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

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Contents.

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I am indebted to the Carnegie Trust for the Universities of Scotland for maintenance during the last three years and to Professor R. A. Raphael, F.R.S., for providing the opportunity and facilities for performing the work.

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SUMMARY.

Structural studies on the methyl esters of the triterpene acids from the polypore Daedalea quercina have established the structure of the major component, methyl carbomethoxyacetyl-quercinate. In common with this compound, three other esters from this fungus are 23-keto-27-carboxylic esters. The first and second of these are methyl 16-hydroxycarbomethoxyacetylquercinate and methyl 16-hydroxyquercinate respectively. The third ester, methyl carbomethoxyacetyldaedaleate, which is the first recorded triterpene based on a C_{32} skeleton, is highly reactive and has not, as yet, yielded recognisable transformation products. The structure advanced for this compound is based on spectroscopic examination. A minor constituent of Daedalea quercina is thought to be methyl 25-hydroxypolyporenate C.

The synthesis and properties of carbomethoxyacetyl and carboxyacetyl derivatives of triterpenes have been studied.

Examination of the methyl esters of the triterpene acids from Leptoporus stipticus has established that the major component, methyl tyromycate, is methyl 3-oxolanosta-7,9(11),24-trien-26-oate. Four other compounds from this fungus have been studied. Three of these have been shown to be triterpene glucosides, the first from a fungal source, whose basic nuclei are similar to

that of methyl tyromycate. The last ester from Leptoporus stipticus, methyl leptoporate, is the second example of a C_{32} triterpene.

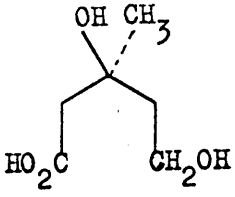
Leptoporus stipticus (Pers.) Quel., when cultured on malt agar medium, produces eburicoic acid and tumulosic acid. Attempts to influence the metabolite production of this latter fungus are described.

INTRODUCTION

PART 1 - FUNGAL TERPENES.

The history of the word 'terpene' provides a perfect example of how definitions must be modified and their terms of reference expanded to keep abreast of the increase in knowledge. The noun was first used by Wallach[1] to describe a group of volatile C_{10} hydrocarbons which could be isolated from plant essential oils. This definition was extended to include the oxygenated analogues of these C_{10} compounds and further modified to include products of a similar origin which contained fifteen or twenty carbon atoms. In modern usage the description 'terpene' applies to hundreds of naturally occurring compounds, widely distributed throughout the plant and animal kingdom, whose basic carbon skeleton may contain from five to forty atoms.

Attempts to define 'terpene' must take into account the observations of Bertholet[2], Wallach[1] and Ruzicka[3] that the framework of all compounds accepted as terpenoid can be built from a single C_5 unit. All terpenes can then be derived by linkage of these units. This '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 has been successfully applied to natural products, a more exact



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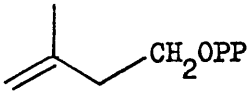

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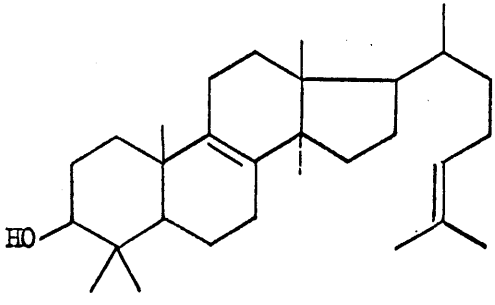

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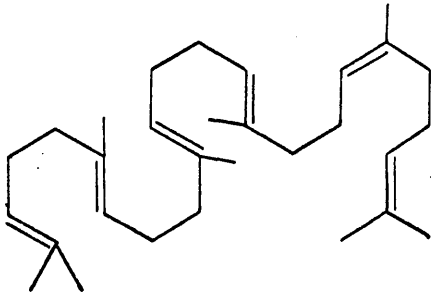

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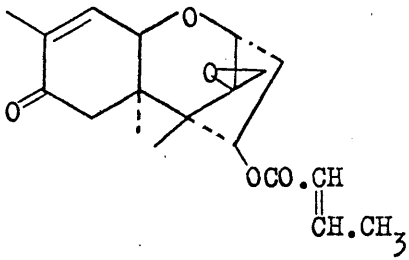
statement should take into account the biological basis for the isoprene rule. It has been shown that mevalonic acid (1) is the precursor of the C_5 building unit and that this exists in nature as isopentenyl pyrophosphate (2). A more rigorous definition of 'terpenoid' would then be, "any part of a compound biosynthetically derivable from mevalonic acid via isopentenyl pyrophosphate, or a polymer thereof." The prefixes hemi, mono, sesqui, di, sester, tri and tetra are then applied when the compound is obtained from the isopentenyl monomer, dimer, trimer, tetramer, pentamer, hexamer or octamer respectively.

Lanosterol (3) may now be described as a triterpene alcohol since it has been shown to be biosynthesised from mevalonic acid[5] via squalene (isopentenyl hexamer) (4)[6]. It will be noted that before a compound may be formally designated as terpenoid it is necessary to show that it arises from mevalonic acid. Since the number of compounds which have been isolated from natural sources is enormous, the amount of tracer studies required by the rigorous definition is prohibitive. Because of this, definitions such as that of Haagen-Smit are still of great value. An important fact allowed for by a biological description is that it includes compounds for which mevalonic acid may provide only part of the structure. There are numerous illustrations of this



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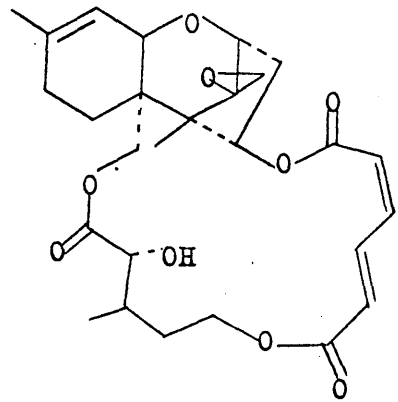

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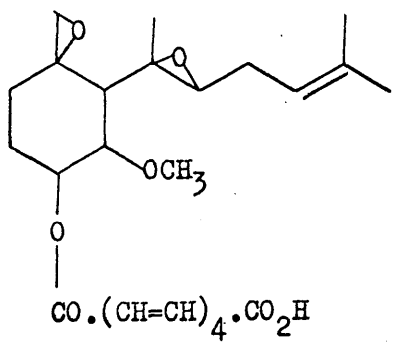

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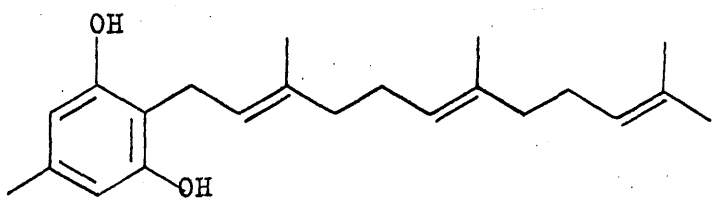

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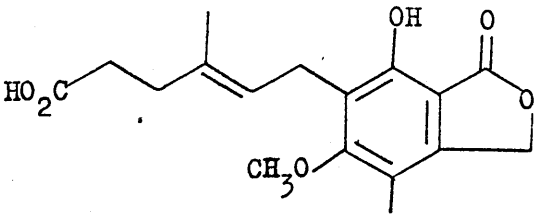

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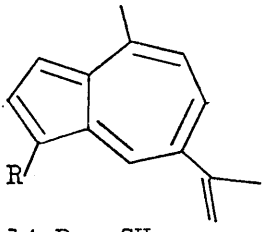

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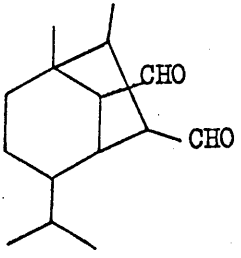
15 R = CHO



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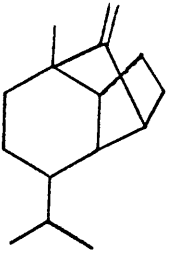

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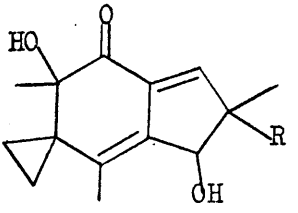
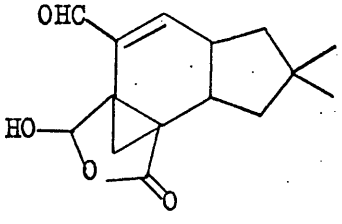


18 R = CH_3 or CH_2OH



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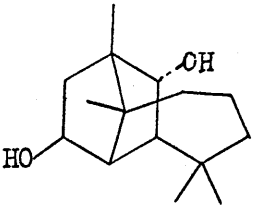

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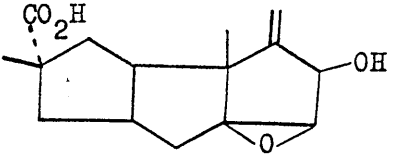

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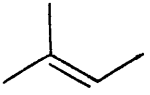

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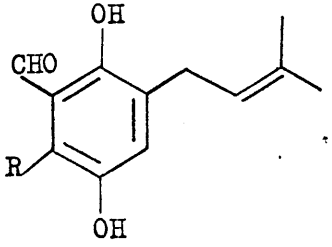
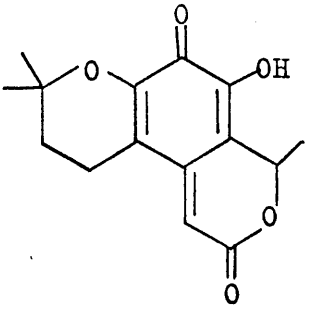


6 R = CH_3-$(CH{=}CH)_3$-

7 R = CH_3-$(CH_2)_6$-



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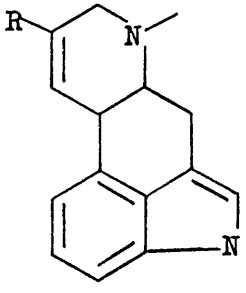
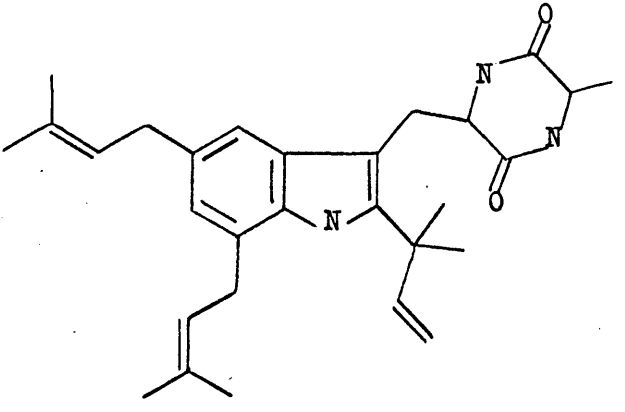


9 R = CH_2OH

10 R = CH_3



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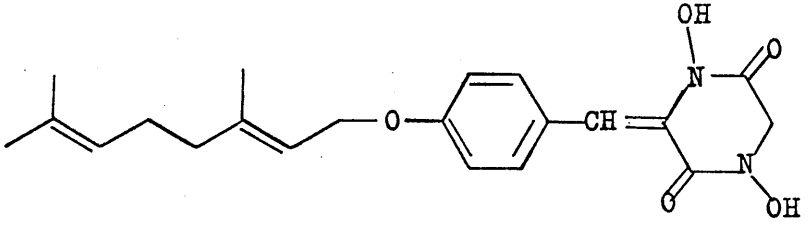

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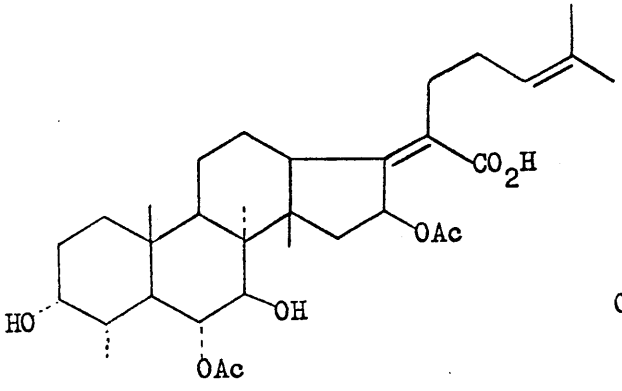
type of 'mixed pathway', some of which will be discussed in the examples of fungal terpenes chosen.

Studies of fungal metabolites have encountered almost all types of terpenes. Puccinia graminis yields 2-methylbut-2-ene (5)[7] as an example of a hemiterpene, while structures incorporating the monomeric isopentenyl unit are exibited by auroglaucin (6)[8] and flavoglaucin (7)[9] from Aspergillus glaucus as well as by fuscin (8)[10], elymoclavine (9), agroclavine (10)[11] and echinulin (11)[12]. No C_{10} compounds based on the isopentenyl dimer have yet been isolated from a mould, but this unit can be seen in the ether substituent of mycelianamide (12)[13] or in the degraded side chain of mycophenolic acid (13)[14].

Fungal sesquiterpenes[15] show a variety of structural forms. In the azulenes from Lactarius delicosus, lactaroviolin (14)[16] and lactarazulene (15)[17] the three C_5 elements are easily visible, whereas in helminthosporal (16)[18], sativene (17)[19], the illudins (18)[20], marasmic acid (19)[21], culmorin (20)[22] and hirsutic acid (21)[23] a more complex pattern is observed. Examples of structures partially derived from three mevalonate molecules are trichothecin (22)[24] and the related verrucarin A (23)[25], and fumagillin (24)[26]. Grifolin (25)[27] represents the case of a compound of complex biosynthetic origin containing the simple



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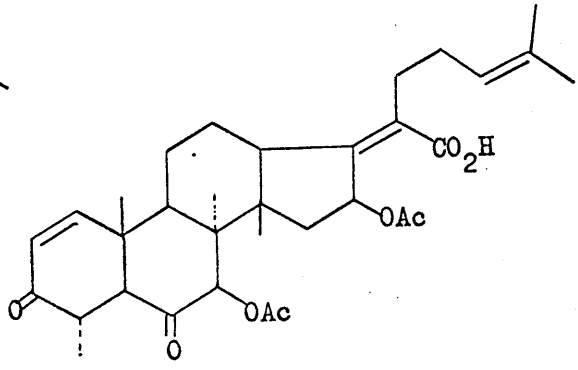

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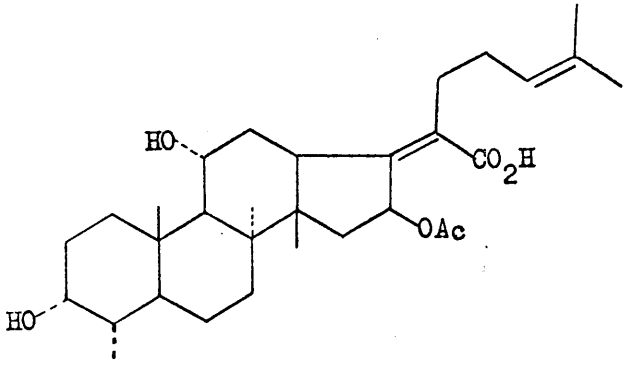

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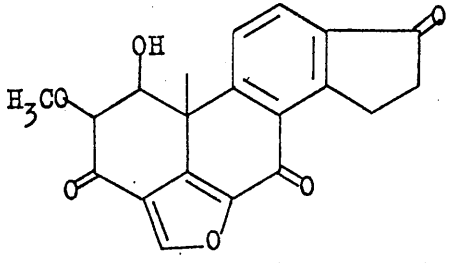

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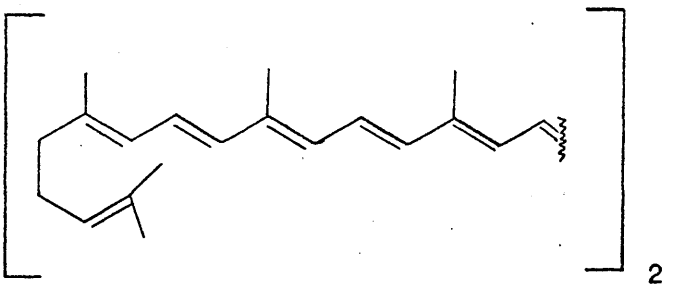

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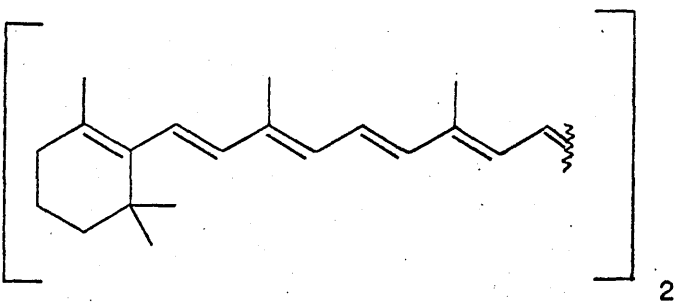

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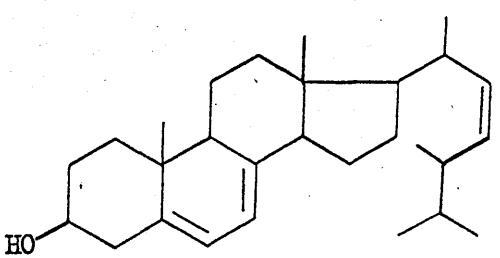

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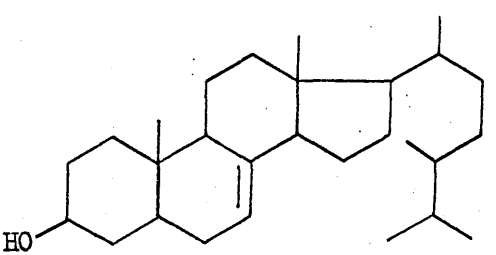

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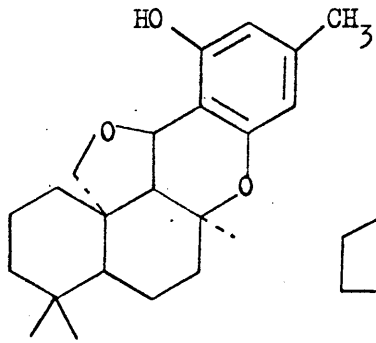

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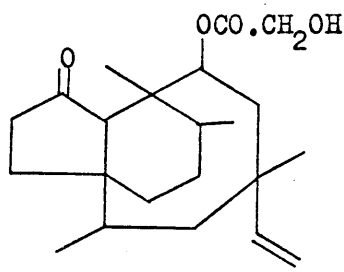

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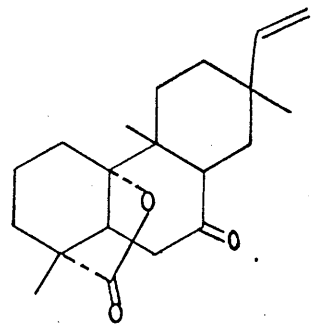

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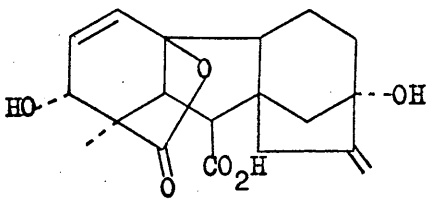

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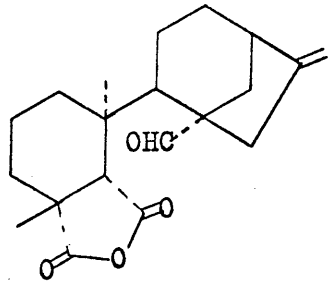

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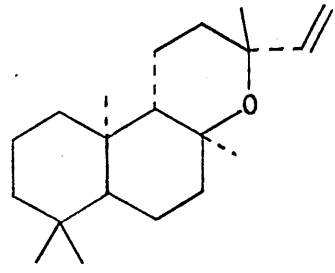

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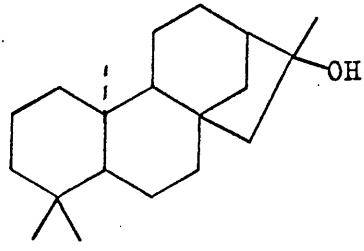

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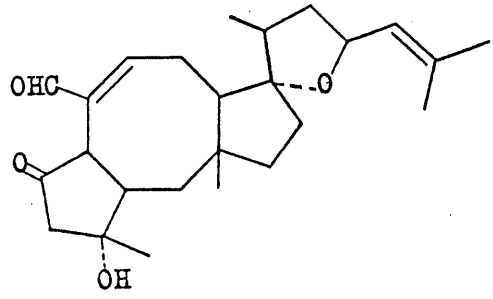

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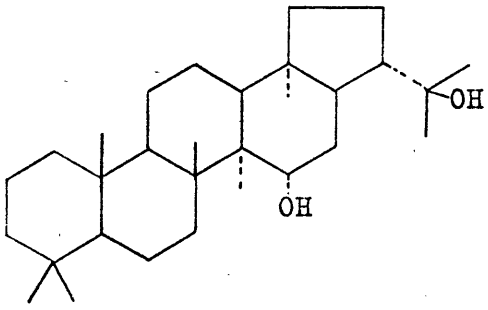
isopentenyl trimer and siccanin (26)[28] is a similarly derived molecule in which cyclisation has occurred. Pleuromutilin (27)[29], rosenonolactone (28)[30] and the metabolites of Gibberella fujikuroi[31], the gibberellins (e.g. gibberellic acid (29)), fungenal (30), olearyl oxide (31) and the kaurene derivatives (e.g. kauranal (32)) illustrate the diversity of structural types available from the isopentenyl trimer.

