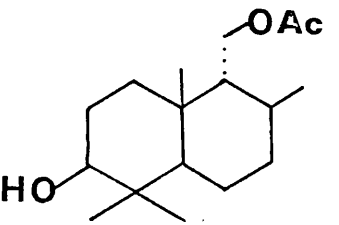

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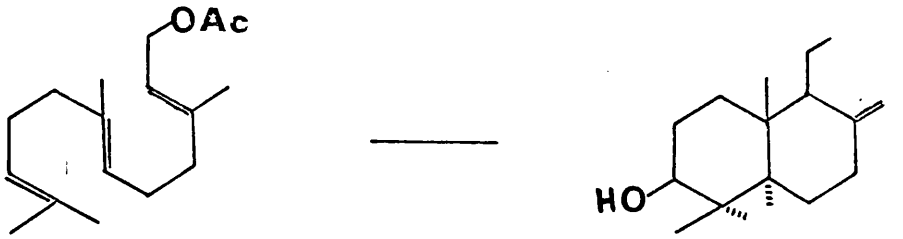
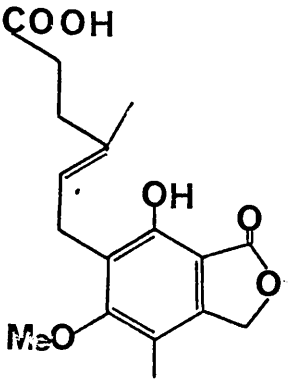


43

the different groups of sesquiterpenes. A well defined group of sesquiterpenes has been isolated which is apparently derived by a non-stop trans antiparallel cyclisation of farnesyl pyrophosphate. for example iresin (37) [37], drimenol (38) [38], polygodial (39) [39], and confertifolin (40) [40]. It was suggested initially that these might in fact be degraded di- and triterpenes, but iresin has been shown to have the opposite stereochemistry from that found in most higher terpenes and steroids, although drimenol, confertifolin, etc., have been shown to possess the "conventional" absolute stereochemistry.

The actual mechanism of cyclisation in these cases has not been proved, but in vitro treatment of the terminal monoepoxide of farnesyl acetate with boron trifluoride-ether complex or mineral acid gave a reasonable yield of the stereoisomers (41) (42) [41] also oxidative cyclisation of farnesyl acetate (43) [42] by a free radical path occurred in a remarkably specific way [42].

INTRODUCTION



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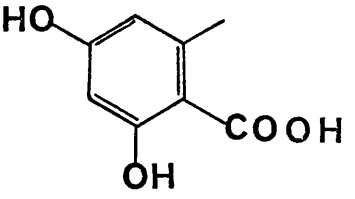

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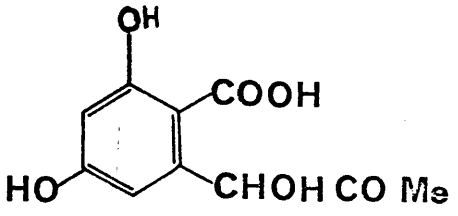
INTRODUCTION

Penicillium brevicompactum belongs to the class of fungi known as Fungi Imperfecti and has been previously studied among others Oxford and Raistrick [43], Godin [44] and Birch [45]. A series of phenolic compounds was isolated, among them mycophenolic acid, which was found by Gosio [46] in 1896 to inhibit *Bacillus antracis*. Although the antibacterial activity/toxicity ratio was too low to allow its use as a therapeutic agent, nevertheless, this marked the discovery that antibacterial substances could be produced by micro-organisms.

Mycophenolic acid (44) has been extensively studied by Birch and co-workers. They suggested that the aromatic nucleus is that of orsellinic acid (45) oxidised to phthalide [47], The C - methyl group at C - 5 and the O - Me groups being derived from methionine [48]. When labelled orsellinic acid was fed to the organism, incorporation was poor and the label turned up in the corresponding position in mycophenolic acid only to an extent of about one fourth, indicating the previous degradation in part to small units [49]. This result may be due to problems of assimilation of the orsellinic acid fed to the organism,



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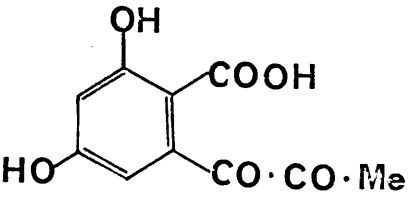

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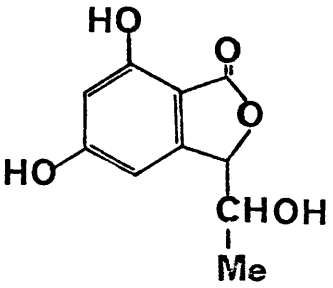

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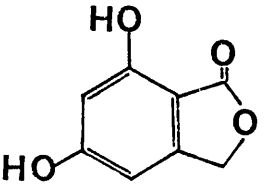

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or it may suggest that the precursor of mycophenolic acid is (46) instead. In support of this the C_{10} compound (47) accompanies mycophenolic acid in P. brevicompactum and in growing experiments was found to reach a maximum concentration in early growth which later decreased [50].

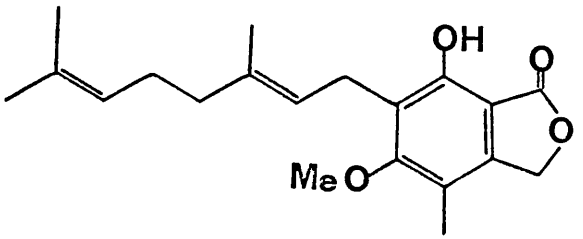
The strain of P. brevicompactum used in the present work was previously shown to afford a mixture of C_{10} compounds including (47)[51] . Another of these compounds has now been tentatively identified as the hydroxyphthalide (48) on the following grounds. It was phenolic and it was noted that its ultraviolet spectrum at neutrality and also at pH 10 was superimposable with that of 5,7-dihydroxyphthalide (49) . Furthermore, both substances possessed similar ultraviolet-induced deep-blue fluorescence and produced the same wine colouration with alcoholic ferric chloride. However, they did not possess exactly the same R_f value in methyl ethyl ketone, water, diethylamine T.L.C. system.

Mass measurement indicated the molecular formula $C_{10}H_{10}O_5$. If the presence of the nucleus (49) is assumed then in the NMR spectrum, the one proton

signal at 4.31 could be assigned to a proton in the lactone ring. The low τ value of this proton (cf 4.8 τ in mycophenolic acid) could be due to the deshielding effect of a group attached at this position. The presence of a multiplet at 5.39 and of a doublet at 8.54 τ suggests that this group could be $-CH\ OH\ -CH_3$ as in (48) . This structure is strongly supported by the cracking pattern which shows an abundant ion at $^{m}/e$ 165 corresponding to a loss of $-CH\ OH-CH_3$ from the molecular ion. T.L.C. evidence of the formation of this compound was obtained following the borohydride reduction of the ketone (47) .

Since the main subject of the present work concerned the terpenoid constituents of P. brevicompactum, this compound has not as yet been studied further. However, since a C_2 unit could be lost by retroaldol cleavage this compound may have significance in the biosynthesis of the nucleus of mycophenolic acid.

DISCUSSION PART I



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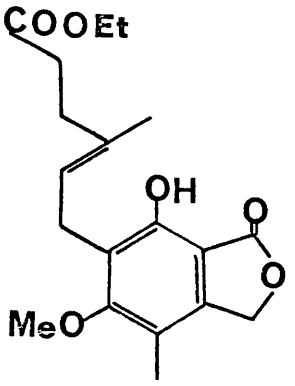

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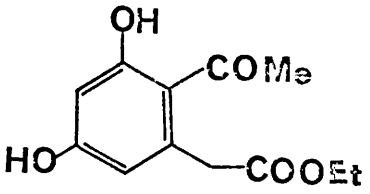
DISCUSSION PART 1

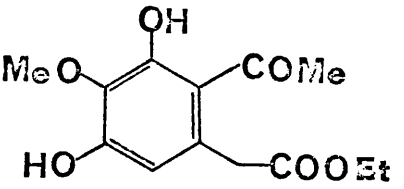
The side chain of mycophenolic acid is derived from mevalonate and has been shown to represent a geranyl group from which the terminal three carbons have been removed by oxidation. Evidence of this has been provided by the isolation from a culture of P. brevicompactum containing 2 - C mevalonolactone, of acetone and mycophenolic acid in approximately equimolecular quantities and of approximately equal, molar, specific activities [52].

In the present work a strain of P. brevicompactum was used which had already been found to produce mycophenolic acid and three metabolites which appeared to be related . Since no analogue of mycophenolic acid with an intact geranyl side chain, e.g. (50) has ever been reported and nothing is known about the mechanism of this oxidative cleavage , , it was of interest to carry out a systematic investigation of these metabolites.

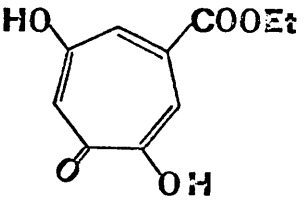
One of these three compounds was the ethyl ester of mycophenolic acid (51)[51] . An interesting feature is the presence of the unusual ethyl


OH
COMe
HO
COOEt

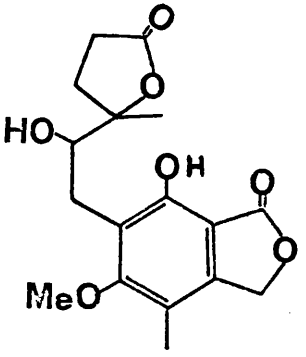
52


OH
MeO
COMe
HO
COOEt

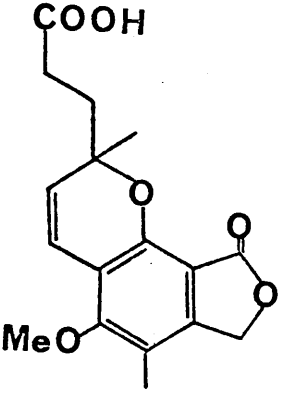
53


HO
COOEt
O
OH

54


O
O
HO
OH
O
O
MeO

55

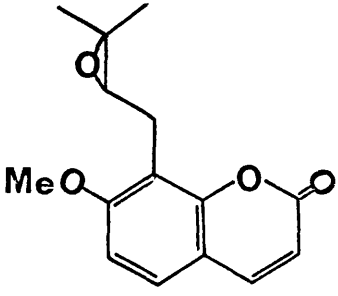

COOH
O
O
O
MeO

56

grouping. The natural occurrence of ethyl esters such as this is rare, previous examples being curvulin (52)[53] curvin (53) [53], ethylacetate[54] and ethyl stipitatonate (54) [55] from various fungi, and monoethyl dipicolinate from various bacilli.[56]

The hydroxylactone (55)[51] was also isolated at the same time. The distinction between the two possible isomers was made by synthesis of both from mycophenolic acid, the threo-isomer via peracid oxidation and the erythro via osmylation of the double bond of mycophenolic acid.

The third compound was named mycochromenic acid (56) [51]. The structure of this compound was proposed on the following grounds : microanalysis and mass spectroscopy indicated the molecular formula $C_{17}H_{18}O_6$. As with that of mycophenolic acid the NMR showed resonances at 7.9, 6.2 and 4.9 τ corresponding to an aromatic methyl group, to a methoxyl group and to the methylene group of the phthalide respectively. However, the phthalide carbonyl group does not have a free phenolic grouping in the peri position in keeping with the UV spectrum being unchanged on basification. The position of the UV maximum at max 3325 Å (cf UV spectrum of mycophenolic acid max 3070 Å) and occurrence in the



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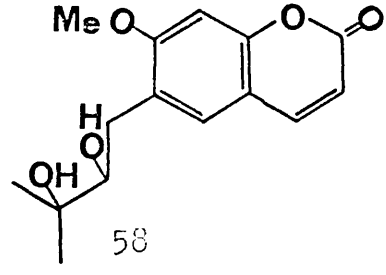

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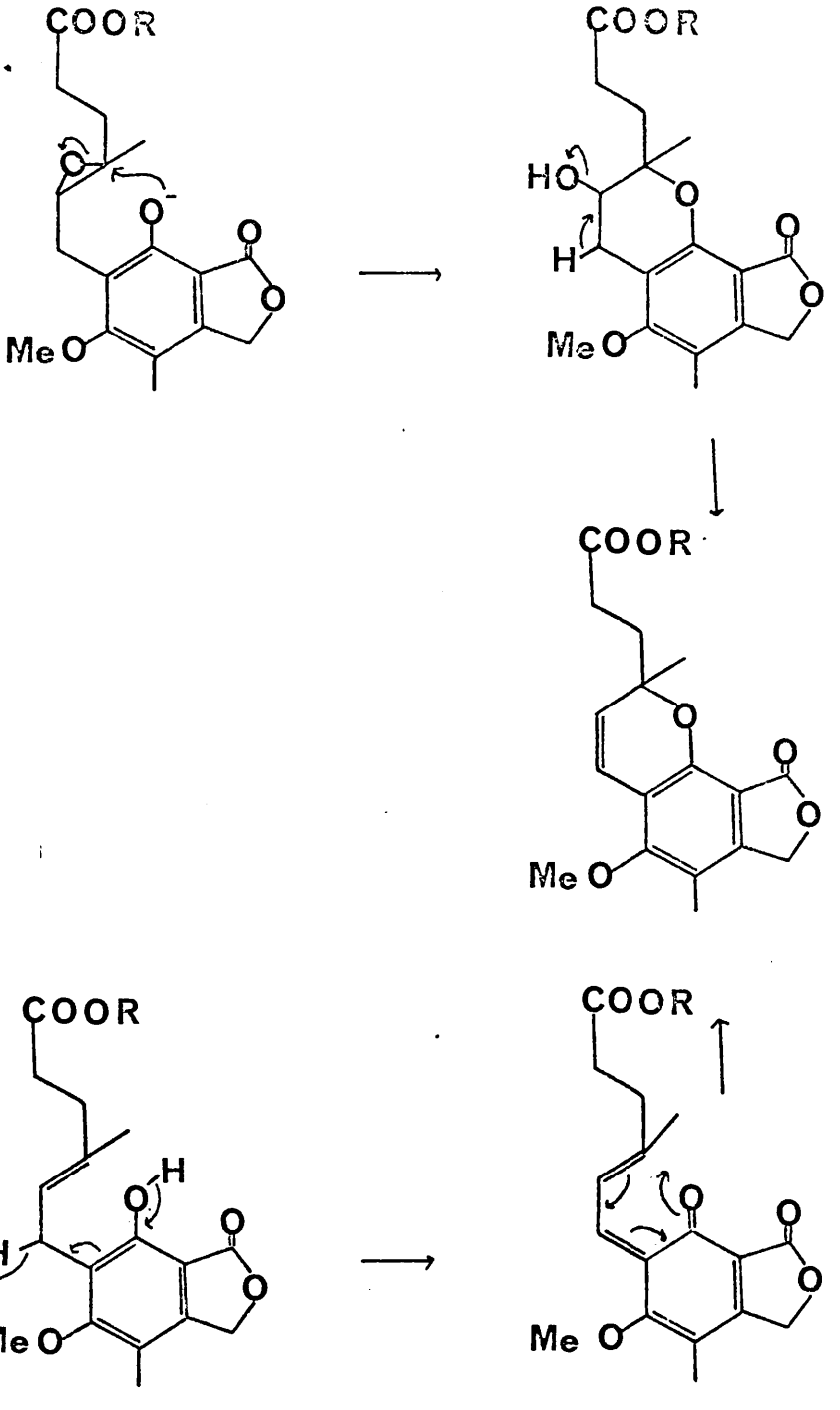
NMR of doublets at 4.37 and 3.32 τ (J = 10.2 $^{c}/_{s}$) suggested the presence of the mycophenolic acid chromophore extended by conjugation with a cis disubstituted double bond. The remaining features of the NMR were also consistent with the proposed structure, a singlet at 8.50 τ being assigned to the CH_3 - C - O - grouping and multiplets at 7.45 and 7.8 τ to methylene groups respectively and to the carbonyl grouping , the latter methylene group suffering additional deshielding by the gem ether function.

In the present work an attempt to synthesise mycochromenic acid from mycophenolic acid has been carried out to prove the suggested structure and to test in vitro the following biogenesis.

The hydroxylactone (55) occurring in P. brevicompactum could be derived from mycophenolic acid by initial formation of a glycol with subsequent cyclisation to the threo - lactone (55) . This has many analogies in plant terpenoids, e.g. in coumarins in which a double bond of an isoprenoid side chain occurs as an oxidised form either as an epoxide as in auropten (57)[57] or as a glycol as in toddalolactone (58)[58] . Similarly mycochromenic acid might be


COOR
O
O
O
O
Me O
COOR
HO
H
O
O
O
Me O
COOR
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Me O
COOR
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H
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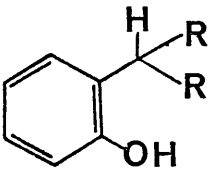
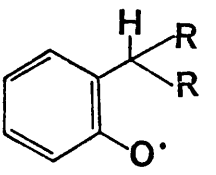
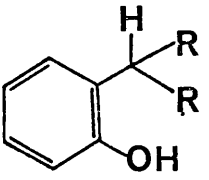
Me O
COOR
O
O
O
Me O

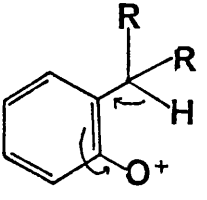
Path A

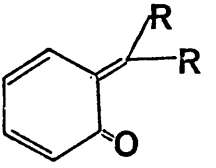




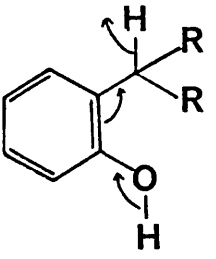
Path B





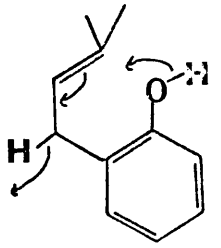


Path B -



derived biogenetically from mycophenolic acid (scheme 58) by attack of the phenolate anion upon the corresponding epoxide followed by dehydration. Alternatively, mycochromenic acid might be derived from mycophenolic acid by abstraction of a hydride ion giving the unstable intermediate quinone methide which would immediately isomerize to the chromene. Three mechanisms have been considered for the formation of the quinone methide (59)[59]. The first mechanism(path A), based on chemical evidence that phenoxy radical formation is the initial step,involves one electron oxidation. If an ionic process is involved, then the methide could be formed by hydride abstraction from the hydroxyl group (path B)or more directly from the benzylic carbon(path B') .

It has been shown that enols are subject to two electron oxidation[60] . Thus in both chemical and biochemical dehydrogenation of steroidal ketones it is the enolic form of the substrate which undergoes hydride abstraction at the carbon atom . In addition suitable enolic compounds are rapidly dehydrogenated by high potential quinone reagents, thought to function by accepting hydride ion [61] . Another mechanism (scheme 60) which excludes a quinone



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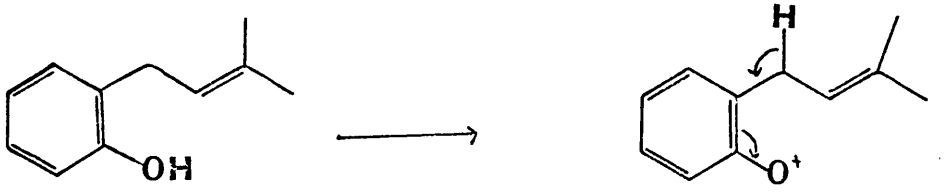

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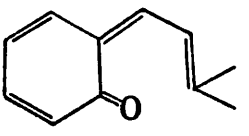

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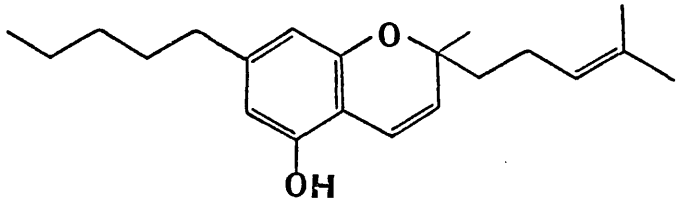
methide intermediate could be proposed, but in one case at least support for the formation of the quinone methide has been found, namely in the occurrence of derivatives of the parent phenol (61) oxygenated at the benzylic position, this being presumed to arise by hydration of the intermediate (62) [59].

In view of the experiments described later, only a brief attempt to test the first mechanism by creating the phenoxy radical was carried out. Methanolic solutions of mycophenolic acid were irradiated separately with ordinary light and with UV light under a stream of oxygen. Only starting material was recovered in both cases.

The second mechanism was tested as follows : Epoxidation of mycophenolic acid with m -chloroperbenzoic acid followed by treatment of the product with base resulted in the formation of the threohydroxy lactone. No cyclic ether could be detected. Similar reaction with the ethyl ester of mycophenolic acid using dry sodium ethoxide again gave only this lactone. This may be formed via hydration of the epoxide during work up.

The failure of this synthetic approach to

mycochromenic acid prompted an investigation of the third route, that is, by hydride abstraction. In order to test this possibility, mycophenolic acid was refluxed in benzene for several hours with 10% palladium charcoal under nitrogen. Examination of the products by T.L.C. showed the presence of a small amount of mycochromenic acid; the yield was increased by using xylene instead of benzene, but was still very low. A substantial improvement was obtained, however, by the use of 2,3 - dichloro - 5,6 dicyano - 1,4 benzoquinone in benzene or even better in xylene. Mycochromenic acid was easily recognisable in T.L.C. by the unusual characteristic deep blue colour that mycochromenic derivatives give when they are sprayed with ceric monium nitrate, baked for a few minutes and allowed to cool. One of the main difficulties in this dehydrogenation experiment lay in the separation of mycochromenic acid from the reaction products. It was thought, particularly in view of the work described later (p17), that one possible source of difficulty might be in the presence of the free carbonyl group which might interact with the double bond. Also one of the main difficulties is the similar R_f value of mycochromenic acid and the decomposition



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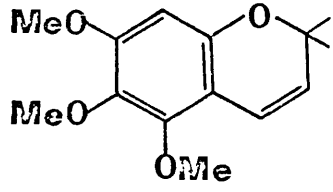

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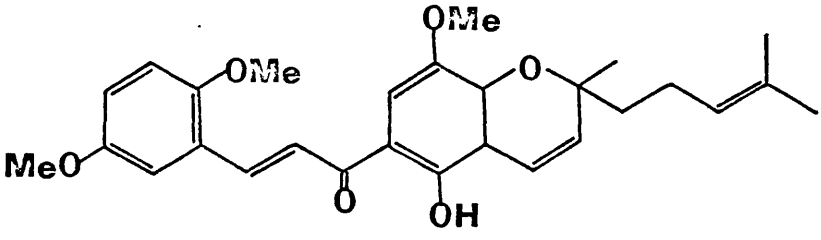

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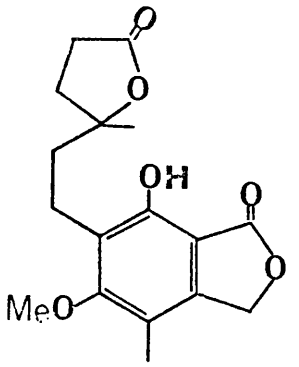
products of D.D.Q. Both these difficulties were avoided by first preparing the ethyl ester of mycophenolic acid. Dehydrogenation with D.D.Q. in xylene now afforded mycochromenic ester in 40% yield this being readily separated from the other reaction products by means of preparative T.L.C.

This appears to be the first experimental reproduction in vitro of the biogenesis of natural chromenes as proposed by Turner [59].

Following the publication of this result, the potential of this method has been explored by other workers[62] in the synthesis of some representative chromenes using cyclodehydrogenation with D.D.Q; these include D L - cannabichrome (63) , evodiononol methyl ester (64) and flemingin C trimethyl ether(65) .

When coupled with prenylation of phenols, this method may be the mildest and most efficient synthesis of 2,2-dimethyl chromenes. The usual methods [63] for synthesis of 2,2-dimethyl chromenes require the use of reagents (L A H or Grignard) which can interfere with other reactive functional groups in the same molecule.

When the NMR spectrum of mycophenolic acid was run



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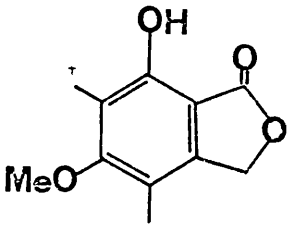

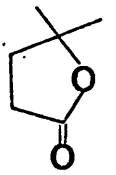

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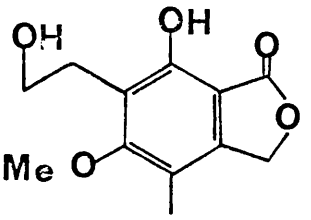
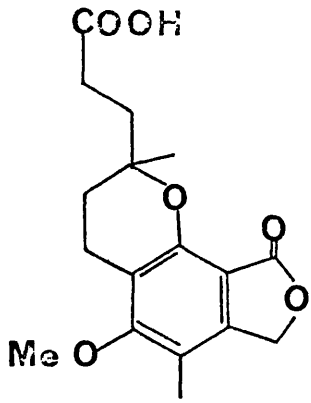


68

in trifluoroacetic acid, it was observed that the spectrum showed a remarkable difference from that in deuterochloroform. Removal of the solvent yielded an isomeric compound that was no longer acid and whose NMR spectrum in deuterochloroform did not show any vinyl protons. It was shown to be a phenolic compound because of the base shift in it's U.V. spectrum and positive test with ferric chloride. The principal features of the mycophenolic nucleus were still present in the NMR spectrum. This also showed the absence of the double bond of the side chain in mycophenolic acid and the methyl appeared as a singlet at 8.86 τ (cf 8.5 τ in mycophenolic acid) corresponding to a methyl group vicinal to an oxygen function. These features indicate the product is the lactone(66) . The remaining features of the NMR spectrum are also in accordance with this structure. Also this structure is in agreement with the mass spectrum whose base peak corresponds to the ions(67) derived by benzylic cleavage. In the case of the hydroxylactone (55) the base peak corresponds to (68) due to the presence of the hydroxyl group. This compound was also obtained on refluxing mycophenolic acid with p-toluensulphonic acid in benzene.



