ASPECTS

OF

FUNGAL METABOLISM

A THESIS PRESENTED BY

IAIN MALCOLM CAMPBELL

TO THE UNIVERSITY OF GLASGOW FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY.

THE CHEMISTRY DEPARTMENT. SEPTEMBER, 1965.

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

TO MY MOTHER

CONTENTS

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to his supervisor Dr. N. J. McCorkindale for his attention and advice at all stages of the work. He is also indebted to Mr. T. Bryce, B.Sc., for obtaining and aiding with the elucidation of mass spectral data, to Miss J. Brown, Miss M. McKenzie and their staff for technical assistance in the preparation of fungal extracts, to Dr. S. Clarke and Miss J. Alexander for bioassays, to Mr. J. Gall for N.M.R. spectra, to Mrs. F. Lawrie for infrared spectra, to Professor W. Klyne for rotary dispersion data, to Mr. J. Cameron, B.Sc., and his staff for microanalyses, to his typist, Miss I. Paton, and to his many laboratory colleagues.

He also wishes to accord his thanks to the Carnegie Trust for the Universities of Scotland for maintainance during the last three years and to Professor R. A. Raphael for providing the opportunity and facilities for performing the work.

SUMMARY

Sporophores of Daedalia quercina have been shown to contain a mixture of tetracyclic triterpene acids. After esterification, methyl polyporenates B and C were isolated together with six labile esters. The most abundant of these was demonstrated to be an half malonate ester and represents the first recorded isolation of such a compound. It has been called methyl methoxycarbonylacetylquercinate. Of the remaining five esters, three have been shown to be triterpene half malonate esters, one to be the acetate corresponding to methyl methoxycarbonylacetylquercinate and one to be a free hydroxy compound. The triterpene moiety in two of these latter malonate esters would appear to be the first reported examples of C_{32} triterpenes.

The instability of the six esters is almost certainly due to the presence in the side chain of a γ-keto-ester system. Tentative structural proposals are advanced for all the compounds. The structures of the C_{32} terpene esters appear to incorporate epidioxide groupings. The "conjugated" triterpenes have also been detected in a culture of Daedalia quercina grown on a synthetic medium.

Studies with extracts derived from the sporophores of Polyporus betulinus revealed that polyporenic acid A is present mainly in the form of ester, one of which constituted a further example of a triterpene half malonate. The other esters encountered

were acetate, caproate, 3-hydroxy-3-methylglutarate, malonate monomethyl ester and 3-hydroxy-3-methylglutarate monomethyl ester.

Possible functional significance for these triterpene "conjugates" is considered.

From cultures of a species of Penicillium, the known substance, mycophenolic acid has been isolated. The ethyl ester and two oxidation products of this latter substance were obtained and characterised.

Samples of a sesquiterpene benzoate, two quinonoid pigments and a phenolic substance were also obtained from the broth of the organism but no definite structures were established for these metabolites.

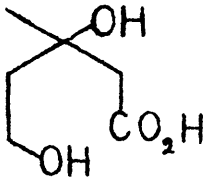
CHAPTER 1

AN INTRODUCTION TO THE STUDY OF FUNGAL TERPENES

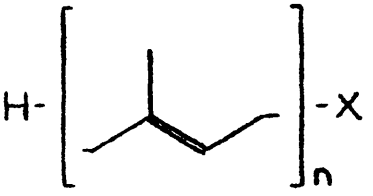
One of the most taxing problems presented by any body of knowledge which is continually increasing in size and scope is that of keeping the laws and definitions which govern the subject in harmony with all current developments. A most striking example of how an early definition was modified and expanded in the light of later findings is to be seen in the history of the term "terpene". Initially the noun was employed by Wallach[1] in 1914 as a generic name for a limited number of C_{10} hydrocarbons which were readily isolated from plant essential oils. This definition was later extended firstly to include the oxygenated derivatives of these C_{10} hydrocarbons (the camphors) then to contain compounds of similar origin possessing carbon contents of fifteen and twenty atoms. Nowadays the term "terpene" is used to describe a class of many thousands of widely distributed naturally occurring compounds whose carbon skeletons can contain from five to forty carbon atoms.

Any modern definition of the word "terpene" must take into account the observations of Bertholet[2], Wallach[1] and Ruzicka[3] that the structural framework of all traditionally accepted terpenoid materials could be nominally derived from linked isoprene units. This, the so-called Isoprene Rule, prompted Haagen-Smit[4] to designate terpenes as " ..... all compounds which have distinct architectural and chemical relationships to the simple C_5H_8 (isoprene) molecule ... " Although such a definition is practically functional and will be used in the subsequent discussion of fungal terpenes, it is far

from being rigorous. A more exacting definition of the term in question, taking into account the biological basis of the Isoprene Rule would be "a naturally occurring substance which can be derived biosynthetically from mevalonic acid (1.1) via an isopentenyl polymer of general formula (1.2, X = $OP_2O_5H_3$, OPO_3H_2, OH, H)".



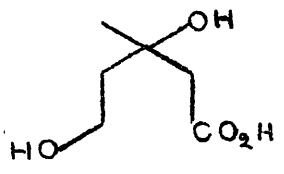
1.1



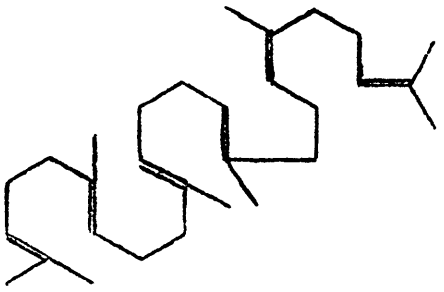
1.2

The stipulated involvement of a polymer in the above definition leads automatically to the classical sub-division of terpenes, the prefixes hemi, mono, sesqui, di, tri and tetra being employed to describe species whose immediate pregenitors are isopentenyl monomers, dimers, trimers, tetramers, hexamers or octamers respectively.

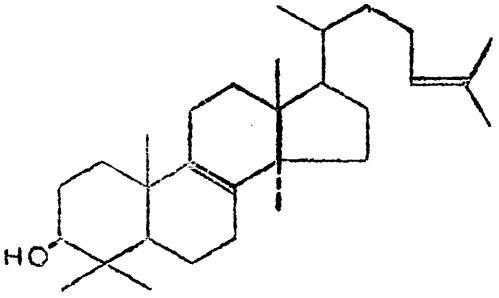
As an example of the operation of the latter definition, lanosterol (1.3) is defined as a triterpene alcohol since it has been shown to be derived biosynthetically from 2-C^{14}-mevalonic acid (1.1)[5] and the isopentenyl hexamer squalene (1.4)[6].



1.1



1.4



1.3

It will be noted, however, that before a substance can be formally defined as a terpene under the conditions proposed above, both mevalonic acid and the appropriate isopentenyl polymer must be shown to be intermediates in their biosynthesis. Since such extensive incorporation data is only available in a limited number of cases, less rigorous, structurally based definitions such as that of Haagen-Smit require to be used until more complete biochemical evidence becomes available.

Representatives of almost all types of terpene are to be found as products of fungal metabolism as can be seen from the examples contained in Tables 1.1 to 1.9. Puccinia graminis provides an example of a fungal hemiterpene in the alkene 2-methylbut-2-ene (1.5)[7], while the monomeric isopentenyl unit can be detected in the molecule of the biogenetically complex diphenolic aldehydes of Aspergillus glaucus, auroglaucin (1.7)[8] and flavoglaucin (1.8)[9] as well as in fuscin (1.6)[10], elymoclavine (1.9), agroclavine (1.10)[11], echinulin (1.11)[12] and novobiocin (1.12)[13].

Although no examples of a simple mould monoterpene have yet been isolated, the isopentenyl dimer characteristic of such compounds is encountered in the ether substituent of mycelianamide (1.13)[14] and in the degraded side chain of mycophenolic acid (1.15)[15].

Sesquiterpenes occur in a variety of structural forms. Simple systems are observed in the azulenes of Lactarius delicosus, lactaroviolin (1.17)[16] and lactarazulene (1.16)[17] while more complex arrangements appear in helminthosporal (1.19)[18], the illudins (1.20)[19] and in the alcoholic portions of trichothecin (1.21)[20] trichodermin (1.22)[21], diacetylscirpenol (1.23)[22] and verrucarin A[23]. Grifolin (1.18)[24] represents the case of a substance of multiple biosynthetic origin containing the isopentenyl trimer.

Rosenonolactone (1.26)[25], pleuromutilin (1.25)[26] and the growth promoting factors produced by Gibberella fujikuroi, the gibberellins (gibberellic acid, 1.24)[27] illustrate admirably the

structural diversity which can arise from the simple isopentenyl tetramer, while the carotenoids[28] contained in Table 1.9 are examples of fungal tetraterpenes.

As far as the present study is concerned the compounds derived from the isopentenyl hexamer squalene (1.4) are of greatest interest. The known fungal metabolites of this class can be arbitrarily divided into three groups according to the number of carbon atoms they possess. The first group contains less than the prescribed thirty carbon atoms and is represented by the fungal sterols such as ergosterol and its derivatives (Tables 1.5, 1.6). The highly oxygenated metabolites cephalosporin P_1 (1.37)[29], helvolic acid (1.38)[30] and fusidic acid (1.39)[31] also belong to this group. In all probability the reduction in carbon content of these compounds is achieved by means of the oxidative-decarboxylation sequence proposed to explain the conversion of lanosterol to cholesterol.[32]

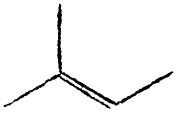
Compounds containing thirty carbon atoms comprise the second group of squalene derived mould metabolites. Squalene itself is known to be a metabolite of Amanita phalloides.[33] The six other known members of this group all possess lanostane skeletons which are oxygenated to varying levels at position C-3 and C-21 and which have $\Delta^{8(9)}$ or $\Delta^{7(9),11}$ unsaturation. The relevant structures are collected in Table 1.7. It is noteworthy that trametenolic acid (1.44) occurs in Trametes odorata,[34] Fomes hartigii[35] and Inonotus obliquus,[36] not in the free state, but as its methyl ester

while 3α-hydroxy-lanosta-8,24-diene-21-oic acid (1.45) appears in Polyporus pinicola[35] as the methyl ester of its acetate.

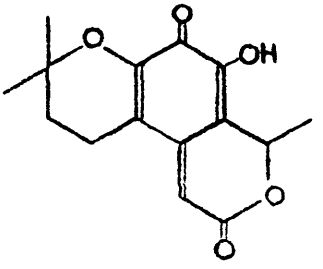
The members of the third group of tetracyclic triterpenes are C_{31} compounds by virtue of the insertion of a C_1 unit at carbon atom C-24 during their biosynthesis. The six known compounds of this type are gathered in Table 1.8. It can be seen that compared with the C_{30} compounds previously examined, there is a greater variability in the positions on the carbon skeleton at which oxidation can take place. There also exists the possibility of having either a monoene or a diene system bridging rings B and C and in this connection it can be mentioned that the monoenes tumulosic acid (1.47)[37] and eburicoic acid (1.51)[38] both co-occur naturally with the corresponding diene. Such monoene/diene mixtures prove very difficult to separate. As with the C_{30} compounds, the C_{31} substances can exist in esterified or "conjugated" form. Thus tumulosic acid can occur as its 3-acetate[39] and its 3,16-diacetate[40] and eburicoic acid as its 3-acetate.[41]

It is relevant to the content of the following chapters to list in Table 1.10 the various fungi which have been shown to give rise to C_{30} and C_{31} tetracyclic triterpenes. It is noted that of the fifteen entries in the Table all but one, Lentinus dactyloides, are members of the family Polyporaceae.

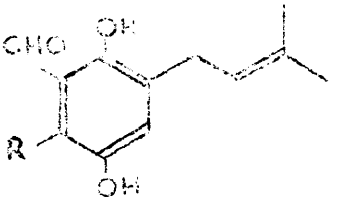
TABLE 1.1 FUNGAL HEMI- AND MONOTERPENES



1.5 2-Methylbut-2-ene.[7]

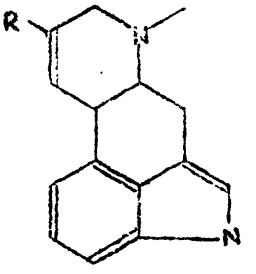


1.6 Fuscin.



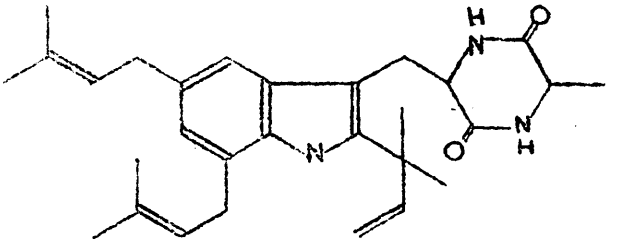
1.7 Auroglaucin. $R=CH_3(CH=CH)_3$[9]

1.8 Flavoglaucin. $R=CH_3(CH_2)_6$[9]



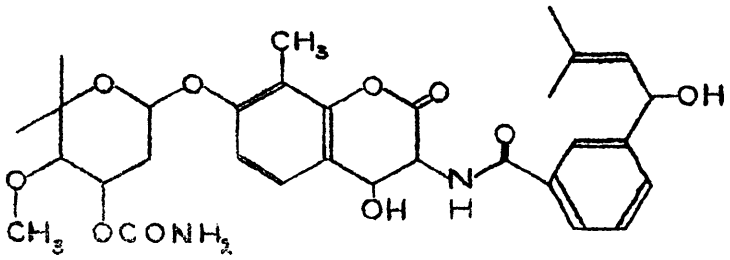
1.9 Elymoclavine. $R=CH_2OH$[11]

1.10 Agroclavine. $R=CH_3$

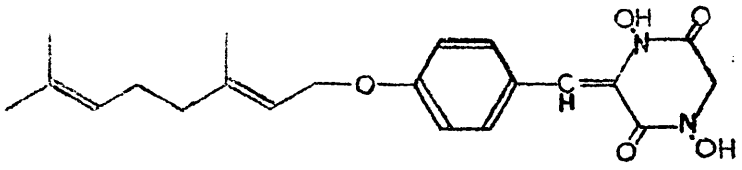


1.11 Echinulin.[12]

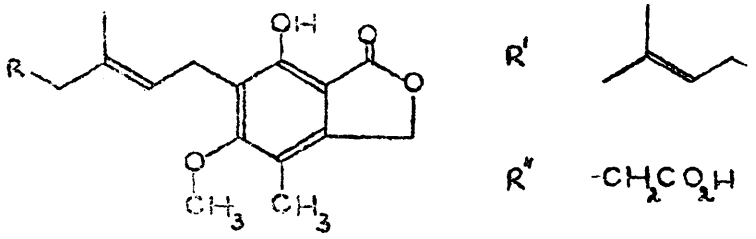
TABLE 1.2. FUNGAL MONO- AND SESQUITERPENES



1.12 Novobiocin[13]

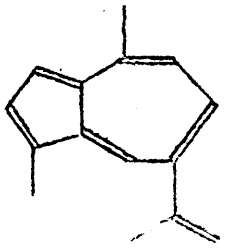


1.13 Mycelianamide[14]

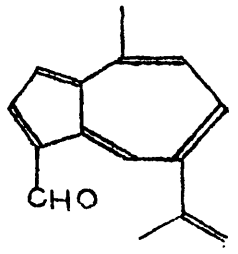


1.15 Mycophenolic acid (R'') and 1.14 the proposed precursor (R')[15]

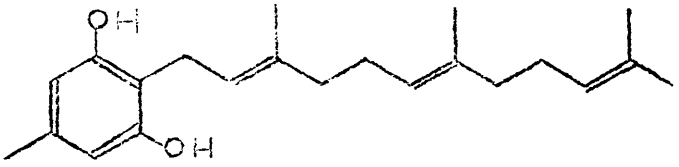
TABLE 1.3. FUNGAL SESQUITERPENES



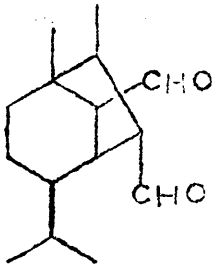
1.16 Lactarazulene[16]



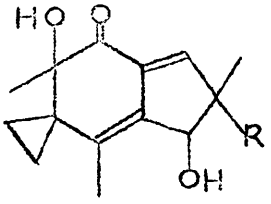
1.17 Lactaroviolin[17]



1.18 Grifolin[24]

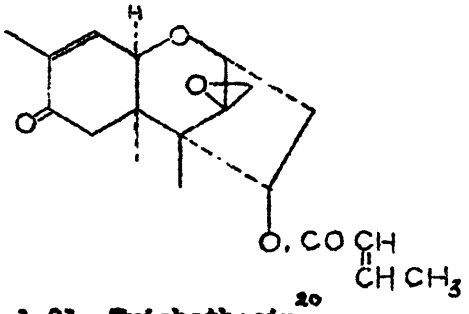


1.19 Helminthosporal[18]



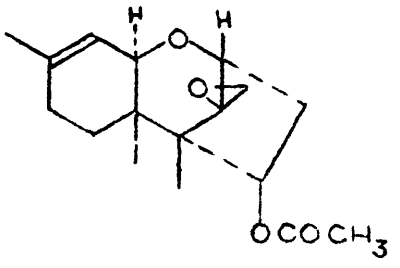
1.20 Iludin M (R = CH_3)

Iludin S (R = CH_2OH)[19]

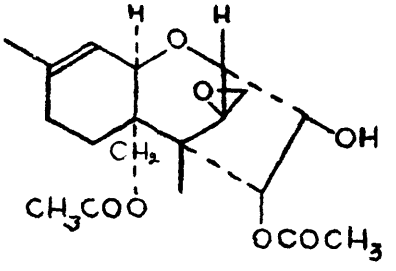


1.21 Trichothecin[20]

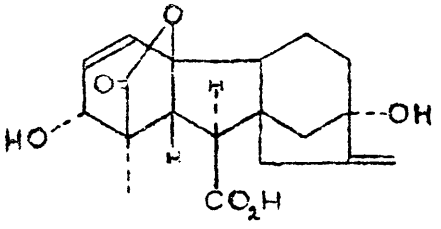
TABLE 1.4. FUNGAL SESQUI- AND DITERPENES



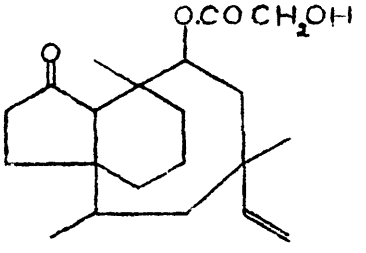
1.22 Trichodermin[21]



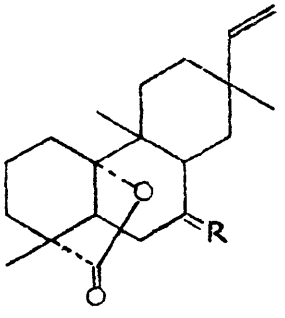
1.23 Diacetylscirpenol[22]



1.24 Gibberellic acid[27]

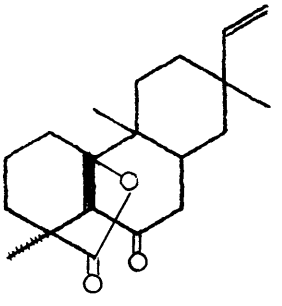


1.25 Pleuromutilin[26]



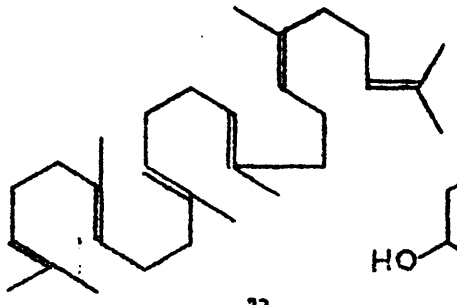
1.26 Rosenonolactone (R = O)[25]

1.27 9-Deoxyrosenonolactone[42] (R = H_2)

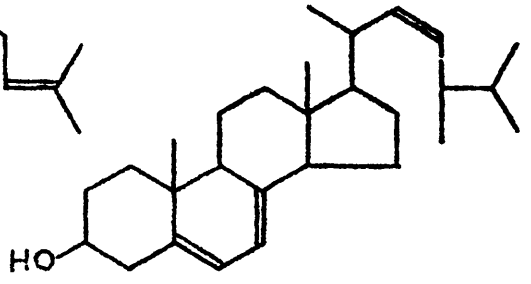


1.28 Rosololactone[43]

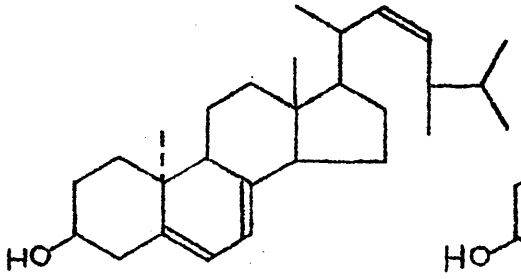
TABLE 1.5. FUNGAL TRITERPENES (STEROIDS)



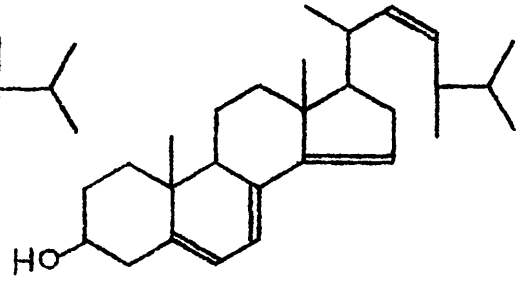
1.29 Squalene[33]



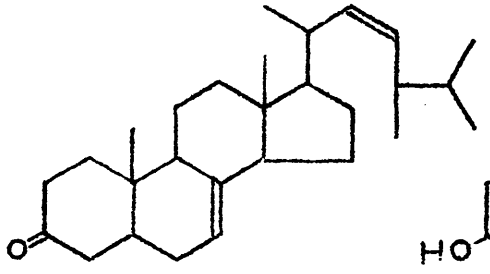
1.30 Ergosterol



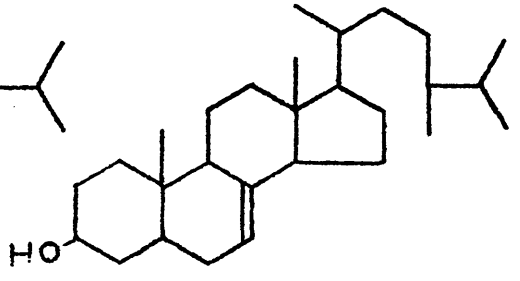
1.31 Pyrocalciferol[44]



1.32 14-Dehydroergosterol[45]

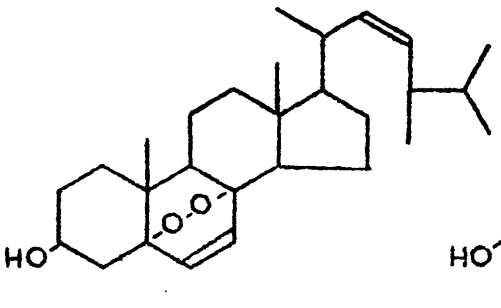


1.33 Ergosta-7,22-diene-3-one[46]

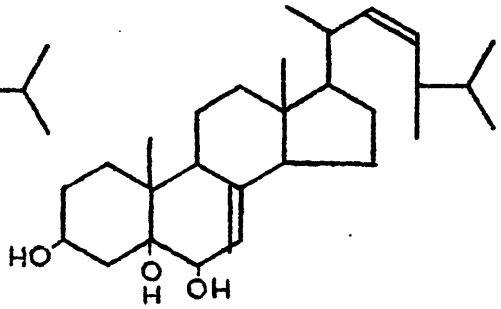


1.34 Fungisterol[47]

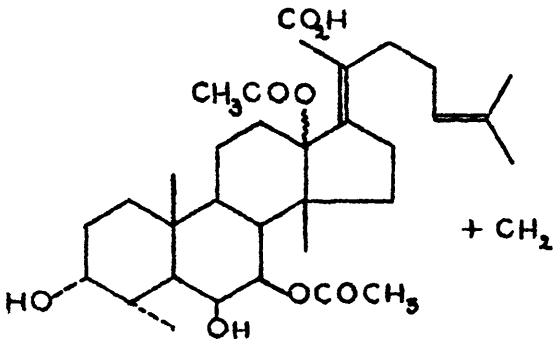
TABLE 1.6. FUNGAL TRITERPENES (STEROIDS, C_{29})



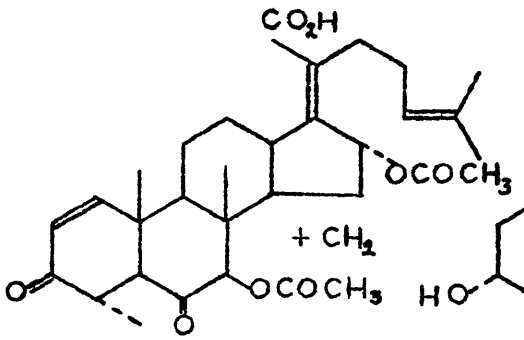
1.35 Ergosterol peroxide[48]



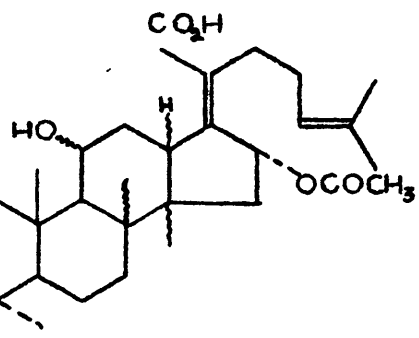
1.36 Cerevisterol[49]



1.37 Cephalosporin P_1[29]

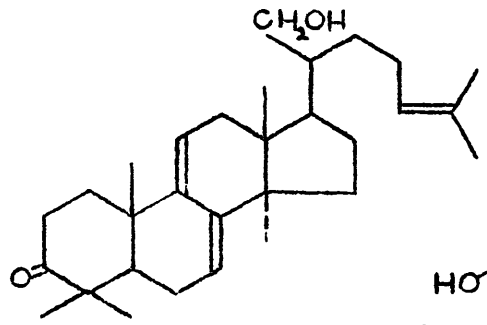


1.38 Helvolic acid[30]

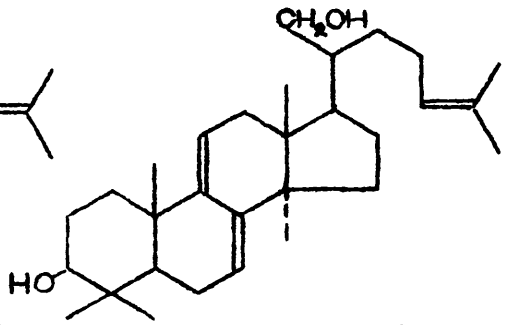


1.39 Fusidic acid[31]

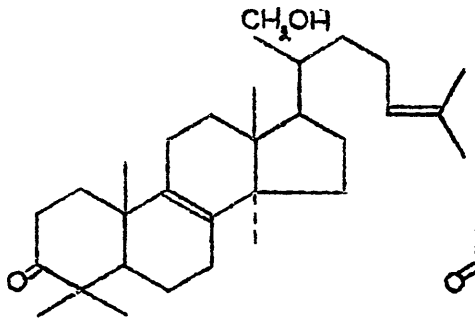
TABLE 1.7. FUNGAL TRITERPENES (C_{30})



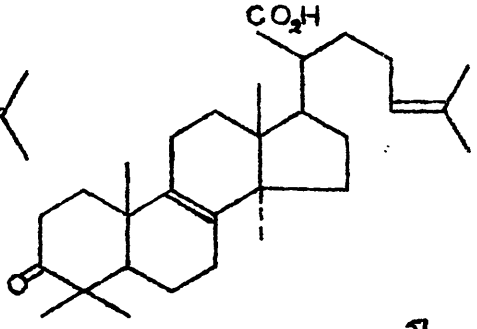
1.40 21-Hydroxylanosta-7,9 (11), 24-triene-3-one [60]



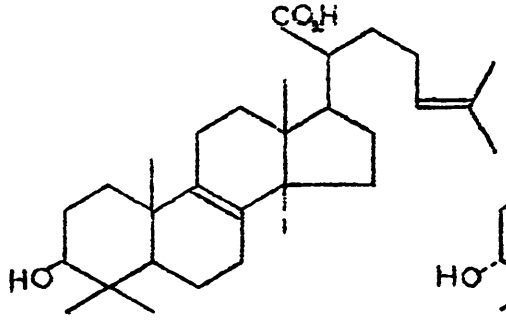
1.41 Lanosta-7,9 (11)-24-triene-3β,21-diol [60]



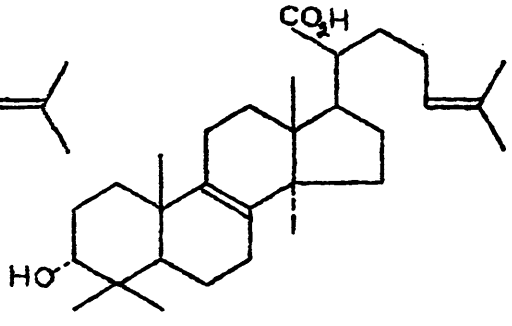
1.42 21-Hydroxylanosta-8,24-diene-3-one [90]



1.43 Pinicolic acid A [57]

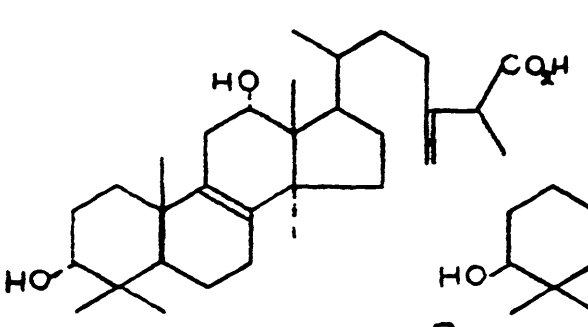


1.44 3β-Hydroxylanosta-3,24-diene-21-oic acid [92]

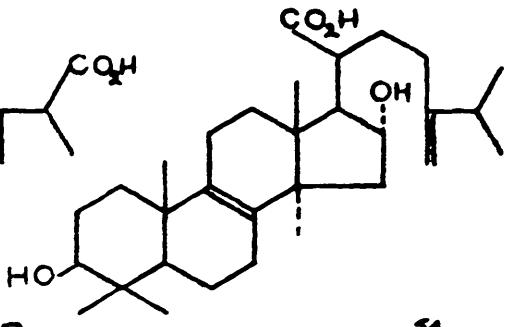


1.45 3α-Hydroxylanosta-8, 24-diene-21-oic acid [36]

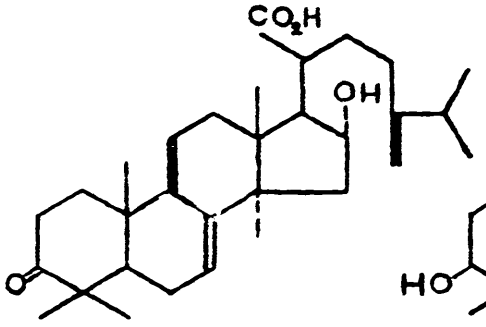
TABLE 1.8. FUNGAL TRITERPENES (C_{31})



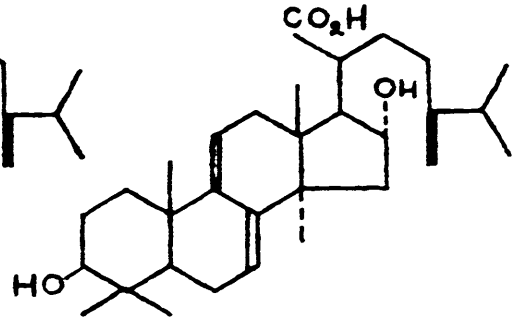
1.46 Polyporenic acid A[53]



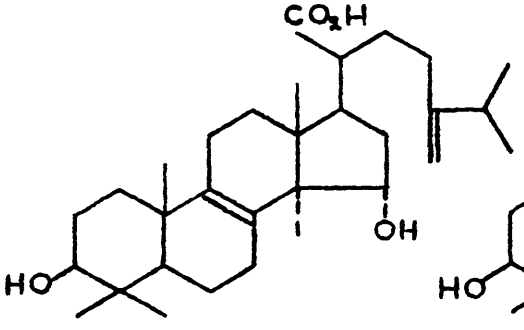
1.47 Tumulosic acid[54]



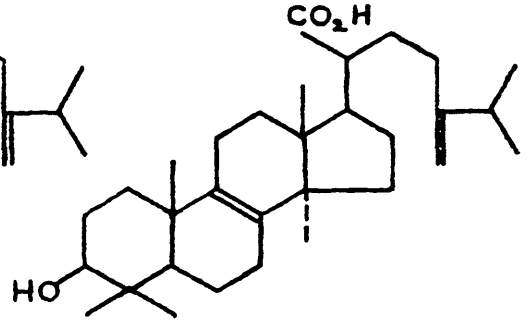
1.48 Polyporenic acid C[55]



1.49 Dehydrotumulosic acid[54]

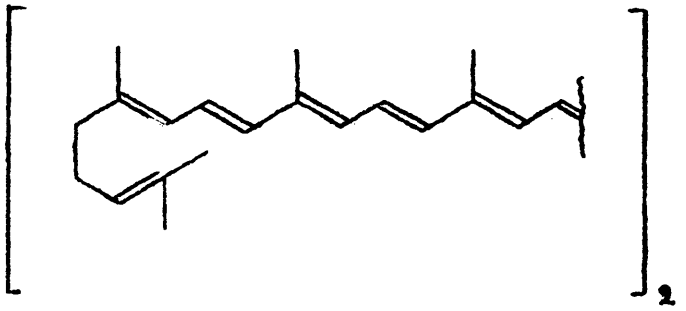


1.50 Sulphurenic acid[56]

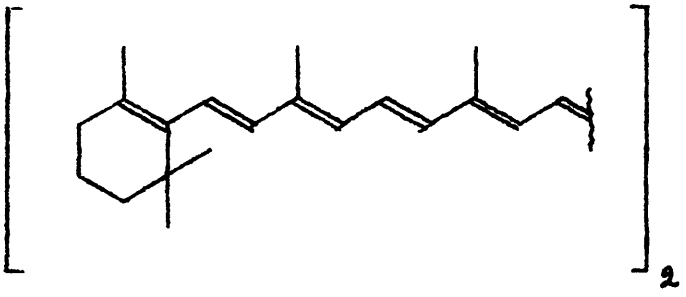


1.51 Eburicoic acid[57]

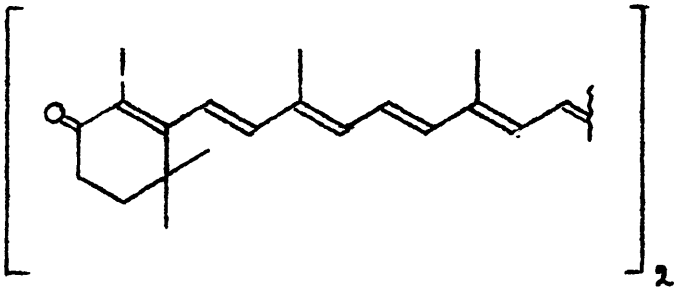
TABLE 1.9. FUNGAL TETRATERPENES[27]



1.52 Lycopene



1.53 β-Carotene



1.54 Cantharanthin

TABLE 1.10. LIST OF FUNGI KNOWN TO PRODUCE C_{30} AND C_{31} TETRACYCLIC TRITERPENES

POLYPORUS PINICOLA	21-Hydroxylanosta-8,24-dien-3-one, lanosta-7,9(11), 24-triene-3β, 21-diol, trametenolic acid A, pinicolic acid, methyl 3α-acetoxylanosta-8-24 dien-21-oate, polyporenic acid C.
POLYPORUS ANTHRACOPHILUS	Eburicoic acid, and its acetate.
TRAMETES ODORATA	Methyl 3β-hydroxylanosta-8,24-dien-21-oate, trametenolic acid B.
FOMES HARTIGII	Methyl 3β-hydroxylanosta-8,24-diene-21-oate, trametenolic acid B.
INONOTUS OBLIQUUS	Lanosterol, methyl 3β-hydroxylanosta-8,24-dien-21-oate, inotodiol.
FOMES OFFICINALIS	Eburicoic acid.
POLYPORUS SULPHUREUS	Eburicoic acid and its acetate, sulphurenic acid.
POLYPORUS HISPIDUS	Eburicoic acid, dehydroeburicoic acid.
PORIA COCCUS	Eburicoic acid, dehydroeburicoic acid, tumulosic acid and its 3-acetate.
LENTINUS DACTYLOIDES	Eburicoic acid, dehydroeburicoic acid.
POLYPORUS EUCALYPTORIUM	Eburicoic acid.
POLYPORUS BETULINUS	Polyporenic acids A and C, tumulosic

	acid and its diacetate.
POLYPORUS AUSTRALIENSIS	Tumulosic acid.
POLYPORUS BENZOINUS	Polyporenic acid C.

CHAPTER 2

AN OUTLINE OF THE BIOGENESIS OF TETRACYCLIC TRITERPENES

Several detailed reviews of the biosynthesis of tetracyclic triterpenes, with special reference to those pathways leading to the steroids, are currently available[58] and only the basic elements of the processes which lead to the construction and modification of the lanostane skeleton will be considered here.

The sequence of reactions commences with the derivation of acetyl coenzyme A from dietary fat or carbohydrate. As is shown in Table 2.1, one molecule of this substance is carboxylated to yield malonyl coenzyme A which is then decarboxylatively coupled with a second molecule of acetyl coenzyme A to give acetoacetyl coenzyme A. Claisen ester condensation with a third molecule of acetyl coenzyme A provides β-hydroxy-β-methylglutaryl coenzyme A. Up to this point, all the reactions are completely reversible and, as will be noted in Chapter 5, the β-hydroxy-β-methylglutaryl derivative is in equilibrium with acetate, this making it a source of terpenes and other acetate derived substances. However the reduction of β-hydroxyl-β-methylglutaryl coenzyme A to mevalonic acid is effectively irreversible and this reaction appears to be one of the points at which terpene synthesis can be controlled.

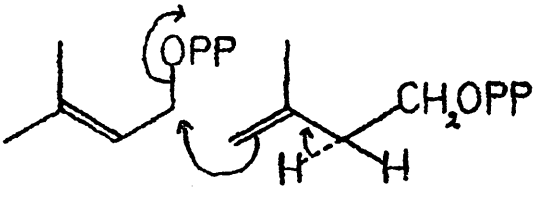
As the result of some recent work, Brodie[59] has proposed that the synthetically involved acetate, malonate, acetoacetate and β-hydroxy-β-methylglutarate species are not coenzyme A ester but enzyme esters. The issue, however, appears to be somewhat confused and further evidence will be required before the validity of the

findings can be assessed.

The conversion of mevalonic acid to isopentenyl pyrophosphate requires three distinct phosphorylation reactions followed by a concerted 1 : 2 elimination of carbon dioxide and the elements of phosphoric acid.

The elaboration of the C_{30} isopentyl polymer squalene from isopentenyl pyrophosphate proceeds via dimethylallyl, geranyl and farnesyl pyrophosphates. Although the detailed mechanism of the reactions involved is not yet fully understood, some of the stereochemical aspects have been elucidated by the Popjak, Cornforth group in an astoundingly beautiful manner.[40] In essence the results are as follows.

a. In the coupling of dimethylallyl pyrophosphate with isopentenyl pyrophosphate to give geranyl pyrophosphate, inversion takes place at carbon atom C_1 of the dimethylallyl pyrophosphate molecule. In addition, it has been shown that the hydrogen removed from carbon atom C-2 of the isopentenyl pyrophosphate molecule comes from the α face thus:-



b. In the coupling of the farnesyl pyrophosphate molecules to give squalene itself, the carbon atom C_1 of one molecule suffers inversion

while that of the other experiences no overall change of stereochemistry.

No totally authenticated mechanism for the union of the two farnesyl units is presently available. In Table 2.1, a cyclic scheme based on the well-known Stevens rearrangement is included. It is due to Popjak and Cornforth[4] and is in accord with all available experimental data.

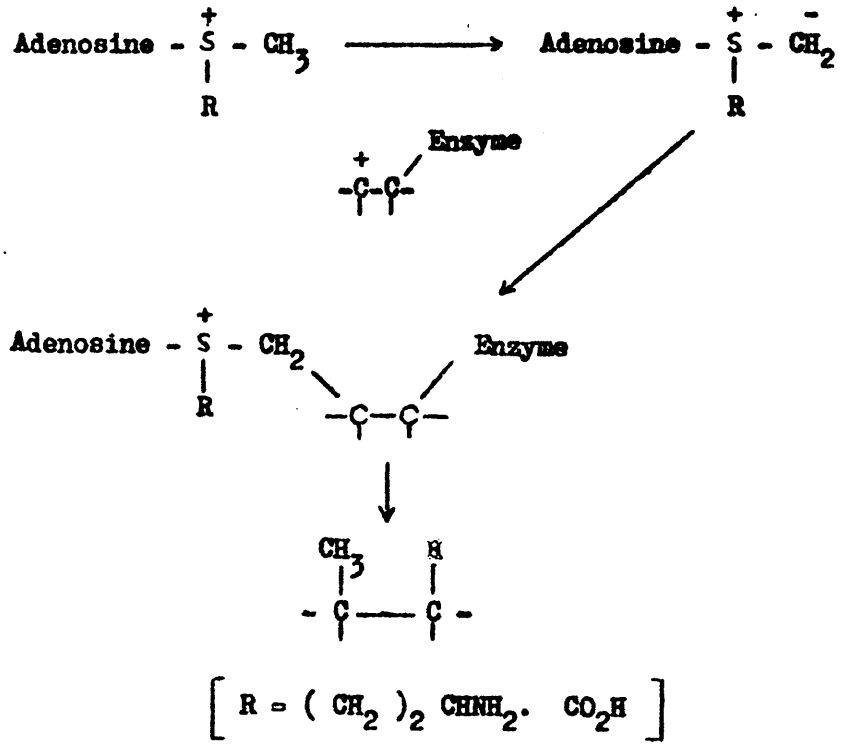
The cyclisation of squalene to lanosterol, the last step in the sequence, involves a series of concerted hydrogen and methyl migrations, all of which have been carefully studied with the aid of radio tracers. However, the precise nature of the entity which initiates the process is not known; it may be the cationic species OH^+.

Lanosterol marks the point at which the biosynthetic pathways branch which lead eventually to the various tetracyclic triterpenes, the steriods, the C_{30} and C_{31} compounds. The C_{30} compounds are immediately available on introduction of the required oxygen functions and double bonds. The steroids require to undergo a series of oxidation and decarboxylation reactions which lead to the removal of the methyl groups on carbon atoms C-4 and C-14.[32] The C_{31} compounds, however, have one additional carbon atom introduced into the skeleton at position C-24 and the processes involved are worthy of further consideration.

It has been shown that the additional carbon atom of ergosterol

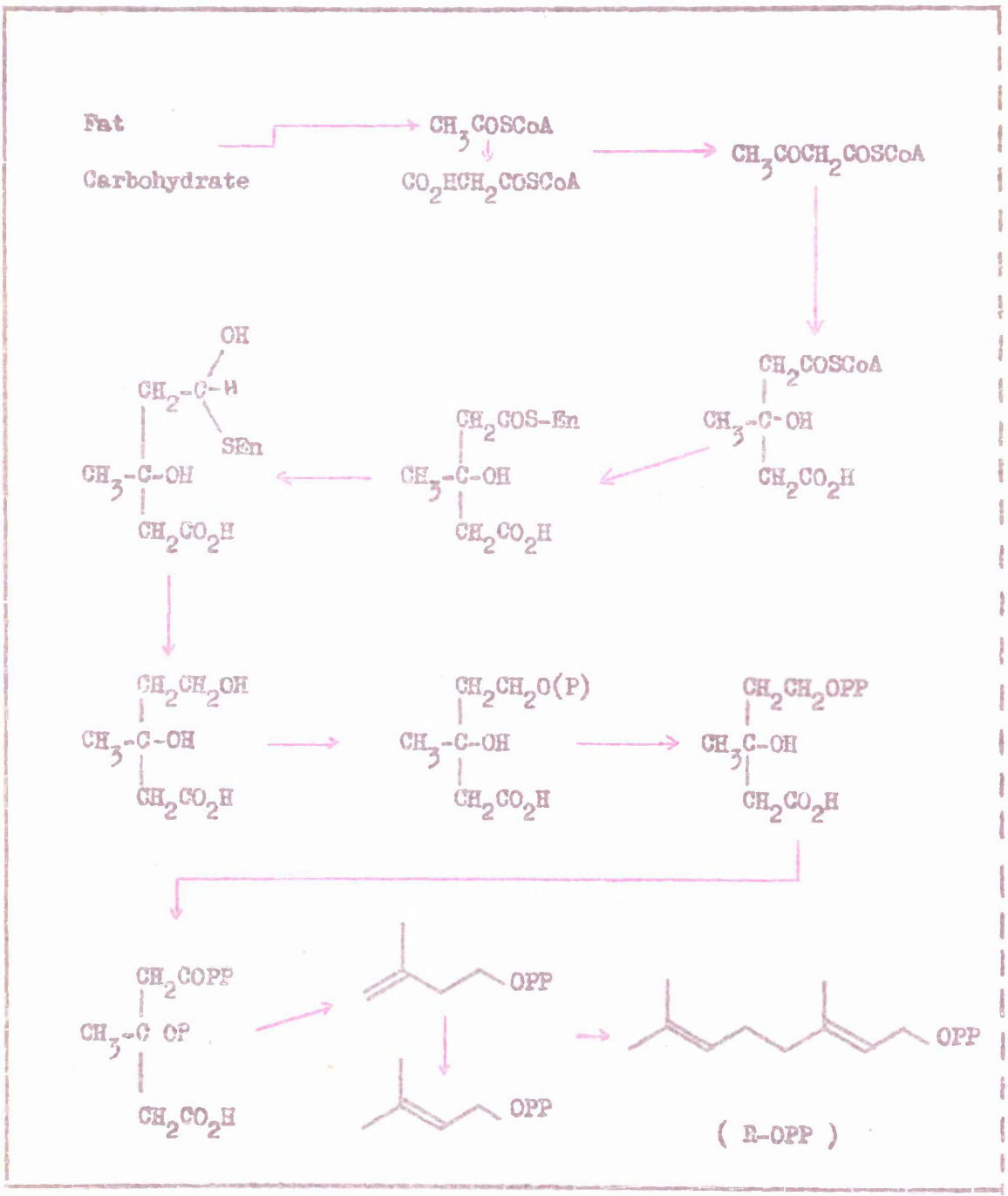
and eburicoic acid are derived from formate and from methionine.[62] Further work with ergosterol, which is presumably relevant to the C_{31} triterpenes, showed that when Me-D_3-methionine was fed to methionine-less strains of Neurospora crassa only two of the three deuterium atoms were incorporated into ergosterol.[63] It has also been shown recently that when ergosterol is biosynthesised from 2-^{14}C-4S-^{3}H-mevalonate, a tritium atom is retained at carbon atom C-24.[64]

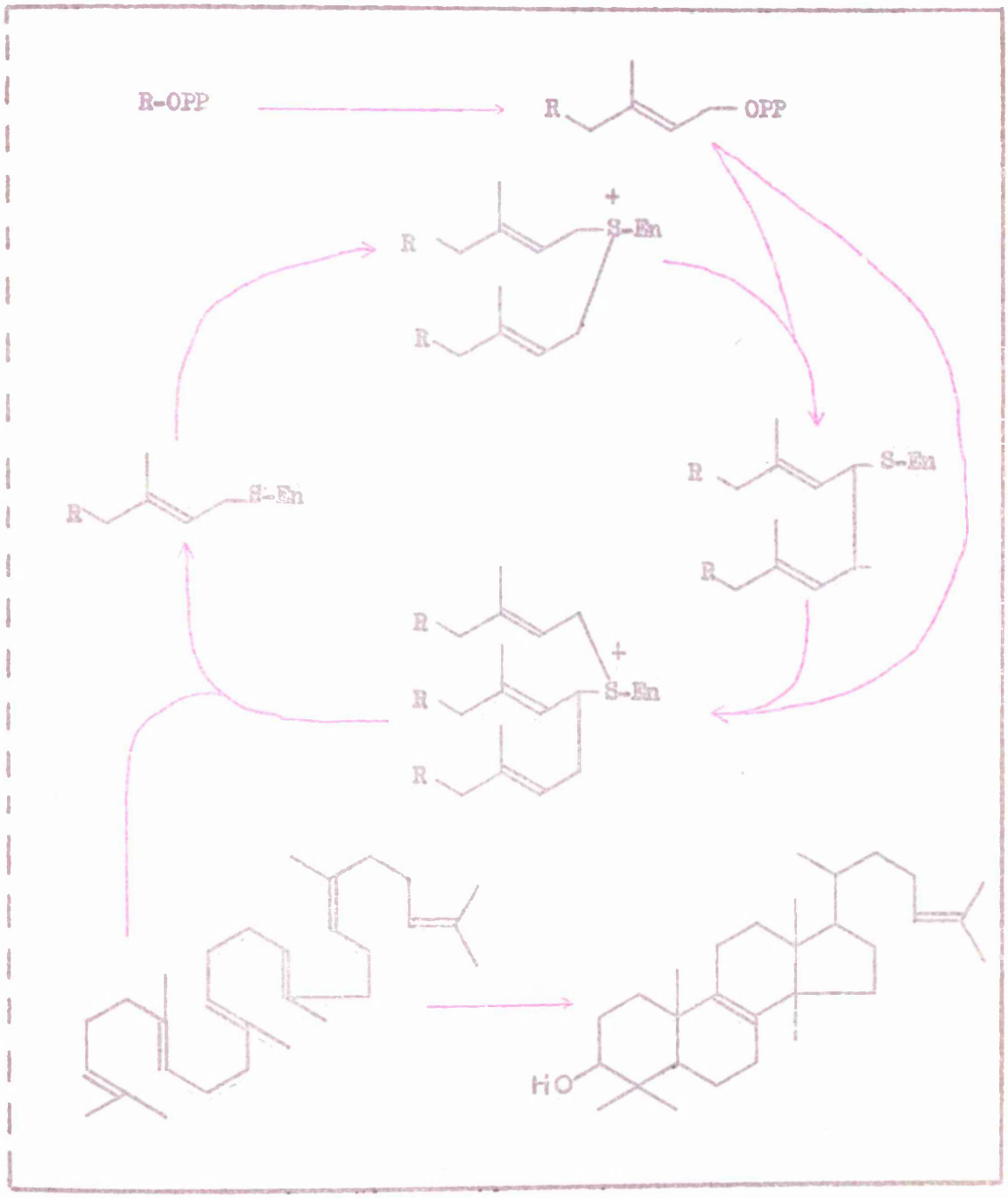
These experimental findings suggest that the "extra" carbon atom of the phytosterols and of the C_{31} tetracyclic triterpenes may be introduced via a 24-25 cyclopropanoid intermediate which may have been formed by the addition of a carbene-like species to the $\triangle^{24(25)}$ double bond.