Recently Nozoe et al. and Canonica et al. have isolated and identified ophliobolin (33)[32] and its analogues. These C_{25} compounds are the first known representatives of the sester-terpenes, the class of terpenes derivable from the isopentenyl pentamer. Examples of fungal tetraterpenes are the carotenoids lycopene (34) and β-carotene (35)[33].

The compounds with which the present study is involved are all (presumably) available from the isopentenyl hexamer. Previously isolated compounds of this type can be arbitrarily classified by the number of carbon atoms in their basic skeleton. The first group consists of metabolites containing less than the prescribed thirty atoms and is exemplified by ergosterol (36) and its derivatives[34] e.g. fungisterol (37), the highly oxygenated antibiotics cephalosporin P_1 (38)[35], helvolic acid (39)[36], fusidic acid (40)[37] and the C_{19} compound viridin (41)[38]. It is very



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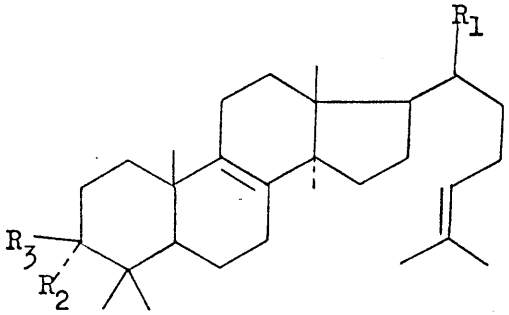


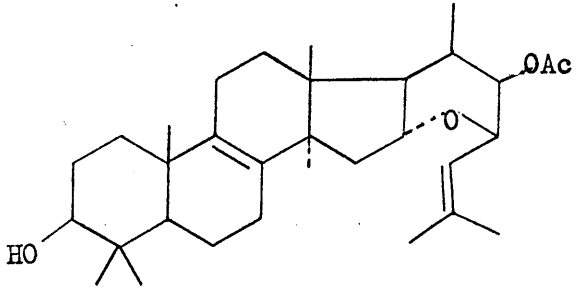
43 $R_1 = CO_2H$, R_2 , $R_3 = O$

44 $R_1 = CH_2OH$, R_2 , $R_3 = O$

45 $R_1 = CH_2OH$, $R_2 = H$, $R_3 = OH$

46 $R_1 = CO_2H$, $R_2 = OH$, $R_3 = H$

47 $R_1 = CO_2H$, $R_2 = H$, $R_3 = OH$



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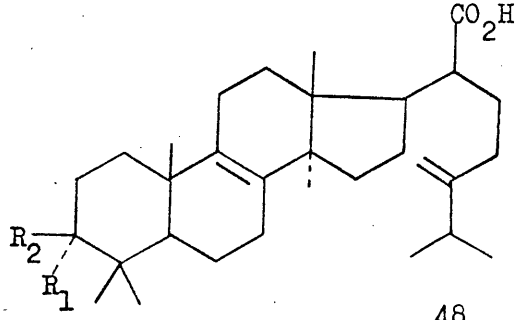
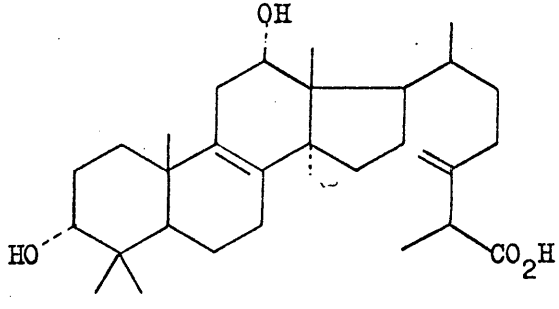


49 $R_1 = H$, $R_2 = OH$ [48]

50 R_1 , $R_2 = O$, with 16α-OH [49]

51 $R_1 = H$, $R_2 = OH$, with 16α-OH [50]

52 $R_1 = H$, $R_2 = OH$, with 15α-OH [51]



53 [52]

probable that these substances arise from squalene by cyclisation and oxidative-decarboxylative loss of carbon atoms in a manner similar to that suggested for cholesterol[39].

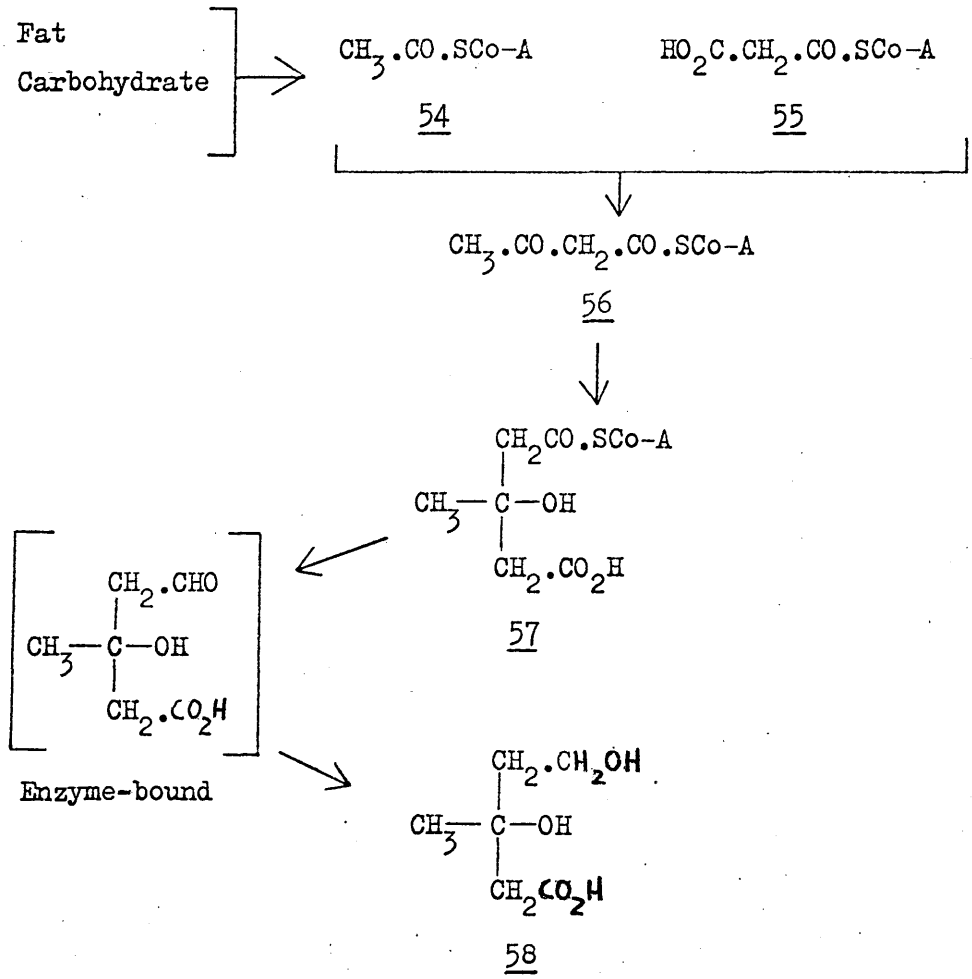
Squalene (4) is produced by Amanita phalloides[40] and seven other C_{30} compounds have been isolated from fungal sources. Dustanin (42)[41] is unique in that, so far, it is the only pentacyclic triterpene to have been isolated from a fungus, although such compounds are common in higher plants[42]. The six other compounds are all based on a lanostane skeleton. These are pinicolic acid (43) and its analogues from Polyporus pinicola (44 - 46)[43], trametenolic acid (47)[44] and echinodol (48)[45]. The latter has been included in this group since it is obviously an acetylated C_{30} skeleton. Similarly 46 occurs in Polyporus pinicola as the acetate of its methyl ester[46], while 47 has been isolated from Trametes odorata[44], Fomes hartigii[46] and Inonotus obliquus[47] as its methyl ester.

The members of the third group of fungal tetracyclic triterpenes all have an extra C_1 unit (cf. introduction, part II). The known examples 49 to 53[48 - 52] are all based on a lanostane skeleton, usually with a carboxyl group attached to C-20. The exception being polyporenic acid A which bears the acid function on C-25. It is worth mentioning that compounds containing the 8,9-

double bond very often co-occur with the corresponding 7,9 (11)-diene and that complete separation of these compounds is extremely difficult. For this reason all the triterpenes 43 to 53 have been represented as having the 8,9-double bond even though some, e.g. 50, have been reported as having been isolated as the pure 7,9 (11)-diene analogue.

C_{31} compounds may also exist in esterified or 'conjugated' form. Examination of Polyporus anthracophilus, allowed to grow on decaying Eucalyptus regnans, showed the presence of the acetate of eburicoic acid (49) but the same fungus, when cultured on a synthetic medium produced the free hydroxy acid 49[53]. A similar situation has been observed by Shibata concerning Poria coccus. Cultured mycelium had been shown to yield free tumulosic acid (51)[50] but harvested schlerotia contained the corresponding 3-acetate, the so-called pachymic acid[54]. In Polyporus betulinus tumulosic acid occurs as its 3,16-diacetate[55] and recent studies on this fungus[56] have indicated that polyporenic acid A (53) is conjugated with at least three different simple acids.

TABLE 1

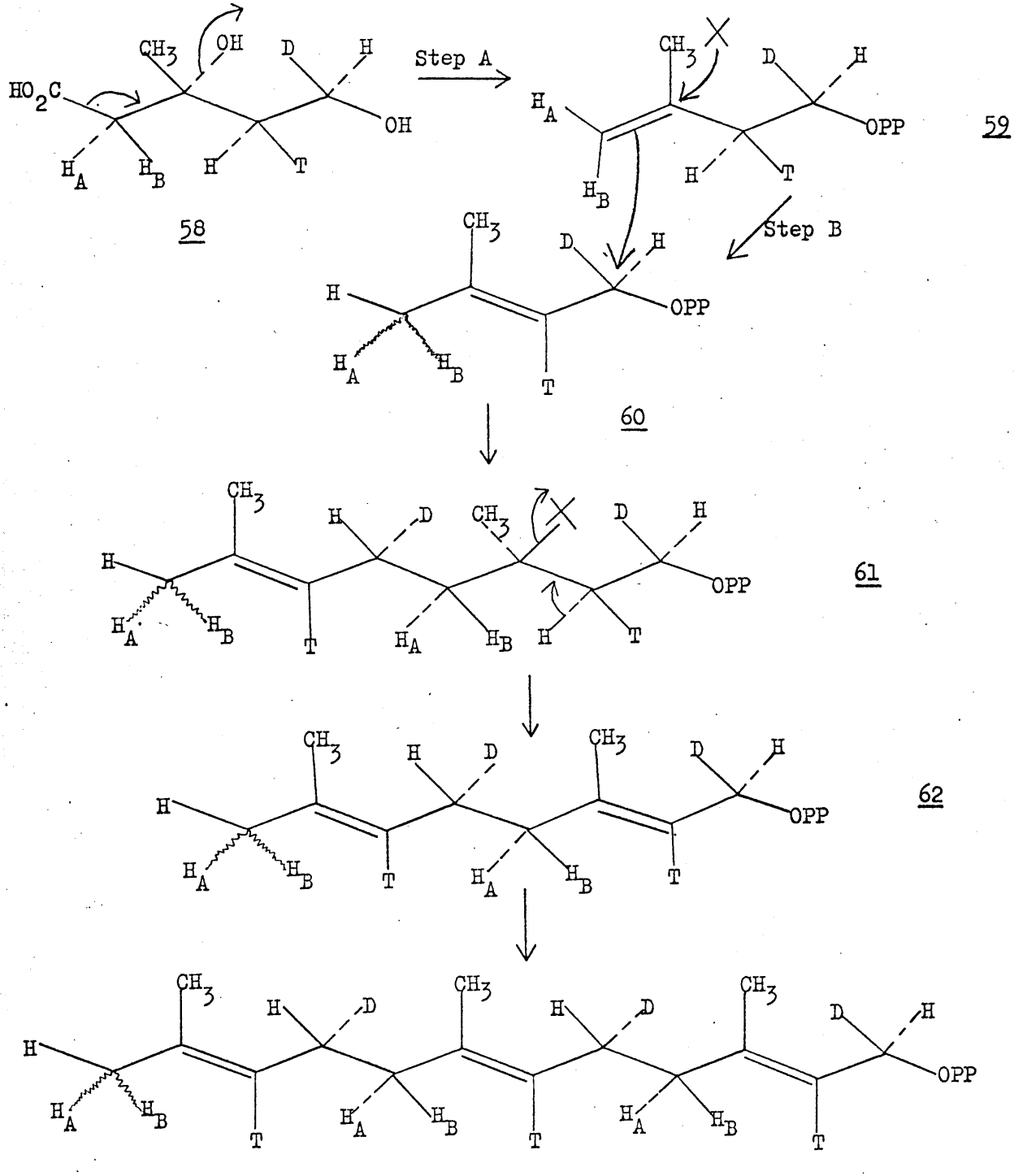

Fat
Carbohydrate
CH3.CO.SCo-A
54
HO2C.CH2.CO.SCo-A
55
CH3.CO.CH2.CO.SCo-A
56
CH2CO.SCo-A
CH3—C—OH
CH2.CO2H
57
CH2.CHO
CH3—C—OH
CH2.CO2H
Enzyme-bound
CH2.CH2OH
CH3—C—OH
CH2CO2H
58

PART II - THE BIOGENESIS OF TETRACYCLIC TRITERPENES.

The biogenesis of tetracyclic triterpenes has been reviewed by Nicholas[57], and more recently by Clayton[58], and only a brief summary of the steps leading to the acyclic intermediate squalene will be given. The cyclisation process and the further modification of the lanosterol molecule will be considered in more detail.

The catabolic breakdown of carbohydrate or fat substrate leads to acetyl coenzyme-A ester (acetyl Co-A) (54). The sequence of reactions up to the accepted precursor of terpenes, mevalonic acid, is shown in Table 1. The acetyl Co-A is carboxylated to give malonyl Co-A (55) which is then decarboxylatively coupled to a second molecule of acetyl Co-A to yield acetoacetyl Co-A (56). Claisen condensation of this species with a third molecule of acetyl Co-A leads to β-hydroxy-β-methylglutaryl Co-A (57). The evidence suggests that, up to this point, the reactions are reversible but that the reduction of 57 to mevalonic acid (58) is effectively irreversible and that this is one of the points at which terpene synthesis may be controlled. Brodie[59] has proposed that the first three steps also involve enzyme-bound species rather than the free Co-A esters and that the final reduction to mevalonic acid releases it from the enzyme surface. This suggestion has not yet been conclusively proved and further work is required

TABLE 2



before any preference for either of the alternatives can be established.

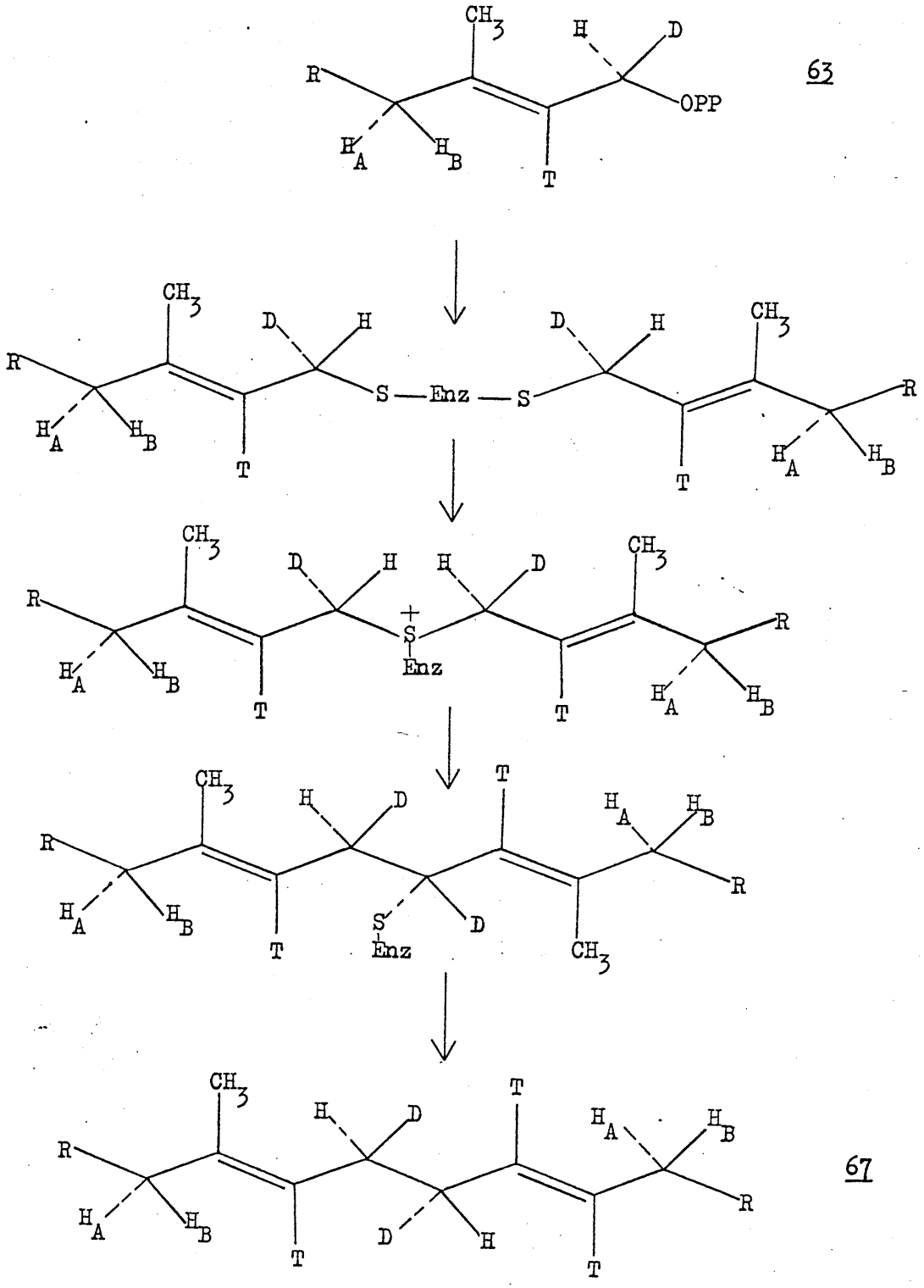
The steps from mevalonic acid to squalene have been elucidated by the Cornforth-Popjak group[60 - 64] using elegant enzyme work and brilliant labelling studies. A precis of their results is shown in Table 2 by using a hypothetical triply labelled mevalonic acid molecule. The important features of the route are as follows:

Step A has been shown to be a concerted trans dehydration-decarboxylation reaction, although whether or not the hydroxyl is phosphorylated before elimination is not yet clear.

In Step B isopentenyl pyrophosphate (59) isomerises to dimethyl-allyl pyrophosphate (60) with removal of the hydrogen at C-2 from the α-face of the molecule and addition of a hydrogen atom of unknown stereochemistry at C-4.