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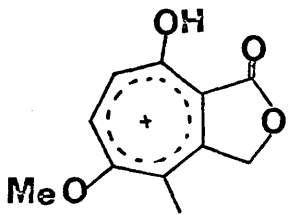

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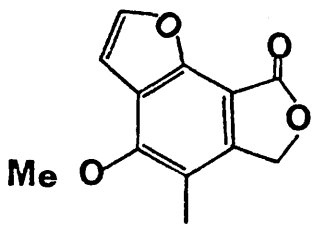

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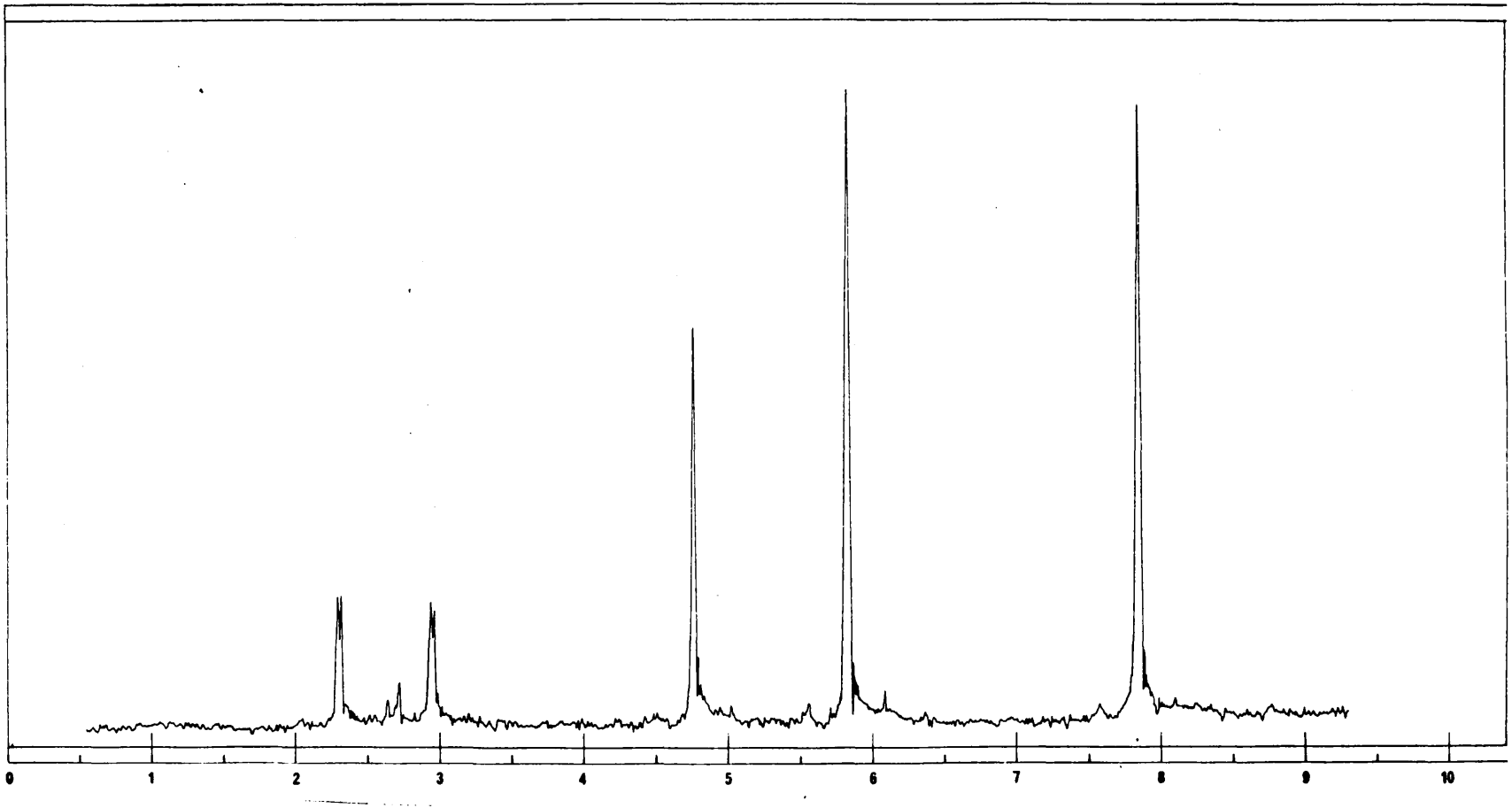
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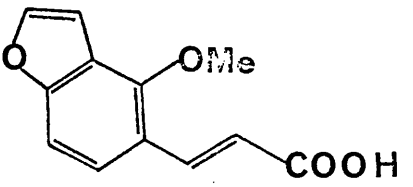
On the other hand the double bond of mycophenolic acid interacts with the phenolic hydroxyl group under different acidic conditions, namely, upon refluxing in acetic acid with concentrated sulphuric acid[64] . The new product was non phenolic, showing no base shift in it's UV spectrum and showed no vinyl proton in NMR spectrum which was closely similar to that of the above lactone. The structure (69) which was assigned to this compound, corresponds to the dihydro derivative of mycochromenic acid and catalytic reduction of ethyl mycochromenate in fact was found to give the ethyl ester of this product.

The mass spectrum is also in agreement with the proposed structure, thus the base peak at $^{m}/e$ 207 corresponding to the fragment (70).

In the present work, two further metabolites related to mycophenolic acid have been isolated. Both of them are present in small amounts and are only found in the later states of growth (28 days).

One of them has been named mycofuranolide (71) and figure (72) shows the NMR recorded for this.

compound. Elemental analysis and mass spectroscopy indicated the molecular formula $C_{12}H_{10}O_4$. The NMR spectrum of the metabolite with resonances at 4.76 τ (phthalide methylene) 5.82 τ (aromatic methoxyl) and 7.84 τ (aromatic methyl) suggested that the mycophenolic nucleus was present in the new compound. In contrast with mycophenolic acid, in the IR spectrum there is only one band in the carbonyl region at 1780 cm^{-1} which corresponds to the γ - lactone, but in this compound the intramolecular hydrogen bonding responsible for the lowering of the frequency of the lactone in mycophenolic acid is absent. It was deduced that the metabolite was non-phenolic from the absence of any hydroxyl absorption in the infrared, from the negative test with ferric chloride and since the ultraviolet spectrum was unchanged in base.
Of the four oxygen atoms known to be present, three have been allocated. Since no other carbonyl absorption was detected in the infrared, this other oxygen atom must then form part of an ether linkage. Apart from the three signals due to the mycophenolic nucleus the NMR spectrum showed two doublets at 2.3 and 2.95 τ (AB quartet $J = 2^c/s$). This pattern is characteristic of the furanoid α and β proton respectively of a benzofuran.



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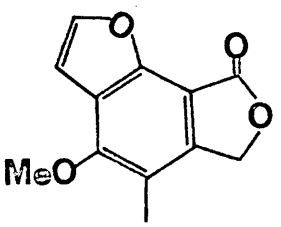

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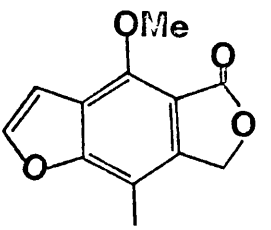

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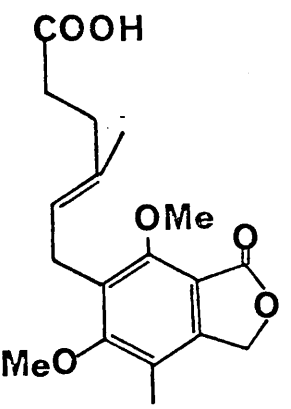
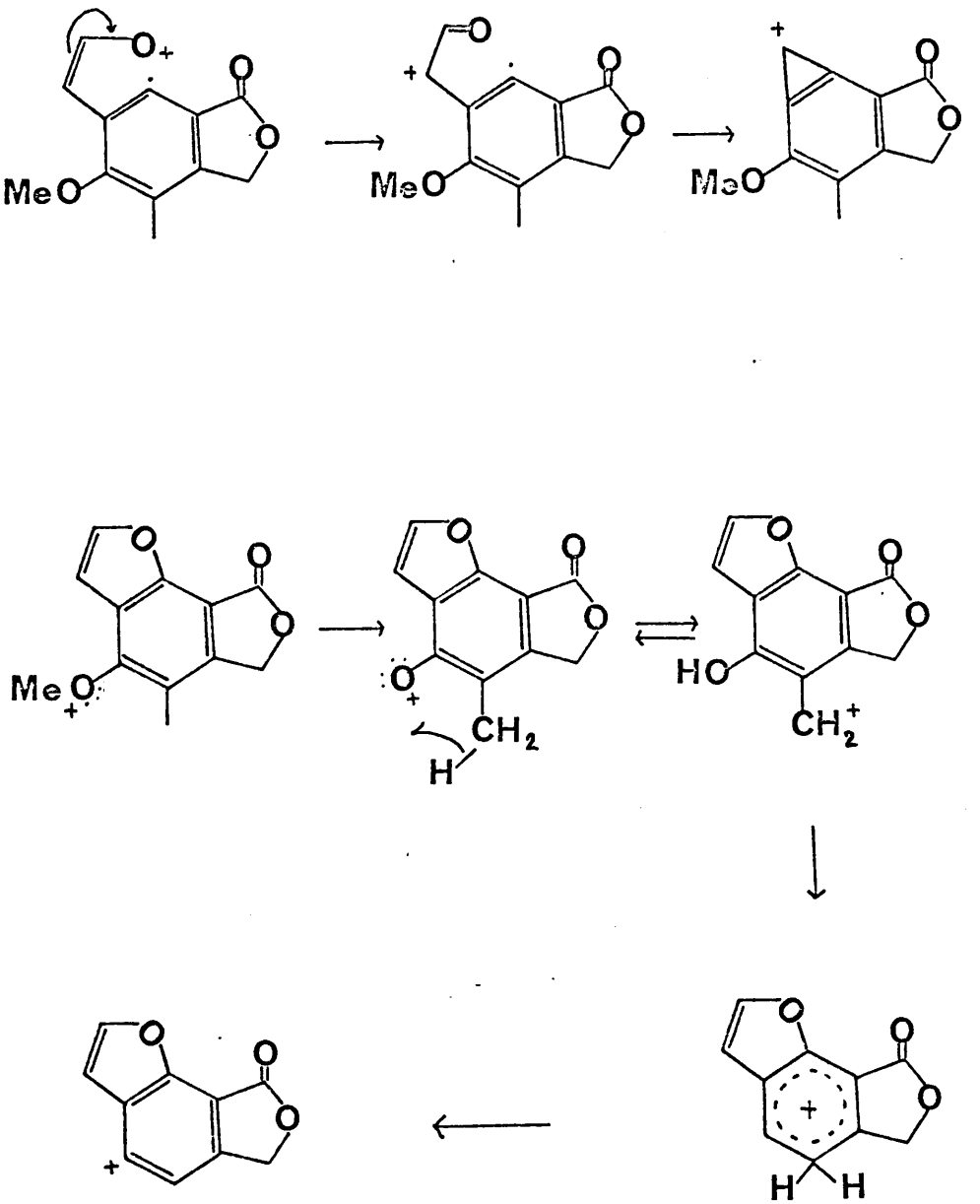
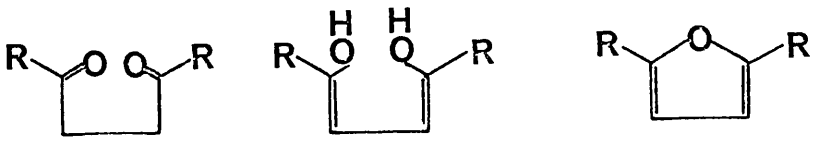


76


MeO
MeO
MeO
MeO
HO
CH2
H
CH2+
H
H

Analogies are found in coumarins e.g.: furocoumarinic acid (73)[65] where the furan protons came at 2.43 and 3.0 τ . The above physical data allowed two structural possibilities (74) and (75) . The low value of the methoxyl group 5.82 might seem to be in favour of the structure (75), by analogy with O-methyl mycophenolic acid (76)[51] where the methoxyl group in the peri position to the lactone carbonyl group appears at 5.97 τ . On the other hand, the model of the structure (74) shows that the methoxyl protons lie in the plane of the double bond of the furan ring. Models also show that in mycochromenic acid the methoxyl protons lie outwith the plane of the double bond of the pyran ring. This interpretation is supported by the resonance of the methoxyl group in furocoumarinic acid which appears at 5.88 τ . The structure (74) was thought more likely because of the co-occurrence of a series of compounds in the same strain of fungus with the methoxyl group at the same position.

The mass spectrum of mycofuranolide shows the characteristic cracking pattern of a benzofuran as represented in figure (77).[66]



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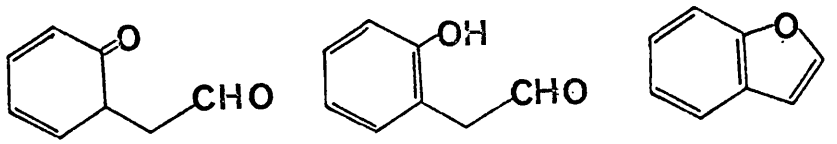
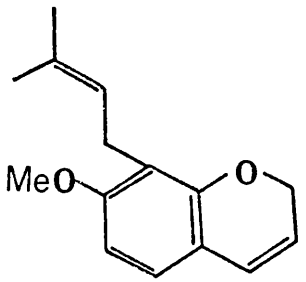


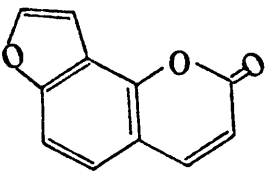
79 80

81 82

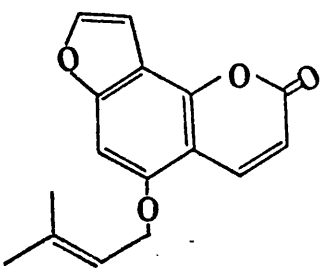
The structure (74) was confirmed by synthesis as follows. Ring closure involving cyclo-dehydration of 1:4 - dicarbonyl compounds represents one of the oldest and most convenient methods for the preparation of furan derivatives (78)[67] . For the synthesis of a simple unsubstituted benzofuran system, the required 1:4 - dicarbonyl derivative would be (79) of which the mono-enol form is O - hydroxyphenyl acetaldehyde (80) . The O - hydroxyphenyl acetaldehyde derivative required for the present work has been prepared from mycophenolic acid. Ozonolysis of mycophenolic acid in chloroform yielded a complex mixture. The acetate of mycophenolic acid was then prepared and ozonolysis in methanol produced a mixture of acetal and aldehyde although in low yield However, ozonolysis in chloroform at -15°C gave the aldehyde (81) in fair yield. Dehydration of the aldehyde (81) with p- toluenesulphonic acid in benzene yielded (82) which was identical to mycofuranolide in its NMR, IR, UV, spectra and R_f value.

The existence in P. brevicompactum of compounds containing isoprenoid units e.g. mycophenolic acid,





83

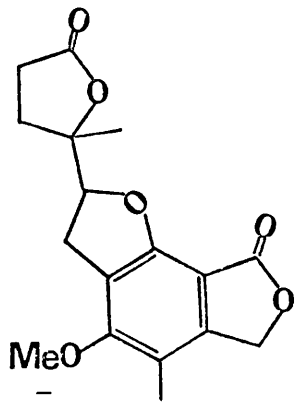


84

with others containing unsubstituted furan ring has many parallels among natural products (83). [68] A significant feature is that frequently, definite isopentane units and unsubstituted furan rings are found incorporated together in one compound (84) [69]. Recently it has been found that in furocoumarins [4-^{14}C] mevalonic acid has been incorporated into the furan ring showing that the two carbons of the furan ring were part of an isoprene unit. [70]

The second new metabolite related to mycophenolic acid was present only in small amounts. It showed all the features of the mycophenolic nucleus (lactone methylene group at 4.95 τ, methoxyl group at 6.11 τ and aromatic methyl group at 7.99 τ) but was not acidic or phenolic. The IR spectrum showed carbonyl absorptions at 1780 and 1795 cm^{-1} indicating the presence of a second γ - lactone function apart from that of the phthalide nucleus. The similarity of its NMR spectrum with that of the lactone (55) and the occurrence of its molecular ion at m/e 318 suggested the structure (85) for this compound.

At this stage we learned from Dr. W.B. Turner that he had obtained a compound with the same gross structure,



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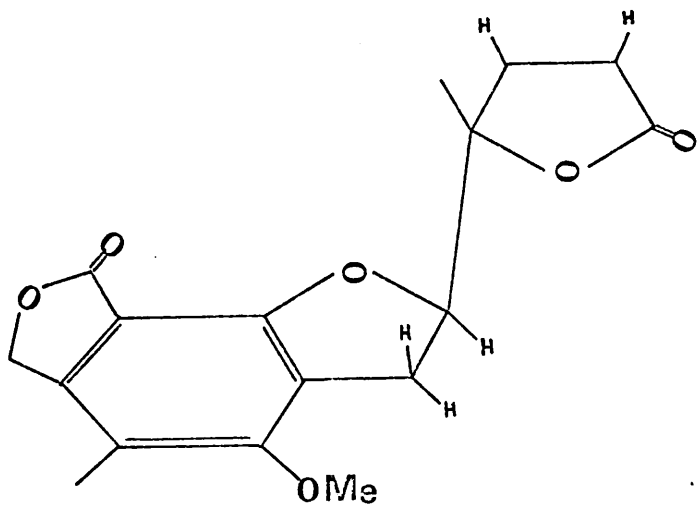
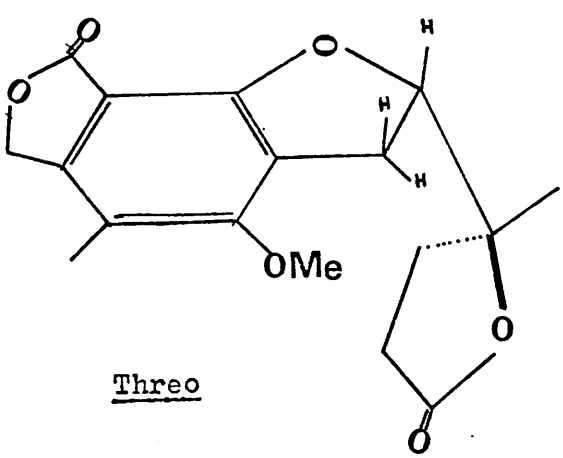
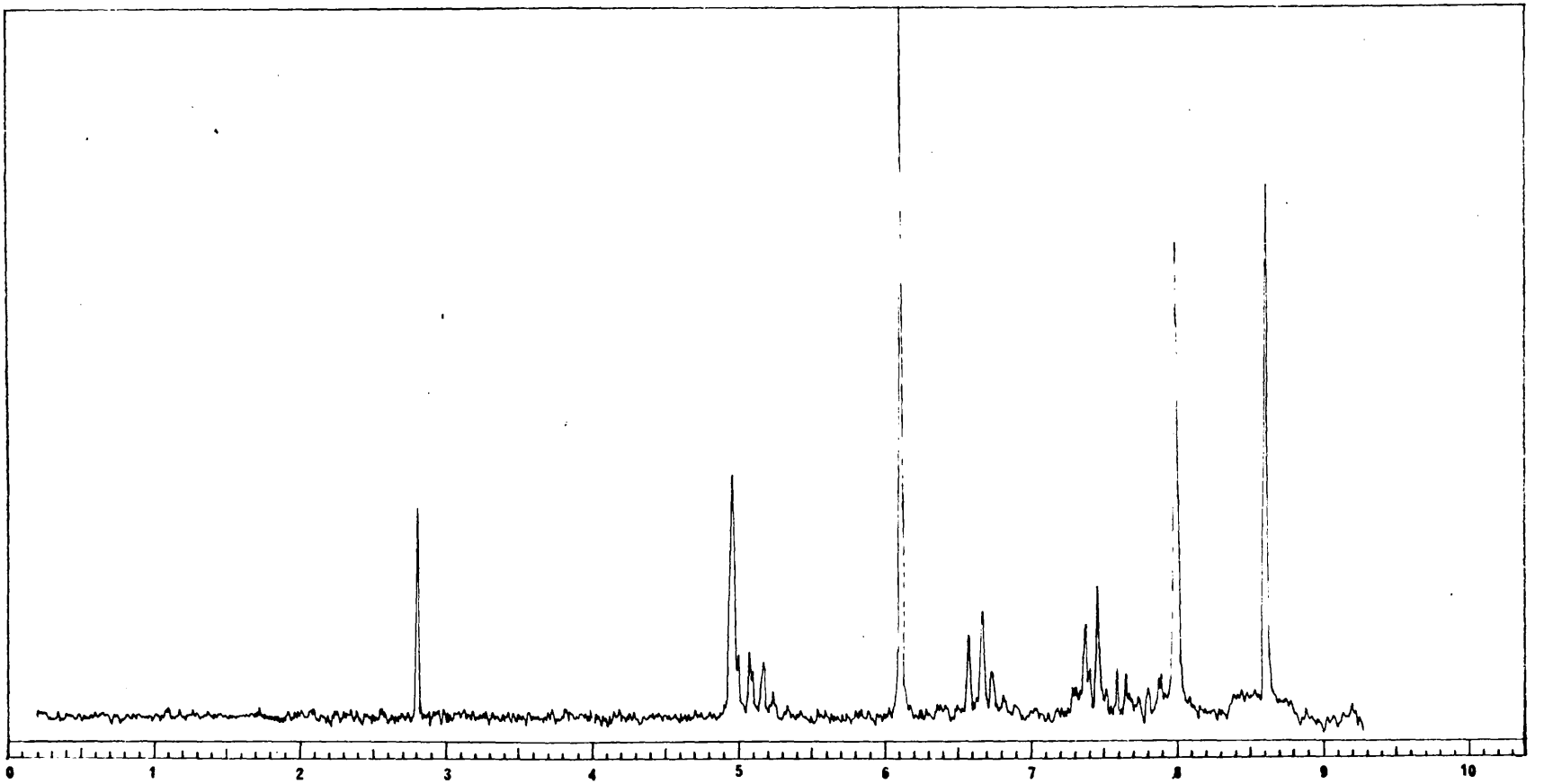


Erythro



Threo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by synthesis from mycophenolic acid by successive treatment with bromine and alkali. A comparison of the NMR spectrum of this product* with that of the isolated product showed that although both had signals in the same region the peaks at 6.65 τ and 5.09 τ corresponding respectively to the methylene protons and the methine proton in the dihydrofuran ring showed a different multiplicity. In the synthetic product the methylene protons appear to be equivalent and are split by the vicinal proton into a doublet, while in the natural compound the methylene protons appear as a multiplet which can be recognised as the AB part of an ABX system.

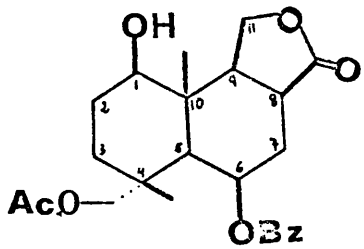
Comparison of models of the erythro and threo forms of this compound show that the erythro should more readily adopt a conformation in which the saturated lactone ring would cause non equivalence of the dihydrofuran CH_2 group. This suggests that the natural compound and the synthetic compound are the erithro and threo isomers respectively. Synthetic studies to confirm this assignment could not be carried out owing to lack of time.

*Kindly provided by Dr. W.B. Turner, I.C.I. Pharmaceuticals Ltd.

DISCUSSION PART II

DISCUSSION PART II

Work by Birch has shown that the side chain of mycophenolic acid is probably a degraded geranyl unit. As described in the foregoing section, other compounds with a degraded geranyl side chain have been isolated from P. brevicompactum but all are related to mycophenolic acid. In the screening of the broth of P. brevicompactum for compounds containing isoprenoid units the fungus was fed with mevalonic acid and a radio T.L.C. scan of the neutral compounds was carried out. This showed the presence of a large number of compounds having radioactivity. Three of these can be distinguished from the rest by their characteristic staining properties with ceric ammonium nitrate solution and were isolated by partition of the broth extract between water and chloroform to remove the most polar compounds, washing with saturated aqueous sodium bicarbonate to remove the acids and finally chromatography of the neutral fraction. These three compounds turned out to be closely related to one another but not to mycophenolic acid. The compound with an R_f of 0.65 in 10% methanol-chloroform was called



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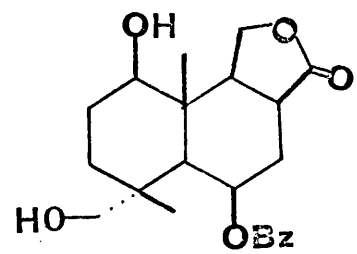

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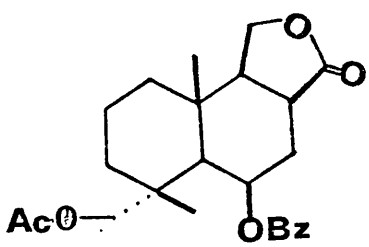

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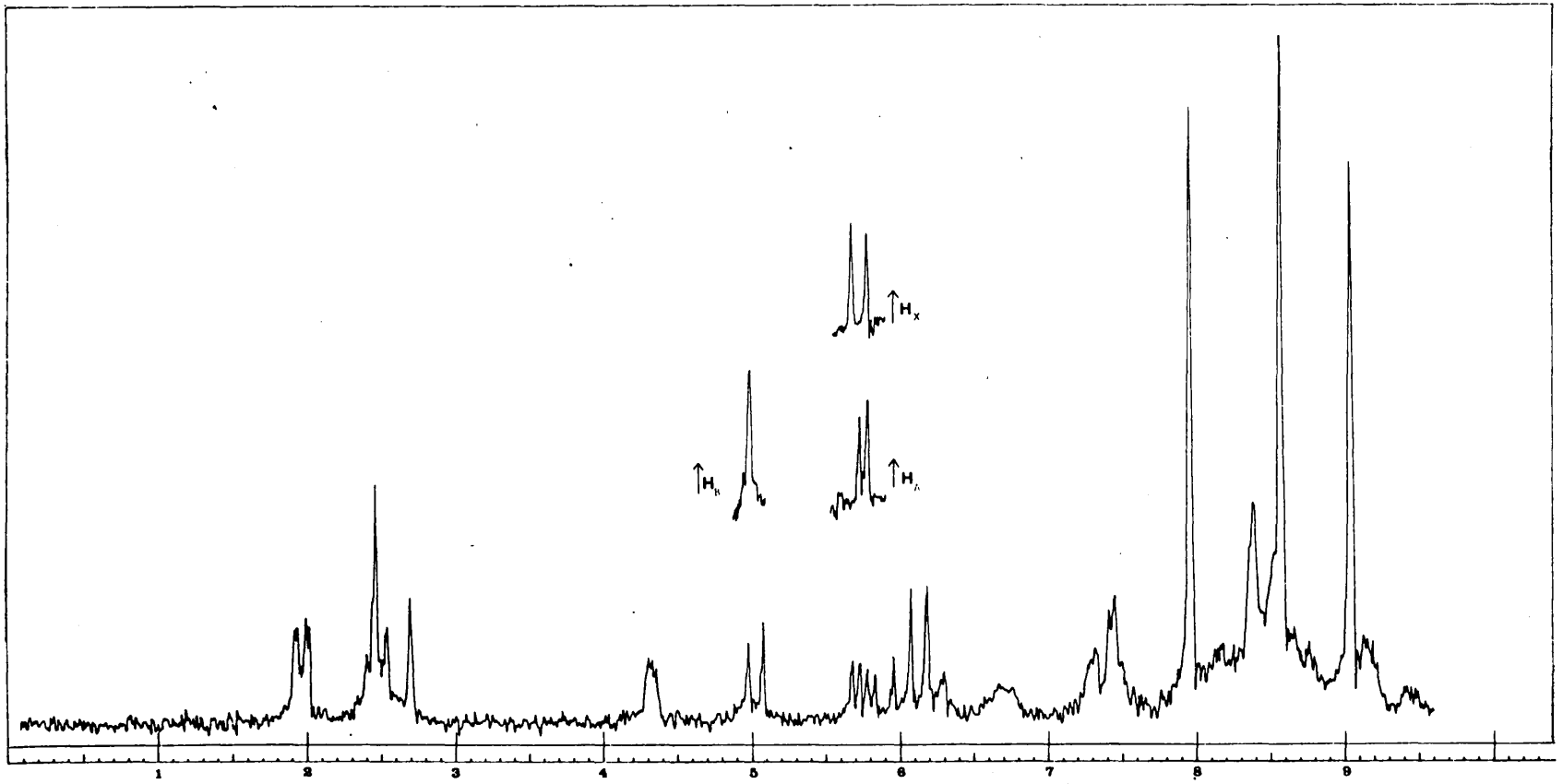
H_X
H_B
H_A
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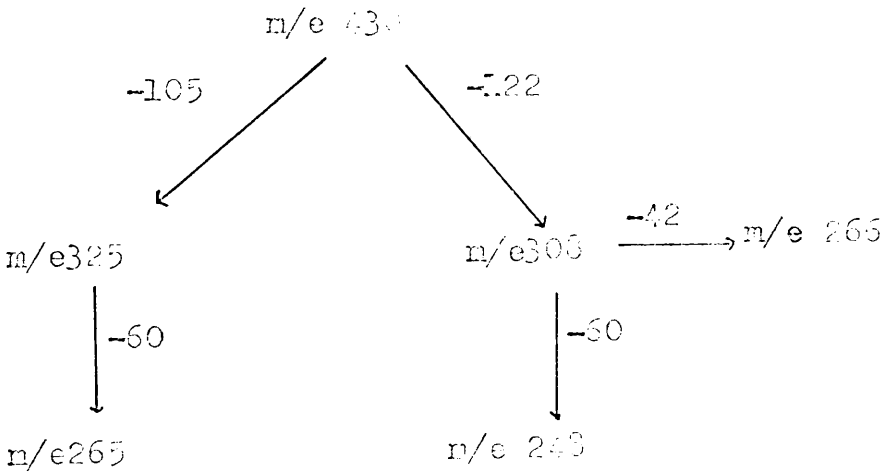
pebrolide shown to have the structure (86) and the others with R_f at 0.87 and 0.28 were shown to be deoxypebrolide (87) and desacetylpebrolide (88) respectively. Deoxypebrolide was produced in small amounts in comparison with the other two.