The above, biologically attractive mechanism for the incorporation of the additional C_1 unit has been proposed by Lederer[65] and involves the addition of an ylide, generated by the oxidation of S-adenosylmethionine, to the $\Delta^{24(25)}$ double bond which has been suitably polarised by association with an enzyme. It will be seen that the so-formed adduct can give the C-methyl compound directly on hydrogenation without actually involving a cyclopropanoid intermediate.

TABLE 2.1. THE BIOGENESIS OF LANOSTEROL




R-OPP
R
OPP
+
S-En
R
R
R
S-En
R
R
S-En
R
R
+
S-En
R
R
HO

CHAPTER 3

THE METABOLITES OF DARDALIA QUERCINA

Since the beginnings of the Science of Botany, systematic classification of the members of the Vegetable Kingdom has been arbitrarily based on considerations of morphology and physiology. This is especially true of the Fungi where a taxonomy based on such features has brought order to the vast multiplicity of fungal forms. Over the last few decades, however, interest has arisen in the chemical nature of the Fungi and it now becomes intriguing to seek chemical reflections of those physiological and morphological characteristics which are the basis of taxonomy.

In this respect it is of interest to examine the species distribution of the known fungal tetracyclic triterpenes. It was noted in Chapter 1 (Table 1.10) that fourteen of the fifteen organisms reported to produce these materials were members of the family Polyporaceae. Before it could be judged how far the corollary were true namely that tetracyclic triterpenes are of general occurrence in the Polyporaceae, many more detailed chemical analyses of the members of this Family would have to be performed. As part of a general programme directed towards this end, the detailed chemical analysis of the organism Daedalia quercina was undertaken. This large, brown, corky, hoof-shaped polypore is commonly found investing the base of dead oak stumps. It is also known to attack fashioned oak timber in buildings and mines. The common name for Daedalia quercina - the Maze Fungus - derives from the fact that

in the sporophore the hymenium lines long, deep, tortuous furrows so giving the under-surface of the fruiting body the aspect of a maze.

In the vast majority of previous studies of both plant and fungal triterpenes, the natural extract was subject to initial saponification. This vigorous procedure has been strongly criticised by Ourisson et al. in the following terms:-[4]

> "It must be remembered that the isolation of substances after treatment with strong alkali gives no indication as to their true native form. Such treatment can produce marked changes in the substituents present; ester groups, in particular, may be hydrolysed."

In order that the study of Daedalia quercina should provide as much valid chemical and biochemical information as possible, it was decided to work with fresh extracts and to avoid saponification.

Healthy, young sporophores of Daedalia quercina were collected from an oak stump in Garscube Estate and, after superficial cleaning were homogenised and extracted with cold methanol. The lipids and light petroleum soluble sterols such as ergosterol were removed from the methanol extract by treatment with light petroleum. Small scale acid-base extraction of the brown solid which constituted the petrol insoluble residue indicated that approximately 86 % of this material was soluble in aqueous base. Since the petrol insoluble

fraction was thus essentially a mixture of acids, preliminary investigation was made with chromatographic methods known to fractionate acids. Since a number of adsorption and partition systems failed to produce any appreciable separation of the components of the acid mixture, it was decided to esterify the petrol insoluble fraction and conduct the remainder of the analysis on the methyl esters of the constituent acids.

The thin layer chromatographic behaviour and staining properties of the methyl esters of the eight principal metabolites of Daedalia quercina are presented in Table 3.1. It has been found useful in this investigation to refer to these methyl esters by the code names D.Q.1, D.Q.4, D.Q.5, D.Q.6, D.Q.7, D.Q.8, D.Q.9 and D.Q.10; these will therefore be used throughout the following text to uniquely represent the substances whose chromatographic and staining properties are defined in Table 3.1. As a result of staining, it was shown that all these compounds except D.Q.5 contained carbonyl groups capable of reacting with 2,4-dinitrophenylhydrazine, while the compounds D.Q.4 and D.Q.5 possessed conjugated diene systems. The latter feature could be deduced from the observation that when the developed T.L.C. plate of the Daedalia quercina esters was sprayed with an 0.005 % aqueous solution of the adsorption indicator rhodamine 6G and viewed in ultraviolet light of wavelength 2560 Å, the compounds D.Q.4 and D.Q.5 appeared as purple areas. Under similar

TABLE 3.1. R_f VALUES OF THE DAEDALIA QUERCINA ESTERS

	R_f Values		Staining	
	100 % $CHCl_3$	99 % $CHCl_3$ 1 % CH_3OH	Rhodamine 6 G.	D.N.P.
D.Q.1	0.58	-	red	+
D.Q.4	0.31	0.72	purple	+
D.Q.5	0.00	0.50	purple	-
D.Q.6	0.61	-	red	+
D.Q.7	0.43	-	red	+
D.Q.8	0.49	-	red	+
D.Q.9	0.00	0.40	red	+
D.Q.10	0.00	0.19	red	+
p-aminoazobenzene	0.60	0.90		
p-hydroxyazobenzene	0.21	0.56		

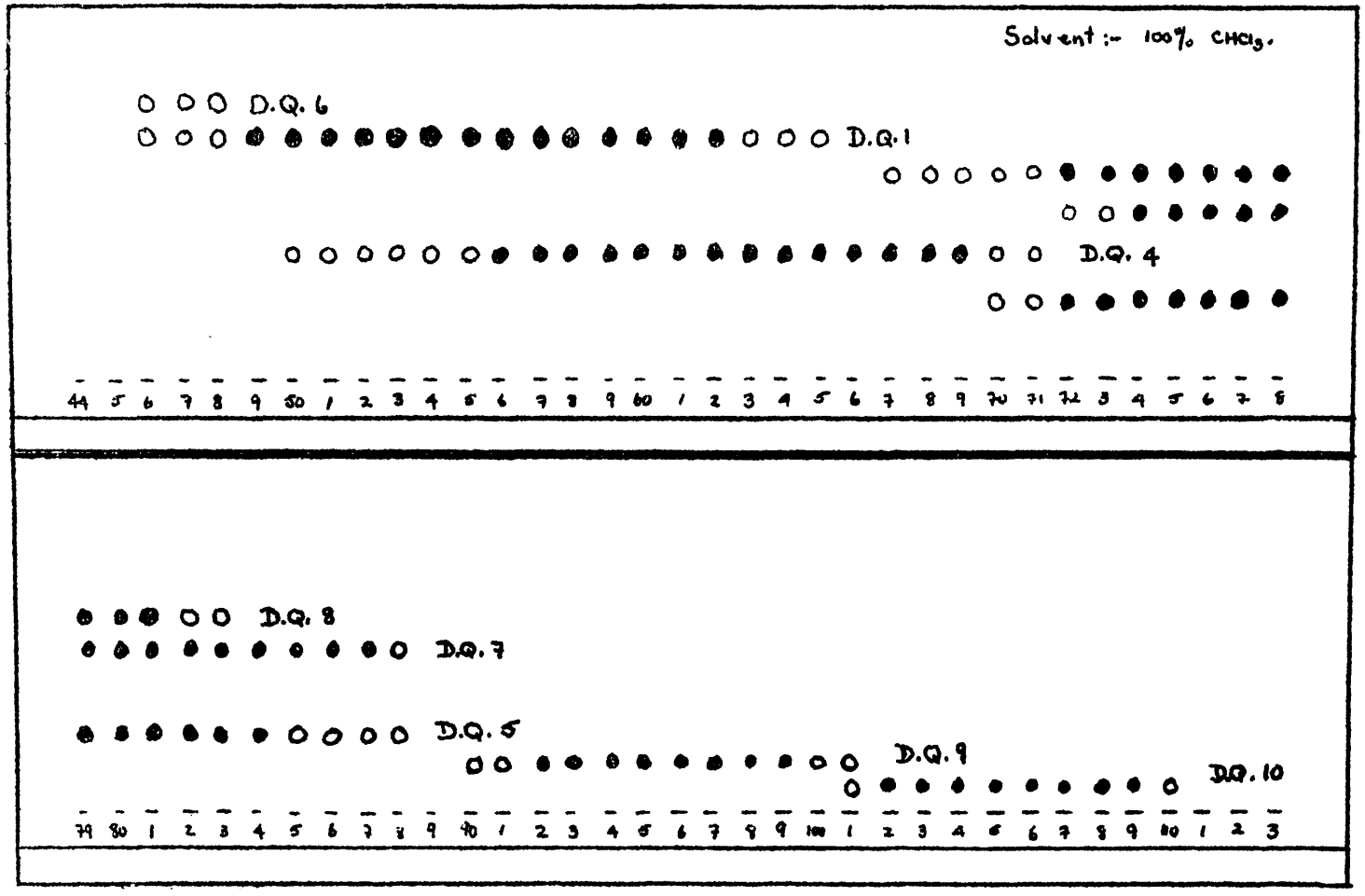
conditions substances containing monoenes or non-conjugated dienes gave rise to red zones. This simple, non-destructive technique for both types of compound has been used extensively in preparative layer chromatography wherein it was found advisable to incorporate the dye directly into the plates at the time of their preparation.

There can be no doubt that the most critical phase in the analysis of the Daedalia quercina metabolites was the development of chromatographic methods which would effectively fractionate the complex mixture of esters.

Initially Woelm Grade IV neutral alumina was employed in conjunction with gradients of light petroleum, ether and ethyl acetate. Although this procedure led to excessive loss of material through decomposition on the adsorbant, pure samples of each of the metabolites were eventually obtained. The use of alumina was, however, discontinued when supplies of Mallinckrodt silicic acid became available. Although this latter adsorbant was not just so selective in fractionation, it is much less destructive than alumina and its use with gradients of light petroleum and ethyl acetate produced well-nigh quantitative separations.

The T.L.C. of a typical silicic acid fractionation is displayed in Table 5.2. It can be seen that pure samples of the compounds D.Q.1, D.Q.4, D.Q.9 and D.Q.10 were immediately available. The remaining four substances, the compounds D.Q.6, D.Q.7, D.Q.8 and D.Q.5 were isolated by means of preparative layer chromatography

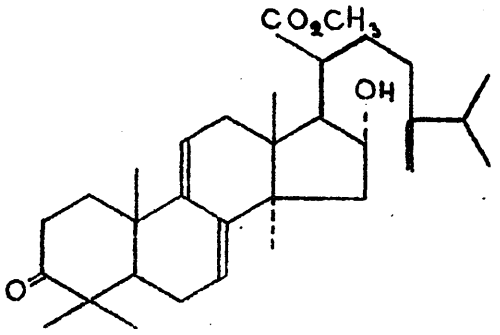
TABLE 3.2. TYPICAL SILICIC ACID FRACTIONATION OF D. QUERCINA ESTERS



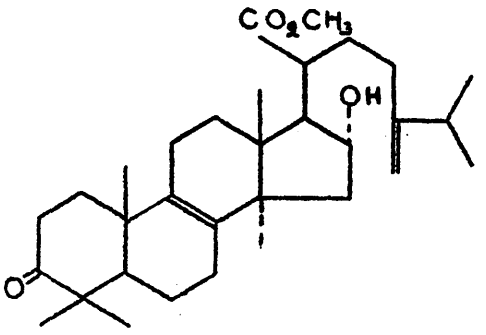
on rhodamine-treated plates. It must be noted, however, that all the compounds, save D.Q.4 and D.Q.5, decompose appreciably on standing at room temperature and further purification may be required immediately prior to the study of any particular compound.

The nature of the substances D.Q.4 and D.Q.5 will be considered first. The substance D.Q.4, m.p. 192-194°C had an analysis in accord with the molecular formula $C_{32}H_{48}O_4$ and was shown osmometrically to have a molecular weight of 492±6. Absorption bands in the infrared spectrum at 3623 cm^{-1} (ε 76.6, $\Delta\nu_{\frac{1}{2}}$ 16), 1736 cm^{-1} (ε 580, $\Delta\nu_{\frac{1}{2}}$ 18) and 1713 cm^{-1} (ε 569, $\Delta\nu_{\frac{1}{2}}$ 14) were compatible with there being in the molecule a secondary hydroxyl group, an alkoxycarbonyl system and a saturated aliphatic or six membered alicyclic ketone function. The first indication that the material might be based on a tetracyclic carbon skeleton came from the ultraviolet spectrum of the substance which contained the triplet characteristic of a steroidal 7,9(11) diene.[67] Unsaturation was also present in the molecule as an exocyclic methylene group as shown by infrared bands at 1642 and 890 cm^{-1} and a two proton multiplet at 4.55 tau in the N.M.R. spectrum. In review, the spectroscopic evidence was wholly consistant with the compound being methyl polyporenate C (3.1).[55] Mixed melting point and chromatographic comparison of the substance D.Q.4 with an authentic sample of methyl polyporenate C seemed to confirm their identity. Careful analysis of the mass spectrum, however, revealed that the substance

D.Q.4 was, in fact, a binary mixture. Together with a parent



3.1



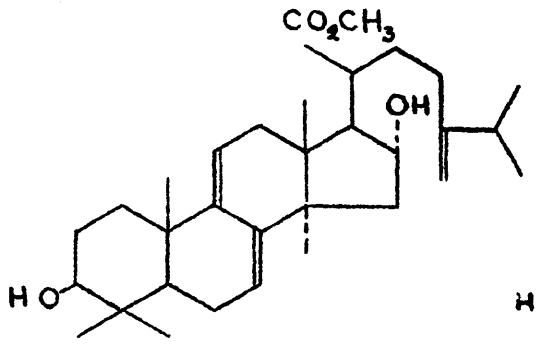
3.2

molecular ion at m/e 496 corresponding to methyl polyporenate C, there occurred a smaller peak (12 % of m/e 496) at m/e 498 which was almost certainly due to contamination of methyl polyporenate C by a small amount of the corresponding 8(9)-monoene, methyl 7,11-dihydropolyporenate C (3.2). Such monoene : diene mixtures are common in the previously encountered triterpenes of the Polyporaceae-tumulosic acid,[54] pinicolic acid[51] and eburicoic acid[57] are all accompanied by their 7,11-dehydroderivatives.

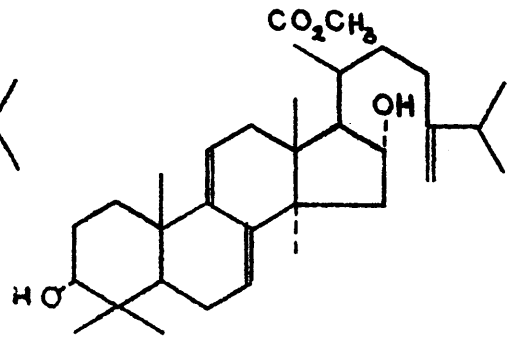
The physical data of the substance D.Q.5 bore a great resemblance to the corresponding values for the substance D.Q.4 as is shown in Table 3.3. The minor differences could be explained if the substance D.Q.5 was either the 3β (3.3) or 3α (3.4) alcohol derived from methyl polyporenate C.

TABLE 3.3. COMPARISON OF PHYSICAL DATA COMPOUND D.Q.4 AND D.Q.5

	D.Q.4	D.Q.5
Molecular formula	$C_{32}H_{48}O_4$	$C_{32}H_{50}O_4$
λ_{max}	2372Å (13,500) 2430Å (15,300) 2510Å (10,600)	2370Å (12,300) 2430Å (14,300) 2510Å (10,800)
ν_{max}	3623 (77) 1736 (580) 1713 (569) 1645 890	3623 (123) 1737 (538) -- 1645 890
Tau values	4.55 (2H, t, J=5.4) 5.25 (2H, d, J=2) 5.90 (1H, m) 6.28 (3H, s) -- --	4.58 (2H, t, J=6) 5.25 (2H, d, J=3) 5.90 (1H, m) 6.28 (3H, s) 6.74 (1H, m)
P.M. Ion	m/e 496	m/e 498

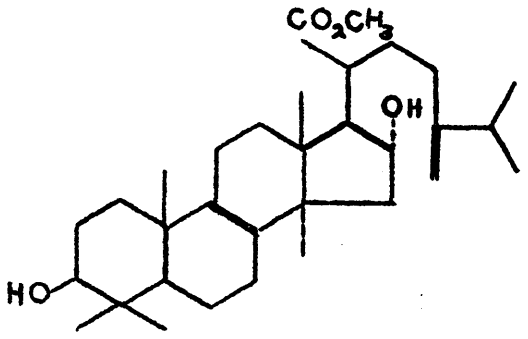


3.3



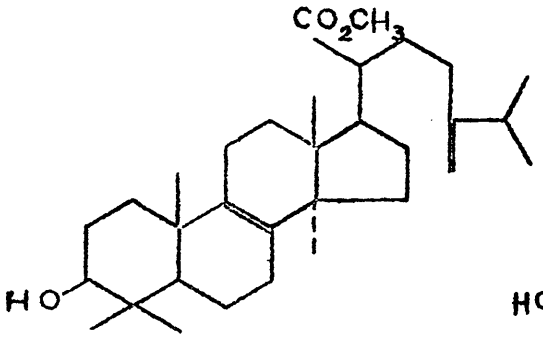
3.4

For comparison purposes, both these alcohols were synthesised unambiguously, the former by sodium borohydride reduction of methyl polyporenate C, the latter by Meerwein-Pondorf reduction of the same compound. To all intents and purposes the substance D.Q.5 was identical to the 3β-alcohol (3.3) as judged by N.M.R. and infrared spectra, R_f value and mixed melting point. However the compound D.Q.5 is also a monoene : diene mixture; two parent molecular ions were detected in the mass spectrum, the major at m/e 498 (71 %) corresponding to methyl 7,11-dehydro-tumulosate (3.3), the minor at m/e 500 (29 %) corresponding presumably to methyl tumulosate (3.5).

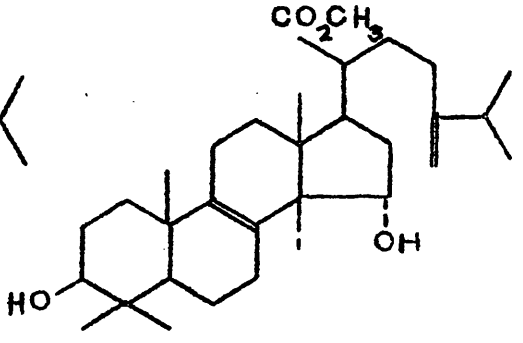


3.5

The N.M.R. signals characteristic of the functional groups present at carbon atoms C-3, C-7, C-11, C-16, C-21 and C-24 in the substances D.Q.4 and D.Q.5 are collected in Table 3.4. The corresponding literature values for methyl eburicoate (3.6)[57] and methyl sulphurenate (3.7)[56] are included for comparison.



3.6



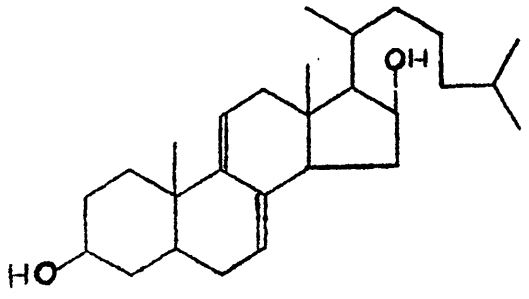
3.7

It is interesting to note that the 16α-hydroxyl group in the substances D.Q.4 and D.Q.5 exercises a slightly greater (0.1 tau) shielding

TABLE 3.4. N.M.R. DATA FOR THE COMPOUNDS D.Q.4 AND D.Q.5.

	3-H	7,9-H	16-H	OCH_3	$=CH_2$
The compound D.Q.4	-	4.56 t J=5.4	5.80	6.29	5.21
The compound D.Q.5	6.75	4.60 t J=6	5.89	6.29	5.20 5.27
Methyl eburicoate	6.78	-	-	6.33	5.33 5.26
Methyl sulphurenate	6.75	-	-	6.33	5.33 5.26

influence on the protons of the 24-exocyclic methylene group than the 15α-hydroxyl group in methyl sulphurenate. The substances D.Q.4 and D.Q.5 were mixtures and thus no detailed study of that part of the N.M.R. spectrum caused by saturated methyl groups was possible. It was noted, however, that the spectra of both compounds contained a peak at approximately 9.40 tau. Construction of scale models of the molecules concerned indicated that in both cases the C-18 methyl group was situated almost directly above the plane of the conjugated double bond. In this position the protons of the methyl group would experience considerable diamagnetic shielding. It is noteworthy that the theoretical value for the C-18 methyl group in 3-keto and 3β,16α-dihydroxycholesta-7,9 (11)-diene (3.8) is 9.49 tau.[63]



3.8

The detailed examination of the mass spectra of the compounds D.Q.4 and D.Q.5 was of considerable assistance in the elucidation of the structures of the remaining metabolites. The mass values

and abundances of the principal ions are gathered in Table 3.5. Both substances readily lost a methyl group and the elements of water from the parent ion to give rise to peaks at P-15, P-18 and P-33. The ready loss of 31 units of mass from the parent ion in both spectra could possibly be due to elision of the ester methoxyl group. A significant proportion of the ion current corresponded to the classical steroid fission whereby the side chain together with the carbon atoms C-15, C-16 and C-17 of ring D were eliminated;[69] in the compounds under present consideration this process was responsible for peak systems centred at m/e 269 (D.Q.4) and m/e 271 (D.Q.5).

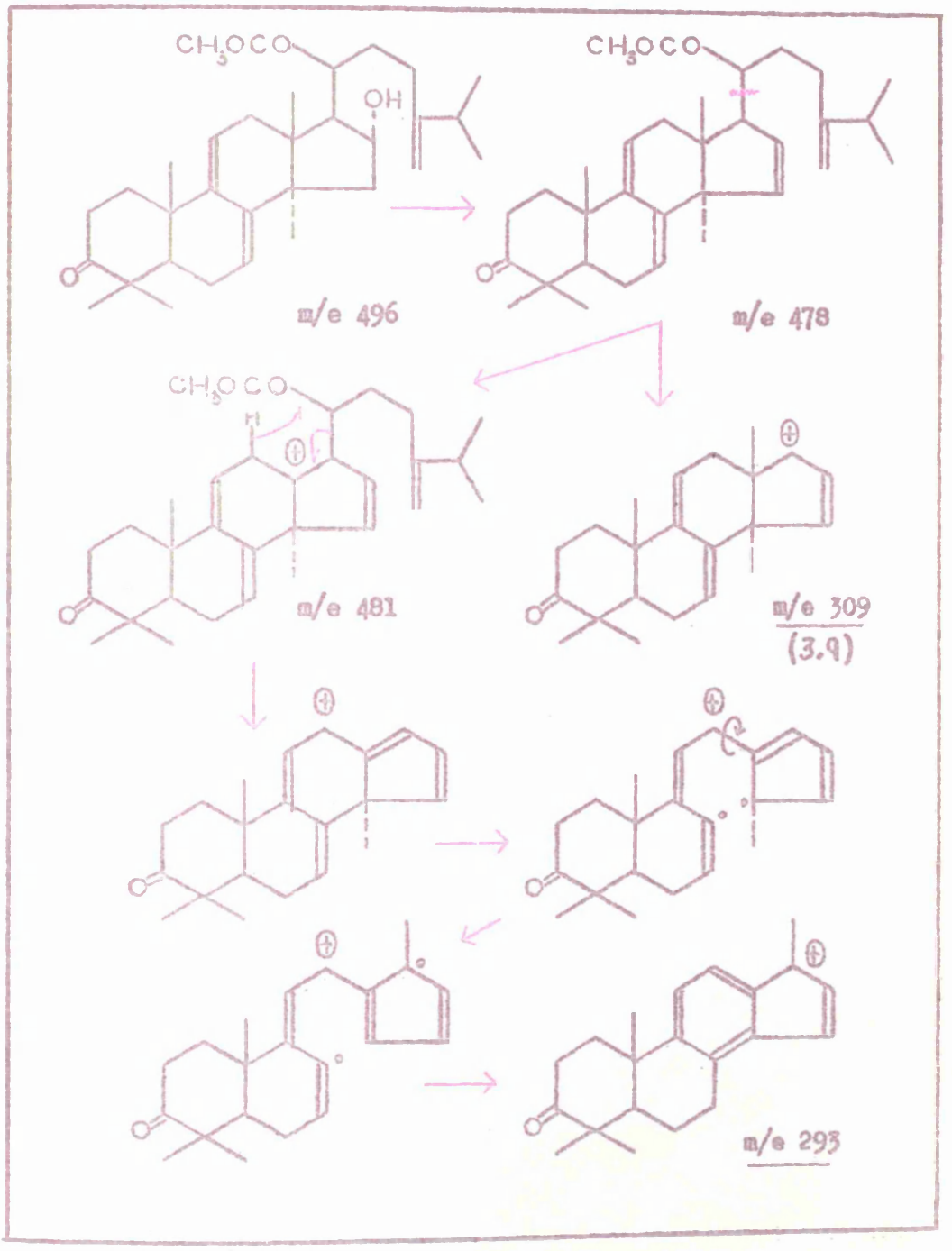
The most abundant ions in the spectrum of the compound D.Q.4 occurred at m/e 309 (86 %) and m/e 293 (100 %). The former peak can be convincingly rationalised in terms of a primary loss of water from ring D followed by a "Type A_3"[70] cleavage between carbon atoms C-17 and C-20 to give the allylic ion 3.9 (Table 3.6).

The genesis of the base peak of the spectrum is more difficult to trace. It was noted that the transition m/e 463 - m/e 293 was marked by an abundant metastable ion at 185.5 and therefore it appeared that the base peak was obtained primarily from a species related to the parent ion by losses of a methyl group and the elements of water. In addition, a doubly charged ion corresponding to the base peak was observed at 147.5. The ability to lose two electrons is normally possessed by ions containing an aromatic

TABLE 3.5. MASS SPECTRAL DATA FOR THE COMPOUND D.Q.4 AND D.Q.5

The compound D.Q.4		The compound D.Q.5	
m/e	%	m/e	%
496	9	498	14
481	9	483	11
478	14	480	14
465	26	467	28
463	30	465	19
309	86	311	49
293	100	295	100
269	51	293	33
		277	46
		271	17
m^*	Transition	m^*	Transition
185.5	463-293	187.2	465-295

TABLE 3.6. PROPOSED CRACKING PATTERN FOR THE COMPOUND D.Q.4



system. The scheme shown in Table 3.6 provides a reasonable genesis for the base peak which takes all the above observations into account.

Similar rationales of fragmentation pattern can be applied to the spectrum of the substance D.Q.5 where, however, the principal ions are shifted to higher mass by two units due to the fact that the compound D.Q.5 is the alcohol derivative of the substance D.Q.4 As will be seen from Table 3.5, the spectrum of the substance D.Q.5 possessed two additional peaks at m/e 293 and m/e 277. These can be readily explained in terms of the elimination of the elements of water from the ions at m/e 311 and m/e 295 respectively.

With the establishment of the chemical nature of the substances D.Q.4 and D.Q.5 as mixtures of tetracyclic triterpenes, it had been demonstrated that one more member of the Polyporaceae contained such compounds. Recent work in this field[71] has shown that two other members of the Polyporaceae, Lenzites striata and Leptoporus stipticus also produce tetracyclic triterpenes. At very least, these observations do not invalidate the hypothesis that tetracyclic triterpenes are of general occurrence in the Polyporaceae.

The compound D.Q.1 was found to be structurally more complex than either methyl polyporenate C or methyl 7,11-dehydrotumulosate and even now only a tentative structure (3.24) can be advanced. At a comparatively early stage in the investigation it was noted that this substance was prone to decomposition and was particularly

sensitive to both heat and alkali. In one experiment in which the compound D.Q.1 was treated with warm aqueous sodium hydroxide, ten distinct decomposition products were detected after a reaction period of only a few minutes. This pronounced lability proved to be a great hindrance to structural elucidation.

Mass spectrometric and classical combustion analysis indicated that the compound D.Q.1 could be represented by the molecular formula $C_{36}H_{56}O_7$ (m/e 600). It was of interest to note that the general appearance of the high field region of the N.M.R. spectrum resembled the pattern normally exhibited by tetracyclic triterpenes. Approximate integration measurements of the signals occurring between 8.80 and 9.30 tau suggested that seven methyl groups attached to saturated centres were present in the molecule. The low field area of the spectrum contained only four peaks, an ill-defined one proton multiplet at 5.30 tau, two three proton singlets at 6.30 and 6.37 tau and a two proton singlet at 6.70 tau. It seemed probable that the two three proton singlets were each due to a methoxyl group.

Examination of the infrared spectrum of the compound D.Q.1 revealed that none of the seven oxygen atoms in the molecular formula were present as hydroxyl groups. Intense absorption was, however, present in the carbonyl area. The lowest frequency band (1716 cm^{-1}, ε 405, $\Delta\nu_{\frac{1}{2}}$ 20) in a set of three was assigned to the stretching vibration of an aliphatic or six membered alicyclic ketone. This

assignment was in accord with ultraviolet absorption at 2820 Å (ε 86) and the positive reaction given by the metabolite with Brady's reagent. In this latter respect it may be interposed that several attempts were made to isolate manageable quantities of the 2,4-dinitrophenylhydrazone of the compound. D.Q.1. The reaction, however, appears to be beset by side reactions and only small amounts of relatively impure material were ever obtained.

Intensity measurements carried out on the second absorption band (1736 cm^{-1}, ε 1272, $\Delta\nu_{\frac{1}{2}}$ 18) suggested that it had been caused by two different carbonyl groups, possibly in ester units. Bearing in mind the facts that the original acidic extract had been methylated and that the N.M.R. spectrum of the compound D.Q.1 contained two signals attributable to the resonance of the protons of methoxyl groups, it seemed possible that the compound D.Q.1 was a dimethyl ester and hence could be represented as:-

$$C_{32}H_{50}O_3\ (CO_2CH_3)_2$$

The remaining band in the carbonyl region of the infrared spectrum (1757 cm^{-1}, ε 766, $\Delta\nu_{\frac{1}{2}}$ 13) was initially attributed to a γ or δ-lactone. This assignment, however, was altered in the light of later work.

Possible relative dispositions of these carbonyl functions were deduced from an analysis of the mass spectrum of the compound D.Q.1. The relative abundances of the major ions in the spectrum

are collected in Table 3.7. A scheme in which the major transitions are supported by the presence of metastable ions is contained in Table 3.8. It can be appreciated that the parent molecular ion (m/e 600), the base peak (m/e 467) and the two principal fragment ions (m/e 585 and 309) in the spectrum are inter-related through losses of neutral fragments of mass 15,118 and 158. Accurate mass measurement of the m/e 309 peak showed that it corresponded uniquely to the oxygen free species $C_{23}H_{33}$. Making the assumption that the initial loss of 15 units of mass corresponded to the fission of an angular methyl group, it was possible to deduce that the combined molecular formula of the neutral species of mass 118 and 158 must be $C_{12}H_{20}O_7$, i.e. together these molecules account for all the oxygen atoms present in the compound D.Q.1. Calculations showed that there were only three non-trivial modes in which the analysis $C_{12}H_{20}O_7$ could be accommodated between two molecules of mass 118 and 158. They are:-

	158	118
A	$C_8H_{14}O_3$	$C_4H_6O_4$
B	$C_7H_{10}O_4$	$C_5H_{10}O_3$
C	$C_6H_6O_5$	$C_6H_{14}O_2$

The third set of values, C, can be immediately excluded since the entity $C_6H_{14}O_2$ is completely saturated and therefore could not be considered as a product of a rearrangement induced by electron

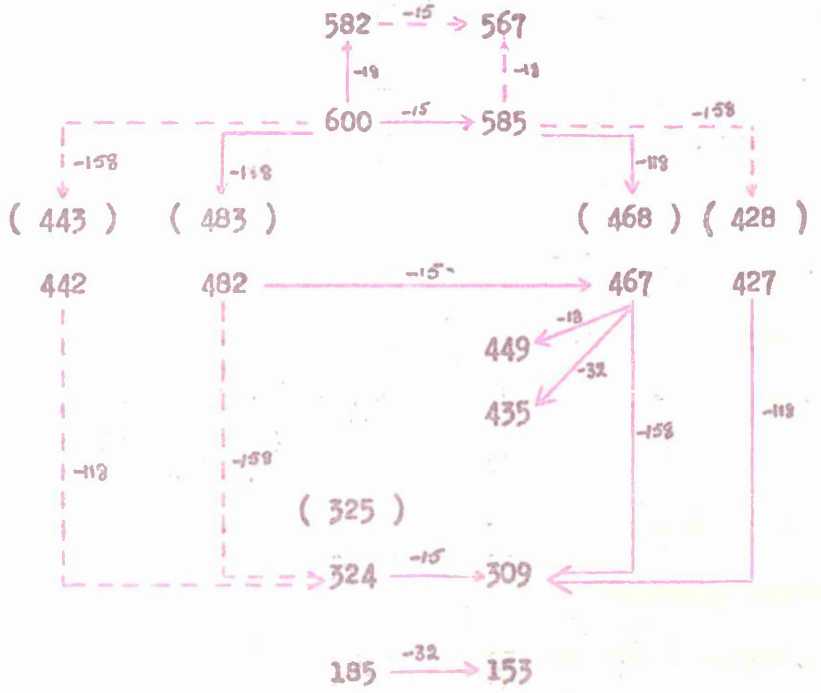
TABLE 3.7. MASS SPECTRAL DATA FOR THE COMPOUND D.Q.1

m/e	%	m/e	%
600	43.7	428	6.0
585	35.7	427	18.8
582	3.0	325	10.8
500	2.0	324	15.2
483	7.0	309	63.4
482	7.4	255	9.0
468	35.1	185	26.6
467	100.0	153	25.8
443	3.5	143	65.4
442	2.8	115	22.7
		59	80.5

also at m/e

189	175	161	149	135	123	109
187	173	159	147	133	121	107
185	171	157	145	131	119	105

TABLE 3.8. PROPOSED MASS SPECTRAL CRACKING PATTERN FOR THE COMPOUND D.Q.1



Full line denotes a process for which metastable ion is available.

m*	Transition
570.4	600-585
564.3	600-582
452.4	482-467
431.7	467-449
405.2	467-435
387.2	600-482
372.6	585-467
294.7	324-309
223.7	427-309
204.4	467-309
126.5	185-153

bombardment. Of the remaining two alternatives, A and B, the second appeared inconsistant with the results of the infrared spectral analysis which had revealed that the compound D.Q.1 contained two ester groups, one ketone group and a lactone (?). Any fragment of the compound D.Q.1 containing three oxygen atoms would therefore require to possess at least two double bond equivalents. Since the entity $C_5H_{10}O_3$ has only one double bond equivalent, the second set of values, B, was not considered to be feasible. It was therefore concluded that the two neutral fragments of mass 158 and 118 were represented by the molecular formulae $C_8H_{14}O_3$ and $C_4H_6O_4$ respectively. Possible structures for these moieties were then considered.

Since the unit $C_4H_6O_4$ contained four oxygen atoms it must have contained at least one of the methoxycarbonyl systems. This allowed only five structures for the neutral molecule of mass 118, namely (3.11, 3.12, 3.13, 3.14, 3.15).

$CH_3O.CO.O.CO.CH_3$ (3.11)

$CH_3O.CO.O.CH_2CHO$ (3.12)

$CH_3O.CO.CH_2CO_2H$ (3.13)

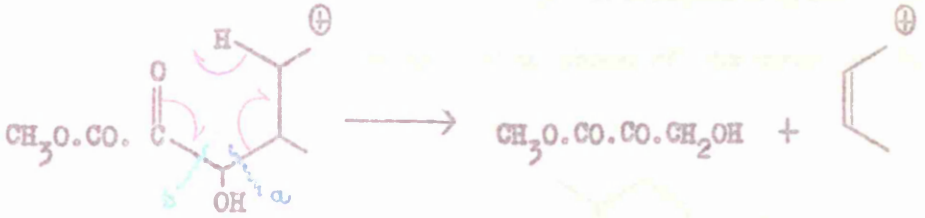
$CH_3O.CO.CO.CH_2OH$ (3.14)

$CH_3O.CO.CO.OCH_3$ (3.15)

Of these structures, it seemed justifiable to exclude the

two carbonate esters (3.11, 3.12) and dimethyl oxalate (3.15) since no reasonable mechanism could be envisaged for their elimination from a larger molecule under electron bombardment. Acceptable mechanisms were available to explain the formation of either of the two remaining possibilities, methoxycarbonylacetic acid (3.13) and methyl hydroxypyruvate (3.14). Both these species could be derived by means of "Type H" [7o] rearrangements as shown (3.16 and 3.17). Furthermore, fission of each at point "a" would give

3.16



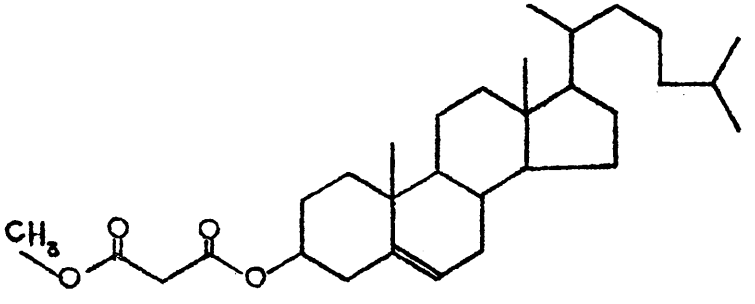
3.17

rise to the loss of 117 units of mass observed to take place from the parent molecular ion of the compound D.Q.1. Two further observations allowed differentiation to be made between the two possible solutions. If the unit (3.17) was part of the structure of the compound D.Q.1, it would be expected that the infrared spectrum of the metabolite ester would contain evidence of a bonded hydroxyl

group and furthermore, that the mass spectrum would contain an ion at m/e 87 corresponding to fission at point "b" (3.17). Since neither of these phenomena were observed it was concluded that the fragment $C_4H_6O_4$ was methoxycarbonylacetic acid (3.13) and that the compound D.Q.1 was a methoxycarbonylacetate.

As immediate confirmation of this finding it was noted that in dimethyl molonate, the protons of the methylene group are responsible for an N.M.R. signal at 6.67 tau. As has already been mentioned, the N.M.R. spectrum of the compound D.Q.1 contained a two proton singlet at 6.70 tau.

Since little literature was available relating to the spectral characteristics of unsymmetrical malonate esters, it was decided to synthesise the model system cholesteryl methoxycarbonylacetate (3.18) and compare its properties with those of the compound D.Q.1.



3.18

Thus methoxycarbonylacetyl chloride was prepared from dimethyl malonate via potassium methoxycarbonyl acetate according to the

method of Staudinger and Becher.[72] This acid chloride was then used in a pyridine catalysed esterification of cholesterol. The product, cholesteryl methoxycarbonylacetate, was a white crystalline compound which, on electron bombardment, was found to loss 118 units of mass from the parent molecular ion (cf 3.16).

The N.M.R. absorption of the methoxycarbonylacetate unit in cholesteryl methoxycarbonylacetate was identified as a three proton singlet at 6.29 tau (CH_3O-) and a two proton singlet at 6.79 tau ($CO.CH_2.CO$). The point of esterification at the 3-position in the steroid was defined in terms of a one proton multiplet at 5.40 tau. These values when compared with the low field aspects of the N.M.R. spectrum of the compound D.Q.1 provided additional confirmation that this latter compound was a methoxycarbonylacetate and suggested that the point of esterification in the main carbon skeleton was marked by a one proton multiplet at 5.30 tau.

A study of the infrared absorption of cholesteryl methoxycarbonylacetate provided some interesting and diagnostically valuable information. Comparison of the lower frequency region of its solid state spectrum with that of cholesterol itself suggested that bands at 1284, 1217, 1150, 1030 and 1014 cm^{-1} might be characteristic of the methoxycarbonylacetate unit. In this regard it was noted that the lower frequency region of the solid state spectrum of the compound D.Q.1 contained intense absorption at 1263, 1222, 1159,

1036 and 1022 cm^{-1}.

In carbon tetrachloride solution the carbonyl region of the infrared spectrum of cholesteryl methoxycarbonylacetate contained two peaks, the first at 1759 cm^{-1} (ε 570, $\Delta\nu_{\frac{1}{2}}$ 17), the second at 1739 cm^{-1} (ε 697, $\Delta\nu_{\frac{1}{2}}$ 17). This phenomenon of twin carbonyl absorption in malonate esters has previously been encountered in simple cases such as dimethyl and diethyl malonate by Abramovitch who concluded that it was caused jointly by rotational isomerism and vibrational coupling.[73]

Since the compound D.Q.1 contained a methoxycarbonylacetyl grouping which should give rise to twin infrared carbonyl absorption at approximately 1759 and 1739 cm^{-1}, the assignment of the bands in the carbonyl region of the spectrum of the compound D.Q.1 was revised as follows:-

1757 and 1736 cm^{-1}	methoxy carbonyl unit
1736 cm^{-1}	a third ester group
1716 cm^{-1}	an aliphatic or six membered alicyclic ketone.

As final proof of this portion of the structure of the compound D.Q.1, transesterification experiments were conducted. Model reactions with cholesteryl methoxycarbonylacetate showed that reaction at room temperature for twelve hours with a preparation of anhydrous sodium methoxide in dry methanol would probably be

sufficient to effect transesterification. It was found, however, that the temperature of refluxing methanol was required for the reaction to proceed satisfactorily. A portion of the reaction mixture from such an operation was analysed by gas-liquid chromatography when it was found that a substance of identical retention time to dimethyl malonate was present.

It thus seemed proved beyond all reasonable doubt that the compound D.Q.1 was a methoxycarbonylacetate. The alcoholic moiety, $C_{32}H_{52}O_4$, itself was the methyl ester of an hydroxy, keto-acid, $C_{31}H_{50}O_4$, which has been named "quercinic acid". The compound D.Q.1 is thus methyl methoxycarbonylacetylquercinate.

The positional relationship between the keto group and the carboxylic acid group in quercinic acid was deduced as a result of the study of the non-volatile products of the transesterification of methyl methoxycarbonylacetylquercinate. Thin layer chromatographic analysis of the reaction mixture revealed that three substances had been formed, their standard R_f values being 0.50, 0.36 and 0.21. These compounds were readily separated by means of light petroleum : ethyl acetate gradient elution chromatography on silicic acid.

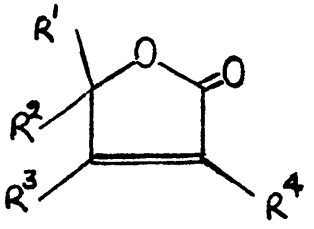
The least polar of the three materials, the compound of R_f value 0.50 was obtained as an intractible gum whose molecular weight was determined mass spectrometrically to be 500. The infrared spectrum of the substance possessed a band at 3630 cm^{-1}

which was assigned to the stretching vibration of a secondary hydroxyl group and bands at 1740 and 1716 cm^{-1} which were attributed to the carbonyl bands of a methoxycarbonyl group and an aliphatic or six membered alicyclic ketone respectively. The first two assignments were corroborated from the N.M.R. spectrum of the substance which contained a three proton signal at 6.33 tau due to the protons of a methoxyl group and a one proton multiplet at 6.58 tau attributable to a single hydrogen under an hydroxyl group. The principal fragmentation sequence induced by electron bombardment proceeded from a parent molecular ion at m/e 500 by loss of a methyl group, the elements of water and the previously encountered neutral fragment of mass 158 units to give a base ion at m/e 309.

In review, the spectral evidence appeared wholly consistant with the substance being the hydroxy, keto-ester which might be expected to arise from the demalonation of methyl methoxycarbonylacetylquercinate, that is methyl quercinate itself. However, treatment of the compound of R_f value 0.50 with excess methoxycarbonylacetyl chloride under conditions which had led to the coupling of this reagent with cholesterol, gave rise to a number of products one of which was shown to have a similar but non-superposable N.M.R. spectrum with methyl methoxycarbonylacetylquercinate. Furthermore, this product of the coupling reaction was found to be slightly more polar than the natural metabolite

ester when chromatographed on layers of silica. It is thus feasible to presume that the compound of R_f value 0.50 was an isomer of methyl quercinate and it therefore has been named methyl neoquercinate. It was presumably formed from methyl quercinate during the transesterification reaction by means of base catalysed inversion at one of the labile asymmetric centres of that molecule.

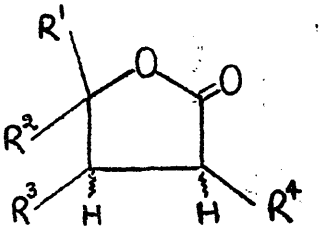
The second product to be isolated from the transesterification reaction had an R_f value of 0.36 and was a white crystalline solid of m.p. 228-229°C. Classical analysis was in accord with the molecular formula $C_{31}H_{48}O_3$ (m/e 468). The material was shown to be an alcohol by virtue of infrared absorption at 3636 cm^{-1} and an observed abundant loss of 18 units of mass from the parent molecular ion. When the infrared spectrum was recorded in chloroform solution, bands were noted at 1740 cm^{-1} (ε 956, $\Delta\nu_{\frac{1}{2}}$ 21) and 1685 cm^{-1}; when carbon tetrachloride was used as solvent, these bands moved to the higher frequencies 1764 and 1689 cm^{-1} respectively. Taken in conjunction with ultraviolet absorption at 2170 Å (ε 14,600) these facts were considered as evidence that the substance contained an α,β-unsaturated γ-lactone system such as (3.19). In harmony



3.19

with this proposal it was noted that when the compound was hydrogenated in glacial acetic acid in the presence of Adam's catalyst, a product was formed whose parent molecular ion appeared at m/e 470 and whose infrared spectrum, recorded in carbon tetrachloride solution, possessed carbonyl absorption solely at 1782 cm^{-1}. Furthermore the dihydro material was transparent in the ultraviolet. Since the above α,β-unsaturated lactone is nominally a dehydration product of quercinic acid, it has been called anhydroquercinic acid.

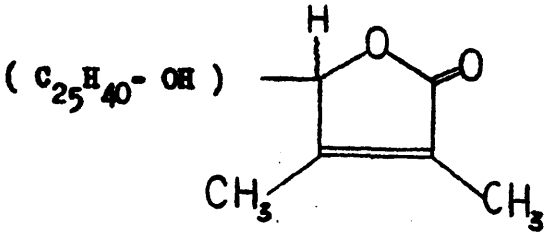
The nature of the substituents R^1, R^3 and R^4 in the formula (3.19) was deduced from a comparison of the N.M.R. spectra of anhydroquercinic acid and its hydrogenation product (3.20). The process of hydrogenation was reflected in the spectra as the movement of a one proton multiplet at 5.25 tau in anhydroquercinic



3.20

acid to a value of 6.32 tau in dihydroanhydroquercinic acid and the replacement of two broad three proton singlets at 8.03 and 8.19 tau in anhydroquercinic acid by two sets of three proton doublets in the saturated methyl area of the dihydro compound. These observations were most acceptably rationalised if, in the formulae (3.19 and 3.20) $R^1 = H$ and $R^3 = R^4 = CH_3$. It was then

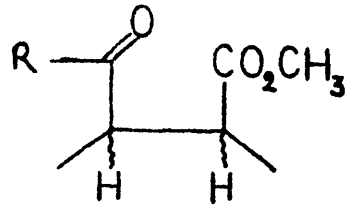
possible to represent the partial structure of anhydroquercinic acid as (3.21).



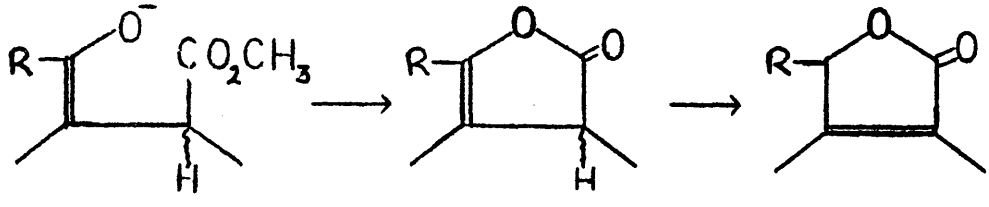
3.21

The third product of the transesterification of methyl methoxycarbonylacetylquercinate appeared to be spectroscopically identical to the first, methyl neoquercinate. However the new compound, methyl isoquercinate was found to be very instable, quickly decomposing with elimination of the elements of methanol to form the new compound isoanhydroquercinic acid which, as its name suggests, was shown by standard methods to be a butenolide isomeric with anhydroquercinic acid.