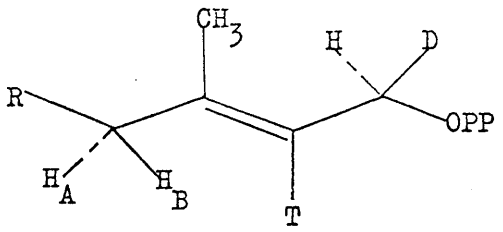
The stereochemistry of the condensation of 59 and 60 is as shown. The involvement of group X (of unknown constitution) and the suggested intermediate 61 has been deduced from the fact that again the α-hydrogen atom is eliminated from C-2. Thus concerted elimination of HX from 59 is a trans process, whereas a completely concerted mechanism would require loss of the β-hydrogen atom at C-2. The stereochemistry at C-1 in 60 is

TABLE 4



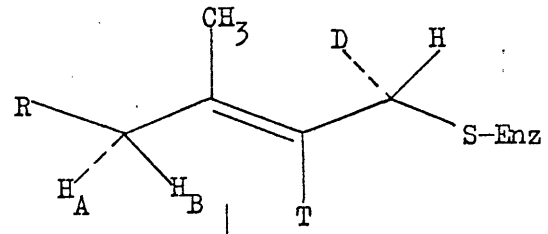
63

67

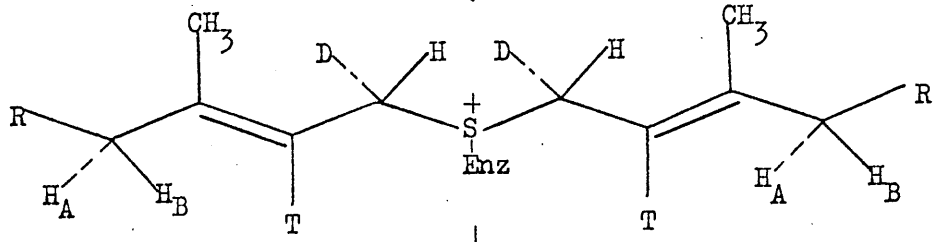


63

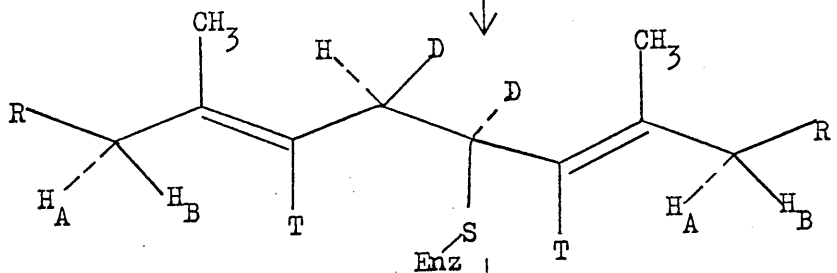
TABLE 3



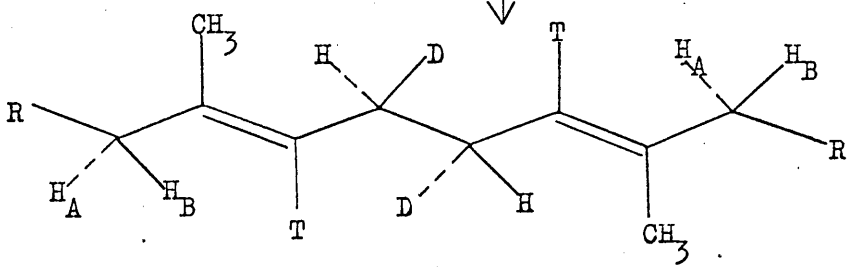
64



65



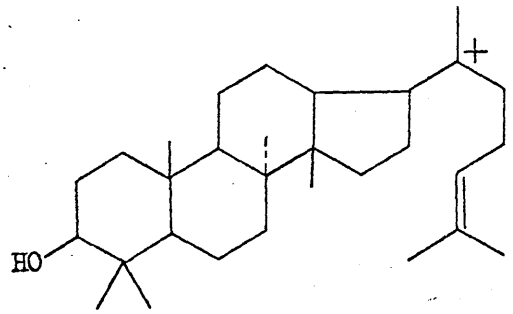
66



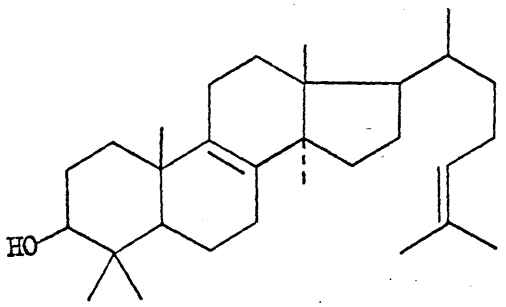
67

inverted in the product 62, as would be expected from an SN_2 type displacement of pyrophosphate. By a similar condensation of 59 on geranyl pyrophosphate (62) farnesyl pyrophosphate (63) is generated.

No fully authenticated mechanism for the 'head to head' linkage of two farnesyl units to give squalene has yet been put forward. The scheme shown in Table 3, based on a Stevens rearrangement, is due to Popjak and Cornforth[65] and is in accord with the available data which requires an inversion of configuration at C-1 of only one of the farnesyl units[66] and a reductive step involving incorporation of one hydrogen from NADPH[67]. It involves displacement of pyrophosphate from farnesyl pyrophosphate by the sulph-hydryl group of an enzyme. Reaction of another molecule of 63 with this complex (64) would yield a difarnesyl-enzyme complex (65). After Stevens rearrangement of this species, forming the required central double bond, reductive breakdown of the squalene-enzyme complex (66) occurs releasing squalene (67) with stereochemistry as shown, providing that the reduction occurs with retention of configuration. The requirement of this latter assumption prompted Martin-Smith[68] to suggest that the initial two steps occur as in Table 4. It will be noted that this mechanism allows for an inversion of configuration on addition of



68



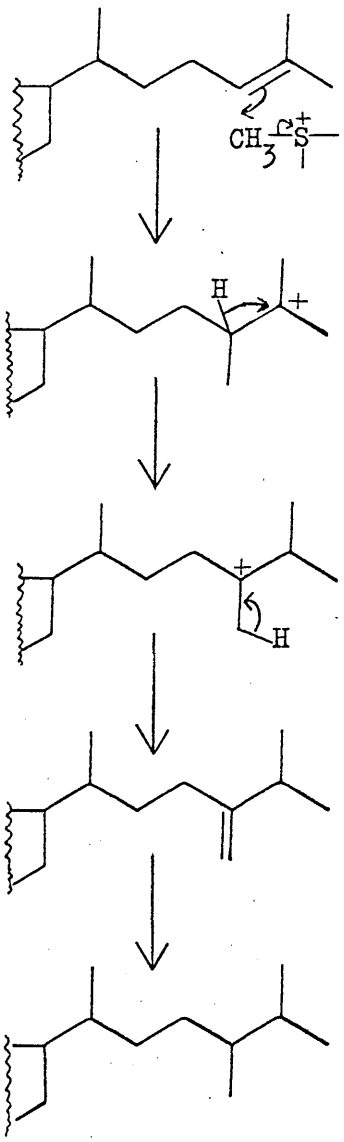
69

the hydrogen from NADPH since it involves an extra inversion at C-1 of one of the farnesyl units.

Production of lanosterol requires the cyclisation of squalene in the enzyme-bound chair-boat-chair-boat configuration[69]. Initiation of the cyclisation leads to the intermediate carbonium ion 68 which, by a series of concerted stereospecific hydrogen and methyl shifts, yields lanosterol (69). Elimination of a proton from 68 without Wagner-Meerwein shifts leads to the fusidane skeleton e.g. fusidic acid (40).

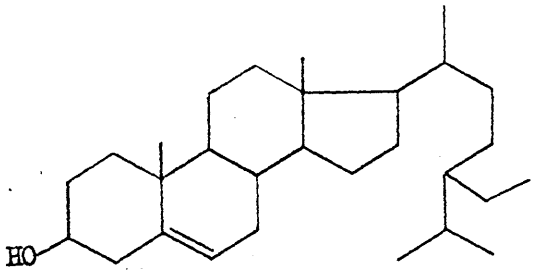
Recent work on the initiation step has cast doubt on the previously accepted theory of attack at C-3 by a reagent which transfers OH^+ with concurrent cyclisation at C-2. The groups of Corey[70] and van Tamelen[71] have shown that the 2,3-epoxide of squalene is most probably the intermediate. However, Barton and Moss[72] have shown that lanosta-8,24-diene can be converted into lanosterol by cell-free yeast extracts and they suggest that, at least in some cases, oxidation at C-3 may occur after cyclisation has occurred and that hydroxylation at C-3 releases the lanosterol from the enzyme surface. Breslow *et al.*[73] have suggested, on the basis of *in vitro* experiments, that a free radical initiator is involved. The dangers of this type of approach are illustrated by van Tamelen's failure[74] to repeat,

TABLE 5

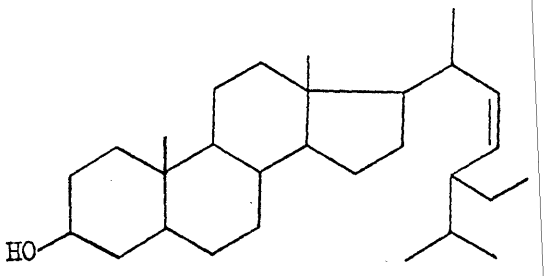


in vitro, the cyclisation of squalene 2,3-epoxide to lanosterol. It appears from the evidence that there may not be one general mechanism of conversion of squalene to lanosterol and that the initiating species may vary between organisms. The 1,2 shifts of hydrogens from C-17 to C-20 and from C-13 to C-17 and of methyl groups from C-14 to C-13 and from C-8 to C-14, as required in the rearrangement of 68 to 69, have been shown to occur[75,76].

Lanosterol has been considered the point at which branching of biosynthetic pathways to tetracyclic triterpenes and sterols occurs. Oxidation and decarboxylation remove the methyl groups at C-4 and C-14 and lead to the sterols[39]. It should be noted, however, that recent work by Ourisson[77] has shown that, at least in some cases, lanosterol is not an intermediate in phytosterol biosynthesis. C_{30} triterpenes are immediately available from lanosterol by the introduction of double bonds and oxygen fuctionality. C_{31} compounds, on the other hand, require the introduction of an 'extra' carbon at C-24. Tracer studies on ergosterol and eburicoic acid showed that this C_1 unit is derived from formate or methionine[78]. Further studies on these compounds have shed light on the probable mechanism. The scheme shown in Table 5 is based on the evidence that two of the three deuterium atoms from CD_3-methionine are incorporated into eburicoic acid and

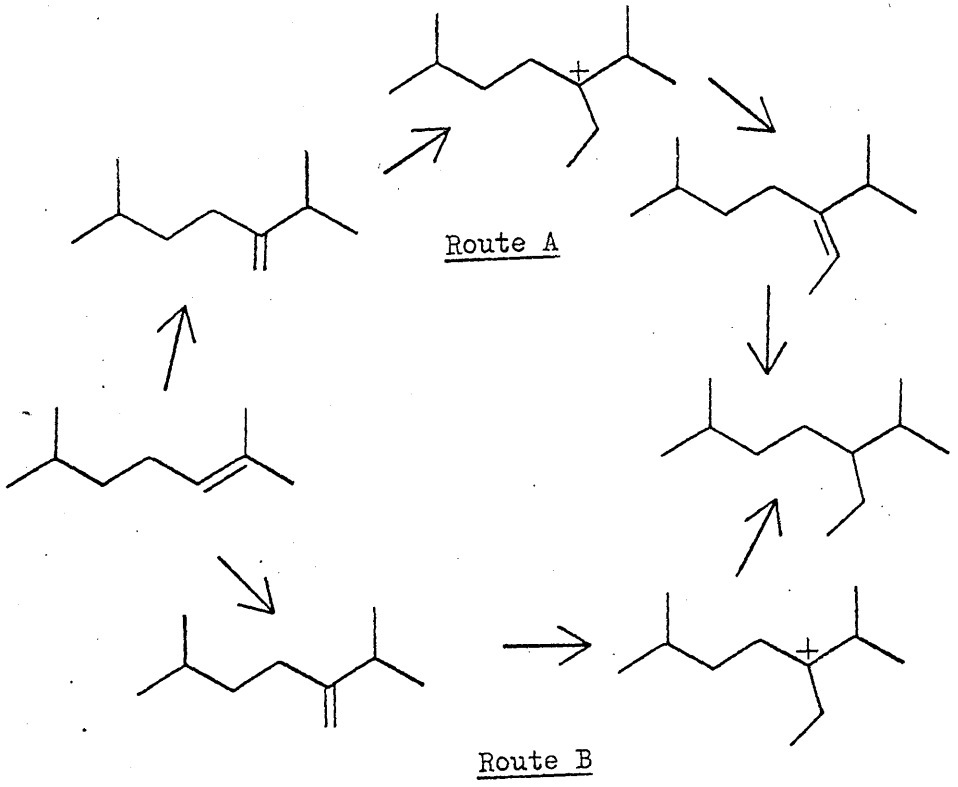


70



71

TABLE 6



ergosterol[79] and the observation that there is a hydride transfer from C-24 to C-25 in the conversion of lanosterol to ergosterol[80]. Barton[81] has postulated that the additional C_1 unit is introduced into lanosterol as a first step before further modifications of these compounds take place.

In view of the evidence to be presented that C_{32} triterpenes have been isolated from Daedalea quercina and Leptoporus stipticus, it is of interest to consider the biosynthetic studies on analogous phytosterols which have an extra two carbon unit at C-24 since it is very likely that a similar process will operate. The ethyl and ethylidene side chain at C-24 are synthesised by two successive C-methylation steps involving methionine[82]. Goodwin's group has studied[83,84] the incorporation of CT_3-methionine into β-sitosterol (70) in maize and larch leaves and conclude, from their result that only four tritium atoms are incorporated, that the pathway is via the ethylidene compound as shown in Table 6, route A. Recent work by Lederer et al.[85] on stigmast-22-en-3β-ol (71) from a slime mould showed that five deuterium atoms were incorporated using CD_3-methionine as substrate and led that group to conclude that the pathway is as shown in Table 6, route B. Further work on this subject is required to determine if either of these pathways is general or if the routes differ in higher and lower organisms.

PART I

THE METABOLITES OF DAEDALEA QUERCINA

The chemical study of fungal products has increased rapidly in recent years, mainly because of advancements in mycological techniques which now enable most organisms to be cultured on synthetic media in the laboratory. Thus large amounts of material may readily be made available for structural elucidation or further consideration. The feeding of radio tracers to fungi has become a relatively simple procedure and biosynthetic pathways may be the more easily studied because of this, and also because of the short life cycle of many fungi. The current wide interest in metabolic processes has made a mycology laboratory an additional feature of many chemistry departments.

The increasingly more sophisticated array of physical tools available to the chemist make structural elucidation considerably easier than previously. Thus the chemical constituents of numerous fungi have been examined and the structures of many of these compounds deduced. Most of the substances studied in this way are described as secondary metabolites. These were defined by Bu'Lock[86], "as having a restricted taxonomic distribution, as being products which are not formed under all circumstances and as having no obvious metabolic function". It would be expected that the presence of any particular compound or group of compounds in an organism would be characteristic of that organism.

It should therefore be possible to ascribe biological significance, even if only taxonomic, to the metabolites produced by a fungus. For maximum return in this direction it should be borne in mind that the same fungus may produce a different pattern of products when grown under varying conditions and that precautions should be taken to ensure that the compounds isolated are as near as possible to the form in which they exist in nature.

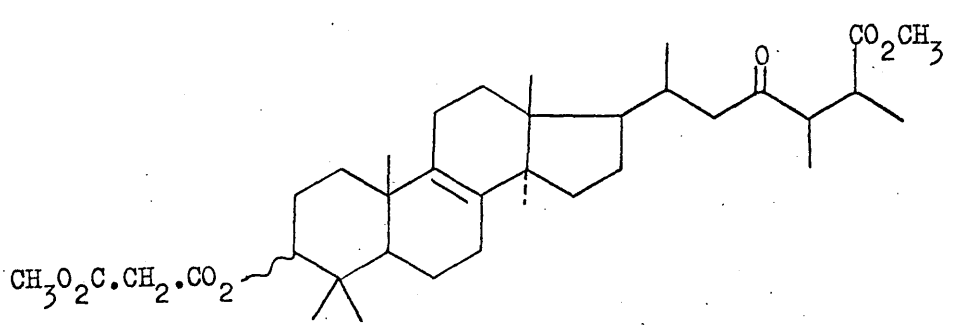
Chemical examinations of the family Polyporaceae in many cases revealed the presence of tetracyclic triterpenes. A recent study of Polyporus betulinus and Daedalea quercina in this department[56], using a mild non-hydrolytic isolation procedure, obtained triterpenoids, of which the majority were conjugated with simple acids. The structural studies of most of the metabolites from the latter fungus were incomplete when this work was commenced.

Daedalea quercina owes its trivial name 'Maze fungus' to the labyrinthiform pore layer on the underside of its hard, corky, hoof-shaped sporophores. It is frequently found on the dead wood of living trees, especially oak, and has been known to grow on fashioned timber.

Healthy young sporophores were collected from an oak stump in Garscube Estate and, after superficial cleaning, were

TABLE I - 1 T.L.C. CHARACTERISTICS OF THE METHYL ESTERS OF D. QUERCINA

	R_f values			
	100%$CHCl_3$	1%MeOH in $CHCl_3$	Ceric ammonium nitrate/H_2SO_4	Rhodamine 6G
D.Q.1	0.58	--	brown	red
D.Q.4	0.31	0.72	brown	purple
D.Q.5	0.00	0.50	brown	purple
D.Q.7	0.43	--	brown	red
D.Q.9	0.00	0.40	yellow	red
D.Q.10	0.00	0.19	yellow	red
D.Q.11	0.00	0.10	brown	purple

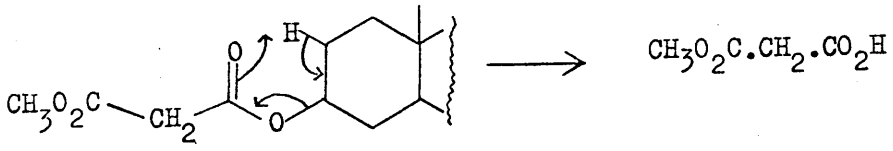


1.1

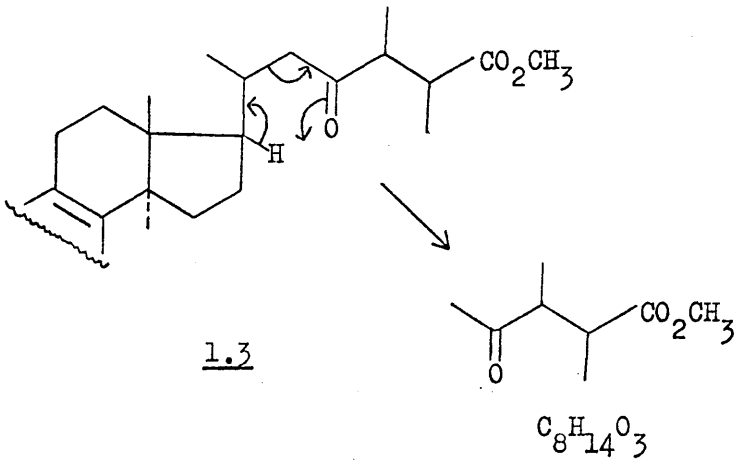
homogenised in methanol. Sterols and lipids (15% by weight) were removed by light petroleum treatment to leave a mixture of acids. For ease of handling and separation this material was methylated with diazomethane. Examination of the crude mixture by thin layer chromatography (t.l.c.) revealed the presence of seven major compounds, to which the code names D.Q.1, D.Q.4, D.Q.5, D.Q.7, D.Q.9, D.Q.10 and D.Q.11 have been assigned to facilitate discussion. These code names refer to the compounds whose t.l.c. behaviour is defined in Table I - 1.

Column chromatography on silicic acid using a light petroleum to ethyl acetate gradient afforded fractions from which could be separated, by crystallisation or preparative layer chromatography (p.l.c.), pure samples of D.Q.1, D.Q.4, D.Q.7, D.Q.9, D.Q.10 and D.Q.11. Isolation of D.Q.5 was more difficult but, since its identity with methyl dehydrotumulosate had been established, no further work was done on it. Similarly D.Q.4 was shown to be methyl polyporenate C. A point of interest with respect to these two compounds was that each was contaminated with about 10% of the corresponding 8,9-monoene.

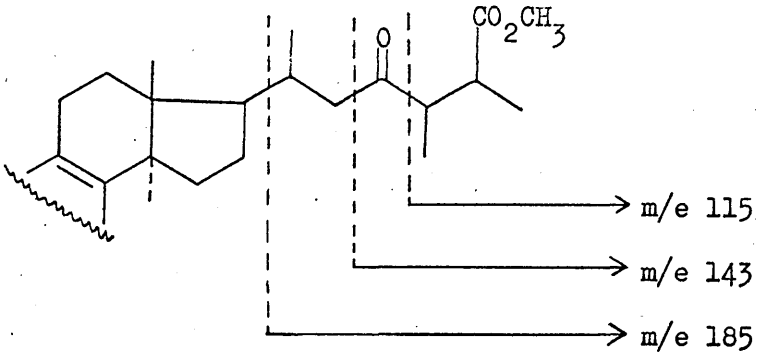
Campbell had tentatively suggested structure 1.1 for the major compound D.Q.1, $C_{36}H_{56}O_7$. The evidence submitted in support of this structure is summarised in Tables I - 2 and I - 3.


CH_3O_2C
CH_2
O
H
O
$CH_3O_2C.CH_2.CO_2H$

1.2

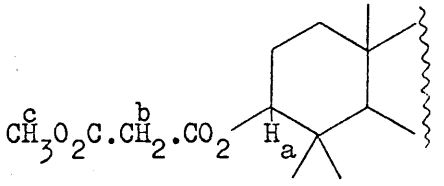

CO_2CH_3
O
H
CO_2CH_3
O
$C_8H_{14}O_3$

1.3

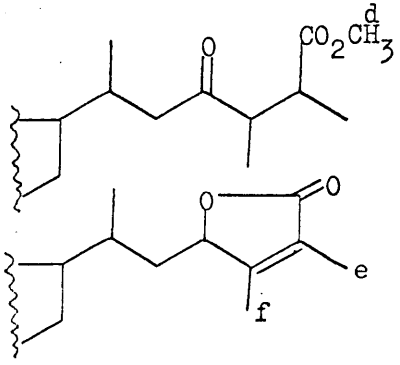

CO_2CH_3
O
m/e 115
m/e 143
m/e 185

1.4

TABLE I - 2 I.R. AND N.M.R. EVIDENCE FOR THE STRUCTURE OF D.Q.1

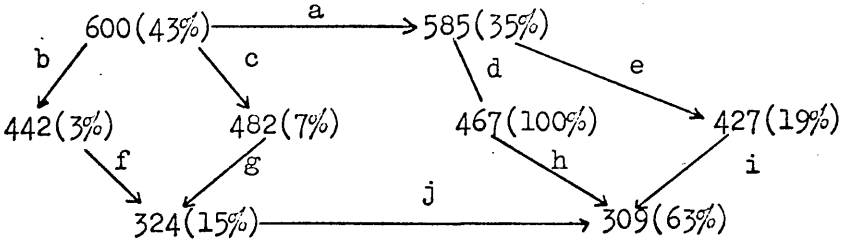


ν (cm^{-1})	τ values
1758	H_a 5.30
1736	H_b 6.30
	H_c 6.70



ν (cm^{-1})	τ values
1736	H_d 6.37
1716	
1740	H_e 8.03
1685	H_f 8.19

TABLE I - 3 MASS SPECTRAL BREAKDOWN OF D.Q.1



185(26%) —k→ 153(26%)

Also at m/e

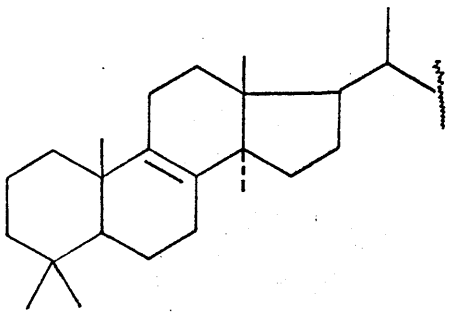
143(65%)

115(22%)

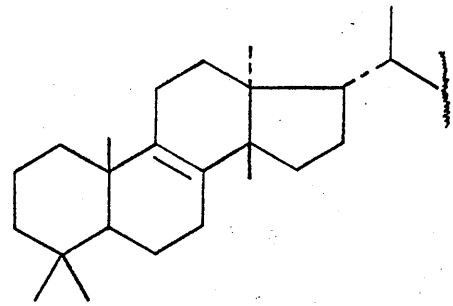
Transition	Δ^m/e
a,j	15
b,e,g,h	158 ≡ $C_8H_{14}O_3$
c,d,f,i	118 ≡ $C_4H_6O_4$
k	32

The major loss in the mass spectrum of a neutral fragment of 118, mass measuring for $C_4H_6O_4$, indicated the presence of the carbomethoxyacetate substituent at C-3 which could be lost by a McLafferty type fission[87] as in 1.2. The signals at 5.30τ, 6.30τ and 6.70τ in the nuclear magnetic resonance (n.m.r.) spectrum were in agreement with this partial structure, as was the doublet absorption at 1758 cm^{-1} and 1736 cm^{-1} in the infra red (i.r.) spectrum. Final confirmation was obtained by transesterification with sodium methoxide and identification of dimethyl malonate by gas-liquid chromatography (g.l.c.). The relative position of the ketone (1716 cm^{-1}) and the methyl ester (1736 cm^{-1}) was determined from one of the non-volatile products of the transesterification reaction. This compound was shown to contain an α,β-dimethyl α,β-unsaturated γ-lactone grouping.