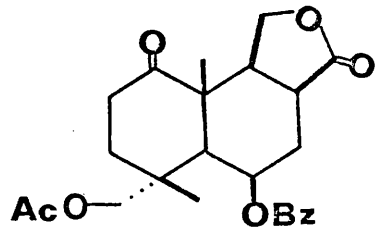
Figure (89) shows the NMR spectrum recorded for pebrolide. Elemental analysis and mass spectroscopy indicated that the molecular formula of pebrolide was $C_{24}H_{30}O_7$. The presence of two tertiary methyl groups in pebrolide was indicated by the two singlets at 9.04 and 8.56 τ in the NMR spectrum. The presence of an acetate grouping was indicated by a peak at 1740 cm^{-1} and 1243 cm^{-1} in the I.R. spectrum, by a singlet at 7.96 τ in the NMR spectrum, and by losses of 42 and 60 mass units in the mass spectrum (90). The alcohol corresponding to this acetate, which will be discussed later, did not show any of these spectral features. The presence of a benzoate grouping was indicated firstly by the IR spectrum with peaks at 1715 cm^{-1} and at 1597, 1580 (C = C stretching) and 711 cm^{-1} (aromatic C-H out of plane), secondly by the NMR spectrum where the typical pattern of benzoyl ring protons appeared at 2.50 and 1.96 τ . The latter


m/e 43
-105
-122
m/e325
m/e308
-42
m/e 266
-60
-60
m/e265
m/e 248

corresponds to the ortho protons, the low τ value being due not only to the withdrawing capacity of the carbonyl group, but also to its anisotropic effect and thirdly by the mass spectrum where losses of 122 and 105 units of mass were favoured processes (figure 90).

The presence of a saturated γ -lactone grouping was indicated by the peak at 1780 cm^{-1} in the IR spectrum and the peak at 3605 cm^{-1} revealed the presence of a hydroxyl group.

With all the oxygens accounted for, pebrolide is thus the acetate benzoate of a trihydroxy γ -lactone of molecular formula $C_{15}\ H_{24}\ O_5$. In this formula two double bond equivalents, apart from the two of the lactone ring, remain unaccounted for. It was established that there were no double bonds present since pebrolide gave a negative reaction with tetranitromethane and since catalytic reduction gave only a hexahydro derivative, M+ at m/e 436, in which only the benzene ring had been reduced, as indicated by the appropriate changes in the NMR spectrum. Pebrolide, therefore, has two rings in addition to the lactone ring.



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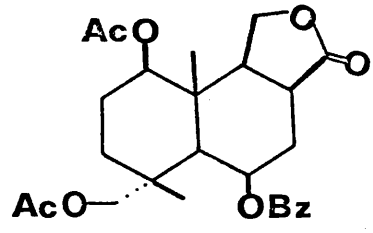
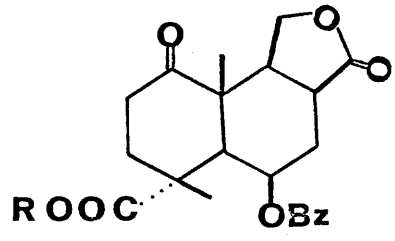


92

That the hydroxyl group in pebrolide was secondary was shown by oxidation of pebrolide with Jones reagent to a ketone (91), molecular formula $C_{24}.H_{28}O_7$ (analysis and mass spectrum) with the consequent disappearence of the multiplet at 6.7 τ in the NMR spectrum assigned to the proton geminal to the hydroxyl group. That the second metabolite deoxypebrolide(87), differed from pebrolide only in the absence of the secondary hydroxyl group was indicated by the similarity of the NMR spectra, the main difference being the absence of the multiplet at 6.7 τ in the spectrum of deoxypebrolide and this was also indicated by the absence of hydroxyl absorption in the I.R. spectrum, by elemental analysis and mass spectroscopy.

The NMR spectrum of the third metabolite desacetyl pebrolide (88), indicated that this compound did not possess an acetate grouping but the other resonance signals were similar to those of pebrolide. Indeed, acetylation of pebrolide and desacetylpebrolide yielded the same diacetate (92). As will be discussed later, it was not possible to interrelate pebrolide and desacetylpebrolide by basic hydrolysis of the former but



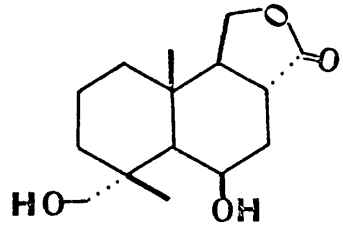
93 R = H

94 R = CH_3

it was found that under controlled conditions acid hydrolysis of pebrolide removed selectively the acetate grouping yielding desacetylpebrolide identical to the natural product.

The alcohol resulting from this hydrolysis was shown to be primary by oxidation with Jones reagent to an acid (93) which was characterised as its methyl ester (94). In the NMR spectrum of pebrolide, the AB system at 6.21 and 6.03 τ with a coupling constant of 12 cps was assigned to the methylene protons in a tertiary acetoxymethyl group. It is well known[71] that protons of this type often show different chemical shifts and a coupling constant of 11 cps showing thereby that they do not freely rotate but have a preferred conformation . These signals are absent in the spectrum of the corresponding methyl ester (94).

Attempts to base hydrolyse the acetate grouping in pebrolide did not yield desacetylpebrolide but a compound less polar which was shown to be its isomer. The same compound was obtained when desacetylpebrolide was treated under the same conditions. This isomer presented similar features in the NMR spectrum to that of desacetylpebrolide but with significant changes in the region 5-6 τ . The double doublet at 5.74 τ in



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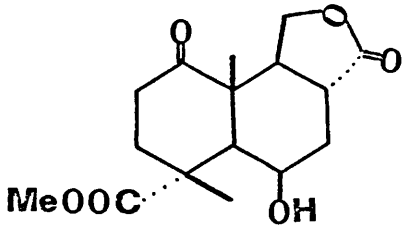
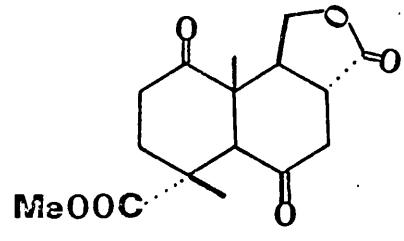


96

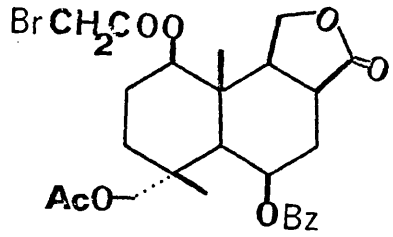


97

pebrolide collapsed to a doublet, J = 10 cps, upon irradiation at 7.48 τ (the middle of superimposed multiplets) and to a doublet, J = 6 cps, upon irradiation at 5.02 τ . This indicated the possibility of these protons forming an ABX system of the type-CH.CH_2.O.CO- where JAX was equal to zero. The isomer of desacetylpebrolide presented similar features in the NMR but JAX was no longer equal to zero.

That the benzoate was secondary was shown by treatment of deoxypebrolide with aqueous sodium hydroxide which gave a diol (95) and benzoic acid. The proton under the benzoate appeared in the NMR spectrum of pebrolide at 4.3 τ and integrated for one proton while in the diol it appeared at 5.6 τ . That the benzoate group was secondary was also indicated by the hydrolysis of the keto acid (93) yielding a hydroxyacid characterised as its methyl ester (96). Oxidation of the ester (96) gave the keto ester (97) with the consequent disappearence in the NMR spectrum of the signal assigned to the proton geminal to benzoate.

Attempts to reduce the carbonyl of the lactone group in desacetylpebrolide with LAH gave a complex mixture from which no pure compounds could be isolated.

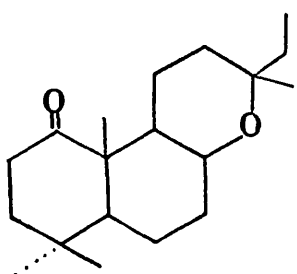


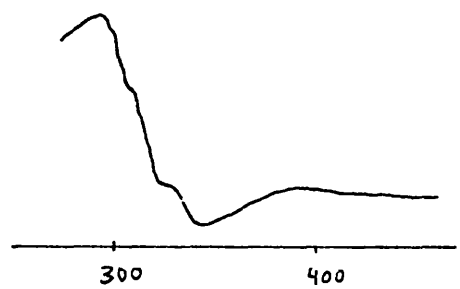
98

Pebrolide ketone was unaffected by attempted catalytic reduction with 10% Pd - charcoal in ethanol. However, when PtO_2 in acetic acid was used, a compound identical with the hydrogenation product of pebrolide was obtained. It thus appears that the secondary hydroxyl group in pebrolide is in the more stable configuration (e.g. equatorial rather than axial).

At this stage in the investigation, attempts were made to prepare a derivative suitable for study by X-rays. Reaction of pebrolide with p-bromobenzene sulphonyl chloride did not afford a crystalline product. However, reaction with bromoacetyl bromide gave the bromoacetate which was in a suitable crystalline form. Its structure was elucidated by means of X-ray analysis (see appendix) and was shown to have the structure (98) .

The absolute configuration was determined by the method of anomalous dispersion and is that shown in (98) . The same conclusion can be reached on the basis of ORD data. The ORD of pebrolide, desacetylpebrolide and deoxypebrolide all show a trough at 259 mμ. The ORD curve of pebrolide ketone showed, in addition to a trough at 259 mμ, a peak at 315 mμ.





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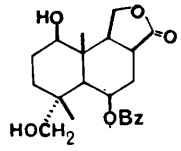
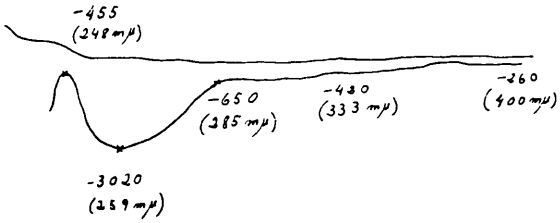
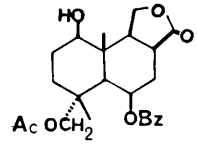
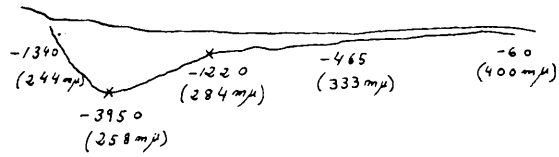
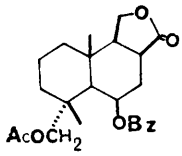
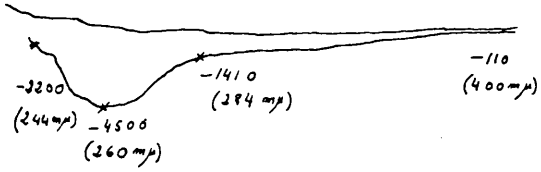

HO
O
O
HOCH2
OBz

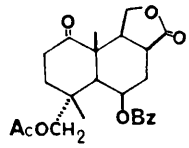

-455
(248 mμ)
-650
(285 mμ)
-430
(333 mμ)
-260
(400 mμ)
-3020
(259 mμ)

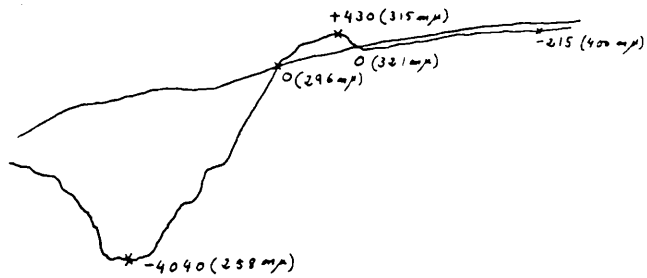

HO
O
O
Ac OCH2
OBz


-1340
(244 mμ)
-3950
(258 mμ)
-1220
(284 mμ)
-465
(333 mμ)
-60
(400 mμ)


O
O
AcOCH2
OBz


-3200
(244 mμ)
-4506
(260 mμ)
-1410
(284 mμ)
-110
(400 mμ)

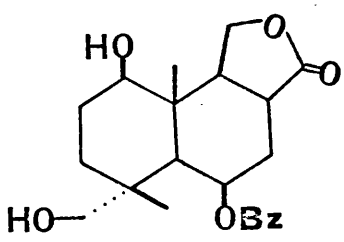

O
O
O
AcOCH2
OBz


+430 (315 mμ)
-215 (400 mμ)
0 (321 mμ)
0 (296 mμ)
-4040 (258 mμ)

Accordingly, the effect of the carbonyl group at C - 1 is to produce a small positive Cotton effect. The absolute stereochemistry shown could be calculated on the basis of the Octant Rule[72] (figure 99) to result in a positive Cotton effect. 1 - ketomanolyl oxide with comparable absolute stereochemistry also gives a small positive Cotton effect (figure100).[73]

Pebrolide isthus a sesquiterpene lactone with a drimane skeleton. Some features of this structure are particularly interesting. Firstly, the C - 1 oxygenation is very unusual not only in this type of sesquiterpenes but also in diterpenes, triterpenes and steroids and secondly, the presence of a benzoate grouping is unique in the sesquiterpene field.

On the basis of this structure the facile base-catalysed isomerisation of desacetylpebrolide can be understood to involve epimerisation at C - 8 of the cis equatorial lactone.[101] The X-ray data show that the two six membered rings are both chairs but with a certain amount of distortion particularly in the B ring which has the fused cis lactone ring and three axial β substituents.



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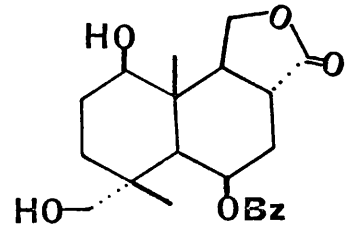

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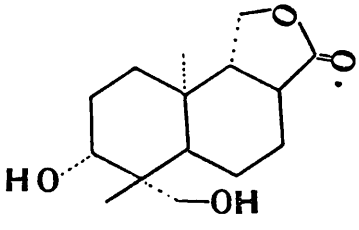

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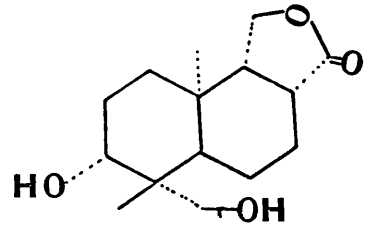

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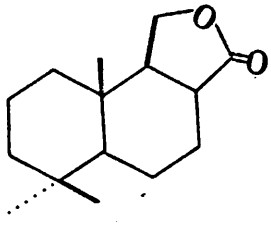

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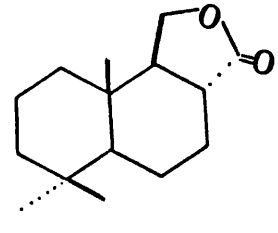
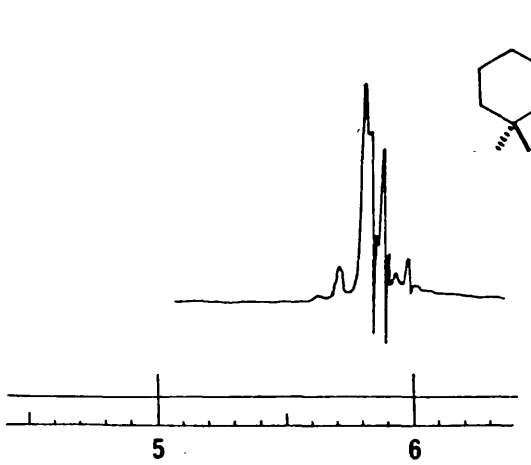
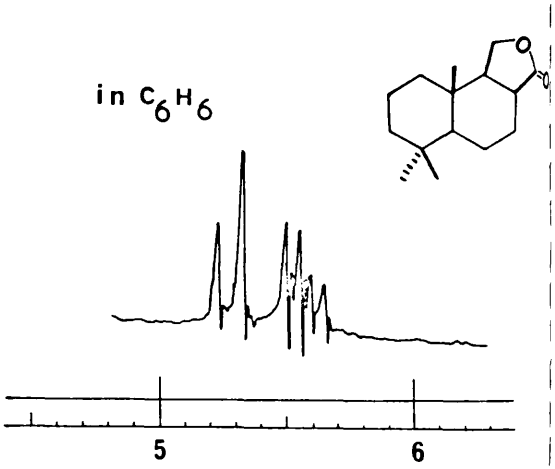


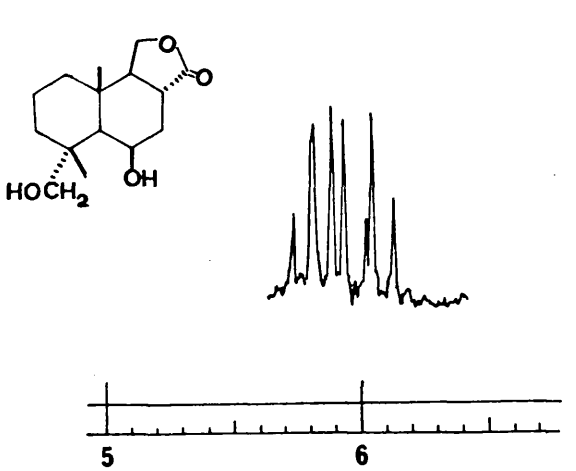
105

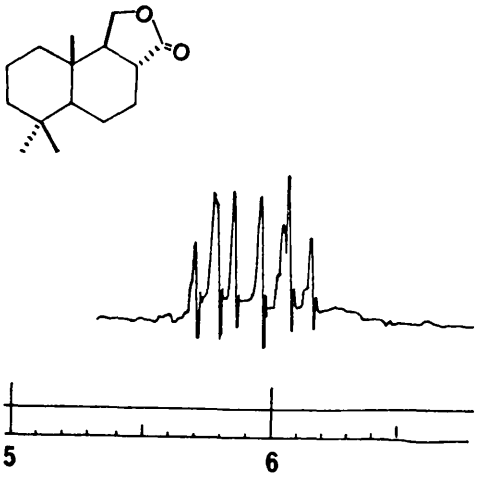
This crowding explains the reaction of desacetyl-pebrolide with base since epimerisation of the axial 8β lactonic substituent to equatorial 8α would give the less crowded trans isomer. Analogous epimerisations have been reported, dehydroiresin (102) giving isodihydroiresin (103)[37] and dihydroconfertifolin (104) giving isodihydro-confertifolin (105)[40]. It would be expected on mechanistic grounds that epimerisation of the lactone would occur prior to opening of the lactone ring. This was confirmed by the detection by T.L.C. of isodesacetyl pebrolide in the alkaline reaction mixture obtained by treatment of desacetypebrolide with a methanolic solution of potassium hydroxide for less than one minute. Two other spots were visible one corresponding to starting material and the other on the base line to potassium salts. Upon acidification, only two compounds appeared, desacetylpebrolide and its isomer.

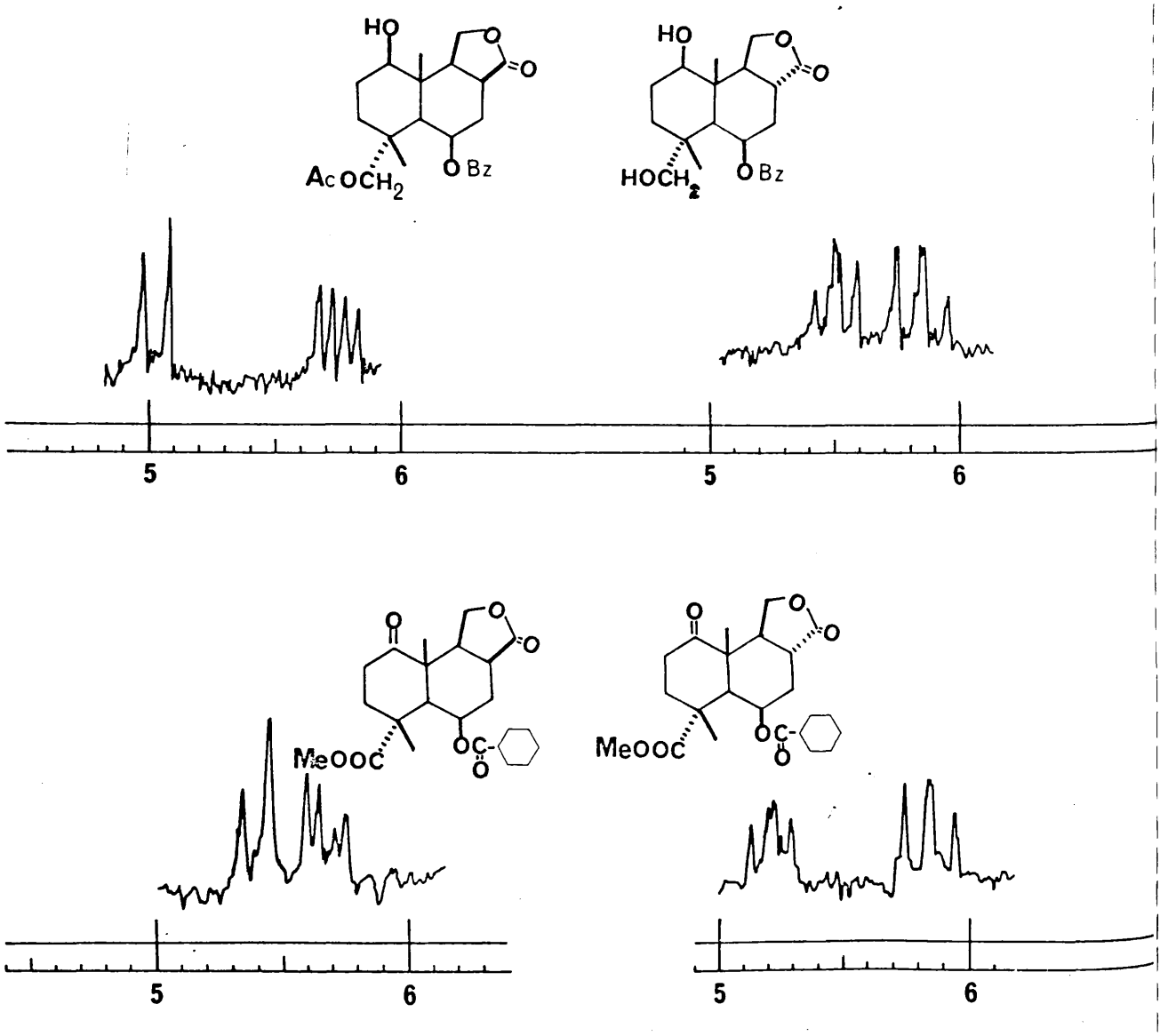
In interpreting the NMR spectra of pebrolide and its derivatives, it is necessary to take into account the crowded nature of the molecule which results in a number of large shielding and deshielding effects.


5
6


in C_6H_6
5
6


HOCH2
OH
5
6


5
6


HO
O
O
O
AcOCH2
OBz
HO
O
O
O
HOCH2
OBz
5
6
5
6
O
O
O
O
MeOOC
OC
O
O
O
O
MeOOC
OC
O
5
6
5
6

This interpretation was assisted by the availability of a number of derivatives of pebrolide, some of which were prepared with a view to degrading biosynthetically labelled metabolite (c f. later section).

In the NMR spectrum of pebrolide, the AB part of the ABX system mentioned above has been assigned to the methylene protons of the γ - lactone and these are coupled to the proton at C - 9. In the case of dihydroconfertifolin the corresponding protons appear as a multiplet at 5.89 τ in deuterochloroform. If the spectrum of dihydroconfertifolin is run in benzene then the multiplet is better spread out showing more clearly the same splitting pattern and coupling constants as in pebrolide (figure 106). It can be seen from models that with ring B as a chair the dihedral angles of the 11α and 11β protons with the proton at C - 9 are 30° and 90° respectively which correspond according to the Karplus equation[74] to coupling constants of 7 and 0 cps, close to the observed values of J_{BX} (5.5 cps) and J_{AX} (0cps). This confirms that ring B adopts a chair conformation in solution. It might seem from models that the steric interaction of the three axial β substituents would be relieved if ring B

were, for example, a twist boat but this would not give appropriate dihedral angles. Ring B is also a chair in the crystal of bromoacetylpebrolide. It may also be seen that the lactone ring is non planar and this could explain the non equivalence of the methylene protons in dihydroconfertifolin. It may be seen from the above that it is the β proton in pebrolide which has almost zero coupling with the vicinal proton and appears at 5.0 τ i.e. down-field 0.89 p.p.m. from that in dihydroconfertifolin. This effect must be due mainly to the lone pair on the hydroxyl group at C - 1 since the corresponding resonance in 1 - deoxypebrolide (table 1) is at 5.62 τ. The 11 α proton is relatively remote from the oxygen atom. X-ray data show that the distance between 11β proton and the oxygen at C - 1 is $\sim$ 2.2 Å while that for the 11 - α proton is $\sim$ 3.3 Å.

It has been shown[75] that when a proton is deshielded by the lone pair of electrons of an oxygen function, an additional downfield shift is observed when its NMR spectrum is run in pyridine. This has been attributed to the lone pair of electrons of the oxygen atom complexing with the solvent. $CDCl_3$ - pyridine downfield

		H-11β	H-11α
desacetylpebrolide	$CDCl_3$	5.02	5.74
	C_5H_5N	4.70	5.72
isodesacetylpebrolide	$CDCl_3$	5.80	5.45
	C_5H_5N	5.66	5.29

Table 2

		τ	
C-1	C-4α	H-11β	H-11α
(86) OH	CH_2OA_c	5.02	5.74
(92) OA_c	CH_2OA_c	5.36	5.78
(91) =O	CH_2OA_c	5.50	5.70

Table 3

shifts are found for several signals in the NMR spectrum of desacetylpebrolide. It may be seen in table 2 that of the two lactone methylene protons the 11β proton shows a shift of 0.3 p.p.m. while the 11α proton shows a shift of only 0.062 p.p.m.

In trans desacetylpebrolide the dihedral angles between the 11α and 11β protons and the proton at C - 9 are 35^{6} and 157^{o}, respectively, corresponding to coupling constant of 6.5 and 13.5 cps. Since the observed values are J_{AX} = 7 cps and J_{BX} = 11 cps, it may be seen that the 11β prpton signal is that at higher field.

In trans desacetylpebrolide the 11α and 11β protons are both 2.7 Å from the oxygen at C - 1 and both show a down field shift from those in trans dihydroconfertifolin (0.35 and 0.25 p.p.m.). Table 2 shows the $CDCl_3$ - pyridine shift for the methylene protons of the lactone in trans desacetylpebrolide. Both protons show the same additional downfield shift of 0.15 p.p.m.