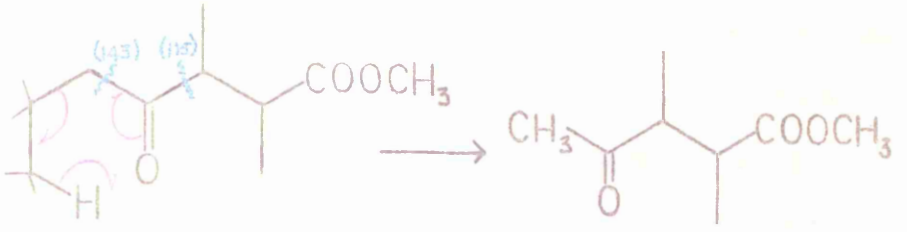
The existence of hydroxybutenolides as primary or secondary degradation products of methyl methoxycarbonylacetylquercinate provided strong evidence that in this latter compound the keto and ester functions were situated 1 : 4 to each other. Such relative disposition (3.22) would allow the following base catalysed reaction to occur converting the keto ester into an α,β-unsaturated γ-lactone.



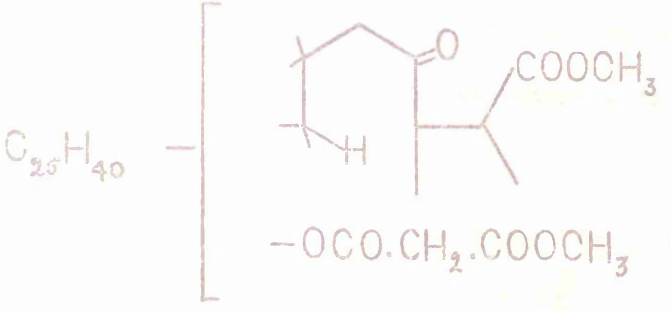
3.22



Further information concerning the immediate environment of the ketone in quercinic acid and its derivatives was obtained by consideration of the structural unit (3.22) in the light of the mass spectrum of the compound D.Q.1. It has already been noted that two major neutral molecules were elided, one of which was identified as methoxycarbonylacetic acid. The other, of mass 158, was shown to have the molecular formula $C_8H_{14}O_3$. Assuming that the three oxygen atoms present in this fragment were those of the ketone and ester and assuming further that the species was eliminated by means of "Type H" rearrangement[70] (3.23), it was possible to deduce that the ketone must have possessed an α-methylene group and at least one γ-hydrogen. The partial formula (3.24) could



3.23

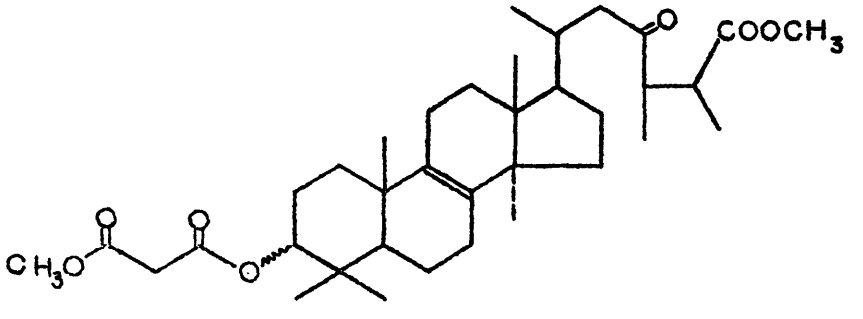


3.24

then be established for methyl methoxycarbonylacetylquercinate. The two abundant ions occurring in the mass spectrum of the compound D.Q.1 at m/e 143 and m/e 115 could be explained in terms of cleavage α to the ketonic carbonyl as shown above (3.23).

Little experimental data is available relating to the structure of the residual $C_{25}H_{40}$ unit other than that it appears to contain a tetrasubstituted double bond. This was deduced from the observation that the compound D.Q.1 gave a slow positive reaction with tetranitromethane but the N.M.R. spectrum of the metabolite contained no evidence of ethylenic proton resonance. Five of the nine double bond equivalents present in the compound D.Q.1 have

thus been accounted for and it may be deduced that a tetra-cyclic system existed in the molecule. Reviewing the facts that quercinic acid is a C_{31} compound, that its methoxycarbonylacetyl derivative was isolated from a preparation which also yielded the methyl esters of four C_{31} tetracyclic triterpenes and that the spectral properties of the compound D.Q.1 bore several close resemblances to those expected of such materials, it was tempting to propose that quercinic acid was itself a tetracyclic triterpene and that the compound D.Q.1 could be represented by structure (3.25).



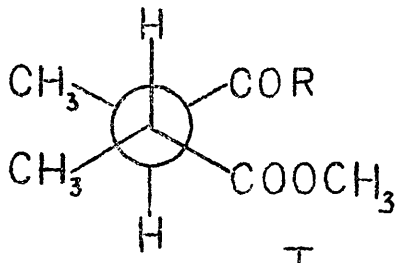
3.25

It can be easily appreciated that such a structure as (3.25) would be particularly sensitive to alkali since in addition to break-down of the malonate unit, there could occur isomerisation at each of the two asymmetric carbon atoms in the side chain, C-24 and C-25. Base catalysed lactonisation of the γ-keto-ester could also occur. The structure also explains the presence in the mass spectrum of the compound D.Q.1 of peaks at m/e 185 and m/e 153 which

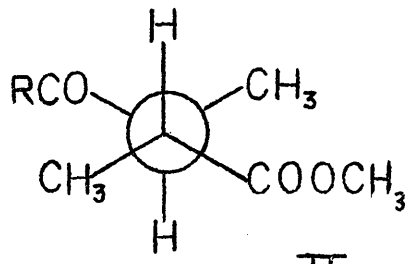
can be considered as arising by elision of the side chain, i.e. cleavage between carbon atoms C-17 and C-20, to give the ion m/e 185 which subsequently loses the elements of methanol. The following pattern of triplets encountered with the compound D.Q.1 has also been found in the mass spectra of other tetracyclic triterpenes.

189	175	161	149	135	123	109
187	173	159	147	133	121	107
185	171	157	145	131	119	105

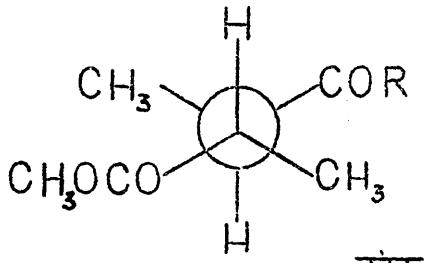
Attempts to deduce the relative stereochemistry of the side chain substituents in methyl quercinate, methyl neoquercinate and methyl isoquercinate have not met with unqualified success. The four possible isomers are shown below in their states of minimum energy.



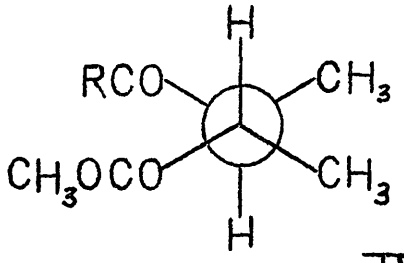
I



II



III



IV

It will be noted that in the two threo isomers (I and IV) the ketone and methoxycarbonyl groups could easily interact to form a butenolide without there arising any serious steric interaction between the methyl groups. It is thus suggested that methyl isoquercinate, which lactonises spontaneously, is one of the threo isomers. Methyl quercinate and methyl neoquercinate, which do not lactonise spontaneously, could therefore be represented by one or other of the erythro isomers (II and III) in which the process of lactonisation would be attended by eclipsing of the methyl groups. Since methyl quercinate appears to be less stable than methyl neoquercinate, models were constructed to see whether the relative stabilities of the two erythro isomers (II and III) could be predicted. Formal considerations of conformational stability, however, shed no light on the problem.

The remaining metabolites of Daedalia quercina, the compounds D.Q.6, D.Q.7, D.Q.8, D.Q.9 and D.Q.10 have only been studied in a superficial manner. They are all prone to decomposition at room temperature and tedious purifications are required immediately prior to any study. With the exception of the compound D.Q.6, all appear to be structurally more complex than methyl methoxycarbonylacetylquercinate.

The compound D.Q.6 was available only in very small amount. It was a white crystalline substance for which the analysis $C_{34}H_{52}O_5$ was obtained by mass spectrometry (parent molecular ion m/e 542).

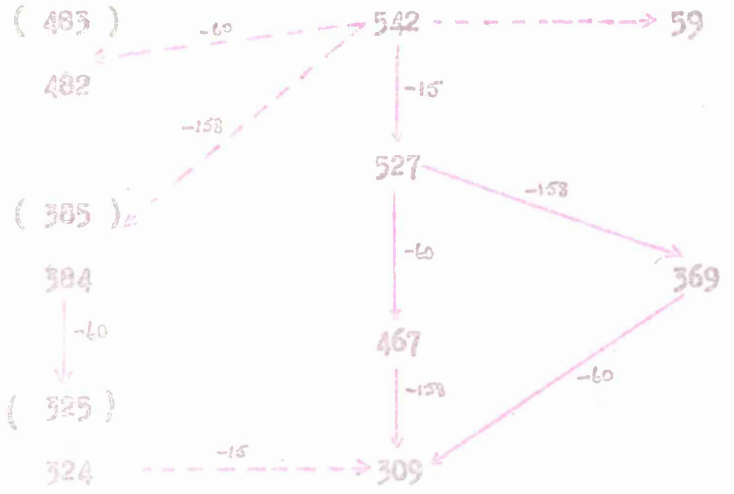
TABLE 3.9. MASS SPECTRAL DATA FOR THE COMPOUND D.Q.6

m/e	%	m/e	%
542	18.6	369	9.8
527	11.5	325	7.1
511	2.7	324	5.3
484	15.1	309	23.9
483	12.4	185	15.1
482	21.3	153	17.8
467	20.4	143	44.3
385	3.5	115	20.6
384	3.5	59	100.0

also at m/e

189	175	161	149	135	123	109
187	173	159	147	133	121	107
185	171	157	145	131	119	105
183	169					

TABLE 3.10. PROPOSED MASS SPECTRAL CRACKING PATTERN FOR THE COMPOUND D.Q.6



(Full line denotes a process for which metastable ion is available)

m*	Transition
512.4	542-527
413.5	527-467
274.5	385-325
258.7	369-309
258.5	527-369
204.4	467-309
126.5	185-153

P.B.6 362 mg.

33. METHYL 3-(4-METHOXYCARBONYL-3-HYDROXY-3-METHYLBUTYRYL)-POLYPORENATE A (4.19). THE COMPOUND P.B.4

The material was isolated as detailed above, the substance being eluted from the silica with ethyl acetate, treated with decolourising charcoal and crystallised from a light petroleum : ethyl acetate system as needles (900 mg.), m.p. 79-81°C.

ν_{max} (KBr) 3598, 3510 (broad), 3084, 2830, 1742, 1726, 1705, 1647, 892 cm^{-1}.

ν_{max} (1.15 mM. CCl_4) 3638 cm^{-1} (ε 55.5, $\Delta\nu_{\frac{1}{2}}$ 14), 3528 cm^{-1} (ε 55.5, $\Delta\nu_{\frac{1}{2}}$ 80), 1742 cm^{-1} (ε 925, $\Delta\nu_{\frac{1}{2}}$ 20), 1715 cm^{-1} (ε 388 sh.) also 3090, 2833 and 1646 cm^{-1}.

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 5.12 (2H, s), 5.30 (1H, t, J=2.4 c/s.), 6.02 (1H, d, J=7.2 c/s.), 7.40 (4H, broad s), 8.70 (3H, s), 8.75 (3H, d, J=7.2 c/s.).

Molecular parent ion at m/e 658 undetected; first peak in mass spectrum at m/e 640 (P - 18).

Analysis	Found	C 71.19 %	H 9.25 %
$C_{39}H_{62}O_8$	requires	C 71.09 %	H 9.48 %

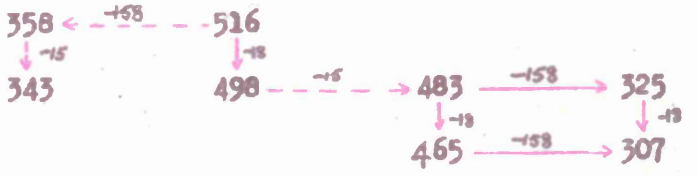
Infrared absorption at 1738 cm^{-1} (ε 941, $\Delta \nu_{\frac{1}{2}}$ 27) and 1718 cm^{-1} (ε 523, $\Delta \nu_{\frac{1}{2}}$ 22) suggested that the compound was a keto-diester From the presence of an intense band at 1246 cm^{-1} in the infrared, it was inferred that one of the esters was an acetate - a finding which was confirmed by the existence in the mass spectrum (Tables 3.9 and 3.10) of losses of 60 units of mass from the parent ion, the P-15 ion and the P-(15 + 158) ion. The presence in the compound D.Q.6 of structural features which could give rise to major ions in the mass spectrum at m/e 309, 185, 153, 143 and 115 and to the elision of a neutral fragment of mass 158 units suggested that the compound D.Q.6 was closely related to methyl quercinate. However, no chemical corroboration is at present available that the compound D.Q.6 is, in fact, methyl acetylquercinate.

The compound D.Q.10 has not been obtained in an high state of purity, nevertheless some interesting structural elucidation has been possible. The consensus of infrared and N.M.R. spectral evidence was that the substance was the methyl ester of a dihydroxy keto-acid. From the features of the mass spectral cracking pattern shown in Table 3.11, it appeared probable that the compound D.Q.10 was methyl hydroxyquercinate or an isomer thereof since the characteristic loss of 158 units of mass and ions at m/e 185, 153, 143, 115 were clearly discernible. A limited amount of information is available relating to the site of this hydroxyl group. A one proton singlet at 5.93 tau in the N.M.R. spectrum of the compound

TABLE 3.11. MASS SPECTRAL DATA FOR THE COMPOUND D.Q.10.

m/e	%	m/e	%
516	23.9	325	43.5
498	27.1	307	22.8
483	100.0	246	30.4
465	26.0	185	26.0
358	22.6	153	47.8
343	10.9	143	45.6
341	19.5	115	17.4

m*	Transition
480.5	516-498
447.8	483-465
289.9	325-307
218.7	465-307
202.6	483-325
126.5	185-153



revealed that it is secondary and, since it did not appear in the side chain fragment of mass 185 units, it must be attached to the tetracyclic nucleus. If it can be assumed that structure (3.25) is in fact valid for methyl methoxycarbonylacetylquercinate, the only possible sites for secondary hydroxylation are carbon atoms C-1, C-2, C-6, C-11, C-12, C-15 and C-16. Since analogies are available for the hydroxylation of fungal tetracyclic triterpenes at carbon atoms C-12, C-15 and C-16, these latter sites seemed more probable. As encountered in other triterpene systems, considerations of splitting patterns proved of little use. However, it may be significant to mention in relation to the value of 5.93 tau obtained for the hydrogen atom in the compound D.Q.10, that the literature values for hydrogens under 12α, 15α and 16α hydroxyl groups are 6.04, 5.73 and 5.90 tau respectively (cf Tables 3.4 and 4.3).

The compound D.Q.9 was also obtained as an intractable gum. Preliminary spectroscopic analysis showed that the easily detected features of a methoxycarbonylacetate unit were present in the substance. Since the parent molecular ion occurred at m/e 616 and since the N.M.R. spectrum of the compound closely resembled that of the compound D.Q.10, it was probable that the metabolite D.Q.9 is the methoxycarbonylacetate of the compound D.Q.10, that is methyl methoxycarbonylacetylhydroxyquercinate.

The two remaining metabolites, the compounds D.Q.7 and D.Q.8 were also shown to be methyl malonate esters. Both compounds were very closely related, possessing almost identical mass spectra

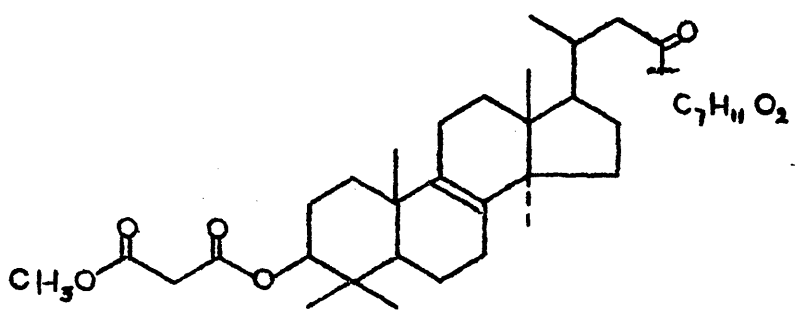
and differing only very little in chromatographic R_f value. On decomposition with base, both compounds give rise to the same T.L.C. pattern of decomposition products and it therefore seems probable that they were, in fact, stereoisomers. For this reason only the properties of the compound D.Q.7 have been examined in detail.

The compound D.Q.7, m.p. 137-138°C was a white crystalline substance whose parent molecular ion at m/e 612 analysed uniquely for $C_{37}H_{56}O_7$. Since it was known to be a methoxycarbonylacetate, the molecular formula of the alcoholic residue must be $C_{33}H_{52}O_4$. N.M.R. and infrared absorption indicated that the residue was the methyl ester of an hydroxy-keto-acid. This being the case, it will be seen that the new metabolite contained a carbon skeleton of 32 atoms, two more than that of the basic triterpenes.

As yet no definite structure has been established for the alcoholic residue of the compound D.Q.7 although it seems certain that it is closely related to methyl quercinate.

It was noted at an early stage in the study of the compound D.Q.7 that its mass spectrum was identical to that of the compound D.Q.1 save for the fact that the peaks which could be attributed to the fragmentation processes involving the side chain were all shifted 12 units to higher mass. Thus the neutral fragment of mass 158 units found in the mass spectrum of the compound D.Q.1 appeared as a loss of 170 units in the compound D.Q.7 while the

ions m/e 185, 153, 143 and 115 in the spectrum of the compound D.Q.1 were replaced in that of the new metabolite by ions at m/e 197, 165, 155 and 127. This observation was taken as evidence that the compounds D.Q.1 and D.Q.7 were structurally identical in rings A, B, C and D and, at least, to that point in the side chain from which the ions of mass m/e 115 and m/e 127 were derived, that is to the ketone bearing carbon atom C-24. A tentative partial structure for the compound D.Q.7 would then be (3.26).



3.26

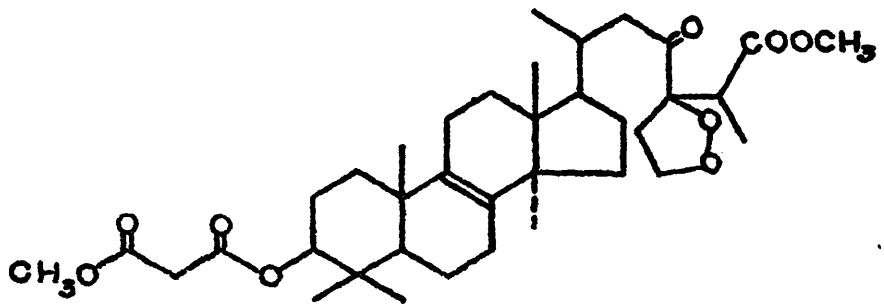
The unit $C_7H_{11}O_2$ must contain the methoxycarbonyl group present in the compound D.Q.7 and its detailed structure should be derivable from a study of those spectral differences which existed between the two compounds D.Q.1 and D.Q.7. It was particularly noticeable that the compound D.Q.7 differred from the compound D.Q.1. in possessing an N.M.R. two proton multiplet at 5.50 tau and in exhibiting intense O.R.D. at 3280Å ($\bar{a}$ 16,200). Despite numerous attempts, no feasible structure could be established for the entity $C_7H_{11}O_2$ which was in keeping with these spectroscopic observations.

The most acceptable rationale of the two pieces of spectroscopic data mentioned above was that the compound D.Q.7 possessed the structural element $-CH_2-CH_2-O-$ and that an oxygen atom was present α to the ketone group as part of an highly asymmetric unit. However, all the oxygen atoms present in the mass spectrally derived formula $C_{37}H_{56}O_7$ have already been successfully assigned and it must therefore be concluded that the ion m/e 612 = $C_{37}H_{56}O_7$ is not, in fact, the parent molecular ion but is derived from a more highly oxygenated species whose molecular ion is not discernible in the mass spectrum.

It was noted that the molecular formula $C_{37}H_{56}O_9$ was more compatible with the results of the microanalysis than was the previously accepted formula $C_{37}H_{56}O_7$:-

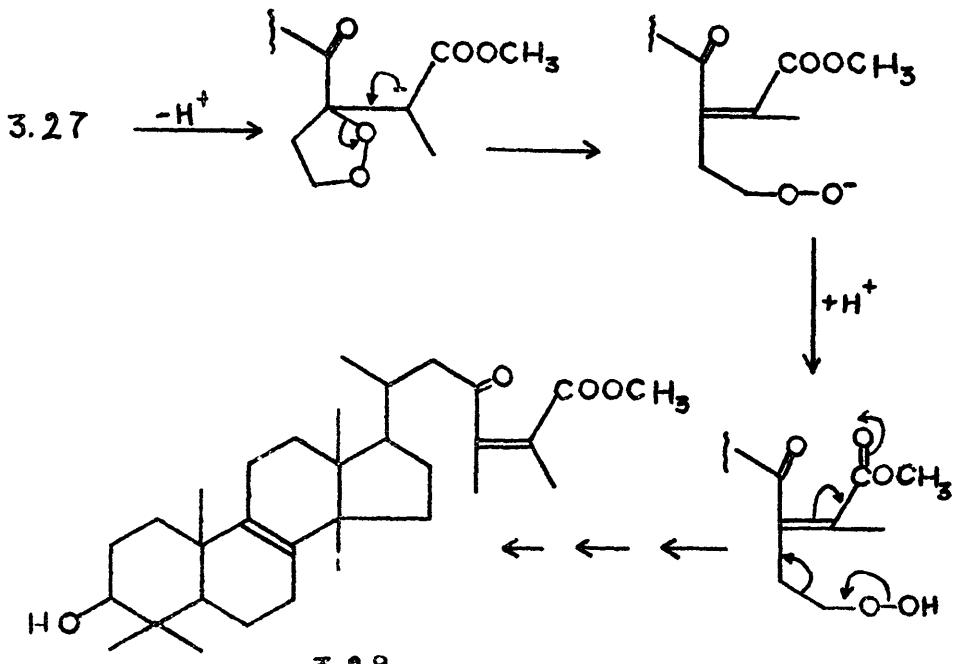
	C %	H %
Found	69.65	8.55
$C_{37}H_{56}O_7$ requires	72.52	9.21
$C_{37}H_{56}O_9$ requires	68.92	8.75.

On the basis of this revised molecular formula, structure (3.27) is proposed as a working hypothesis for the nature of the compound D.Q.7. It provides some measure of explanation for the observed N.M.R. and O.R.D. spectral phenomena and may conceivably lose the elements of molecular oxygen thermally in the mass spectrometer.



3.27

The above structure is not inconsistant with the results of preliminary experiments on the demalonation of the compound D.Q.7. T.L.C. of the product obtained from such a reaction suggested that only two substances had been formed. However, mass spectrometric analysis of a crystalline sample of one of these "substance" revealed that it was a mixture of at least six compounds whose parent molecular ions were detected at m/e 498, 500, 510, 512, 514, 526 and 528. The most abundant of these ions, that at m/e 498, may correspond to the species (3.28) which could be derived from structure (3.27) as shown.



3.28

It must be emphasised, however, that these proposals are highly speculative and much more detailed study will be required before any firm conclusions relating to the structures of the compounds D.Q.7 and D.Q.8 can be made.

In summary it can be said that the analysis of the methyl esters of the principal acidic metabolites present in the sporophore of Daedalia quercina had led to the isolation of methyl polyporenate C, methyl 7,11-dihydropolyporenate C, methyl tumulosate and methyl 7,11-dehydrotumulosate together with four new, apparently tetracyclic, triterpene acids. All four of these latter materials occurred as their malonate esters and, in addition, one was

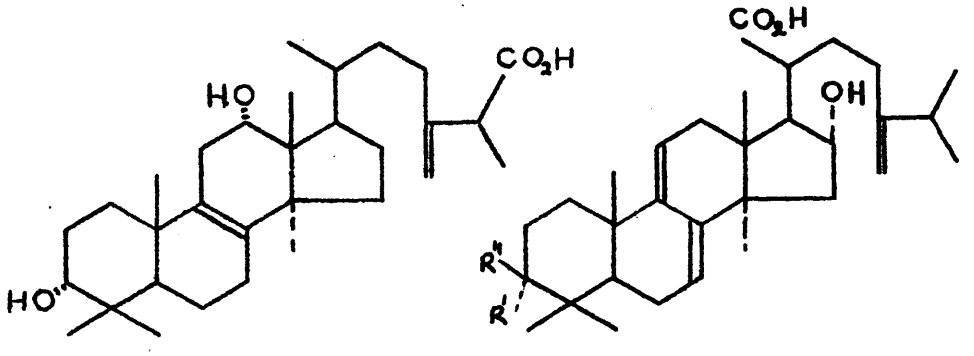
encountered as its acetate and another in the free state. In the text which follows such compounds as contain triterpene nuclei esterified with small organic acids will be referred to as "triterpene conjugates".

Recently Daedalia quercina has been successfully grown in pure culture on a synthetic medium. T.L.C. and N.M.R. analyses of an extract of the mycelium produced indicated that all the compounds save D.Q.6 were present. Under the conditions of the assay, however, the small amount of this latter substance which might have been present would not be detected.

CHAPTER 4

THE METABOLITES OF POLYPORUS BETULINUS

The most significant result of the foregoing work was the demonstration of the presence in Daedalia quercina of triterpene acids esterfied with small organic molecules such as acetic and malonic acids - the isolation of these conjugates necessarily involving mild non-hydrolytic methods. These observations raised the question whether such conjugates occur more generally in triterpene producing fungi, their chemical sensitivity being the cause of their non-isolation in previous analyses. With this possibility in mind, the great, white "tinder fungus", Polyporus betulinus (Ungulina betulina) was subject to intensive examination. As has been stated in Chapter 1, Polyporus betulinus is an excellent source of the tetracyclic triterpenes, polyporenic acids A, B and C (4.1, 4.2, 4.3).



4.1

4.2 R'=H, R"=OH.

4.3 R',R"=O.

Fresh, young sporophores of Polyporus betulinus were collected in mid-Autumn from the birch woods around Milngavie and were homogenised in methanol within six hours of harvesting. After

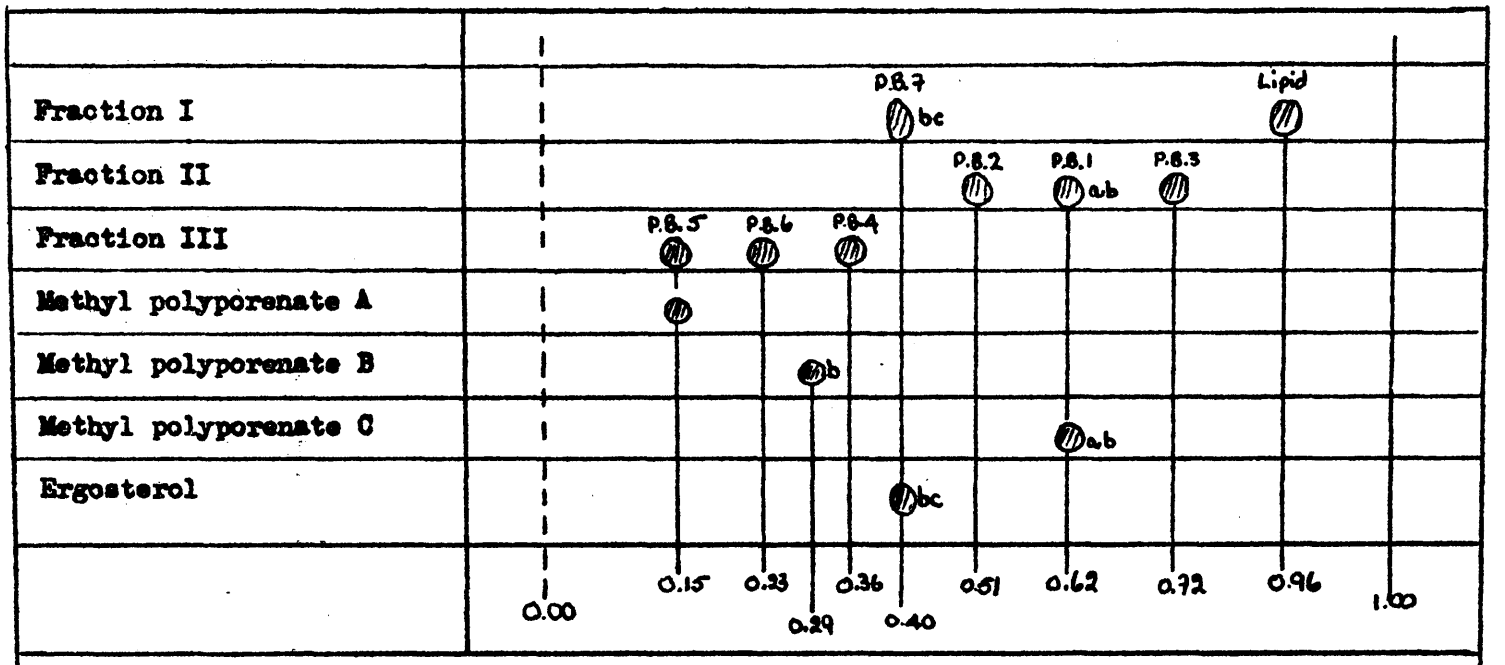
extraction for eight weeks at room temperature, the methanol extract was evaporated and the fat content of the oily residue reduced by extraction with low boiling light petroleum. The petrol insoluble fraction was subsequently esterified with diazomethane and the product fractionated on a column of silica employing light petroleum: ethyl acetate gradient elution.

As a result of systematic thin layer chromatography (T.L.C.), the eluant was divided into three fractions. Details of the T.L.C. behaviour of these three fractions (I, II, III) are presented in Table 4.1, together with the corresponding data for ergosterol and the methyl esters of the known metabolites of Polyporus betulinus, polyporenic acids A, B and C. In accordance with the system employed in the analysis of the constituents of Daedalia quercina, the metabolites of Polyporus betulinus were given the code names P.B.1, P.B.2, P.B.3, P.B.4, P.B.5, P.B.6 and P.B.7. The structures of three of these metabolites were immediately suggested by virtue of a correspondence in R_f value and staining behaviour on thin layers between the compound P.B.1 and methyl polyporenate C, the compound P.B.5 and methyl polyporenate A and the compound P.B.7 and ergosterol respectively. In this preliminary analysis no trace of methyl polyporenate B could be detected.

After standing at room temperature for several days, long slender needles of the compound P.B.7 were deposited from the ethyl acetate solution of Fraction I. The spectral and chroma-

TABLE 4.1. R_f VALUES AND STAINING BEHAVIOUR OF P.B. METABOLITES

Solvent 1 % CH_3OH in $CHCl_3$



STANDARDS

p-Amino-azobenzene R_f 0.67

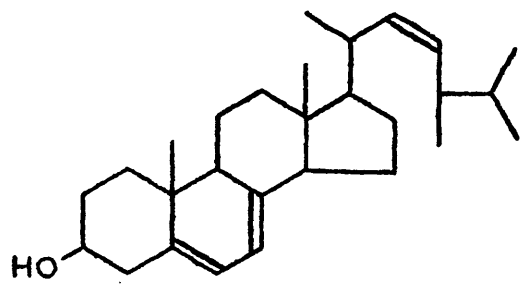
p-Hydroxy-azobenzene R_f 0.35

a positive test with D.N.P

b purple stain with rhodamine 6G

c intensely black spot with ceric reagent

tographic properties of this product were found to be in close accord with the data quoted for ergosterol (4.4)[54]. The identity



4.4

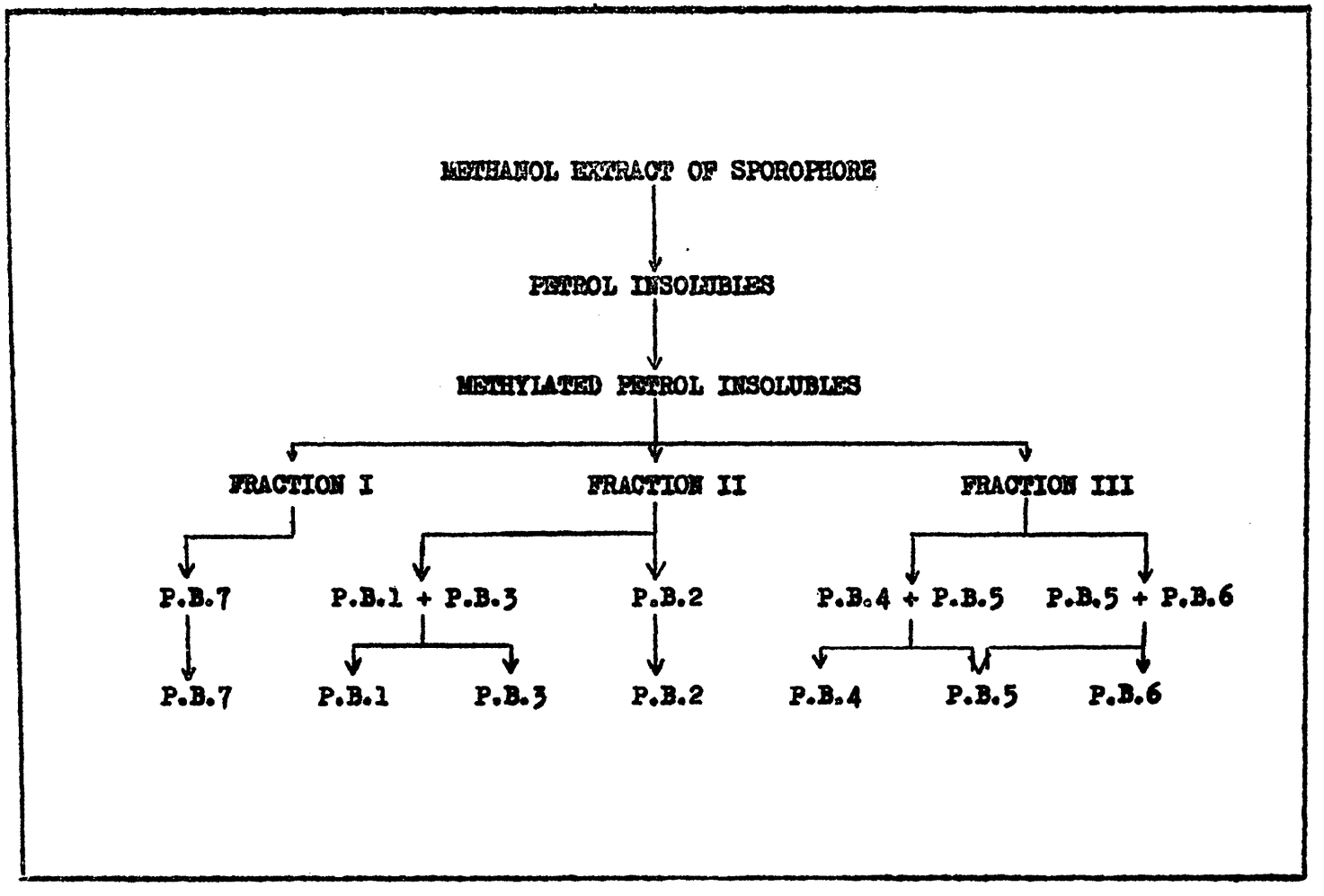
of the compound P.B.7 was confirmed by mixed melting point and infrared spectral comparison with an authentic sample of ergosterol.

Ergosterol has previously been isolated from the fruiting body of Polyporus betulinus by Jones.[74] This sterol occurs very widely in the fungal world and therefore seems likely to have some important role to play in mould metabolism.

The separation techniques employed in isolating the six remaining metabolites are summarised in Table 4.2. Gradient elution chromatography of Fraction II on silicic acid succeeded in separating the compound P.B.2 from the compounds P.B.1 and P.B.3. Fractional crystallisation of this latter mixture led to the isolation of the substance P.B.1, while preparative layer chromatography of the crystallisation mother liquors afforded the substance P.B.3 in a state of purity.

Fraction III was treated in similar fashion. Gradient

TABLE 4.2. SEPARATION OF THE P. BETULINUS METABOLITE ESTERS



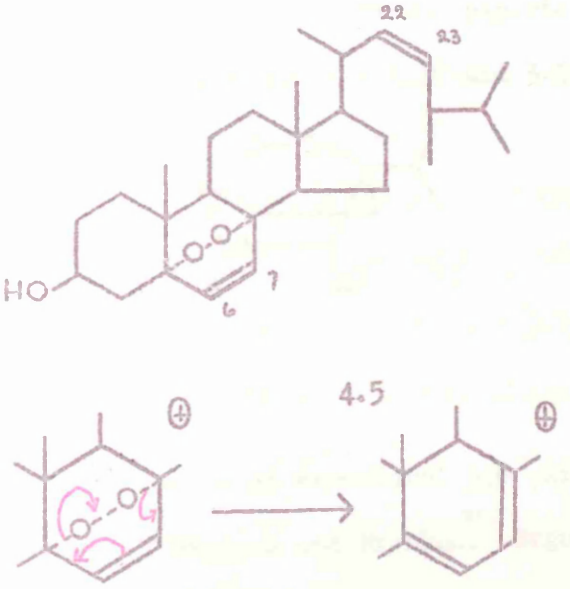
elution chromatography on neutral alumina gave two binary mixtures, namely of the compounds P.B.4 and P.B.5 and of the compounds P.B.5 and P.B.6. Despite numerous attempts using finely adjusted gradients on both alumina and silicic acid, column chromatography did not resolve either of these mixtures. Separation was finally achieved by means of preparative layer chromatography on large rhodamine-treated plates adopting the technique of multiple elution. By this means pure samples of the species P.B.4, P.B.5 and P.B.6 were obtained.

The melting points and molecular formulae of the six metabolites as determined by micro-analysis, and mass spectroscopy were as follows:-

P.B.1	193-194°C	$C_{32}H_{46}O_4$
P.B.2	88-89°C	$C_{36}H_{56}O_7$
P.B.3	130-132°C	$C_{34}H_{52}O_5$
P.B.4	79-81°C	$C_{39}H_{62}O_8$
P.B.5	148-149°C	$C_{32}H_{50}O_4$
P.B.6	177-178°C	$C_{28}H_{44}O_3$

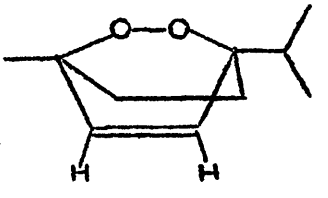
Systematic structural analysis commenced with the simplest member of the group, the compound P.B.6. This white crystalline substance possessed no carbonyl groups nor any chromophore capable of absorbing in the visible or ultraviolet. It was noted that its molecular formula corresponded to the introduction of two atoms of oxygen into the formula of ergosterol ($C_{28}H_{44}O$). A possible relationship between the compound P.B.6 and ergosterol was suggested

by the fact that both substances were rapidly oxidised to intensely black spots when their thin layer chromatograms were developed with ceric ammonium nitrate-sulphuric acid. Furthermore, the mass spectral cracking pattern of the compound P.B.6 differed from that of ergosterol solely in the addition of two peaks at m/e 410 and m/e 428. These observations could be rationalised if the compound P.B.6 were ergosterol 5,8-peroxide (4.5) or a structural isomer thereof. Such a substance would have no ultraviolet absorption above 2200 Å and, on electron bombardment, could lose molecular oxygen by a "Type D"[70] rearrangement of the parent ion (4.6 to 4.7). It will be noted that the ion (4.7) is the parent molecular ion of ergosterol.



The peak at m/e 410 results from the elimination of the elements of water from the m/e 428 parent ion.

The N.M.R. spectrum of the compound P.B.6 was consistent with its being that of ergosterol peroxide. In the low field region of the spectrum two independent sets of ethylenic absorption were detected. The first system, centred at 4.80 tau with a coupling constant of 6 c/s., was identified by comparison with the spectrum of ergosterol as the ethylenic protons of carbon atoms C-22 and C-23 (4.5). The remaining set of peaks constituted a well-defined A.B. system centred at 3.56 tau and 3.83 tau with a coupling constant of 7.8 c/s. and was readily accommodated by the hydrogens on carbon atoms C-6 and C-7 of ergosterol peroxide. These values are in close agreement with the reported data for the vinylic protons of ascaridole (4.8) - 3.58 and 3.53 tau and



4.8

a coupling constant of 7.8 c/s.[75]

For comparison, a sample of ergosterol 5,8-peroxide was prepared by the method of Windaus and Brunken.[76] Ergosterol was irradiated at room temperature in the presence of molecular oxygen and a small amount of eosin - this latter substance acting as a photo-sensitising dye in the coupling of oxygen with the ergosterol

diene system. Spectral, chromatographic and mixed melting point comparison of the synthetic sample with the compound P.B.6 provided final evidence of identity.

Ergosterol peroxide has previously been reported as a metabolite of Aspergillus fumigatus[77] and Trichophyton schonleini[78]. Considering its ease of production by photo-catalysed oxidation of ergosterol, it is surprising that none of the authors concerned seriously considered the possibility that ergosterol peroxide might be an artefact. In examining this situation we have discounted the possibility of autoxidation during chromatography or crystallisation since many other ergosterol containing fungi have been subject to similar methods without the appearance of ergosterol peroxide.

It was, however, feasible that Polyporus betulinus contained a substance capable of promoting the photo-oxidation of endogenous ergosterol during the extraction process. To test this, a sample of the methanol extract of a Polyporus betulinus sporophore was supplemented with pure ergosterol and irradiated under high oxygen tension. Over a period of twelve hours, the concentration of ergosterol peroxide was observed to rise from an initial value of less than one per cent to approximately eighty per cent; in a control experiment involving ergosterol, pure methanol and oxygen only a trace of the peroxide was detected after a similar reaction period. It must therefore be concluded that Polyporus betulinus does in

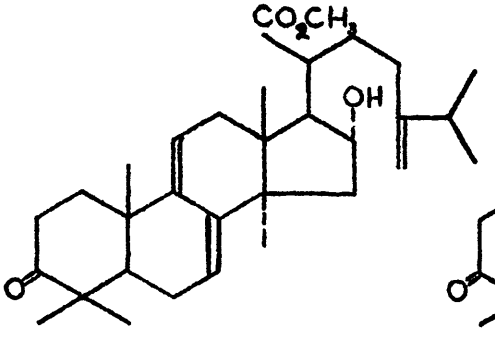
fact produce a material capable of simulating the photo-sensitising effect of eosin.

This observation casts new light on the question of the metabolic authenticity of the isolated peroxide since it must now be accepted that non-enzymic, photo-catalytic processes will make a contribution to the synthesis of the compound. The extent of this contribution was determined as follows. A section of the context of a fresh sporophore was frozen by immersion in liquid nitrogen and quickly pulverised. The resultant fine powder was extracted in cold methanol for three minutes and the extract immediately assayed for ergosterol and its peroxide by T.L.C. Only the former compound could be detected; no trace of the peroxide was apparent.

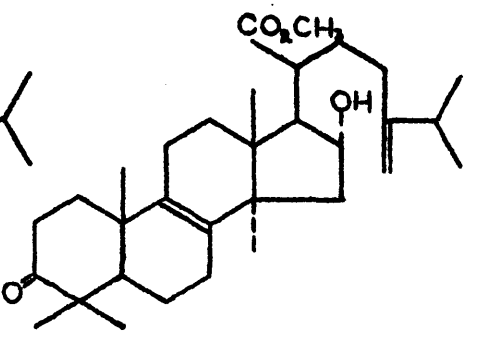
It thus appears that the ergosterol peroxide isolated from the fruiting bodies of Polyporus betulinus is an artefact. It had been produced during the extraction process by the joint action on ergosterol of molecular oxygen and some other metabolite of the organism which was able to effect photo-catalysis. In view of this finding it would be of interest to reinvestigate the production of ergosterol peroxide by Aspergillus fumigatus and Trichophyton schonleini.

The preliminary T.L.C. analysis had indicated that the compounds P.B.1 and P.B.5 might be methyl polyporenate C and A respectively. These indications were subsequently confirmed as will now be described. The compound P.B.1, $C_{32}H_{46}O_4$, possessed

spectral properties identical to those of methyl polyporenate C (4 .9) and its melting point, 193-194°C was undepressed by



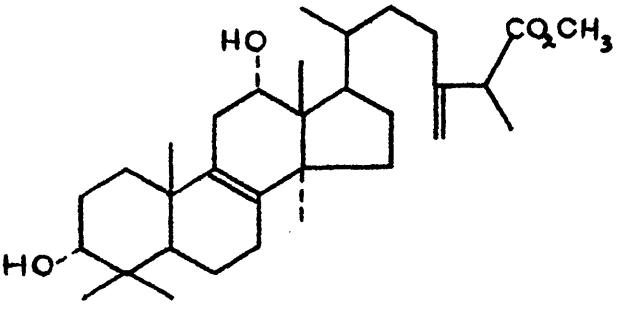
4.9



4.10

admixture with an authentic sample of that material. However, mass spectrometric analysis indicated that the substance P.B.1 was methyl polyporenate C contaminated by approximately 12 % of a dihydrocompound, presumably the 8(9)-mono-ene (4.10). In this respect it resembled the substance previously isolated from Daedalia quercina.

The compound P.B.5 had an analysis in accord with the formula $C_{32}H_{50}O_4$ and was shown to be methyl polyporenate A (4.11)[63]



4.11

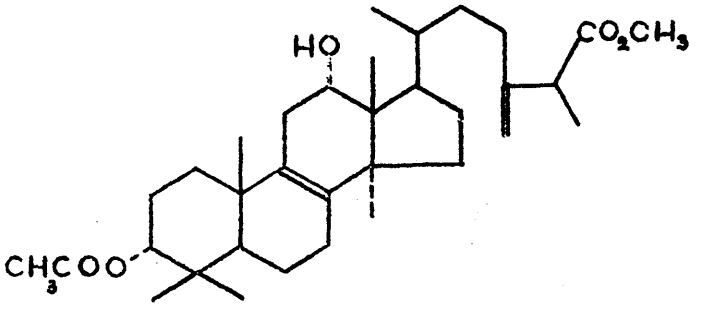
on the basis of the physical measurements described below. In the infrared, the two hydroxyl groups of the compound P.B.5 absorbed at 3639 cm^{-1} (ε 93.2, $\Delta\nu_{\frac{1}{2}}$ 18) while the methoxycarbonyl system gave rise to peaks at 2832 cm^{-1} and 1744 cm^{-1} (ε 510, $\Delta\nu_{\frac{1}{2}}$ 23). Further absorption at 3090, 1648 and 900 cm^{-1} was attributable to the exocyclic methylene group. This latter functional unit was reflected in a two proton N.M.R. signal at 5.10 tau. Additional features of the N.M.R. spectrum were the singlet at 6.33 tau due to the protons of the methyl ester and the resonances at 6.90 (quartet) and 8.75 (doublet) produced by the hydrogen and methyl group at carbon atom C-25.

Two sets of peaks at 6.63 and 6.04 tau both integrated for one proton and were assigned to the hydrogens under the hydroxyl groups at carbon atoms C-3 and C-12. By analogy with the value of 6.60 tau observed for the 3β hydrogen in methyl 3α, 16α-dihydroxy-24-methylenelanost-7,9(11)-diene-21-oate (Experimental Section 7), the peak at 6.63 tau in the spectrum of the compound P.B.5 could be assigned to the 3β hydrogen. As confirmation of this assignment a sample of the compound P.B.5 was acetylated under conditions which, applied to methyl polyporenate A, yielded solely the 3-acetate.[53] The N.M.R. spectrum of the acetylated product was wholly consistant with its structure being methyl 3-acetylpolyporenate A (4.12). It contained no absorption at 6.63 tau but contained a new peak at 5.35 tau. From these

observations it could be concluded that the 3β hydrogen of methyl polyporenate A gave rise to an ill-defined triplet at 6.63 tau while the 12β hydrogen appeared as a doublet centred at 6.04 tau. This latter value falls into the range 5.59 to 6.12 tau quoted by Smith for the 12β hydrogen of the 12α-hydroxy bile acids.[99]

The mass spectra of isolated and authentic samples of methyl polyporenate A were identical and showed the loss of the elements of water and a methyl group from the parent ion to give the base peak at m/e 467.

The substance P.B.3 represented the next most complex molecular system. The infrared spectrum of this substance was indicative of a system containing one hydroxyl group (3631 cm^{-1}, ε 54.5, $\Delta\nu_{\frac{1}{2}}$ 16), two alkoxycarbonyl groups (1739 cm^{-1}, ε 845, $\Delta\nu_{\frac{1}{2}}$ 23) and an exocyclic methylene group (3080, 1640, 890 cm^{-1}). During the examination of the compound P.B.5, the 3-acetate of methyl polyporenate A (4.12) had been prepared.



4.12

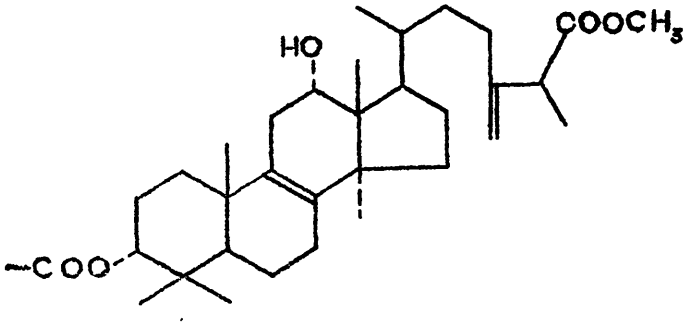
It was noted that the N.M.R. spectrum of the substance P.B.3 was virtually identical with the spectrum of this acetate. Furthermore, the micro-analysis of the substance P.B.3 corresponded to the formula of methyl 3-acetylpolyporenate A ($C_{34}H_{54}O_5$). The T.L.C. R_f values of the substance P.B.3 and of methyl 3-acetylpolyporenate A were identical and also the infrared spectra were superposable. The only property of the substance P.B.3 which was not consistant with its being methyl 3-acetylpolyporenate A was a melting point discrepancy of over ten degrees with the synthetic material. Despite numerous preparative layer chromatograms and repeated crystallisations from various solvent systems, the melting point of the isolated metabolite could not be elevated to the literature value (138-139°C).