If it was assumed that D.Q.1 is a C_{31} tetracyclic triterpene of the previously encountered type then structure 1.1 became the most probable. The mass spectrum provided evidence confirming this proposal. The loss of a neutral fragment, $C_8H_{14}O_3$, could be accounted for by a McLafferty fission of the C-20, C-22 bond involving removal of the C-17 proton as shown in 1.3. Similarly abundant ions at m/e 143 and m/e 115 could be explained by cleavage α to the ketonic carbonyl at C-23 as shown in 1.4.



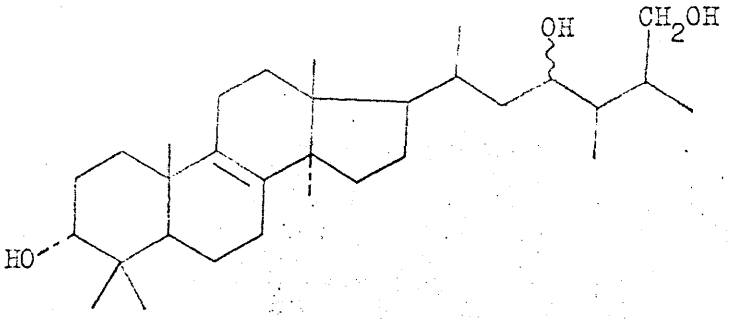
1.5



1.6

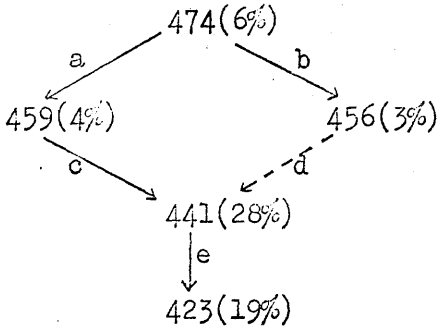
In the present work the first aspect in structural investigation of D.Q.1 to be studied concerned the nature of the nucleus since no evidence had been obtained other than spectral similarities with compounds known to be based on the lanostane skeleton and a positive reaction with tetranitromethane.
The two most commonly encountered tetracyclic nuclei are the lanostene (1.5) and the euphene (1.6) types. These may be readily distinguished by conversion of each into the corresponding 7,9(11)-dienoid system. Ultra violet (u.v.) spectral data indicate that the electrons in the lanostadiene system are the more easily excited, evidently because of the difference in stereochemistry between rings C and D. Lanosta-7,9(11)-diene has absorption at 237, 243 and 252 mμ, whereas eupha-7,9(11)-diene has absorption at 232, 240 and 247 mμ[88].

Treatment of D.Q.1 with selenium dioxide yielded a mixture of at least four products but encouragement was derived from the appearance of the chromophore expected for the lanosta-7,9(11)-diene skeleton. Accordingly it was decided to carry out the oxidation on a more stable derivative of D.Q.1. D.Q.1, when treated with a large excess of lithium aluminium hydride in ether at room temperature for 24 hours, yielded a mixture of D.Q.1-triols (1.7) epimeric at C-24. The n.m.r. spectrum of this



1.7 1.8

TABLE I - 4 HIGH m/e MASS SPECTRAL FRAGMENTATION OF THE D.Q.1-TRIOLS (1.7)



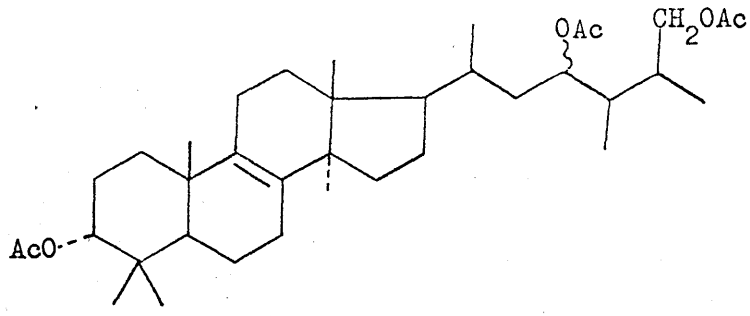
Transition	Δ^m/e	Metastable ion
a	15	444.1
b	18	438.5
c	18	422.8
d	15	
e	18	405.6

TABLE I - 5 GROUPS OF IONS AT LOWER m/e

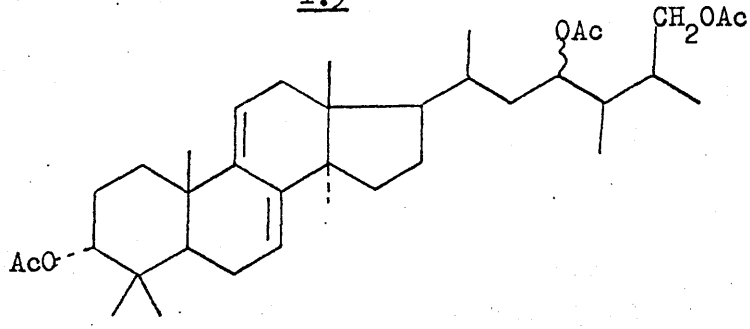
189	175	161	149	135	123	109
187	173	159	147	133	121	107
185	171	157	145	131	119	105

material showed the low field resonances expected for one primary alcohol (6.74τ, 2H, multiplet) and two secondary alcohols (5.76τ, 2H superimposed, multiplets). The removal of all carbonyl functions and the presence of the hydroxyl groupings were confirmed by the i.r. spectrum which showed no absorption between 1700 - 1800 cm^{-1} and a hydroxyl peak at 3617 cm^{-1}. On consideration of the mass spectrum of this material it was found that it agreed with the structure 1.7. The two most abundant ions at m/e 99 (100%) and m/e 139 (55%) could be explained in terms of a cyclic dehydration of the 1,4-diol and then cleavage α to the ether linkage to give the ion 1.8, or fission of the C-17, C-20 bond with transfer of one hydrogen. Normal dehydrations, characteristic of alcohols, and the loss of the methyl radical, which is a common occurrence in terpenes, accounted for the peaks at high m/e values as illustrated in Table I - 4. It was noted that groups of ions, which also occurred in the mass spectrum of D.Q.1, were visible at lower mass numbers. These values are collected in Table I - 5. These ions must be due to fragmentation of the tetracyclic nucleus, and no rational explanation of the pathways leading to them could be suggested.

Acetylation with pyridine and acetic anhydride led to the triacetates 1.9. Reaction of this material with selenium dioxide



1.9



1.10

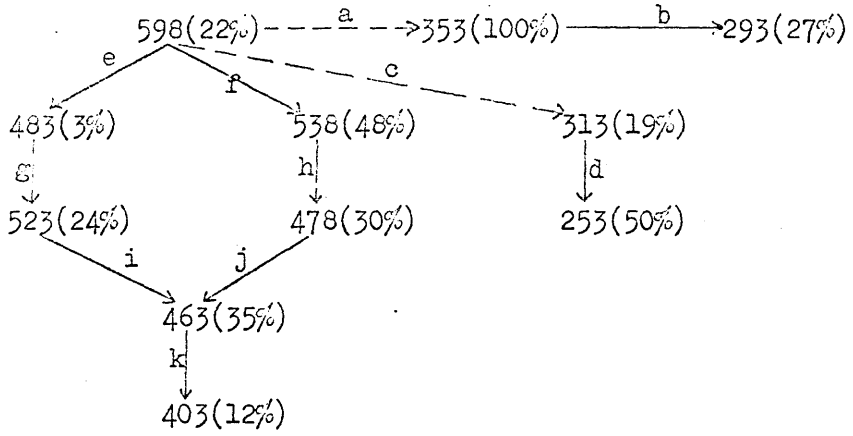
in acetic acid gave the required dienoid triacetates 1.10. The u.v. spectrum of this substance showed absorption at 236.5, 243.5 and 252 mμ, characteristic of the lanosta-7,9(11)-diene system. This series of reactions confirmed that D.Q.1 was based on a lanost-8-ene skeleton and therefore had gross structure 1.1.

Comparison of the spectroscopic data of the monoene triacetates and the diene triacetates established characteristic differences. Examination of the n.m.r. spectra showed the appearance in 1.10 of the two vinyl protons at C-7 and C-11 as a multiplet at 4.60τ, and slight differences in the methyl contour due to the shielding effect of the 7,9(11)-diene system. N.m.r. studies of the methyl groups of lanostane derivatives[89, 90] have shown that the transformation from 8,9-monoene to 7,9(11)-diene affects mainly the C-18 methyl group since it lies directly over the double bond system. Its resonance position is raised by ca. 0.11 p.p.m.. A similar, smaller effect causes the C-32 methyl to be shielded to the extent of 0.06 p.p.m.. The tau values for the nuclear methyl groups in the two compounds are shown below.

8,9-monoene	1.9	8.98,	9.07,	9.12,	9.18,	9.26
7,9(11)-diene	1.10	9.00,	9.07,	9.12,	9.24,	9.41
		C-19	C-30,C-31		C-32	C-18

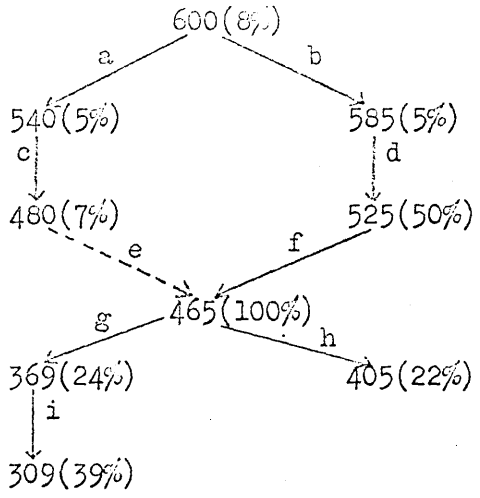
It will be seen from this table that the values in this case were

TABLE I - 7 PROPOSED FRAGMENTATION OF 1.10



Transition	Δ^m/e	Metastable ion
a	245	
b	60	243.4
c	285	
d	60	204.6
e	15	567.3
f	60	472.8
g	60	
h	60	425.3
i	60	411.9
j	15	458.6
k	60	352.9

TABLE I - 6 PROPOSED FRAGMENTATION OF 1.9



Transition	Δ^m/e	Metastable ion
a	60	486.8
b	15	572.3
c	60	427.1
d	60	471.3
e	15	
f	60	412.1
g	96	272.6
h	60	353.0
i	60	258.8

+0.06 and +0.15 and that these were in reasonable agreement with the expected differences. The other values were, as predicted, almost unaffected. It was therefore possible to assign the peaks at 9.18τ and 9.26τ in the n.m.r. spectrum of 1.9 to the C-32 and C-18 methyls respectively. Possible assignments for the other methyl groups, based on literature interpretations of similar systems, are included in the table.

The important peaks in the mass spectrum of 1.9 are shown in Table I - 6. The major ion at m/e 465 arose by loss of two molecules of acetic acid and a methyl radical. The ion at m/e 369 could be reasonably assumed to contain an intact ring A, since it lost a further molecule of acetic acid. It could then be suggested that this ion arose from the ion at m/e 465 by elimination of the neutral fragment C_7H_{12} by a McLafferty type rearrangement involving the side chain which had been desaturated by two losses of acetic acid.

The mass spectrum of the doubly unsaturated triacetate, shown in Table I - 7, was then considered. The fragment ions at high mass number probably arose by processes similar to those encountered in the breakdown of 1.9 as shown in Table I - 6. However the base peak of the spectrum at m/e 353 and the second most abundant ion at m/e 253 had no counterparts. It was

considered that these probably involved mainly loss of side chain. The former required removal of an additional two protons, the ion formed losing an acetic acid molecule. The latter required 'steroid-type' fission of ring D[91] to yield the ion of mass 313 and then further loss of 60 units. These fragmentations and the increased abundance of ions corresponding to those in Table I - 6 seemed to indicate that the 7,9(11)-diene system conferred increased stability to electron bombardment on the tetracyclic nucleus. It was also noted that the first three groups of peaks in Table I - 5 were all moved down by 2 mass units in the spectrum of 1.10.

There remained to be determined the stereochemistry of 1.1 at C-3, C-24 and C-25. Consideration of the n.m.r. spectrum decided between the two possibilities at C-3. The proton at C-3 is split by both protons of the C-2 methylene group. The size of the coupling to these protons depends on the dihedral angle[92]. Using measurements of the appropriate dihedral angles on models it can be predicted that a proton in the α, or axial, configuration at C-3 will be coupled with $J \simeq 10$ c/s to the axial proton at C-2 and with $J \simeq 3$ c/s to the equatorial proton at C-2. Although these coupling constants cannot be measured, the net effect is to broaden the signal due to the 3α-proton. For a 3β-proton, on the other hand, both couplings are in the region of

TABLE I - 8 τ VALUES OF PROTONS AT C-3

3β-alcohols	τ	$\nu_{\frac{1}{2}}$	3α-alcohols	τ	$\nu_{\frac{1}{2}}$
Methyl eburicoate	6.73	12c/s	Methyl polyporenate A	6.63	5c/s
Dihydrolanosterol	6.78	13c/s	Methyl epidehydro-tumulosate	6.60	5c/s
Dihydrolanosteryl carbomethoxyacetate	5.39	14c/s	Methyl 3-carbomethoxy-acetylpolyporenate A	5.34	6c/s
γ-Lanosteryl acetate	5.43	15c/s	D.Q.1	5.30	5c/s
			Triacetates 2.9	5.30	4c/s

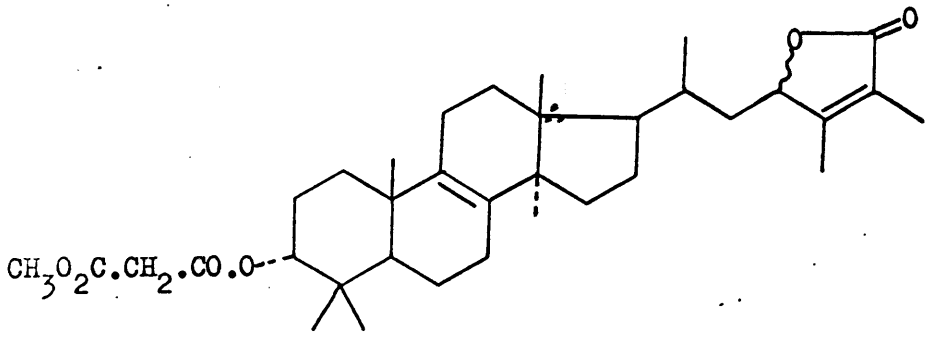
3 c/s. Thus the resonance due to a 3β-proton will be relatively sharp. The half-band width of the signal due to the proton at C-3 will then be characteristic of its configuration. In Table I - 8 are shown the τ values and half-band widths for some 3-hydroxy triterpenes and their esters. By consideration of these values it will be seen that D.Q.1 had a 3α-carbomethoxyacetyl substituent. Lithium aluminium hydride reduction of an ester leads to retention of configuration of the alcohol[93]. Thus the triacetates further confirmed the assignment of stereochemistry. It will be noted that the τ values for a C-3 proton also help to distinguish between the two configurations, the α-proton resonating at higher field.

Attempts to define the stereochemistry at C-24 and C-25 were unsuccessful. The signals in the n.m.r. spectrum due to the protons at these positions could be readily determined by decoupling experiments involving the deshielded methyl groups at the same positions. These occured as doublets at 8.76 and 8.82τ and 8.82 and 8.91τ. Irradiation of the broad multiplet at 7.3τ caused these doublets to collapse to sharp singlets at 8.79τ and 8.87τ respectively. However, reversing the procedure in an attempt to see the vicinal coupling between the two protons under consideration failed to simplify the complex of peaks at

7.3τ to any recognisable form.

It was at this stage in the work that it was noted that there was a distinct difference, in the 60 Mc/s spectrum, in the heights of the peaks due to the two methoxyl groups. On obtaining the 100 Mc/s spectrum it was seen that the methoxyl region was resolved into a singlet at 6.37τ, integrating for three protons, and two peaks at 6.42 and 6.44τ, in the ratio of 1 : 3, whose total integration was three protons. Two possibilities were considered to explain this phenomenon. Firstly that the two latter signals were due to unequal amounts of closely related compounds, inseparable after repeated purification proceedures, and probably stereoisomers. Alternatively they might have arisen from restricted rotation about one or other of the bonds in the side chain.

The latter explanation was favoured initially since material extracted at various different times showed the same relative intensities for the peaks in question. Temperature studies in tetrachloroethane appeared to suggest that restricted rotation was present since the peaks at 6.37τ and 6.44τ were of almost equal intensity when the spectrum was determined at 130°C. However, the situation was complicated by loss of resolution and by spinning side-bands which developed at high temperature. The situation was clarified by repeating the temperature studies



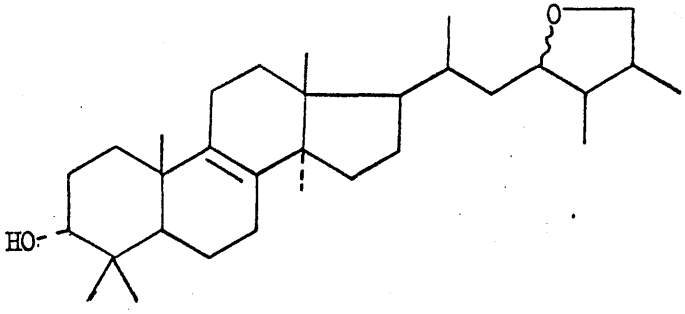
1.11

with a sweep width of 500 c/s. It could then be clearly seen that, although the peak at 6.44τ did increase slightly in intensity, the small peak at 6.42τ was present, even at 130°C. Thus the substance D.Q.1 was most probably a mixture of stereoisomers. No t.l.c. conditions could be found to separate the two compounds.

In view of the fact that most of the other compounds of Daedalea quercina, namely D.Q.7, D.Q.9 and D.Q.10, appeared to contain the γ-keto ester function in the side chain in a position analogous to that of D.Q.1, it was decided to re-investigate the cyclisation reaction with a view to improving the recovery of butenolide. The yield from methoxide treatment was very poor, and this reaction was therefore not applicable to the small amounts of the other substances available. D.Q.1, on treatment with base or mineral acid, gave numerous products because of the sensitivity of its functional groups to these reagents. But refluxing in dry benzene with a catalytic quantity of p-toluene-sulphonic acid led to an almost quantitative yield of cyclised material, shown below to have structure 1.11. Investigation of this material using a number of solvent systems showed that 50% light petroleum in ether separated it into two components. Separation by p.l.c. gave equal amounts of the two compounds. The structures of these isomers followed from their spectral

data, which were almost identical, showing the expected i.r. and n.m.r. peaks for the carbomethoxyacetate. The α,β-unsaturated lactone absorbed at 1687 and 1764 cm^{-1} in the i.r. and at 213 mμ in the u.v., while the α,β-dimethyl substitution on this function gave the signals at 8.08 and 8.22τ in the n.m.r. spectrum. In the mass spectrum the base peak at m/e 435 arose by loss of 118 and 15 mass units, while an abundant ion at m/e 111 corresponded to cleavage α to the lactone grouping at C-23.