Table 3 shows the effect on the lactone methylene protons of various functions at C - 1. It may be seen that the deshielding effect of the hydroxyl group in

pebrolide is modified by acetylation. This is due to the delocalisation of the lone pair of electrons of the oxygen atom by the carbonyl group of the acetate. The 11α proton does not vary much from 5.75τ in all these derivatives.

As mentioned earlier, the methyl resonances in pebrolide appear at 9.05 and 8.51 τ although it is not immediately obvious which methyl appears at low field. However, a comparison of the NMR spectra of the methyl ester (94) and the ketone (91) show that the resonance at 9.03τ in pebrolide is due to the methyl group at C - 4 since the higher field resonance is affected by the conversion of CH_2OAc to COOMe . The deshielding by 0.19 p.p.m. of the methyl at C - 4 in the ketone (91) is in agreement with the effects found in steroids where a ketone at position C - 3 and C - 7 deshields the C - 19 methyl group by 0.24 p.p.m. and 0.27 p.p.m. respectively.

The angular methyl group in pebrolide thus appears in the NMR at unexpectedly low field (8.5 τ). It is well known that an axial hydroxyl group will deshield an axial methyl group situated 1,3 on the same ring[76]. In the diol(95) derived by vigorous hydrolysis of deoxypebrolide, both

				Me τ	
C-1	4∝	C-6		C-4	C-10
(104) H	H	H	cis	9.16	9.03
(95) H	CH_2OH	OH	trans	8.87	8.75
(86) OH	CH_2OA_c	OB_z	cis	9.04	8.56
* OH	CH_2OA_c	$O\text{-}COC_6H_{11}$	cis	8.99	8.67
(88) OH	CH_2OH	OB_z	cis	9.12	8.53
(101A)OH	CH_2OH	OB_z	trans	9.06	8.55
(126)=O	CH_2OH	OB_z	cis	9.0	8.4
(91) =O	CH_2OA_c	OB_z	cis	8.85	8.35
(94) =O	CO_2Me	OB_z	cis	8.51	8.31
** OH	CH_2OH	$O\text{-}COC_6H_{11}$	cis	9.10	8.65

* Hexahydropebrolide.

** Hexahydro-desacetylpebrolide.

Table 4

methyl groups are deshielded by about 0.3 p.p.m. relative to the methyl groups in either cis or trans dihydroconfertifolin. In the table 4 it is shown that the stereochemistry of the lactone ring makes little difference to the τ values of the methyl groups (cf Nos. 88 and 101A); thus comparison of cis and trans is permissible. The cyclohexane carboxylate group appears to deshield one of the methyl groups and shield the other and the benzoate does this to a greater degree.

If partial double bond character is attributed to the - O.CO - bond in the benzoate group, it may be seen in the slightly distorted configuration resulting from the repulsion of the B substituents, that the methyl group at C - 10 will lie in the plane of the pseudo double bond and will be deshielded while the methyl group at C - 4 will lie at right angles to this plane and will be shielded. This double bond character may be expected to be greater in the benzoate than in the cyclohexanecarboxylate and this could explain the more marked shielding and deshielding effect in the benzoate.

Another signal in the NMR spectrum of pebrolide which appeared at an unexpectedly low field is that at 4.3 τ

	4α	H–6α τ
(88)	CH_2OH	4.25
(86)	CH_2OAc	4.28
(99)	COOMe	4.46

desacetylpebrolide	$CDCl_3$	4.25
	C_5H_5N	3.97

Table 5

assigned to the proton geminal to the benzoate. Removal of the benzoate grouping as in the diol (95) produced an up-field shift of 1.26 p.p.m. but the value is still very low for an equatorial proton geminal to a hydroxyl group. It appears that this deshielding effect is produced by the lone pair of electrons of the oxygen of the primary alcohol. Delocalisation of the electron pair of the oxygen by acetylation as in pebrolide or conversion of the primary alcohol to the methyl ester reduces the deshielding effect. The deshielding effect due to the electron pair of the oxygen atom upon the proton geminal to the benzoate was again enhanced when the NMR spectrum of desacetylpebrolide was run in pyridine (table 5).

The absence of any significant additional down-field shift in pyridine of the methyl at C - 4 in some diterpenoids having a 4α CH_2OH has been used as evidence that the CH_2OH group has a preferred conformation in which the OH group is far removed from the axial C - 4 methyl group and is perpendicular to the plane of the ring.[77] This would also seem to apply to the 4β methyl group in desacetylpebrolide which shows a down-field shift in pyridine of only 0.1 p.p.m.

The proximity of one of the protons of the hydroxy methyl group and the 6 α proton is shown by Nuclear Overhauser effect. Thus, irradiation at 6.2 τ resulted in an enhancement of the signal at 4.3 τ by about 9%.

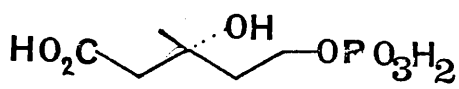
It was found that in the bromoacetyl pebrolide crystal, the distance between the oxygen of the primary alcohol and the hydrogen at C - 7 is 2.56 Å. This suggests that there is little difference between the conformation in the crystal and the preferred conformation in the solution.

Conclusion.

From the NMR spectrum of pebrolide and its derivatives and the X-ray analysis of bromoacetyl - pebrolide it may be concluded that the preferred conformation in solution and the conformation in the crystal are very similar, in particular, with respect to the conformation of ring B, of the benzoate function and of the 4α substituent.

INTRODUCTION

BIOSYNTHESIS OF PEROXIDE



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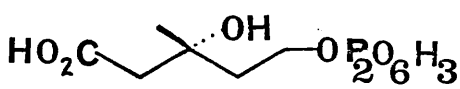

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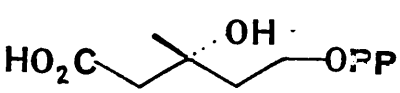

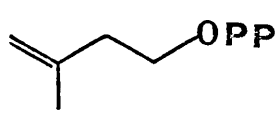

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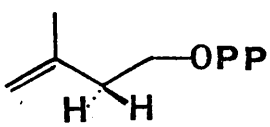

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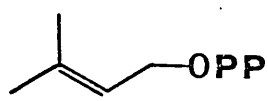
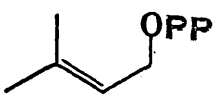


109

In recent years progress on the study of the biogenesis of natural products has been greatly advanced by the use of tracers other than ^{14}C, in particular, ^{3}H, ^{18}O, ^{13}C and ^{2}H. The use of hydrogen and oxygen isotopes lead to an understanding of many of the processes leading from mevalonic acid to terpenoids.

It has been shown that the first two enzymic processes both phosphorylations which successively produce mevalonic acid 5 - phosphate (106) and mevalonic acid 5 - pyrophosphate (107)[78]. The last one gives inorganic phosphate, carbon dioxide and 3 - methyl 3 -butenyl pyrophosphate (108) . The oxygen atom from the tertiary hydroxyl group is found in inorganic phosphate after the reaction.[79] It was deduced that the hydroxyl group is phosphorylated before elimination. The formation of the new double bond is thought to occur by a concerted elimination and not, for example, by dehydration followed by decarboxylation, for no hydrogen from the aqueous medium appears in the product. Stereochemically, the process is a <u>trans</u> elimination.[80] Next, 3 - methyl - 3 butenyl pyrophosphate (108) is converted



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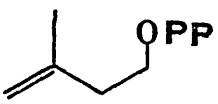

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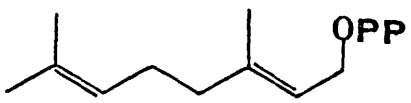

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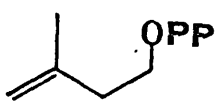

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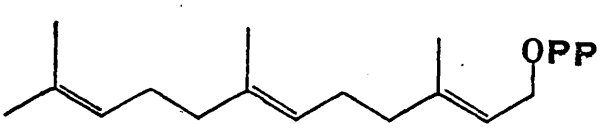

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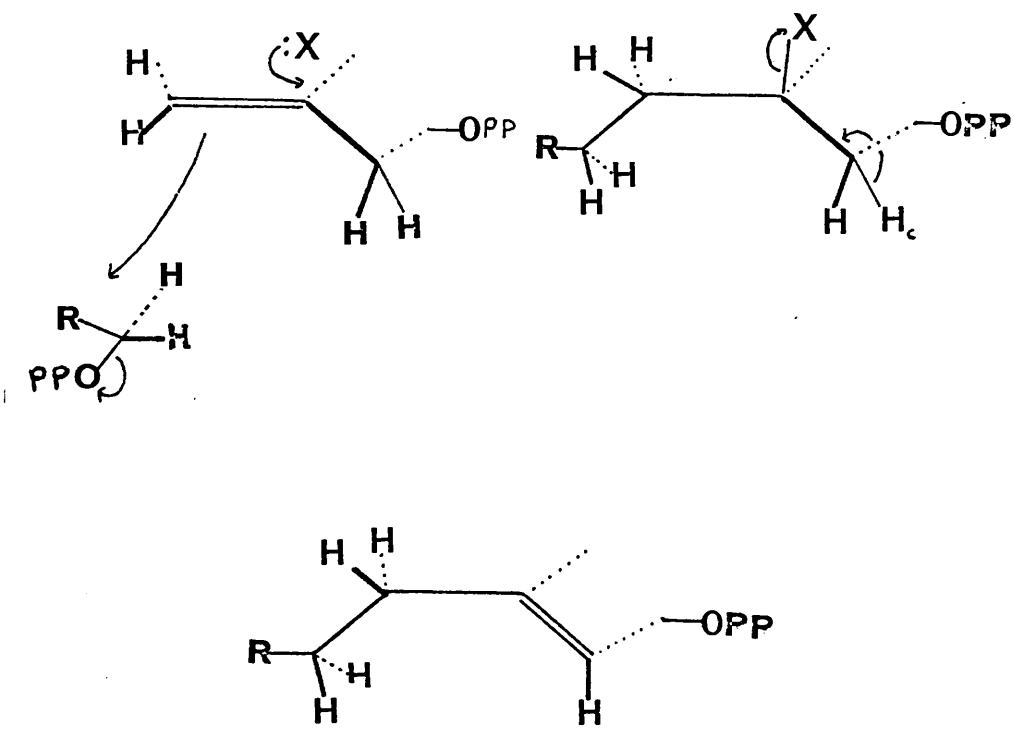
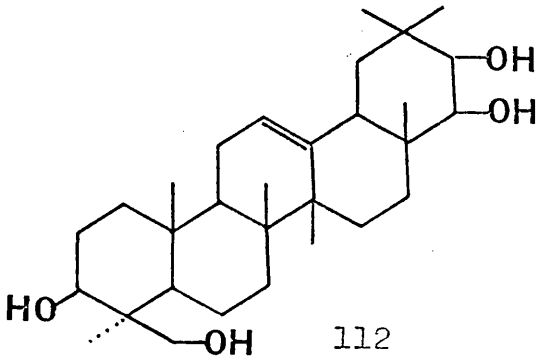


Figure A

by a prototropic shift into 3 - methyl 2 - butenyl pyrophosphate (109)[81]. The elimination of a proton in the change (108) --→ (109) is stereospecific, the hydrogen marked Hc being the one eliminated. These two molecules (108) and (109) are condensed with loss of pyrophosphate ion from one of them and a hydrogen ion from the other to form geranyl pyrophosphate (110). This product then replaces (109) in a further, exactly similar, reaction with (108) to form farnesyl pyrophosphate (111)[82].

Experiments using asymmetric labelling[83] with hydrogen isotopes showed that the establishment of a new carbon to carbon bond is accompanied by complete inversion of configuration at the allylic carbon atom; such inversion is characteristic of an SN2 reaction rather than of a carbonium ion reaction. Further, the fixed stereochemical relation between addition of the allylic C_5 unit and elimination of the hydrogen ion (cf figure A) is taken to indicate that the enzymic process proceeds in two distinct steps, first, trans addition of the allylic unit and of an electron donating group X and, second, trans elimination of X and the hydrogen ion.

A number of terpenes appear to arise by a non-stop



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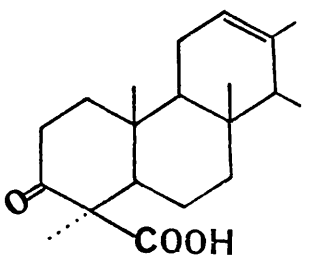

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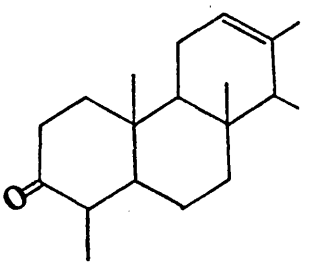
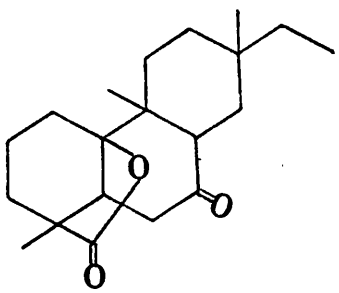


114

trans-anti-parallel cyclisation of farnesyl pyrophosphate (three isopentenyl units), geranyl geraniol pyrophosphate (4 isopentenyl units), and squalene (six isopentenyl units) to give sesquiterpenes, diterpenes and triterpenes, respectively. Proof that the methyl carbons in the terminal isopropylidene groups of squalene retain their individual identity in the course of cyclisation has been presented by Arigoni[84]. Soya-bean seedlings were supplied with (2 ^{14}C) mevalonate and the labelled soya - sapogenols subsequently isolated. The 1,3 glycol structure in ring A (112) was oxidised to give the unstable 3 - oxo-24-carboxylic acid (113) which was readily decarboxylated to the ketone (114) giving CO_2 that contained no ^{14}C. Hence, in the formation of this pentacyclic triterpene, the axially oriented hydroxymethyl group at $C_{(4)}$ was derived from the methyl carbon of mevalonic acid. There is also retention of individual identity by the corresponding gem disubstituted carbon atoms in rosenonolactone[84]. Removal of the keto group from the dihydro compound (115) under Wolff - Kishner conditions took place accompanied by opening of the lactone ring. The resulting unsaturated acid decarboxylated smoothly when heated at 250° to give CO_2 and the unsaturated hydrocarbon (116). No radioactivity



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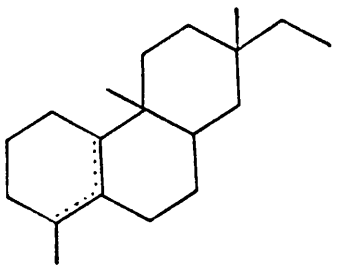

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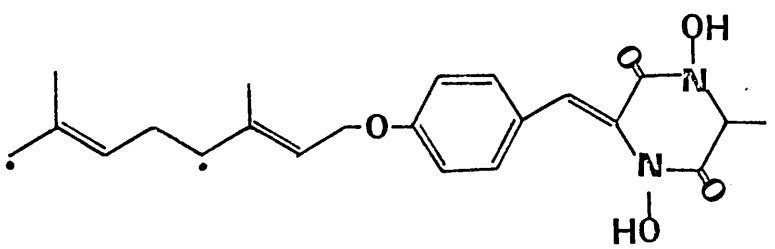

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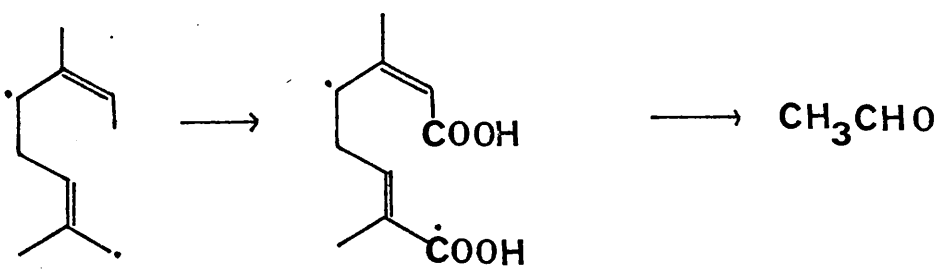
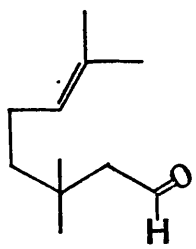


118

was found in CO_2. The absence of radioactivity in CO_2 derived from the lactone ring parallels the findings in the triterpene series and makes it probable that although different initiators may be involved, the mechanism for the ring closure is essentially of the same type for the di and triterpenes.

The retention of stereochemical individuality by the apparently identical terminal geminal dimethyl groups in an open chain terpenoid structure has been demonstrated in the case of mycelianamide (117) by Birch et al[85] . This compound was biologically labelled as shown from (2 ^{14}C) mevalonic acid and the terpenoid chain cleaved by treatment with sodium in liquid ammonia. The resulting hydrocarbon was administered to a rabbit and recorded from the urine in the form of its dicarboxylic acid metabolite (118) . Ozonolysis of this material yielded acetaldehyde that was free from radioactivity, indicating that enzymatic attack on one of the gem - dimethyl groups had been confined to that which was labelled. This is an example of dissymetric reactivity of a symmetrical substrate in association with enzymic site.

In contrast to this Yeowell and Schmid[86]



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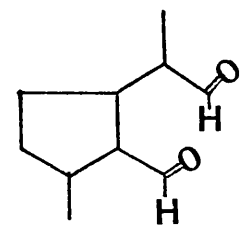

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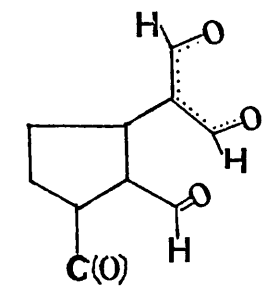

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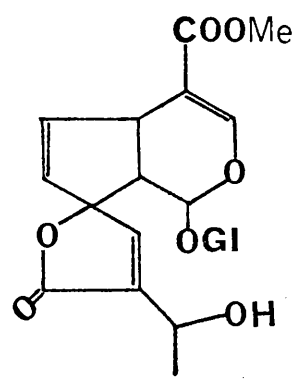

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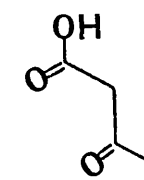


123

have reported evidence for randomisation of the isopropylidene methyl carbons of citronellal (119) in the course of its presumed conversion via iridodial (120) into the plant glucoside plumieride(122). Comparative studies of the distribution of label from (1 ^{14}C) acetate and (2 ^{14}C) mevalonate respectively led the authors to postulate the union of aceto-acetate(123) with an intermediate of the type (121) in which the isopropylidene carbons become indistinguishable from each other.

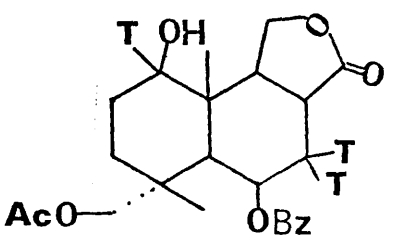
DISCUSSION

Fungi appear to offer some advantages for the investigation of terpene biosynthesis for the reason that they are readily grown under conditions which may be rigorously defined and labelled substrates are often readily incorporated into metabolites as compared with higher plants.

Examination of the structure now elucidated for pebrolide showed that the distribution of oxygen functions would be favourable for determination of labelling patterns in different parts of the molecule. Accordingly, we were led to study the biosynthesis of pebrolide by Penicillum brevicompactum.

One of the principal aims of this study was to establish whether the cyclisation involved in the biosynthesis of pebrolide occurs with the same stereospecificity as in di- and triterpenes with respect to the gem dimethyl at the C - 4 and to find which of the carbons at that position is derived from the C - 2 of mevalonic acid.

It is expected that if the fungus is fed with 2 - ^{3}H mevalonic acid, the tritium will be incorporated into pebrolide as shown in (124). If the β methyl group



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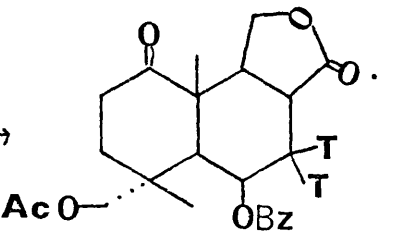

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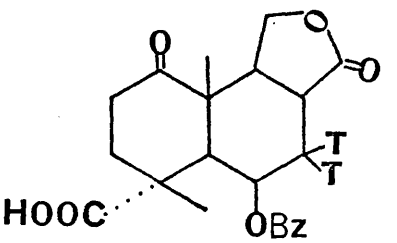

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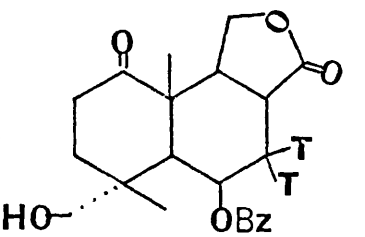
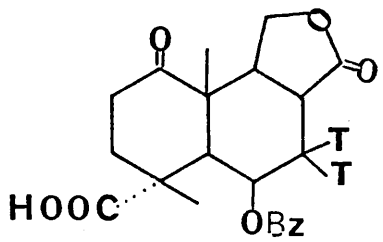


126

at C - 4 is derived from C - 2 of mevalonic acid then 2 tritium atoms will be expected in this group, but if the CH_2OAc group is the one which is derived from C - 2 of mevalonic acid then there will be only $1\frac{1}{3}$ tritium atom in this group, this being the statistical residue of tritium resulting from oxygenation of a doubly tritiated methyl group.

It would be predicted from this labelling pattern that oxidation of the hydroxyl group at C - 1 will result in a drop of the ratio $^3H/\ ^{14}C$. If the carbon at C - 4 is derived from C - 2 of mevalonic acid and there is no scrambling, then conversion of the CH_2O Ac group of pebrolide ketone to a carboxyl function will result in the loss of $1\frac{1}{3}$ tritium atoms with the consequent change in the ratio of tritium to carbon. On the other hand, if it is the β substituent at C - 4 which is derived from C - 2 of mevalonic acid, then no change in the ratio $^3H/\ C^{14}$ will be observed. The oxygenation at C - 6 should also allow the confirmation of tritium atoms at C - 7. Hydrolysis of the benzoate, oxidation to the ketone and equilibration with base should result in removal of the remaining tritium atoms.



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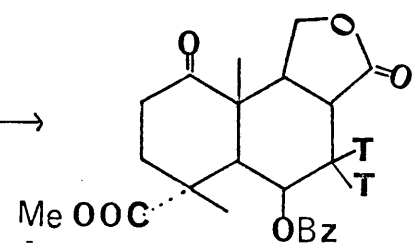

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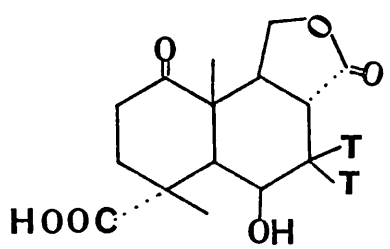

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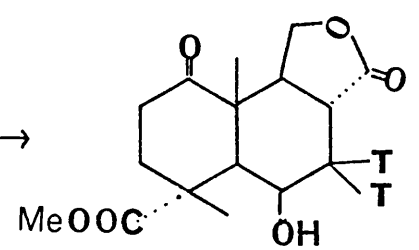

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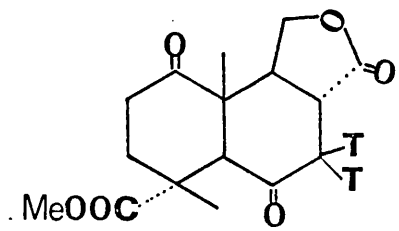


131

The transformation necessary to carry out this degradative scheme were all achieved using inactive material as follows. Pebrolide ketone (125), obtained by oxidation of pebrolide with Jones reagent, was selectively hydrolysed to the corresponding alcohol (126) using acid. Oxidation of this with Jones reagent and esterification of the resultant acid (127) gave the methyl ester (128). Basic hydrolysis of the benzoate function of the acid (127) was accompanied by epimerisation of the lactone giving the ketol acid (129) which was characterised as its methyl ester (130). Oxidation with Jones reagent now gave the diketo ester (131). The compounds obtained thus were useful as reference compounds in the sesquence with active material.

Preliminary studies of the type and amount of sesquiterpenes produced in relation to the time of growth were carried out using T.L.C. It was shown that in six day cultures, the fraction contained mainly pebrolide. At nine days desacetylpebrolide was present. Because the acetate group of pebrolide was necessary to allow this selective degradation, the fungus was harvested after six days.

A four day old culture of P.brevicompactum was fed

with 10 ml. of a 11 ml. aqueous solution of D L - Mevalonic acid - 2 -^{14}C lactone and D L - Mevalonic acid - 2T-lactone of 0.05 mc and 2 mc of activity respectively. At the end of 48 hours, the fungus was collected and the broth separated and worked up in the usual manner. To the chloroform solution of the neutrals 187 mg. of inactive pebrolide was added and re-isolated by means of crystallisation and purified to constant activity. The specific activity of pebrolide with respect to tritium was found to be 0.001 µc. The incorporation in pebrolide was 0.007 % (or 0.014 % based on L - mevalonate).

The low incorporation could be due among other factors to the substantial amount of mycophenolic acid produced by the fungus and present not only in the broth but in the mycelium. The side chain of mycophenolic acid is formed formally by two isopenoid units.

Another factor which might be responsible for the low incorporation was revealed by carrying out a TLC radio scan of the crude mixture of neutrals. This showed that an unidentified compound less polar than pebrolide having a very high activity relative to pebrolide was present. It is known[87] that steroids incorporate mevalonic acid

much more readily than sesquiterpenes and this compound could correspond to a steroid ester. In addition, ergosterol has been found in substantial amounts in the mycelium of P. brevicompactum, hence the low incorporation of mevalonate into pebrolide may be partly due to channeling of this precursor into steroids.

If the number of tritium atoms in pebrolide is 5, a $^{3}H/^{14}C$ ratio of 33.3 would be expected, but on the other hand, if the number of tritium atoms is 13/3 then the ratio should be 28.8. The $^{3}H/14_{C}$ ratio in pebrolide was found to be 29.8 in agreement with the latter. This is evidence of the possible presence of $^{4}/3$ of tritium in the group at C - 4.

Oxidation of pebrolide gave pebrolide ketone and the ratio $^{3}H/^{14}C$ was found to be 25.2, which represents the loss of one tritium atom. The - CH_2.OAc group in pebrolide ketone was transformed into a carbomethoxy function and the ratio $^{3}H/^{14}C$ was found to be 11.7, which represents the loss of $^{4}/3$ tritium atoms with respect to pebrolide ketone and $^{7}/3$ to pebrolide.

This result indicates that during the biosynthesis

of pebrolide, each one of the methyl groups at C - 4 keeps its identity and that the carbon atom at C - 4 position is derived from C - 2 of mevalonic acid. It is possible that although different initiators may be involved, the mechanism for the ring closure is essentially of the same type for this kind of sesquiterpene as for di- and triterpenes.

INTRODUCTION

X-RAY ANALYSIS OF BROMOACETYLPEBROLIDE

INTRODUCTION

Since the first successful diffraction of X-rays by Von Laue in 1912, the study of crystalline structures, on an atomic basis, has developed rapidly. A great variety of crystal structures have now been determined ranging from the very simple, in the case of NaCl, to the complex structures of the proteins.

The procedure of a structure determination employed in this work involved recording the diffraction of the X-rays by the crystal, photographically; estimating the intensities of the diffracted beams; determining the structure by the heavy atom method and refining the parameters of the atoms in the molecule by Fourier and least-square method.

The following is a brief account of the theory underlining this method.