These observations could be readily rationalised on the basis of the substance P.B.3 being a mixture in which methyl 3-acetylpolyporenate A was the major component. This was confirmed by mass spectroscopy which showed that, in addition to the parent molecular ion at m/e 542 expected of methyl 3-acetylpolyporenate A, there occurred a further parent ion at m/e 598. The ratio of acetate to contaminant was estimated from the mass spectrum to be 87 : 13.

The problem of the chemical constitution of the contaminant of mass 598 was now tackled. Comparison of the infrared spectra of the mixture P.B.3 and methyl 3-acetylpolyporenate A showed no

significant differences between the two; examination of the N.M.R. spectra of the two metabolites revealed only small divergences in the saturated methylene region. From that region of the mass spectrum which was not contaminated by the fragmentation pattern of the acetate it could be seen that the minor component suffered loss of a methyl group and the elements of water from the parent ion. The peak structures and relative abundances of the ions at m/e 598, 583, 580 and 565 closely resembled the first four ions in the spectrum of methyl 3-acetylpolyporenate A. Numerous attempts to resolve the mixture by normal T.L.C. methods or by the "wedge" modification[80] were uniformly unsuccessful.

In view of the evident similarity in chromatographic and spectral properties which existed between the two components of the mixture, it seemed probable that the minor component was also an ester of methyl polyporenate A. On the basis of this assumption it was possible to suggest the partial structure (4.13)



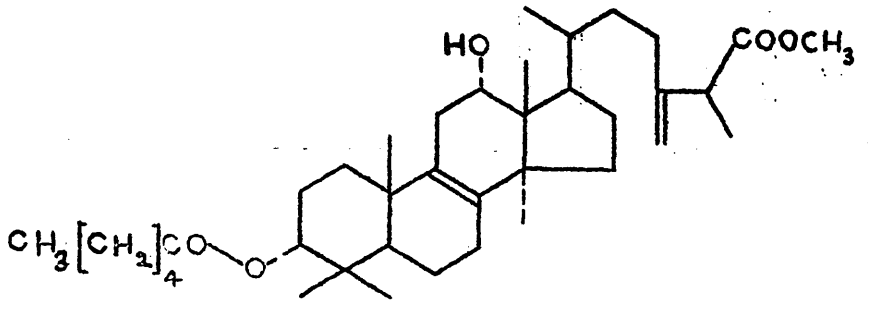
4.13

which accounted for 527 units of mass. Since the mixture P.B.3 gave negative tests for nitrogen, sulphur and halogen, the remaining 71 units of mass must be defined by one of the following formulae:-

C_5H_{11} C_4H_7O $C_3H_3O_2$

Of these possibilities the last two seemed a little less probable since additional oxygen functions might be expected to confer on the whole molecule a polarity greater than that of methyl 3-acetylpolyporenate A; it had already been noted from T.L.C. work that the components of the P.B.3 mixture were closely allied in polarity. The remaining formula, C_5H_{11}, corresponded to the esterification of methyl polyporenate A by the fatty acid $C_6H_{12}O_2$. Thus a sample of the substance P.B.3 was treated with anhydrous sodium methoxide in dry methanol and a portion of the product analysed for C_6 methyl esters by gas-liquid chromatography using a temperature programme which would uniquely define the normal series of fatty acid methyl esters. Under those conditions a peak corresponding to methyl caproate was detected in the transesterification product. This established that a caproate ester must have been present in the substance P.B.3. T.L.C. of a sample of the reaction mixture against authentic methyl isopolyporenate A (4.16, cf later discussion) indicated that material of the same R_f value as methyl isopolyporenate A was the sole non-volatile product of the methanolysis. It must be emphasised, however, that the proof that

the minor component of the P.B.3 mixture was methyl 3-caproylpolyporenate A (4.14) is not as rigorous as would be desired.



4.14

The N.M.R. spectrum of a methyl polyporenate A 3-ester proved to be characteristic. The salient features are presented as a line diagram in Table 4.3. The 3β hydrogen comes into resonance as an ill-defined triplet at 5.33 tau with an estimated coupling constant of 2.8 c/s. The C-24 exocyclic methylene group gives rise to a fine multiplet centred at 5.10 tau while the methoxycarbonyl methyl group appears as a three proton singlet at 6.32 tau. As has already been proved, the 12β hydrogen appears as a doublet at 6.04 tau. The hydrogen and methyl group at carbon atom C-25 are responsible for a quartet-doublet pattern at 6.90 and 8.76 tau respectively, the coupling constant being 7.2 c/s. Complex absorption due to the saturated methyl groups is defined by peaks at 8.91, 9.01, 9.04, 9.11 and 9.42 tau.

Analysis of the N.M.R. spectrum of the two remaining metabolites

TABLE 4.3. N.M.R. SPECTRUM OF A METHYLPOLYPORENATE A 3-ESTER

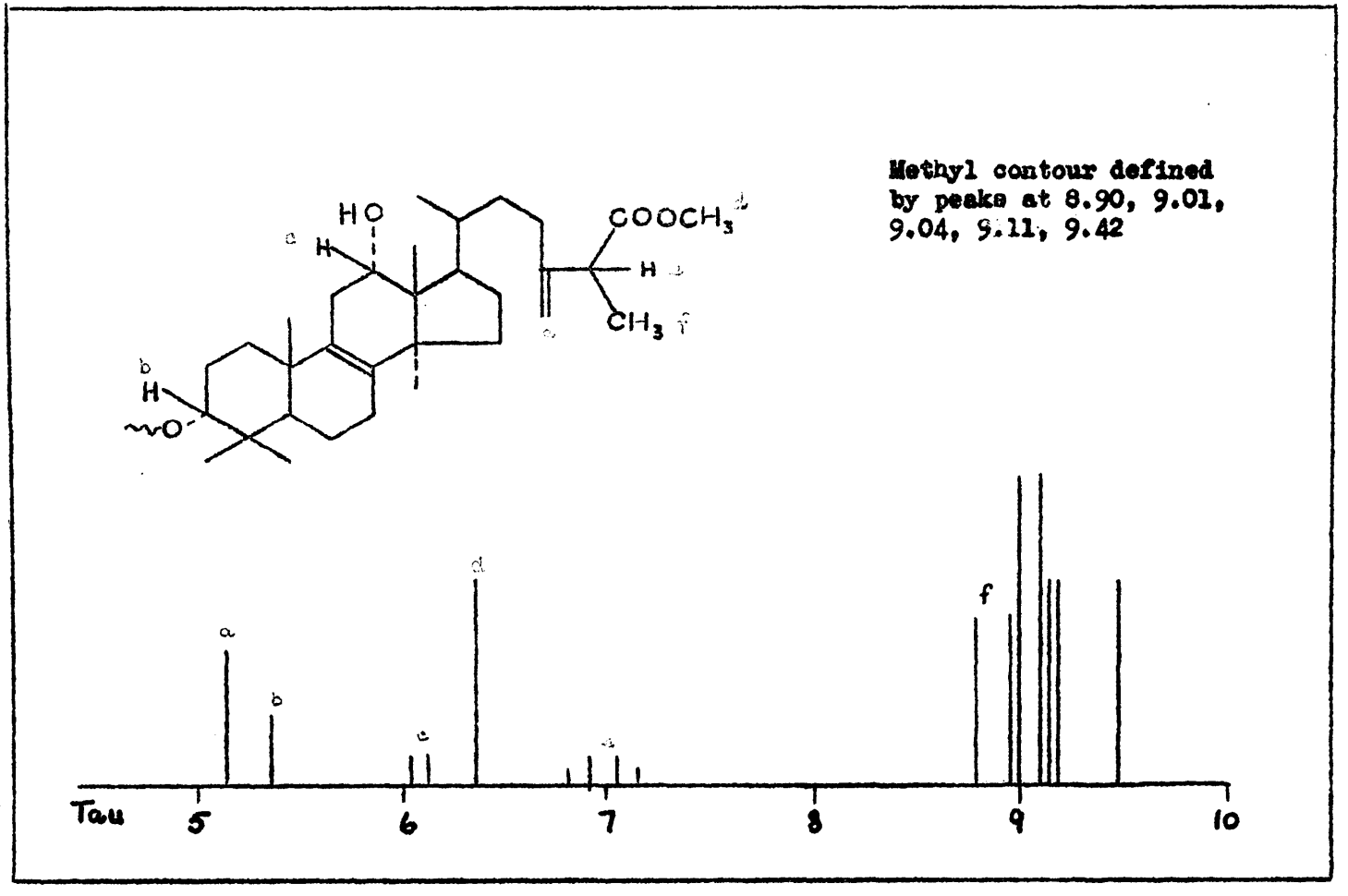
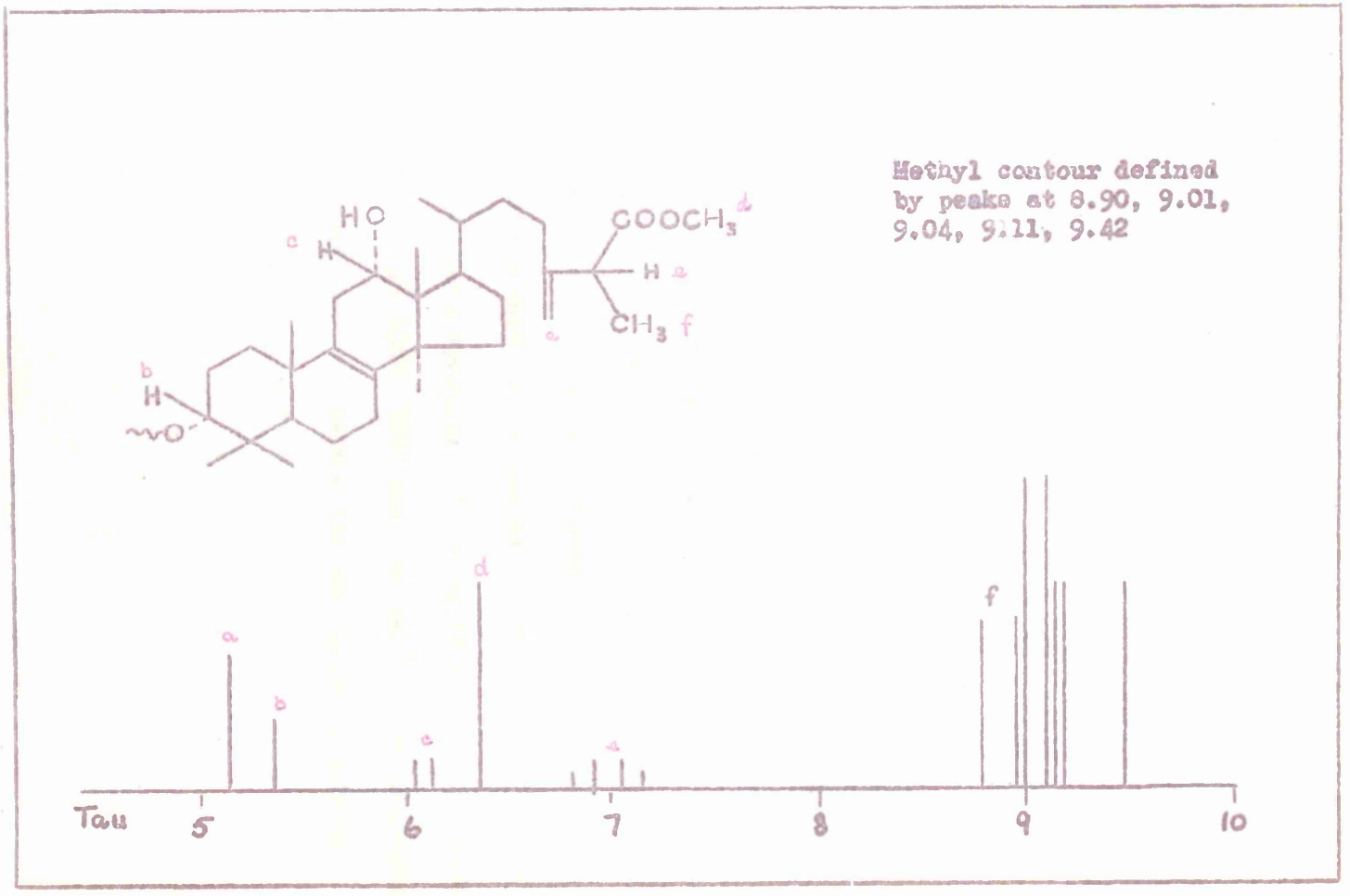


TABLE 4.3. N.M.R. SPECTRUM OF A METHYLPOLYPORENATE A 3-ESTER

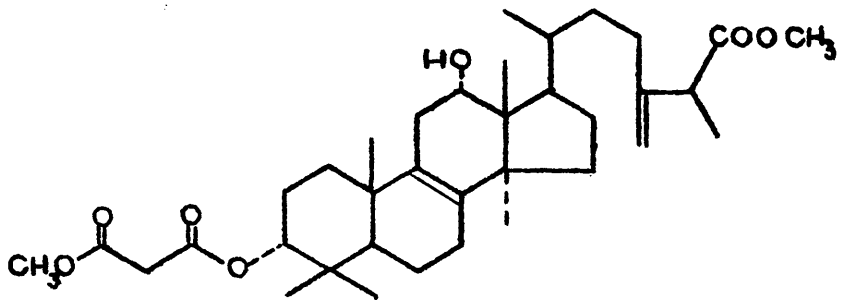


of Polyporus betulinus, the compounds P.B.2 and P.B.4, revealed that both these substances possessed the above characteristics of a methyl polyporenate A 3-ester.

The compound P.B.2 melted over the range 88-89°C and analysed in accord with the formula, $C_{36}H_{56}O_7$. The probability that this substance was a methyl polyporenate A 3-ester enabled the formula to be expressed in the alternative form:

$$C_{32}H_{50}O_3 \cdot C_4H_6O_4$$

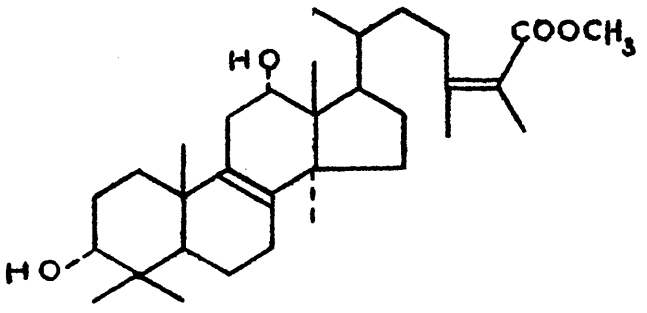
The nature of the entity $C_4H_6O_4$ was readily determined since the N.M.R. spectrum of the compound P.B.2 contained the three proton singlet at 6.36 tau and two proton singlet at 6.70 tau characteristic of the methoxycarbonylacetyl unit. The presence of this structural grouping was further confirmed by the observation of a loss of 118 units of mass from the parent ion in the mass spectrometer and the occurrence, in the infrared spectrum, of the typical malonate bands at 1757, 1739, 1273, 1236, 1162, 1037 and 1020 cm^{-1}. Spectrographic analysis thus suggested that the metabolite was methyl 3-methoxycarbonylacetylpolyporenate A (4.15).



4.15

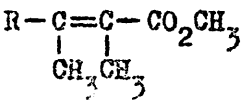
The following chemical transformations proved this to be correct.

The compound P.B.2 was treated with the reagent which had smoothly transesterified the Daedalia quercina malonates - anhydrous sodium methoxide in dry methanol. A portion of the product of this reaction was analysed by gas-liquid chromatography and was shown to contain an appreciable quantity of dimethyl malonate. The non-volatile product of the reaction was isolated by P.L.C. as a white crystalline compound and was deduced to be methyl isopolyporenate A (4.16) as follows.



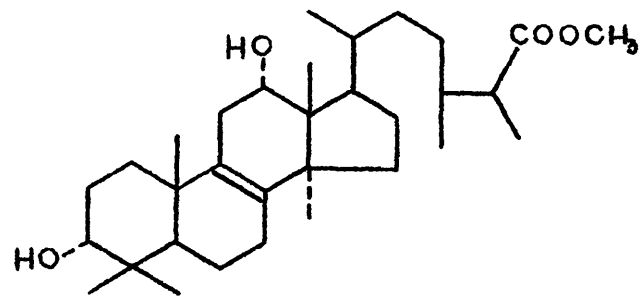
4.16

Analysis and molecular weight (parent molecular ion at m/e 500) indicated that the compound was isomeric with methyl polyporenate A. The N.M.R. spectrum of methyl polyporenate A was very similar to that of the transformation product except that the latter showed no evidence of an exocyclic methylene group nor any $A.X_3$ system at carbon atom C-25. Instead there appeared a six proton singlet at 8.20 tau. This latter feature together with an infrared band at 1718cm^{-1} (ε 430, $\Delta\nu_{\frac{1}{2}}$ 18) and ultraviolet absorption at 2260 Å (ε 8,900) was in agreement with the presence of a tri-substituted α,β- unsaturated ester such as:-



Since, under basic conditions, the β,γ double bond of methyl polyporenate A is known[53] to move into conjugation with the ester carbonyl to give isopolyporenate A, the fact that the compound P.B.2 suffered a base catalysed double bond migration during demalonation was further evidence that it was a methyl polyporenate A 3-ester.

Final proof of structure was obtained by hydrogenating the compound P.B.2 to remove the exocyclic methylene group and repeating the transesterification. The non-volatile product of the reaction was isolated and was found to correspond in R_f value, spectra and mixed melting point with a sample of methyl 24,28-dihydropolyporenate A (4.17) obtained by hydrogenation and methylation of an authentic



4.17

sample of polyporenic acid A. The compound P.B.2 was thus proved to be methyl 3-methoxycarbonylacetylpolyporenate A (4.15).

Finally attention was focussed on the structure of the compound P.B.4 which was a white crystalline substance, m.p. 79-81°C, possessing analytical data consistant with a molecular formula $C_{39}H_{62}O_8$. The material gave rise to no significant absorption in the ultraviolet. As detailed in the case of the compound P.B.2, it was possible to deduce that the compound P.B.4 was a methyl polyporenate A 3-ester and, as such, its molecular formula could be expressed in the form:-

$$C_{32}H_{50}O_3 \cdot C_7H_{12}O_5$$

in which the latter unit represented the formula of the conjugating acid.

Subtraction of the N.M.R. spectrum of the compound P.B.4 from that of a methyl polyporenate A 3-ester (Table 4.3), gave rise to the following simple spectrum:-

6.34 tau	3 protons	singlet
7.40 tau	4 protons	broad singlet
8.70 tau	3 protons	singlet

From this it was concluded that the conjugating acid contained two methyl groups and two chemically slightly dissimilar methylene groups, all in environments which would lead to unsplit signals. It seemed probable that the singlets at 6.34 and 8.70 tau were caused respectively by a methoxyl group and by a methyl group which was deshielded in some way such as being β to oxygen. Bearing in mind that the conjugating acid would require the unit $-CO_2H$ for union with the basic nucleus, it was possible to write down the principal features of its structure as:-

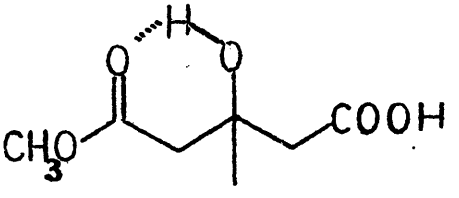
$$C_2HO_2 \left\{ \begin{array}{l} -CH_3 \\ -OCH_3 \\ -CH_2- \\ -CH_2- \\ -CO_2H \ (B^n) \end{array} \right.$$

In the infrared the compound P.B.4 exhibited two independent hydroxyl absorptions. The first of these, appearing at 3635 cm^{-1} (ε 55.5, $\Delta\nu_{\frac{1}{2}}$ 14) was assigned to a non-bonded hydroxyl group. The second band (3528 cm^{-1}, ε 55.5, $\Delta\nu_{\frac{1}{2}}$ 18) was diffuse and appeared lower in the spectrum at a frequency characteristic of hydrogen bonded hydroxyl groups. Dilution studies indicated that the hydrogen bond was intramolecularly established. Subtracting from this spectrum the absorption expected of the 12α hydroxyl group

(3639 cm^{-1}) and the C-26 carbomethoxy group (1740 cm^{-1}) and assuming that the ester link between conjugating acid and basic nucleus gave rise to absorption at the normal value of 1740 cm^{-1}, it was possible to deduce that the conjugating acid contained the structural features responsible for the absorption at 1715 and 3528 cm^{-1} and propose that the unit C_2HO_2 in the above scheme contained an hydroxyl group and a carbonyl function. The structural elements of the conjugating acid could then be tabulated as:-

-CO-	$-CO_2H$ (B^n)
$-OCH_3$	$-CH_2-$
-OH	$-CH_3$
$-CH_2-$	C

These elements had then to be assembled to give a structure which would have no vicinal protons, would have two methylene groups in similar but non-identical environments, would have a deshielded methyl group and would have provision for intramolecular hydrogen bonding. There is a unique solution, the half methyl ester of β-hydroxy-β-methyl-glutaric acid (4.18).

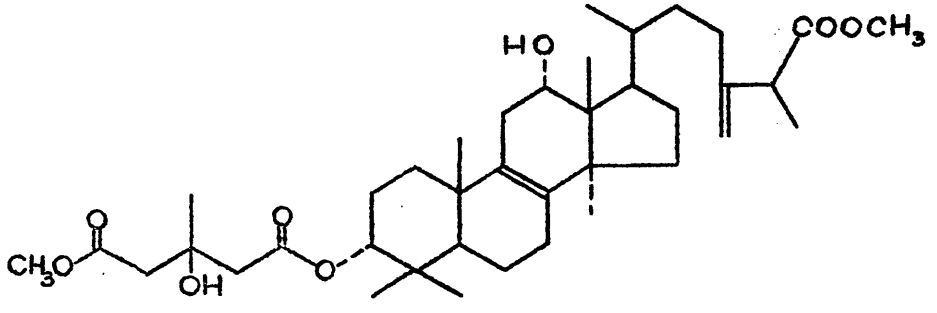


4.18

It may be pointed out at this point that hydrogen bonding should also be possible between the hydroxyl group and the carbonyl oxygen of the conjugating linkage.

As a preliminary check on these spectroscopic findings, the N.M.R. spectrum of an authentic sample of dimethyl β-hydroxy-β-methylglutarate was run. It was found to be superposable with the spectrum derived from the compound P.B.4 by subtraction of the absorption due to the methyl polyporenate A unit, save for a slight down-field shift (0.01 tau) of one of the methylene groups. This observation was easily rationalised in terms of a slight difference in the chemical environment of the two methylene groups in the asymmetrically substituted ester - the compound P.B.4.

On the basis of spectroscopic measurements it was thus proposed that the compound P.B.4 was methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A (4.19). This hypothesis was then subject to chemical verification.



4.19

As would be expected the substance readily absorbed one mole of hydrogen to give a product which showed no infrared absorption at 1648 or 890cm^{-1} but gave a slow positive reaction with tetranitromethane. The N.M.R. of the dihydro compound showed no A.X_3 pattern at 6.92 and 8.76 tau but instead possessed a doublet at 8.81 tau attributable to a methyl group placed β to an alkoxycarbonyl system.

When the compound P.B.4 was transesterified with anhydrous sodium methoxide in dry methanol the non-volatile product of the reaction was found to be methyl isopolyporenate A (4.16). The volatile product of the reaction was identified by gas-liquid chromatography as dimethyl β-hydroxy-β-methylglutarate. Furthermore, transesterification of the dihydro derivative of the compound P.B.4 gave a non-volatile product identical in all respects with methyl 24,28-dihydropolyporenate A (4.17).

With the establishment of the structure of the compound P.B.4 the analysis of the methyl esters of the principal acid constituents of Polyporus betulinus was complete. It had resulted in the isolation from the sporophore tissue of the following triterpenes:-

methyl polyporenate C (0.2 %)
methyl polyporenate A (0.7 %)
methyl 3-acetylpolyporenate A }
methyl 3-caproylpolyporenate A } (0.1 %)
methyl 3-methoxycarbonylacetylpolyporenate A (0.4 %)
methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A (0.7 %)

and had convincingly demonstrated that, in at least one other triterpene producing fungus, the triterpenes occurred in conjugated form. The figures in parenthesis in the above table represent the yield of the ester concerned expressed as a percentage of the original sporophore weight. Before describing work performed with non-esterified extracts of Polyporus betulinus, it is apposite to consider some of the characteristic spectral properties of methyl polyporenate A and its conjugates.

The features of the N.M.R. spectrum attributable to the functionality at carbon atoms C-3, C-12, C-24 and C-25 have already been considered. It was mentioned at that juncture that the pattern of absorption produced by the saturated methyl groups was also highly characteristic of the series. As will now be shown, detailed examination of the changes produced in this pattern as a result of chemical reaction led to the assignment of specific resonance frequencies to particular methyl groups. Data relating to the methyl resonance pattern of the conjugates and their principal transformation products is presented in Table 4.4. It will be seen that two distinct patterns emerged. Firstly there was the pattern of the conjugates themselves defined by peaks at 8.91, 9.01, 9.04, 9.11 and 9.42 tau. Removal of the ester group from carbon atom C-3 caused this pattern to collapse to the more simple four element system defined by peaks at 8.91, 9.05, 9.15 and 9.42 tau. Integration measurements indicated that the process of hydrolysis was reflected in the N.M.R. spectrum as the up-field shift of two three proton

TABLE 4.4. METHYL RESONANCE PATTERN OF THE POLYPORENIC ACID A (POLY. A) DERIVATIVES

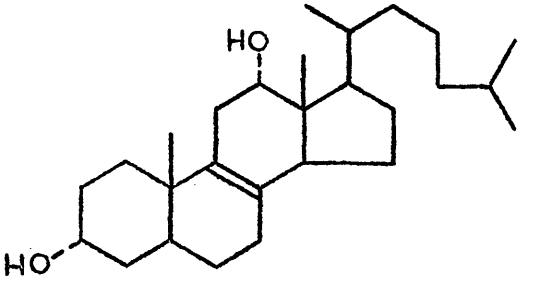
	Tau Values					
Methyl polyporenate A.	8.91		9.05		9.15	9.42
Methyl dihydropolyporenate A	8.91		9.05		9.15	9.42
Methyl isopolyporenate A.	8.91		9.04		9.14	9.41
Integration of above (CH_3)	1.5		2.5		1	1
Dimethyl poly. A malonate	8.91	9.01	9.04	9.10		9.41
Dimethyl dihydro poly A malonate	8.91	9.02	9.06	9.10		9.42
Dimethyl poly. A glutarate	8.90	9.01	9.04	9.11		9.41
Dimethyl dihydropoly. A glutarate	8.90	9.02	9.05	9.12		9.43
Methyl poly. A acetate	8.86	9.00	9.03	9.11		9.41

singlets from 9.02 and 9.11 tau to 9.05 and 9.15 tau. Since an alteration in the resonance frequency of a proton is normally indicative of a change in its immediate chemical environment it was feasible to state that the hydrolysis of the ester function at carbon atom C-3 would primarily influence the chemical shift of the gem-dimethyl group at carbon atom C-4. It was thus concluded that the 4,4-dimethyl system in methyl polyporenate A, methyl 24,28-dihydropolyporenate A and methyl isopolyporenate A was responsible for signals at 9.05 and 9.14 tau, while in the 3-esters of methyl polyporenate A and methyl 24, 28-dihydropolyporenate A it was responsible for signals at 9.01 and 9.11 tau.

The chemical shifts of the methyl groups C-18, C-19, C-21 and C-32 were deduced from an analysis of the spectrum of methyl isopolyporenate (4.16). This substance possessed two vinylic methyl groups and hence its saturated methyl resonances were less complex, being caused by only six entities. As is shown in Table 4.4 integration revealed that the peaks at 9.41 and 9.14 tau were occasioned by single methyl groups while the remaining four methyl groups were distributed between peaks at 8.91 and 9.04 in the ratio 1.5 : 2.5. The two half units of intensity contained in these latter two peaks was ascribed to a doublet centred at 8.98 tau (J 7.8 c/s) produced by the splitting of the methyl group C-21 by the hydrogen on carbon atom C-20.

The singlet at 9.14 tau and part of the signal at 9.04 tau

have already been assigned to the 4,4-dimethyl group, hence each of the remaining singlet methyl absorptions at 8.91, 9.04 and 9.42 must be due to one or other of the angular methyls C-18, C-19, C-32. Making the assumption that the compound $\Delta^{8(9)}$-cholesten-3α, 12α-diol (4.20) was a valid model for the C-18 and C-19



4.20

methyl groups in methyl isopolyporenate A it was possible to compute theoretical values for their resonance frequencies. Calculations based on data provided by Bhacca and Williams[91] and by Cohen and Roch[63] are shown in Table 4.5. The latter authors make no provision for a $\Delta^{8(9)}$ double bond and thus the Bhacca and Williams value was employed. The results indicated that a value of 9.39 - 9.36 tau could be expected for the C-18 methyl resonance and a value of 9.01 - 9.10 tau for the C-19 methyl resonance. On the basis of these calculated values it is proposed that the peaks at 9.04 and 9.41 tau in the spectrum of methyl isopolyporenate A and therefore in the related compounds are due to the C-19 and C-18 methyl groups respectively. By a

TABLE 4.5. CALCULATED RESONANCES FOR C-18, C-19 METHYL GROUPS

	BHACCA & WILLIAMS[31]		COHEN & ROCK[48]	
	C-18	C-19	C-18	C-19
5α,14α Androstane	0.692	0.792	9.31	9.23
3α OH	+0.008	0.000	+0.03	+0.04
$\Delta^{8(9)}$	-0.083	0.125	+0.08	-0.13
12α OH	+0.042	-0.008	-0.11	-0.04
17β C_8H_{17}	-0.050	-0.017	+0.05	0.00
Result (tau)	9.391	9.008	9.36	9.10

process of elimination it would appear that the C-32 methyl group was responsible for the peak at 8.91 tau. This relatively low value may be caused by the deshielding of the protons of the C-32 methyl group by the electrons of the 8(9) double bond and of the 12α hydroxyl group. These tentative assignments of resonance signals to specific methyl groups are summarised in Table 4.6.

Examination of the solid state infrared spectra of methyl polyporenate A and the related compounds suggested that peaks at 1457, 1437, 1375, 1097, 1086, 1071, 1050, 988 and 977 cm^{-1} were characteristic of the methyl polyporenate A nucleus. The latter five bands were found to be of particular use in structural analysis.

On electron bombardment methyl polyporenate A and its derivatives suffer a characteristic sequence of fragmentation reactions. The abundances of the principal ions in the spectra of the compounds are collected in Table 4.7. Since the compounds all behave similarly, the fission sequence will be discussed in terms of methyl 3-methoxycarbonylacetylpolyporenate A (4.15). From the position of meta-stable ions in the spectrum it was possible to deduce that the parent molecular ion (m/e 600) was linked to the base peak (m/e 467) by means of two independent pathways. The first pathway proceeded by means of fission of an angular methyl group to give the ion P-15 = m/e 585 and thence to the base peak by "Type H"[70] elimination of the elements of methoxycarbonylacetic acid (4.21 to 4.22).

TABLE 4.6. PROPOSED ALLOCATION OF N.M.R. SIGNALS TO PARTICULAR METHYL GROUPS

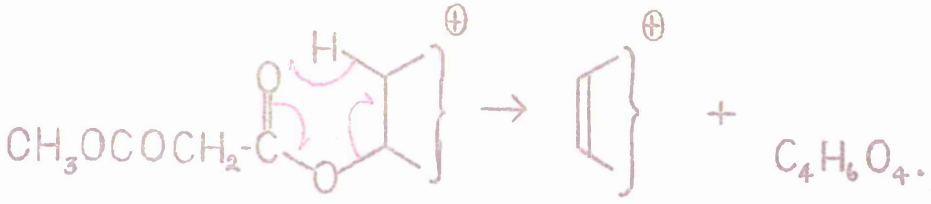
	C-30 and C-31		C-19	C-18	C-32	C-21		(J)
M. poly A	9.05	9.15	9.05	9.42	8.91	8.98	d	8.4
M. H_2-poly A	9.05	9.15	9.05	9.42	8.91	8.98	d	8.4
M. iso-poly A	9.04	9.14	9.04	9.41	8.90	8.97	d	8.4
M. mca poly A	9.01	9.11	9.04	9.41	8.91	8.97	d	5.4
M. mhmb poly A	9.01	9.12	9.03	9.42	8.89	8.98	d	8.4
M. Acetyl poly A	9.00	9.11	9.03	9.41	8.86	8.96	d	5.4
M. mhmb-H_2 poly A	9.00	9.12	9.05	9.43	8.90	8.98	d	8.4
M. mca-H_2 poly A	9.02	9.10	9.06	9.42	8.91	8.98	d	9.6

mca = methoxycarbonylacetyl

mhmb = 4-methoxycarbonyl-3-hydroxy-3-methylbutyryl

TABLE 4.7. COLLECTED MASS SPECTRAL DATA FOR THE P. BETULINUS METABOLITES

	Methyl Poly A	Methyl dihydro Poly A	Methyl iso Poly A	Acetate Conjugate	Malonate Conjugate	Glutarate Conjugate
P	500 28.6 %	502 29.3 %	500 17.6 %	542 38 %	600 3.4 %	--
P-15	485 2.7 %	487 2.1 %	485 1.5 %	527 7 %	585 1.0 %	--
P-18	482 11.6 %	484 13.3 %	482 7.2 %	524 3 %	582 3.5 %	640 83.3 %
P-33	467 100 %	469 100 %	467 100 %	509 100 %	567 34.2 %	625 13.0 %
482	11.6 %	484 13.3 %	7.2 %	6 %	13.5 %	6.90 %
467	100 %	469 100 %	100 %	12 %	100 %	35.9 %
449	31.7 %	451 31.1 %	26.6 %	62 %	81.6 %	100 %
313	10.7 %	11.3 %	9.50 %	6 %	9.3 %	5.7 %



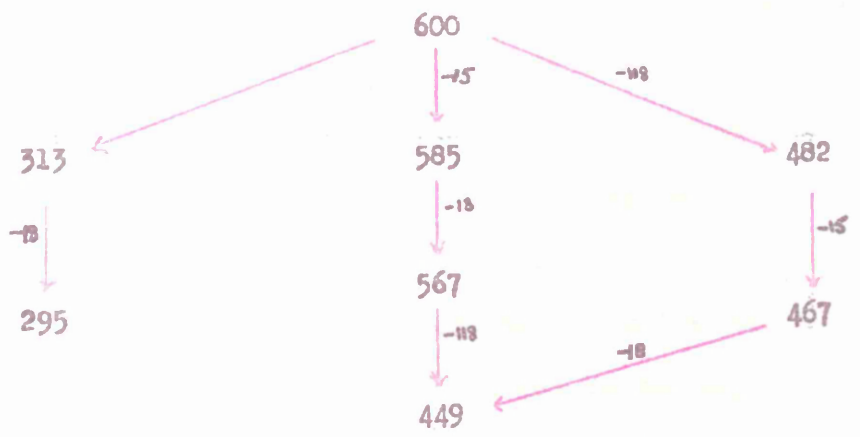
4.21 m/e 585 4.22 m/e 467

The second pathway involved the same fission reactions but executed them in reverse order. An ion corresponding to complete loss of the side chain due to fission between carbon atoms C-17 and C-20 was detected in 10 % abundance at m/e 313. Losses of the elements of water, presumably from the 12α hydroxyl group, were observed to take place from the ions m/e 585, m/e 467 and m/e 313. These findings are summarised in Table 4.8.

The various other derivatives and transformation products of methyl polyporenate A, with the exception of the hydroxymethyl-glutarate conjugate, undergo similar fission sequences. In all cases a methyl group, the substituent at carbon atom C-3 (OH, CH_3OCOCH_2COO) and a proton are lost to give the base peak at m/e 467. This latter ion subsequently loses the elements of water to yield an abundant ion at m/e 449. The spectrum of each substance contained an ion of mass m/e 313.

Some slight divergence from this typical behaviour was noted in the case of the compound methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A. Under the conditions in which the spectrum was obtained no parent ion could be detected for this

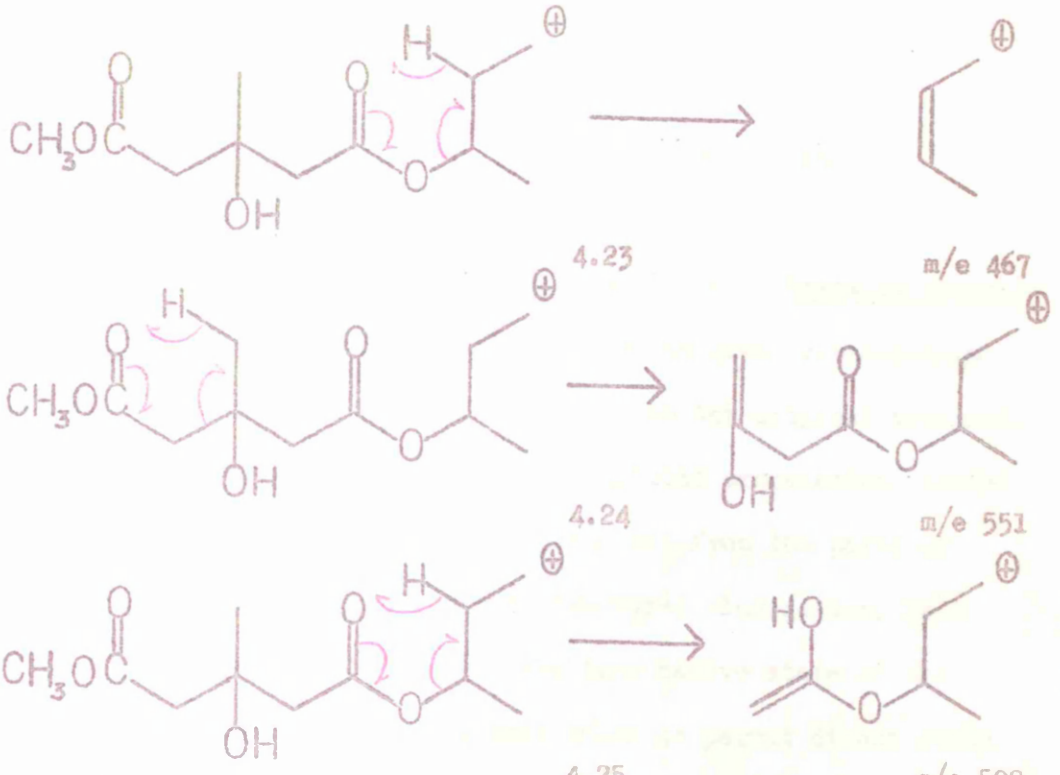
TABLE 4.8. PROPOSED MASS SPECTRAL FISSION SEQUENCE FOR THE COMPOUND P.B.2.



m*	transition
549.5	585-567
387.2	600-482
373.6	585-467
431.6	467-449
355.5	569-449

compound. The first ion in the spectrum occurred at m/e 640 and corresponded to a dehydration product of the polyporenic acid A ester. In similar manner the base peak of the spectrum was shifted down by eighteen units of mass from the expected value of m/e 467 to m/e 449. Since there was no reasonable doubt as to the validity of the structure of the compound it must be concluded that the conjugate underwent thermal dehydration. It can be shown that the hydroxyl group which was eliminated as water was that on carbon atom C-12 since the ion formed by the dehydration process (m/e 640) subsequently lost the other hydroxyl group present in the molecule as part of 4-methoxycarbonyl-3-hydroxy-3-methylbutyric acid. Although the other polyporenic acid A derivatives all showed a loss of water from the parent ion the corresponding peak had only an abundance of approximately 10 %, evidently not such a dominant process as in the case of the hydroxymethylglutarate. This can be attributed to the promotion of thermal dehydration by the higher probe temperature required to volatilise this latter compound.

Before leaving the mass spectrum of the hydroxymethylglutarate it is of interest to refer to two fission processes which do not figure in any of the other conjugates. In addition to the "Type H" rearrangement causing complete removal of the hydroxymethylglutarate unit (4.23),



two further "Type H" rearrangements can occur. The first (4.24) involves fission α,β to the methoxycarbonyl system of the hydroxymethylglutarate ester and leads to the loss of 74 units of mass and an ion at m/e 551 (P-18-15-74). The second process (4.25) involves fission α,β to the conjugate ester linkage and gives rise to a loss of 116 units of mass and an ion at m/e 509 (P-18-15-116).

The "finger print" region of the mass spectrum of all the methyl polyporenate A series of compounds contained an impressive group of doublet, triplet and quartet peak systems. Although the structure of the ions producing them could not be deduced, the pattern seemed to be characteristic of the tetracyclic triterpene

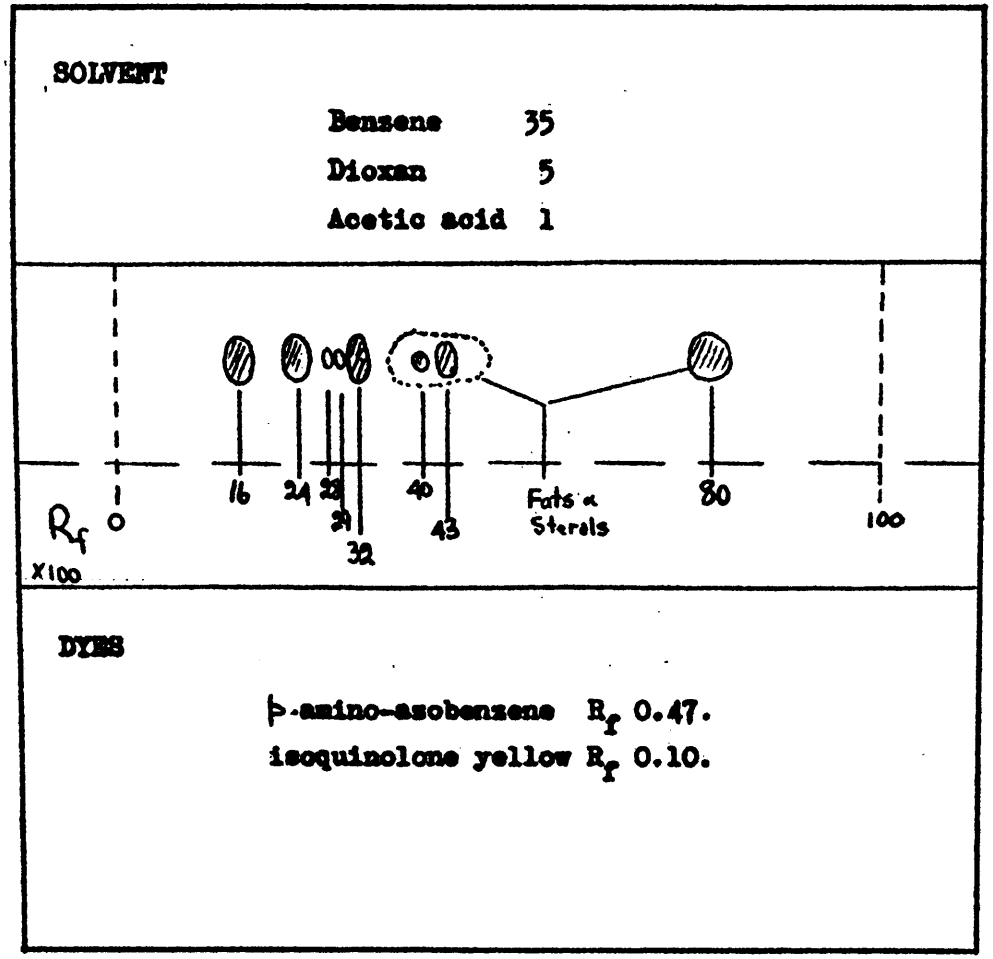
carbon skeleton. The peaks concerned occurred at

187	175	161	149	137	123	109
185	173	159	147	135	121	107
	171	157	145	133	119	105
	169		143	131		

The previous work on both the metabolites of Daedalia quercina and Polyporus betulinus had suffered from the great disadvantage that the analyses had been conducted, not on the original methanol extract of the sporophore, but on a methylated preparation derived from it. This procedure, although excellent from the point of view of the initial separation and structural elucidation, left some measure of uncertainty as to the true native state of the fungal metabolites. Steps were thus taken to permit direct study of the methanol extract of Polyporus betulinus. A benzene : dioxan : glacial acetic acid (B.D.A.) T.L.C. system was developed which was able to produce good resolution of the constituents of the methanol extract. As is shown in Table 4.9, eight distinct substances were detected and their standard R_f values determined. The material of R_f value 0.43 was immediately identified as ergosterol by virtue of a correspondence in R_f value and staining behaviour with an authentic sample of that compound.

Of the seven remaining substances it seemed reasonable to assume that four would be related to methyl polyporenate A, its acetate, methoxycarbonylacetate and 4-methoxycarbonyl-3-methyl-butyrate. To ascertain the precise nature of the relationship the

TABLE 4.9. T.L.C. OF METHANOL EXTRACT OF P. BETULINUS SPOROPHORE

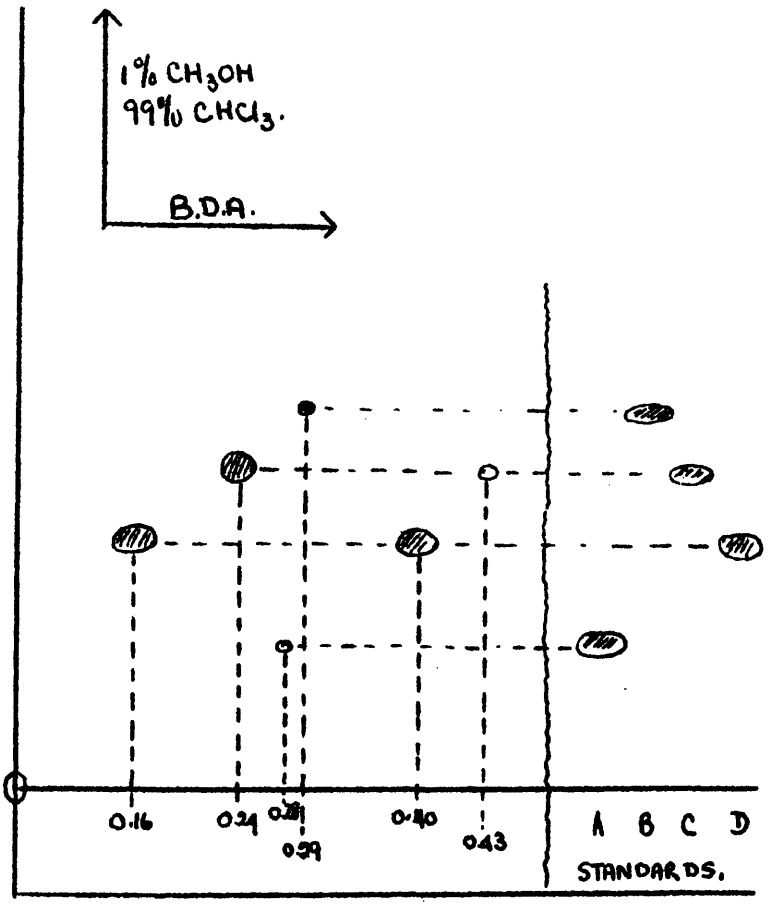


following two dimensional T.L.C. operation was performed. A sample of the methanol extract was placed at the origin of a two dimensional chromatogram (Table 4.10) and developed in the x-direction with the B.D.A. solvent system. After in situ esterification with diazomethane the plate was developed in the y-direction with 1 % methanol in chloroform against standard samples of the previously isolated methyl esters. As a result, polyporenic acid A and 3-acetylpolyporenic acid A were located as the compounds of R_f value 0.28 and 0.29 respectively.

It was noted, however, with some surprise that the hydroxymethylglutarate and the malonate were each formed by methylation of two distinct acidic compounds. Accordingly, a sample of the methanol extract was subject to column chromatography on Mallinckrodt silicic acid. The T.L.C. record of the separation so produced is shown in Table 4.11. For ease of discussion, the chemical constitution of the more polar of the four compounds concerned will be considered first.