Since these compounds were similar in most respects it was assumed that they were epimeric at C-23. The establishment of which compound corresponded to which isomer would depend on the n.m.r. spectrum and the size of the coupling constants. Unfortunately the signal due to the proton under the lactone at C-23 was superimposed on the resonance of the 3β-proton and therefore no assignment of stereochemistry could be made. That equal amounts of the two isomers was obtained was expected from the probable mechanism of the cyclisation. This involved attack at C-23 by the tosylate anion to generate an oxy-anion which would form the lactol tosylate by attack of the carbomethoxyl group. Elimination of the catalyst p-toluenesulphonic acid would then yield the β,γ-unsaturated lactone which should rearrange to the butenolide very readily by a hydrogen transfer. Since



1.12

there was no steric or electronic driving force to direct the addition of hydride at C-23, equal amounts of the two epimers were observed.

A more attractive method of interrelating the two carbonyl groups in the side chain was reduction of both of them to an alcohol function and then cyclisation to the tetrahydrofuran. This had the advantage of converting the side chain to a relatively inert state, thus allowing further reactions to be carried out on the nucleus. Studies[94] on this type of cyclic dehydration have shown that it occurred stereospecifically under the catalytic effect of mineral acid in methanol. Attempts to separate the D.Q.1-triols (1.7) by fractional crystallisation or by chromatography were unsuccessful. The mixture was treated with hydrochloric acid in methanol in the hope that the tetrahydrofuranoid compounds would be separable. This was achieved by p.l.c. using chloroform as eluent. The component of R_f 0.71 was a crystalline solid $C_{31}H_{52}O_2$ whose properties corresponded to those expected for the desired product 1.12. T.l.c. showed that it was completely free of the other product (R_f 0.56) but g.l.c. revealed the presence of two peaks of relative intensities 3 : 1. The mass spectra of these peaks were determined by combined g.l.c-mass spectrometry (g.c.-m.s.). It was found that

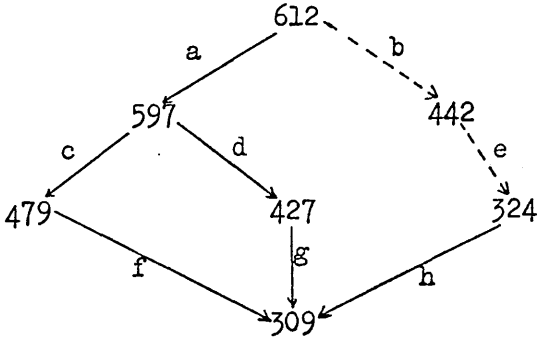
minor variations in peak intensities were the only differences between the two spectra. It could thus be stated with reasonable certainty that the two peaks on the gas chromatogram were due to two stereoisomers of structure 1.12. The other component, R_f 0.56, was an oil. T.l.c. showed only a single spot but g.l.c. indicated that two compounds were present, again in the ratio of 3 : 1. G.c.-m.s. on these two peaks gave two spectra with M^+ at 456 which were almost identical to each other and to the two spectra obtained from the material of R_f 0.71. Thus two other isomers of 1.12 were present. No detailed study of the physical data of the two materials was carried out, other than to confirm that the anticipated cyclic dehydration had occurred. A single hydroxyl was present (3620 cm^{-1}), while the presence of the sharp multiplet at 6.57τ confirmed that it must be at C-3. The tetrahydrofuran ring gave a complex of peaks between 6.0 and 6.7τ due to the C-23 proton (H-C(-O)-) and the C-26 protons ($-CH_2-O-$). No analysis of this system was attempted because of the mixtures involved. The mass spectra were similar to that of the D.Q.l-triols in that the base peak at m/e 99 and the second most abundant ion at m/e 139 could be explained in the same manner, but differed by having only one loss of 18 mass units at high molecular weight.

The isolation from this reaction of four products provided

convincing evidence that the D.Q.1-triols were a mixture of four diastereoisomers. On the assumption that epimerisation did not occur under the conditions of the lithium aluminium hydride reduction, it was concluded that D.Q.1, as the n.m.r. had suggested (cf. p. 24), was a mixture consisting of a main component together with 20 - 30% of a stereoisomer, probably the C-24 epimer.

The compound D.Q.7 was, as previously reported[56], a crystalline solid, m.p. 137-138°C. High resolution mass spectrometry gave the formula $C_{37}H_{56}O_7$ for the highest ion in the spectrum at m/e 612, but no satisfactory elemental analysis in agreement with this formula could be obtained. From the close similarities in spectral data between this compound and D.Q.1, it appeared that D.Q.7 was a 3α-carbomethoxyacetate of a tetracyclic triterpene hydroxy-keto-ester. These conclusions were based on the presence of the spectral characteristics of the carbomethoxyacetate grouping (signals in the n.m.r. spectrum at 5.30τ (1H, multiplet), 6.28τ (3H, singlet) and 6.60τ (2H, singlet) and peaks in the i.r. spectrum at 1758 cm^{-1} and 1740 cm^{-1}). This was confirmed by the loss of 118 mass units in the mass spectrum. Additional absorption at 1740 cm^{-1} and 1719 cm^{-1} was ascribed to the methyl ester and ketone respectively, the methoxyl group of the former giving rise to a 3H singlet at 6.34τ in the n.m.r.

TABLE I - 10 PROPOSED FRAGMENTATION OF D.Q.7



Transition	Δ^m/e	Metastable ion
a	15	582.3
b	170	
c	118	384.5
d	170	306.0
e	118	
f	170	199.5
g	118	223.9
h	15	294.8

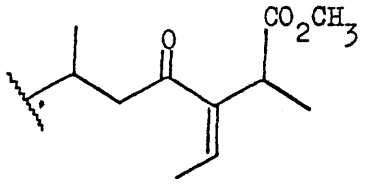
TABLE I -9 MASS SPECTRUM OF D.Q.7

m/e	%
612	24
597	42
553	8
495	7
479	34
442	7
427	34
383	9
324	16
309	100
197	80
165	30
155	31
127	50

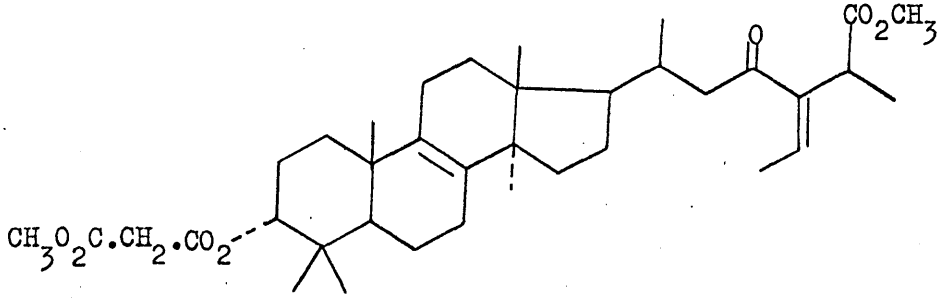
spectrum. On subtraction of $C_4H_5O_4$ (carbomethoxyacetate) from the molecular formula, it was seen that, if the assumption that D.Q.7 was a tetracyclic triterpene was correct, the residual unit was the methyl ester of a C_{32} triterpene acid. At the commencement of this work no firm structure for this compound had been advanced.

Re-examination of the mass spectrum of D.Q.7 (Table I - 9) led to the breakdown scheme, supported by the presence of appropriate metastable ions, shown in Table I - 10. It has been noted that, in the mass spectrum of D.Q.1, fragmentation of the γ-keto-ester function gave ions at m/e 185, 153, 143 and 115, while total loss of the side chain occurred as a neutral fragment of 158 mass units. It can be seen that the spectrum of D.Q.7 contains abundant ions at m/e 197, 165, 155 and 127, and that a major loss of 170 mass units occurs. The difference of twelve mass units in all these fission processes could be explained in terms of a C_2 unit attached to C-24, as might be predicted on biogenetic grounds. Mass measurement of the ions shown below confirmed that the side chain contained one more carbon than that of D.Q.1.

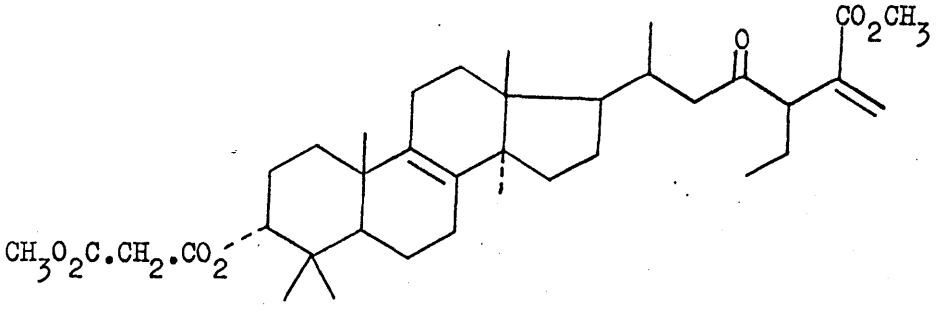
Approximate m/e	Formula
612	$C_{37}H_{56}O_7$
597	$C_{36}H_{53}O_7$



1.13



1.14

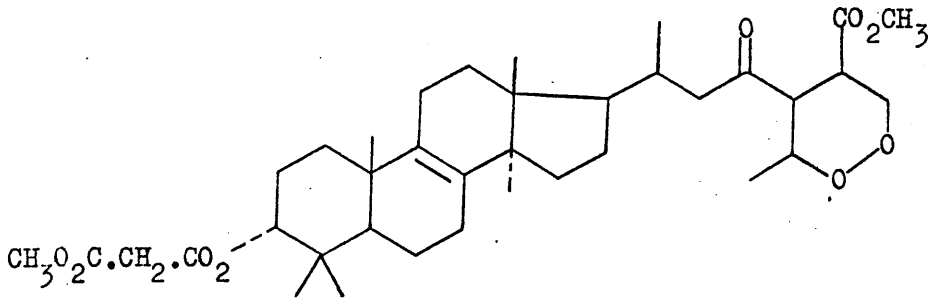


1.14a

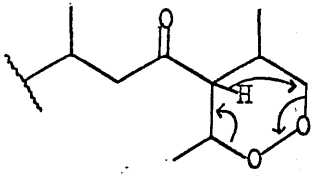
Approximate m/e	Formula
497	$C_{32}H_{47}O_3$
427	$C_{27}H_{39}O_4$
309	$C_{23}H_{33}$
197	$C_{11}H_{17}O_3$

Thus the ion at m/e 197, which represents the complete side chain, has formula $C_{11}H_{17}O_3$. Possible structures for the side chain would be as in 1.13, or an isomer thereof. The difference in mass between the ions at m/e 597 and m/e 427 and between ions at m/e 479 and m/e 309 corresponded to the fragment $C_9H_{14}O_3$ and could be explained by a McLafferty rearrangement of a similar side chain. The mass spectral evidence agreed with structure 1.14, or a double bond isomer of this e.g. 1.14a corresponding to the parent ion at m/e 612.

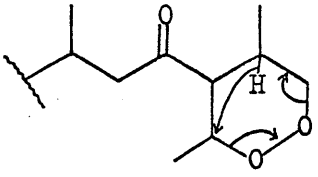
The similarity of D.Q.1 and D.Q.7 was obvious from their carbonyl absorptions in the i.r. which were superposable, but the n.m.r. spectrum of D.Q.7 differed from that of D.Q.1 in three ways. Firstly in containing an additional signal at 5.48τ (2H, multiplet). This suggested that D.Q.7 might contain a terminal methylene grouping but this was discounted since suitable i.r. or u.v. absorption was not observed and attempts at catalytic hydrogenation led only to the recovery of the starting material.



1.15



1.16



1.16a

An alternative explanation of this signal as being due to a $-CH_2-O-$ grouping was apparently contradicted by the fact that all seven oxygen atoms of the molecular formula had been accounted for. However, an oxygenated system might be present which was susceptible to an elimination reaction of some kind to yield 1.14 or its isomer. The most obvious possibility, a hydroxyl group which could undergo elimination, thermally or on electron impact, was however ruled out in view of the complete lack of hydroxyl absorption (free or bonded) in the i.r. spectrum.

A further difference in the n.m.r. spectra of D.Q.1 and D.Q.7 was that only one low field methyl doublet was present (8.72 and 8.84τ) in D.Q.7. This was to be expected if a C_2 unit was present at C-24. The hydrogen atom to which this methyl was coupled was found, rather surprisingly, under the methoxyl peaks at 6.31τ. Irradiation at this point caused the doublet to collapse to a sharp singlet at 8.78τ. The last difference between the spectra of D.Q.7 and D.Q.1 was that the multiplet at 7.3τ was considerably altered in the n.m.r. of D.Q.7. Attempts to decouple this region or to explain the variation were unsuccessful.

A tentative structure 1.15 based on the available physical evidence may be put forward. This compound could conceivably lose O_2 on electron impact by the processes 1.16 or 1.16a.

Such a structure could explain the low field resonances at 5.48 and 6.31τ. The results of several elemental analyses were in better agreement with the formula $C_{37}H_{56}O_9$ (1.15) than with the mass spectrometrically determined $C_{37}H_{56}O_7$.

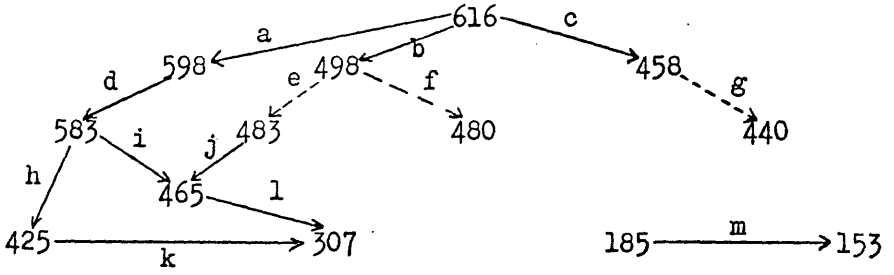
D.Q.7 did not give a positive peroxide test, even under the forcing conditions recommended for a dialkyl peroxide[95]. Treatment with p-toluenesulphonic acid in benzene, zinc dust in glacial acetic acid, lithium aluminium hydride in ether, or warming to 80° at 0.01 mm., all produced mixtures of at least five products.

Work is at present being initiated which, it is hoped, will establish, beyond all doubt, the structure of D.Q.7.

The component D.Q.9 could only be isolated as a gum after purification by plate chromatography. All attempts to induce this substance to solidify failed, as did attempts to prepare solid derivatives. The formula $C_{36}H_{56}O_8$ was derived from mass measurement of the parent peak at m/e 616.

Examination of the spectral data of D.Q.9 almost completely defined its structure. Signals at 5.36τ (3β-proton), 6.43τ (methoxyl) and 6.69τ(deshielded methylene) in the n.m.r. and peaks at 1737 cm^{-1} and 1757 cm^{-1} in the i.r. spectrum suggested the 3α-carbomethoxyacetate grouping. The i.r. spectrum also showed the presence of a ketone (1717 cm^{-1}), another methyl ester

TABLE I - 12 PROPOSED BREAKDOWN OF D.Q.9



Transition	Δ^m/e	Metastable ion
a	18	580.2
b	118	403.4
c	158	342.6
d	15	567.7
e	15	
f	18	
g	18	
h	158	309.9
i	118	370.6
j	18	447.3
k	158	221.8
l	118	202.6
m	32	126.7

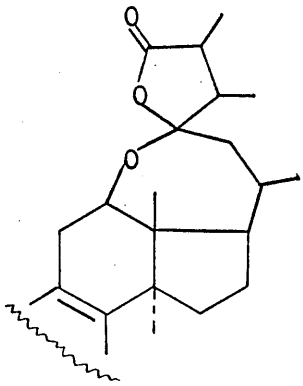
TABLE I - 11 MASS SPECTRUM OF D.Q.9

m/e	%
616	14
598	36
583	74
498	18
483	36
480	13
465	50
458	13
455	13
440	17
425	26
346	43
325	23
307	43
213	35
201	26
199	32
185	60
153	65
143	100
115	43

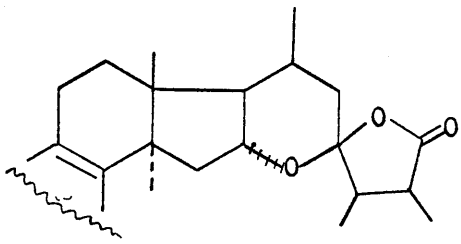
(1737 cm^{-1}) and a hydroxyl grouping (3619 cm^{-1}). That this alcohol was secondary was deduced from the fact that only one proton geminal to hydroxyl was present in the n.m.r. spectrum as a triplet at 5.96τ.

The close similarity between the rest of the spectrum and that of D.Q.1 strongly suggested that D.Q.9 was a hydroxylated analogue of the main component. On examination of the mass spectrum this correlation was amply borne out. The pertinent ions are shown in Table I - 11. It will be seen that the breakdown pattern of D.Q.9 (Table I - 12) is very similar to that of D.Q.1 (Table I - 6). Losses of 118 and 158 mass units (for the carbomethoxyacetate and side chain respectively), the methyl radical and the elements of water can explain most of the breakdown, while cleavage of the side chain gives rise to ions at m/e 185, 153, 143 and 115. It was also noteworthy that all the ions of Table I - 5 were again present. The ion at m/e 346 could be due to a fragmentation of the tetracyclic nucleus directed by the presence of the hydroxyl function, since no corresponding abundant ion was present in the spectrum of D.Q.1.

On consideration of the previously isolated fungal tetracyclic triterpenes the occurrence of lanostane derivatives substituted by 12α-, 15α- and 16α- hydroxyl groupings was noted.



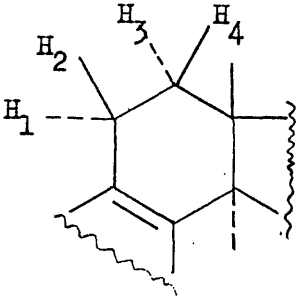
1.17



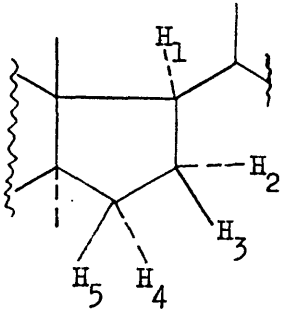
1.18

These positions were therefore favoured as sites for the alcohol grouping in D.Q.9. The correctness of this assumption and the elimination of C-15 as a possible site was demonstrated by reaction with p-toluenesulphonic acid in benzene at room temperature. The single product was obtained as a gum. In contrast with the product obtained from D.Q.1, it was shown as follows that this product was not a butenolide. The i.r. spectrum of this substance showed three peaks in the carbonyl region. Two of these, those at 1737 and 1758 cm^{-1}, demonstrated the retention of the carbomethoxyacetate grouping. This was confirmed by the characteristic signals in the n.m.r. spectrum. The third peak at 1783 cm^{-1} was assigned to a saturated γ-lactone which had arisen by interaction of the ketone and the methyl ester since the absorptions due to these functions had disappeared. The lack of a hydroxyl peak in the i.r. spectrum and the observation that the proton formerly geminal to this group and resonating at 5.96τ, was now centered at 5.66τ suggested an internal attack on the ketone carbonyl by the hydroxyl. The anion formed then attacked the carbomethoxyl grouping to form a spiro-lactone. From models it was seen that a hydroxyl on C-15 could not be involved in such a transformation, whereas a hydroxyl group at C-12 and C-16 could cyclise in this manner to give a seven membered ether (1.17) or a six membered

TABLE I - 13 CALCULATION OF J FOR PROTONS AT C-12 AND C-16



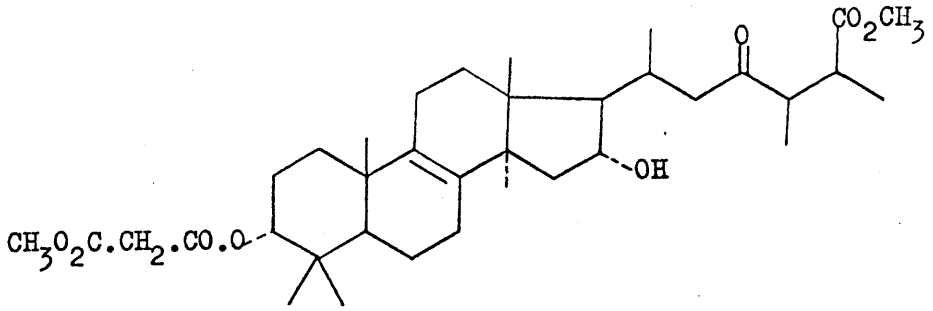
Protons	Dihedral angle	J
H_1H_3	10°	9c/s
H_1H_4	110°	2c/s
H_2H_3	120°	4c/s
H_2H_4	0°	10c/s



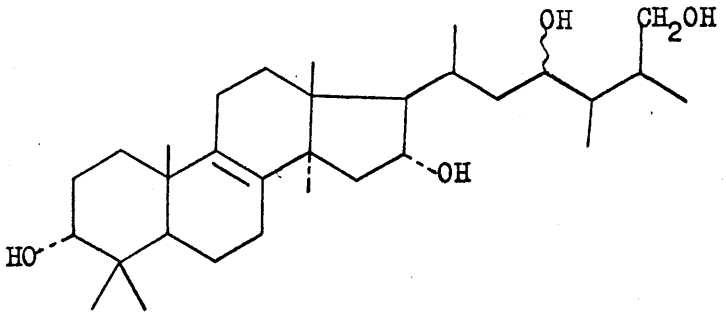
Protons	Dihedral angle	J
H_1H_2	125°	5c/s
H_1H_3	5°	10c/s
H_2H_5	110°	2c/s
H_2H_4	30°	8c/s
H_3H_5	20°	9c/s
H_3H_4	95°	1c/s

ether (1.18) respectively. The molecular weight, determined from M^+ at m/e 548, agreed with either structure, loss of a methyl radical to give the base peak and a further loss of 118 mass units being the major fragmentation pattern. Ions at m/e 153 (48%) and m/e 69 (35%) were thought to be due to side-chain fragmentation. The 16-position was favoured because of the ring size and because the 12-position is known to be sterically hindered[96]. A further argument for the former position came from a detailed study of the resonance of the proton under hydroxyl. This had the outline of a broadened triplet (apparent J=8 c/s). The spectra of methyl polyporenate C, methyl tumulosate and methyl dehydrotumulosate, all of which bear an α-hydroxyl on C-16, exhibited the same pattern in every case. On the other hand, methyl polyporenate A, which has a 12α-hydroxyl, showed a broad doublet, as did all its derivatives. This then distinguishes between the two positions.