A beam of X-rays incident on a crystal is scattered by the electrons within the crystal, the scattered wave recombining in various directions to give the observed diffraction maxima. Diffraction theory shows that the amplitude and phase of the wavelength from a scattering point in a three demensional array is

$$F_j \exp 2 \pi i (hx_j + ky_j + lz_j) \quad .. (1)$$

where f_j is the scattering power of the point, $x_j\ y_j\ z_j$ are the fractional co-ordinates of the point with respect to the cell edge and h, k, l, are integers. At the maximum of the diffraction spectrum, hkl, the wave resulting from a combination of all waves from all scattering points within the unit cell is given by,

$$F_{hkl} = f_1 \exp 2 \pi i (hx + ky_1 + lz_1) + f_2 \exp 2 \pi i (hx_2 + ky_2 + lz_2) + ... + f_i \exp 2 \pi i (hx_i + ky_i + lz_i) =$$

$$= \sum_{J=1}^{m} f_j \exp 2 \pi i (hx_j + ky_j + lz_j) \quad .. (2)$$

F_{hkl}, a complex quantity is known as the structure factor, its modulus $|F_{hkl}|$, as the structure amplitude. The structure factor is defined as the ratio of the amplitude of the radiation scattered in the order hkl by the contents of one unit cell to that scattered by a single electron under the same conditions. In practice, it is only possible to measure directly the structure amplitude and not the phase for any given diffraction maximum.

The scattering units of a crystal are the electrons associated with each atom. Since atoms of different

chemical types each have their own characteristic electronic distribution, each exhibits different scattering properties, these scattering properties being described by the f - curve. Thermal motion of the atoms within the crystal causes the effective f - curve to fall off more rapidly with sin θ/λ than for the same atom at rest.

In the simple case

$$f = f_o \exp - B (\sin \theta/\lambda)^2 \quad \ldots\ (3)$$

where f and f_o are the scattering functions for the atom at rest and undergoing vibrational motion, respectively. The quantity, B, is called the temperature co-efficient and its value is given by

$$B = 8 \pi^2 U \quad \ldots\ (4)$$

where U is the mean square displacement normal to the reflecting plane of the atom from its mean position; U is known as the temperature factor. In the case of isotropic vibration of a given atom the temperature co-efficient, B is adequately described by equation (4). In practice such an ideal case is seldom encountered and it is necessary to describe the temperature co-efficient vibration in terms of anisotropy temperature factor U_{ij} as expressed by :

$$\exp.\ 2\pi i\ (U_{11}\ h^2\ a^* + U_{22}\ k^2\ b^{*2} + U_{33}\ l^2\ c^{*2} + 2\ U_{12}\ hka^*b^* + 2\ U_{23}\ klb^*c^* + 2\ U_{31}\ zlc^*a^*) \quad .. (5)$$

where a^*, b^* and c^* are reciprocal lattice parameters. In the course of the X-ray analysis the vibrational motions of the atoms must be taken into consideration. This necessitates the calculation and subsequent refinement of temperature factors.

The number of electrons in a volume element dxdydz is given by $(xyz)\ \frac{V}{abc}\ d\ x\ dydz$ where V is the volume of the unit cell, thus the structure factor expression may be written.

$$F_{hkl} = \frac{V}{abc} \int_a^a \int_o^b \int_o^c (xy2)\ \exp\ 2\pi i\ (hx + ky + lz)\ dxdydz \quad .. (6)$$

where h,k,l are integers. The density of scattering matter throughout the unit cell can also be expressed in terms of a Fourier Summation

$$xyz = \sum \sum \sum A(pqr)\ \exp\ 2\pi i\ (px + qy + yz) \quad ..(7)$$

p,q and r being integers and A(pqr) the unknown general term. Substitution of equation (6) into (7) leads to

$$A(pqr) = F(hkl)/V \quad ..(8)$$

Subsequent substitution of equation (8) into (7) yields the expression of the distribution and density of

scattering matter in the unit cell as a Fourier summation.

$$\rho(xyz) = \sum\sum\sum \frac{F(hkl)}{V} \exp 2\pi i (hx + ky + lz) \quad ..(9$$

This can be expressed in terms of a phase angle as

$$(xyz) = \sum\sum\sum \frac{F(Hkl)}{V} (\exp 2\pi hx + 2\pi ky + 2\pi lz - (hkl)) \quad ..(1,$$

Examination of equation (10) reveals that determination of a structure using only observed phaseless structure amplitudes is not immediately possible on account of the unavailability of the phases $\alpha(hkl)$. This difficulty has been called the phase problem and its solution or evasion has been the object of many crystallographers' work. One of the more general techniques due to Robertson was employed in this analysis. The key to the Heavy Atom Method lies in the Patterson summation

$$P(xyz) = \sum\sum\sum |F(hkl)|^2 \cos 2\pi (hx + ky + lz).$$

The square of the phaseless structure amplitudes derived from the observed diffraction data are included in the Patterson summation and the resulting function P(xyz) is a description of the interatomic vectors within the unit cell. The height of these vectors is proportional to the square of the atomic number of the atom involved in the vector. Consequently a heavy atom to heavy atom vector would be expected to be much larger than

any other vector; this is indeed found in practice. From the position of such identifiable vectors and a knowledge of the space group of the crystal lattice system, the co-ordinates of the heavy atom may be calculated. The contribution of the heavy atom, F_h (hkl), to each reflection can then be found. If F_h (hkl) constitutes a large percentage of the total observed amplitude then the heavy atom phase angle may be taken as a good approximation to the (unknown) phase angle of the reflection. On the other hand, if F_h(hkl) makes but a small contribution to the observed structure amplitude, assignment of phase is uncertain. Employing the phases derived in this manner in conjunction with the appropriate observed structure amplitudes, Fourier summation leads to an electron density distribution revealing some, perhaps all, of the atoms of the structure. Inclusion of the atomic co-ordinates thus found into subsequent structure factor calculations and Fourier summations eventually leads to complete determination of the structure.

On elucidation of the structure, it is important to know its degree of correctness. A measure of correctness

is found by evaluating R where

$$R = \Sigma \, |Fo| - |Fc| \; / \; \Sigma \, |Fo| \qquad .. \; (12)$$

and where Fc is the structure amplitude calculated from atomic co-ordinates of the molecule and Fo is the observed structure amplitude.

The last stage of an X-ray crystallographic analysis is the adjustment of atomic co-ordinates such that the calculated structure factors agree as closely as possible with those observed, that is, minimisation of R or some similar function.

There are a considerable number of refinement techniques available based on Fourier methods. Unfortunately they may be subject to termination -of- series errors, and can be rather time consuming. Since high-speed digital computers have come into common use, the refinement of structures by least-squares method is not a formidable undertaking. Programmes for these least - square calculations are now freely available.

In the least-squares method, new co-ordinates, scale factors and vibrational parameters are derived such that $\Sigma w \Delta^2$ is minimised. To perform this task the expansion of a Taylor series is necessary and the concomitant end-of-series errors require more than one

cycle of least squares calculations to be performed before final minimisation is achieved. In a refinement where each atom is considered to have isotropic vibrational properties, the parameters x,y,z, and U_{iso}, as well as scale factors must be included in the normal equations. For a structure such as ours containing 35 atoms, a matrix of equations about 145 x 145 must be solved in the isotropic least-squares refinement. An anistropic refinement requires x,y, z, six Uij's for atoms as well as the scale factor to be included in the normal equation. Solution of the resulting matrix was a task too great for the computing facilities available, and a block diagonal approximation was used. The function minimised by the least-squares method is

$$W\ (\ Fo\ -\ Fc\ )^2$$

where W is a weighting function, and

$$\sqrt{w} = \left\{ \frac{1 - \exp\ -P_1(\sin\ ^{\circ}/_{\lambda})^2}{1 + P_2\ |Fo| + P_3\ |Fo|^2} \right\}^{\frac{1}{2}}$$

with $P_1 = 100$, $P_2 = 0.001$ and $P_3 = 0.0001$. Introduction of a weighting scheme is necessary in order to downweight the less reliable reflexions. The least-squares method also refines scale factors which are subsequently used to place all the data upon an approximately absolute scale necessary for the anisotropic refinement. Measures of

correctness of the refined structure given by R' factor where

$$R' = \frac{\Sigma\ W\ (\ |Fo|\ -\ |Fc|\ )^2}{\Sigma\ \ W\ |Fo|^2}$$

are used rather than the R factors as defined earlier.

DISCUSSION

Crystal Data.

The preparation and physical properties of the bromoacetate of pebrolide have been described in the experimental section.

A single crystal was grown from an ethereal solution and mounted so as to rotate about the a axis. Oscillation, rotation and Weissenberg photographs were recorded for the crystal using Cu - K_α (λ = 1.5418Å) radiation. Precession photographs were recorded using Mo - K_α (λ = 0.7107Å), radiation. Calculation based on these photographs yielded crystal data as follows :

Pebrolide Derivative $C_{26}H_{31}O_8Br$ M = 552

monoclinic, a = 9.08, b = 9.41, c = 15.16Å.

β = 98.2

V = 1282 Z = 2 Dc = 1.43

The only systematic absence in the X-ray data was 0k0 if k is odd. Thus the space group must be $P2_1$ since the derivative is optically active.

Intensity Data.

A small crystal bathed in a uniform X-ray beam was employed for the intensity measurements. The data were collected on a Nonius camera using Robertson's multiple - film technique, reciprocal lattice nets 0kl to 7kl being recorded. The intensities were estimated using a Joyce- Lobel flying spot integrating microdensitometer, intensity values being corrected for appropriate Lorentz polarisation and rotation factors. The various nets of Fo's were placed on an approximately absolute scale at a later stage of the refinement; some 879 independent reflexions were measured and used in the structure solution and refinement.

Solution of the structure.

The x and y co-ordinates of the bromine atom were determined from three dimensional Patterson synthesis. Since the space group is polar the y co-ordinate was set at zero. In the first electron-density distribution calculated with the observed structure amplitudes and the bromine phase angles there was, as expected, a false mirror plane which made interpretation of the map rather difficult.

However, it proved possible to select a few peaks as genuine atoms.

Successive cycles of structure factors and electron density distribution calculations allowed more and more atoms of the molecular framework to be distinguished and eventually after seven cycles, the complete structure was revealed as (98). The R factor was 16.97.

Refinement.

Employing the programme devised by Prof. D.W.J. Cruickshank and J.G.F. Smith, structure factor least-squares methods were used for the refinement process. Isotropic temperature factors U_{iso}, of 0.05 for bromine and carbon or oxygen atoms, respectively, were used in the initial states of the refinement. The progress of the refinement and the various values of R and R' are given in table 6. The final co-ordinates and thermal parameters are in table 7. These are sufficient to unambiguously establish the structure as (98). No great accuracy is claimed in this analysis. The bond length and the angles are in table (8). No value differs significantly from the expected value. There appears to be some disorder of the acetate group as revealed by bond length and atom density of the type $O - C\leq^{O}_{CH_3}$ --- $O - C\leq^{CH_3}_{O}$

H	K	L	I_k	$I_{\bar{k}}$	F_k	$F_{\bar{k}}$
1	1	$\bar{12}$	<		<	
1	1	$\bar{8}$	>		>	
1	1	5	>		>	
2	1	$\bar{10}$	<		<	
4	1	5	<		<	

Table 9

but this in no way affects the validity of the structure

Absolute configuration. The absolute configuration of the bromoacetylpebrolide was determined by means of Bijvoet's anomalous dispersion method [88]. The intensities of six Bijvoet pairs(9) were estimated visually and structure factors were calculated taking into account the anomalous dispersion corrections for bromine given in the International Tables. The results of the calculatio are presented in table (9). It follows that (98) and figure correctly represent the absolute stereochemistry of the compound.

Computing.

The calculation for this X-ray crystallographic analysis were performed using the English Electric KDF9 computer in conjunction with programmes devised and written by the following:

Fourier Synthesis	J.G. Sime
Structure Factor Least Squares	D.W.J.Cruickshank and J.G.F. Smith
Bond lengths and Angles	K.W. Muir

Numerous other small programes were used for the

structure factors suitable for publication, etc. The authors of these and other programmes were K. Islam, W. Oberhansli, D. Pollard and D. McGregor.

Table 8

Br(1).....C(8)	1.8221	C(19).....C(9)	1.623
C(8).....C(11)	1.4810	C(9).....C(12)	1.642
C(11).....O(6)	1.275	C(22).....C(12)	1.466
C(11).....O(5)	1.274	C(12).....O(1)	1.454
O(5).....C(3)	1.531	O(1).....C(27)	1.581
C(3).....C(10)	1.565	C(27).....O(2)	0.984
C(10).....C(2)	1.593	C(27).....C(13)	1.420
C(2).....C(18)	1.519	C(13).....C(23)	1.447
C(18).....C(22)	1.585	C(23).....C(26)	1.361
C(22).....C(5)	1.648	C(26).....C(21)	1.449
C(5).....C(3)	1.428	C(17).....C(7)	1.437
C(5).....C(1)	1.349	C(7).....C(13)	1.355
C(5).....C(4)	1.775	C(18).....C(14)	1.657
C(4).....C(6)	1.537	C(18).....C(20)	1.433
C(4).....C(19)	1.537	O(7).....C(25)	1.270
C(6).....C(4)	1.430	C(25).....C(24)	1.4415
C(19).....C(15)	1.518	C(25).....C(16)	1.416
O(5).....C(6)	3.054	C(1).....O(1)	3.020
C(1).....C(14)	3.1538	O(7).....C(12)	3.1774
C(15).....C(3)	1.089		

Table 8

C(1)...C(5)...C(3)	=	116°
C(1)...C(5)...C(4)	=	116°
C(1)...C(5)...C(22)	=	120°
C(14)..C(18)..C(22)	=	110°
O(1)...C(12)..C(22)	=	111°
C(12)..O(1)...C(27)	=	113°
O(1)...C(12)..C(9)	=	106°
C(3)...C(10)..C(2)	=	103°
C(10)..C(2)...C(18)	=	113°
C(2)...C(18)..C(22)	=	110°
C(18)..C(22)..C(5)	=	111°
C(22)..C(5)...C(3)	=	101°
C(5)...C(4)...C(19)	=	114°
C(4)...C(19)..C(9)	=	122°
C(19)..C(9)...C(12)	=	108°
C(9)...C(12)..C(22)	=	117°

H	K	L	Fo	Fc
0	0	2	37.1	15.5
0	0	3	56.1	50.8
0	0	4	60.0	54.3
0	0	5	42.4	41.1
0	0	6	44.5	46.6
0	0	7	9.1	4.4
0	0	8	13.6	10.9
0	0	9	44.8	39.7
0	0	10	12.0	9.3
0	0	11	8.2	13.0
0	0	12	20.7	20.6
0	0	13	5.5	8.6
0	1	2	15.8	11.0
0	1	3	12.3	5.9
0	1	4	79.1	54.6
0	1	5	47.4	45.0
0	1	6	23.6	20.2
0	1	7	25.4	25.3
0	1	8	23.3	27.3
0	1	9	14.0	16.0
0	1	10	15.2	7.4
0	1	11	13.4	10.4
0	1	12	8.1	8.9
0	1	13	10.7	17.6
0	1	14	14.2	10.0
0	2	1	80.6	75.2
0	2	2	50.4	45.2
0	2	3	21.9	22.8
0	2	4	10.4	13.5
0	2	5	23.4	25.3
0	2	6	26.9	28.3
0	2	7	19.3	17.6
0	2	8	20.9	22.0
0	2	9	25.4	26.7
0	2	10	11.3	9.0
0	2	11	9.0	10.7
0	2	12	12.2	9.2
0	2	13	11.9	10.1
0	2	14	12.4	7.8
0	3	1	31.9	30.2
0	3	2	44.7	40.6
0	3	3	17.6	11.0
0	3	4	25.6	32.0
0	3	5	30.8	34.5
0	3	6	9.3	7.4
0	3	7	25.5	23.3
0	3	8	21.6	18.3
0	3	9	5.6	6.9
0	3	10	22.2	21.7
0	3	11	8.2	5.6
0	3	12	4.6	7.0
0	3	13	16.9	16.9
0	3	14	5.2	9.3
0	4	0	36.0	43.0
0	4	1	14.8	12.5
0	4	2	35.4	33.9
0	4	3	30.9	35.2
0	4	4	38.8	32.7
0	4	5	21.2	23.9
0	4	6	29.0	34.2
0	4	7	5.8	5.2
0	4	8	7.7	7.3
0	4	9	12.6	11.8
0	4	10	12.2	12.2
0	4	11	6.7	2.2
0	4	12	8.0	9.2
0	4	13	8.2	7.6
0	4	14	4.4	1.9
0	4	15	4.1	3.9
0	5	1	38.7	42.2
0	5	2	9.6	9.3
0	5	3	9.6	5.6
0	5	4	19.1	22.4
0	5	5	10.4	12.8
0	5	6	14.4	15.7
0	5	7	13.7	15.8
0	5	8	12.8	13.7
0	5	9	8.3	7.3
0	5	10	16.4	17.2
0	5	13	7.6	8.4
0	5	14	7.2	6.0
0	6	0	20.0	21.3
0	6	1	12.1	16.5
0	6	3	18.0	26.5
0	6	4	12.6	13.9
0	6	5	13.7	17.4
0	6	6	23.1	28.3
0	6	7	7.9	9.7
0	6	8	5.5	2.1
0	6	9	11.3	10.1
0	6	10	6.4	3.5
0	6	12	8.4	8.1
0	7	1	8.9	15.9
0	7	2	4.9	17.1
0	7	3	13.7	13.1
0	7	4	13.9	10.0
0	7	5	8.8	9.8
0	7	6	10.7	8.0
0	7	8	4.8	8.4
0	7	9	5.9	3.5
0	7	10	5.2	7.1
0	7	11	4.5	3.5
0	8	0	11.8	10.7
0	8	1	4.6	1.0
0	8	2	4.6	7.6
0	8	3	11.0	3.4
0	8	4	7.5	8.9
0	8	5	11.4	11.7
0	8	6	12.5	15.5
0	9	1	6.1	11.5
0	9	5	5.1	4.7
0	9	6	4.6	1.7
0	9	8	4.7	6.0
0	10	0	5.5	4.3
0	10	1	4.6	2.2
1	0	11	6.3	5.4
1	0	10	7.6	3.6
1	0	9	22.4	21.3
1	0	8	42.7	43.3
1	0	7	24.8	21.9
1	0	6	50.7	43.3
1	0	5	61.6	62.2
1	0	4	16.6	5.7
1	0	3	72.4	63.6
1	0	2	44.1	40.3
1	0	-5	21.1	27.0
1	0	-6	33.6	39.3
1	0	-7	28.0	9.4
1	0	-9	8.9	9.5
1	0	-10	10.6	5.3
1	0	-12	12.8	15.1
1	0	-13	8.5	7.3
1	0	-15	7.5	7.0
1	0	-17	7.0	4.3
1	1	13	10.6	6.8
1	1	12	7.5	4.9
1	1	11	13.9	7.8
1	1	10	37.4	31.0
1	1	9	21.6	17.5
1	1	8	13.9	13.0
1	1	7	14.6	17.7
1	1	6	36.3	34.4
1	1	5	33.2	26.9
1	1	4	54.1	48.8
1	1	3	13.9	13.6
1	1	2	75.0	74.5
1	1	-2	38.3	40.4

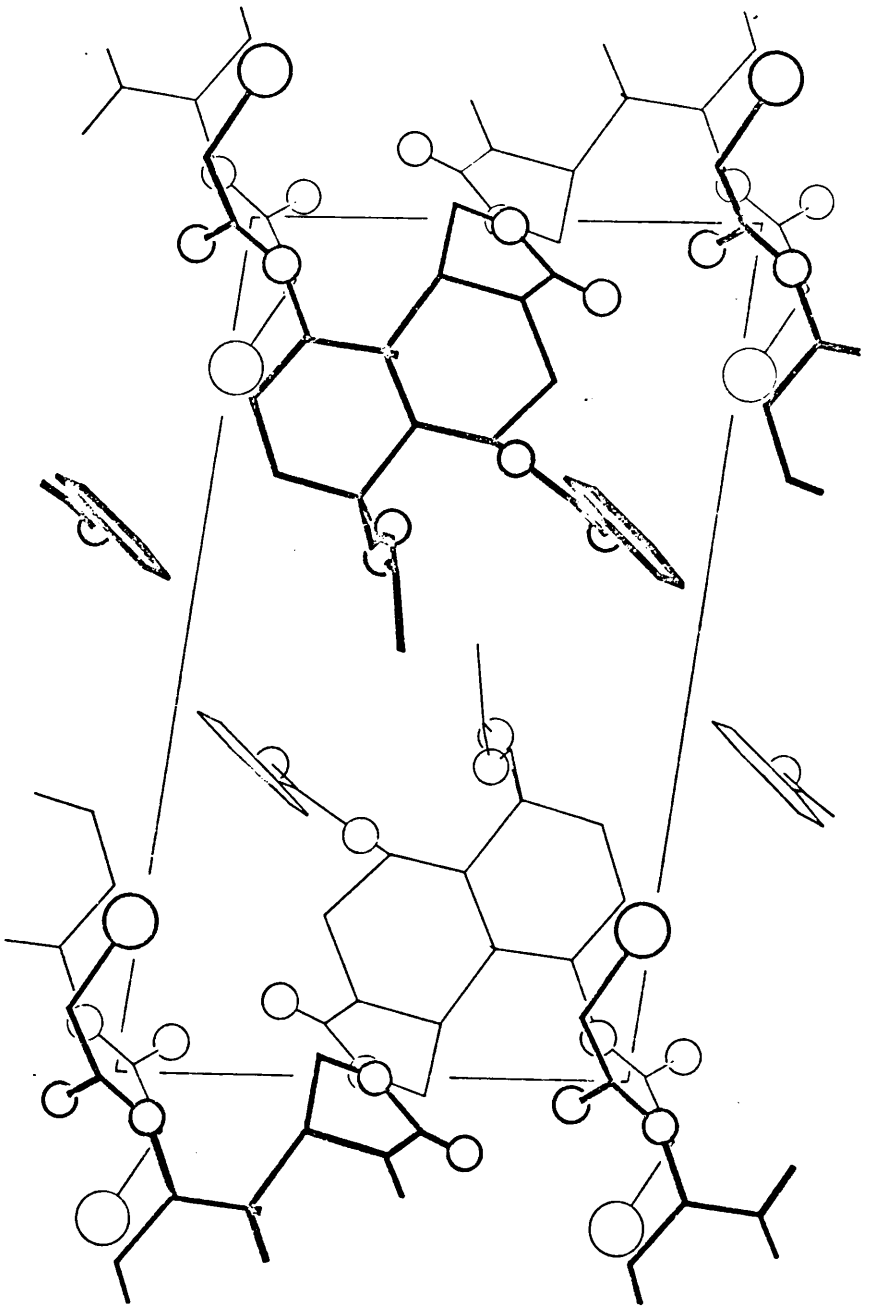
H	K	L	Fo	Fc
1	1	-3	47.8	51.1
1	1	-4	54.1	56.9
1	1	-5	32.9	30.4
1	1	-6	8.1	3.8
1	1	-7	27.6	32.8
1	1	-8	13.6	11.8
1	1	-9	12.0	12.5
1	1	-10	37.8	38.5
1	1	-11	35.6	45.9
1	1	-12	6.5	6.7
1	1	-13	22.0	20.9
1	1	-14	9.2	4.4
1	2	12	15.7	12.5
1	2	11	16.4	13.3
1	2	10	8.6	9.0
1	2	9	15.4	12.7
1	2	8	27.4	24.8
1	2	7	37.4	34.0
1	2	6	67.7	55.4
1	2	5	30.5	23.4
1	2	4	20.2	25.8
1	2	3	59.6	53.3
1	2	2	64.1	61.5
1	2	1	8.5	11.0
1	2	0	73.2	80.2
1	2	-1	42.1	40.7
1	2	-2	33.5	25.3
1	2	-3	45.1	47.6
1	2	-4	57.7	53.6
1	2	-5	37.6	41.2
1	2	-6	41.8	45.4
1	2	-7	21.4	18.2
1	2	-8	12.4	14.4
1	2	-9	37.7	29.1
1	2	-12	6.9	5.3
1	2	-14	6.8	6.0
1	2	-15	8.3	5.9
1	3	-14	7.4	6.5
1	3	-13	15.6	15.7
1	3	-11	24.9	21.9
1	3	-10	16.6	15.1
1	3	-9	6.4	4.7
1	3	-8	15.3	12.1
1	3	-7	18.9	23.8
1	3	-6	15.4	5.8
1	3	-5	34.6	37.7
1	3	-4	9.4	16.8
1	3	-3	9.0	7.9
1	3	-2	39.6	44.0
1	3	-1	44.6	55.6
1	3	0	28.8	30.4
1	3	1	7.5	10.2
1	3	2	37.2	40.3
1	3	3	16.9	7.0
1	3	4	47.7	44.9
1	3	5	42.2	35.3
1	3	6	25.6	25.6
1	3	7	19.9	18.1
1	3	8	23.2	21.1
1	3	9	8.9	7.2
1	3	10	16.3	15.9
1	3	11	7.8	5.0
1	3	13	12.6	10.2
1	3	14	7.3	6.7
1	4	12	13.1	9.9
1	4	11	8.6	11.5
1	4	10	5.9	5.9
1	4	9	12.0	13.9
1	4	8	13.9	14.1
1	4	7	20.7	20.3
1	4	6	30.4	31.0
1	4	5	22.6	18.1
1	4	4	18.3	16.1
1	4	3	15.1	27.7
1	4	2	48.5	48.5
1	4	1	29.6	27.9
1	4	0	73.4	76.9
1	4	-1	44.9	46.8
1	4	-2	40.3	29.7
1	4	-3	21.9	29.4
1	4	-4	23.1	12.9
1	4	-5	22.6	27.8
1	4	-6	20.3	25.4
1	4	-7	30.9	31.1
1	4	-8	7.6	8.4
1	4	-9	15.5	25.3
1	4	-10	6.8	6.8
1	4	-11	7.5	8.0
1	4	-12	8.6	7.9
1	5	-14	7.9	6.2
1	5	-11	12.9	11.8
1	5	-10	9.0	10.1
1	5	-7	19.4	26.0
1	5	-6	12.1	16.7
1	5	-5	28.2	30.5
1	5	-4	10.9	24.2
1	5	-3	12.4	12.9
1	5	-2	9.2	19.7
1	5	-1	21.5	25.4
1	5	0	20.6	13.6
1	5	1	26.9	21.2
1	5	2	28.4	29.0
1	5	3	29.8	6.6
1	5	4	32.1	28.9
1	5	5	11.3	19.2
1	5	6	9.1	6.3
1	5	8	18.3	16.8
1	5	10	10.4	7.0
1	5	13	8.2	7.2
1	6	11	11.8	12.7
1	6	9	6.8	11.2
1	6	8	8.8	9.6
1	6	7	8.3	7.1
1	6	6	12.5	15.4
1	6	5	12.8	16.3
1	6	4	14.1	11.4
1	6	2	19.5	21.2
1	6	1	12.6	17.9
1	6	0	29.7	34.4
1	6	-1	26.2	27.3
1	6	-2	16.4	16.7
1	6	-3	15.8	20.2
1	6	-4	10.7	11.2
1	6	-5	8.2	4.5
1	6	-6	13.2	17.2
1	6	-7	13.5	15.6
1	6	-9	7.0	9.4
1	6	-10	6.1	8.8
1	7	-10	5.2	7.5
1	7	-8	5.3	5.7
1	7	-7	9.4	12.4
1	7	-5	17.4	21.9
1	7	-4	14.3	16.2
1	7	-3	12.2	9.1
1	7	-2	9.2	7.4
1	7	-1	8.0	5.5
1	7	0	5.6	10.0
1	7	2	6.4	11.1
1	7	4	9.2	12.5
1	7	5	17.8	21.0
1	7	6	8.2	6.6
1	8	9	5.4	4.3
1	8	8	6.6	5.8
1	8	7	6.7	5.8
1	8	6	5.3	7.7
1	8	4	7.2	4.5
1	8	3	10.8	17.2