The substance of R_f value 0.24 was a white, crystalline, acidic compound, m.p. 184-185°C, which analysed in accord with the molecular formula $C_{34}H_{52}O_7$. The N.M.R. spectrum of the compound contained a two proton singlet at 6.53 tau together with all the peaks expected of a polyporenic acid A 3-ester. Suspicions that the material was 3-carboxyacetylpolyporenic acid A (4.26) were

TABLE 4.10. 2D. T.L.C. OF P. BETULINUS SPOROPHORE METHANOL EXTRACT



A Methyl polyporenate A

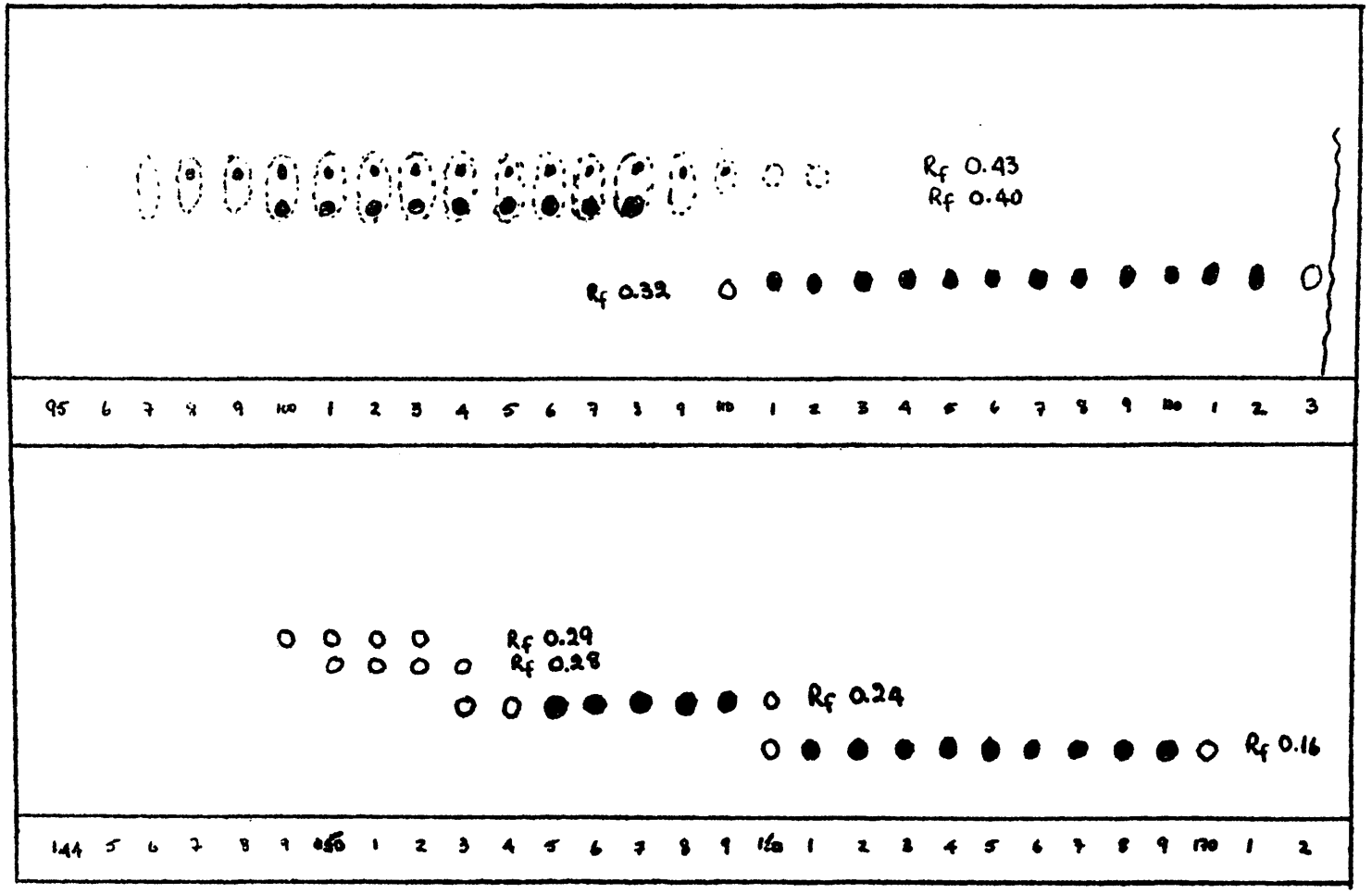
B Methyl 3-acetylpolyporenate A

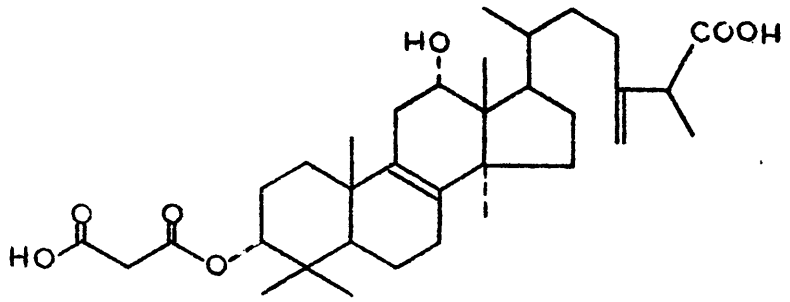
C Methyl 3-methoxycarbonylacetylpolyporenate A

D Methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A.

TABLE 4.11. T.L.C. RECORD OF CHROMATOGRAPHIC FRACTIONATION OF P. BETULINUS ACIDS

Solvent B.D.A.

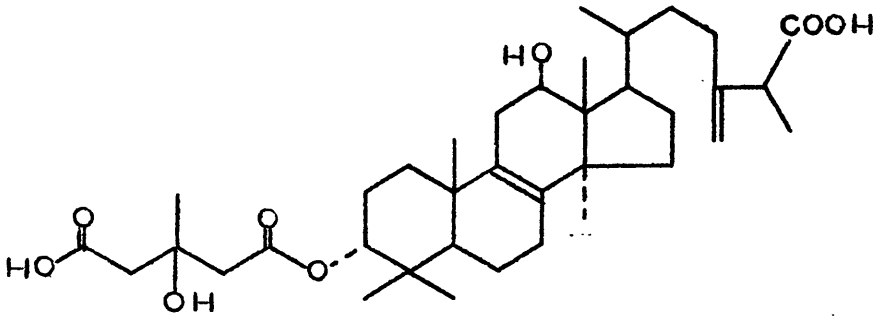




4.26

confirmed when, after methylation, the substance gave a product identical in R_f value with methyl 3-methoxycarbonylacetylpolyporenate A.

An exactly analogous sequence of events led to the identification of the second substance (R_f value 0.16) as 3-(4-carboxy-3-hydroxy-3-methylbutyryl)-polyporenic acid A (4.27).



4.27

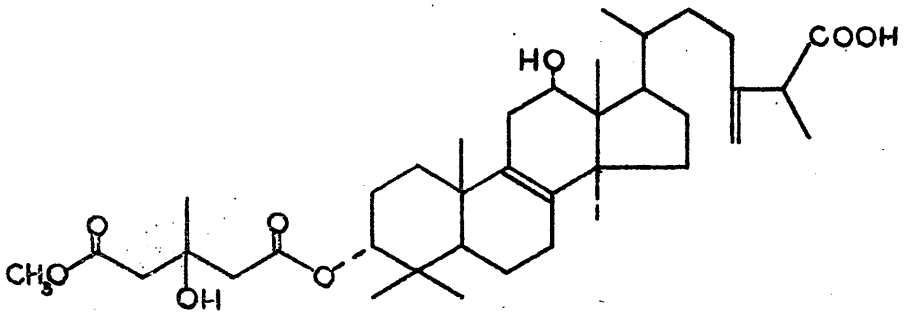
The third material (R_f value 0.32) was eluted from the column in fractions 111-124. It was a clear gum which methylated to give a substance identical in R_f value to methyl 3-(4-methoxy-

carbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A. Examination of the N.M.R. spectrum of the gum revealed that it contained the resonances expected of a polyporenic acid A - hydroxymethylglutaric acid conjugate together with a three proton singlet at 6.30 tau. The infrared spectrum of the material was indicative of a substance containing two alkoxycarbonyl groups. The gum thus appeared to be a monomethyl ester of 3-(4-carboxy-3-hydroxy-3-methylbutyryl)-polyporenic acid A. The decision as to which of the two carboxylic acid groups in the molecule was esterified was made on the basis of mass spectral measurements.

As pointed out earlier, the mass spectrum of methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A showed three fragment ions which could be formed by "Type H" rearrangement of the (P-15-18) ion involving different portions of the hydroxymethylglutarate unit. It was noted that in each case the neutral molecule eliminated (mass 74, 116, 176) contained within it the methyl ester of the glutarate moiety (cf. 4.23, 4.24, 4.25). It was thus argued that, if in the half ester of R_f value 0.32 the free carboxyl group occurred in the side chain rather than in the hydroxymethylglutarate system, similar losses of 74, 116 and 176 units of mass should be detected in its mass spectrum. If, on the other hand, the free carboxyl group occurred in the hydroxymethylglutarate grouping, no such losses would be observed.

Unfortunately there were no metastable peaks in the spectrum

of the substance but a plausible fragmentation sequence can nevertheless be suggested (Table 4.12). It is evident that, apart from losses of methyl groups and the elements of carbon dioxide, the principal fragmentations involve elimination of neutral fragments of mass 74, 116 and 176. This indicated that the half ester of R_f value 0.32 was 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenic acid A (4.28).

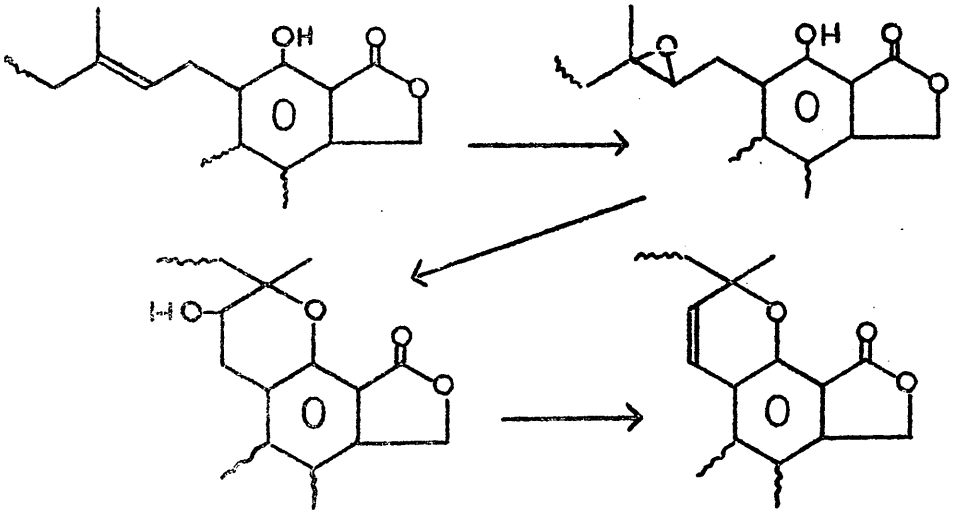


4.28

Some difficulty was experienced in separating the fourth compound (R_f value 0.40) from the complex mixture of sterols and fats which had been simultaneously eluted from the column. Although preparative layer chromatography with the B.D.A. solvent system was able to produce adequate separation, it was found impossible to obtain recovery of better than 10 % of the pure compound from the silica. From the small, relatively impure sample that was obtained the following results were gleaned. The substance methylated to give a product identical in R_f value

with the mass spectrum of the compound P.V.9 of which the salient features are collected in Table 8.4. It will be seen that apart from the loss of carbon dioxide from the carboxylic acid and lactone groupings, the principal fragment ions were derived from "Type A_4^{26}" fission of the methyl and propionic acid moieties from the chromene nucleus (8.13).

Preliminary attempts to synthesise the compound (8.12) were not successful. Epoxidation of ethyl mycophenolate with m-chloroperbenzoic acid followed by treatment of the resulting epoxy-phenol with strong base or buffer pH 9 in the hope of effecting the sequence shown below resulted in both cases in the formation of the threo-



hydroxylactone (8.10b). It thus appeared that, even in mild base, there is a greater tendency towards ester hydrolysis and attack of

with methyl 3-methoxycarbonylacetylpolyporenate A while its N.M.R. spectrum contained signals at 6.28 and 6.69 tau. These observations suggested that the compound might be a monomethyl ester of 3-carboxyacetylpolyporenic acid A. No conclusive evidence is available to indicate which of the two carboxylic acids groups was methylated, however, by analogy with the hydroxymethylglutarate conjugate, it would appear more probable that the ester group was located in the malonate unit.

Evidence that these methyl esters were authentic metabolites and not artefacts produced by interaction of the diacids with the methanol used as solvent in the primary extraction, was obtained by repeating the isolation and separation using ethanol as solvent. Both esters were again detected and the more easily obtained, the half ester of 3-(4-carboxy-3-hydroxy-3-methylbutyryl)-polyporenic acid A, was isolated and identified as the methyl derivative by N.M.R. spectroscopy. This observation proved that the monomethyl ester of the hydroxymethylglutarate was authentic and it was assumed by analogy that the methyl ester of the malonate conjugate was also genuine.

As a result of these analyses of both methylated and non-methylated extracts of the sporophore tissue of Polyporus betulinus it was possible to draw up the following list of metabolites shown to occur therein:-

ergosterol,

polyporenic acid C,

polyporenic acid A,

3-acetyl-polyporenic acid A,

3-carboxyacetylpolyporenic acid A,

3-(4-carboxy-3-hydroxy-3-methylbutyryl)-

polyporenic acid A,

3-caproylpolyporenic acid A,

a mono-methyl ester of 3-carboxyacetylpolyporenic

acid A.

It was noteworthy that at no time in this investigation was there any evidence of the presence of polyporenic acid B.

Lately a culture of Polyporus betulinus has been grown under controlled conditions on a synthetic medium. Preliminary T.L.C. and N.M.R. analyses of a methanol extract of the mycelium produced, failed to reveal the presence of any of the conjugates.

CHAPTER 5

THOUGHTS ON THE METABOLIC SIGNIFICANCE OF TRITERPENE CONJUGATES

There can be little doubt that one of the most interesting and challenging aspects of modern biological chemistry centres around attempts to discover the functional importance in the living cell of the various chemical compounds which can be isolated therefrom. Considerable success has already been achieved in this direction with mammalian systems. It is common knowledge that the elements of the genetic code are the purine and pyrimidine bases and that acetyl choline is the chemical agent whose action at the synapse is responsible for nerve transmission. Little progress has, however, been made with non-mammalian systems and it appears that in this field the majority of natural product structural elucidations are viewed as an end in themselves rather than the beginning of a fuller study of the role of metabolites in metabolism.

The isolation from Daedalia quercina and Polyporus betulinus of groups of triterpene acids conjugated with such metabolically significant molecules as acetic, malonic and hydroxymethylglutaric acids led to some interesting speculation as to the possible function or functions of these conjugates in the tissue of the organisms concerned. Seen in general terms, three possibilities appeared worthy of consideration, the compounds could be functionally defensive, structural or metabolic. These roles will be illustrated below with special reference to the triterpene conjugates.

Defensive. It seemed possible that the conjugates might be broad

spectrum antifungal or antibacterial agents which would permit Daedalia quercina and Polyporus betulinus to compete more successfully in their natural environment and would preserve them against attack by fungal parasites.

Structural. None of the isolated native conjugates were appreciably soluble in water and since all possessed a moderate number of functional groups which could bond or complex with other molecules, it seemed possible that the conjugates might compose part of the insoluble structural network of the cells themselves.

Metabolic. It has already been pointed out that the acids found in conjugation with the tetracyclic triterpenes are of great metabolic importance being involved in the biosynthesis of both terpenes and fats. The possibility that the conjugates were involved in the storage, transport, activation or chemical recognition of such entities appeared worthy of further consideration.

The results of bioassays suggest that the conjugates are not broad spectrum antifungal or antibacterial agents. 3-Carboxyacetylpolyporenic acid A, 3-(4-carboxy-3-hydroxy-3-methylbutyryl) polyporenic acid A and 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl) polyporenic acid A were tested against standard preparations of Baccilus subtilis (Gram negative bacterium), Escherchia coli (Gram positive bacterium) and Botrytus alia (Fungus) and were not found to inhibit growth or spore germination.

Before considering the possibility of metabolic or structural

functionality it is suitable to describe two small experiments whose results are apposite to the discussion. In the first, a portion of the context tissue of a Polyporus betulinus sporophore was homogenised in water, and successively extracted with water and methanol before being allowed to stand in methanol overnight. Thereafter the residual tissue was saponified and the ethyl acetate solubles extracted. The weights of the various extracts are shown below:-

water	623 mg.
methanol	3185 mg.
methanol (overnight)	15 mg.
ethyl acetate	7 mg.

T.L.C. analysis of the various extracts showed that the conjugates were only present in the initial methanol extract.

In the second experiment two equally sized pieces of context tissue were removed from the same Polyporus betulinus sporophore, were frozen in liquid nitrogen and pulverised. The powder from the first block of tissue was treated with ethereal diazomethane and the resulting esters extracted. The residual tissue was then extracted with methanol but since it was shown to contain no conjugates, it was pooled with the ester fraction and the joint weight recorded. The powder from the second block of tissue was immediately extracted with methanol and the resultant extract methylated. Comparison of the product from both blocks of tissue indicated

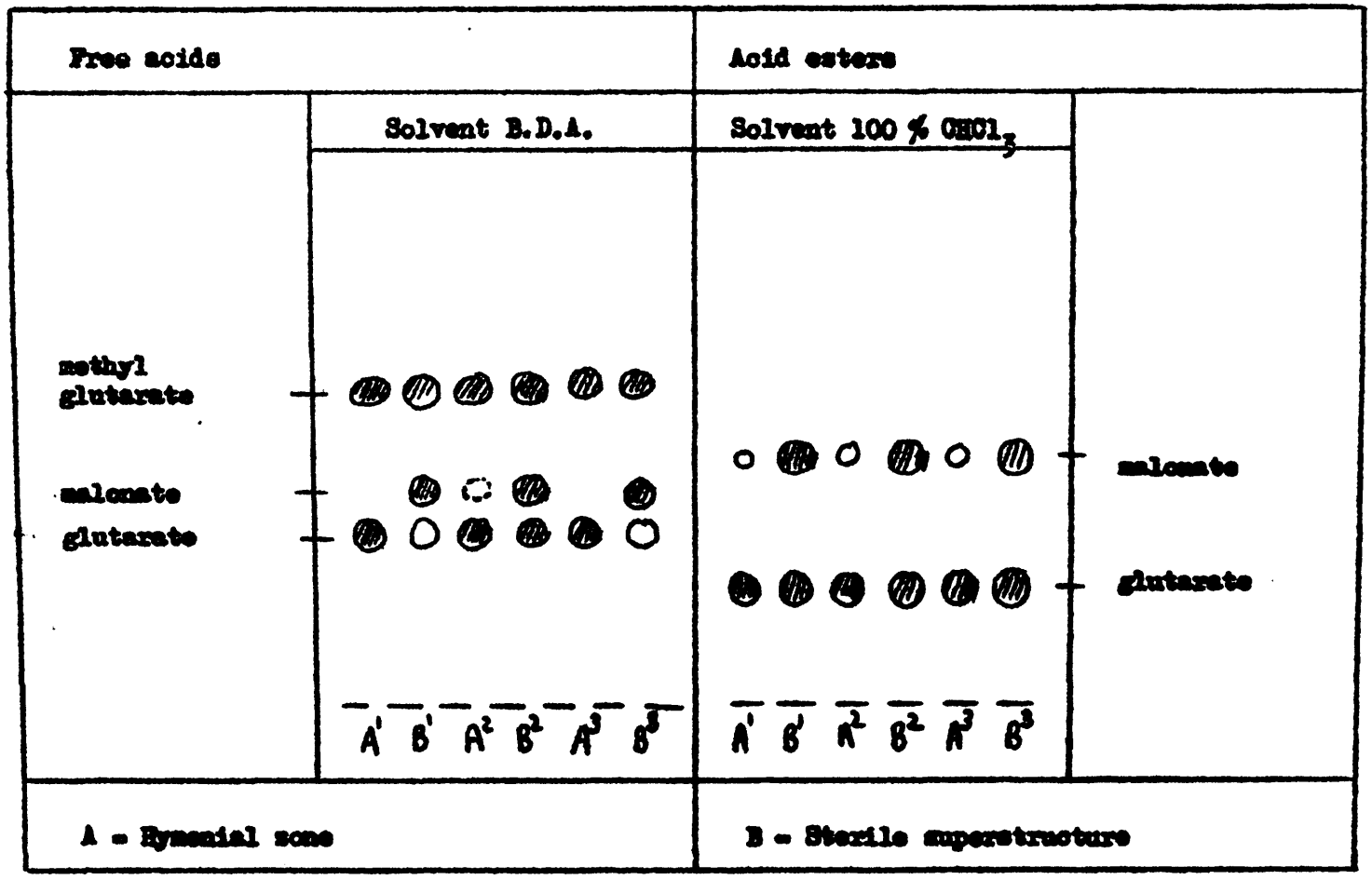
not only that the weights were comparable but also that the spectrum of metabolites present as revealed by T.L.C. was the same.

These experiments demonstrate in a convincing manner that the conjugates must be present as such and not as glycerides, glycosides or as elements in a polymer or copolymer. This latter observation requires that, if the conjugates are structurally involved, they must be present as loosely complexed, interstitial occlusions in the main chitin framework of the cell wall. Such a situation is quite acceptable since fat appears to be incorporated in a similar way into mammalian cell walls.[92]

Before any firm conclusions can be made on the validity of possible metabolic involvement of the conjugates, studies will need to be made employing conjugates labelled both in the conjugating acid and in the terpene nucleus.

However, one last experimental observation may be relevant. Before the sporophore of Polyporus betulinus becomes fertile, an autolytic process leads to the separation of the hymenium from the main body of the sporophores and thus, in mature specimens, the pore area can be cleanly separated from the infertile superstructure. Detailed examination of three sporophores taken at different times from different geographical areas indicated that there was a pronounced disparity in the ratio of total malonate to total hydroxymethylglutarate between the two zones described above. As can be

TABLE 5.1. T.L.C. COMPARISON OF MALONATE: HYDROXYMETHYLGLUTARATE RATIO BETWEEN HYMENIUM AND STERILE SUPERSTRUCTURE



seen from the T.L.C. record shown in Table 5.1, there was very little malonic acid conjugate in the pore area; the biogenetically more complex hydroxymethylglutarate conjugate was in high preponderance. Recalling the statement made by Corner in 1953 that [93] ...

"It is not impossible that all the material from which the spores are made is stored in the subhymenial hyphae, perhaps, too, in the walls of the skeletal and binding hyphae ...."

it is tempting to suggest that the glutaric acid conjugate represents a portion of this material and that the other conjugates are intermediates in its synthesis.

CHAPTER 6

EXPERIMENTAL

I N D E X

INTRODUCTION

INSTRUMENTATION

Melting points were determined on a Kofler Hot-Stage apparatus and are uncorrected. Ultraviolet spectra (u.v.) were obtained on a Unicam S.P.800 recording spectrophotometer while infrared spectra (i.r.) were measured on a Unicam S.P.100 spectrophotometer (quantitative) and on a Perkin Elmer 237 spectrophotometer (qualitative). Nuclear magnetic resonance spectra (n.m.r.) were determined on a Perkin Elmer R.10 60 Mc/s. spectrophotometer, tetramethylsilane being used as internal standard. Mass spectra were obtained by direct insertion into an A.E.I. M.S. 9 double focusing mass spectrometer; precise mass measurements were made relative to perfluorotributylamine. In two cases molecular weights were determined on a Mechrolab 301.A vapour phase osmometer. Gas-liquid chromatography was performed on Pye Argon and Perkin Elmer F.11 Chromatographs.

CHROMATOGRAPHY

a. Column. As an aid to reproducibility, all column chromatography was conducted in standard vessels with a height to diameter ratio of 15:1. Approximately exponential elution gradients were produced by the method described by Lederer,[24] the relationship between solvent

composition and fraction number being determined by placing water in upper and lower vessels, adding methylene blue to the lower one, allowing the gradient to develop directly into tubes and assaying the dye composition therein spectrophotometrically (7000 Å) as a function of time.

b. Layer. Merck Kieselgel G in 0.25 mm. layers was used in thin layer chromatography (T.L.C.); Merck kieselgel H and Merck Kieselgel $H_{256/366}$ in both 1.00 and 0.60 mm. layers were used in preparative layer chromatography (P.L.C.). All P.L.C. plates were pre-eluted with the developing solvent before application of the mixture. Detection was achieved by means of ceric ammonium nitrate-sulphuric acid (1 % in 10 %) oxidation, iodine vapour adsorption or by observation in ultraviolet light (2540 and 3500 Å) where the plates had been subject to prior impregnation with 0.005 % Rhodamine 6G. The dye stuffs, azobenzene, sudan red, p-amino-azobenzene, p-hydroxy-azobenzene and 2-methyl-6, 7-methylenedioxy-3(2H)-isoquinolone,[95] were used as standards in all layer chromatography.

"Multiple Elution" is a method of obtaining maximum resolution of mixtures of compounds of closely allied polarity. A solvent system is first selected in which the desired compound has an R_f value of between 0.1 and 0.2. This solvent is then used to effect repeated development of plates loaded with the mixture until the desired component has been eluted to a position of R_f 0.5 and

maximum separation from the other constituents has been achieved.

MISCELLANEOUS

Diazomethane was prepared from bis(N-methyl-N-nitroso)-terephthalamide by the method of Moore and Reed.[96] All solvents were removed on a rotary film evaporator. Unless otherwise stated, "light petroleum" refers to petroleum ether of b.p. 60-80°C. All solutions were dried over magnesium sulphate. The following abbreviations are used in reporting the n.m.r. data:-

s.	singlet	d.	doublet
t.	triplet	q.	quartet
m.	multiplet	H.	proton

THE METABOLITES OF DAEDALIA QUERCINA

3. EXTRACTION OF METABOLITES AND THEIR METHYLATION.

Fresh sporophores of Daedalia quercina were collected in mid-Autumn from an oak stump in Garscube Estate. The cleaned, dried tissue (900 g.) was homogenised in a Waring Blender in methanol (12 l.). After standing at room temperature for two months with this solvent the extracted tissue was filtered off and the filtrate evaporated to give a light brown solid (42.6 g., m.p. 175-188°C) which represented 4.7 % of the original sporophore weight. This substance was titurated with boiling light petroleum (b.p. 40-60°C, 500 ml.) to remove lipids (ν_{max}-liquid film- 1720, 1240 cm^{-1}), ergosterol (λ_{max}-methanol-2420, 2830, 2930 Å) and other petrol soluble substances.

A portion (1 g.) of the petrol insoluble residue (37.8 g., m.p. 183-188°C) was dissolved in ethyl acetate (100 ml.) and extracted successively with water (2 x 100 ml.), 6N aqueous hydrochloric acid (2 x 100 ml.) and aqueous sodium carbonate (4 x 100 ml.). The aqueous extract was lyophilised, while the acidic and basic solutions were neutralised, extracted with ethyl acetate, dried and evaporated. As a result, the proportion of acidic, basic and neutral material in the original methanol extract was determined to be as follows:-

Water soluble	0 %
acid soluble	9 %
base soluble	86 %
neutral	5 %

Attempts to fractionate the petrol insolubles by adsorption

chromatography on silica, silicic acid, silicic acid: oxalic acid or silicic acid: acetic acid or by partition chromatography on caster oil or with the Berstrom[91] system, methanol, water: chloroform, heptane were essentially unsuccessful. The petrol insolubles were therefore methylated and the chemical analysis conducted on the methyl esters of the metabolites. Thus a portion of the petrol insolubles (32.6 g.) was dissolved in methanol (750 ml.), cooled to 0°C and treated with excess ethereal diazomethane to give the Daedalia quercina esters as an oil (33.0 g.)

2. T.L.C. ANALYSIS OF THE DAEDALIA QUERCINA ESTERS.

THE "D.Q." REFERENCE CODE.

A sample of the Daedalia quercina esters was analysed by T.L.C. in the two solvent systems:-

A. 100 % chloroform,

B. 1 % methanol : 99 % chloroform.

The eluted plates were developed by spraying with iodine vapour, ceric ammonium nitrate-sulphuric acid solution, rhodamine 6G solution and a solution of Brady's reagent. The staining characteristics and standard R_f values of the eight principal substances are collected in Table 3.1.

For ease of reference, the code names D.Q.1, D.Q.4, D.Q.5, D.Q.6, D.Q.7, D.Q.8, D.Q.9 and D.Q.10 will be used throughout the text to uniquely represent the compounds whose chromatographic properties are defined in Table 3.1.

3. CHROMATOGRAPHIC FRACTIONATION OF THE D. QUERCINA ESTERS ON ALUMINA.

A portion of the D. quercina esters (24.1 g.) was adsorbed from light petroleum onto a column of Woelm grade IV neutral alumina (560 g., 4 x 60 cm., R_v 710 ml.) and eluted with a gradient extending from light petroleum (1.5 l.) through 20 % ether in light petroleum (2 l.) and ether (1 l.) to ethyl acetate (1 l.). Fractions (26 ml.) were taken from a flow rate of 104 ml. per hour and were assayed for metabolite content by T.L.C. in chloroform and 1 % methanol in chloroform. The following table summarises the fractionation produced.

Fraction	Weight (mg.)	Content	Eluant
57-69	285	D.Q.1, D.Q.6	8 % ether in petrol
70-90	872	D.Q.1	11 % " " "
91-100	388	D.Q.7, D.Q.8,	14 % " " "
101-126	288	D.Q.4, D.Q.7, D.Q.8	15 % " " "
127-152	621	D.Q.5	42 % " " "
153-159	415	D.Q.9	18 % ethyl acetate
160-176	742	D.Q.10	25 % ethyl acetate
	3,611 i.e. 15 % recovery		

Pure samples of the compounds D.Q.1 and D.Q.5 were obtained by crystallisation of the material contained in fractions 70-90 and 127-159 respectively from light petroleum: ethyl acetate mixtures. The compound D.Q.-4 was separated from the compounds D.Q.7 and D.Q.8 by fractional crystallisation from light petroleum: ethyl acetate systems. P.L.C. on rhodamine-treated plates with

1 % methanol in chloroform as solvent led to the purification of the compounds D.Q.7 and D.Q.8 while a similar operation was used to purify the compounds D.Q.9 and D.Q.10. The trace of the compound D.Q.6 present in fractions 57-69 was isolated by P.L.C. in chloroform of the material remaining after the bulk of the compound D.Q.1 had been removed by crystallisation. See Table 6.1 for yields.

4. CHROMATOGRAPHIC FRACTIONATION OF THE D. QUERCINA ESTERS ON SILICIC ACID.

A portion of the D. quercina esters (21.269 g.) was adsorbed from light petroleum onto a column of Mallinckrodt silicic acid (490 g., 4 x 60 cm., R_v 460 ml.) and eluted with a gradient of light petroleum (1 l.) to ethyl acetate (3 l.). Fractions (22 ml.) were taken from a flow rate of 88 ml. per hour and their T.L.C. assay in chloroform and 1 % methanol in chloroform permitted the following groupings to be made:-

Fraction	Weight (g.)	Content	Eluant (petrol in ethyl acetate)
45-49	2.406	D.Q.1, D.Q.6	37 % to 33 %
50-56	5.508	D.Q.1	33 % to 29 %
57-66	2.517	D.Q.1, D.Q.-4	29 % to 25 %
67-87	5.371	D.Q.5, D.Q.7, D.Q.8	25 % to 15 %
88-100	2.847	D.Q.9	15 % to 11 %
101-110	1.675	D.Q.10	11 % to 9 %
	20.324	i.e. 95 % recovery	

The compound D.Q.1 was obtained from fractions 45-49 and from fractions 50-56 by means of crystallisation from light petroleum. This method also permitted the separation of the binary mixture of the compounds D.Q.1 and D.Q.4 present in fractions 57-66. The materials D.Q.9 and D.Q.10 were purified by P.L.C. on sets of rhodamine-treated plates employing a three fold elution with 1 % methanol in chloroform.

Fractionation of the ternary mixture contained in fractions 67-87 was achieved by the more complex procedure described as follows. The material from the grouped fractions 67-87 (5.371 gm.) was adsorbed from light petroleum onto a column of Mallinckrodt silicic acid (380 g., 4 x 55 cm., R_v 400 ml.) and eluted with a gradient of light petroleum (1.5 l.) to 50 % light petroleum: 50 % ethyl acetate (1.5 l.). Fractions (20 ml.) were taken from a flow rate of 80 ml. per hour. T.L.C. analysis indicated that fractions 52-59 (1.625 g.) contained a mixture of the compounds D.Q.5 and D.Q.7, while fractions 60-68 contained a mixture of the three substances D.Q.5, D.Q.7 and D.Q.8 (1.584 g.).

Samples (300 mg.) of the material provided by the combined fractions 52-59 were subject to P.L.C. on six rhodamine-treated plates (200 x 200 x 1 mm.) employing a threefold elution with chloroform. The pure compounds D.Q.5 and D.Q.7 were removed from the silica by elution with ethyl acetate and were treated with decolourising charcoal prior to crystallisation.

A further sample of the substance D.Q.5 was obtained by

TABLE 6.1. YIELDS OF THE D. QUERCINA METABOLITES OBTAINED BY CHROMATOGRAPHY.

With the exception of the compounds D.Q.4 and D.Q.5, the esters of the metabolites of Daedalia quercina decompose appreciably on standing at room temperature. This fact makes it difficult to accurately assess the yields of each metabolite obtained from the described separational procedure. The following table, however, gives an indication of the amounts of pure substance which might be expected.

(mg.)	Adsorbent	
	Alumina	Silicic Acid
Initial crudeweight	24,010	21,270
D.Q.1	900	5,260
D.Q.4	220	1,820
D.Q.5	500	1,210
D.Q.6	6	10
D.Q.7	50	280
D.Q.8	40	120
D.Q.9	100	540
D.Q.10	100	580

similar methods from the material of the combined fractions 60-68. Under these conditions, however, no resolution between the compounds D.Q.7 and D.Q.8 was obtained. These substances were finally separated by P.L.C. of samples (50 mg.) of the mixture on ten rhodamine-treated plates (200 x 200 x 0.5 mm.) employing multiple elution with the solvent system, 30 % benzene: 70 % chloroform. Pure samples of each component were obtained only after three such plating operations. For yields see Table 6.1.

5. METHYL POLYPORENATE C (3.1) AND METHYL 7,11-DIHYDROPOLY-PORENATE C (3.2). THE SUBSTANCE D.Q.4.

This substance was isolated as described in Sections 3 and 4. It crystallised from a light petroleum : ethyl acetate system as needles, m.p. 193-194°C.

ν_{max} (KBr) 3500 (broad), 3086, 3030, 2832, 1734, 1711, 1642, 890 cm^{-1}

ν_{max} (1.43 mM., CCl_4) 3623 cm^{-1} (ε 76.6, $\Delta\nu_{\frac{1}{2}}$ 16), 1736 cm^{-1} (ε 580, $\Delta\nu_{\frac{1}{2}}$ 18), 1713 cm^{-1} (ε 569, $\Delta\nu_{\frac{1}{2}}$ 14), also 3083, 3031, 2842, 1642 cm^{-1}

λ_{max} (CH_3OH) 2372 Å (ε 13,500), 2430 Å (ε 15,300), 2510 Å (ε 10,600).

Tau values (CCl_4) 4.55 (2H, t, J=5.4 c/s.), 5.25 (2H, d, J=3 c/s.), 5.90 (1H, m), 6.28 (3H, s).

R. D. (methanol, c=0.5559) $[I]_{500}$ + 198, $[I]_{263}$ + 15,400.

Molecular weight 492 ± 6 (isothermal distillation from methyl ethyl ketone)

Parent molecular ions at m/e 496 (88 %) and m/e 498 (12 %).

Analysis	Found	C	77.20 %	H	9.80 %	CH_3O	6.58 %
Calculated for	$C_{32}H_{48}O_4$	C	77.37 %	H	9.74 %	CH_3O	6.25 %

This material was shown to be identical (R_f, mass spectrum, mixed melt) with a sample of methyl polyporenate C generously provided by Dr. T. G. Halsall. Both the isolated and authentic samples contained approximately 12 % of the related dihydro compound.

6. METHYL 7,11-DEHYDROTUMULOSATE (3.3) AND METHYL TUMULOSATE (3.5). THE SUBSTANCE D.Q.5.

a. A sample of the mixture methyl polyporentate C and methyl dihydropolyporenate C (200 mg.) was dissolved in methanol (5 ml.) and cooled to 0°C. To this solution solid sodium borohydride (50 mg.) was added over 5 minutes. After standing at room temperature for two hours, the reaction solution was added to water (20 ml.) and extracted with ethyl acetate (2 x 25 ml.). The combined ethyl acetate extracts were washed with water (2 x 30 ml.), dried and evaporated to give a white solid (129 mg., 98 %) which crystallised from aqueous methanol in needles, m.p. 176.5-178°C.

ν_{max} (KBr) 3450 (broad), 3086, 3030, 1735, 1642, 890 cm^{-1}.

ν_{max} (1.47 mM., CCl_4) 3623 cm^{-1} (ε 123, $\Delta\nu_{\frac{1}{2}}$ 23), 1737 cm^{-1} (ε 538, $\Delta\nu_{\frac{1}{2}}$ 15) also 3080, 3028, 2846, 1645 cm^{-1}

λ_{max} (CH_3OH) 2370 Å (ε 10,400), 2430 Å (ε 14,700), 2510 Å (ε 12,700).

Tau values (CCl_4) 4.58 (2H, t, J=6 c/s.), 5.25 (2H, d, J=2 c/s.), 5.90 (1H, m), 6.30 (3H,s), 6.74 (1H, m).

b. The compound D.Q.5 was isolated as described in Sections 3 and 4. It crystallised from aqueous methanol as needles, m.p. 175-177°C.

λ_{max} (CH_3OH) 2370 Å (ε 12,100), 2430 Å (ε 14,300), 2520 Å (10,800)

Parent molecular ions at m/e 498 (71 %) and m/e 500 (29 %).

Analysis	Found	C	76.68 %	H	10.30 %	OCH_3 7.01 %
Calculated for	$C_{32}H_{50}O_4$	C	77.06 %	H	10.10 %	OCH_3 6.25 %

This material was found to be similar (R_f, i.r. and mixed melt) with the sample prepared in 'a' above. The ratio of monoene to diene would appear to have little effect on melting points, mixed melting points or R_f values.

7. METHYL 3α,16α - DIHYDROXY-24-METHYLENELANOST-7,9(11) - DIENE-21-OATE (3.4).

Clean aluminium foil (22 g.) was suspended in redistilled isopropanol (100 ml.), mercuric chloride (100 mg.) and carbon tetrachloride (2 ml.) were added and the reaction was refluxed for 17 hours. To a portion of this preparation of aluminium isopropoxide (50 ml.) was added the naturally occurring mixture of methyl polyporenate C and methyl 7,11-dihydropolyporenate C (1.058 g.). After a further reflux period of 8 hours, the reaction solution was added to cold 6N aqueous hydrochloric acid (25 ml.) and extracted with ethyl acetate (2 x 25 ml.) The combined ethyl acetate extracts were washed with water (2 x 50 ml.), dried and evaporated to give a white solid (981 mg.) which was shown by T.L.C. in 1 % methanol in chloroform to be a binary mixture. This mixture was separated by P.L.C. on four rhodamine-treated plates (200 x 200 x 1 mm.) employing a two-fold elution with 1 % methanol in chloroform.

The less polar compound (421 mg., 40 %) was removed from the silica by elution with ethyl acetate, treated with decolourising charcoal and crystallised from aqueous methanol in needles, m.p. 176-177°C. It was shown (R_f and mixed melt) to be the β-alcohol.

The corresponding α-alcohol (290 mg., 27 %) was eluted from the silica in similar manner and crystallised from aqueous methanol in needles, m.p. 171-173°C.

ν_{max} (KCl) 3440 (broad), 3079, 3026, 2840, 1735, 1718, 1640, 888 cm^{-1}.

Tau values (CCl_4) 4.60 (2H, t, J=6 c/s.), 5.28 (2H, d, J= 2 c/s.),

5.99 (1H, m), 6.32 (3H, s), 6.60 (1H, m).

Analysis Found C 76.76 % H 10.40 %

Calculated for $C_{32}H_{50}O_4$ C 77.06 % H 10.10 %

8. METHYL METHOXYCARBONYLACETYLQUERCINATE. THE COMPOUND D.Q.1.

This substance was isolated from the mixture of D. quercina esters as described in Sections 3 and 4. It crystallised from light petroleum as needles, m.p. 101-103°C.

ν_{max} (KBr) 2835, 1751, 1733, 1706, 1468, 1456, 1437, 1418, 1390, 1366-75, 1348, 1339, 1292, 1263, 1222, 1199, 1179, 1159, 1036, 1022, 978, 893, 880, 847 cm^{-1}.

ν_{max} (0.809 mM., CCl_4) 1758 cm^{-1} (ε 632, $\Delta\nu_{\frac{1}{2}}$13), 1736 cm^{-1} (ε 1272, $\Delta\nu_{\frac{1}{2}}$18), 1716 cm^{-1} (ε 405, $\Delta\nu_{\frac{1}{2}}$20) also 2837, 1470, 1435, 1408, 1390, 1377, 1370, 1357, 1327, 1310, 1254, 1242, 1200, 1156, 1058, 1034, 1025 cm^{-1}.

ν_{max} (4.16 mM., CS_2) 1758 cm^{-1} (ε 766, $\Delta\nu_{\frac{1}{2}}$13), 1736 cm^{-1} (ε 1500, $\Delta\nu_{\frac{1}{2}}$16), 1715 cm^{-1} (ε 486, $\Delta\nu_{\frac{1}{2}}$18).

λ_{max} (CH_3OH) 2820 Å (ε 86.1).

Tau values (CCl_4) 5.30 (1H, t, J=2.7 c/s.), 6.30 (3H, s), 6.37 (3H, s), 6.70 (2H, s). Methyl contour defined by peaks at 8.82, 8.87, 8.97, 9.00, 9.08, 9.12 and 9.25.

R. D. (methanol, c=0.137) $[\bar{x}]_{400}$ -210, $[\bar{x}]_{303}$ -2,700, $[\bar{x}]_{256}$ +4,590, $[\bar{x}]_{250}$ +4,380.

Parent molecular ion at m/e 600 = $C_{36}H_{56}O_7$.

Analysis Found C 72.16 % H 9.22 % CH_3O 10.63 %

$C_{36}H_{56}O_7$ requires C 71.96 % H 9.39 % CH_3O 10.30 %.

The compound D.Q.1 normally decomposes when treated with Brady's reagent; however, on one occasion a crystalline 2,4-dinitrophenylhydrazone was isolated from the reaction. It crystallised from methanol as fine orange needles, m.p. 150-162°C (dec.)

ν_{max} (0.89 mM., CCl_4) 1757 cm^{-1} (ε 511, $\Delta\nu_{\frac{1}{2}}$15), 1738 cm^{-1} (ε 1038, $\Delta\nu_{\frac{1}{2}}$19).

λ_{max} (CH_3OH) 3630 Å (ε 23,500).

9. POTASSIUM METHOXYCARBONYLACETATE

Redistilled dimethyl malonate (61.295 g., 0.464 M.) was dissolved in methanol (100 ml.) and added dropwise over a period of 90 minutes to a stirred solution of potassium hydroxide (24.784 g., 0.450 M.) in methanol (200 ml.). After a reaction period of 22 hours, the white solid was filtered off, the filtrate treated with diethyl ether to precipitate the remaining product and the combined crude potassium methoxycarbonylacetate (48.011 g., 84 %) washed with ether (500 ml.). Potassium methoxycarbonylacetate crystallised from methanol in plates, (42.22 g.) m.p. 205-206°C (dec.).

ν_{max} (KCl) 1735, 1605, 1375, 1304 cm^{-1}.

Analysis	Found	C	31.34 %	H	3.72 %
Calculated for	$C_4H_5O_4K$	C	31.01 %	H	3.23 %

10. METHOXYCARBONYLACETYL CHLORIDE.

A solution of thionyl chloride (34.8 g., 0.255 M.) in anhydrous ether (70 ml.) was added dropwise over 2 hours to a chilled suspension of potassium methoxycarbonylacetate (47.9 g., 0.306 M.) in dry ether (200 ml.). The reaction temperature was maintained at 0°C for 6 hours and then allowed to rise to 23°C for a further period of 6 hours. Thereafter the precipitated potassium sulphate was removed by filtration through glass paper, and the solvent evaporated to give a brown oil (33.98 g.). Low pressure distillation gave pure methoxycarbonylacetyl chloride, b.p. 54-60°C, 15 mmHg. (9.508 g., 30 %).
ν_{max} (liquid film) 1798, 1740, 1440, 2880 cm^{-1}.

11. CHOLESTERYL METHOXYCARBONYLACETATE (3.19).

A solution of cholesterol (1.001 g., 2.6 mM.) and dry pyridine (4 ml.) in anhydrous ether (10 ml.) was added dropwise at room temperature over 1 hour to a solution of methoxycarbonylacetyl chloride (6 g., 28.5 mM.) in dry ether (10 ml.). Stirring was maintained for a further two hours. Thereafter the ether solution was treated with water (25 ml.), washed with 6N aqueous hydrochloric

acid (6 x 25 ml.), dried and evaporated to give an oily solid (1.491 g.). This solid was adsorbed from light petroleum onto a column of B.D.H. silica (100 g., 3 x 24 cm.); elution with 5 % ether in light petroleum (500 ml.) provided pure cholesteryl methoxycarbonylacetate (1.162 g., 92 %) which crystallised from a light petroleum: ethyl acetate system as needles, m.p. 108-108.5°C.

ν_{max} (KCl) 1765, 1736, 1296, 1284, 1216, 1150, 1030, 1014 cm^{-1}.

ν_{max} (1.15 mM., CCl_4) 1759 cm^{-1} (ε 570, $\Delta\nu_{\frac{1}{2}}$ 17), 1739 cm^{-1} (ε 697, $\Delta\nu_{\frac{1}{2}}$ 17).

Tau values (CCl_4) 5.40 (1H, m), 6.25 (3H, s), 6.79 (2H, s).

Parent molecular ion at m/e 486.

Analysis Found C 76.47 % H 10.50 %

$C_{31}H_{50}O_4$ requires C 76.50 % H 10.35 %

12. METHANOLYSIS OF CHOLESTERYL METHOXYCARBONYLACETATE

In a reaction conducted in a small dry box cholesteryl methoxycarbonylacetate (244 mg.) was dissolved in anhydrous methanol (10 ml.) and treated with a preparation of sodium methoxide (50 mg. sodium) in anhydrous methanol (10 ml.) at room temperature for 12 hours. Thereafter the reaction solution was quickly added to 16N aqueous hydrochloric acid (10 ml.) and immediately extracted with ether (2 x 25 ml.). The combined ether layers were washed with water (2 x 40 ml.), dried and

evaporated to give a white solid (156 mg., 81 %) which was found to be identical (i.r., R_f, mixed melt) with an authentic sample of cholesterol.

13. METHANOLYSIS OF METHYL METHOXYCARBONYLACETYLQUERCINATE.

PART I - THE VOLATILE PRODUCT

Again under anhydrous conditions, methyl methoxycarbonylacetylquercinate (162 mg.) was dissolved in dry methanol (10 ml.) and treated with an anhydrous preparation of sodium methoxide (10 mg. sodium) in dry methanol (10 ml.). After a reflux period of 8 hours, the reaction solution was quickly added to chilled 16N aqueous hydrochloric acid (20 ml.) and immediately extracted with chloroform (20 ml.). The washed, dried chloroform extract was reduced in volume to approximately 1 ml. by careful fractional distillation before being analysed by gas-liquid chromatography on a 4 foot 10 % polyethyleneglycol adipate column (Pye Argon, flow 32 ml. argon per minute, temperature 75°C). The reaction solution was found to give rise to a major peak of retention time 29.8 minutes; the retention time of an authentic sample of dimethyl malonate was 29.2 minutes. Co-application of dimethyl malonate and the reaction solution gave rise to a single well-formed peak of retention time 30.1 minutes. Minor components were detected in the reaction solution with retention times of 20.1, 16.0 and 10.3 minutes.

14. METHANOLYSIS OF METHYL METHOXYCARBONYLACETYLQUERCINATE.

PART II - THE NON-VOLATILE PRODUCTS

In a reaction conducted in a small dry-box, methyl methoxycarbonylacetylquercinate (2.40 g.) was dissolved in anhydrous methanol (20 ml.) and treated with a preparation of sodium methoxide (100 mg. sodium) in methanol (20 ml.) at room temperature for 13 hours. Thereafter a small aliquot was removed from the reaction vessel, acidified, extracted with ethyl acetate and examined by T.L.C. in chloroform. Since unreacted starting material was detected, the complete reaction was allowed to proceed for a further 12 hours at the temperature of refluxing methanol. At the conclusion of this second period, the reaction solution was quickly added to 16N aqueous hydrochloric acid (60 ml.) and immediately extracted with ethyl acetate (2 x 100 ml.). The combined ethyl acetate extracts were washed with water (2 x 100 ml.) and evaporated to give a white solid (1.801 gm.) which was shown by T.L.C. in chloroform to be a mixture of three substances, R_f values 0.21, 0.36 and 0.50 (p-amino-azobenzene, R_f value 0.56, p-hydroxy-azobenzene, R_f value 0.21).

These substances were fractionated on a column of Mallinckrodt silicic acid (80 g., 2 x 30 cm.) employing a gradient of light petroleum (1 l.) to 50 % of light petroleum: 50 % ethyl acetate. Fractions (20 ml.) were taken from a flow rate of 40 ml. per hour and were assayed by T.L.C. in chloroform.

a. The substance of R_f value 0.51. Methyl neoquercinate.

Fractions 23-31 of the above column, eluted with solvent varying in composition from 15 % to 21 % ethyl acetate, contained the compound of R_f value 0.51 (673 mg.). It was further purified by P.L.C. on three $HF_{256/366}$ plates (200 x 200 x 1mm.) employing a twofold elution with chloroform. Removal of the compound from the silica by means of ethyl acetate elution provided methyl neoquercinate as an oil which could not be induced to crystallise.

ν_{max} ($CHCl_3$) 1729, 1710 cm^{-1}.

ν_{max} (CCl_4) 3630, 1740, 1716 cm^{-1}.