A rationalisation of these facts may be obtained by an examination of atomic models and determination of the approximate values for the dihedral angles and the corresponding coupling constants according to Karplus[97], are as indicated in Table I - 13. It will then be seen that for a 12β-proton the two coupling constants involved are 10 c/s and 2 c/s, resulting in a broadened doublet, as was observed. For a 12α-proton a similar, but



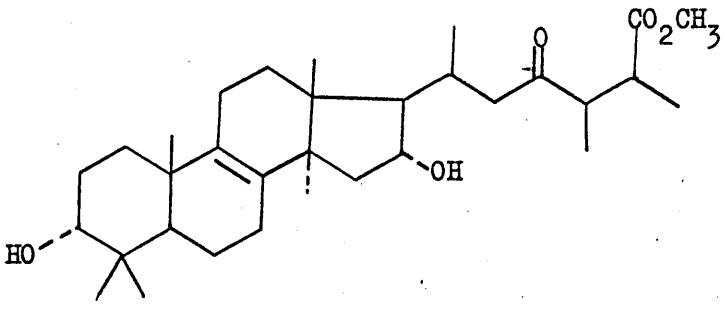
1.19



1.20

broader doublet would be expected. A 16β-proton, whose coupling constants with adjacent protons are 10,9 and 1 c/s, would be expected to give a broadened triplet. This treatment is not sufficiently accurate to distinguish between the 16α- and 16β-protons since these would be expected to give similar patterns, but it does, in conjunction with the other evidence, strongly suggest that D.Q.9 has structure 1.19.

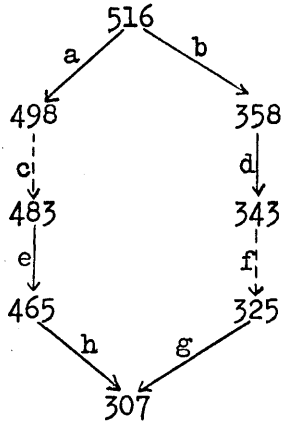
Attempts to derivatise D.Q.9 as the 3,5-dinitrobenzoate[98], the α-naphthylurethan[99], or as its p-iodobenzoate all resulted in low yields of gummy material. Solid material could be obtained by reduction to the tetraol 1.20 with an excess of lithium aluminium hydride. Again what was presumed to be an epimeric mixture was obtained. The i.r. spectrum of this material, $C_{31}H_{54}O_4$, showed no carbonyl functions but contained hydroxyl absorption at 3625 cm^{-1}. In the n.m.r. spectrum the most notable feature was a broad signal at 7.34τ, due to the four hydroxyl protons, which disappeared on D_2O addition, while the five protons under the alcohol functions all appeared at low field. The reduction of the carbonyl functions caused the methyl groups deshielded by them in the starting material to move to higher field. The mass spectrum was similar to that of 1.7 in having abundant ions at m/e 139 and m/e 99, while losses of water and the methyl



1.21

TABLE I - 14 MASS SPECTRAL DATA OF D.Q.10

m/e	%
516	24
498	29
483	100
465	25
451	14
433	12
358	23
343	9
341	20
325	42
307	23
246	32
185	37
153	46
143	42
115	17

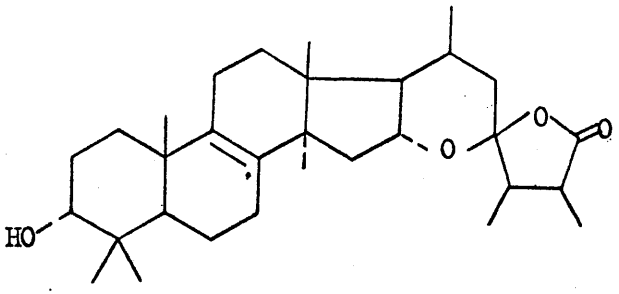


Transition	Δ^m/e	Metastable ion
a	18	480.6
b	158	248.6
c	15	
d	15	328.0
e	18	447.8
f	18	
g	18	290.1
h	158	202.6

radical from the parent ion at m/e 490 accounted for all other important ions.

The compound D.Q.10 could only be obtained as a clear gum, despite rigorous chromatographic purification. Mass measurement of the parent ion in the mass spectrum gave the formula $C_{32}H_{52}O_5$. Consideration of the spectral data of this compound led to the conclusion that it was closely related to D.Q.9, the obvious difference being that it lacked the carbomethoxyacetate grouping at C-3. The n.m.r. signals characteristic of this latter function were replaced by a multiplet at 6.59τ (1 proton). The half-band width of this signal suggested that it was due to the 3β-proton. Similarly the i.r. spectrum showed only an ester (1735 cm^{-1}) and a ketone (1715 cm^{-1}) in the carbonyl region, but two hydroxyl groupings at 3640 cm^{-1} (ε 79). The mass spectrum and the principal fragmentation patterns are shown in Table I -14. These account for all the major ions, other than that at m/e 246. It will be seen that this is analogous to the ion at m/e 346 in the mass spectrum of D.Q.9. This and the similarity in breakdown of the two compounds further emphasises their close relationship.

Reduction of D.Q.10 with lithium aluminium hydride yielded a product, m.p. 108-110°C, which was identical in all respects to 1.20, the tetraols obtained from D.Q.9. Structure 1.21 could



1.22

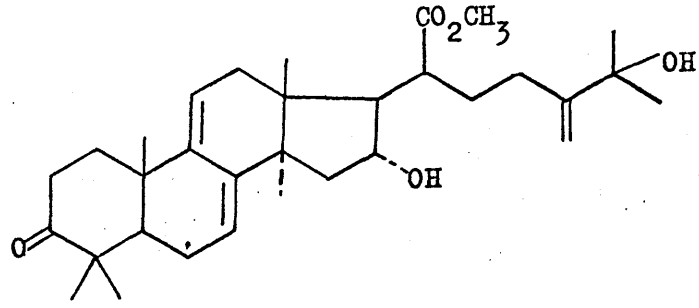
then be proposed for D.Q.10.

Reaction of this compound with p-toluenesulphonic acid in benzene at room temperature led to a solid $C_{31}H_{48}O_4$ m.p. 197-199°C, to which the structure 1.22 was assigned on the following evidence. The i.r. spectrum showed one hydroxyl grouping at 3619 cm^{-1} and a single carbonyl peak at 1765 cm^{-1}. The n.m.r. spectrum showed that the 3α-hydroxyl grouping was still present since the narrow multiplet was visible at 6.58τ. The signal of the proton geminal to the other secondary alcohol in D.Q.10 (5.87τ, triplet) had moved downfield to 5.66τ. This value was the same as that encountered in 1.18, the spiro-lactone from D.Q.9. The mass spectrum showed the parent ion to be at m/e 484, (mass measurement $C_{31}H_{48}O_4$), while loss of 15 mass units to give the base peak at m/e 469 was the major fragmentation. Ions at m/e 153 (50%) and m/e 69 (39%), similar to those encountered in the spiro-lactone from D.Q.9, were also present.

The quantity of the last methyl ester from Daedalea quercina to be examined was such that only a tentative structure can be put forward, based on the spectroscopic evidence. That D.Q.11, $C_{32}H_{48}O_5$, was based on the lanosta-7,9(11)-diene skeleton was demonstrated by its u.v. absorption at 252.5, 244.5 and 237.5 mμ. The nature of the oxygen functions was deduced from the i.r.

TABLE I - 15 MASS SPECTRUM OF D.Q.11

m/e	%
512	22
497	18
494	9
479	10
469	15
391	8
325	25
309	100
307	22
291	18
203	38



1.23

spectrum. Two carbonyl groups were present, an ester at 1738 cm^{-1} and an acyclic or six-membered ring ketone at 1718 cm^{-1}. That the other two oxygens were present as alcohol functions was apparent from absorption at 3624 cm^{-1} and 3609 cm^{-1}. The n.m.r. spectrum, as well as confirming the presence of a methyl ester (6.32τ) and the diene system (4.60τ), showed that one alcohol grouping was secondary (1H, triplet at 5.78τ), while the other was tertiary. The siting of the latter group at C-25 followed from the presence of a six-proton singlet at 8.68τ (Me-C-O). A terminal methylene grouping (1625 cm^{-1}) caused a doublet at 5.28τ.

Examination of the methyl region of the n.m.r. spectrum showed that all five nuclear tertiary methyl groups were clearly visible at tau values 8.84, 8.94, 9.01, 9.05 and 9.42. These values bore a distinct resemblence to those of methyl polyporenate C which were 8.88, 8.94, 8.96, 9.00, 9.03 and 9.42τ. Replacement of the signals due to the gem-dimethyl at C-25, a doublet with J=6 c/s centred at 9.0τ in this latter compound, with a six-proton singlet at 8.68τ led to an n.m.r. spectrum which was almost superposable with that of D.Q.11. It may then be proposed that D.Q.11 had structure 1.23.

The mass spectral data of this compound is shown in Table I-15. It can be seen that the base peak at m/e 309 corresponds to

dehydration followed by cleavage of the side chain. The peaks at high mass number can be explained by loss of a methyl radical or the elements of water. Analogous processes to these fragmentations occur in the mass spectrum of methyl polyporenate C.

In summary, it can be said that Daedalea quercina produces five previously unencountered triterpenes, four of which have a novel γ-keto-acid side chain. One of these is certainly based on a C_{32} skeleton, although its complete structure is somewhat tentative. If the basic nucleus of D.Q.1 may be termed quercinic acid, then the five compounds isolated were:

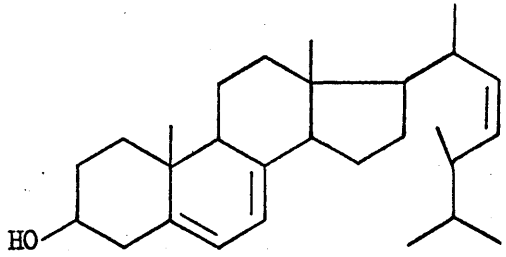
D.Q.1 methyl 3α-carbomethoxyacetylquercinate.

D.Q.7 methyl 3α-carbomethoxyacetyldaedaleate.

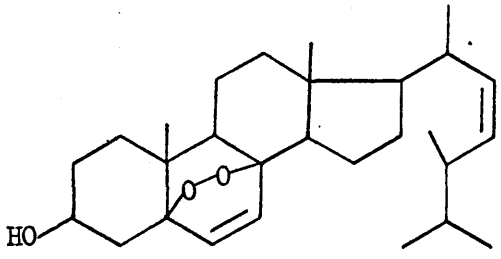
D.Q.9 methyl 16α-hydroxy-3α-carbomethoxyacetylquercinate.

D.Q.10 methyl 16α-hydroxyquercinate.

D.Q.11 methyl 25-hydroxypolyporenate C.



1.24



1.25

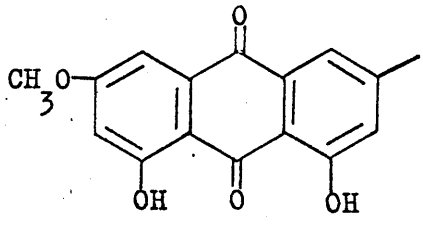
No detailed examination of the light petroleum soluble material of Daedalia quercina has been undertaken. The u.v. spectrum of this crude material exhibited absorption at 242, 283 and 293 mμ. This was characteristic of the ergosta-5,7-dienoid system[100]. T.l.c. examination in 1% methanol in chloroform showed the presence of material giving the characteristic intense blue-black colouration at R_f 0.40 when sprayed with acidic ceric ammonium nitrate and heated. This indicated that ergosterol (1.24) was present. Only very slight traces of ergosterol peroxide (1.25), easily detectable by a similar treatment (when a dark green colour developed), were present. Ergosterol peroxide has been reported as a metabolite of Daedalea quercina[101]. It has also been detected in extracts of Aspergillus fumigatus[102], Trichophyton schoenleini[103], Penicillium schlerotigenum[104] and Polyporus betulinus[56].

In view of the fact that Windaus[105] showed that ergosterol could be converted into its epidioxide by photosensitized oxidation, it was decided to investigate Polyporus betulinus and Daedalea quercina to determine whether or not ergosterol peroxide was a genuine metabolite. Methanol extracts of Polyporus betulinus sporophores, when examined immediately by t.l.c., showed that ergosterol was present whereas none of its peroxide could be

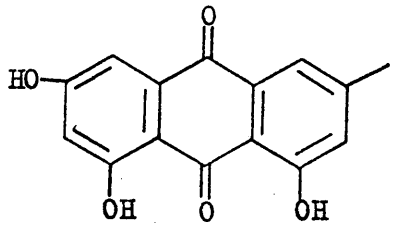
detected. But, after standing in daylight at room temperature for only three days, the dark green staining peroxide could be easily seen. Examination showed that the methanol extract of Daedalea quercina exhibited similar behaviour. These results strongly suggest that 1.25 does not arise from metabolic processes.

Further experiments were designed to prove this theory. Small quantities of ergosterol were added separately to methanol extracts of each fungus in which neither this compound nor its oxygenation product was present in detectable amounts. These solutions were irradiated for 18 hours with white light under a slow stream of oxygen. An almost quantitative conversion to the peroxide was obtained. A control experiment using only a methanolic solution of ergosterol showed no similar conversion.

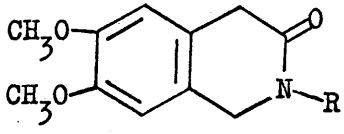
It has been shown that a photosensitizing agent (e.g. eosin[105]) can accomplish the transformation under examination. That such a system must be present in the fungal extracts was confirmed by repeating the above experiments (a) with oxygen but with exclusion of light, and (b) without the stream of oxygen. Nitrogen was first bubbled through the solution in (b) to remove dissolved oxygen. In both cases the ergosterol remained unchanged. Thus ergosterol peroxide from Daedalea quercina and Polyporus betulinus was an artefact formed by photosensitized oxidation of



1.26

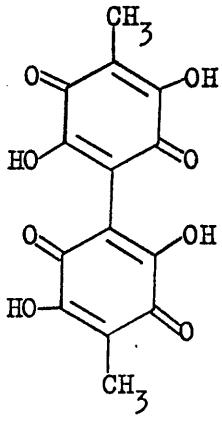


1.27

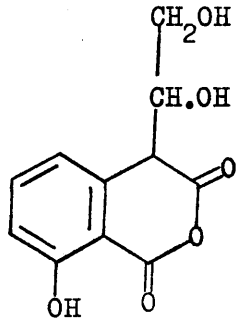


1.28 R = CH_3

1.29 R = OH



1.30



1.31

the metabolite ergosterol. The agent, or agents, responsible for this conversion were unaffected by thermal, base or acid treatment.

Investigation of possible models for these agents showed that eosin, physcion (1.26)[106], emodin (1.27)[107], N-methyl-6,7-dimethoxy-3-isoquinolone (1.28) and N-hydroxy-6,7-dimethoxy-3-isoquinolone (1.29)[108] could all accomplish the required transformation whereas oosporein (1.30)[109] and oospoglycol (1.31)[110] could not. These results indicate only that a range of compounds may act as the photosensitizing agent and in no way attempts to define this range. Quantitative experiments with ergosterol (10 mg.) in methanol (10 ml.) showed that eosin was the most efficient catalyst requiring only 0.01 mg. to achieve complete conversion. The activity of the anthraquinones was $^{1}/_{5}$ of this, while the isoquinolones required 0.1 mg.. Similar quantitative experiments with crude mould extracts showed that the minimum quantity required was 100 mg.. T.l.c. examination of this material showed a number of polar coloured compounds, one or several of which could be responsible for the photosensitizing property. Even if it were assumed that the activity of these materials was equal to the least active of the model compounds, the maximum amount available was 25 mg. per Kg. of fungus. Because of the difficulties involved in

dealing with small quantities of a mixture of polar compounds this matter was not pursued.

The major methyl esters of Daedalea quercina bear the novel carbomethoxyacetate grouping, and it has been shown[56] that this grouping also occurs in one of the main products of Polyporus betulinus. These facts, coupled with the paucity of data in the literature, make the study of such compounds a valuable one. In addition, future plans involving radio tracer work require a synthetic pathway which is economical on the triterpene alcohol portion of such conjugates.

Since the work up method employed led to the isolation of compounds bearing the carbomethoxyacetate grouping, it was decided to attempt the synthesis of model compounds in this system as a pointer to a general method. A previous synthesis of this type of system[111] suffered from the drawback of low yields in stages where the ester being varied had already been incorporated. Carbomethoxyacetyl chloride was prepared from dimethyl malonate via potassium carbomethoxyacetate by the method of Staudinger and Becker[112]. Condensation of this acid chloride with cholesterol or dihydrolanosterol in the presence of pyridine gave the corresponding carbomethoxyacetates. The yields (based on the amount of sterol) were 86% and 90% respectively.

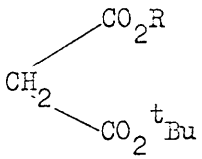
However, it is known that the compound isolated from Polyporus betulinus occurred as the free carboxyacetyl conjugate.

It was felt that some indication as to stability and properties of this system could be obtained by synthesising model compounds. Again a prerequisite for the route was that the yields, in steps after the sterol nucleus has been added, must be good.

The first method attempted was analogous to that used above for the corresponding methyl esters, but, despite rigid adherence to the conditions specified by the authors, carboxyacetyl chloride could not be prepared from malonic acid and thionyl chloride by the method of Staudinger and Ott[113]. Similarly, adaption of the method of Wilds and Shunk[114] using oxalyl chloride led to polymeric products.

The approach of half-hydrolysis of the corresponding malonyl diester was tried. Malonyl dichloride was prepared from malonic acid and phosphorus pentachloride according to Black et al.[115], and condensed with cholesterol in the presence of pyridine. A good yield of dicholesteryl malonate was obtained but, although a variety of hydrolysis procedures were tried, in all cases about 50% of the starting material was recovered, while half the material had been completely hydrolysed.

A fresh line of attack was the thought that the required compounds might be obtained from an unsymmetrical ester of the form 1.32.



1.32

Treatment of such a compound with acid should allow preferential hydrolysis of the t-butyl group to release the requisite carboxyacetate. Mono-t-butyl malonate was obtained by carbonation of t-butyl acetate by the method of Hauser et al.[111]. The acid chloride of this compound could not be obtained by the action of thionyl chloride or oxalyl chloride but direct coupling of the free acid with cholesterol was achieved by a mixed anhydride technique using p-toluenesulphonyl chloride in pyridine [116]. Treatment of the resulting cholesteryl carbo-t-butoxyacetate with dry hydrogen chloride[117] gave the desired free acid. A similar sequence of reactions led to the isolation of the corresponding derivative of dihydrolanosterol.

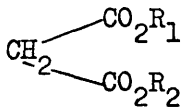
The compounds bearing the carbomethoxyacetate grouping which have been isolated from D. quercina and P. betulinus are the first of their kind. A previous examination of P. betulinus[52] failed to detect any carboxyacetyl polyporenic acid A, and it was considered that this might be due to the method of work up employed, since compounds with this structural feature would be expected to be sensitive. Accordingly both cholesteryl carboxyacetate and dihydrolanosteryl carboxyacetate were subjected to

treatment designed to simulate an extraction procedure from natural sources. Refluxing in methanol for 24 hours and treatment with ethereal diazomethane yielded >90% recovery of the corresponding methyl ester. Thin layer chromatography before methylation showed that the major decomposition product was the free alcohol and that there was almost no conversion to the methyl ester by the action of refluxing methanol.

Treatment with 2N methanolic sodium hydroxide at room temperature was found, by t.l.c., to require 30 minutes for complete hydrolysis of both carboxyacetates and both carbomethoxy-acetates. By comparison, treatment of the simple acetate of dihydrolanosterol for a similar time showed that only 50% hydrolysis had occurred. The conclusion from these experiments is that acid-base extraction procedures such as have been used previously in the isolation of fungal triterpene acids[118] would destroy any carboxyacetates which may be present in the natural extract, but that such compounds should be recoverable even after soxhlet extraction of the natural tissue, as long as no saponification step is employed.