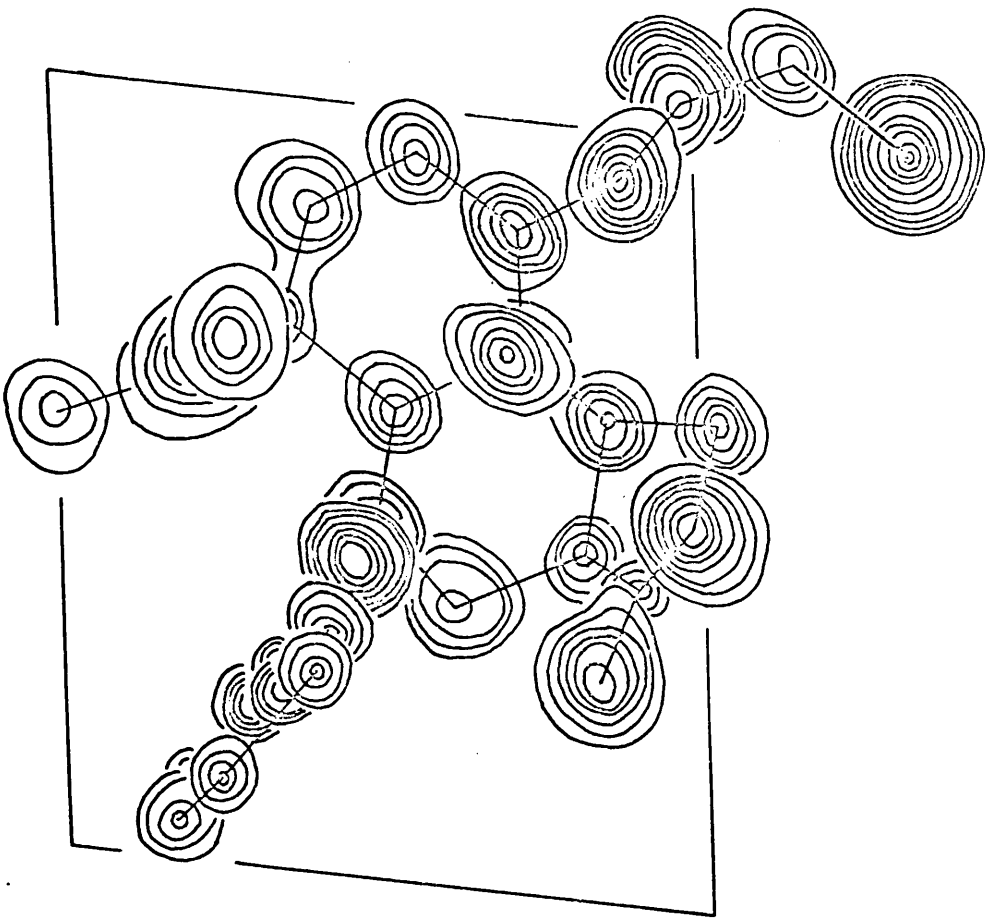
H	K	L	Fo	Fc
1	8	2	10.1	9.9
1	8	1	10.2	14.9
1	8	-2	8.3	10.4
1	8	-6	10.0	14.5
1	8	-7	9.8	8.7
1	9	-7	4.9	4.5
1	9	-5	5.5	6.5
1	9	-4	6.3	8.6
1	9	-3	6.4	3.6
1	9	4	5.9	3.2
1	9	5	8.3	7.2
2	0	11	12.9	8.2
2	0	10	10.0	14.9
2	0	9	8.7	6.4
2	0	8	17.2	14.9
2	0	6	36.7	34.0
2	0	5	18.6	16.2
2	0	4	16.6	20.2
2	0	2	71.0	66.1
2	0	-3	82.9	111.2
2	0	-4	114.1	85.6
2	0	-5	104.0	79.3
2	0	-6	30.6	28.5
2	0	-7	59.1	50.1
2	0	-8	8.2	7.6
2	0	-9	45.3	45.3
2	0	-11	21.9	17.5
2	0	-12	22.2	21.2
2	0	-14	9.3	8.2
2	1	-14	11.6	9.7
2	1	-11	19.9	17.2
2	1	-10	7.2	5.4
2	1	-9	13.9	10.3
2	1	-8	10.6	13.0
2	1	-7	36.0	33.0
2	1	-6	20.7	24.3
2	1	-5	80.8	53.5
2	1	-4	77.3	69.0
2	1	-3	37.1	35.8
2	1	-2	26.1	29.1
2	1	1	83.4	73.8
2	1	2	37.5	40.9
2	1	3	40.4	32.8
2	1	4	44.2	45.2
2	1	5	45.5	47.5
2	1	6	26.8	27.5
2	1	7	32.5	25.7
2	1	8	20.0	21.6
2	1	9	11.6	44.6
2	1	10	19.7	16.2
2	1	11	12.1	12.5
2	1	12	10.0	10.6
2	2	12	8.3	8.0
2	2	11	15.0	11.8
2	2	10	6.6	6.0
2	2	9	16.1	15.6
2	2	8	27.9	25.1
2	2	7	11.7	10.7
2	2	6	17.7	16.6
2	2	5	44.5	38.9
2	2	3	45.3	50.1
2	2	2	46.8	40.7
2	2	1	19.6	22.6
2	2	0	68.4	59.7
2	2	-1	82.9	73.3
2	2	-2	23.7	21.4
2	2	-3	41.0	45.1
2	2	-4	70.7	55.3
2	2	-5	72.7	55.9
2	2	-6	31.1	27.5
2	2	-7	15.3	22.6
2	2	-8	12.5	8.0
2	2	-9	18.7	20.6
2	2	-10	15.2	12.7
2	2	-11	17.9	15.0
2	2	-12	23.7	21.1
2	3	-12	9.7	9.0
2	3	-11	17.2	16.9
2	3	-10	16.3	17.1
2	3	-8	21.5	23.9
2	3	-7	29.5	29.1
2	3	-6	19.3	17.3
2	3	-5	28.0	35.5
2	3	-4	53.4	47.9
2	3	-3	14.7	15.5
2	3	-2	12.1	22.1
2	3	-1	20.0	25.0
2	3	0	22.3	15.1
2	3	1	17.2	24.6
2	3	2	17.7	26.4
2	3	3	25.5	19.5
2	3	4	27.2	31.4
2	3	5	39.5	35.4
2	3	6	24.1	18.5
2	3	7	15.5	17.9
2	3	9	19.1	10.2
2	3	10	9.0	10.4
2	3	11	16.1	7.2
2	3	12	7.7	10.9
2	4	12	8.4	7.3
2	4	11	6.3	4.9
2	4	10	6.0	8.7
2	4	9	15.5	16.0
2	4	8	22.7	21.7
2	4	7	14.8	8.2
2	4	6	14.2	13.3
2	4	5	15.1	18.4
2	4	4	13.5	11.6
2	4	3	40.5	43.9
2	4	2	33.9	35.3
2	4	1	32.0	30.9
2	4	0	19.3	23.9
2	4	-1	21.8	20.1
2	4	-2	14.5	8.7
2	4	-3	23.4	33.2
2	4	-4	22.4	23.3
2	4	-5	23.6	23.5
2	4	-6	12.3	15.4
2	4	-8	13.5	12.1
2	4	-9	12.4	12.3
2	4	-10	8.9	7.0
2	4	-11	13.7	14.2
2	5	-11	9.4	14.6
2	5	-10	13.4	17.1
2	5	-8	10.7	23.1
2	5	-7	20.0	20.9
2	5	-6	12.6	9.6
2	5	-5	9.0	14.7
2	5	-4	22.6	27.4
2	5	-3	11.6	7.1
2	5	-2	31.3	37.8
2	5	-1	8.0	11.1
2	5	0	17.5	12.3
2	5	1	32.1	35.4
2	5	2	9.3	21.3
2	5	3	19.3	12.1
2	5	4	9.4	14.7
2	5	5	22.9	23.7
2	5	7	13.3	17.0
2	5	8	3.4	7.6
2	5	10	12.9	13.2
2	5	12	5.4	3.9
2	6	9	8.3	8.7
2	6	8	6.1	2.3
2	6	7	13.0	14.5
2	6	6	10.7	17.5
2	6	5	19.3	21.3

H	K	L	Fo	Fc
2	6	4	10.2	7.3
2	6	3	7.2	16.3
2	6	1	10.1	9.1
2	6	0	8.9	13.3
2	6	-1	10.1	7.8
2	6	-3	7.5	14.1
2	6	-4	7.0	9.2
2	6	-5	12.1	10.8
2	6	-6	16.3	24.0
2	6	-7	7.6	5.3
2	6	-8	15.0	16.3
2	6	-9	7.1	4.9
2	6	-11	6.1	7.5
2	7	-7	17.9	17.3
2	7	-5	8.6	10.5
2	7	-2	10.3	15.6
2	7	-1	5.8	16.2
2	7	0	12.1	13.3
2	7	1	13.7	13.9
2	7	2	7.0	13.9
2	7	3	14.7	8.4
2	7	4	11.0	10.3
2	7	5	7.1	7.8
2	8	7	8.5	8.9
2	8	6	12.8	13.6
2	8	5	10.6	10.3
2	8	4	8.7	6.9
2	8	2	7.1	8.4
2	8	1	7.9	8.1
2	8	-2	7.8	7.3
2	8	-3	14.4	15.3
2	8	-5	9.7	8.7
2	8	-6	8.7	10.5
2	8	-7	6.1	8.2
3	0	9	16.1	12.2
3	0	6	8.4	12.8
3	0	5	42.1	47.7
3	0	4	43.5	35.9
3	0	3	45.1	46.9
3	0	2	22.6	25.5
3	0	-4	12.6	22.0
3	0	-5	14.1	14.9
3	0	-6	55.2	46.7
3	0	-8	51.9	49.8
3	0	-9	34.8	33.0
3	0	-10	15.0	10.5
3	0	-12	6.7	7.4
3	1	-14	8.5	8.7
3	1	-13	11.0	6.2
3	1	-11	14.6	13.5
3	1	-10	14.8	14.2
3	1	-9	15.5	15.0
3	1	-8	31.4	31.5
3	1	-7	14.0	12.3
3	1	-6	47.0	32.7
3	1	-5	13.7	15.9
3	1	-4	47.9	41.3
3	1	-3	14.3	13.7
3	1	-2	34.3	33.9
3	1	1	55.8	52.4
3	1	2	29.5	21.5
3	1	3	16.8	26.4
3	1	4	16.8	24.5
3	1	5	44.7	37.4
3	1	7	25.6	31.7
3	1	8	15.1	15.7
3	1	9	12.0	11.4
3	1	10	9.9	3.0
3	2	12	17.5	16.7
3	2	10	11.1	12.6
3	2	8	18.2	20.0
3	2	7	11.9	5.9
3	2	6	24.0	24.7
3	2	5	31.7	29.0
3	2	4	20.8	16.2
3	2	3	43.5	43.9
3	2	2	27.6	25.1
3	2	1	16.7	15.2
3	2	0	45.6	43.5
3	2	-1	41.2	45.4
3	2	-2	12.0	10.6
3	2	-3	35.7	37.0
3	2	-4	12.7	9.7
3	2	-5	42.8	35.8
3	2	-6	48.6	41.3
3	2	-7	22.3	26.0
3	2	-8	22.6	19.0
3	2	-9	20.7	21.2
3	2	-12	9.7	7.0
3	2	-14	8.5	6.0
3	3	-14	11.4	9.8
3	3	-11	10.1	11.1
3	3	-10	20.9	19.5
3	3	-8	19.5	25.7
3	3	-6	13.3	16.7
3	3	-5	24.4	26.6
3	3	-4	26.4	22.1
3	3	-3	23.0	19.8
3	3	-2	43.3	41.3
3	3	-1	60.2	39.2
3	3	0	11.3	10.5
3	3	1	53.3	49.1
3	3	2	14.2	17.1
3	3	3	16.2	15.7
3	3	4	37.7	37.2
3	3	5	35.1	34.4
3	3	6	13.3	3.3
3	3	7	20.4	20.3
3	3	8	9.6	9.8
3	3	9	13.4	7.7
3	3	10	10.3	7.6
3	3	12	8.5	13.1
3	4	9	9.0	8.0
3	4	6	10.8	19.3
3	4	5	17.8	19.3
3	4	4	12.7	10.1
3	4	3	8.1	14.7
3	4	1	13.2	11.8
3	4	0	12.8	25.0
3	4	-1	44.9	45.7
3	4	-2	11.0	8.4
3	4	-3	13.4	23.0
3	4	-4	17.8	19.7
3	4	-5	22.3	27.0
3	4	-6	41.3	39.2
3	4	-7	27.9	27.7
3	4	-10	11.5	8.6
3	4	-12	11.0	11.4
3	5	-10	11.0	9.4
3	5	-9	6.8	5.9
3	5	-6	15.5	15.5
3	5	-5	5.9	15.9
3	5	-4	11.7	13.4
3	5	-3	7.6	10.5
3	5	-2	9.1	13.9
3	5	-1	20.7	24.9
3	5	0	9.0	2.3
3	5	1	13.8	19.3
3	5	2	8.6	12.1
3	5	4	15.6	22.1
3	5	5	13.5	14.8
3	5	6	24.4	21.5
3	5	7	9.1	11.5
3	5	8	12.1	10.9
3	5	9	11.0	7.2
3	5	10	9.9	9.3

H	K	L	Fo	Fc
3	5	12	9.0	8.5
3	6	7	15.4	14.9
3	6	6	9.3	15.0
3	6	3	9.0	13.3
3	6	2	11.3	14.3
3	6	1	8.2	6.2
3	6	0	10.1	18.3
3	6	-1	23.1	21.0
3	6	-2	11.3	6.9
3	6	-3	11.4	14.9
3	6	-4	6.3	11.0
3	6	-5	7.2	10.8
3	6	-7	8.3	10.2
3	7	-7	7.4	3.7
3	7	-4	14.9	17.3
3	7	1	7.5	10.1
3	7	2	8.3	12.9
3	7	3	13.6	9.4
3	7	7	9.2	10.6
3	8	5	9.7	8.1
3	8	1	8.6	4.9
3	9	0	9.0	8.8
4	0	6	30.0	25.9
4	0	5	20.5	23.0
4	0	4	15.8	10.3
4	0	3	23.9	26.4
4	0	2	22.0	25.3
4	0	1	43.8	31.8
4	0	-4	10.3	5.8
4	0	-5	14.0	14.7
4	0	-6	64.0	61.7
4	0	-7	17.2	23.5
4	0	-8	14.0	8.8
4	0	-9	22.2	19.2
4	0	-10	20.1	21.1
4	0	-12	16.0	17.3
4	1	-11	10.0	12.8
4	1	-10	19.9	19.4
4	1	-8	26.3	27.1
4	1	-7	16.8	21.8
4	1	-6	19.0	16.2
4	1	-5	18.2	23.7
4	1	-4	21.4	19.6
4	1	-3	13.1	15.2
4	1	-2	29.2	32.6
4	1	-1	37.7	28.0
4	1	0	45.8	29.6
4	1	1	64.4	57.1
4	1	2	21.4	43.6
4	1	3	31.1	28.8
4	1	4	23.0	22.8
4	1	5	7.6	6.9
4	1	6	22.0	21.7
4	1	7	30.8	24.1
4	1	8	23.2	15.6
4	2	9	7.4	4.0
4	2	8	13.8	13.3
4	2	7	8.9	8.7
4	2	6	18.6	17.5
4	2	5	15.9	16.7
4	2	4	22.1	14.2
4	2	3	34.0	25.8
4	2	2	20.9	26.7
4	2	1	37.3	29.9
4	2	0	22.8	22.8
4	2	-1	43.6	38.2
4	2	-2	25.0	10.0
4	2	-4	13.3	14.3
4	2	-5	24.1	21.6
4	2	-6	36.0	44.3
4	2	-7	16.7	13.6
4	2	-8	14.1	15.0
4	2	-9	21.5	18.1
4	2	-10	11.8	12.8
4	3	-11	15.3	13.5
4	3	-10	10.3	13.4
4	3	-6	17.5	20.8
4	3	-5	21.0	25.3
4	3	-4	21.6	21.0
4	3	-3	16.9	14.1
4	3	-2	31.8	25.4
4	3	-1	26.4	34.1
4	3	0	15.3	10.8
4	3	1	19.1	21.6
4	3	2	22.6	18.1
4	3	3	23.5	21.5
4	3	4	16.0	22.9
4	3	5	16.8	14.9
4	3	7	20.1	20.9
4	3	8	7.5	8.4
4	3	9	15.7	16.2
4	4	9	14.1	8.8
4	4	5	15.1	14.9
4	4	4	9.5	5.9
4	4	3	28.8	26.0
4	4	2	25.7	29.7
4	4	1	23.1	16.0
4	4	0	15.1	21.1
4	4	-1	13.9	21.8
4	4	-2	14.5	16.2
4	4	-3	15.4	16.1
4	4	-4	7.5	7.0
4	4	-6	19.9	26.2
4	4	-7	15.5	16.6
4	4	-8	13.1	13.4
4	4	-9	14.6	17.3
4	5	-5	13.6	17.9
4	5	-4	11.1	13.8
4	5	-3	10.2	5.9
4	5	-2	15.0	21.2
4	5	-1	8.4	8.0
4	5	1	12.2	17.9
4	5	2	12.4	10.4
4	5	5	11.7	14.8
4	5	6	12.1	8.2
4	5	7	12.4	13.1
4	6	6	13.5	9.7
4	6	4	12.1	9.1
4	6	3	21.2	19.6
4	6	2	21.1	19.8
4	6	1	11.3	9.7
4	6	0	14.7	20.5
4	6	-1	10.6	14.2
4	6	-4	11.0	15.8
4	6	-5	13.4	12.2
4	7	0	10.1	10.5
4	7	1	9.8	13.7
4	7	2	11.7	12.8
5	0	7	19.2	19.0
5	0	5	10.6	11.0
5	0	4	16.1	14.9
5	0	3	16.5	13.5
5	0	2	48.3	45.0
5	0	1	16.9	11.7
5	0	-3	10.0	34.0
5	0	-4	15.5	24.6
5	0	-5	8.7	13.5
5	0	-6	20.4	23.3
5	0	-7	16.3	17.7
5	0	-9	21.8	14.7
5	0	-10	12.1	8.6
5	0	-11	15.6	16.1
5	1	-11	14.7	14.3
5	1	-9	11.5	6.5
5	1	-7	14.3	17.9
5	1	-6	13.6	15.6

H	K	L	Fo	Fc
5	1	-5	15.7	21.2
5	1	-4	13.0	25.1
5	1	-3	14.0	13.3
5	1	-2	30.5	36.5
5	1	-1	8.9	12.1
5	1	0	30.7	29.5
5	1	1	30.8	25.5
5	1	2	15.1	16.7
5	1	4	24.2	22.4
5	1	5	24.7	20.1
5	1	6	7.8	0.1
5	1	7	33.6	25.0
5	1	8	24.3	20.9
5	2	7	15.4	10.9
5	2	6	7.0	5.2
5	2	5	26.5	26.4
5	2	4	9.0	10.0
5	2	3	18.2	22.2
5	2	2	14.1	14.3
5	2	1	29.9	15.6
5	2	0	21.6	24.2
5	2	-1	16.6	23.9
5	2	-3	19.0	24.5
5	2	-4	19.8	17.7
5	2	-6	19.9	27.5
5	2	-7	20.3	23.7
5	2	-9	13.7	9.3
5	2	-12	13.4	15.5
5	3	-11	9.9	9.6
5	3	-8	15.3	16.0
5	3	-6	14.4	14.7
5	3	-5	22.6	20.0
5	3	-4	15.0	17.2
5	3	-3	11.0	4.7
5	3	-2	15.6	20.8
5	3	-1	6.6	6.2
5	3	0	19.3	12.2
5	3	1	26.0	28.2
5	3	2	11.8	12.6
5	3	3	22.3	18.5
5	3	4	22.5	16.7
5	3	6	15.3	8.8
5	3	7	22.8	21.6
5	3	8	20.0	14.9
5	4	7	14.5	7.4
5	4	6	11.4	12.5
5	4	5	8.6	15.5
5	4	2	13.5	11.3
5	4	1	23.7	17.7
5	4	0	15.1	20.0
5	4	-1	23.4	21.7
5	4	-2	13.4	8.2
5	4	-3	13.8	12.0
5	4	-4	9.9	12.1
5	4	-5	9.9	13.0
5	4	-6	19.8	25.3
5	5	-6	10.2	10.4
5	5	-2	8.1	15.8
5	5	0	13.4	14.3
5	5	1	27.6	27.8
5	5	2	23.2	22.3
5	5	3	22.5	17.2
5	5	4	10.4	7.5
5	5	5	18.6	13.7
5	5	6	12.9	8.1
5	5	7	17.0	15.3
5	5	8	12.2	8.5
5	6	4	9.3	5.4
5	6	1	22.9	15.0
5	6	0	11.0	5.4
5	6	-3	9.7	11.6
5	6	-5	10.6	10.2
5	6	-6	11.2	12.7
5	7	-4	9.0	2.5
5	7	-1	8.1	10.7
5	7	2	12.9	16.5
6	0	7	12.9	17.5
6	0	6	12.6	12.1
6	0	4	14.3	7.8
6	0	3	23.1	15.5
6	0	2	14.9	16.1
6	0	-4	12.3	24.0
6	0	-5	31.6	31.5
6	0	-6	35.5	36.9
6	0	-9	13.9	9.5
6	1	-10	11.0	10.5
6	1	-8	11.3	12.6
6	1	-6	14.6	18.6
6	1	-4	16.1	16.4
6	1	-2	25.5	29.8
6	1	-1	23.5	24.3
6	1	0	12.0	12.4
6	1	1	6.1	10.1
6	1	2	13.2	6.5
6	1	3	6.1	8.0
6	1	4	13.2	15.7
6	1	5	15.0	10.2
6	1	6	30.1	27.4
6	1	7	24.0	18.5
6	1	8	9.5	5.9
6	2	8	9.9	11.5
6	2	5	17.1	12.6
6	2	4	8.9	2.3
6	2	3	5.5	5.3
6	2	2	15.9	21.0
6	2	1	5.4	11.6
6	2	0	5.4	14.1
6	2	-2	9.8	7.9
6	2	-3	20.6	25.2
6	2	-4	17.4	22.0
6	2	-5	12.9	15.0
6	2	-6	23.3	21.1
6	2	-7	12.7	[illegible]
6	2	-10	9.6	6.6
6	3	-6	15.2	12.5
6	3	-5	7.7	8.9
6	3	-2	8.4	13.1
6	3	0	7.2	8.4
6	3	1	8.9	9.5
6	3	2	8.5	8.0
6	3	4	19.3	21.1
6	3	5	10.7	7.3
6	3	6	24.3	19.8
6	4	7	9.5	7.5
6	4	5	11.3	5.7
6	4	4	12.3	9.1
6	4	2	15.5	14.6
6	4	1	5.9	2.1
6	4	0	14.8	15.1
6	4	-1	13.1	10.5
6	4	-2	5.2	5.0
6	4	-3	16.9	18.7
6	4	-4	18.2	16.1
6	4	-6	10.0	12.5
6	4	-8	10.2	8.8
6	4	-9	8.3	10.8
6	4	-10	16.1	11.5
6	5	-7	9.6	11.3
6	5	-6	11.7	9.8
6	5	-5	9.6	11.0
6	5	-3	8.2	7.5
6	5	0	12.6	10.7
6	5	4	17.1	16.7
6	5	6	11.7	10.3
6	6	-1	13.9	10.0
6	6	-2	12.6	13.5





EXPERIMENTAL

INSTRUMENTATION

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Infra red spectra were measured with a Unicam S.P. 200 instrument and for high resolution (KBr disc and solution spectra, in the solvent as stated) with a Unicam S.P. 100 double beam infra red spectrometer equipped with an S.P. 130 sodium chloride prism grating double monochrometer, operated under vacuum. Ultraviolet spectra were obtained, in ethanol solutions, on a Unicam S.P. 800 recording spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Perkin-Elmer R 10 60 Mc/s spectrometer and with a Varian HA - 100 100 Mc/s spectrometer. Unless otherwise stated all values quoted are recorded at 100 Mc/s in deuterochloroform with tetramethylsilane as internal standard. Mass spectra were obtained with A.E.I. MS9 and MS12 mass spectrometers. Analytical gas - liquid chromotography was performed on a Pye Argon chromatograph.

Thin layer chromatography.

Rf values were determined from elution on 0.25 mm layer of Kieselgel G, the compounds being located by spraying with ceric ammonium nitrate (1%)

in sulphuric acid (10%) and baking, 10% methanolic ferric chloride, and 10% ethanolic 2,4 - dinitrophenylhydrazine.

General.

Diazomethane was prepared by the method of More and Reed[89] from bis (N - methyl - N -nitroso) - terephthalamide. All organic extracts were dried over anhydrous magnesium sulphate and solvents were removed using a rotatory film evaporation. "Light petroleum", unless otherwise stated, refers to light petroleum, b.p. 40 - 60°C.

Culture and extraction of the mould.

Spores of Penicillium brevicompactum (strain P-1), suspended in sterilised water, were added to 100 Roux bottles each containing 200 ml of sterile medium Czapek - Dox with the addition of 1% cornsteep liquor). Cultures were allowed to grow at 25° for four weeks. The filtered medium was stirred with charcoal (10g/litre of filtrate) for 1 - 2 hours and the charcoal was extracted in a Soxhlet apparatus with acetone for 24 hours. The extract was partitioned between chloroform and water. The chloroform solution was then

extracted with a saturated aqueous solution of sodium bicarbonate. Neutralisation of the bicarbonate solution with dilute HCl precipitated mycophenolic acid which was purified by crystallisation from methanol/water. The chloroform was then dried and the solvent removed. The residue was chromatographed on silica gel (30g).

Fractions eluted with light petroleum - benzene (1:4) gave mycofuranolide (4 mg).

Fractions eluted with benzene gave a mixture of unidentified compounds (100 mg).

Fractions eluted with benzene - chloroform (9:1) gave deoxypebrolide (4 mg). Isolation of deoxypebrolide was hindered by the presence of an oil in the same fractions. However, the crystals of deoxypebrolide were physically separated from this oil and recrystallised from ether.

Fractions eluted by benzene - chloroform (9:1 - 1:9) gave ethyl mycophenolate (51) and the hydroxy - lactone (55) identified by comparison (T.L.C., IR) with an authentic sample. Other unidentified compounds were also present.

Fractions eluted by benzene-chloroform (1:9) gave the dihydrofuran lactone (10mg) (85) .

Factions eluted with chloroform gave pebrolide (30 mg) (86) after trituration of the mixture with light - petroleum.

Fractions eluted with chloroform - methanol (20:1) contained desacetylpebrolide (45 mg) (88) . Some difficulty was met in the isolation of this compound due to the presence in the earlier fractions of a gelatinous compound which interfered in the crystallization of desacetylpebrolide. This gelatinous substance which was slightly less polar than desacetylpebrolide, could not be obtained in crystalline form even though chromatographically pure has not been characterised as yet.

Isolation of 2,4 dihydroxy - 6 pyruvylbenzoic acid (47).

The broth extracts (6g) from two 15 day cultures of P. brevicompactum were combined and partitioned between chloroform and water. The water was removed and the resultant solid (4g) placed on a column of Mallinckrodt silicic acid which had been thoroughly washed with the

solvent system benzene (90) - dioxane (45) - acetic acid (4). The fourth column volume of solvent eluted 2,4 dihydroxy - 6 pyruvylbenzoic acid (90mg), identical with authentic sample (RF and I.R. spectrum.)

3,5 - Dihydroxy -α- (α' - hydroxyethyl) phthalide (48).