Tau values ($CDCl_3$) 6.33 (3H, d, J=1.2 c/s.) 6.58 (1H,m), methyl contour defined by peaks at 8.82, 8.87, 8.90, 9.00, 9.01, 9.11, 9.26.

Tau values (CCl_4) 6.38 (3H, d, J=1.2 c/s), 6.69 (1H, m), methyl contour defined by peaks at 8.94, 9.03, 9.10, 9.18, 9.28.

Parent molecular ion at m/e 500.

A sample of this oil (82 mg.) and pyridine (0.2 ml.) were dissolved in anhydrous ether (5 ml.) and added dropwise over 15 minutes to a stirred solution of methoxycarbonylacetyl chloride (982 mg.) in dry ether (10 ml.). After a further two hours at room temperature, the solution was treated with water (30 ml.) and the ethereal layer washed with 6N aqueous hydrochloric acid (6 x 20 ml.), dried and evaporated to give an oil (180 mg.). T.L.C. analysis in chloroform revealed that

at least four components were present, R_f value 0.52, 0.46, 0.36 and 0.00, (p-amino-azobenzene, R_f 0.59, p-hydroxy-azobenzene, R_f 0.18). The least polar of these was isolated by P.L.C. on two rhodamine treated plates (200 x 200 x 0.6 mm.) employing chloroform as eluant. It was an oil (32 mg.) which was found to be similar (n.m.r., R_f value) but not identical with an authentic sample of methyl methoxycarbonylacetylquercinate.

b. <u>The substance of R_f value 0.21. Methyl isoquercinate</u>

Fractions 37-44 of the above column, eluted with solvent varying in polarity from 23 % to 28 % ethyl acetate, provided an oil (457 mg.) which contained both the substance of R_f value and 0.36 and that of R_f value 0.21. Fractional crystallisation of the mixture from a light petroleum: ethyl acetate system provided a pure sample (201 mg.) of the more polar substance. It crystallised from light petroleum: ethyl acetate mixtures as needles, m.p. 284-286°C.

ν_{max} (CCl_4) 3630, 1740, 1716 cm^{-1}.

Tau values ($CDCl_3$) 6.31 (3H, d, J=1.2 c/s), 6.58 (1H, m), methyl contour defined by peaks at 8.75, 8.89, 9.01, 9.03, 9.13 and 9.28.

Parent molecular ion at m/e 500.

Within hours of isolation this substance decomposed to the new compound anhydro-isoquercinic acid which crystallised from a light petroleum: ethyl acetate system as needles, m.p. 270-274°C.

ν_{max} (KBr) 3530 (broad), 1752 (s), 1731, 1684, 1094, 1058, 1047, 1007, 986, 978, 958, 765 cm^{-1}.

ν_{max} (1.15 mM., $CHCl_3$) 1742 cm^{-1} (ε 956, $\Delta\nu_{\frac{1}{2}}$ 21), 1683 cm^{-1} (ε 113, $\Delta\nu_{\frac{1}{2}}$ 12).

λ_{max} (CH_3OH) 2170 Å (ε 14,600).

Tau values ($CDCl_3$). 5.20 (1H, m), 6.58 (1H, m) 8.04 (3H, d, J=1.2 c/s), 8.18 (3H, d, J=1.2 c/s), methyl contour defined by peaks at 8.73, 8.89, 9.00, 9.01, 9.10, 9.25.

Parent molecular ion at m/e 468.

Analysis Found C 79.05 % H 10.10 %

$C_{31}H_{48}O_3$ requires C 79.44 % H 10.32 %

c. <u>The substance of R_f value 0.36. Anhydroquercinic acid</u>

The mother liquor from the crystallisation of methyl isoquercinate was evaporated to give a solid (251 mg.) from which a sample of the compound of R_f value 0.36 was isolated by P.L.C. on one $HF_{256/366}$ plate (200 x 200 x 1mm.) using chloroform as eluant. The substance was recovered from the silica with ethyl acetate, treated with decolourising charcoal and crystallised from ethyl acetate as needles (126 mg.), m.p. 228-229°C.

ν_{max} (KBr) 3543, 3470, 1754 (s), 1740, 1684, 1093, 1062, 1048, 1009, 990, 977, 960, 768 cm^{-1}.

ν_{max} (5.56 mM., $CHCl_3$) 1742 cm^{-1} (ε 480, $\Delta\nu_{\frac{1}{2}}$ 22), 1684 cm^{-1}.

ν_{max} (CCl_4), 3638, 2836, 1764, 1689 cm^{-1}.

λ_{max} (CH_3OH) 2170 Å (ε 12,700).

Tau values ($CDCl_3$) 5.25 (1H, d, J=7.8 c/s). 6.57 (1H, m), 8.03 (3H, s), 8.19 (3H, d. J=1.2 c/s), methyl contour defined by peaks at 8.90, 9.00, 9.01, 9.10, 9.13, 9.28.

Parent molecular ion at m/e 468.

Analysis Found C 79.73 % H 9.99 %

$C_{31}H_{48}O_3$ requires C 79.44 % H 10.32 %

15. HYDROGENATION OF ANHYDROQUERCINIC ACID

Anhydroquercinic acid (37 mg.) was dissolved in ethanol (5 ml.) and shaken for two hours with hydrogen at room temperature and atmospheric pressure in the presence of platinum oxide (10 mg.). Removal of the catalyst and evaporation of the solvent yielded a white solid (32 mg.) whose i.r. spectrum was identical to that of anhydroquercinic acid.

However, hydrogenation was effected at room temperature and atmospheric pressure with glacial acetic acid (5 ml.) as solvent and platinum oxide as catalyst (10 mg.). After a five hour period, the catalyst was removed by filtration through glass paper and the solvent evaporated to give a solid (26 mg.) which was purified by P.L.C. on two rhodamine treated plates (200 x 200 x 0.25 mm.) using chloroform as eluant. The pure dihydroanhydroquercinic acid was removed from the silica by elution with ethyl acetate and crystallised from a light petroleum:

ethyl acetate system as rosettes (7 mg.), m.p. 221-223°C

ν_{max} (CCl_4) 3638, 1782 cm^{-1}.

λ_{max} (CH_3OH) no absorption above 2200 Å

Tau values ($CDCl_3$) 6.32 (1H, m), 6.60 (1H, m), methyl contour defined by peaks at 8.76, 8.80, 8.92, 9.01, 9.04, 9.10, 9.14, 9.21 and 9.30.

Parent molecular ion at m/e 470.

16. ATTEMPTED PREPARATION OF THE ETHYLENE KETAL OF METHYL ISOQUERCINATE

Methyl isoquercinate (200 mg.) was dissolved in dry benzene (10 ml.) and refluxed with redistilled ethylene glycol (1 ml.) and p-toluenesulphonic acid (3 mg.) for 16 hours with azeotropic removal of water. Thereafter the solution was cooled, washed with water (4 x 20 ml.), dried and evaporated to give a solid (65 mg.) which crystallised from an ethyl acetate: light petroleum system as needles (42 mg.), m.p. 270-274°C.

This material was identical (R_f, i.r., n.m.r. and mixed melt) with isoanhydroquercinic acid.

17. THE COMPOUND D.Q.6

This substance was isolated as described in Sections 3 and 4. The small amount of compound available crystallised as needles from petrol, m.p. 184-192°C.

ν_{max} (1.57 mM., CCl_4) 1738 cm^{-1} (ε 941, $\Delta\nu_{\frac{1}{2}}$ 27), 1718 cm^{-1} (ε 523, $\Delta\nu_{\frac{1}{2}}$ 22), also 2834, 1470, 1455, 1388, 1376, 1371, 1357, 1246, 1195, 1178, 1159, 1060, 1036, 1017 cm^{-1}.

λ_{max} (CH_3OH). No significant absorption above 2200 Å

Parent molecular ion at m/e 542.

18. THE COMPOUND D.Q.7

The isolation of the compound is detailed in Sections 3 and 4. It crystallised from ethyl acetate : light petroleum as needles m.p. 136-138°C.

λ_{max} (1.43 mM, CCl_4) 1758 cm^{-1} (ε 656, $\Delta\nu_{\frac{1}{2}}$ 15), 1740 cm^{-1} (ε 1110, $\Delta\nu_{\frac{1}{2}}$ 20); 1719 cm^{-1} (ε 425, $\Delta\nu_{\frac{1}{2}}$ 17), also 2833, 1468, 1457, 1434, 1408, 1389, 1263, 1245, 1200, 1167, 1151, 1143, 1116, 1103, 1057, 1047, 1033, 1024, 1013 cm^{-1}.

λ_{max} (CH_3OH) 3300 Å (ε 209)

Tau values (CCl_4) 5.30 (1H, t, J=2.7 c/s.), 5.5 (2H, m), 6.30 (3H, s), 6.36 (3H, s), 6.69 (2H, s), 7.38 (2H, m), methyl contour defined by peaks at 8.85, 8.98, 9.06, 9.11, 9.20, 9.24.

R.D. (methanol, C 0.0247) $[\alpha]_{500}$ - 703, $[\alpha]_{341}$ + 16,200, $[\alpha]_{314}$ - 16,200, $[\alpha]_{279}$ - 3,480, $[\alpha]_{254}$ - 4,460

Parent molecular ion at m/e 612 = $C_{37}H_{56}O_7$.

Analysis	Found	C 69.65 %	H 8.55 %
$C_{37}H_{56}O_7$	requires	C 72.52 %	H 9.21 %
$C_{37}H_{56}O_9$	requires	C 68.92 %	H 8.75 %

19. PRELIMINARY RESULTS FROM THE METHANOLYSIS OF THE COMPOUND D.Q.7

In a reaction conducted in a small dry box the compound D.Q.7 (313 mg.) was dissolved in dry methanol (10 ml.) and refluxed with an anhydrous preparation of sodium methoxide (20 mg. sodium) in dry methanol (10 ml.) for 11.5 hours. Thereafter the reaction solution was added to chilled 16N aqueous hydrochloric acid (40 ml.) and immediately extracted with ether (2 x 50 ml.). The combined ethereal extracts were washed with water (2 x 100 ml.), dried and evaporated to give a white solid (257 mg.) which was shown by T.L.C. analysis in 1 % methanol in chloroform to contain two substances of closely allied polarity, R_f values 0.50 and 0.45 (p-amino-azobenzene, R_f 0.81, p-hydroxy-azobenzene, R_f 0.51).

P.L.C. on two rhodamine-treated plates (200 x 200 x 1 mm.) employing a sixfold elution with chloroform led to a partial separation of the components (R_f 0.50, 48 mg., m.p. 103-109°C, R_f 0.45, 42 mg., oil); in each case one substance was slightly contaminated by the

other. Both isolated samples shared the following spectroscopic data:-

ν_{max} (CCl_4) 3634, 3390, 1734, 1715 cm^{-1}

Tau values (CCl_4) 6.34 (3H, s), 6.65 (1H, m) methyl contour defined by peaks at 9.01, 9.06, 9.15, 9.25.

The compound of R_f value 0.50 exhibited the following spectroscopic data:-

λ_{max} (CH_3OH) Maxima at 2250 Å, 2320 Å, 2410 Å all with ε = 4,250 based on a molecular weight of 510.

Parent molecular ions at m/e 498, m/e 500, m/e 510, m/e 512, m/e 514, m/e 526, m/e 528.

20. THE COMPOUND D.Q.8

This compound was isolated as described in Section 3 and 4. It crystallised from ethyl acetate : light petroleum as needles, m.p. 136.5-138°C.

ν_{max} (1.55 mM., CCl_4) 1757,cm^{-1} (ε 664, $\Delta\nu_{\frac{1}{2}}$ 15), 1739 cm^{-1} (ε 1004, $\Delta\nu_{\frac{1}{2}}$ 22), 1722 cm^{-1} (ε 420, $\Delta\nu_{\frac{1}{2}}$ 21), 2833, 1468, 1456, 1434, 1408, 1389, 1197, 1164, 1153, 1115, 1104, 1068, 1061, 1033, 1024, 1012 cm^{-1}.

λ_{max} (CH_3OH) no absorption above 2200 Å

Parent molecular ion at m/e 612.

Analysis	**Found**	**C**	**69.55 %**	**H**	**8.83 %**
$C_{37}H_{56}O_7$	**requires**	**C**	**72.52 %**	**H**	**9.21 %**
$C_{37}H_{56}O_9$	**requires**	**C**	**68.92 %**	**H**	**8.75 %**

21. THE COMPOUND D.Q.9

This substance was isolated as detailed in Sections 3 and 4. It was a clear gum.

ν_{max} (CCl_4) 3620, 3500 (broad), 1755, 1738, 1714 cm^{-1}.

λ_{max} (CH_3OH) no appreciable absorption above 2200 Å.

Tau values (CCl_4) 5.34 (1H, m), 5.94 (1H, m), 6.34 (3H, s), 6.40 (3H, s), 6.71 (2H, s), methyl contour defined by peaks at 8.74, 8.84, 8.94, 8.99, 9.06, 9.12, 9.30.

Parent molecular ion at m/e 616.

22. THE COMPOUND D.Q.10

The isolation of this compound is described in Sections 3 and 4. It was a clear gum.

ν_{max} (CCl_4) 3620, 3480 (broad), 1740, 1715, 1679 cm^{-1}.

Tau values (CCl_4) 5.93 (1H, m), 6.38 (3H, s), 6.63 (1H, m), methyl contour defined by peaks at 8.74, 8.83, 8.92, 9.01, 9.08, 9.15, 9.30.

Parent molecular ion at m/e 516, also ions at m/e 510, m/e 512, m/e 514, m/e 528 and m/e 530.

23. CULTURE OF DAEDALIA QUERCINA ON A SYNTHETIC MEDIUM. DETECTION OF THE CONJUGATES IN THE MYCELIUM

Spores were collected from two fresh sporophores of D. quercina and were allowed to germinate and grow for 29 days on malt agar plates at 25°C and 70 % relative humidity. Portions of the mycelial mat so produced were transferred to malt agar slopes and allowed to grow for a further 36 days. Thereafter the slope cultures were homogenised and the homogenate used to inoculate 76 Roux surface culture bottles which had previously been steam sterilised containing 250 ml. of 5 % aqueous malt extract. Cultures were allowed to grow undisturbed at 25°C and 70 % relative humidity, artificial illumination being provided by Mazda fluorescent tubes for 12 hours per day. After 56 days, the broth was filtered off and the mycelium collected, dried at 40°C, powdered and extracted with methanol at room temperature for 7 days. From 57.0 gms of dry mycelium, 3.12 gms of methanol extract was obtained.

T.L.C. analysis in 100 % chloroform indicated that the compounds D.Q.1, D.Q.4, D.Q.5, D.Q.7, D.Q.8, D.Q.9 and D.Q.10 were present. The assay was not sufficiently sensitive to detect any of the compound D.Q.6 that might be present.

The N.M.R. spectrum of a methylated sample of the methanol extract exhibited absorption at 6.70 tau as well as at 6.30 and 6.37 tau.

THE METABOLITES OF POLYPORUS BETULINUS

24. EXTRACTION AND PRIMARY FRACTIONATION

Fresh sporophores of Polyporus betulinus were collected in mid-Autumn from the woods around Milngavie and were processed within six hours of harvesting. The cleaned, dried tissue (992 g.) was homogenised in a Waring Blender in methanol (10 l.). The slurry was filtered free of tissue after two months extraction at room temperature. The solvent was evaporated to give a brown semi-solid (116 g.) which by extraction with boiling light petroleum (40-60°C, 2 l.) gave a petrol soluble oil (10.5 g.). The insoluble residue was dissolved in methanol (500 ml.), cooled to 0°C and treated with excess ethereal diazomethane. The ether soluble fraction of the product (75.5 g.) was slurried in ether with B.D.H. silica (50 g.), placed on a column of the same adsorbant and eluted with a gradient of light petroleum (2 l.) to ethyl acetate (2 l.). T.L.C. analysis of the fractions permitted the following groupings to be made.

Fraction	Weight (g.)	Eluant (ratio of petrol to ethyl acetate)
I	1.345	100:0 to 90:10
II	17.924	90:10 to 50:50
III	28.003	50:50 to 0:100

The standardised T.L.C. R_f values of the seven principal components of these Fractions are presented in Table 4.1.

25. ERGOSTEROL (4.4). THE COMPOUND P.B.7.

After standing for two weeks at room temperature the ethyl acetate solution of Fraction I (1.345 g.) deposited a solid (160 mg.) which crystallised from light petroleum : ethyl acetate as needles (90 mg.), m.p. 164-165°C (lit. 165°C).[5k]

ν_{max} (KBr) 3400 (broad), 3034, 1060, 1036, 1025, 980, 968 cm^{-1}.

λ_{max} (hexane) 2520 Å (ε 3,500), 2620 Å (ε 6,200), 2720 Å (ε 10,200), 2820 Å (ε 10,800), 2940 Å (ε 5,900).

Tau values (CCl_4) 4.50 (2H, m), 4.80 (2H, m).

This material was identical (i.r., R_f, mixed melt) with a sample of authentic ergosterol.

26. COLUMN CHROMATOGRAPHY OF FRACTION II

A portion (8.291 g.) of Fraction II was adsorbed from light petroleum onto a column of Mallinckrodt silicic acid (480 g., 4 x 60 cm., R_v 490 ml.) and eluted with a gradient of light petroleum (1.5 l. to 50 % light petroleum : 50 % ethyl acetate (1.5 l.). Fractions (25 ml.) were taken from a flow rate of 60 ml. per hour.

a. Methyl polyperenate C (4.9) and methyl 7,11-dihydropolyperenate C (4.10). The substance P.B.1.

Fractions 63-75 of the above column, eluted with solvent varying in composition from 21 % to 26.5 % ethyl acetate, were found by T.L.C.

analysis to contain two substances. Their R_f values in 1 % methanol : 99 % chloroform were 0.72 and 0.62 respectively (p-amino-azobenzene, R_f 0.67, p-hydroxy-azobenzene R_f 0.35). Fractional crystallisation of the mixture (2.082 g.) from a light petroleum : ethyl acetate system produced a pure sample of the slower running material (986 mg.) as needles, m.p. 193-194°C (lit. 192-194°C)[65]

ν_{max} (KBr) 3500 (broad), 3086, 3030, 2832, 1734, 1711, 1642, 890 cm^{-1}.

ν_{max} (1.43 mM. CCl_4) 3623 cm^{-1} (ε 76.6, $\Delta\nu_{\frac{1}{2}}$ 16), 1736 cm^{-1} (ε 580, $\Delta\nu_{\frac{1}{2}}$ 18) 1713 cm^{-1} (ε 569, $\Delta\nu_{\frac{1}{2}}$ 14).

λ_{max} (CH_3OH) 2372 Å (ε 13,500), 2430 Å (15,300), 2510 Å (ε 10,600).

Tau values (CCl_4) 4.55 (2H, t, J=5.6 c/s.), 5.25 (2H, d, J=3 c/s.), 5.90 (1H, m), 6.28 (3H, s).

R.D. (methanol c=0.5559) $[\Phi]_{500}$ + 198, $[\Phi]_{263}$ + 15,400.

Molecular weight 492 ± 6 (isothermal distillation from methyl ethyl ketone solution).

Parent molecular ions at m/e 496 (88 %) and m/e 498 (12 %).

Analysis	Found	C 77.20 %	H 9.80 %	CH_3O 6.58 %
Calculated for $C_{32}H_{48}O_4$		C 77.37 %	H 9.74 %	CH_3O 6.25 %

This material was shown to be identical (R_f, mass spectrum, mixed melt) with a sample generously provided by Dr. T. G. Halsall. Both samples showed the presence of approximately 12 % of the

related dihydro compound.

b. Methyl methoxycarbonylacetylpolyporenate A (4.15).

The compound P.B.2.

Fractions 79-93 of the column previously described, eluted with solvent varying in composition from 27 % to 30 % ethyl acetate, were found by T.L.C. analysis to contain one single substance. Its R_f value was 0.51 in 1 % methanol : 99 % chloroform (p-amino-azobenzene, R_f 0.67, p-hydroxy-azobenzene, R_f 0.31). The white solid (2.081 g.) crystallised from a light petroleum : ethyl acetate system as needles (1.500 g.), m.p. 88-89°C.

ν_{max} (KBr) 3602, 3090, 2832, 1755, 1736, 1645, 1236, 1163, 1037, 1020, 890 cm^{-1}

ν_{max} (1.16 mM. CCl_4) 3630 cm^{-1} (ε 60, $\Delta\nu_{\frac{1}{2}}$ 17), 1756 cm^{-1} (ε 705, $\Delta\nu_{\frac{1}{2}}$ 16) 1736 cm^{-1} (ε 1220, $\Delta\nu_{\frac{1}{2}}$ 19) also 3090, 2832 and 1646 cm^{-1}.

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 5.12 (2H, s), 5.34 (1H, t, J=2.4 c/s.), 6.04 (1H, d, J=7.2 c/s.), 6.32 (3H, s), 6.34 (3H, s), 6.70 (2H, s), 6.92 (1H, q, J=7.2 c/s.), 8.74 (3H, d, J=7.2 c/s.).

Parent molecular ion at m/e 600 = $C_{36}H_{56}O_7$.

R. D. (methanol c=0.433) $[\Xi]_{500}$ +300, $[\Xi]_{400}$ +480, $[\Xi]_{300}$ + 1110, $[\Xi]_{250}$ +2640, $[\Xi]_{238}$ + 4170, $[\Xi]_{233}$ +4230, $[\Xi]_{227}$ +3060.

Analysis	Found	C	71.69 %	H	9.54 %
$C_{36}H_{56}O_7$	requires	C	71.97 %	H	9.37 %

27. METHYL 3-METHOXYCARBONYLACETYL-24,28-DIHYDROPOLYPORENATE A (4.17)

Methyl 3-methoxycarbonylacetylpolyporenate A (131 mg.) was dissolved in ethanol (10 ml.) and hydrogenated at room temperature and atmospheric pressure for two hours with platinum oxide (31.9 mg.) as catalyst. When the hydrogen absorption was complete (5 ml.), the catalyst was removed by filtration through glass paper and the resulting solution evaporated to give a white solid (128 mg.) which crystallised from aqueous methanol as needles (98 mg.), m.p. 140-141°C.

ν_{max} (KBr) 3560, 2830, 1756, 1736, 1262, 1232, 1153, 1037, 1019 cm^{-1}.

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 5.32 (1H, t, J=2.4 c/s.), 6.04 (1H, d, J=7.2 c/s.), 6.32 (3H, s), 6.38 (3H, s), 6.71 (2H, s), 8.91 (3H, d, J=7.2 c/s.).

Parent molecular ion at m/e 602 = $C_{36}H_{58}O_7$.

Analysis	Found	C	71.43 %	H	9.45 %
$C_{36}H_{58}O_7$	requires	C	71.73 %	H	9.70 %

28. METHANOLYSIS OF METHYL 3-METHOXYCARBONYLACETYLPOLYPORENATE A.

a. Isolation of methyl isopolyporenate A (4.16)

In a reaction conducted in a small dry-box, sodium methoxide, prepared from sodium (50 mg.) and anhydrous methanol (10 ml.), was added to a solution of methyl 3-methoxycarbonylacetylpolyporenate A (318 mg.) in methanol (8 ml.). After a reflux period of 16 hours, the reaction mixture was quickly added to chilled 16N aqueous hydrochloric acid (20 ml.) and immediately extracted with ether (2 x 20 ml.). The combined ethereal extracts were washed with water (2 x 20 ml.), dried and evaporated to give a white solid (191.8 mg., m.p. 151-155°C, 73 %). This material was purified by P.L.C. on two rhodamine-treated plates (200 x 200 x 1 mm.) using 2 % methanol : 98 % chloroform as the developing solvent. The pure methyl isopolyporenate A was removed from the silica by elution with ethyl acetate, treated with decolourising charcoal and crystallised from aqueous methanol as needles (52 mg.), m.p. 158-163°C (lit. 163-165°C).[53]

ν_{max} (KBr) 3500 (broad), 2838, 1708, 1633 cm^{-1}.

ν_{max} (1.20 mM. CCl_4) 3637 cm^{-1} (ε 91.5, $\Delta\nu_{\frac{1}{2}}$ 22), 1718 cm^{-1} (ε 430, $\Delta\nu_{\frac{1}{2}}$ 18).

λ_{max} (CH_3OH) 2260 Å (ε 8,900).

Tau values (CCl_4) 6.04 (1H, d, J=7.2 c/s.), 6.30 (3H, s), 6.63 (1H, t, J=2.8 c/s.) 8.20 (6H, s).

Molecular parent ion at m/e 500

Analysis	Found	C	76.52 %	H	10.39 %
Calculated for	$C_{32}H_{52}O_4$	C	76.75 %	H	10.47 %

b. Detection of dimethyl malonate

The procedure outlined above was repeated with a further sample of methyl 3-methoxycarbonylacetylpolyporenate A (493.6 mg.). The washed, dried ethereal solution was reduced in volume by careful fractional distillation and a sample analysed by gas-liquid chromatography on a 4 foot 10 % polyethyleneglycol adipate column (Pye Argon, flow of 26 ml. of argon per minute, temperature 75°C). For the reaction solution a single peak was observed with retention time 20.9 minutes; the retention time for an authentic sample of dimethyl malonate was 20.8 minutes. Co-application of a sample of the reaction solution and a sample of authentic dimethyl malonate gave rise to a single well-formed peak of retention time 20.7 minutes.

29. METHYL 24,28-DIHYDROPOLYPORENATE A (4.17)

a. Polyporenic acid A (132.3 mg.) was dissolved in ethanol (10 ml.) and hydrogenated at room temperature and atmospheric pressure for two hours with platinum oxide as catalyst (32.6 mg.). When the hydrogen absorption was complete (6 ml.), the catalyst was removed by filtration through glass paper and the resulting solution treated with excess ethereal diazomethane, at 0°C. Removal of the solvent provided

an oil (128 mg.) which was purified by P.L.C. on two rhodamine-treated plates (200 x 200 x 1 mm.) using 2 % methanol : 98 % chloroform as the developing solvent. The pure methyl 24,28-dihydropolyporenate was removed from the silica by elution with ethyl acetate, treated with decolourising charcoal and crystallised from an aqueous methanol system as needles (81 mg.), m.p. 140-142°C (lit. 140-142°C).[53]

ν_{max} (KBr) 3500 (broad), 2832, 1742, 1727 cm^{-1}.

ν_{max} (1.47 mM. CCl_4) 3638 cm^{-1} (ε 97.2, $\Delta\nu_{\frac{1}{2}}$ 23), 1738 cm^{-1} (ε 605, $\Delta\nu_{\frac{1}{2}}$ 14).

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 6.05 (1H, d, J=7.2 c/s.), 6.35 (3H, s) 6.65 (1H, m), 8.91 (3H, d, J= 7.8 c/s.).

Molecular parent ion at m/e 502 = $C_{32}H_{54}O_4$.

Analysis	Found	C 76.56 %	H 10.83 %
Calculated for $C_{32}H_{54}O_4$		C 76.45 %	H 10.83 %

b. In a reaction conducted in a small dry-box, sodium methoxide, prepared from sodium (50 mg.) and anhydrous methanol (10 ml.), was added to a solution of methyl 3-methoxycarbonylacetyl-24,28-dihydropolyporenate A (128 mg.) in anhydrous methanol (8 ml.). After a reflux period of 10 hours, the mixture was quickly added to chilled 16N aqueous hydrochloric acid (20 ml.) and immediately extracted with ether (2 x 20 ml.). The combined ethereal extracts

were washed with water (2 x 30 ml.), dried and evaporated to give a white solid (92 mg., 87 %). This substance was purified by P.L.C. on a rhodamine-treated plate (200 x 200 x 1 mm.) using 2 % methanol : 98 % chloroform as the developing solvent. The pure methyl 24,28-dihydropolyporenate was removed from the silica by elution with ethyl acetate, treated with decolourising charcoal and crystallised from aqueous methanol as needles (50 mg.) m.p. 139-141°C.

This material was found to be identical (i.r., n.m.r., R_f, mixed melt) with that prepared in "a" above.

30. METHYL 3-ACETYLPOLYPORENATE A. THE SUBSTANCE P.B.3

a. Methyl polyporenate A (62 mg.) was dissolved in anhydrous pyridine (5 ml.) and treated with acetic anhydride (1 ml.) for 72 hours at 21°C. Thereafter the reaction mixture was poured into ice-cold 6N aqueous hydrochloric acid (20 ml.) and extracted with ether (2 x 20 ml.). The combined ethereal extracts were washed with 6N hydrochloric acid (6 x 20 ml.), then water (2 x 20 ml.), dried and evaporated to give an oil (78 mg.). This substance was purified by P.L.C. on a rhodamine-treated plate (200 x 200 x 1 mm.) using 100 % chloroform as the developing solvent. Methyl 3-acetyl-polyporenate (62 mg., 93 %) was removed from the plate by elution with ethyl acetate, treated with decolourising charcoal and crystallised

from aqueous methanol as needles, m.p. 136-137°C (lit. 137-138°C).[53]

ν_{max} (KBr) 3629, 3080, 2830, 1738, 1640, 1375, 1249, 890 cm^{-1}.

ν_{max} (1.37 mM. CCl_4) 3631 cm^{-1} (ε 54.5, $\Delta\nu_{\frac{1}{2}}$ 16), 1739 cm^{-1} (ε 845, $\Delta\nu_{\frac{1}{2}}$ 23) also 3090, 2832 and 1645 cm^{-1}.

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 5.10 (2H, s), 5.35 (1H, m), 6.04 (1H, d, J=7.2 c/s.), 6.33 (3H, s), 6.90 (1H, q, J=7.2 c/s.), 7.96 (3H, s) 8.72 (3H, d, J=7.2 c/s.).

Parent molecular ion at m/e 542.

Analysis	Found	C	74.99 %	H	10.04 %
Calculated for	$C_{34}H_{54}O_5$	C	75.23 %	H	10.03 %

b. The mother liquors from the crystallisation of methyl polyporenate C contained approximately 50 % of a material of R_f value 0.72 in 1 % methanol : 99 % chloroform (p-amino-azobenzene R_f 0.67, p-hydroxy-azobenzene R_f 0.31). The mixture was resolved (1.061 g.) by P.L.C. on four rhodamine-treated plates (200 x 200 x 1 mm.) employing a four fold elution with 50 % benzene : 50 % chloroform. The substance was removed from the silica by elution with chloroform and successive crystallisation from light petroleum and aqueous methanol gave needles (59 mg.), m.p. 130-132°C.

This material was almost identical (i.r., n.m.r., R_f) with that prepared in "a" above.

31. DETECTION OF METHYL CAPROATE AS A PRODUCT OF THE TRANSESTERIFICATION OF THE SUBSTANCE P.B.3

A sample of the substance P.B.3 (181 mg.) was dissolved in anhydrous methanol (5 ml.) and treated with a preparation of sodium methoxide (10 mg. sodium) in anhydrous methanol (5 ml.). After a reflux period of 13 hours, the reaction solution was poured into 16N aqueous hydrochloric acid (10 ml.) and immediately extracted with ether (2 x 20 ml.). The combined ethereal extracts were dried and carefully freed from solvent at 40°C. The residue was dissolved in chloroform and subjected to gas-liquid chromatography on a 6 foot 10 % polyethyleneglycol adipate column (Perkin Elmer F.11, flow of 32 ml. of nitrogen per minute, temperature 8 minutes at 50°C, thereafter to 90°C at a rate of 3°C per minute). The reaction sample gave a single peak with retention time of 16.0 minutes; under identical conditions, authentic methyl caproate had a retention time of 16.1 minutes. Co-application of the reaction sample and authentic methyl caproate gave rise to a single well defined peak of retention time 16.0 minutes.

32. CHROMATOGRAPHIC SEPARATION OF FRACTION III

Fraction III (28.003 g.) was slurried in light petroleum with Woelm grade IV neutral alumina (50 g.), placed on a column of

the same adsorbant (1000 g., 5 x 75 cm.) and eluted with a gradient of light petroleum (1.5 l.) to ethyl acetate (1.5 l.). Fractions (23 ml.) were taken; the flow rate being 100 ml. per hour.

Fractions 107-124, eluted with solvent varying in composition from 52 % to 57 % ethyl acetate, provided an oil (12.086 g.) which was shown by T.L.C. analysis to contain the compounds P.B.4 and P.B.5 (R_f values 0.36 and 0.15 respectively in 2 % methanol : 98 % chloroform ; p-amino-azobenzene R_f 0.67 p-hydroxy-azobenzene 0.31). No separation of the compounds had been produced.

The oil was placed on a column of Mallinckrodt Silicic acid (1000 g.) and eluted with the same solvent system; again no separation was effected. A column of the same adsorbant, eluted with the less polar gradient, light petroleum (1.5 l.) to 50 % light petroleum : 50 % ethyl acetate (4.5 l.), also failed to fractionate the components. Thus samples (440 mg.) of the oil were submitted to P.L.C. on eight rhodamine-treated plates (600 x 200 x 1 mm.) employing a threefold elution with 2 % methanol in chloroform. By this means the compounds P.B.4 (1.654 g.) and P.B.5 (802 mg.) were obtained in pure form.

Fractions 125-131 of the original column, eluted with solvent varying in composition from 57 % to 59 % ethyl acetate also contained a binary mixture (1.371 g.). The compound P.B.5 and P.B.6 were separated by P.L.C. on two rhodamine-treated plates (600 x 200 x 1 mm.) using a 2 % methanol in chloroform as solvent. Yields, P.B.5 - 462 mg.,

P.B.6 362 mg.

33. METHYL 3-(4-METHOXYCARBONYL-3-HYDROXY-3-METHYLBUTYRYL)-POLYPORENATE A (4.19). THE COMPOUND P.B.4

The material was isolated as detailed above, the substance being eluted from the silica with ethyl acetate, treated with decolourising charcoal and crystallised from a light petroleum : ethyl acetate system as needles (900 mg.), m.p. 79-81°C.

ν_{max} (KBr) 3598, 3510 (broad), 3084, 2830, 1742, 1726, 1705, 1647, 892 cm^{-1}.

ν_{max} (1.15 mM. CCl_4) 3638 cm^{-1} (ε 55.5, $\Delta\nu_{\frac{1}{2}}$ 14), 3528 cm^{-1} (ε 55.5, $\Delta\nu_{\frac{1}{2}}$ 80), 1742 cm^{-1} (ε 925, $\Delta\nu_{\frac{1}{2}}$ 20), 1715 cm^{-1} (ε 388 sh.) also 3090, 2833 and 1646 cm^{-1}.

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 5.12 (2H, s), 5.30 (1H, t, J=2.4 c/s.), 6.02 (1H, d, J=7.2 c/s.), 6.06 (1H, s), 6.32 (3H, s), 6.35 (3H, s), 6.92 (1H, q, J=7.2 c/s.), 7.40 (4H, broad s), 8.70 (3H, s), 8.75 (3H, d, J+7.2 c/s.).

Molecular parent ion at m/e 658 undetected; first peak in mass spectrum at m/e 640 (P - 18).

Analysis Found C 71.19 % H 9.25 %

$C_{39}H_{62}O_8$ requires C 71.09 % H 9.48 %

34. METHYL 3-(4-METHOXYCARBONYL-3-HYDROXY-3-METHYL-BUTYRYL)-24,28-DIHYDROPOLYPORENATE A

Methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methyl-butyryl)-polyporenate A (833.8 mg.) was dissolved in ethanol (25 ml.) and hydrogenated at room temperature and atmospheric pressure for three hours with platinum oxide (157 mg.) as catalyst. When the hydrogen absorption was complete (30 ml.), the catalyst was removed by filtration through glass paper and the resulting solution evaporated to give an oil (910 mg.) which crystallised from light petroleum as prisms (810 mg.), m.p. 91-93°C.

ν_{max} (KBr) 3400 (broad), 2825, 1734, 1700 (sh),

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 5.30 (1H, m), 6.08 (1H, d, J=7.2 c/s.), 6.35 (3H, s), 7.40 (4H, broad s), 8.71 (3H, s), 8.90 (3H, d, J=7.2 c/s.).

Parent molecular ion at m/e 660 undetected; first ion in spectrum at m/e 642 (P - 18).

Analysis	Found	C 71.19 %	H 9.70 %
$C_{39}H_{64}O_8$	requires	C 70.89 %	H 9.70 %

35. METHANOLYSIS OF METHYL 3-(4-METHOXYCARBONYL-3-HYDROXY-3-METHYLBUTYRYL)-POLYPORENATE A

a. Isolation of methyl isopolyporenate A (4.16)

The procedure outlined in Section 28a for the preparation of this compound from methyl 3-methoxycarbonylacetylpolyporenate A was followed with a sample of methyl 3-(4-methoxycarbonyl-3-methylbutyryl)-polyporenate A (420 mg.). The ethereal extract, on evaporation, provided an oily solid (182 mg.) which was purified by P.L.C. on two rhodamine-treated plates (200 x 200 x 1 mm.) employing 2 % methanol : 98 % chloroform as developing solvent. The purified methyl isopolyporenate A was removed from the silica by elution with ethyl acetate, treated with decolourising charcoal and crystallised from aqueous methanol as needles (100 mg.) m.p. 165 (lit. 163-165°C)[53].

This substance was identical (i.r., n.m.r., R_f) with that obtained by methanolysis of methyl 3-methoxycarbonylacetylpolyporenate A.

b. Detection of dimethyl 3-hydroxy-3-methyl-glutarate

The procedure described above was repeated with a further sample of methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methyl-butyryl)-polyporenate A (443.5 mg.). The washed, dried ethereal solution was freed from solvent by careful distillation and the residue was taken up in chloroform. This solution was analysed by gas-liquid chromatography on a 4 foot 2 % Versamide 900 column (Perkin Elmer F.11, flow of 38 ml. of nitrogen per minute, temperature 5 minutes at 80°C, thereafter to 200°C at a rate of 5°C per minute). The reaction

sample gave rise to a single peak of retention time 16.0 minutes; authentic dimethyl 3-hydroxy-3-methyl-glutarate had a retention time of 16.6 minutes, under the same conditions. Co-application of the reaction product and authentic dimethyl 3-hydroxy-3-methyl-glutarate gave a single well defined peak of retention time 16.4 minutes.

36. METHYL 24-28-DIHYDROPOLYPORENATE A (4.17)

The method outlined in Section 29b for the transesterification of methyl 3-methoxycarbonylacetylpolyporenate A was repeated with a sample of methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-24,28-dihydropolyporenate A (280 mg.). The product was purified by P.L.C. in similar fashion to yield a substance which crystallised as needles from aqueous methanol (60 mg.), m.p. 139-141°C (lit. 140-142°C).

This compound was found to be identical (i.r., n.m.r. R_f; mixed melt) with the sample of methyl 24,28-dihydropolyporenate A prepared from authentic polyporenic acid (cf Section 29a).

37. METHYL POLYPORENATE A (4.11). THE COMPOUND P.B.5.

The isolation of this compound has already been described

(Section 32). It was eluted from the P.L.C. plates with ethyl acetate, treated with decolourising charcoal and crystallised from aqueous methanol as needles (720 mg.), m.p. 148-149°C (lit. 148.5-149.5°C).

ν_{max} (KBr) 3500 (broad), 3090, 1742, 1727, 1648, 900 cm^{-1}.

ν_{max} (1.50 mM. CCl_4) 3639 cm^{-1} (ε 93.2, $\Delta\nu_{\frac{1}{2}}$ 18), 1744 cm^{-1} (ε 510, $\Delta\nu_{\frac{1}{2}}$ 23), also 3090 and 1648 cm^{-1}.

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 5.10 (2H, s), 6.04 (1H, d, J=7.2 c/s.), 6.33 (3H, s), 6.63 (1H, m), 6.90 (1H, q, J=7.0 c/s.), 8.75 (3H, d, J=7.0 c/s.).

Parent molecular ion at m/e 500.

Analysis Found C 76.54 % H 10.40 %

Calculated for $C_{32}H_{52}O_4$ C 76.75 % H 10.47 %

This substance was identical (i.r., n.m.r., R_f, mixed melt) with an authentic sample of methyl polyporenate A generously provided by Dr. T. G. Halsall.

38. ERGOSTEROL 5,8-PEROXIDE (4.5). THE COMPOUND P.B.6.

a. Ergosterol (1.516 g.) was dissolved in ethanol (100 ml.), eosin was added (2 mg.), and the solution was illuminated at room temperature by two 100 watt Mazda bulbs with a slow stream of oxygen passing (50 ml. per minute). After twelve hours,

the solvent was removed and the solid (1.528 g.) purified by P.L.C. on three rhodamine-treated plates (600 x 200 x 1 mm.) using 2 % methanol : 98 % chloroform as eluant. The peroxide was removed from the plate by elution with ethyl acetate, treated with decolourising charcoal and crystallised from a light petroleum : ethyl acetate system as needles, m.p. 178°C (0.882 g., 58 %, lit.[77] 178°C).

ν_{max} (KBr) 3434 (broad), 2960, 2874, 1075, 1042, 1027, 968 cm^{-1}.

λ_{max} (CH_3OH) No absorption above 2200 Å.

Tau values (CCl_4) 3.56 (1H, d, J=7.8 c/s.), 3.83 (1H, d, J=7.8 c/s.), 4.80 (2H, m.).

Parent molecular ion at m/e 428.

Analysis	Found	C	78.27 %	H	10.18 %
Calculated for	$C_{28}H_{44}O_3$	C	78.46 %	H	10.35 %

b. The compound P.B.6 was isolated as described in Section 32. The substance was eluted from the silica with chloroform and crystallised from aqueous methanol as needles, m.p. 177-178°C.

It was found to be identical (i.r., n.m.r., R_f., mixed melt) with that prepared in "a" above.

c. Ergosterol (30 mg.) was added to a portion (20 ml.) of the methanol extract of the sporophores (Section 24) which itself contained only traces of ergosterol and its peroxide. The solution

was illuminated and oxygenated for twelve hours in the manner described in "a" above. At the end of this period the reaction solution was assayed for ergosterol peroxide by T.L.C. in 2 % methanol : 98 % chloroform. Approximately 80 % of the product had the same R_f value as ergosterol peroxide.

In a control experiment involving ergosterol (30 mg.) and pure methanol (20 ml.) only a trace of the peroxide was detected after a twelve hour reaction period.

39. PREPARATION AND T.L.C. ANALYSIS OF A METHANOL EXTRACT OF A POLYPORUS BETULINUS SPOROPHORE

A clean, dry, whole sporophore of Polyporus betulinus (48 g.) was homogenised in methanol (500 ml.) at room temperature over a period of five minutes. The resultant slurry was filtered free of tissue and the filtrate evaporated to give a brown semi-solid (1.251 g.). A portion of this solid was subject to T.L.C. in the low pH solvent system (B.D.A.):-

benzene	35
dioxan	5
glacial acetic acid	1

Eluted plates were developed separately with B.D.H. "Universal" indicator, iodine vapour and ceric ammonium nitrate-sulphuric acid when the results shown in Table 4.9 were obtained.

The relationship between the six principal acidic substances and the previously encountered esters was established by two dimensional T.L.C. A sample of the methanol extract was placed at the origin of a two dimensional T.L.C. plate (200 x 200 x 0.25 mm.) and eluted in the x-direction with the solvent system B.D.A. When the solvent had travelled 10 cm. the plate was removed from the tank, carefully freed from solvent and treated along the length of the x-axis with an ethereal solution of diazomethane. The esters so produced were then chromatographed in the y-direction with the solvent system 1 % methanol in chloroform against authentic samples of methyl polyporenate A, methyl 3-acetylpolyporenate A, methyl 3-methoxy-carbonylacetylpolyporenate A and methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A. The results are tabulated in Table 4.10.

40. CHROMATOGRAPHIC FRACTIONATION OF THE ACIDS OF POLYPORUS BETULINUS

A portion (2.552 g.) of the methanol extract of a Polyporus betulinus sporophore (cf Section 39) was adsorbed from chloroform onto a column of Mallinckrodt silicic acid (300 g. 4 x 50 cm., R_v 340 ml.) and eluted firstly with chloroform then with 1 % methanol in chloroform. Fractions (25 ml.) were taken from a flow rate of 60 ml. per hour and were assayed by T.L.C. in the

B.D.A. solvent system. The results appear in Table 4.11.

a. Monomethyl ester of 3-carboxyacetylpolyporenic acid A

Fractions 98-109 (746 mg.), eluted from the above column with chloroform, contained the acidic material of R_f value 0.43 (Table 4.9) together with fats and sterols. It was isolated in a relatively impure form by P.L.C. on four rhodamine-treated plates (200 x 200 x 1 mm.) employing elution with the B.D.A. solvent system. It was with some difficulty that the substance was removed from the silica by elution with ethyl acetate. The compound proved to be a gum (32 mg.)

The n.m.r. spectrum of this crude substance contained absorption at 6.23 and 6.60 tau. On methylation it produced a material identical in R_f value with methyl 3-methoxycarbonylacetyl-polyporenate A.

b. 3-(4-Methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenic acid A (4.28)

Fractions 111-124 (307 mg.) eluted from the above column with chloroform contained the acidic substance of R_f value 0.32 (Table 4.9). It proved to be a gum.

ν_{max} ($CHCl_3$) 1736, 1710 cm^{-1}.

Tau values ($CDCl_3$) 5.01 (2H, m), 5.21 (1H, m), 5.98

(1H, d, J=7.8 c/s.), 6.30 (3H, s) 7.29 (4H, s), 8.70 (3H, s), methyl contour defined by peaks at 8.87, 9.00, 9.04, 9.10 and 9.37.

Parent molecular ion at m/e 644 undetected; first ion in spectrum occurs at m/e 626.

On methylation this substance gave a product of identical R_f value to methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenate A.

c. 3-Carboxyacetylpolyporenic acid A (4.26)

Fractions 154-160 (243 mg.) eluted from the above column with 1 % methanol in chloroform contained the acidic substance of R_f value 0.24 (Table 4.9). It was a white crystalline compound which crystallised from light petroleum : ethyl acetate as needles (200 mg.), m.p. 184-185°C.

ν_{max} (6.1 mM. $CHCl_3$) 3614 cm^{-1} (ε 49, $\Delta\nu_{\frac{1}{2}}$ 30), 3505 cm^{-1} (ε 59, $\Delta\nu_{\frac{1}{2}}$ 54), 1763 cm^{-1} (ε 262), 1734 cm^{-1} (ε 565), 1710 (ε 785, $\Delta\nu_{\frac{1}{2}}$ 28).

Tau values ($CDCl_3$) 5.00 (2H, s), 5.22 (1H, m), 5.93 (1H, d, J=6.6 c/s.), 6.53 (2H, s), 6.80 (1H, q, J=7.2 c/s.), 8.67 (3H, d, J=7.2 c/s.)

Methyl contour defined by peaks at 8.89, 9.01, 9.06, 9.08, 9.38.

Analysis Found C 71.29 % H 9.55 %

$C_{34}H_{52}O_7$ requires C 71.30 % H 9.15 %

On methylation this substance gave a product identical in R_f value to methyl 3-methoxycarbonylacetylpolyporenate A.

d. 3-(4-Carboxy-3-hydroxy-3-methylbutyryl)-polyporenic acid A (4.27)

Fractions 161-170 (381 mg.), eluted from the above column with 1 % methanol in chloroform, contained the acidic substance of R_f value 0.16 (Table 4.9). It was a white crystalline material which crystallised from light petroleum : ethyl acetate as needles (120 mg.), m.p. 165-166°C.

ν_{max} ($CHCl_3$) 3620, 3518, 1742, 1710 cm^{-1}.

Tau values ($CDCl_3$) 5.00 (2H, m), 5.24 (1H, m), 5.96 (1H, d, J=7.2 c/s.), 6.80 (1H, q, J=7.2 c/s.), 7.28 (4H, s), 8.62 (3H, s), methyl contour defined by peaks at 8.80, 9.02, 9.08, 9.10, 9.39.

Analysis Found C 70.23 % H 9.50 %

$C_{37}H_{58}O_8$ requires C 70.44 % H 9.27 %

On methylation this compound gave a product identical in R_f value with methyl 3-(4-methoxycarbonyl-3-hydroxy-3-methyl-butyryl)-polyporenate A.

N.B. Material (104 mg.) giving rise on methylation to methyl polyporenate A and methyl 3-acetylpolyporenate A were noted in fractions 150-153 of the above column. These substances were not

examined in further detail.

41. ISOLATION OF 3-(4-METHOXYCARBONYL-3-HYDROXY-3-METHYL-BUTYRYL)-POLYPORENIC ACID A FROM AN ETHANOL EXTRACT OF POLYPORUS BETULINUS SPOROPHORES

The procedures detailed in Section 40 for the isolation of 3- (4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenic acid A from a methanol extract of Polyporus betulinus sporophores were repeated with an ethanol extract therefrom. The half ester of R_f value 0.32 so obtained was shown by N.M.R. spectroscopy to be 3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenic acid A and not the ethyl analogue.

42. EXPERIMENTS INVOLVING DIRECT METHYLATION OF POLYPORUS BETULINUS SPOROPHORE TISSUE

Two blocks (3.41 g.) of fresh tissue, "a" and "b", were cut from the same Polyporus betulinus sporophore and were frozen in liquid nitrogen before being separately pulverised. The fine powder from block "a" was suspended in ether and immediately treated with diazomethane. After one hour the tissue was removed, washed with ether and extracted at room temperature for 0.5 hours with methanol. The ether and methanol extracts were evaporated to give 244 mg. and

30 mg. of material respectively.

The powder from block "b" was extracted with methanol at room temperature for 1.5 hours. Thereafter the tissue was removed by filtration and the filtrate evaporated and methylated to give 268 mg. of material.

Since T.L.C. examination of the methanol extract from block "a" revealed it to contain no polyporenic acid A conjugates, it was united with the ether extract from the same source. T.L.C. comparison of the combined extract from block "a" with the extract from block "b" indicated that the same esters were present in the same proportion in both.