Abramovitch[119] has studied the carbonyl absorption of symmetrical diesters of malonic acid. He showed that all the compounds he studied exhibited complex bands in the 1700 - 1800 cm^{-1}

TABLE I - 16 N.M.R. AND I.R. VALUES FOR ESTERS OF MALONIC ACID



R_1	R_2	ν in cm^{-1} in CCl_4	τ value of CH_2
Me	Me	1747 , 1763	6.67
Et	Et	1740 , 1757	6.63
n-Pr	n-Pr	1738 , 1755	6.76
iso-Pr	iso-Pr	1734 , 1751	6.82
n-Bu	n-Bu	1739 , 1755	6.75
sec-Bu	sec-Bu	1733 , 1750	6.80
Me	Cholesteryl	1739 , 1759	6.79
t-Bu	Cholesteryl	1731 , 1748	6.88
Me	Dihydrolanosteryl	1738 , 1758	6.59
t-Bu	Dihydrolanosteryl	1737 , 1748	6.72
Cholosteryl	Cholesteryl	1737 , 1755	6.67

region in the i.r.. He ascribed these signals to a combination of rotational isomerism and vibrational coupling. In Table I - 16 are shown the values for some symmetrical diesters and some unsymmetrical esters of malonic acid. It will be seen that all the compounds show, under low resolution, two bands at about 1755 cm^{-1} and 1735 cm^{-1}. It was noticeable that, in all the cases examined, the band at lower wave number was more intense. It appears that the doublet carbonyl peak and the relative intensities of these two peaks are characteristic of esters of malonic acid. Consideration of the 'fingerprint region' of such esters established that they all exhibited strong absorption at 1150 cm^{-1}, and that all four unsymmetrical esters showed additional peaks at ca. 1280, 1215, 1030 and 1010 cm^{-1}. In this respect it is worth noting that the carbomethoxyacetates isolated from Daedalea quercina all had similar absorption. The relevant data are shown below:

D.Q.1. 1292, 1227, 1159, 1036 and 1022 cm^{-1}.

D.Q.7. 1284, 1211, 1160, 1035 and 1013 cm^{-1}.

D.Q.9. 1277, 1212, 1157, 1044 and 999 cm^{-1}.

In the n.m.r. spectrum the tau value of the methylene group is fairly constant. The values for the substances studied are included in Table I - 16. The mean figure of 6.7τ

is thus of great use in diagnosing the presence of a malonate ester. This has been used in the following section.

The mass spectral cleavage of the synthetic compounds was as expected in that all esters exhibited loss by a McLafferty rearrangement from the 3-position, carbomethoxyacetates losing 118 mass units while carbo-t-butoxyacetates lost 160 mass units. Apart from loss of the methyl radical, these were the only important fragmentations.

Since the first two members of the family Polyporaceae, which had been examined by a mild non-hydrolytic isolation procedure, contained sensitive conjugated species, it was decided to conduct a survey of other fungi of the same genus. Crude methanol extracts of P. betulinus or D. quercina, when methylated, clearly showed, when the n.m.r. spectrum of the mixture was determined, the peak at ca 6.7τ due to the methylene group of the carbomethoxyacetate function. On this basis it seemed reasonable, for a rapid survey, to examine the result of a similar procedure on other fungi. Hopes that conjugates would prove to be of wide occurrence were dashed by the results of this survey which were, without exception, negative. For the sake of completeness a list of the members of the Polyporaceae examined by this n.m.r. study are shown below. They are divided into those which were cultured (A) and those which were field-collected (B).

(A)	(B)
Lenzites betulina	Polyporus sulphureus
Lenzites subferrugina	Polyporus hispidus
Lenzites thermophila	Polyporus frondosus
Lenzites repanda	Polyporus giganteus
Lenzites trabea	Fomes ignareus
Lenzites striata	Fomes annosus

(A) contd.

Daedalea biennis

Daedalea juniperina

Daedalea unicolor

Daedalea confragosa

(B) contd.

Ganoderma applanatum

Oxyporus populinus

Polystictus versicolor

It was noted that all the above fungi appeared to contain triterpene acids since, in every case, the saturated methyl region (8.5-9.5τ) exhibited the pattern expected, while the methoxyl groups of the methyl esters were visible at ca 6.3τ. Also visible was a peak at ca 8.7τ, of very variable intensity, thought to be due to the presence of fatty acids.

EXPERIMENTAL

Instrumentation

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected, boiling points are uncorrected. Ultraviolet spectra were obtained, in ethanol solutions, on a Unicam S.P. 800 recording spectrophotometer. Infra red spectra were measured with a Unicam S.P. 200 instrument and, for high resolution (KBr discs and solution spectra, in the solvent as stated) with a Unicam S.P. 100 double beam infra red spectrometer equipped with an S.P. 130 sodium chloride prism grating double monochromater, operated under vacuum. Nuclear magnetic resonance spectra were recorded with a Perkin-Elmer R. 10 60 Mc/s spectrometer and with a Varian HA-100 100 Mc/s spectrometer. Unless otherwise stated all τ values quoted are recorded at 100 Mc/s in chloroform with tetramethylsilane as internal standard. Mass spectra were obtained with an A.E.I. MS9 double focussing mass spectrometer, precise mass measurements were made relative to perfluorotributylamine. Analytical gas-liquid chromatography was performed on a Pye Argon chromatograph, while combined mass spectrometry-gas chromatographic determinations were obtained from an LKB-9000 instrument. Optical rotations, in chloroform solution, were measured on a Hilger and Watts microptic photoelectric polarimeter.

Thin Layer Chromatography.

R_f values were determined from elution on 0.25 mm. layers of Kieselgel G, the compounds being located by spraying with ceric ammonium nitrate/sulphuric acid (1% in 10%) and baking. The dyes p-aminoazobenzene and p-hydroxyazobenzene were used to standardise all R_f values. The R_f values of these dyes in the solvents employed are given below.

Solvent	p-aminoazobenzene	p-hydroxyazobenzene
100% $CHCl_3$	0.60	0.21
1% MeOH in $CHCl_3$	0.90	0.56
10% MeOH in $CHCl_3$	-	0.92
100% benzene	0.35	0.10
50% light petroleum in ether	0.46	0.20
25% light petroleum in ether	0.64	0.48

All p.l.c. plates were pre-washed with the developing solvent before application of the mixture.

General

Diazomethane was prepared by the method of Moore and Reed [120] from bis(N-methyl-N-nitroso)-terephthalamide. All organic extracts were dried over anhydrous magnesium sulphate and solvents were removed using a rotary film evaporator. 'Light petroleum', unless otherwise stated, refers to light petroleum ether b.p. 60-

80°C.

The following abbreviations are used in reporting spectral data: s, singlet: d, doublet: t, triplet: q, quartet: m, multiplet: br., broad: and infl, point of inflexion.

1. EXTRACTION OF THE METABOLITES AND METHYLATION

Fresh, healthy sporophores of Daedalea quercina were collected in mid-Autumn from an oak stump in Garscube Estate. The clean, dry tissue (600 g.) was homogenised in a Waring Blender in methanol (10 l.). After standing at room temperature for two months the extracted tissue was filtered off and the filtrate evaporated to give a brown gum (36 g.). Treatment of this material with light petroleum (300 ml.) removed lipids and fats (5.4 g.). The petrol insolubles were dissolved in methanol and treated with an excess of ethereal diazomethane to give the methyl esters as an oil (30.3 g.).

2. "D.Q." REFERENCE CODE.

A sample of the methyl esters of Daedalea quercina was analysed by t.l.c. in two solvent systems:

A 100% chloroform.

B 1% methanol in chloroform.

Plates eluted with these systems were sprayed with ceric ammonium nitrate in sulphuric acid solution and rhodamine 6G solution. The staining characteristics and standard R_f values of the seven principal substances are shown in Table I - 1. For ease of reference the code names D.Q.1, D.Q.4, D.Q.5, D.Q.7, D.Q.9, D.Q.10 and D.Q.11 will be used in the text to uniquely represent the

compounds whose chromatographic properties are defined by Table I - 1.

3. CHROMATOGRAPHIC SEPARATION OF D. QUERCINA ESTERS.

The esters of Daedalea quercina (30.3 g.) were adsorbed on to silicic acid (15 g.) which was placed on top of a column of silicic acid (650 g.). This was eluted with light petroleum (1.5 l.) to which ethyl acetate (3.5 l.) was gradually added and fractions (20 ml.) were taken at the rate of four per hour. T.l.c. analysis of the fractions revealed the groupings shown below.

Fractions	Weight (g.)	Main constituents
103 - 109	7.52	D.Q.1, D.Q.4
110 - 114	3.8	D.Q.4, D.Q.7
115 - 120	3.15	D.Q.7, D.Q.5
121 - 130	4.12	D.Q.5, D.Q.9
131 - 140	2.54	D.Q.9
141 - 143	0.53	D.Q.9, D.Q.10
144 - 162	2.39	D.Q.10, D.Q.11
163 - 200	3.2	mixture of polar constituents
Total	27.25	i.e. 90% recovery

D.Q.1 was obtained from fractions 103 - 109 by fractional

crystallisation from light petroleum. Trituration of fractions 110 - 114 or 115 - 120 with ether permitted isolation of pure D.Q.7. Further chromatography was required to obtain pure D.Q.9, D.Q.10 and D.Q.11. Elution of fractions 121 - 130 or 131 - 140 on Kieselgel H plates using 1% methanol in chloroform allowed separation of pure D.Q.9. D.Q.10 was also separated from fractions 141 - 143 or from a similar treatment of fractions 144 - 162. D.Q.11 was recovered from fractions 144 - 162 by p.l.c. with the same solvent system employing Kieselgel HF_{254} as adsorbent. This was found to be more convenient than spraying with rhodamine 6G solution. The total yields of each of the components under study is shown below.

		% weight of dry sporophores
D.Q.1	5.1 g.	8.52
D.Q.7	0.37 g.	0.06
D.Q.9	0.62 g.	0.10
D.Q.10	0.57 g.	0.09
D.Q.11	0.03 g.	0.005

4. D.Q.1 (METHYL 3-α-CARBOMETHOXYACETYL QUERCINATE.)

This substance was isolated as detailed in Section 3. It crystallised from light petroleum as needles, m.p. 101-103°C. $[\alpha]_D$ -2.8 (c 0.8). R_f ($CHCl_3$) 0.58.

ν_{max} (KBr) 2950, 2877, 2835, 1751, 1733, 1706, 1456, 1437, 1418, 1390, 1375-1366, 1348, 1339, 1292, 1263, 1227, 1199, 1179, 1159 1060, 1036, 1022, 988, 893, 881 and 847 cm^{-1}.

ν_{max} (CCl_4) 1758 cm^{-1} (ε 632), 1736 cm^{-1} (ε 1272), 1716 cm^{-1} (ε 405)

τ values (CCl_4) (60 Mc/s) 5.30 (1H, m), 6.30 (3H, s), 6.37 (3H, s), 6.70 (2H, s), methyl contour 8.82, 8.87, 8.97, 9.00, 9.08, 9.12 and 9.25.

τ values 5.38 (1H, m), 6.37 (3H, s), 6.42 and 6.44 (3H), 6.69 (2H, s), methyl contour 8.84, 8.92, 9.01, 9.10, 9.14 and 9.28.

Analysis Found C 72.16% H 9.22% M^+ at m/e 600 $C_{36}H_{56}O_7$

$C_{36}H_{56}O_7$ requires C 71.96% H 9.39% M.W. 600

5. REDUCTION OF D.Q.1 WITH LITHIUM ALUMINIUM HYDRIDE TO D.Q.1-TRIOLS (1.7)

To lithium aluminium hydride ($LiAlH_4$) (1 g. 20 fold excess) in dry ether (200 ml.) was added, slowly over 1 hour, D.Q.1 (1.6 g.) in dry ether (50 ml.) and the reaction stirred at room temperature for 24 hours. The excess reagent was removed by dropwise addition of mineral acid. After further addition of 2N hydrochloric acid (50 ml.), extraction of the ethereal layer and washing with sodium carbonate (4 x 25 ml.) and brine (2 x 25 ml.), a white

solid (1.4 g.) was obtained. Crystallisation of this material from ethyl acetate yielded the D.Q.1-triols (1.7) (0.9 g., 71%) as granules, m.p. 199.5-201°C. R_f (1% MeOH in $CHCl_3$) 0.10. ν_{max} (KBr) 3400 (br.), 2940, 2868, 1466, 1451, 1375, 1367, 1284, 1257, 1154, 1124, 1110, 1060, 1037, 1026, 1011, 985, 971, 958, 915 and 864 cm^{-1}.

ν_{max} ($CHCl_3$) (1.1 mM.) 3623 cm^{-1} (ε 98), 3430 (br.)
(0.1 mM.) 3617 cm^{-1} (ε 131), 3460 (br.)

τ values (60 Mc/s D.M.S.O.) 5.76 (2H, m), 6.74 (2H, m), methyl contour 9.06, 9.13, 9.20, 9.30 and 9.41.

Analysis Found C 78.18% H 11.67% M^+ at m/e 474

$C_{31}H_{54}O_3$ requires C 78.43% H 11.46% M.W. 474

6. ACETYLATION OF D.Q.1-TRIOLS (1.7)

The D.Q.1-triols (1.7) (200 mg.) were treated with pyridine (0.5 ml.) and acetic anhydride (0.5 ml.) at room temperature for 12 hours. The reaction mixture was poured into a mixture of 4N hydrochloric acid (5 ml.) and iced water (10 ml.). Ether (25 ml.) extraction of this suspension and washing with 4N hydrochloric acid (5 ml.) and saturated sodium carbonate (2 x 10 ml.) gave, after drying, the triacetates 1.9 (210 mg., 83%) which crystallised

from methanol as needles, m.p. 159-160°C. R_f ($CHCl_3$) 0.77.

ν_{max} (KBr) 2938, 2866, 2821, 1732, 1469, 1450, 1431, 1371, 1272, 1243-1230, 1179, 1054, 1033, 1018, 975 and 959 cm^{-1}.

ν_{max}($CHCl_3$) 1739 cm^{-1} (ε 1210)

τ values (60 Mc/s) 4.97 (1H, m), 5.30 (1H, m), 6.03 (2H, d, J=7 c/s), 7.93 (9H), methyl contour 8.98, 9.07, 9.12, 9.18 and 9.26.

Analysis	Found	C 73.74%	M^+ at m/e 600
		H 10.18%	
$C_{37}H_{60}O_8$	requires	C 73.96%	M.W. 600
		H 10.06%	

7. OXIDATION OF D.Q.1-TRIOL TRIACETATES (1.9) WITH SELENIUM DIOXIDE.

The triacetates 1.9 (100 mg.) were refluxed with selenium dioxide (120 mg.) in glacial acetic acid (10 ml.) for 12 hours. After addition of a small quantity of sodium acetate, the metallic selenium was filtered off by glass paper. Evaporation of the solvent yielded a white solid (71 mg.) which, on crystallisation from methanol, gave the diene triacetates 1.10 (51 mg., 52%), m.p. 141-143°C. R_f ($CHCl_3$) 0.77.

ν_{max} (KBr) 2941, 2871, 1734, 1471, 1458, 1444, 1371, 1273, 1240-1236, 1178, 1066, 1034, 1016, 988 and 962 cm^{-1}.

ν_{max} ($CHCl_3$) 1724 cm^{-1} (ε 1170)

λ_{max} 236.5 mμ (log ε 4.13), 243.5 mμ (log ε 4.19), 252 mμ (log ε 4.02) τ values (60 Mc/s) 4.60 (2H, m), 4.98 (1H, m), 5.31 (1H, m), 6.04 (2H, d, J=7 c/s), 7.94 (9H), methyl contour 9.00, 9.07, 9.12, 9.24, and 9.41.

Analysis	Found	C 74.46%	M^+ at m/e 598
		H 10.08%	
$C_{37}H_{58}O_6$	requires	C 74.21%	M.W. 598
		H 9.76%	

8. ISOMERIC BUTENOLIDE MALONATES 1.11 FROM D.Q.1 BY TREATMENT WITH p-TOLUENESULPHONIC ACID.

D.Q.1 (300 mg.) and p-toluenesulphonic acid (30 mg.) were refluxed in dry benzene (100 ml.) for 18 hours. The mixture was then cooled and washed with water (2 x 25 ml.). After drying and evaporation of the solvent a white solid (280 mg.) was obtained. P.l.c. on five 20 x 20 x .1 cm layers of Kieselgel H using 50% light petroleum ether allowed the separation of the products, R_f 0.53 and R_f 0.47.

Isomer α, R_f 0.53, crystallised from light petroleum (b.p. 40-60°) as fine needles, m.p. 152.5-154°C.

ν_{max} (KBr) 2940, 2870, 1757, 1732, 1687, 1436, 1370, 1326, 1272, 1238, 1172, 1153, 1096, 1055, 1035, 1016 and 999 cm^{-1}.

ν_{max} (CCl_4) 1764 cm^{-1} (ε 1340), 1740 cm^{-1} (ε 578), 1687 cm^{-1} (ε 98)

λ_{max} 213 mμ (ε 10,100)

τ values 5.28 (2H, m), 6.29 (3H, s), 6.63 (2H, s), 8.08 (3H, s), 8.22 (3H, s), methyl contour 8.93, 9.01, 9.10, 9.13 and 9.29.

Analysis Found C 73.76% H 9.17% M^+ at m/e 568

$C_{35}H_{52}O_6$ requires C 73.91% H 9.21% M.W. 568

Isomer β, R_f 0.47, crystallised from light petroleum as plates, m.p. 141-143°C.

ν_{max} (KBr) 2953, 2870, 1760-1735, 1686, 1433, 1370, 1330, 1274, 1205, 1172, 1146, 1075, 1055, 1034, 1014, 993 and 989 cm^{-1}.

ν_{max} (CCl_4) 1763 cm^{-1} (ε 1670), 1739 cm^{-1} (ε 843), 1688 cm^{-1} (ε 109)

λ_{max} 214 mμ (ε 9,750)

τ values 5.28 (2H, m), 6.28 (3H, s), 6.63 (2H, s), 8.10 (3H, s) 8.20 (3H, s), methyl contour 8.94, 9.01, 9.08, 9.12 and 9.28.

Analysis Found C 74.00% H 9.21% M^+ at m/e 568

$C_{35}H_{52}O_6$ requires C 73.91% H 9.21% M.W. 568

9. ACID CATALYSED DEHYDRATION OF THE D.Q.1-TRIOLS (1.7).

The triols 1.7 (100 mg.) were dissolved in methanol (20 ml.) to which 36N hydrochloric acid (2 ml.) had been added. The

mixture was refluxed on a steam bath for 4 hours. After cooling and neutralisation with 4N sodium hydroxide (18 ml.) the methanol was removed and the aqueous solution extracted with ether (2 x 20 ml.). On drying and evaporation of the solvent an amorphous solid (90 mg.) was obtained. P.l.c. on 20 x 20 x .1 cm Kieselgel H with chloroform as solvent separated the two components of R_f 0.71 and R_f 0.53.

Product of R_f 0.71 crystallised from light petroleum as needles, m.p. 188-190°C.

ν_{max} (KBr) 3470 (br.), 2965, 2941, 2877, 1464-1458, 1386, 1378, 1076, 1048, 1015, 994 and 966 cm^{-1}.

ν_{max} (CCl_4) 3620 cm^{-1} (ε 43)

τ values 6.57 (1H, m), 6.06, 6.57, 6.72 (2 x 1H, m), methyl contour 8.99, 9.03, 9.06, 9.08 and 9.11.

Analysis	Found	C 81.76%	M^+ at m/e 456
		H 11.74%	
$C_{31}H_{52}O_2$	requires	C 81.52%	M.W. 456
		H 11.48%	

G.l.c. on a 4 foot column of 1% SE 30 at 250° with a flow rate of 40 ml./min. showed two peaks of retention times 12.25 min. and 15.0 min. (relative intensities 3 : 1). Cholesterol had a retention time of 5.5 min. under the same conditions.

The mass spectra of both peaks were similar with M^+ at m/e 456.

Product of R_f 0.56 was isolated as a gum.

ν_{max} (film) 3420 (br.), 2980, 2819, 1467-1459, 1391, 1322, 1297, 1241, 1111, 1073, 1048, 1043, 997 and 973 cm^{-1}.

ν_{max} (CCl_4) 3621 cm^{-1} (ε 50)

τ values 6.55 (1H, m), 5.90, 5.98, 6.73 (2 x 1H, m) methyl contour 8.97, 9.00, 9.01, 9.05 and 9.11.

M^+ at m/e 456.

G.l.c. under the same conditions as above gave two peaks of retention times 13.75 min. and 14.0 min. (relative intensities 3 : 1).

The mass spectra of both peaks were similar with M^+ at m/e 456.

10. D.Q.7.

This compound was isolated as described in Section 3. It crystallised from ethyl acetate/light petroleum as needles, m.p. 136-138°C. R_f ($CHCl_3$) 0.43. $[\alpha]_D$ -63 (c 0.8)

ν_{max} (KBr) 2943, 2867, 2840, 1751, 1737, 1722, 1457, 1435, 1409, 1376, 1370, 1328, 1284, 1273, 1262, 1240, 1232, 1211, 1160, 1050, 1035, 1013, 984 and 885 cm^{-1}.

ν_{max} (CCl_4) 175 cm^{-1} (ε 657), 1740 cm^{-1} (ε 1092), 1719 cm^{-1} (ε 438)

τ values 5.30 (1H, m), 5.48 (2H, m), 6.28 (3H, s), 6.31 (1H, m), 6.34 (3H, s), 6.60 (2H, s), methyl contour 8.72, 8.84, 8.94, 9.00,

9.04, 9.12 and 9.24.

Highest ion at m/e 612 ≡ $C_{37}H_{56}O_7$.