The broth extracts (6g) from two 15 days cultures of P. brevicompactum were combined and partitioned between chloroform and water. The water was removed and the resultant solid (4g) placed on a column of Mallinckrodt silicic acid which had been thoroughly washed with the solvent system benzene (90) - dioxane (45) - acetic acid (4). The tenth column volume of solvent eluted 3,5 - dihydroxy -α- (α' - hydroxy ethyl) phthalide (335 mg) which was further purified by P.L.C. (pre-washed with methanol) using methanol 30% - chloroform (70%) as developing solvent. The product was removed from the silica by elution with methanol as a gummy solid. ν max (KCl) 1725, 1611, 1475, 1160 cm^{-1} λ max (CH_3OH) (mμ), ph7: 219 (O.D. 141), 258 (O.D. 0.81), 293(O.D. 0.25) λ max (CH_3OH) (mμ), ph 10: 234 (O.D. 1.11), 285 (O.D. 0.89), 3160 (O.D. 0.51). Mass spectrum : Ions at m/e 210 (20%) 166 (100%) 165 (70%) 137 (80%). m* corresponding to 210 - 166, 165 - 137. τ CF_3COOH (60 Mc/s) 8.55 and 8.36 (0.7 and 0.3 H,

d. J = 6 cps - CHOH - CH_3), 5.40 (1H, m, - CHOH) 4.4 and 4.31 (0.3 and 0.7 H, d. J = 3 cps, Ar. CH.O.CO -) 3.20, 3.27 (each IH, S, phenolic ring H).

Synthesis of 3,5 - dihydroxy - α - (α' - hydroxy ethyl) phthalide (48).

2,4 Dihydroxy - 6 pyruvylbenzoic acid (90mg) was dissolved in methanol and Na BH_4 (93 mg) added and stirred overnight. The solution was then acidified, filtered and chromatographed in a methanol pre-washed 20 x 20 cm silicagel plate of 0.7 mm thickness. The plate was eluted with methanol (30%) - chloroform (70%). The major band was removed and extracted with methanol. Removal of the solvent gave 3,5 dihydroxy - α(α' - hydroxy ethyl) phthalide (20mg) as a gummy solid. τ CF_3 COOH (60 M $^c/_s$) 8.55 and 8.36 (0.5 and 0.5H, d. J = 6 cps - CHOH CH_3), 5.40 (IH, m, - CHOH), 4.4 and 4.31 (0.5 and 0.5H, d. J = 3 cps, Ar. CH.O.CO-) 3.20, 3.27 (each 1H, s, phenolic ring H).

The IR spectrum and T.L.C. properties were identical with those of a sample of the phthalide isolated as in the previous section.

Attempted synthesis of mycochromenic acid via ethyl mycophenolate epoxide.

i. Ethyl mycophenolate (134 mg) was dissolved in chloroform (4ml) and m-chloroperbenzoic acid (86mg) added. The reaction was allowed to proceed at room temperature for 7 hours. After this period the solvent was removed, and 4 N NaOH (5ml) added. The reaction was left to stand overnight and then acidified and extracted with chloroform. Removal of the solvent gave a solid which crystallised from chloroform - light petroleum as needles (79mg 64%) m.p. 218°C.

ν max (K Br) 3438, 1763, 1739, 1620, 1199, 1160, 1136, 1075, 1032, 968 cm^{-1}.

ν max ($CH Cl_3$) 3620, 3451, 1768, 1741 cm^{-1}.

λ max (mμ) 215 (346), 250 (860), 304(420).

τ_{CDCl_3} (60 M c/s) 8.50 (3H, s, $-O.CO.CH_3$), 7.9 (3H,s, $Ar - CH_3$), 6.2 (3H,s, $- O CH_3$), 6.1 (2H, m, $Ar . CH_2-$) 4.85 (2H, s, $Ar.CH_2.O.CO-$).

This compound was identical (IR, UV, NMR, m.p.) with an authenic sample of hydroxylactone (55) .

ii. The ethyl ester of mycophenolic acid (56mg) was dissolved in chloroform (3 ml) and m-chloroperbenzoic acid (33 mg) was added. The solution was left to stand overnight. Removal of the solvent then gave a

solid which was dissolved in benzene and sodium ethoxide added. The mixture was left to stand for 24 hours. Removal of the solvent now gave a solid which was dissolved in water and neutralized with dilute HCl. The product was extracted with ethyl acetate Evaporation of the solvent gave the lactone (55) . This compound was identical (IR, UV, T.L.C.) with an authentic sample of the hydroxylactone (55) .

Ethyl mycophenolate (51)

Mycophenolic acid (506 mg) in ethanol and 3 drops of concentrated H_2SO_4 were refluxed for 8 hours, the solvent removed and the remaining oil dissolved in chloroform and washed with sodium bicarbonate. Removal of the chloroform gave ethyl mycophenolate (530 mg) which crystallised from chloroform - light petroleum as needles m.p. 88 - 90°C.

ν max (KCl) 3420, 1736, 1624, 1167 cm^{-1}.

ν max ($CHCl_3$) 1737 (ε 1349) cm^{-1}.

τ_{CDCl_3} (60 M c/s) 8.81 (3H,t, J = 7cps - CH_3), 820 (3H, vinyl methyl) 7.88 (3H, s, Ar.CH_3), 7.67 (4H, s, - CO.CH_2.CH_2.C =), 6.61 (2H,d,

Ar.CH_2-), 6.25 (3H, s, - $COCH_3$), 5.91 (2H, , 5 = 7 cps. - CH_2.O.CO -), 4.80 (2H, s, - Ar. CH_2.O.CO - and 1H, diffuse t, = CH -), 2.3 (1H, s, Ar. OH).

This compound was identical (IR, NMR, mixed m.p.).

Mycophenolic acid (44) (69 mg) was refluxed in xylene (25ml) with 10% Pd. charcoal (85mg) for 13 hours. A small amount of mycochromenic acid (10%) was detected then by T.L.C. with an authentic sample of ethyl mycophenolate.

Ethyl mycochromenate (Ethyl ester, of 56).

Ethyl mycophenolate (51) (350mg) was dissolved in xylene and 2,3-dichloro - 5,6 - dicyano - 1,4 - benzoquinone (319 mg) added and the mixture was refluxed for 24 hours. The solution was filtered and the oil recovered after removal of the solvent, was purified by P.L.C. on two 20 x 20 cm. Kieselgel plates with chloroform as eluting solvent. Ethyl mycochromenate was obtained as an oil (138 mg: 40%) b.p. 140°C at 0.02 mm.

(Found; C, 65.79, H, 6.53, M^+ at $^m/_e$ 346. $C_{19}H_{22}O_6$ requires C, 65.88, H, 6.40, M.W. 346).

νmax (film) 1765, 1733, 1635, 1604, 1596 cm^{-1},

νmax (C Cl_4) 1782, 1740 cm^{-1},

λmax (mμ) 240 (496), 321 (= 1045), 332 (= 1045),

τ_{CDCl_3} (60Mc/s) 8.80 (3H, t, J = 7 cps $-CH_3$),

8.52 (3H, s, $-CH_3$), 7.9 (3H, s, $Ar.CH_3$),

6.22 (3H, s, $-OCH_3$), 5.9 (3H, 9, J = 7cps -

CU_2-), 4.9 (2H, s, $Ar.CH_2.O.CO-$) ,

4.35 (1H, d, J = 10 cps = CH. C. O -),

3.32 (1H, d, J = 10 cps Ar. CH =).

Mass spectrum : Ions at $^m/_e$ 346 (30%), 301 (20%),

259 (80%), 257 (60%), 245 (100%), 230 (60%). 221 (20%)

201 (40%).

m* corresponding to 346 → 245, 245 → 230, 230 → 201.

Mycochromenic Acid (56)

To ethyl mycochromenate (5 mg) in methanol a methanolic solution of potassium hydroxide was added and left to stand for 30 minutes. After acidification with dilute HCl, the solvent was removed and the remaining solid dissolved in chloroform and extracted with a saturated aqueous solution of sodium bicarbonate. The alkaline solution was acidified and extracted with chloroform. Removal of the solvent gave a solid which crystallised from chloroform - light petroleum as prisms m.p. 164 - 165°. Identity with an authentic sample of mycochromenic acid was established by T.L.C.,

mixed melting point and comparison of I.R. spectra.

Ethyl dihydromycochromenate (Ethyl ester of 69).

Mycochromenic ethyl ester (5mg) was dissolved in ethanol (10ml) and hydrogenated at room temperature and atmospheric pressure for three hours with 5% Pd. charcoal as catalyst. Filtration of the solution and removal of the solvent yielded ethyl dihydromycochromenate as an oil. This compound was shown to be identical with a sample obtained as described below, by comparison of T.L.C. and I.R. spectra.

Dihydromycochromenic acid (69) .

Mycophenolic acid (149 mg) was dissolved in acetic acid (10ml),5 drops of concentrated H_2SO_4 added and the solution refluxed for one hour. The solvent was removed and the remaining oil dissolved in chloroform and washed with water. Removal of the chloroform gave the acid (69) which crystallised from chloroform - light petroleum as needles (132mg,88%) m.p. 172-175°C,

(Found: C, 63.77; H, 6.19; M^+ at $^m/_e$ 320,

$C_{17}H_{20}O_6$ requires C, 63,74; H, 6.29;m.w 320).

ν max (nujol) 3250, 1725, 1705, 1600 cm^{-1},

λ max (mμ) 250 (ε = 3200), 308 (ε = 1920),

τ_{CDCl_3} (60 M $^{c}/s$) 8.64 (3H, s, CH_3), 8.1 (4H, m, $-CH_2.C.O-$), 7.89 (3H, s, Ar. CH_3), 7.40 (4H, m, Ar.CH_2. and $CH_2.CO.O-$), 6.19 (3H, s, $-OCH_3$), 4.9 (2H, s , Ar - $CH_2.O.CO-$).

Mass spectrum: Ions at $^{m}/e$ 320 (50%), 302 (40%), 247 (70%), 207 (100%), 159 (60%).

m * corresponding to 320 → 302, → 302 → 207.

Ethyl dihydromycochromenate (Ethyl ester of 69).

Dihydromycochromenic acid (^{69}) (12mg) was dissolved in ethanol (10ml) and three drops of conc. $H_2 SO_4$ added. The mixture was refluxed for three hours, the solvent removed and the remaining oil dissolved in ether and washed with sodium bicarbonate. Removal of the ether yielded an oil.

ν max (nujol)1760, 1720, 1605, 1320, 1130, 1105, 1040, 800 cm^{-1}.

τ_{CDCl_3} (60 Mc $^{c}/s$) 8.75 (3H, t, J = 8 cps - CH_3), 8.64 (3H, s, $CH_3.C.O-$), 8.1 (4H, m, $CH_2.C.O-$),

7.88 (3H, s, Ar. CH_3), 7.4 (4H, m, Ar. CH_3 and CH_2.CO.O -), 6.2 (3H, s, -O.CH_3), 5.90 (2H, 9, J = 8 cps, - O.CH_2-), 4.9 (2H, s, Ar. CH_2.O.CO -).

Mycofuranolide (71).

This substance was present in the fractions eluted with petroleum - benzene (4:1) as described earlier and crystallised from chloroform - light petroleum as needles m.p. 174 - 176°C.

R_f 0.75 in 10% methanol - chloroform,

(Found; C, 66.87, H, 4.63, M^+ at $^m/_e$ 218),

$C_{12}H_{10}O_4$ requires C, 66.05, H, 4.62, M.W. 218),

λ max (mμ) 277 (ϵ = 5450), 226 (ϵ = 10.000),

ν max (KBr) 1770, 1649, 1608, 1470, 1390, 1250 cm^{-1},

ν max (CCl_4) 1780, (γ lactone ϵ = 1000),

$CDCl_3$ 7.84 (3H, s, Ar. CH_3), 5.82 (3H, s, -OCH_3), 4.76 (2H, s, Ar. - CH_2 - O - CO -), 2.95 (CH J = 2 cps - H of furan), 2.3 (IH, d, J = 2 cps - H of furan).

Mass spectrum : Ions at $^m/_e$ 218 (60%), 203 (30%), 189 (100%), 175 (30%).

m^* corresponding to 218 → 189.

O-Acetylmycophenolic acid.

To mycophenolic acid (657mg) in pyridine (10ml), acetic anhydride (2ml) was added and the mixture was left to stand overnight at room temperature. To the solution ice-water was added and removal of the solvent then gave a white solid which crystallised from chloroform - light petroleum as needles (647 mg, 90%) m.p. 156 - 158°C.

ν max (KBr) 1768, 1727, 1624, 1612, 1196, 1188, 1210, 1133, 1070 cm^{-1},

λ max (mμ) (CH_3OH) 2150 (2.900), 246 (1.040), 279 (= 190), 280 (= 190),

τ $CDCl_3$ (60 Mc/s) 8.17 (3H, s, $-C{:}C.CH_3$), 7.75 (3H, s, $Ar.CH_3$), 7.58 (3H, s, $-O.CO.CH_3$ 4H, s, $-CH_2.CH_2.CO.O$), 6.59 (2H, d J - 6.6 cps $Ar.CH_2-$), 6.16 (3H, s, $-O.CH_3$), 4.80 (1H, diffuse t - $CH{:}C-$), 4.80 (2H, s, $Ar.CH_2.O.CO-$).

The arylacetaldehyde (81).

O-Acetyl mycophenolic acid (200mg) was dissolved in chloroform and ozonised for three hours at - 15°C. To the solution, a suspension of zinc in acetic acid was added and the mixture stirred for one hour.

The solution was filtered and the solvent removed. The remaining oil was dissolved in chloroform and washed with a saturated aqueous solution of sodium bicarbonate. The chloroform solution was reduced to a small volume and the product isolated by means of P.L.C. in silicagel. The band with a positive reaction with 2,4 - dinitrophenylhydrazine was extracted with chloroform. Removal of the solvent gave the acetaldehyde (81) which crystallised from ether as rods (47 mg, 26%) m.p. 112-115°C.

(Found: C, 60.44; H, 504; M^+ at $^m/_e$ 278

$C_{14}H_{14}O_6$ requires C, 60.43; H, 5.07; M.W.278)

ν max (mujol) 1750, 1705, 1610, 1605, 1180 cm^{-1},

λ max (mμ) 210 (O.D.I.6), 246 (O.D.O.5) 285 (O.D.O.2),

τ_{CDCl_3} (60 M^c/s) 7.78 (3H, s, Ar.CH_3), 7.65 (3H, s, -O.CO.CH_3), 6.3 (2H, m, Ar-CH_2 . CO-) 6.24 (3H, s, - OCH_3), 4.83 (2H, s, Ar.CH_2.O.CO-), 0.34 (IH, t, J = 1cps - CHO).

Mass spectrum : Ions at $^m/_e$ 278 (5%), 236 (10%), 218 (30%), 208 (100%), 207 (60%), 190(50%), 179 (10%), 159 (60%).

m* corresponding to 278 → 218, 236 → 208, 208 → 190.

Synthesis of mycofuranolids.

To the aldehyde (81) (28mg) in benzene (5ml) p - toluene sulphonic acid was added (30mg) and the mixture refluxed for three hours. Filtration of the solution and removal of the solvent gave a solid which was dissolved in ether and washed with water. The solution was then reduced to small volume and chromatographed in a 10 x 20 cm silicagel plate using chloroform as eluent. The band corresponding to the desired product was removed and extracted with chloroform. Removal of the solvent gave the furan (82) which crystallised from chloroform - light petroleum as needles (15mg 68%) m.p. 174 - 176°C.

ν max (KBr) 1770, 1649, 1608, 1470, 1390, 1250 cm^{-1},

τ $CDCl_3$ 7.84 (3H, s, Ar. CH_3), 5.82 (3H, s, - OCH_3O, 4.76 (2H, s, Ar.CH_2.O.CO-), 2.95 (IH J = 2cps β - H of furan), 2.3 (IH, d, J = 2 cps - H of furan).

This compound was identical (IR, NMR, m.p. with an authentic sample of mycofuranolide.

Lactonisation of mycophenolic acid.

i. Mycophenolic acid (96 mg) was dissolved in

trifluoroacetic acid (10ml) and allowed to stand for two hours at room temperature. After removal of the solvent, the resulting solid was dissolved in chloroform and washed with a saturated aqueous solution of sodium bicarbonate. After drying and evaporating the chloroform, the lactone (66) was obtained. Crystallisation from chloroform - light petroleum gave colorless needles (66 mg, 68%) m.p. 161 - 162°C.

(Found; C, 63.79, H, 6.7, M+ at $^{m}/e$ 320,

$C_{17}H_{20}O_6$ requires C, 63.74, H, 6.29, M.W.320),

λ max (mμ) 215 (14.400), 250 (= 4.000), 304 (= 2.400),

ν max (KBr) 1770, 1735, 1630, 1465, 1292, 1140 cm^{-1},

τ_{CDCl_3} (60 M $^{c}/s$), 8.5 (3H, s, -O. C.CH_3), 8(4H, m, -CH_2.C.O -), 7.87 (3H, s, Ar.CH_3), 7.3 (4H, m, -Ar.CH_2-, -CH_2-CO.O), 6.2 (3H, s, - O.CH_3), 4.8 (2H, s, Ar. CH_2.O.CO-), 2.3 (IH, s, Ar. OH),

Mass spectrum: Ions at $^{m}/e$ 320 (30%), 302 (28%), 247 (50%), 207 (100%), 159 (40%).

m^{*} corresponding to 320 → 302, 302 → 207.

ii. To mycophenolic acid (49mg) in benzene (20ml) p-toluenesulphonic acid (50mg) was added and the mixture was refluxed for three hours. After filtering the solution, evaporation of the solvent gave a solid which

was dissolved in chloroform and washed with a saturated aqueous solution of sodium bicarbonate and then water. Removal of the solvent gave the lactone (66) which crystallised from chloroform - light petroleum as colorless needles (30mg) m.p. 161 - 162°C. A mixed melting point with the compound obtained by the action of trifluoracetic acid on mycophenolic acid showed no depression.

Pebrolide (86).

This substance was isolated as described earlier. It crystallised from light petroleum - chloroform as colorless needles m.p. 167 - 170°C.

$(\alpha)_D = -41°$. R_f 0.65 in 10% methanol in chloroform,

(Found ; C, 66.63, H, 6.95, M^+ at $^m/_e$ 430,

$C_{24}H_{30}O_7$ requires C, 66.97, H, 6.97, M.W. 430)

R.D: $(\Phi)_{244}$ - 1340, $(\Phi)_{258}$ (trough) - 3950, $(\Phi)_{284}$ - 1220 $(\Phi)_{333}$ -465, $(\Phi)_{400}$ -60.

λ max 230 mμ (ε = 9,729),

ν max (KCl) 3500, 1764, 1710, 1597, 1580, 1243, 711 cm^{-1}.

ν max ($CHCl_3$) 3605, 1780 (lactone; ε = 1108), 1740 (acetate; ε = 621), 1715 (Benzoate; ε = 907) cm^{-1},

τ_{CDCl_3} 9.04, 8.56each (3H, s, CH_3), 7.96 (3H, s,

$-O.CO.CH_3$), 6.70 (IH, m, H.C.OH), 6.21, 6.03 (2H, AB J = 12 cps. - $CH_2O.Ac$), 5.74 (IH, dd, J_1 = 10 cps, J_2 = 5.5 cps - $CH_2.O.CO$), 5.02 (IH, d, J = 10cps - CH_2.).C)-), 432 (IH,m, H.C.OB_z), 2.50 and 1.96 (3H and 2H, m, Ph . CO.O -).

Mass spectrum: Ions at m/e 430 (0.1%), 357 (5%), 325 (10%), 308 (30%), 266 (40%), 265 (70%), 248 (20%), 247 (18%), 235 (40%), 230 (30%), 217 (50%), 105 (100%). m^* corresponding to 325 → 265, 308 → 290, 266 → 248, 265 → 247, 248 → 230, 235 → 217.

Desacetylpebrolide (88)

This substance was isolated as described earlier. It crystallised from chloroform - light petroleum as colourless needles m.p. 252 - 255°C,

$(\alpha)_D$ = - 25°, R_f 0.28 in 10% methanol in chloroform.

(Found; C, 68, 41, H, 7.08, M^+ at m/e 388, $C_{22}H_{28}O_6$ requires C, 68.02, H, 7.27.M.W. 388).

R.D.: $(\Phi)_{248}$ - 455, $(\Phi)_{259}$ (trough) - 3020, $(\Phi)_{285}$ -650, $(\Phi)_{333}$ - 420, $(\Phi)_{400}$ - 260

λmax 230 mμ (ε = 11.860)

νmax (K.Br) 3420, 1756, 1713, 1602, 1582, 711 cm^{-1}.

νmax ($CHCl_3$) 1771 (γ lactone; ε = 539), 1711 (Benzoate; ε = 472) cm^{-1}.

$\tau_{C_5H_5N}$ 9.0, 8.20 (each 3H, s, CH_3), 6.46 (IH,m, H.C.OH), 6.74, 6.16 (2H, AB, J = 11 cps, - CH_2.O.H), 5.74 (1H,dd J, = 10 cps J_2 = 6 cps - CH.O.CO-), 4.70 (IH, d, J = 10cps - CH_2.O.CO-), 3.7 (IH, m, H.C.OBz),

Mass spectrum: Ions at m/e 388 (0.1%), 358 (1%), 357 (1%), 283 (25%), 266 (15%), 248 (3%), 236 (20%), 218 (12%), 192 (10%), 105 (100%),

m^* corresponding to 388 → 299, 388 → 266, 358 → 304, 266 → 235, 236 → 218.

Deoxypebrolide (87) .

This compound was isolated as described earlier. It crystallised from ether as colourless needles m.p. 171 - 173°C.

(Found: C, 69.58, H, 7.28, M^+ at m/e 414,

$C_{24}H_{30}O_6$ requires C, 69.56, H, 7.24, M.W. 414),

R_f 0.87 in 10% methanol in chloroform.

R.D.: $(\Phi)_{244}$ - 220, $(\Phi)_{260}$ (trough) - 460, $(\Phi)_{284}$ -1410, $(\Phi)_{400}$ -110.

νmax (KBr) 1775, 1738,1710, 1605, 1588, 1249, 711 cm^{-1}.

νmax ($CHCl_3$) 1783 (γ- lactone, ε = 784) 1735 (acetate; ε = 603) 1717 (Benzoate; ε = 733),

τ_{CDCl_3} 9.04, 8.56 (each 3H, s, CH_3), 7.96 (3H, s, - O.CO.CH_3), 6.23, 6.03 (2H, ABq J = 12 cps - CH_2.OAc), 5.82 (1H, dd, J_1 = 10, J_2 = 6, - CH_2.O.CO -), 5.62 (1H, d, J = 10cps - CH_2.O.CO -), 4.30 (1H, m, H.C.OB_z), 2.50 and 2.00 (3H and 2H, m, Ph.CO.O -).

Mass spectrum : Ions at $^m/_e$ 414 (0,1%), 292 (5%), 258 (30%), 249 (30%), 232 (10%), 219 (10%), 167 (100%), 105 (50%),

m^* corresponding to 292 → 219.

Isodesacetylpebrolide (101) .

i. Desacetyl pebrolide (88) (19mg) was allowed to stand with a methanolic solution of potassium hydroxide for three hours and then neutralised with dilute hydrochloric acid. The product was extracted with chloroform, washed with water and dried. Evaporation gave isodesacetylpebrolide (101) which crystallised from ether as plates (17 mg 89%) m.p. 192 - 193°C.

(Found: C, 68, 31, H, 7.36, M^+ at $^m/_e$ 388,

$C_{22}H_{28}O_6$ requires C, 68.02, H, 7.27, M.W.388,

ν max (KCl) 3460, 1760, 1718, 1607, 1590, 717 cm^{-1},

ν max ($CHCl_3$) 1778 (lactone; ϵ = 710) 1719 (Benzoate; ϵ = 609),

τ_{CDCl_3} 9.06, 8.55 (each 3H, s, CH_3), 6.58 (1H, m, H.C.OH), 6.37, 6.83 (2H, AB q J = 12 cps- CH_2OH), 5.8- (1H, dd, J_1 = 8 cps, J_2 = 11 cps, $-CH_2.O.CO-$), 5.45 (1H, dd, J_1 = 8 cps, J_2 = 7 cps, $-CH_2.O.CO-$), 4.18 (1H, m, $H.C.OBz$), 2.43 and 1.90 (3H and 2H m, Ph.CO.O-).

Mass spectrum: Ions at m/e 388 (0.1%), 358 (1%), 357 (1%), 283 (25%), 266 (15%). 248 (3%). 236 (20%), 218 (12%), 192 (10%), 105 (100%),

m^* corresponding to 388 → 299. 388 → 266, 358 → 204, 266 → 235, 236 → 218.

ii Pebrolide was treated under the same conditions and gave a compound identical with the product obtained in i. This was established by comparison of R_f, NMR and IR spectra.

Desacetylpebrolide from pebrolide.

To a solution of pebrolide (86) (18mg) in acetone , 6N sulphuric acid (2ml) was added and the mixture left to stand overnight. The solution was extracted with chloroform. The chloroform solution was washed with water, dried and evaporated to give a solid which crystallised from chloroform - light petroleum as needles (12mg.74%).

Identity with desacetylpebrolide was established by R_f mixed m.p., IR and NMR spectra.

O-Acetyl pebrolide (92)

i. From pebrolide.

To a solution of pebrolide (86) (61mg) in pyridine (5ml), acetic anhydride (0.5ml) was added and the mixture left to stand at room temperature for 12 hours. The reaction mixture was poured into ice-water and the product extracted with ether. The ethereal solution was washed with water, dried and evaporated to give o-acetyl pebrolide (92) which crystallised from ether as needles (44mg 66%) m.p. 178 - 180°C.

(Found: C,65.82, H, 6.92, M^+ at $^m/_e$ 472. $C_{26}H_{32}O_8$ requires C, 66.10, H, 6.78, M.W. 472).

ν_{max} (KCl) 1781, 1739, 1709, 1599, 1580, 1247, 711 cm^{-1}.

τ_{CDCl_3} 9.0, 8.44 each (3H, s, CH_3), 7.92, 7.89 (each 3H,s,-O.CO.CH_3), 5.99, 6.19 (2H, AB q J = 12 cps - CH_2.O.CO-), 5.36 (IH, d, J = 10 cps - CH_2.O.CO - and IH, m, H.C.OAc), 4.28 (IH,m, H.C.OB_z), 2.44 and 1.96 (3H and 2H, m, Ph,CO.O-),

Mass spectrum: Ions at $^m/_e$ 472 (1%), 399 (8%), 367 (7%), 350 (30%), 308 (50%), 307 (48%), 290 (45%), 265 (12%), 247 (25%), 235 (25%), 230 (70%), 227 (90%),

m^{*} corresponding to 367 → 307, 307 → 247, 307 → 265, 235 → 217, 265 → 247.

ii From desacetyl pebrolide.

To a solution of desacetyl pebrolide (88) (7mg) in pyridine (10ml), acetic anhydride (0.5ml) was added and the mixture left to stand overnight. The reaction mixture was poured into ice-water and the product extracted with ether. The ethereal solution was washed with water, dried and evaporated to give a solid which crystallised from ether as needles (5mg 59%) m.p. 178 - 180°C. This was shown to be identical with o-acetylpebrolide by comparison of R_f, mixed m.p. and IR spectra.

Desacetylpebrolide (3.6mg) in acetic acid (5ml) was refluxed for two hours. T.L.C. analysis of the reactions products showed the presence of two compounds corresponding to pebrolide and o-acetyl pebrolide in similar amounts.