43. EXTRACTION EXPERIMENTS WITH POLYPORUS BETULINUS SPOROPHORE

A clean, dry sporophore of Polyporus betulinus (38.7 g.) was homogenised, extracted five times with water (300 ml.) and three times with methanol (3 x 300 ml.) before being allowed to stand overnight in methanol (300 ml.). Thereafter the tissue was filtered off and refluxed with 4N aqueous sodium hydroxide for 12 hours. The resultant sludge was filtered to remove the tissue remnants (22.3 g.) and the filtrate acidified and extracted with ethyl acetate (2 x 500 ml.). The combined ethyl acetate extracts were dried and evaporated, the methanol extracts individually evaporated and the pooled aqueous extracts lyophilised

to give the following yields of material.

Total water	623 mg.
1st methanol	2,626 mg.
2nd methanol	553 mg.
3rd methanol	6 mg.
Overnight methanol	15 mg.
Ethyl acetate (base hydrolysis)	7 mg.

44. COMPARISON OF THE MALONATE : HYDROXYMETHYL-GLUTARATE RATIO IN CONTEXT AND PORE TISSUE OF A POLYPORUS BETULINUS SPOROPHORE

The pore tissue of three sporophores of Polyporus betulinus collected from different geographical areas was carefully removed from the context tissue and all six samples separately homogenised in methanol. The methanol extracts so produced were examined by T.L.C. in the B.D.A. solvent system before being methylated and re-examined by T.L.C. in the solvent system 1 % methanol in chloroform. The results of these analyses are shown in Table 5.1.

45. BIOASSAYS WITH THE MALONATE AND HYDROXYMETHYLGLUTARATE CONJUGATES

3-Carboxyacetylpolyporenic acid A, 3-(4-carboxy-3-hydroxy-3-methylbutyryl)-polyporenic acid A and

3-(4-methoxycarbonyl-3-hydroxy-3-methylbutyryl)-polyporenic acid A were tested separately at a level of 10^3ppm. against the following organisms

Baccilus subtilis,

Escherichia coli,

Botrytus alia.

No inhibition of growth was detected with any of the metabolites.

46. CULTURE OF POLYPORUS BETULINUS ON SYNTHETIC MEDIUM. FAILURE TO DETECT MALONATE OR HYDROXYMETHYLGLUTARATE CONJUGATES.

A culture of Polyporus betulinus (N° 4C) was obtained from Forestry Products Research Laboratories and was subcultured onto 12 5 % malt agar slopes. After 7 days the mycelial mat was homogenised and the homogenate used to inoculate 24 Roux surface culture bottles which had previously been steam sterilised containing 250 ml. of a 5 % malt extract medium. The cultures were allowed to grow at 25°C and 70 % relative humidity for 56 days, artificial illumination being provided by Mazda fluorescent tubes for 12 hours per day.

After the prescribed growth period the broth was removed and the mycelium collected, dried, pulverised and extracted overnight with methanol at room temperature. The 45 gm. of dry

mycelium provided 1.78 g. of methanol solubles.

T.L.C. and N.M.R. spectral analyses failed to detect the presence in the methanol extract of any of the polyporenic acid A conjugates.

BIBLIOGRAPHY

1. O. Wallach, "Terpene und Campher", Leipzig, 1914

2. M. P. E. Bertholet, "Chemic Organique Fondée sur la Synthese", Paris, 1860, p. 733.

3. L. Ruzicka, Experientia, 9 357 (1953).

4. A. J. Haagen-Smit, "The Essential Oils", Van Nostrand, New York, 1948 1 p 17.

5. T. T. Tchen, K. Bloch, J. Biol. Chem., 226 921 (1957), J. Biol. Chem., 226 931 (1957)

6. R. K. Maudgal, T. T. Tchen, K. Bloch, J. Am. Chem. Soc., 80 2589 (1958).
J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popjak, Y. Shimizu, S. Ichii, E. Forchielli, E. Caspi, J. Am. Chem. Soc. 87 3224 (1965).

7. F. R. Forsyth, Can. J. Botany, 33 363 (1955).

8. A. Quilico, C. Cardani, L. Panizzi, Atti accad. nazl. Lincei Rend., Classe sci. fis., Mat. e nat. sci., 14 358 (1953).

9. A. Quilico, C. Cardani, G. Stagno d'Alcontres, Gazzetta, 83 754 (1953).

10. D. H. R. Barton, J. B. Hendrickson, J. Chem. Soc., 1028 (1956).

11. A. Hofmann, R. Brunner, H. Kobel, A. Brack, Helv. Chim. Acta, 40 1358 (1957).

12. C. Cardani, G. Casnati, F. Piozzi, A. Quilico, Tetrahedron Letters, 16 1 (1959). A. J. Birch, G. E. Blance, S. David, H. Smith, J. Chem. Soc., 3128 (1961).

13. H. Hoeksema, E. Caron, J. W. Hinman, J. Am. Chem. Soc., 78 2019 (1956).

14. R. B. Bates, J. H. Schauble, M. Soucek, Tet. Lets., 1683 (1963).

15. J. H. Birkinshaw, H. Raistrick, D. J. Ross, Biochem. J., 50 630 (1952).

16. E. Heilbronner, R. W. Schmid, Helv. Chim. Acta, 37 2018 (1954).

17. F. Sorm, V. Benesova, V. Herout, Chem. Listy, 47 1856 (1953).

18. P. de Mayo, E. Y. Spencer, J. Am. Chem. Soc., 84 494 (1962).

19. T. C. McMorris, M. Auchel, J. Amer. Chem. Soc., 87 1594 (1965).

20. J. Gutzwiller, R. Mauli, H. P. Sigg, Ch. Tamm, Helv. Chim. Acta, 47 2234 (1964).

21. S. Abrahamsson, B. Nilsson, Proc. Chem. Soc., 188 (1964).

22. E. Flury, R. Mauli, H. P. Sigg, Chem. Comm., 26 (1965).
A. W. Dawkins, J. F. Grove, B. K. Tidd, Chem. Comm., 27 (1965).

23. J. Gutswiller, Ch. Tamm, Helv. Chim. Acta, 48 157 (1965).

24. T. Goto, H. Kakisawa, Y. Hirata, Tetrahedron, 8 2079 (1963)

25. A. Harris, A. Robertson, W. B. Whalley, J. Chem. Soc., 1799 (1958).

26. D. Arigoni, Gazzetta, 92 884 (1962). A. J. Birch, D. W. Cameron, C. W. Holzapfel, R. W. Richards, Chem. Ind., 374 (1963).

27. J. F. Grove, Quat. Rev., 15 56 (1961).

28. For leading references see M. W. Miller "PFIZER Handbook of Microbial Metabolites", McGraw-Hill, 1961, ch. 5.

29. A. Melera, Experientia, 19 565 (1963)

30. S. Okuda, S. Iwasaki, Y. Nakayama, K. Tsuda, IUPAC. Symposium Abstracts, Kyoto, 1964, p. 62.

31. D. Arigoni, W. von Daehne, W. O. Godtfredsen, A. Merera, S. Vangedal, Experientia, 19 521 (1963).

32. J. A. Olson, M. Lindberg, K. Bloch, J. Biol. Chem., 226 941 (1957).

33. K. Taufel, H. Thaler, H. Schreyegg, Fettchem. Umschau, 43 26 (1936)

34. T. G. Halsall, R. Hodges, G. C. Sayer, J. Chem. Soc., 2036 (1959).

35. L. Canonica, E. Fedeli, A. Fiecchi, Gazzetta, 89 818 (1959)

36. K. Kempska, R. S. Ludwiczak, U. Wrzeciono, Roczniki Chem., 36 1453 (1962).

37. L. A. Cort, R. M. Gascoigne, J. S. E. Holker, B. J. Ralph, A. Robertson, J. J. H. Simes, J. Chem. Soc., 3713 (1954).

38. J. S. E. Holker, A. D. G. Powell, A. Robertson, J. J. H. Simes, R. S. Wright, R. M. Gascoigne, J. Chem. Soc., 2422 (1953).

39. S. Shibata, S. Natori, K. Fujita, I. Kitagawa, K. Watanabe, Chem. Pharm. Bull. Japan, 6 608 (1958)

40. J. M. Guider, T. G. Halsall, R. Hodges, E. R. H. Jones, J. Chem. Soc., 3234 (1954).

41. F. N. Lahey, P. H. A. Strasser, J. Chem. Soc., 873 (1951).

42. W. B. Whalley, B. Green, D. Arigoni, J. J. Britt, C. Djerassi, J. Am. Chem. Soc., 81 5520 (1959).

43. A. Harris, A. Robertson, W. B. Whalley, J. Chem. Soc., 1807 (1958).

44. J. Castells, E. R. H. Jones, G. D. Meakins, R. W. J. Williams, J. Chem. Soc., 1159 (1959).

45. D. H. R. Barton, T. Bruun, J. Chem. Soc., 2728 (1951).

46. H. R. Arthur, T. G. Halsall, R. D. Smith, J. Chem. Soc., 2603 (1958).

47. H. Wieland, G. Coutelle, Ann., 548 270 (1941).

48. P. Wieland, V. Prelog Helv. Chim. Acta, 30 1028 (1947).

49. G. H. Alt, D. H. R. Barton, J. Chem. Soc., 1356 (1954).

50. T. G. Halsall, G. C. Sayer, J. Chem. Soc., 2031 (1959).

51. J. M. Guider, T. G. Halsall, E. R. H. Jones, J. Chem. Soc., 4471 (1954).

52. T. G. Halsall, E. R. H. Jones, Fortschr. Chem. org. Naturst. 12 95 (1955).

53. R. G. Curtis, I. Heilbron, E. R. H. Jones, G. F. Woods, J. Chem. Soc., 3019 (1953).

T. G. Halsall, R. Hodges, E. R. H. Jones, J. Chem. Soc., 3019 (1953).

54. L. A. Cort, R. M. Gascoigne, J. S. E. Holker, B. J. Ralph, A. Robertson, J. J. H. Simes, J. Chem. Soc., 3713 (1954). J. M. Guilder, T. G. Halsall, E. R. H. Jones, J. Chem. Soc., 4471 (1954).

55. A. Bowers, T. G. Halsall, E. R. H. Jones, G. C. Sayer, J. Chem. Soc., 2385 (1954) also J. Chem. Soc., 3070 (1954).

56. J. Fried, P. Grabowich, E. F. Sabo, A. I. Cohen, Tetrahedron 20 2297 (1964).

57. J. S. E. Holker, A. D. G. Powell, A. Robertson, J. J. H. Simes, R. S. Wright, R. M. Gascoigne, J. Chem. Soc., 2422 (1953).

58. P. Bernfeld, "Biogenesis of Natural Compounds", Pergamon (1963).
R. B. Clayton, Quat. Rev., 21 168 (1965).

59. J. D. Brodie, G. Wasson, J. W. Porter, J. Biol. Chem., 238 1294 (1963), 239 1346 (1964).

60. G. Popjak, 6th International Congress of Biochemistry, New York (1964), Abstract p 545.

61. G. Popjak, NATC-sponsored symposium; "Metabolism and Physiological Significance of Lipids", ed. Dawson, Rhodes, Wiley, 1964, p 45.

62. W. G. Dauben, G. J. Fonken, G. A. Boswell, J. Am. Chem. Soc., 79 1000 (1957).
G. J. Alexander, A. M. Gold, E. J. Schwenk, J. Am. Chem. Soc., 79 2967 (1957).

H. Danielsson, K. Block, J. Am. Chem. Soc., 79 500 (1957)

63. G. Jauriéguiberry, G. Law, J. H. McCloskey, E. Lederer, Compt. Rendu, 258 3587 (1964).

64. K. J. Stone, F. W. Hemming, Biochem. J., 96 14c (1965)

65. E. Lederer, Biochem. J., 93 449 (1964).

66. G. Ourisson, P. Crabbé, O. Rodig, "Tetracyclic Triterpenes", Hermann, 1964, p 21.

67. A. I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products", Pergamon, 1964, p. 51.

68. A. I. Cohen, S. Rock, Steroids 3 243 (1964).

69. K. Biemann, "Mass Spectroscopy", McGraw-Hill, 1962, p. 339

70. K. Biemann, "Mass Spectroscopy", McGraw-Hill, 1962, p. 76-77.

71. H. Adam, N. J. McCorkindale, unpublished work.

72. H. Staudinger, H. Becher, Berichte, 50 1019 (1917).

73. R. A. Abramovitch, Canada. J. Chem., 37 1146 (1959).

74. L. C. Cross, C. G. Elliot, I. M. Heilbron, E. R. H. Jones, J. Chem. Soc., 632 (1940).

75. N. S. Bhacca, L. F. Johnson, J. N. Shoolery, "N.M.R. Spectra Catalogue", Varian Associates (1962), no. 276.

76. A. Windaus, J. Brunken, Ann., 460 225 (1928).

77. P. Wieland, V. Prelog, Helv. Chim. Acta, 30 1028 (1947).

78. G. Bauslaugh, G. Just, F. Blank, Nature 202 1218 (1964)

79. L. L. Smith, Steroids, 4 395 (1964)

80. K. Randerath, "Thin Layer Chromatography", Verlag-Chemie

(1963), p. 49.

81. N. S. Bhacca, D. H. Williams, "Applications of N.M.R. Spectroscopy in Organic Chemistry", Holden Day, 1964, p. 19.

82. J. C. Finerty, E. V. Cowdry, "A Textbook of Histology", Kimpton, 1960, p.40.

83. E. J. H. Corner, Phytomorphology 3 152 (1953).

84. E. Lederer, M. Lederer, "Chromatography: A Review of Principles and Applications", Elsevier, 2nd Ed. (1957) p.43.

85. A. W. McCulloch, Ph.D. Thesis, Glasgow, 1965.

86. J. A. Moore, D. E. Reed, Organic Synthesis 41 16 (1961).

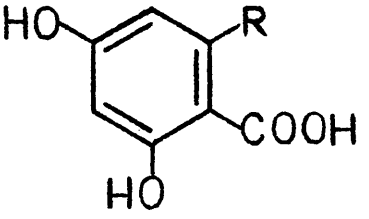
87. S. Bergatröm, J. Sjovall, Acta Chem. Scand., 14 1693 (1960).

CHAPTER 7

AN INTRODUCTION TO THE STUDY OF THE METABOLITES OF PENICILLIUM BREVI-COMPACTUM

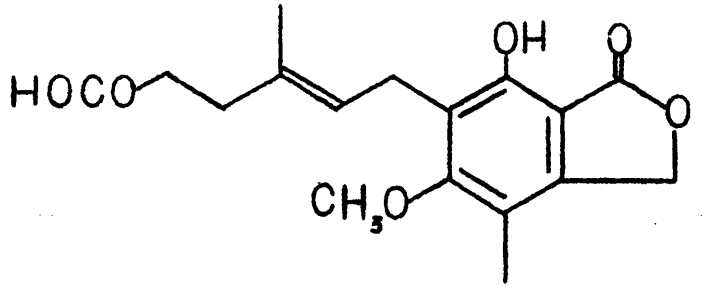
The organism Penicillium brevi-compactum is widely distributed in Nature and can be isolated from soil, from all manner of decaying vegetable matter and from spoilt or mouldy grain. It is distinguished from other members of the genus in possessing a relatively short, compact base to the penicillus or spore-bearing organ.

The detailed chemical analysis of the metabolites produced by this mould was first undertaken in 1932 by Clutterbuck, Oxford, Raistrick and Smith[1] who studied the principal chemical constituents of the broth of some fifteen strains of Penicillium brevi-compactum. These authors succeeded in isolating from fourteen of the strains, four water soluble phenolic compounds whose structures were shown to form the following biogenetically interesting series,[2,3]



R = $CH_2.CO.CH_3$ 7.1
= $CHOH.CO.CH_3$ 7.2
= $CO.CO.CH_3$ 7.3
= CO_2H 7.4.

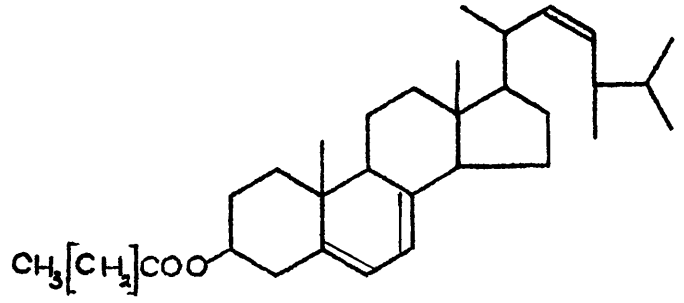
A further phenolic acid was isolated at that time by the Raistrick Group from twelve strains of Penicillium brevi-compactum. It was shown to be identical to the substance mycophenolic acid (7.5) which had previously been encountered by Arlsberg and Black[4]



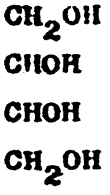
7.5

and possibly by Gosio[6] as a metabolite of Penicillium stoloniferum and "Penicillium glaucum" respectively. As the result of detailed degradative work by Birkinshaw et al.[6] the structure of the metabolite was finally elucidated in 1952. Mycophenolic acid has been the subject of biosynthetic study by Birch who demonstrated that the side chain was terpenoid in origin while the aromatic nucleus was derived from an acetate-malonate polymer.[7] The aromatic methyl and methoxyl groups were shown to come from methionine.[8]

The conjugated sterol, ergosterol palmitate (7.6)[9] and i-erythritol (7.7)[10] have also been obtained by Raistrick et al.



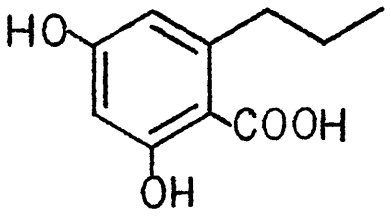
7.6



7.7

from cultures of Penicillium brevi-compactum.

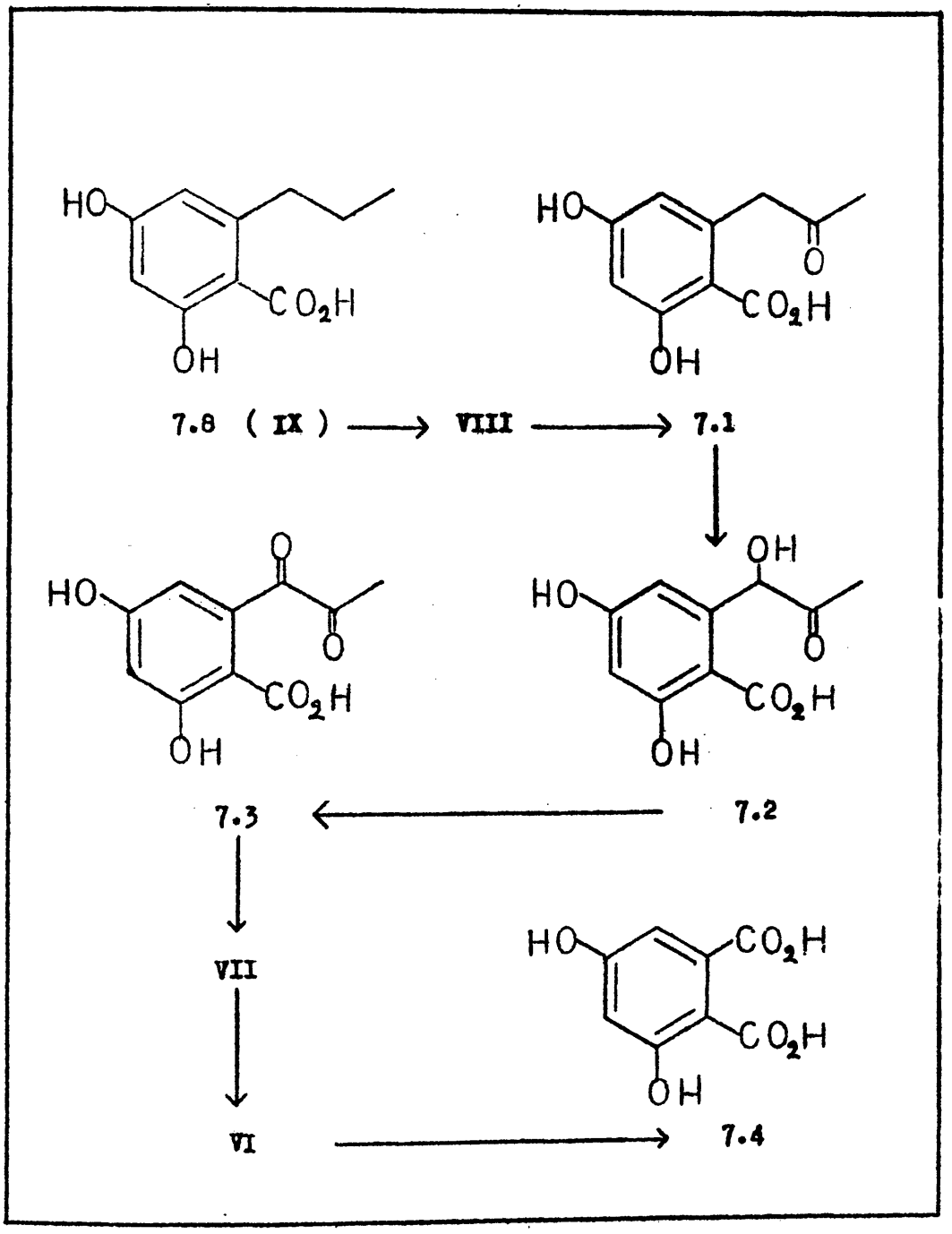
Without any doubt the most comprehensive survey of the metabolites of Penicillium brevi-compactum has been conducted by Godin in Belgium.[11-17] He succeeded in isolating mycophenolic acid and the other four "Raistrick phenols" from cultures of the mould and noted the presence of eight other phenolic products which he designated IV, V, VI, VII, VIII. IX, X and XI.[11] Reflooding experiments with a mature mycelial preparation seemed to indicate that the latter two substances, X and XI were biosynthetically related to mycophenolic acid while the materials IX, VIII, VII, VI were related to the "Raistrick phenols" in the manner shown in Table 7.1. Only compound IX was present in sufficient quantity to permit further study and, on the basis of what must be considered the most fragmentary experimental data, Godin proposed structure (7.8) for this substance.[12]



7.8

In a second phase of study Godin isolated the glucose polymer compactose from the mycelium of cultures of Penicillium brevi-compactum[14] and conducted an extensive and detailed paper chromatographic survey of the sugars and Krebs cycle acids present

TABLE 7.1 BIOGENETIC SEQUENCE PROPOSED BY GODIN TO INTERRELATE THE C_{10} METABOLITES OF PENICILLIUM BREVI-COMPACTUM.[12]



in the broth of a culture of the growing mould. His results, summarised in Table 7.2 indicate that there is considerable variation in the metabolite content depending on whether cultures are static or agitated.[15] Godin has also studied the variation produced in the pattern of acids observed in static cultures when calcium carbonate is added to the medium.[16]

It may be pointed out that the phosphorylated sugars present in Penicillium brevi-compactum have been the subject of study by Simonart, Bommers and Parmentier[17] and that 5-ribosylisocytosine and 3-ribosylisocytosine have been synthesised in reactions catalysed by the cytoplasmic supernatant fluid from a strain of Penicillium brevi-compactum.[18]

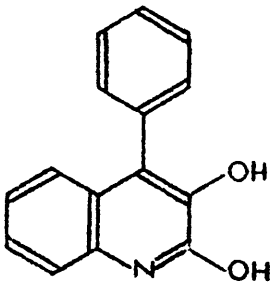
TABLE 7.2. THE SUGARS AND ACIDS OF P. BREVI-COMPACTUM DETECTED BY GODIN.[15]

CULTURES STATIC	CULTURES AGITATED
Arabinose	
Ribose	
Glucuronic acid	
2-Ketogluconic acid	
Gluconic acid	Gluconic acid
Citric acid	Citric acid
Malic acid	Malic acid
Dihydroxyacetone	Dihydroxyacetone
	α-Ketoglutaric acid
	Succinic acid
	Fumaric acid

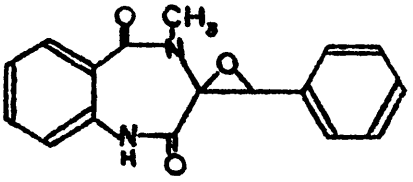
CHAPTER 8

SOME NEW METABOLITES OF PENICILLIUM BREVI-COMPACTUM

At the commencement of this work, a strain of Penicillium viridicatum was being examined for the production of such heterocyclic compounds as viridicatin (8.1)[19] and cyclopenin (8.2)[20] whose biogenetic origins were at that time completely unexplored.[21] Although



8.1



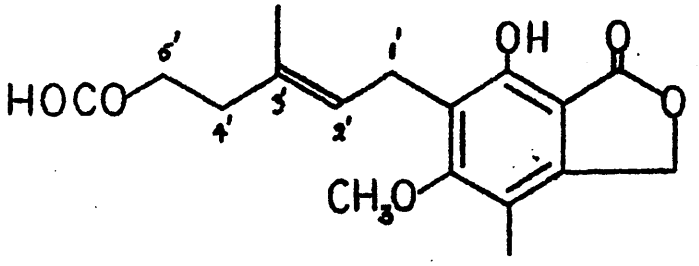
8.2

preliminary investigations revealed that a good yield of a complex mixture of ultraviolet-active metabolites was produced by the mould, none of these were the heterocyclic systems to which we have referred. The structure of many of the substances actually produced was revealed by the detailed analysis now to be described.

The strain of Penicillium viridicatum catalogued by the Commonwealth Mycological Institute as C.M.I.49162 was grown on a standard Czapek-Dox medium for fifteen days as a surface culture. Thereafter the mycelium was separated from the broth, dried, pulverised and extracted with acetone. The metabolites present in the broth were adsorbed onto charcoal and then removed therefrom by means of soxhlet extraction with acetone. Both acetone preparations were studied independently, attention being principally focussed

on the metabolites present in the broth.

Treatment of the acetone extract of the broth with an equal volume of water caused the precipitation of an appreciable amount of a light brown crystalline solid, which, after purification, melted over the range 140-141°C and analysed in accord with the molecular formula $C_{17}H_{20}O_6$ (m/e 320). Microtitration studies indicated that this material possessed two readily ionised hydrogens with ionisation constants (pK_a^1 2.3, pK_a^2 6.2) which suggested that the substance was a phenolic acid. The phenolic nature of the compound was confirmed by the observation that it gave an intensely blue coloration when treated with alcoholic ferric chloride solution. In the light of the spectroscopic evidence now to be presented, the isolated substance was shown to be mycophenolic acid (8.3).



8.3

The hydroxyl and carbonyl regions of the infrared spectrum were rendered complex by virtue of the coexistence in solution of carboxylic acid monomer and dimer. Nevertheless dilution studies and comparison with the absorption characteristics of ethyl myco-

phenolate (8.4) suggested that the band at 3516 cm^{-1} and part of the miximum at 1740 cm^{-1} were due to the carboxylic acid monomer and the broad band between 3225 and 3100 cm^{-1} and the peak at 1714 cm^{-1} due to the carboxylic acid dimer. The strong intramolecular hydrogen bond established between the phenolic hydroxyl group and the γ-lactone carbonyl was responsible for a lowering in the frequency of both. The stretching frequency of the γ-lactone occurred at 1740 cm^{-1} rather than the expected value of 1768 cm^{-1} [23] while the phenolic hydroxyl band appeared at 3450 cm^{-1} rather than 3610 cm^{-1}.[24]

The phenolic hydroxyl group had also a profound influence on the ultraviolet spectrum of the substance. At neutrality or at low pH, a three peak pattern was observed with maxima at 2160, 2505 and 3040 Å. When the pH was raised to such a level that the phenol was ionised, in effect the first and third bands suffered bathochromic shifts to 2280 and 3430 Å respectively.

The N.M.R. spectrum of the isolated metabolite was fully consistent with the compound being mycophenolic acid. Singlet resonance signals at 6.23, 7.86 and 8.19 tau were respectively assigned to the protons of the methoxyl group, of the aromatic methyl group and of the allylic methyl function found in the side chain. The hydrogens of the phthalide methylene group appeared as a two proton singlet at 4.80 tau while the resonance due to the hydrogens on carbon atoms $C\text{-}4^1$ and $C\text{-}5^1$ were superposed to give a broad four proton singlet at 7.61 tau. The vinylic hydrogens

and allylic methylene in the side chain produced a triplet-doublet pattern at 4.70 and 6.50 tau respectively, the coupling constant being 6 c/s. Slight broadening of the signals due to the C-1^1 methylene group and the methyl group on carbon atom C-3^1 was attributed to their allylic coupling to the ethylenic hydrogen on carbon atom C-2^1.

The spectroscopic properties of the corresponding acetate, methyl ether, dihydro derivative and methyl ester methyl ether further corroborated the structure of the isolated metabolite and their melting points conformed to the quoted literature values.

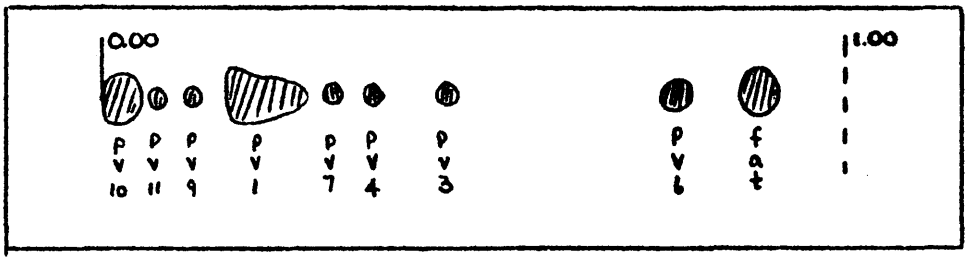
Mycophenolic acid has never before been isolated from any culture of Penicillium viridicatum but it is a recorded metabolite of twelve strains of Penicillium brevi-compactum. The coloration it produces with ferric chloride solution is, in fact, the basis of one of the principal tests used to detect the presence of Penicillium brevi-compactum in spoilt maize.[4] Suspicions that the organism C.M.I. 49162 had been erroneously classified were heightened when chromatographic comparison was made between the broth and mycelial extracts of the organism C.M.I. 49162 and similar preparations derived from nine authentic strains of Penicillium brevi-compactum and six authentic strains of Penicillium veridicatum grown under similar conditions as have been detailed for the culture of the organism C.M.I. 49162. The thin layer chromatogram of the extract of the latter mould closely resembled those of the brevi-

compacti but bore no relation whatsoever to those derived from the viridicati. Following these observations, the organism in question has been reclassified by the Commonwealth Mycological Institute as a Penicillium brevi-compactum.

Despite the fact that strains of this mould have previously been the subject of extensive investigation, the T.L.C. analysis shown in Table 8.1 suggested that a closer study of this strain would be of interest. From the Table it can be seen that there were nine principal metabolites, that of R_f value 0.15 being mycophenolic acid itself. In harmony with previous practice, the code names P.V.1, P.V.3, P.V.4, P.V.6, P.V.7, P.V.8, P.V.9, P.V.10 and P.V.11 were used to define the compounds whose chromatographic properties are defined in Table 8.1. In was noted with some interest that the compounds P.V.6, P.V.7 and P.V.9 possessed similar fluorescence and ferric chloride staining properties to mycophenolic acid.

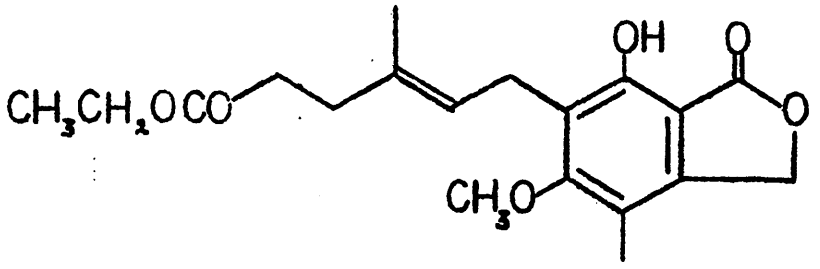
Samples of the compounds P.V.6, P.V.7 and P.V.9 were isolated from the broth extract by means of a combination of column and plate chromatography. The compound P.V.6 was a low melting white solid, m.p. 88-89°C, which provided analytical data compatible with the molecular formula $C_{19}H_{24}O_6$. It was easily identified as ethyl mycophenolate (8.4) from its N.M.R. spectrum which

TABLE 8.1. T.L.C. PROPERTIES OF THE NINE MAJOR METABOLITES OF P. BREVI-COMPACTUM.



Solvent 10 % CH_3OH in $CHCl_3$

	R_f	colour vis	colour u.v.	$FeCl_3$	D.N.P.
P.V.1	0.15	-	blue	blue	-
P.V.3	0.60	yellow	yellow	-	-
P.V.4	0.42	yellow	yellow	-	-
P.V.6	0.94	-	blue	blue	-
P.V.7	0.39	-	blue	blue	-
P.V.8	0.22	-	-	-	-
P.V.9	0.21	-	blue	blue	-
P.V.10	0.00	-	black	brown	+
P.V.11	0.08	-	blue	wine	-
p-Hydroxy azo-benzene isoquinolone yellow	0.75 0.42	- -	- -	- -	- -



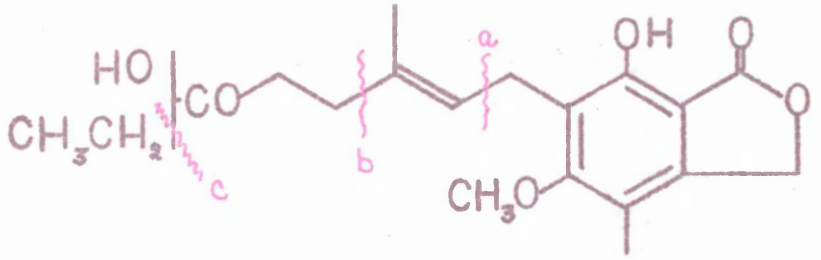
8.4

possessed the previously described pattern of absorption due to the mycophenolic nucleus together with a two proton quartet at 5.95 tau and a three proton triplet at 8.80 tau. Final confirmation of identity was obtained by spectral and mixed melting point comparison of the isolated metabolite with an authentic sample of ethyl mycophenolate prepared by synthesis.

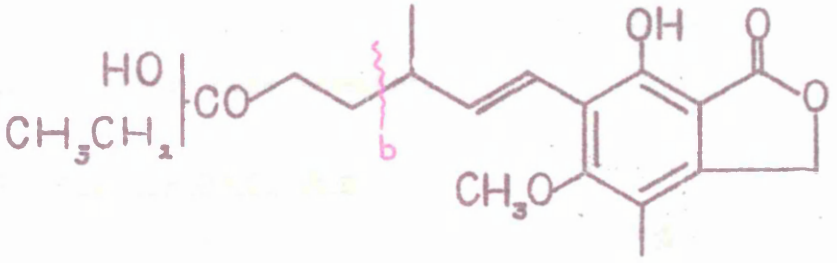
Ethyl mycophenolate has previously been encountered in extracts derived from Penicillium brevi-compactum by Raistrick et al[28] who showed that it had been formed during the extraction process by interaction of mycophenolic acid with ethanol. T.L.C. of samples taken from the broth of a growing culture of the organism under present study indicated that ethyl mycophenolate was present therein. It may also be of interest to mention that N.M.R. spectroscopy revealed that ethyl esters were also present in the complex mixtures of lipids produced by the mycelium of the organism under study.

Details of the mass spectral cracking pattern exhibited by

mycophenolic acid and its ethyl ester are shown in Table 8.2. The base peak of both spectra occurred at m/e 207 and could be explained in terms of a "Type A_4" fission[25] at point "a" (8.5). Fission at point "b" gave rise to the other major ion (m/e 247) but since cleavage β to a double bond is much more favourable than cleavage α to it, it may well be from the double bond isomer (8.6) that the fragmentation takes place.



8.5



8.6

The exceptionally large m/e 44 ion in the spectrum of mycophenolic acid is presumably due to the loss of carbon dioxide from the carboxyl group. The two ions in the spectrum of the ethyl ester at m/e 303 and m/e 302 represented the losses of ethylene and an ethyl group from the ion (P-18). Such losses are typical of carbethoxy compounds[26]. The origin of the ions P-18 and m/e

TABLE 8.2. MASS SPECTRAL DATA FOR MYCOPHENOLIC ACID AND ETHYL MYCOPHENOLATE.

a. Mycophenolic acid

m/e	%
320	46.6
302	42.0
247	97.9
207	100.0
159	39.0
139	39.0
44	370.0

No metastable ions detected.

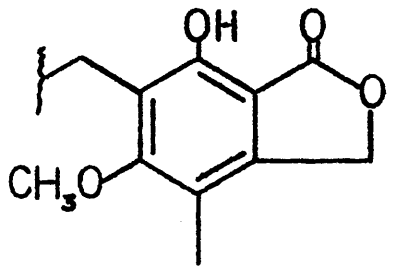
b. Ethyl mycophenolate

m/e	%
348	15.2
330	13.3
303	31.6
302	27.8
247	31.2
207	100.0
159	27.5
128	29.8

No metastable ions detected.

159, 137 and 128 was not immediately evident.

The highly insoluble substance P.V.7 (8.10b) analysed in accord with the formula $C_{17}H_{20}O_7$. Infrared, ultraviolet, N.M.R. and mass spectral similarities with mycophenolic acid indicated that the substituted dihydroxyphthalide nucleus (8.7) was also



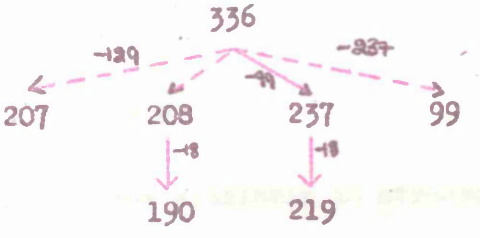
8.7

present in this new metabolite. Infrared spectroscopy further suggested that the three oxygen atoms unaccounted for in the above structure could be accommodated in a γ-lactone function (1768 cm^{-1}, ε 784, $\Delta\nu_{\frac{1}{2}}$ 30) and as an hydroxyl group (3620 cm^{-1}, ε 54, $\Delta\nu_{\frac{1}{2}}$ 56). The hydroxyl group appeared to be secondary since there occurred in the N.M.R. spectrum at 5.63 tau a double-doublet attributable to a single hydrogen under a hydroxyl group. The existence in the compound P.V.7 of a tertiary methyl group was also inferred from the N.M.R. spectrum.

The relative disposition of methyl, lactone and hydroxyl groups was deduced from the observation that on electron bombardment the molecule split into two parts, m/e 99 and m/e 237. The latter ion was, in fact, the base peak of the spectrum (Table 8.3). Assuming

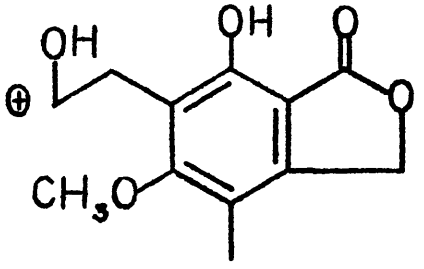
TABLE 8.3. MASS SPECTRAL DATA FOR THE COMPOUND P.V.7

m/e	%
336	15.5
237	100.0
219	6.5
208	38.8
207	50.5
190	11.9
99	51.1

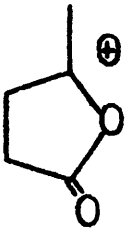


m*	Transition
202.4	237-219
173.6	208-190
167.2	336-237

that the larger fragment contained the structural element (8.7) it was possible to argue that the additional thirty units of mass must correspond to the moiety CH_2O and must represent the substitution in the side chain β to the benzene ring. In view of the fact that the compound P.V.7 contained an hydroxyl group, it was proposed that the charged fragment of mass 237 units possessed structure (8.8). This required that the fragment of mass 99 must contain the γ-lactone and the tertiary methyl group and must have the formula $C_5H_7O_2$.

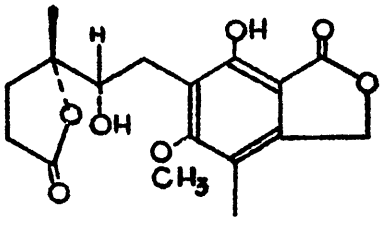


8.8

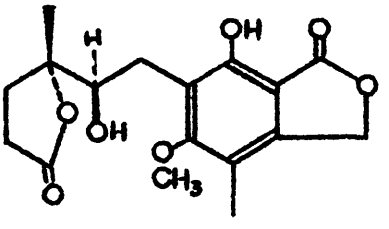


8.9

A unique solution was available in structure (8.9). Assuming that both ions have been formed by the cleavage of a 1,2 di-oxygenated bond, it was possible to propose structure (8.10) as a representation of the compound P.V.7.



8.10a

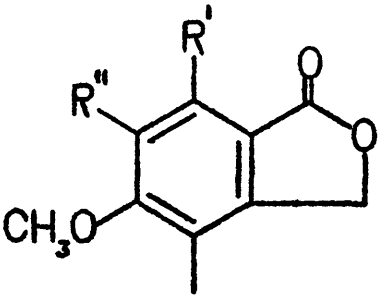


8.10b

There were, however, two possible geometrical isomers for the above structure (8.10 a and 8.10 b). For comparison purposes both isomers were synthesised, the former by osmylation and lactonisation, the latter by epoxidation and lactonisation of mycophenolic acid. It was found that the compound P.V.7 was identical in all respects to the threo-isomer (8.10b).

Attempts to ascertain whether the natural metabolite was optically active have met with no success since insufficient material was available to permit exhaustive classical determination and since the compound's high level of ultraviolet absorption rendered rotary dispersion studies valueless. If the substance were shown to be optically active it would be of interest to determine its absolute stereochemistry and thus shed some light on the stereochemistry of the processes leading to the biosynthesis of the hydroxylactone system.

Micro-analysis and mass spectroscopy indicated that the molecular formula of the compound P.V.9 (8.12), $C_{17}H_{18}O_6$ corresponded formally to that of a dehydromycophenolic acid. The N.M.R. spectrum of the metabolite with resonances at 4.76 tau (phthalide methylene), 6.17 tau (aromatic methoxyl) and 7.84 tau (aromatic methyl) confirmed that the mycophenolic nucleus (8.11) was present in the new compound. It was construed that there was no free



8.11

phenolic hydroxyl at R^1 since the ultraviolet spectrum of the compound P.V.9 was pH independent and since the stretching frequency of the phthalide carbonyl group appeared in the infrared spectrum at the normal value (1763 cm^{-1}, ε 930, $\Delta\nu_{\frac{1}{2}}$ 25). The acidic nature of the substance was in keeping with further infrared absorption at 3528, 1748 and 1714 cm^{-1}.

Of the six atoms of oxygen known to be present in the molecule, five had already been accounted for. Since no unexplained carbonyl or hydroxyl absorptions were detected in the infrared spectrum, it was assumed that the remaining oxygen atom was involved in an ether linkage. It seemed highly probable that this ether might be located at carbon atom C-2 of the aromatic ring (R^1, 8.11).

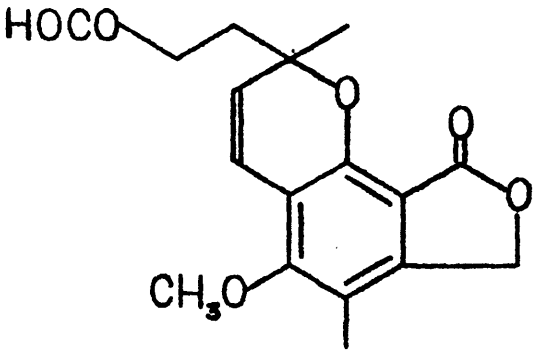
Three of the resonances in the N.M.R. spectrum have already been considered, the remaining features of that spectrum are listed below:-

8.50 tau multiplet (t)	$-C-CH_3$
7.80 tau multiplet (let)	$-CH_2-CH_2-$
7.45 tau multiplet (let)	

4.37 tau	doublet	J	-CH=CH-
3.32 tau	doublet	10.2 c/s.	

The most feasible explanation of this data was that the compound P.V.9 contained two vicinal methylene groups, one tertiary, deshielded methyl group and a double bond system possessing no allylic hydrogens.

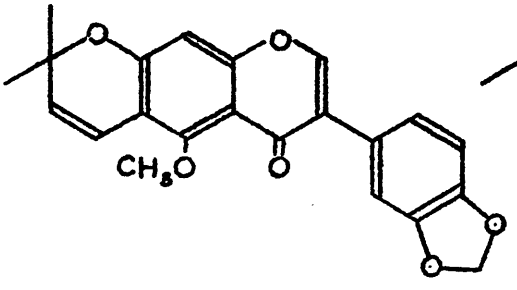
Ultraviolet spectroscopy revealed that in the metabolite there occurred a chromophore more extensive in nature than that possessed by mycophenolic acid and its simple derivatives. This fact suggested that the double bond was in direct conjugation with the aromatic ring and explained admirably why one of the peaks in the A.B system (3.32 tau) was so deshielded. The remaining part of the structure was easily solved. Since the ethylenic unit can have no allylic hydrogen atoms it must be linked directly to the carbon bearing the tertiary methyl group. Furthermore, since neither of the two methylenes had a chemical shift low enough (circa 6.0 tau) to be directly joined to oxygen, it must be assumed that the ether oxygen was also united to the quaternary carbon and that the two methylene groups and the carboxylic acid function existed as a β-substituted propionic acid unit (8.12). The structural relationship of this 2,2-disubstituted chromene



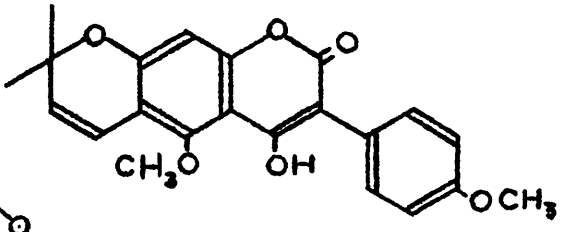
8.12

to mycophenolic acid is immediately evident; the substance has been named mycochromenic acid.

Close analogies for the N.M.R. absorption of the ethylenic protons are available in the chromene systems (8.13) and (8.14)



8.13



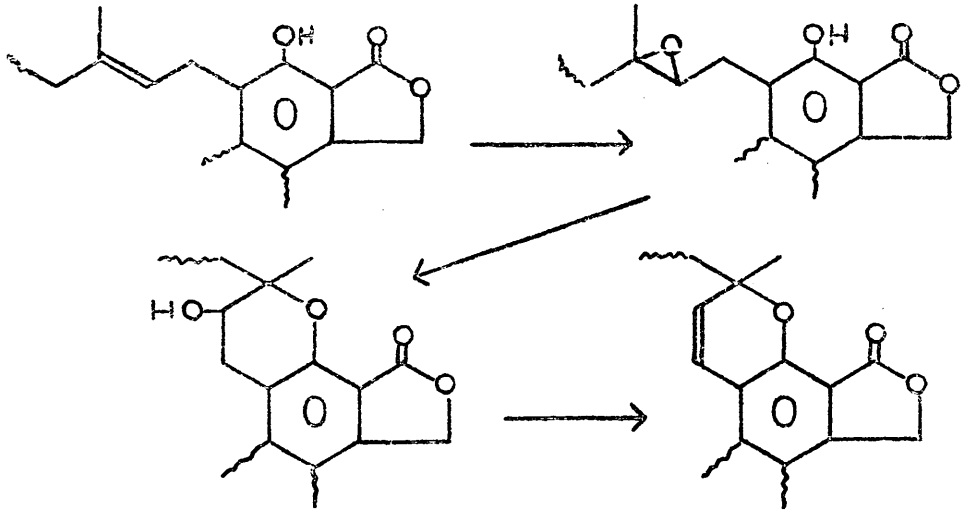
8.14

which exhibit resonance frequencies at 3.23 and 4.28 tau and 3.42 and 4.21 tau respectively, the coupling constant being 10 c/s.[27]

The above structure (8.12) was also completely consistant

with the mass spectrum of the compound P.V.9 of which the salient features are collected in Table 8.4. It will be seen that apart from the loss of carbon dioxide from the carboxylic acid and lactone groupings, the principal fragment ions were derived from "Type A_4"[26] fission of the methyl and propionic acid moieties from the chromene nucleus (8.13).

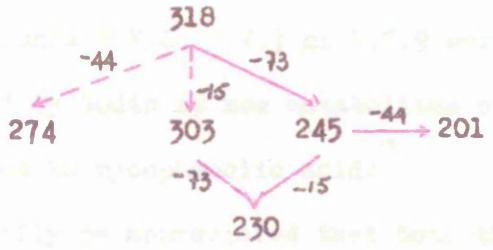
Preliminary attempts to synthesise the compound (8.12) were not successful. Epoxidation of ethyl mycophenolate with m-chloroperbenzoic acid followed by treatment of the resulting epoxy-phenol with strong base or buffer pH 9 in the hope of effecting the sequence shown below resulted in both cases in the formation of the threo-



hydroxylactone (8.10b). It thus appeared that, even in mild base, there is a greater tendency towards ester hydrolysis and attack of

TABLE 8.4. MASS SPECTRAL DATA FOR THE COMPOUND P.V.9.

m/e	%
318	30.8
303	5.5
274	6.4
245	100.0
230	55.5
201	34.6



m*	Transition
215.9	245-230
188.8	318-245
174.6	303-230
158.4	245-201

the epoxide by the carboxylate anion rather than for direct attack of the epoxide by the phenolate anion. The use of a less labile acid blocking group such as an amide and the employment of the dibromide rather than the epoxide may well lead to a successful synthesis.