Pebrolide (3.2mg) in acetic acid (5ml) was refluxed for two hours. T.L.C. analysis of the reaction products shows the presence of starting material and a compound corresponding to o-acetylpebrolide. Similarly

cholesterol and lanosterol were only partially converted to their acetates.

Pebrolideketone (91) .

Pebrolide (86) (106mg) was dissolved in acetone and treated with a slight excess of chromium trioxide in sulphuric acid (Jones reagent) at room temperature for one minute. After addition of ice-water, the mixture was extracted with chloroform. The extract was washed with brine, dried and evaporated to give the ketone which crystallised from ether as needles (94mg 89%), m.p. 187 - 190°.

(Found; C, 67.02, H, 6.62, M^+ at m/e 428, $C_{24}H_{28}O_7$ requires C, 67.29, H, 6.54, M.W.428).

R.D.: $(\Phi)_{258}$ (trough) - 404, $(\Phi)_{296}$ 0, $(\Phi)_{315}$ (peak) + 430, $(\Phi)_{321}$ 0, $(\Phi)_{400}$ -215.

νmax (KCl) 1787, 1732, 1711, 1598, 1582, 711 cm^{-1}.

νmax (CCl_3) 1778 (γ lactone; ε = 676), 1736 (acetate; ε = 517), 1710 (ketone, benzoate; ε = 845).

τ_{CDCl_3} 8.85, 8.35 (each 3H, s, CH_3), 7.91 (3H, s, $CH_3.CO.O-$), 6.10, 5.92 (2H, AB q J = 11 cps - $CH_2.OAc$), 5.62 (IH, dd, J_1 = 10 cps, J_2 = 5 cps - $CH_2.O.CO-$), 5.40 (IH, d, J = 10 cps - $CH_2.O.CO$ -), 4.35 (IH,m, H - C.OBz), 2.65 and 2.15 (3H and 2H, m, Ph, CO.O-).

Mass spectrum: Ions at $^{m}/e$ 428 (0.1%), 323 (10%), 306 (20%), 263 (30%), 246 (10%), 233 (8%), 223 (9%), 197 (15%), 105 (100%).

M^{*} corresponding to 428 → 323, 323 → 263.

Hydrogenation of Pebrolide Ketone - Hexahydropebrolide.

Pebrolide ketone (91) (12 mg) was dissolved in ethanol (10ml) and hydrogenated at room temperature and atmospheric pressure for three hours with 5% Pd-charcoal as catalyst. Filtration of the solution through glass paper and removal of the solvent gave unchanged pebrolide ketone.

Pebrolide ketone (91) (30mg) was dissolved in acetic acid (10ml) and hydrogenated at room temperature and atmospheric pressure for three hours with PtO_2 as catalyst. After filtration of the solution, removal of the solvent gave a solid which crystallised from ether as plates (10mg) m.p. 165 - 167°C.

This compound proved to be identical with hexahydropebrolide by comparison of R_f mixed m.p. and IR spectra.

Hexahydropebrolide.

Pebrolide (86) (80mg) was dissolved in glacial acetic

acid (10ml) and hydrogenated at room temperature and atmospheric pressure for four hours with platinum oxide (50mg) as catalyst. Filtration of the solution through glass paper and removal of the solvent gave hexahydropebrolide which crystallised from ether as plates (72mg, 89%) m.p. 165 - 167°C.

(Found; C,65 .87, H, 8.44, M^+ at $^m/_e$ 436).

$C_{24}H_{36}O_7$ requires C, 66.03, H, 8.31, M.W. = 436).

ν max (KBr) 3500, 1760, 1730, 1260 cm^{-1},

ν max ($CHCl_3$) 1780 (lactone; ε = 667), 1732 (Acetate, cyclohexanecarboxylate; ε = 764),

τ_{CDCl_3} 8.99, 8.67 (each 3H, s, $-CH_3$), 6.7 (IH, m, H.C.OH), 6.1, 6.22 (2H, AB q J, AB = 12 cps, - CH.O Ac), 5.76 (IH, dd, J_1 = 10, J_2 = 6 cps - CH_2.O.CO -), 5.06 (IH, d, J =10 cps, - CH_2.O.CO -), 4.65 (IH, m, H.C.O.CO. C_6H_{11}),

Mass spectrum : Ions at $^m/_e$ 436 (1%), 325 (20%), 309 (40%), 308 (45%), 265 (100%), 249 (45%). 235 (30%), m^* corresponding to 309 → 249, 308 → 235.

Hexahydrodesacetylpebrolide.

Desacetylpebrolide (88) (160mg) was dissolved in glacial acetic acid (14ml) and hydrogenated at room temperature and atmospheric pressure for 20 hours with platinum oxide (144mg) as catalyst. Filtration of the solvent

through glass paper and removal of the solvent gave hexahydrodesacetylpebrolide which crystallised from ether as plates (152 mg, 92%) m.p. 210 - 215°.

(Found; C, 67.16, H, 8.82 M^+ at $^m/_e$ 394,

$C_{22}H_{34}O_6$ requires C, 66.98, H, 8.69, M.W.394).

νmax (KBr) 1740, 1715, 1445 cm^{-1},

νmax ($CHCl_3$) 1772 (lactone; ϵ = 997), 1717 (cyclohexanecarboxylate ϵ = 621) cm^{-1}.

τ_{CDCl_3} 9.1 8.65 (each 3H, s, $-CH_3$), 6.70 (IH, m, -H.C.OH), 6.86, 6.42 (2H, AB, J = 11 cps $-CH_2OH$) 5.73 (IH, dd, J = 10 cps J_2 = 6 cps, $-CH_2.O.CO-$), 5.05 (IH, d, J = 10 cps $-CH_2.O.CO-$), 4.50 (IH, m, $H.C.OB_z$).

Mass spectrum: Ions at $^m/_e$ 394 (1%), 363 (3%), 283 (100%), 267 (80%), 266 (85%). 249 (30%), 248 (30%), 237 (50%), 236 (75%), 235 (70%), 218 (50%), 217 (75%).

m* corresponding to 267 → 249, 266 → 248, 266 → 235.

Ketoacid (93)

Desacetyl pebrolide (88)(166mg) was dissolved in acetone and treated with a slight excess of chromium trioxide in sulphuric acid at room temperature for five minutes. After addition of ice - water, the mixture was

extracted with chloroform. The extract was washed with brine, dried and evaporated to give the keto acid (93) which crystallised from aqueous methanol as needles (166mg, 97%), m.p. 123 - 125°C.

νmax (KBr) 1786, 1755, 1717, 1606, 1582, 723 cm^{-1},

νmax ($CHCl_3$) 1784 (γ lactone ε = 800), 1718 (Ketone acid, benzoate; ε = 1150) cm^{-1}.

Keto ester (94)

The keto acid (93) (33mg) was dissolved in methanol and treated with an ethereal solution of diazomethane. After one hour the solution was filtered. Removal of the solvent gave the keto ester which crystallised from chloroform - light petroleum as needles (29mg, 82%) m.p. 227- 232°C.

(Found; C, 66.66, H, 6.45, M^+ at $^m/_e$, 414 $C_{23}H_{26}O_7$ requires C, 66.66, H, 6.28, M.W. 414).

νmax (KBr) 1779, 1731, 1712, 1604, 1585, 723 cm^{-1},

νmax ($CHCl_3$) 1785, 1722 cm^{-1},

τ_{CDCl_3} 8.52, 8.32 (each 3H, s, CH_3), 6.23 (3H, s, CH_3.O.CO), 5.60 (1H, dd, J_1 = 10 cps, J_2 = 5 cps - CH_2.O.CO-), 5.32 (1H, d, J = 10 cps, - CH_2.O.CO-), 4.46 (1H, m, H.C.OB_z), 2.44 and 1.96 (3H and 2H, m, Ph.O.CO-),

Mass spectrum: Ions at $^m/_e$ 414 (1%), 383 (1%),

355(1%), 309 (48%), 292 (50%), 277 (30%), 260 (5%), 259 (15%), 249 (70%), 233 (40%), 232 (45%), 231 (48%), 209 (60%),

m^* corresponding to 414 → 309, 309 → 277, 277 → 249

Hydrolysis of deoxypebrolide

1 - Deoxypebrolide (87) (34mg) was dissolved in methanol and 5N aqueous sodium hydroxide added (5ml). The solution was refluxed for three hours and then neutralised with dilute hydrochloric acid. Extraction with chloroform gave the dehydroxytranslactone (95) which crystallised from chloroform - light petroleum as needles (16 mg, 73%), m.p. 149 - 154°C.

(Found; C, 66.94, H, 8.80, M^+ at $^m/_e$ 268,

$C_{15}H_{24}O_4$ requires C, 67.14, H, 9.01, M.W.268).

ν_{max} (KBr) 3,500, 1745 cm^{-1},

ν_{max} ($CHCl_3$) 3610 (hydroxyl; ε = 200), 1770 (γ lactone ε = 487) cm^{-1}.

τ_{CDCl_3} 8.87, 8.75 (each 3H, s, CH_3), 6.75, 6.53 (2H, AB q, J = 11cps, - CH_2.OH), 5.91 (1H, dd, J = 8 cps, J_2= 12 cps -CH_2.O.CO-), 5.89 (1H, dd, J_1 = 8cps, J_2= 7 cps, CH_2.O.CO-), 5.56 (1H, m, H.C.OH).

Mass spectrum; $^m/_e$ at 268 (1%), 250 (2%), 237 (30%), 232 (10%), 221 (15%). 220 (17%), 219 (100%), 191 (15%),

173 (20%),

m^* corresponding to 237 - 219, 219 - 201.

The sodium bicarbonate solution was neutralised with dilute hydrochloric acid and extracted with chloroform. The chloroform extract was washed with water, dried and evaporated to give a solid which crystallised from aqueous methanol as needles (11 mg), m.p. 122°C. Identity with benzoic acid was established by R_f, mixed m.p. and IR spectrum.

Ketoalcohol (126)

Pebrolide ketone (125) (69mg) in acetone (25ml) was refluxed with 6N sulphuric acid (5ml) for one hour, cooled and extracted with chloroform. The extract was washed with water, dried and evaporated to give the keto alcohol (126) which crystallised from chloroform - light petroleum as needles (50 mg, 80%), m.p. 208 - 210°C.

(Found; C, 68.22, H, 6.78, M^+ at $^m/_e$386, $C_{22}H_{26}O_6$ requires C, 68.39, H, 6.73, M.W. 386),

ν max (KBr) 3500, 1704, 1596, 1580, 704 cm^{-1},

νmax ($CHCl_3$) 1775 (lactone; ϵ = 526), 1705 (Ketone, benzoate; ϵ = 614),

τ_{CDCl_3} (60 Mg^c/s) 9.0, 8.4 (each 3H, s, CH_3), 6.78, 6.32 (2H, AB q J = 10 cps - $CH_2.OH$),

5.6 (2H, m, - CH_2.O.CO -), 4.3 (1H, m, H.C.OB_z), 2.5 and 2.07 (3H and 2H, m, PhCO.O-).

Mass spectrum: Ions at $^m/_e$ 386 (1%), 281 (10%), 264 (8%), 263 (5%), 234 (11%), 233 (5%), 216 (5%), 192 (5%), 105 (100%).

m^* corresponding to 386 → 264.

Ketohydroxy ester (130)

The keto acid (93) (63mg) was refluxed with 6N aqueous sodium hydroxide (5ml) for four hours and then neutralised with dilute aqueous hydrochloric acid and extracted with ether. The ethereal solution was washed, dried and evaporated to give a solid which was washed with hot ether to remove the benzoic acid present. The remaining solid crystallised from chloroform - light petroleum to give the ketohydroxyacid (129) as needles (30 mg, 63%) m.p. 251 - 254°C.

The ketohydroxyacid (129) (30mg) was dissolved in methanol and treated with an excess of an ethereal solution of diazomethane and left to stand for two hours. After filtration, removal of the solvent gave the ketohydroxyester (130) which crystallised from ether-light petroleum as plates (30mg, 95%) m.p. 175 - 179°C.

(Found; C, 62.04, H, 7.17, M^+ at $^m/_e$ 310 $C_{16}H_{22}O_6$ requires C, 61.92, H, 7.15, M.W. 310).

ν max (KBr) 3550, 1768, 1712, 1692 cm^{-1},

ν max ($CHCl_3$) 1770, (lactone), 1709 (ketone and ester) cm^{-1},

τ (60M $^c/_s$) 8.5, 8.28 (each 3H, s, $-CH_3$), 6.25 (3H, s, $-O.CH_3$), 5.71 (IH, m, H.C.OH), 5.75 (IH, dd, J_1 = 8 cps, J_2 = 12 cps - $CH_2.O.CO-$), 5.19 (IH, dd J_1 = 8 cps, J_2 = 7 cps - $CH_2.O.CO-$).

Mass spectrum: Ions at $^m/_e$ 310 (1%), 295 (1%), 292 (1%), 259 (20%), 251 (100%), 233 (25%), 232 (25%), 205 (20%), 184 50%).

m^* corresponding to 251 → 233, 310 → 259.

Diketoester (131)

The keto-hydroxyester (130) (24mg) was dissolved in acetone (0.5 ml) and treated with a slight excess of chromium trioxide in sulphuric acid at room temperature for two minutes. To the reaction mixture ice-water was added (2ml) and the mixture was extracted with chloroform; the extract was washed with brine, dried and evaporated to give diketone (131) which crystallised from chloroform - light petroleum as needles (20 mg, 80%) m.p. 180 - 185°C.

(Found; C, 61.91, H, 6.49, M^+ at $^m/_e$ 308

$C_{16}H_{20}O_6$ requires C, 62.33, H, 6.54, M.W. 308).

ν max (Nujol) 1770, 1700, 1230, 1000 cm^{-1},

τ_{CDCl_3} (60Mc/s) 8.73, 8.28 (each 3H, s, CH_3), 6.30 (3H, s, - O.CH_3), 5.70 (IH, dd, J_1 = 8 cps, J_2= 12 cps, - CH_2.O.CO-), 5.04 (IH, dd, J_1 = 8 cps, J_2 = 7 cps - CH_2.O.CO -).

Mass spectrum: Ions at $^m/_e$, 308 (10%), 276 (30%), 249 (100%), 248 (30%). 220 (90%), 208 (10%), 203 (10%).

m^* corresponding to 308 → 276, 276 → 248.

O - Bromoacetylpebrolide (98)

Pebrolide (24mg) in benzene (10ml)/pyridine (5 drops) was treated with bromoacetylbromide (1ml). After an hour, a precipitate appeared which was removed by filtration. Removal of the solvent gave an oil which was dissolved in chloroform and washed with aqueous sodium bicarbonate and then water. The removal of the solvent gave O - bromoacetylpebrolide (98), which crystallised from ether as prisms (10 mg, 30%) m.p. 150 - 151°C.

Found: M^+ at m/e 552; $C_{26}H_{31}O_8Br$ requires M.W. 552.

ν max (K.Br) 1780, 1730, 1607, 1590, 1250, 720 cm^{-1},

τ_{CDCl_3} 8.98, 8.4 (3H, s, CH_3), 7.92 (3H, s, - O.CH_3), 6.14 (2H, s, $Br.CH_2.CO.O$ -), 6.17, 6.0 (2H, AB q J = 11 cps - CH_2.OAc), 5.72 (IH, dd, J_1 = 10 cps, J_2 = 5, HC.O.CO -), 5.22 (IH, d, J = 9 cps - CH.O.CO -), 5.0(IH, m, $HC.OCO.CH_2Br$) 4.3 (IH, m, H.C.O.B_z), 2.54, 1.95 (3H and 2H each m, Ph - CO.O -).

Mass spectrum: Ions at m/e 552 (1%), 447 (1%), 445 (1%), 430 (5%), 428 (5%), 387 (25%), 385 (25%). 307 (15%), 290 (12%), 230 (40%), 277 (50%), 105 (100%).

The dihydrofuran lactone (85).

This compound was isolated as described earlier. It crystallised from chloroform light petroleum as needles m.p. 187 - 190°C, R_f 0.7 in 10% methanol chloroform.

max (nujol) 1750, 1620 cm^{-1}.

max ($CHCl_3$) 1795, 1780 cm^{-1}.

($CDCl_3$) 8.6 (3H, s, $-CH_3$), 7.89 (3H, s, $Ar.CH_3$), 6.65 (2H, m, $Ar.CH_2-$), 6.11 (3H, s, $-OCH_3$), 5.09 (1H, m, - CH.O -), 4.95 (2H, s, $Ar.CH_2.O.CO-$).

Radioactive Substrates and their Introduction into Cultures of P. brevicompactum.

Radioactive materials used were 2-^{14}C mevalonic acid (0.05 mc) of specific activity 5.03 mc/mM and 2-^{3}H mevalonic acid (2 mc.) of specific activity 90 mc /mM. These radioactive precursors were dissolved in sterilised water (11 ml.). 10ml. of these solutions were spread evenly over 5 Roux bottles containing 4-day old cultures of P. brevicompactum grown under the usual conditions. These cultures were harvested after a further 48 hr.

Isolation of ^{3}H, ^{14}C pebrolide.

The broth extracts were dissolved in chloroform and washed first with water and then with saturated aqueous sodium bicarbonate. To the dried chloroform solution, inactive pebrolide (187mg.) was added and this was re-isolated by addition of light petroleum and purified by crystallisation. After 3 crystallisations, pebrolide was chemically pure as indicated by TLC. A further 3 crystallisations gave material of constant activity.

^{3}H, ^{14}C Pebrolide ketone.

Crystals and material from the mother liquors from the last 3 crystallisations were combined (79 mg.) and

oxidised to pebrolide ketone under the usual conditions. Yield 68 mg. After crystallisation from ether the ketone was chemically pure as indicated by TLC. A further 3 crystallisations gave material of constant ^{3}H / ^{14}C ratio.

^{3}H, ^{14}C Keto-ester (94).

Pebrolide ketone (57 mg.) from the last 3 crystallisations indicated above was hydrolysed to the corresponding alcohol (43 mg.), which was oxidised and esterified under the usual conditions to give the keto-ester (30 mg.). Crystallisation from chloroform- light petroleum gave pure material as indicated by TLC and the ^{3}H /^{14}C ratio was unchanged by further crystallisation from the same solvent.

Assays for Radioactivity.

Radioactive assays were carried out with a Packard Tri-Carb Liquid Scintillation Spectrometer (Series 3000). Efficiency for counting was 54% for ^{14}C and 30% for ^{3}H. All the samples were counted on the same day to avoid errors due to decay of tritium. All were counted for 100 min.

The samples (ca. 2 mg.) were dissolved in 10 ml. of scintillator solution in a vial. 5 ml. of solution were transferred to a second vial by means of a pippette. Both solutions were diluted to 10 ml. with scintillator solution.

$^{3}H/^{14}C$ ratio of material from the last crystallisations, based on 20 minute counts.

Pebrolide	31.4	27.86	27.4 *
Pebrolide ketone	26.7	26.5	26.1
Keto-ester (94)	12.1	11.7	

* A duplicate experiment has recently been carried out (R.Baxter) involving 8 crystallisations , longer counting times and giving a similar value (28.5)

Counting of Samples

1. Pebrolide.

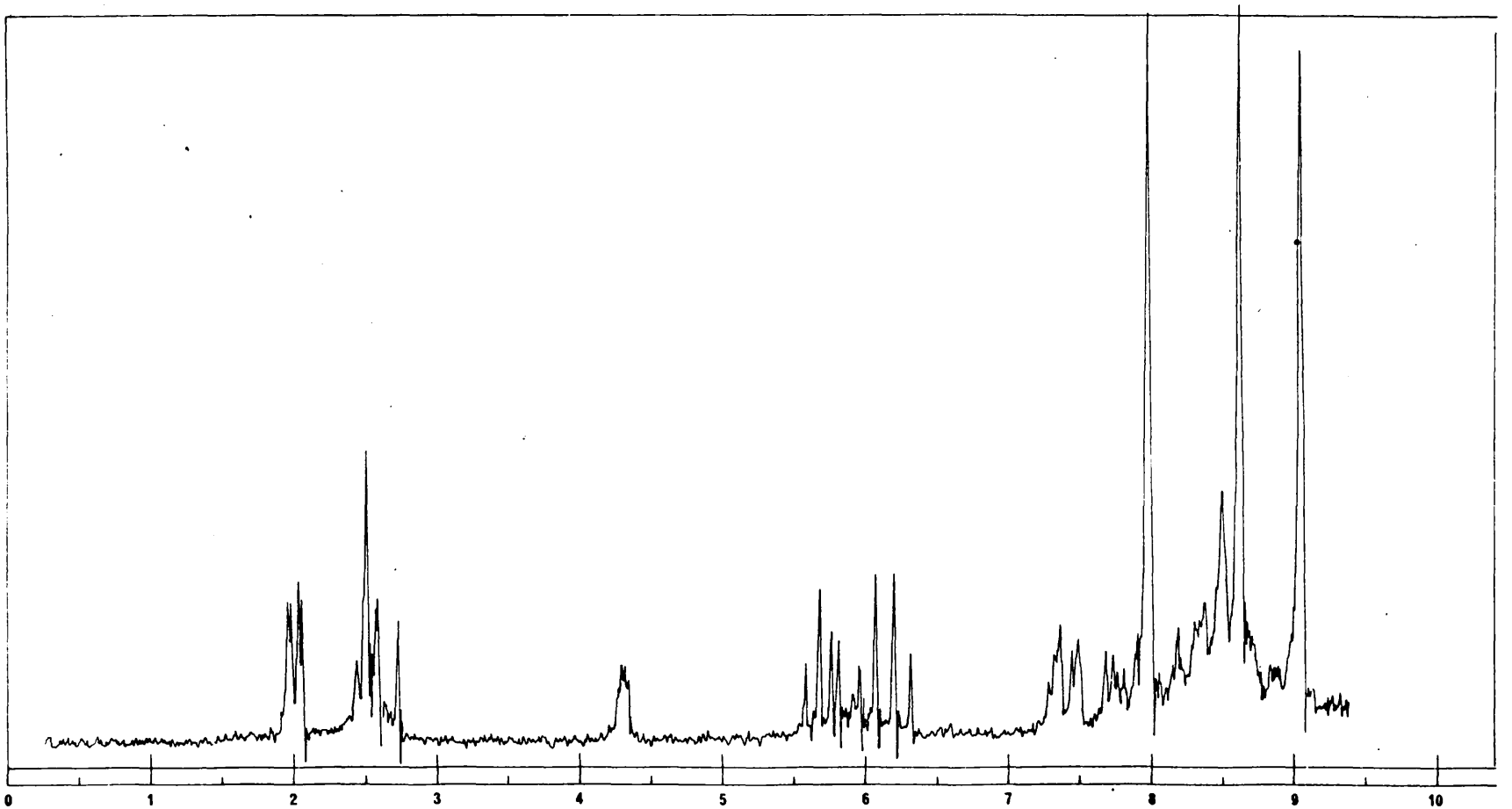
	^{3}H	^{14}C	
	75,547	6,147	
	76,000	6,289	A
	75,047	6,289	
	76,902	6,268	B
	76,745	6,390	
Average	76,048	6,277	
Less background	74,449	4,491	
C.p.m.	744.5	44.91	
D.p.m.	2,481.6	83.16	
^{3}H / ^{14}C ratio	29.86		

2. Pebrolide ketone.

	^{3}H	^{14}C	
	73,367	6,720	
	72,704	6,969	A
	73,249	6,764	
	73,364	7,096	B
	73,119	6,889	
Average	73,161	6,887	
Less background	71,562	5,101	
C.p.m.	715.6	51.01	
D.p.m.	2,385	94.5	
^{3}H / ^{14}C ratio	25.2		

3. Keto-ester (94)

	^{3}H	^{14}C	
	34,033	6,839	
	33,944	6,699	A
	33,821	6,763	
	33,283	6,657	
	33,125	6,491	B
Average	33,641	6,689	
Less background	32,042	4,903	
C.p.m.	320.42	49.03	
D.p.m.	1,067	91.25	
^{3}H / ^{14}C ratio	11.7		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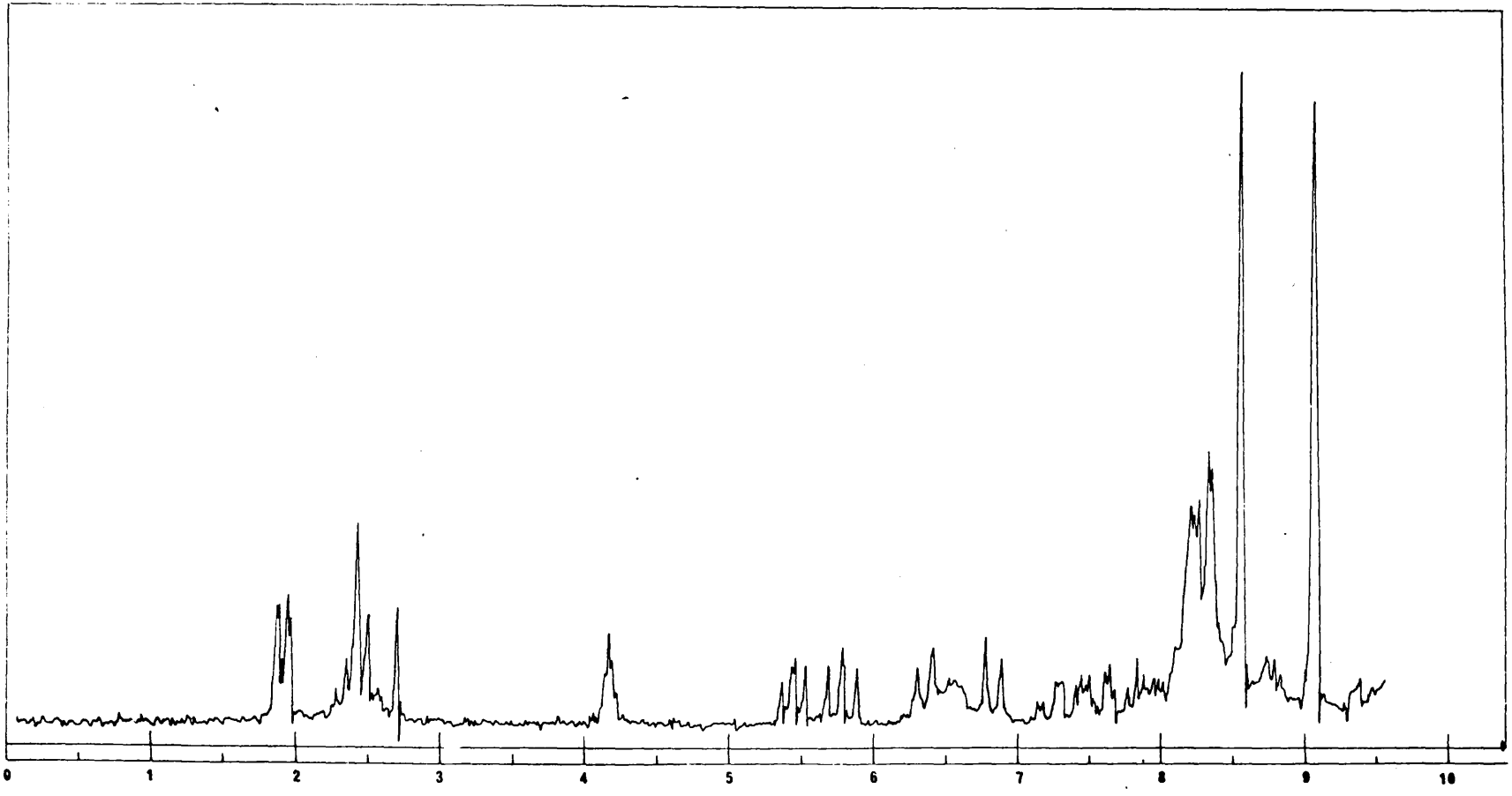

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