Before attempting any further work with the compounds P.V.7 and P.V.9 it was important to establish that they were not artefacts of isolation. This has been done by means of direct chromatography of samples of the broth itself and has unambiguously shown the presence of both metabolites therein. It was interesting to note that all three of the compounds P.V.6, P.V.7 and P.V.9 begin to appear about the tenth day after inoculation and rise to a maximum at about the twenty-eighth day. Chromatography also showed that none of the compounds P.V.6, P.V.7 or P.V.9 were the substances X and XI reported by Godin as new metabolites of Penicillium brevi-compactum related to mycophenolic acid.[12]

It may readily be appreciated that both the compounds P.V.7 and P.V.9 could be derived biogenetically from mycophenolic acid by means of epoxidation and lactonisation or ether formation along the lines of the synthetic approach described above. Since such a sequence is relatively simple it was considered possible that the enzymes responsible might be present in the broth. Accordingly methods were developed under which samples of broth could be removed, filtered and dialysed under sterile conditions. Several

attempts were made to use such preparations to convert mycophenolic acid to the two new metabolites without any measure of success. The failure of this series of experiments may indicate that the required oxidases were not present in the extra-mycelial broth or that essential cofactors had been removed during dialysis. Be that as it may, they do provide conclusive evidence that the compounds P.V.7 and P.V.9 were not spontaneous aerial oxidation artefacts of mycophenolic acid since the experiments involved the exposure of aqueous solutions of mycophenolic acid for twenty days to the conditions prevailing in the broth during the culture of the mould and did not result in the formation of chromene or hydroxylactone.

It was noted during these experiments that the dialysed preparation of the broth contained a species with ultraviolet absorption similar to that of a mycophenolic acid derivative. The probability that it might be a protein-bound form of mycophenolic acid was heightened when it was noted that electrophoresis of the dialysed preparation revealed the presence of a protein with the characteristic ultraviolet induced blue fluorescence of mycophenolic acid and its derivatives.

Labelled mycophenolic acid, derived biosynthetically from C^{14}-formate, is currently being produced and it is hoped to use this material to further investigate the biogenetic problems associated with the production of the compounds P.V.7 and P.V.9 in Penicillium brevi-compactum.

Before leaving mycophenolic acid and the related substances, it is apposite to summarise their main spectroscopic properties. This is done in Tables 8.5, 8.6 and 8.7. It is interesting to examine the variation in chemical shift of the protons of mycophenolic acid and its ethyl ester occasioned by a solvent change from deuterochloroform to trifluoroacetic acid. If it can be assumed that the increment of -0.14 tau observed in the case of the aromatic methyl group and in the methyl group of the ethyl ester is the "normal" increment for such a solvent change, it then appears that the various protons possessing β oxygen atoms (CH_2-O-, CH_3-O-) experienced a larger shift than normal (-0.25, -0.30 tau) while the protons β to double bonds suffered a shift of opposite sign (+ 0.14-+ 0.31 tau). Both these phenomena can be explained in terms of protonation in the solvent of high acidity. Partial protonation of the oxygen β to the methyl and methylene hydrogens can be considered as causing a drift of electrons away from the hydrogens; thus leading to deshielding. Protonation of the double bond will lead to a decrease in its deshielding influence on the protons of allylic methyl and methylene groups and therefore their resonance frequency will rise.

The details of the solution infrared spectra of the compounds have already been discussed. It may be pointed out, however, that as potassium bromide discs the metabolites and their derivatives give rise to a sharp band at 3420-3430 cm^{-1} due to the intramolecular

TABLE 8.5. N.M.R. SPECTRA OF MYCOPHENOLIC ACID AND ITS DERIVATIVES ($CDCl_3$ solution)

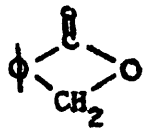
	ϕ-OCH_3	ϕ-CH_3		C_5	C_4	CH_3 C_3	C_2	C_1	Misc.
P.V.1	6.23	7.86	4.80	7.61	6.61	8.19	4.70(d) J=6 c/s	6.60(t)	--
P.V.6	6.22	7.87	4.83	7.70	7.70	8.20	4.73(d) J=7.2 c/s	6.62(t)	5.95 q J=7.2 8.80 t J=7.2
P.V.1/H_2	6.17	7.84	4.76			8.86(d) J=13.2			remainder complex
P.V.1 $CH_3CO.O.$	6.16	7.75	4.80	7.58	7.58	8.17	4.80(d) J=6.6 c/s	6.59(t)	7.58 $CH_3CO.O$
P.V.1 $(CH_3O)_2$	6114	7.75	4.81	7.98	7.58	8.14	4.78(d) J=7.2 c/s	6.50(t)	6.31 5.80
P.V.1 (CH_3O)	6.21	7.81	4.84	7.61	7.61	8.19	4.78(d) J=6.6 c/s	6.58(t)	5.96
P.V.9	6.20	7.90	4.90	7.75	7.75	8.50	3.32(d) J=10.2 c/s	4.37(t)	

TABLE 8.6. N.M.R. SPECTRA OF MYCOPHENOLIC ACID AND ITS DERIVATIVES (CF_3CO_2H)

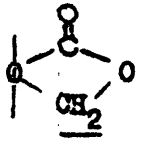
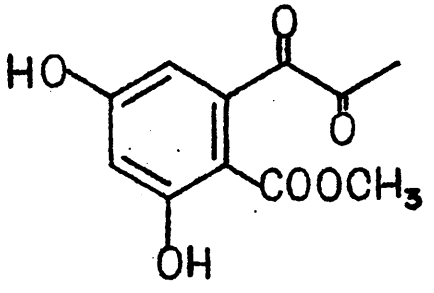
		ϕ-OCH_3	ϕ-CH_3		CH_3 / C_3	Miscellaneous
Three-P.V.7		5.99	7.70	4.50	8.27	5.63 dd J' 9.6 J" 4.2
Erythro-P.V.7		6.00	7.72	4.56	8.28	5.75 dd J' 9.0 J" 4.2
P.V.1	CF_3CO_2H	5.97	7.72	4.59	8.33	
P.V.1	$CDCl_3$	6.23	7.86	4.80	8.19	
P.V.6	CF_3CO_2H	5.93	7.73	4.58	8.51	5.65 q 7.2 c/s 8.60 t
P.V.6	$CDCl_3$	6.22	7.87	4.83	8.20	5.95 q 7.2 c/s 8.80 t

TABLE 8.7. UV OF MYCODERIVATIVES

	neutral acidic					Basic		
Mycophenolic acid	2160	2510	3040			2280	2500	3430
Ethyl mycophenolate	2190	2500	3030			2280	2510	3410
Dihydromycophenolic acid	2180	2510	3050			2300	2520	3450
Methyl mycophenolate, methyl ether	2160	2490	2940			--	--	--
Mycophenolic acid, methyl ether	2200	2500	2950			--	--	--
Mycophenolic acid acetate	2150	2470	2790	2880				
erythro P.V.7	2170	2490	3030			2270	2500	3360
threo P.V.7	2160	2500	3040			2280	2490	3380
P.V.9	--	2460	2800	3220	3330	--	--	--

hydrogen bond and to a characteristic triplet system at 1075, 1036, 966 cm^{-1} presumably due to ring vibrations.

The chromatographic behaviour of the compound P.V.10 suggested that it was an acid; it was therefore esterified and isolated as its methyl ester. This yellow, phenolic substance analysed in accord with the molecular formula $C_{11}H_{10}O_6$ and was shown by the normal spectroscopic methods to be the methyl ester of 3,5-dihydroxy-2-carboxybenzoyl methyl ketone (8.15) - a known metabolite of Penicillium brevi-compactum.[3]



8.15

Most of the work now to be described is of a fragmentary nature and relates to the remaining metabolites of the mould, the compounds P.V.8, P.V.3, P.V.4 and P.V.11. The former substance appears to be the most interesting and from the small amount of material that was isolated the following data were gleaned. The compound P.V.8 is a white crystalline, neutral material, m.p. 252-255°C which analysed in accord with a molecular formula $C_{22}H_{28}O_6$. Evidence to the fact that the substance was a benzoate ester was obtained from the N.M.R. spectrum of the substance which contained

the typical absorption pattern of the benzoyl group and from the mass spectrum in which losses of 122 and 105 units of mass were prominant processes. Thus the formula of the compound could be expressed in the form:-

$$(C_{15}H_{23}O_4) \; OCOC_6H_5$$

The nature of the four remaining oxygenatoms could be deduced from infrared spectroscopy. The carbonyl region of the spectrum contained two band, the first of which at 1711 cm^{-1} (ε 414, $\Delta\nu_{\frac{1}{2}}$ 21) could be attributed to the benzoate ester. The second occurring at 1775 cm^{-1} (ε 480, $\Delta\nu_{\frac{1}{2}}$ 22) was assigned to the stretching of the carbonyl of a γ-lactone. A band in the hydroxyl region at 3629 cm^{-1} (ε 127, $\Delta\nu_{\frac{1}{2}}$ 43) was found to integrate for two hydroxyl groups. The compound P.V.8 thus appeared to be the benzoate ester of a trihydroxy γ-lactone of molecular formula $C_{15}H_{24}O_5$. This latter substance contained four double bond equivalents, two of which would be required for the lactone and hence it may well be a bicyclic sesquiterpene. The N.M.R. spectrum of the compound was extremely complex and spin decoupling techniques will certainly be required for its complete analysis. However, it was noted that three tertiary methyl groups appeared to be present in the molecule.

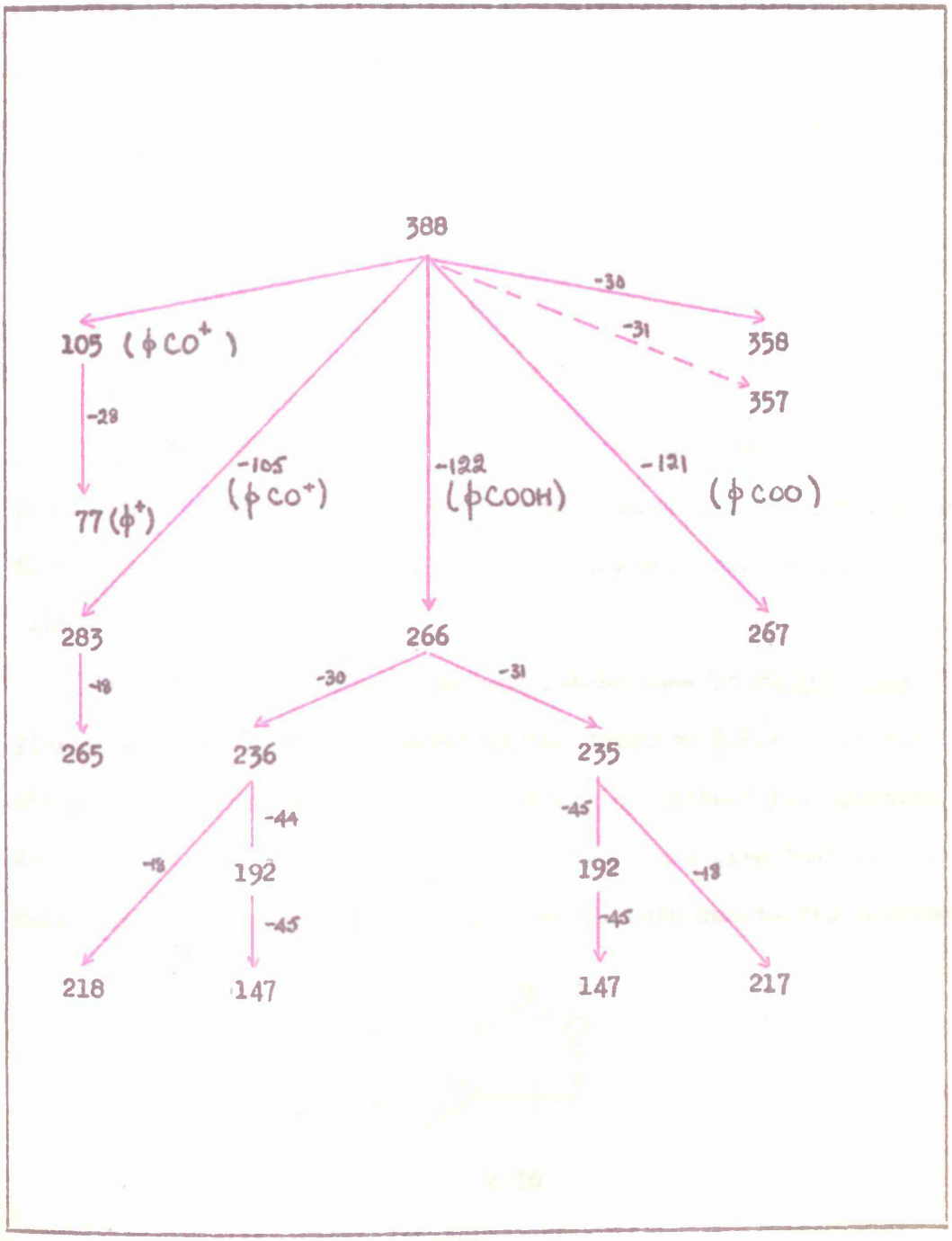
A synopsis of the principal features of the mass spectrum of the compound P.V.8 is presented in Table 8.8 while in Table 8.9 an attempt has been made to construct a fragmentation scheme

TABLE 8.8. MASS SPECTRAL DATA FOR THE COMPOUND P.V.8

m/e	%	m/e	%
388	0.3	235	9.5
358	1.1	219	5.8
357	1.1	218	12.0
283	29.3	217	7.6
266	14.2	192	8.1
265	3.6	142	11.3
237	8.4	105	100.0
236	21.2	77	34.0

m*	Transition
330.3	388-358
240.0	283-265
209.3	266-236
207.6	266-235
206.5	388-283
202.3	237-219
201.3	236-218
200.4	235-217
182.4	388-266
156.9	237-192
156.3	236-192
155.5	235-192
125.6	192-147
56.5	105- 77

TABLE 8.9. PROPOSED FISSION SEQUENCES OF THE COMPOUND P.V.8 ON ELECTRON BOMBARDMENT

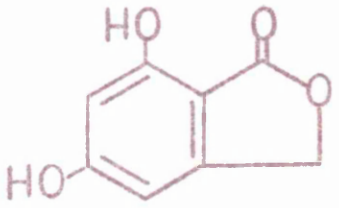


consistant with the positions of metastable ions in the spectrum. Apart from the losses of 122, 105, 18, 15 units of mass which have been previously discussed and an elimination of 44 units of mass presumably from the Y-lactone, there appeared distinctive losses of 30, 31 and 45 units of mass. The latter two fragments may point to the existence in the compound P.V.8 of the primary and secondary alcoholic units shown below.

mass 31 mass 45

The compounds P.V.3 and P.V.4 were isolated in very small quantities indeed and no headway has been made with their structural elucidation. It appears possible that they are both aromatic quinonoid pigment.

The last of the nine principal metabolites of Penicillium brevi-compactum to be considered is the compound P.V.11. It was phenolic in nature and it was noted that its ultraviolet spectrum at neutrality and also at pH 10 was superposable with that of 5,7 dihydroxyphthalide (8.16). Furthermore, both substances possessed



8.16

similar ultraviolet induced deep-blue fluorescence and produced the same wine coloration with alcoholic ferric chloride. However, although T.L.C. comparison in three solvent systems showed the materials to be identical, they did not possess exactly the same R_f value in a methyl ethyl ketone, water, diethylamine T.L.C. system. It seems probable that the compound P.V.11 is, nevertheless, closely related to the phthalide (8.16).

As a conclusion to this chapter there is presented in Table 8.10, details of the T.L.C. R_f value of the isolated metabolites recorded in four solvent systems. It may also be mentioned that during the preparation of C^{14} mycophenolic acid from C^{14} sodium formate, autoradiography of T.L.C. plates indicated that a compound of high specific activity was present in the broth of a 10 day old culture. It was not present in the culture medium after 19 and 28 days growth.

TABLE 8.10. R_f VALUES OF THE P. BREVICOMPACTUM METABOLITES.

	SOLVENT			
	A	B	C	D
P.V.1	0.00	0.15	0.55	0.34
P.V.3	0.00	0.60	0.30	0.80
P.V.4	0.00	0.42	--	--
P.V.6	0.32	0.98	0.79	1.00
P.V.7	0.00	0.39	0.34	0.43
P.V.8	0.00	0.22	0.20	0.28
P.V.9	0.00	0.12	0.36	0.43
P.V.10	0.00	0.00	0.36	0.35
P.V.11	0.00	0.08	0.11	0.83
Radioactive compound	--	--	--	0.22
p-hydroxyazobenzene	0.11	0.75	0.63	1.00
"isoquinolone yellow"	0.00	0.42	0.07	0.24

Solvents

A Chloroform.

B Methanol (10), Chloroform (90).

C Benzene (90), dioxan (45), acetic acid (4).

D Methylethylketone (460), water (40), diethylamine (1)

CHAPTER 9

EXPERIMENTAL

I N D E X

1. GROWTH AND EXTRACTION OF THE MOULD

Penicillium brevi-compactum (Commonwealth Mycological Institute No. 49162.) was sub-cultured onto agar slants and thence to agar seed bottles (15 x 9 cm.). A spore suspension, prepared from 24 such bottles and distilled water (2 l.), contained approximately 7.7×10^7 spores per millilitre. This suspension was used to inoculate 100 Roux surface culture bottles which had previously been sterilised (0.5 hours with steam at 242°C and 12 p.s.i.) containing 200 ml. of the following Czapek Dox medium:-

sodium nitrate		0.2
dipotassium hydrogen phosphate		0.1
potassium chloride		0.05
magnesium sulphate		0.05
ferric sulphate		0.001
glucose		5.0
corn steep liquor		1.0
distilled water	to	100.0

Cultures were allowed to grow undisturbed at 25°C and 70 % relative humidity, artificial illumination being provided by Mazda fluorescent tubes for 12 hours per day. After the prescribed period of growth had elapsed, the mycelial mats were separated from the broth, dried at 40°C, powdered in a mortar and subject to soxhlet extraction with acetone for 24 hours. The broth was stirred with charcoal (10 gm. per litre of broth) at room temperature for 1.5 hours. Thereafter the charcoal was filtered off (glass paper) and extracted in a soxhlet apparatus for 24 hours with acetone.

The mycelial weights were determined after growth periods of 8, 15 and 28 days and, as shown below, indicated that an incubation period of approximately 15 days was required to give maximal tissue production.

Culture age (days)	Mycelial weight (g. / l. broth)
8	9.4
15	16.5
28	12.3

The average 15 day culture provided 11 gm. of mycelial extract from 250 gm. tissue (i.e. 4.4 % extraction) and 3.2 gm. of broth extract from 25 litres of broth (i.e. 1.3 % extraction).

2. PRELIMINARY T.L.C. ANALYSIS. R_f VALUES AND STAINING CHARACTERISTICS OF THE NINE PRINCIPAL METABOLITES

The constituents of both broth and mycelial extracts were examined by T.L.C. in each of the following four solvent systems:-

A. Chloroform (100).

B. Methanol (10), chloroform (90).

C. Benzene (90), dioxan (45), acetic acid (4).

D. Methyl ethyl ketone (460), water (40), diethylamine (1).

The developed plates were viewed in U.V. light (2540 and 3510 Å) and sprayed with 10 % methanolic ferric chloride solution, 1 %

Brady's reagent and a solution of ceric ammonium nitrate (1 %) in 10 % aqueous sulphuric acid. The chromatographic mobilities and staining characteristics of the nine principal metabolites are collected in Table 8.10.

The R_f values were corrected for plate and solvent variation by standardisation relative to the dye mixture of p-hydroxy-asobenzene and 2-methyl-6,7-methylenedioxy-3(2H)-isoquinolone.

The code names P.V.1, P.V.3, P.V.4, P.V.6, P.V.7, P.V.9, P.V.10 and P.V.11 will be used throughout the text to uniquely represent the metabolites with R_f values as defined in Table 8.10.

3. MYCOPHENOLIC ACID (8.3). THE COMPOUND P.V.1.

A sample of 15 day broth extract (3.41 gm.) was dissolved in acetone (20 ml.), filtered through glass paper and treated with water (20 ml.). The precipitated solid was centrifuged off, re-dissolved in acetone (20 ml.) and reprecipitated with water (20 ml.). Gradient precipitation of the resultant solid from aqueous methanol gave brownish needles (2.16 g., m.p. 138-140°C) which were treated with decolourising charcoal and crystallised from a light petroleum: ethyl acetate system as needles (1.08 g.), m.p. 140-141°C (lit.[25] 141°C). The substance titrated as a dibasic acid, pK_a^1 2.3, pK_a^2 6.2. ν_{max} (KBr) 3420, 1738, 1711, 1627, 1207, 1167, 1132, 1100, 1075,

1031, 966 cm^{-1}.

ν_{max} (6.65 mM. $CHCl_3$) 3516 cm^{-1} (ε 55.5, $\Delta\nu_{\frac{1}{2}}$ 44), 3454 cm^{-1} (ε 95.6, $\Delta\nu_{\frac{1}{2}}$ 55), 1740 cm^{-1} (ε 1363, $\Delta\nu_{\frac{1}{2}}$ 24), 1714 cm^{-1} (ε 490, $\Delta\nu_{\frac{1}{2}}$ 19).

λ_{max} (CH_3OH) pH 2 and 7, 2160 Å (ε 41,500), 2505 Å (ε 8,200), 3040 Å (ε 3,900).

λ_{max} (CH_3OH) pH 10, 2280 Å (ε 31,000), 2500 Å (ε 6,000), 3430 Å (ε 6,000).

Tau values ($CDCl_3$) 4.70 (1H, t, J=7 c/s.), 4.80 (2H, s), 6.23 (3H, s), 6.60 (2H, d, J=7 c/s.), 7.61 (4H, m), 7.86 (3H, s), 8.19 (3H, s)

Tau values (CF_3COOH) 4.59 (2H, s), 5.97 (3H, s), 7.72 (3H, s), 8.33 (3H, s), complex absorption between 7.0 and 7.7.

Parent molecular ion at m/e 320 = $C_{17}H_{20}O_6$

Analysis	Found	C 63.90 %	H 6.32 %
Calculated for	$C_{17}H_{20}O_6$	C 63.74 %	H 6.29 %

4. MYCOPHENOLIC ACID ACETATE

Mycophenolic acid (537 mg.) was dissolved in dry pyridine (10 ml.) and acetic anhydride (5 ml.) was added to the chilled solution. The reaction was allowed to stand at 0°C for 63 hours before being poured onto ice-cold water. The organic materials

were extracted into ethyl acetate (100 ml.) and the ethyl acetate solution washed with 6N aqueous hydrochloric acid (6 x 100 ml.) before being dried and evaporated to give a white solid (560.3 mg., 92 %). Mycophenolic acid acetate crystallised from a light petroleum: ethyl acetate system in needles (291 mg.), m.p. 156-158°C (lit.[28] 158-160°C).

ν_{max} (KBr) 1768, 1727, 1624, 1612 (s.), 1196, 1188, 1210, 1133, 1070, 1033 and 970 cm^{-1}.

λ_{max} (CH_3OH) 2150 Å (ε 29,500), 2465 Å (ε 10,400), 2790 Å (ε 1,900), 2830 Å (ε 1,900). On basification the spectrum of the parent acid is obtained.

Tau values ($CDCl_3$) 4.80 (2H, s), 4.80 (1H, t, J=6.6 c/s.), 6.16 (3H, s), 6.59 (2H, d, J=6.6 c/s.), 7.58 (4H, m), 7.75 (3H, s), 8.17 (3H, s).

Analysis	Found	C 63.11 %	H 6.07 %
Calculated for	$C_{19}H_{22}O_7$	C 62.97 %	H 6.12 %

5. DIHYDROMYCOPHENOLIC ACID

Mycophenolic acid (320 mg.) was dissolved in glacial acetic acid (50 ml.) and hydrogenated at room temperature and atmospheric pressure for two hours with platinum oxide (100 mg.) as catalyst. When the hydrogen uptake was complete (23 ml.), the catalyst was

removed by filtration through glass paper. Removal of the solvent gave a white solid (318 mg., 99 %) which crystallised from an ethyl acetate: light petroleum system as needles (290 mg.), m.p. 140-141°C (lit.[28] 139°C).

ν_{max} (KBr) 3430, 1740, 1718, 1623, 1210, 1160, 1102, 1074, 1034, 965 cm^{-1}.

λ_{max} (CH_3OH) pH=7 and 2 2180 Å (ε 33,200), 2510 Å (ε 8,800), 3050 Å (ε 4,200).

λ_{max} (CH_3OH) pH=10 2300 Å (ε 32,800), 2520 Å (ε 7,100), 3450 Å (ε 7,100).

Tau values ($CDCl_3$) 4.76 (2H, s), 6.17 (3H, s), 7.84 (3H, s), 8.86 (3H, d, J=12 c/s.) complex absorption at 7.2-7.8 and at 8.4.

Analysis	Found	C 63.24 %	H 6.84 %
Calculated for	$C_{17}H_{22}O_6$	C 63.34 %	H 6.88 %

6. METHYL MYCOPHENOLATE METHYL ETHER

Mycophenolic acid (320 mg.) was dissolved in methanol (10 ml.) and treated with excess ethereal diazomethane for 24 hours at 0°C. The crude product (329 mg.) was purified by P.L.C. on a plate (200 x 200 x 1 mm.) using 100 % chloroform as the developing solvent. Methyl mycophenolate, methyl ether was removed from the silica by elution with chloroform, was sublimed (180-185°C, 0.3 mmHg.) and crystallised from a light petroleum: ethyl acetate system

as needles (108 mg.), m.p. 57-58°C (lit.[23] 58°C).

ν_{max} (KBr) 1759, 1750, 1602, 1211, 1165, 1125, 1105, 1074, 1033, 966 cm^{-1}.

λ_{max} (CH_3OH) 2160 (ε 48,300), 2490 Å (ε 10,700), 2940 Å (ε 3,200).

Tau values ($CDCl_3$) 4.78 (1H, t, J=7.2 c/s.), 4.81 (2H, s), 5.80 (3H, s), 6.14 (3H, s), 6.31 (3H, s) 6.50 (2H, d, J=7.2 c/s.), 7.58 (4H, m), 7.75 (3H, s), 8.14 (3H, s).

Analysis	Found	C 65.55 %	H 6.81 %
Calculated for	$C_{19}H_{24}O_6$	C 65.50 %	H 6.94 %

7. MYCOPHENOLIC ACID, METHYL ETHER

A solution of methyl mycophenolate, methyl ether (220 mg.) in methanol (5 ml.) was treated at 20°C for 16 hours with 4N aqueous sodium hydroxide (2 ml.). After this period the reaction solution was acidified with 6N aqueous hydrochloric acid (4 ml.) and extracted with ethyl acetate (2 x 20 ml.). The combined organic layers were washed with water (2 x 40 ml.), dried and evaporated to give a white solid (196 mg.) which was purified by P.L.C. on a plate (200 x 200 x 1 mm.) using 10 % methanol in chloroform as eluant. Mycophenolic acid methyl ether was removed from the silica by elution with ethyl acetate, treated with decolourising charcoal and crystallised from an ethyl acetate: light petroleum

system as needles (58 mg.), m.p. 110-111°C (lit.[18] 112°C).

ν_{max} (KBr) 1760, 1716, 1603, 1295, 1205, 1163, 1120, 1102, 1074, 1036, 964 cm^{-1}.

λ_{max} (CH_3OH) 2200 Å (ε 24,500), 2500 Å (ε 9,700), 2950 Å (ε 2,600).

Tau values ($CDCl_3$) 4.78 (1H, t, J=6.6 c/s.), 4.84 (2H, s), 5.96 (3H, s) 6.21 (3H, s), 6.58 (2H, d, J=6.6 c/s.), 7.61 (4H, s), 7.81 (3H, s), 8.19 (3H, d, J=1.2 c/s.).

8. ETHYL MYCOPHENOLATE (8.4). THE COMPOUND P.V.6

A portion of the broth extract (3.57 g.) of P. brevicompactum was placed on a column of Mallinckrodt silicic acid (167 g. 4 x 30 cm., R_v = 242 ml.) and eluted with a gradient of 50 % benzene : 50 % chloroform (1 l.) to 50 % chloroform : 50 % methanol (1 l.). Fractions (20 ml.) were taken from a flow rate of 20 ml. per hour and were assayed by T.L.C. in the usual manner.

The material (418 mg.) eluted from the column with solvent varying in composition from 0.5 % to 2 % methanol in chloroform contained the compound P.V.6 which crystallised from ethyl acetate: light petroleum as plates (262 mg.), m.p. 88-90°C.

ν_{max} (KCl) 3420, 1736, 1624, 1167, 1130, 1098, 1070, 1031, 970 cm^{-1}.

ν_{max} (6.0 mM., $CHCl_3$) 3460 cm^{-1} (ε 100, $\Delta\nu_{\frac{1}{2}}$ 61), 1737 cm^{-1}

(ε 1349, $\Delta\nu_{\frac{1}{2}}$ 25).

λ_{max} (CH_3OH) pH 7 and 2 2190 Å (ε 36,100), 2500 Å (ε 9,900), 3030 (ε 4,700).

λ_{max} (CH_3OH) pH 10 2280 Å (ε 34,500), 2510 Å (ε 6,500), 3410 Å (ε 7,900).

Tau values (CCl_4) 4.73 (1H, t, J=7.2 c/s.), 4.83 (2H, s), 5.95 (2H, q, J=7.2 c/s.), 6.22 (3H, s), 6.62 (2H, d, J=7.2 c/s.), 7.70 (4H, s), 7.87 (3H, s), 8.20 (3H, s), 8.80 (3H, t, J=7.2 c/s.).

Tau values (CF_3CO_2H) 4.58 (2H, s), 5.65 (2H, q, J=7.2 c/s.), 5.93 (3H, s), 8.51 (3H, s), 8.65 (3H, t, J=7.2 c/s.) complex pattern at 7.2 and 7.9.

Parent molecular ion at m/e 348.

Analysis Found C 65.66 % H 7.00 %

$C_{19}H_{24}O_6$ requires C 65.50 % H 6.90 %

This compound was identical (i.r., n.m.r., mixed melt) with a sample of ethyl mycophenolate prepared in 82 % yield by treating mycophenolic acid with ethanolic hydrochloric acid for 24 hours at room temperature.

9. Threo-3- (5-CARBOXY-2,3-DIHYDROXY-3-METHYLPENT-2-ENYL Y-LACTONE)-2-HYDROXY-6-HYDROXYMETHYL-5-METHYL-p-ANISIC

ACID γ-LACTONE (8.10b). THE COMPOUND P.V.7

a. Mycophenolic acid (380 mg., 1.19 mM.) was dissolved in chloroform (10 ml.) and treated with excess m-chloroperbenzoic acid (392 mg., 2.29 mM.) at room temperature for 5.5. hours. Thereafter the solvent was removed and the crude product refluxed with 6N aqueous hydrochloric acid (10 ml.) for five minutes. The resulting product was extracted into ethyl acetate (50 ml.) washed with water (2 x 50 ml.), dried and evaporated to give a white solid (430 mg.) which yielded the lactone (107 mg., 26 %, m.p. 218-220°C) after two crystallisations from aqueous acetic acid.

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

ν_{max} (1.71 mM., $CHCl_3$) 3620 cm^{-1} (ε 54, $\Delta\nu_{\frac{1}{2}}$ 56), 3451 cm^{-1} (ε 120, $\Delta\nu_{\frac{1}{2}}$ 83), 1768 cm^{-1} (ε 786, $\Delta\nu_{\frac{1}{2}}$ 30), 1741 cm^{-1} (ε 1340, $\Delta\nu_{\frac{1}{2}}$ 24).

λ_{max} (CH_3OH) pH 7 2155 Å (ε 34,600), 2500 Å (ε 8,600), 3040 Å (ε 4,200).

λ_{max} (CH_3OH) pH 10 2270 Å (ε 38,000), 2500 Å (ε 7,100), 3360 Å (ε 7,100).

Tau values (CF_3CO_2H) 4.54 (2H, s), 5.63 (1H, d.d, J^{1}= 9.6, J^{11}=4.2 c/s.), 5.99 (3H, s), 7.70 (3H, s), 8.27 (3H, s), complex pattern between 6.7 and 7.4.

Parent molecular ion at m/e 336.

Analysis	Found	C 60. 46 %	H 5.87 %
$C_{17}H_{20}O_7$	requires	C 60.71 %	H 5.99 %

b. The fractions of the column described in Section 8 eluted with solvent varying in composition from 2 % to 13 % methanol contained mycophenolic acid and the compound P.V.7 (1.62 g.). The former substance (1.02 g.) was removed by crystallisation from ethyl acetate: light petroleum and the compound P.V.7 isolated from the mother liquor by means of two successive P.L.C. operations on plates (200 x 200 x 0.5 mm.) using 10 % methanol in chloroform as eluant. The metabolite was recovered from the silica by ethyl acetate elution and crystallised from ethyl acetate: light petroleum as needles (28 mg.), m.p. 218-220°C.

This substance was shown to be identical (i.r. n.m.r., R_f, mixed melt) with the material prepared in "a" above.

10. Erythro-3-(5-CARBOXY-2,3-DIHYDROXY-3-METHYLPENT-2-ENYL Y-LACTONE)-2-HYDROXY-6-HYDROXYMETHYL-5-METHYL-p-ANISIC ACID Y-LACTONE

Mycophenolic acid (580 mg., 1.81 mM.) was dissolved in dry ether (50 ml.) and pyridine (1 ml.) and osmium tetroxide (500 mg., 1.97 mM.) were added. The reaction was allowed to stand in the absence of light and at room temperature for 72 hours before the

precipitated osmate ester was removed by filtration, suspended in methanol (100 ml.) and treated for one hour with gaseous hydrogen sulphide. The osmium sulphide was filtered off and the methanol solution evaporated to give a white solid (562 mg.) which was treated for two hours under reflux with 10 % aqueous hydrochloric acid (20 ml.). Thereafter the reaction solution was extracted with ethyl acetate (2 x 30 ml.) and the combined ethyl acetate extracts washed with water (2 x 60 ml.), dried and evaporated to give a solid (501 mg.) which after four successive crystallisations from ethyl acetate: methanol systems provided the erythrolactone (52 mg.) as needles, m.p. 215.5-217°C.

ν_{max} (KBr) 3436, 1760, 1740, 1620, 1196, 1160, 1135, 1103, 1073, 1036, 967 cm^{-1}.

λ_{max} (CH_3OH) pH 7 2170 Å (ε 31,200) 2490 Å (ε 8,100), 3030 Å (ε 4,000).

λ_{max} (CH_3OH) pH 10 2280 Å (28,600), 2490 Å (ε 6,400), 3380 Å (ε 6,700).

Tau values (CF_3CO_2H) 4.56 (2H, s), 5.75 (1H, d.d, J^{I}= 9.0, J^{II}=4.2 c/s.), 6.00 (3H, s), 7.72 (3H, s) 8.28 (3H, s), complex pattern between 6.6 and 7.4.

Analysis Found C 60.55 % H 5.97 %

$C_{17}H_{20}O_7$ requires C 60.71 % H 5.99 %

The spectroscopic data for this compound was not identical with data derived from the compound P.V.7 (Section 9).

The melting point of a 1 : 1 mixture of the two substances was 199-211°C.

11. 2-(2-CARBOXYETHYL)-2-METHYL-5-METHOXY-6-METHYL-7-HYDROXY-METHYLENE-8-CARBOXYCHROMENE γ-LACTONE (8.13). THE COMPOUND P.V.9

A sample of broth extract from P. brevi-compactum (7.16 g.) was adsorbed onto a column of Mallinckrodt silicic acid (200 g. 4 x 46 cm., R_v = 198 ml.) and eluted with a gradient ranging from benzene (1 l.) through chloroform (1 l.) to ethyl acetate (1 l.). Fractions (30 ml.) were taken from a flow rate of 40 ml. per hour.

Fractions 85-92 provided mycophenolic acid (564 mg.) while fractions 93-95 provided the threo-hydroxylactone (120 mg.).

Fractions 96-101, eluted with solvent varying in composition from 60-64 % ethyl acetate contained a white solid (182 mg.) which after three successive crystallisations from ethyl acetate: light petroleum provided pure compound P.V.9 as needles (36 mg.), m.p. 163-165°C.

ν_{max} (KCl), 3230, 1770, 1750, 1640, 1615, 1604, 1131, 1073, 1036, 967.

ν_{max} (6.45 mM., $CHCl_3$) 3528 cm^{-1} (ε 58, $\Delta\nu_{\frac{1}{2}}$ 61) 1763 (ε 930, $\Delta\nu_{\frac{1}{2}}$ 25), 1748 cm^{-1} (s), 1714 cm^{-1} (ε 310, $\Delta\nu_{\frac{1}{2}}$ 28).

λ_{max} (CH_3OH) 2460 Å (ε 20,500), 2800 Å (ε 3,200), 3215 Å (ε 3,500), 3325 Å (ε 3,000).

Tau values ($CDCl_3$) 3.32 (1H, d, J=10.2 c/s.), 4.37 (1H, d, J=10.2 c/s.), 4.90 (2H, s), 6.20 (3H, s), 7.90 (3H, s), 7.45 (2H, m), 7.89 (2H, m), 8.50 (3H, s).

Parent molecular ion at m/e 318.

Analysis	Found	C	64.24 %	H	5.70 %
$C_{17}H_{18}O_6$	requires	C	64.14 %	H	5.70 %

12. ATTEMPTED CONVERSION OF ETHYL MYCOPHENOLATE TO THE COMPOUND P.V.9

Ethyl mycophenolate (135 mg., 0.387 mM.) was dissolved in chloroform (4 ml.) and treated with m-chloroperbenzoic acid (86 mg., 0.502 mM.) for 7 hours at room temperature. Thereafter the solvent was removed and the resulting solid treated with 4N aqueous sodium hydroxide overnight at room temperature. Acidification and extraction with ethyl acetate provided a product (79.2 mg., 64 %, m.p. 219-221°C) which was shown to be identical (R_f, i.r.) with the threo-hydroxylactone described in Section 9.

A further attempt in which the sodium hydroxide was replaced by a borate buffer pH-9 similarly provided the threo-hydroxylactone.

13. EXPERIMENTS WITH DIALYSED P. BREVI-COMPACTUM BROTH

Under sterile conditions, the broth (250 ml.) from a 15 day culture of P. brevi-compactum was separated from the mycelium, filtered through glass paper and transferred to a length of Visking tubing which had previously been sterilised by irradiation in u.v. light for 72 hours. A similar procedure was adopted with one of the "control" flasks set up with nuterient medium at the time of the original inoculation. Both preparations were dialysed against tap water for 32 hours before being separately placed in two sterile incubation flasks (500 ml.). A sterile, aqueous solution of mycophenolic acid (300 mg. in 100 ml.) buffered with phosphate to pH 7.8 was added to each of the flasks and incubation was allowed to proceed for 20 days. Microscopic examination at the end of this period indicated that both preparations were still sterile.

Both solutions were acidified with hydrochloric acid and extracted with ethyl acetate (2 x 400 ml.). On evaporation of the dried extracts white solids were obtained (reaction 256 mg., control 280 mg.) which were shown by T.L.C. in solvents B, C and D (Section 2) to be pure mycophenolic acid uncontaminated by the compounds P.V. 7 or P.V.9.

It may be noted that an ultraviolet spectral comparison of the two dialysates revealed that the broth contained an entity absorbing at 2910 and 3090 Å. This species may bear a relationship to a protein detected in an eight day old broth sample by disc electrophoresis on polyacrylamidegel in tris-glycine buffer.

The protein had approximately the same mobility as the single protein in the broth sample which absorbs methylene blue.

14. 3,5-DIHYDROXY-2-METHOXYCARBONYL-BENZOYL METHYL KETONE. THE METHYL ESTER OF THE COMPOUND P.V.10

A sample of 15 day broth extract (7.880 gm.) was placed on a column of Mallinckrodt silicic acid (480 g., 4 x 60 cm.) and eluted with a gradient of 99 % chloroform : 1 % glacial acetic acid (1.5 l.) to 80 % chloroform : 19 % methanol : 1 % glacial acetic acid (1.5 l.). Fractions (28 ml.) were taken from a flow rate of 56 ml. per hour. The column eluant was monitored by the normal methods of chess plate and thin layer analysis.

Mycophenolic acid (2.854 gm.) was eluted from the column with solvent varying in composition from 1 % to 3 % methanol and was crystallised from a light petroleum : ethyl acetate system in needles, m.p. 140-141°C.

The substance (5.735 gm.), eluted with solvent varying in polarity from 3 % to 5 % methanol was found by T.L.C. analysis to be predominantly the compound P.V.10. A sample of this substance (2.303 gm.) was dissolved in methanol (100 ml.) cooled to 0°C and treated with excess ethereal diazomethane. Removal of the solvent after a period of 12 hours gave a brown oil (2.813 gm.) which was adsorbed onto a column of Mallinckrodt silicic acid

(100 gm., 3 x 28 cm.) and eluted with a gradient of ether (1 l.) to chloroform (1 l.). Fractions (15 ml.) were taken from a flow rate of 30 ml. per hour and were assayed in the usual manner.

The fractions corresponding to elution with solvent varying in composition from 8 % to 10 % chloroform were evaporated to give a solid (1.146 gm.) which, after treatment with decolourising charcoal, crystallised from a light petroleum : ethyl acetate system as yellow needles (982 mg.), m.p. 137-138°C.

ν_{max} (KBr) 3300, 1708, 1676, 1328, 1268, 1242, 1164, 1121, 745 cm^{-1}.

ν_{max} (1.18 mM., $CHCl_3$) 3585 cm^{-1} (ε 156, $\Delta\nu_{\frac{1}{2}}$ 41), 3440 cm^{-1} (ε 29, $\Delta\nu_{\frac{1}{2}}$ 56), 1723 cm^{-1} (ε 342, $\Delta\nu_{\frac{1}{2}}$ 14), 1707 cm^{-1} (ε 536, $\Delta\nu_{\frac{1}{2}}$ 26), 1683 cm^{-1} (ε 584, $\Delta\nu_{\frac{1}{2}}$ 25).

λ_{max} (CH_3OH) pH 7 and 2 2150 Å (ε 13,500), 2250 Å (ε 11,800) 2540 Å (ε 12,500)

λ_{max} (CH_3OH) pH 10 2300 Å (ε 12,100), 2540 Å (ε 12,500) 2910 Å (ε 5,320).

Tau values (CF_3CO_2H) 3.11 (1H, d, J=3.6 c/s.), 3.30 (1H, d, J=3.6 c/s.), 5.97 (3H, s), 7.32 (3H, s).

Analysis	Found	C 55.34 %	H 4.25 %
$C_{11}H_{10}O_6$	requires	C 55.47 %	H 4.23 %

15. <u>THE COMPOUND P.V.8</u>

A sample (3.57 g.) of broth extract derived from a 28 day culture of P. brevi-compactum was placed on a column of Mallinckrodt silicic acid (167 g., 4 x 30 cm., R_v 250 ml.) and eluted with a gradient of 50 % benzene : 50 % chloroform (1 l.) to 50 % chloroform : 50 % methanol (1 l.) Fractions (10 ml.) were taken from a flow rate of 20 ml. per hour and were assayed by T.L.C. in the usual manner. Solvent with an estimated composition of 15 % methanol eluted the compound P.V.3 and P.V.4. Two of the nine fractions which contained these metabolites also contained the substance P.V.8 (23 mg.) which after treatment with decolourising charcoal, crystallised from ethyl acetate petrol as needles (15 mg.), m.p. 252-255°C.

ν_{max} (KBr) 3420 (broad), 1756, 1713, 1602, 1582, 1276, 1200, 1160, 1108, 1055, 1035, 964 cm^{-1}.

ν_{max} (12.8 mM., $CHCl_3$) 3629 cm^{-1} (ε 127, $\Delta\nu_{\frac{1}{2}}$ 43), 1777 cm^{-1} (ε 480, $\Delta\nu_{\frac{1}{2}}$ 21.5), 1711 (ε 414, $\Delta\nu_{\frac{1}{2}}$ 21).

Parent molecular ion at m/e 388.

Analysis	Found	C 68.41 %	H 7.08 %
$C_{22}H_{28}O_6$	requires	C 68.02 %	H 7.27 %

16. THE COMPOUND P.V.11

The broth extracts (6.8 g.) from two 15 day cultures of P. brevi-compactum were combined and partitioned between ethyl

acetate (250 ml.) and water (250 ml.). The aqueous extract, which was shown by T.L.C. to contain the compounds P.V.10 and P.V.11, was lyophylised and the resultant solid (4.12 g.) placed on a column of Mallinckrodt silicic acid (100 g., 3 x 45 cm., R_v 120 ml.) which had been thoroughly washed with the solvent system benzene (90), dioxan (45), acetic acid (4). The same solvent was then used to develop the column and fractions (10 ml.) were taken from a flow rate of 25 ml. per hour. The tenth column volume of solvent eluted the compound P.V.11 (335 mg.) which was further purified by P.L.C. on four plates (200 x 200 x 1 mm.) using 30 % methanol : 70 % chloroform as developing solvent. The product (101 mg.) was removed from the silica by elution with methanol as a gummy solid.

ν_{max} (nujol) 3400 (broad), 1700, 1680, 1600 cm^{-1}

λ_{max} (CH_3OH) pH 7 and 2 2190 Å (O.D. 1.41), 2580 Å (O. D. 0.81), 2930 Å (O. D. 0.21),

λ_{max} (CH_3OH) pH 10 2340 Å (O. D. 1.11), 2850 Å (O. D. 0.89), 3160 Å (O. D. 0.51).

17. THE COMPOUNDS P.V.3 AND P.V.4

Both these substances are highly coloured and their presence on chromatographic plates and columns can easily be detected. The

combined yield of both these substances from fourteen production batches of P. brevi-compactum amounted to 142 mgs. A relative pure sample of the compound P.V.3 was isolated from this mixture by P.L.C. on two plates (200 x 200 x 1 mm.) using 10 % methanol in chloroform. The substance (28 mg.) was removed from the silica by elution with ethyl acetate; it proved to be an oil.

ν_{max} ($CHCl_3$) 3590, 3410, 3320 (broad), 1777, 1737 (s), 1712, 1690, 1663, 1618, 1603 cm^{-1}.

λ_{max} (CH_3OH) 2380 Å (O. D. 1.91), 2560 Å (O.D. 0.71), 4040 Å (O. D. 0.32).

Insufficient of the compound P.V.4 was obtained to permit spectroscopic examination.

18. DETECTION OF THE COMPOUNDS P.V.6, P.V.7 and P.V.9 IN THE BROTH OF A GROWING CULTURE OF P. BREVI-COMPACTUM

Samples were removed from the broth of a growing culture of P. brevi-compactum every two days and were assayed for mycophenolic acid and the compounds P.V.6, P.V.7 and P.V.9 by T.L.C. with the solvent systems described in Section 2. Mycophenolic acid was detected after two days growth; the other substance began to appear after 10 days growth.

BIBLIOGRAPHY

1. P. W. Clutterbuck, A. E. Oxford, H. Raistrick, G. Smith, Biochem. J., 26 1441 (1932).
2. A. E. Oxford, H. Raistrick, Biochem. J., 26 1902 (1932)
3. A. E. Oxford, H. Raistrick, Biochem. J., 27 634 (1933).
4. C. L. Alsberg, O. F. Black, Bur. Plant Ind. Bull., 270 7 (1913); Chem. Abs., 7 3992[3] (1913)
5. B. Gosio, Rivista d'Igiene e Sanita pubblica, Ann, 7 825, 869, 961 (1896).
6. J. H. Birkinshaw, H. Raistrick, D. J. Ross, Biochem. J., 50 630 (1952).
7. A. J. Birch, Proc. Chem. Soc., 10 (1962)
8. A. J. Birch, R. J. English, R. A. Massy-Westropp, M. Slaytor, H. Smith, J. Chem. Soc., 365 (1958).
9. A. E. Oxford, H. Raistrick, Biochem. J., 27 1170 (1933)
10. A. E. Oxford, H. Raistrick, Biochem. J., 29 1599 (1935).
11. P. Simonart, P. Godin, Bull. Soc. Chim. Belg., 60 446 (1951).
12. P. Godin, Biochem. Biophy. Acta, 11 114 (1953). Antonie van Leeuvenhoek, 21 215 (1955)
13. P. Godin, Antonie van Leeuvenhoek, 21 362 (1955).
14. P. Godin, Biochem. Biophys. Acta, 12 528 (1953).
15. P. Godin, Biochem. Biophys. Acta, 11 114 (1953).
16. P. Godin, Biochem. Biophys. Acta, 11 119 (1953).
17. P. Godin, Antonie van Leeuvenhoek, 21 83, 94, 177 (1955).
18. P. Simonart, G. Bommers, G. Parmentier, Zentr. Bakteriol.,

Parasitenk., Abt. II 110 194 (1957), Chem. Abs., 51 12223^{h} (1957)

19. A. W. Lis, F.W. Allen, Biochem. Biophy. Acta, 61 250 (1962)

20. Y. S. Mohammed, M. Luckner, Tet. Letts., 28 1953 (1963)

21. M. Luckner, K. Mothes, Arch. Pharm., 296 18 (1963)

22. R. Anderson, M.Sc. Thesis, Glasgow, 1965.

23. K. Nakanishi, "Infrared Absorption Spectroscopy", Holden Day, 1962, p. 30.

24. K. Biemann, "Mass Spectrometry", McGraw-Hill, 1962 p. 339.

25. R. M. Silverstein, G. C. Bassler, "Spectrometric Identification of Organic Compounds", Wiley, 1963, p.14.

26. R. Wheeler, Ph.D. Thesis, Bristol, 1964, p. 91.

27. P. W. Clutterbuck, H. Raistrick, Biochem. J. 27 654 (1933).