Analyses	Found	C 69.65%	69.92%	69.76%	69.79%
		H 8.55%	8.54%	8.91%	8.66%
$C_{37}H_{56}O_7$ requires		C 72.52%		M.W. 612	
		H 9.21%			
$C_{37}H_{56}O_9$ requires		C 68.92%		M.W. 644	
		H 8.75%			

11. D.Q.9. (16-HYDROXY- D.Q.1.)

This compound was isolated as described in Section 3. It was a clear gum. R_f (1% MeOH in $CHCl_3$) 0.40. $[\alpha]_D$ -4.0 (c 1.7). ν_{max} (film) 3540, 2920, 2870, 1741, 1725, 1707, 1467, 1456, 1380 1335, 1291, 1277, 1212, 1157, 1122, 1066, 1044, 1032 and 999 cm^{-1}. ν_{max} (CCl_4) 3619 cm^{-1} (ε 40), 1757 cm^{-1} (ε 628), 1737 cm^{-1} (ε 1460), 1717 cm^{-1} (ε 448)

τ values 5.36 (1H, m), 5.96 (1H, t, J=8 c/s), 6.36 (3H, s), 6.43 (3H, s), 6.69 (2H, s), methyl contour 8.76, 8.82, 8.91, 8.99, 9.00, 9.07, 9.12 and 9.28.

M^+ at m/e 616 ≡ $C_{36}H_{56}O_8$.

12. REACTION OF D.Q.9 WITH p-TOLUENESULPHONIC ACID.

D.Q.9 (75 mg.) and p-toluenesulphonic acid (10 mg.) were dissolved in dry benzene (15 ml.) and left at room temperature

for 24 hours. Water (10 ml.) was added and the layers separated. The benzene layer, after further washing with water (5 ml.), was dried and evaporated to yield a clear gum (70 mg.). P.l.c. on a 20 x 20 x 0.07 cm. layer of Kieselgel H with chloroform as solvent permitted separation of the product 1.18 (50 mg., 68%) which was a clear gum. R_f ($CHCl_3$) 0.73.

ν_{max} (film) 2943, 2863, 1775, 1750, 1728, 1467, 1455, 1443, 1390, 1306, 1279, 1239, 1217, 1174, 1156, 1114, 1074, 1041, 1034, 1009 and 958 cm^{-1}.

ν_{max} (CCl_4) 1783 cm^{-1} (ε 496), 1758 cm^{-1} (ε 467), 1737 cm^{-1} (ε 598)

τ values 5.30 (1H, m), 5.66 (1H, t, J=8 c/s), 6.31 (3H, s), 6.63 (2H, s), methyl contour 8.89, 8.95, 9.00, 9.04, 9.13 and 9.24.

M^+ at m/e 584.

13. LITHIUM ALUMINIUM HYDRIDE REDUCTION OF D.Q.9.

To $LiAlH_4$ (150 mg.) in dry ether (10 ml.) was added D.Q.9 (100 mg.) in dry ether (5 ml.). The mixture was stirred at room temperature for 36 hours, then the excess reagent destroyed by dropwise addition of 1N hydrochloric acid. Washing of the ethereal layer with 4N hydrochloric acid (2 x 10 ml.) and brine (3 x 5 ml.) and drying yielded, after evaporation of the solvent, the tetraols 1.20 (65 mg., 50%) which crystallised from methanol as needles, m.p. 107-109°C. R_f (2% MeOH in $CHCl_3$) 0.05.

ν_{max} (KBr) 3400 (br.), 2964, 2890, 2845, 1472-1462, 1442, 1390, 1382, 1147, 1070, 1045, 1024, 997, 991 and 965 cm^{-1}.

ν_{max} (CCl_4) (saturated soln.) 3624 cm^{-1}, 3400 cm^{-1} (br.)

1 : 5 dilution 3625 cm^{-1}.

τ values 5.86 (1H, t, J=8 c/s), 6.32 (1H, m), 6.50 (2 x 1H, m), 7.34 (4H, m), methyl contour 8.95, 8.98, 9.01, 9.05, 9.07, 9.12, 9.19 and 9.24.

On addition of D_2O the peak at 7.34 disappeared.

Analysis	Found	C 75.56%	M^+ at m/e 490
		H 10.77%	
$C_{31}H_{54}O_4$	requires	C 75.87%	M.W. 490
		H 11.09%	

14. D.Q.10 (DESMALONYL-16-HYDROXY-D.Q.1.)

This substance was isolated as detailed in Section 3. It was a clear gum. R_f (1% MeOH in $CHCl_3$) 0.19. $[\alpha]_D$ +18 (c 0.9)

ν_{max} (film) 3500, 2900, 2830, 1731, 1705, 1462, 1439, 1383, 1263, 1211, 1128, 1106, 1077, 1043 and 972 cm^{-1}.

ν_{max} (CCl_4) 3637 cm^{-1} (ε 52), 3540 cm^{-1} (br.), 1735 cm^{-1} (ε 421), 1715 cm^{-1} (ε 362)

1 : 20 dilution 3640 cm^{-1} (ε 79)

τ values 5.87 (1H, t), 6.26 (3H, s), 6.59 (1H, m), methyl contour 8.73, 8.81, 8.89, 8.99, 9.02, 9.07, 9.12 and 9.26.

M^+ at m/e 516 $\equiv C_{32}H_{52}O_5$.

15. ESTABLISHMENT OF D.Q.10 AS DESMALONYL- D.Q.9 BY LITHIUM ALUMINIUM HYDRIDE REDUCTION OF D.Q.10.

To $LiAlH_4$ (140 mg.) in dry ether (10 ml.) was added D.Q.10 (100 mg.) in dry ether (5 ml.). The reaction was stirred at room temperature for 36 hours. The excess reagent was destroyed by addition of 1N hydrochloric acid. The ethereal layer was washed successively with 4N hydrochloric acid (2 x 5 ml.) and brine (3 x 5 ml.) and then dried. Evaporation of the solvent gave a product (75 mg., 52%), m.p. 108-110°C. This was identical with a sample of the tetraols 1.20 prepared from D.Q.9.(i.r., n.m.r., mass spectrum, R_f., m.p. and mixed m.p.)

16. REACTION OF D.Q.10 WITH p-TOLUENESULPHONIC ACID.

D.Q.10 (100 mg.) and p-toluenesulphonic acid (10 mg.) were dissolved in dry benzene (20 ml.) and kept at room temperature for 36 hours. Water (10 ml.) was added and the layers separated. The benzene layer, after further washing with water (2 x 5 ml.) was dried and evaporated to yield a gum (80 g.). P.l.c. on a 20 x 20 x 0.1 cm. layer of Kieselgel H using chloroform as solvent separated the product 1.22 (40 mg., 43%) which crystallised from benzene as fine needles, m.p. 197-199°C. R_f ($CHCl_3$) 0.46. ν_{max} (KBr) 3550, 2970, 2947, 2885, 2820, 1767, 1467-1463, 1441,

1393, 1387, 1335, 1306, 1245, 1228, 1207, 1188, 1177, 1154, 1114, 1072, 1054, 1025 and 998 cm^{-1}.

ν_{max} (CCl_4) 3619 cm^{-1} (ε 41), 1765 cm^{-1} (ε 672)

τ values 5.66 (1H, t, J=8 c/s), 6.58 (1H, m), methyl contour 8.88, 8.94, 9.00, 9.04, 9.13 and 9.23.

Analysis Found C 76.57%
H 9.83%
M^+ at m/e 484 $C_{31}H_{48}O_4$

$C_{31}H_{48}O_4$ requires C 76.82%
H 9.98%
M.W. 484

17. D.Q.11 (METHYL 24-HYDROXYPOLYPORENATE C ?).

This compound was isolated as explained in Section 3. It crystallised from methanol as needles, m.p. 209-211°C.

$[\alpha]_D$ +102 (c 0.3). R_f (1% MeOH in $CHCl_3$) 0.10.

ν_{max} (KBr) 3350 (br.), 3025, 2850, 1710, 1689, 1625, 1447-1434, 1420, 1364, 1242, 1188-1184, 1145, 1089, 1030, 1003, 997, 976, 876 and 830 cm^{-1}.

ν_{max} (CCl_4) 3624 cm^{-1} (ε 72), 3609 cm^{-1} (ε 42), 1738 cm^{-1} (ε 640) 1718 cm^{-1} (ε 648)

τ values (60 Mc/s) 4.60 (2H, m), 5.28 (2H, d, J=4 c/s), 5.78 (1H, t, J=8 c/s), 6.32 (3H, s), 8.68 (6H, s), methyl contour 8.84, 8.94, 9.01, 9.05 and 9.42.

λ_{max} 228 mμ (infl.) (log ε 3.87), 237.5 mμ (log ε 4.03), 244.5 mμ (log ε 4.15), 252.5 mμ (log ε 3.91).

Analysis	Found	C 74.78%	M^+ at m/e 512
		H 9.57%	
$C_{32}H_{48}O_5$	requires	C 74.96%	M.W. 512
		H 9.44%	

18. PHOTOSENSITIZED OXYGENATION OF ERGOSTEROL.

All experiments in this series were carried out under similar conditions. Ergosterol (10 mg.) in a solution of methanol (10 ml.) containing the materials detailed below was irradiated for 18 hours with a 150 watt pearl bulb while oxygen was bubbled through the solution. The solution was then examined by t.l.c. Ergosterol and ergosterol peroxide could be readily detected by spraying with ceric ammonium nitrate in sulphuric acid solution and heating for 3 minutes at 140°C. With 1% methanol in chloroform as solvent, ergosterol gave a characteristic blue-black spot at R_f 0.40, while its peroxide appeared as a dark green spot at R_f 0.23. The results of these irradiation experiments are given in the following table, where the conversion factor is estimated from the relative amounts of ergosterol and its peroxide after 18 hours.

Solute in mg./10ml. of methanol.		Conversion factor (%).
Extract of D. Quercina	500 mg.	100
	100 mg.	100
	50 mg.	75
	10 mg.	25
Extract of P. Betulinus	500 mg.	100
	100 mg.	100

Solute in mg./10ml. of methanol.			Conversion factor (%).
Extract of P. Betulinus	50 mg.		80
	10 mg.		30
Eosin	0.1 mg.		100
	0.01 mg.		100
	0.001 mg.		50
Physcion or emodin	0.05 mg.		100
	0.01 mg.		75
Isoquinolones	0.1 mg.		100
	0.05 mg.		50
	Special Treatment.		
Extract of P. Betulinus	500 mg.	With exclusion of light	0
	500 mg.	No oxygen	0
	500 mg.	Refluxed at pH 1 for 24 hours	100
	500 mg.	Refluxed at pH 12 for 24 hours	100

19. SYNTHESIS OF CARBOMETHOXYACETYL CHLORIDE

a. Potassium carbomethoxyacetate

To dimethyl malonate (60 g.) in methanol (100 ml.) was added, dropwise, a solution of potassium hydroxide (24 g.) in methanol (200 ml.). After 24 hours ether (50 ml.) was added. The precipitated potassium carbomethoxyacetate was recovered by filtration (40 g., 75%), m.p. 204-206°C.

b. Carbomethoxyacetyl chloride.

Redistilled thionyl chloride (25 g.) in dry ether (100 ml.) was dropped into a chilled suspension of potassium carbomethoxyacetate (32 g.) in anhydrous ether (200 ml.). The mixture was kept at 0°C for a further 6 hours and then allowed to stand at room temperature for 18 hours. Removal of the potassium sulphate by filtration yielded a brown oil (22g.). Distillation gave carbomethoxyacetyl chloride, b.p. 56-60°C. (15 mm.) (6 g., 29%). (Literature[112] b.p. 57-59°C. (12 mm.))

ν_{max} (film) 1795 cm^{-1}, 1740 cm^{-1}.

20. CHLOESTEROL CARBOMETHOXYACETATE.

A solution of cholesterol (0.5 g.) and dry pyridine (2 ml.) in anhydrous ether (5 ml.) was added, dropwise, to a solution of carbomethoxyacetyl chloride (3 g.) in ether (5 ml.). The reaction was stirred for 2 hours at room temperature. Water (15 ml.) was

added and, after washing with 6N hydrochloric acid (4 x 10 ml.) and drying, evaporation of the solvents yielded a semi-solid (0.6 g.). P.l.c. on four 20 x 20 x 0.1 layers of Kieselgel H using benzene as solvent gave pure cholesteryl carbomethoxyacetate (0.42 g., 86%) which crystallised from ethyl acetate/light petroleum as needles, m.p. 108-109°C. R_f ($CHCl_3$) 0.78.

ν_{max} (nujol) 1753, 1728, 1283, 1219, 1150, 1037 and 1020 cm^{-1}.

ν_{max} (CCl_4) 1759 cm^{-1} (ε 570), 1739 cm^{-1} (ε 697)

τ values (60 Mc/s, CCl_4) 540 (1H, m), 6.25 (3H, s), 6.79 (2H, s)

Analysis	Found	C 76.47%	M^+ at m/e 486
		H 10.50%	
$C_{31}H_{50}O_4$	requires	C 76.50%	M.W. 486
		H 10.35%	

21. DIHYDROLANOSTERYL CARBOMETHOXYACETATE.

An esterification similar to that described in Section 20 was carried out using dihydrolanosterol (0.5 g.). P.l.c. yielded pure dihydrolanosteryl carbomethoxyacetate (0.46 g., 96%) which crystallised from methanol as needles, m.p. 94-96°C.

R_f ($CHCl_3$) 0.78.

ν_{max} (nujol) 1751, 1728, 1282, 1220, 1150, 1027 and 1007 cm^{-1}.

ν_{max} (CCl_4) 1758 cm^{-1} (ε 446), 1738 cm^{-1} (ε 490)

τ values (60 Mc/s, CCl_4) 5.46 (1H, m), 6.22 (3H, s), 6.59 (2H, s)

Analysis	Found	C 77.03%	M^+ at m/e 528
		H 10.57%	
$C_{34}H_{56}O_4$	requires	C 77.22%	M.W. 528
		H 10.67%	

22. ATTEMPTED PREPARATION OF MALONYL MONOCHLORIDE.

a. To malonic acid (10.4 g.) in dry ether (30 ml.) was added redistilled thionyl chloride (11.8 g.) in anhydrous ether (15 ml.). The mixture was refluxed gently for 6 hours under an atmosphere of nitrogen. Evaporation of the solvents yielded a brown gum (15 g.). This was extracted with a chloroform : light petroleum mixture (2 : 1) (30 ml.) and the extract cooled. When no solid appeared the extract was evaporated to yield malonic acid, identified by i.r. (nujol).

b. To malonic acid (10.4 g.) in water (50 ml.) was added 4N sodium hydroxide (25 ml.). Removal of the water by distillation yielded the disodium salt (14.1 g., 96%). This was washed with methanol and thoroughly dried. Oxalyl chloride (1.2 g.) was added, dropwise with cooling, to disodium malonate (2.2 g.) suspended in dry benzene (15 ml.) with a trace of pyridine (0.1 ml.) After gentle heating for 30 minutes the mixture was allowed to stand at room temperature for 12 hours. Evaporation of the solvents yielded an intractable brown oil which could not be

distilled or solidified. The i.r. spectrum (film) showed no high carbonyl peak.

23. MALONYL DICHLORIDE.

Under a nitrogen atmosphere, phosphorus pentachloride (20 g.) was added to malonic acid (10 g.) in dry benzene (5 ml.). When the vigorous reaction had subsided the solution was refluxed for 45 minutes. Distillation removed the benzene and yielded pure malonyl dichloride b.p. (15 mm.) 49-51°C. (3.2 g., 43%). (Literature[115] b.p. 47°C. (15 mm.))

ν_{max} (film) 1790 cm^{-1}.

24. DICHOLESTERYL MALONATE.

To cholesterol (9.5 g.) in anhydrous ether (100 ml.) and pyridine (0.7 g.), cooled in ice, was added, dropwise with stirring, malonyl dichloride (1.4 g.) in dry ether (5 ml.). After allowing it to warm to room temperature, the purple solution was stirred for 12 hours. Evaporation of the solvents yielded a pale pink solid (8.25 g.) which was adsorbed from light petroleum on to silicic acid (500 g.). Elution with 50% benzene in light petroleum gave dicholesteryl malonate (6.3 g., 84%) which crystallised from ethyl acetate/light petroleum as needles, m.p. 180-182°C. R_f ($CHCl_3$) 0.82.

ν_{max} (nujol) 1755, 1728, 1150, 1035 and 1020 cm^{-1}.

ν_{max} (CCl_4) 1755 cm^{-1} (ε 523), 1737 cm^{-1} (ε 684)

τ values 5.25 (2H, m), 6.67 (2H, s)

Analysis Found C 81.17%

H 11.27%

$C_{57}H_{92}O_4$ requires C 81.36%

H 11.02%

25. ATTEMPTED PARTIAL HYDROLYSIS OF DICHOLESTERYL MALONATE.

A solution of potassium hydroxide (0.01 g.) in methanol (20 ml.) was added, dropwise, to dicholesteryl malonate (0.5 g.) in methanol (10 ml.). The reaction was stirred for 6 hours at room temperature. The solid (0.21 g.) was filtered off and shown, by t.l.c., to be cholesterol. R_f ($CHCl_3$) 0.37. Evaporation of the filtrate yielded unchanged dicholesteryl malonate (0.24 g.). Upon variation of the solvent to benzene-methanol or the base to barium hydroxide similar results were obtained.

26. t-BUTYL CARBOXYACETATE.

Sodamide was freshly prepared by dissolving sodium (13.8 g.) in an excess of liquid ammonia (400 ml.). To this solution was slowly added t-butyl acetate (34 g.) in dry ether (100 ml.). When addition was complete the mixture was placed on a steam bath and the remaining ammonia boiled off, as ether (300 ml.) was added. The reaction was cooled to room temperature and, after

addition of powered carbon dioxide (150 g.), was allowed to stand at room temperature for 12 hours. Water (200 ml.) was added and the aqueous phase separated. This was neutralised, with cooling, with 36N hydrochloric acid (100 ml.) and then extracted with ether (2 x 75 ml.). After washing with saturated sodium carbonate (2 x 30 ml.) and brine (25 ml.) the solution was dried and evaporated to yield a clear oil (26 g.). Distillation under reduced pressure gave pure t-butyl carboxyacetate (32 g., 66%), b.p. (0.09 mm.) 78-80°C.

ν_{max} (film) 3300 (br.), 1740, 1720, 1150 and 985 cm^{-1}.

τ values 1.18 (1H, m), 6.65 (2H, s), 8.52 (9H, s).

27. CHOLESTERYL CARBO-t-BUTOXYACETATE.

p-Toluenesulphonyl chloride (1.3 g.) was added to t-butyl carboxyacetate (0.6 g.) in dry pyridine (10 ml.). This mixture was chilled in ice and a solution of cholesterol (1.3 g.) in anhydrous ether (10 ml.) was added, dropwise with stirring. After stirring at 0°C. for a further 4 hours, the reaction mixture was poured into iced water (50 ml.). The gummy solid was filtered off, taken up in ether (25 ml.), washed with water (2 x 10 ml.) and dried. Removal of the solvent yielded a clear gum which, on trituration with methanol, gave a white solid (1.6 g.).

Chromatography on silicic acid (50 g.), eluting with 25% light petroleum in benzene, gave cholesteryl carbo-t-butoxyacetate (1.2 g., 61%) which crystallised from methanol as plates, m.p. 87-88.5°C. R_f ($CHCl_3$) 0.79.

ν_{max} (nujol) 1733, 1719, 1282, 1213, 1152, 1036 and 1021 cm^{-1}.

ν_{max} (CCl_4) 1748 cm^{-1} (ε 592), 1731 cm^{-1} (ε 693)

τ values 5.45 (1H, m), 6.88 (2H, s), 8.57 (9H, s)

Analysis Found C 77.29% H 10.48% M^+ at m/e 518

$C_{34}H_{56}O_4$ requires C 77.22% H 10.67% M.W. 518

28. CHOLESTERYL CARBOXYACETATE.

Cholesteryl carbo-t-butoxyacetate (0.2 g.) in anhydrous benzene (2 ml.) was cooled in ice and dry hydrogen chloride bubbled through the solution for 20 minutes. The flask was stoppered and the reaction was kept at 0°C. for a further 6 hours. Removal of the solvent gave cholesteryl carboxyacetate (165 mg., 94%) which crystallised from benzene as needles, m.p. 155-156°C. R_f (10% MeOH in $CHCl_3$) 0.42.

ν_{max}(nujol) 3400 (br.), 1744, 1712, 1693, 1204, 1182, 1032 and 1018 cm^{-1}.

ν_{max}(CCl_4) 1749 cm^{-1} (ε 494), 1719 cm^{-1} (ε 568)

τ values 0.80 (1H, br.s), 6.55 (2H, s)

Analysis Found C 76.40%

H 10.24%

$C_{30}H_{48}O_4$ requires C 76.23%

H 10.24%

29. DIHYDROLANOSTERYL CARBO-t-BUTOXYACETATE.

Dihydrolanosterol (1.4 g.) was treated in a similar manner to that described in Section 27. After chromatography of the crude product (1.5 g.) on silicic acid (50 g.), using 50% benzene in light petroleum as eluent, dihydrolanosteryl carbo-t-butoxyacetate (1.1 g., 54%) was obtained from methanol as needles, m.p. 81-83°C. R_f ($CHCl_3$) 0.80.

ν_{max} (nujol) 1732, 1713, 1281, 1217, 1153, 1024 and 1009 cm^{-1}.

ν_{max} (CCl_4) 1748 cm^{-1} (ε 593), 1732 cm^{-1} (ε 687)

τ values 5.43 (1H, m), 6.72 (2H, s), 8.54 (9H, s)

Analysis Found C 78.14%

H 11.12%

M^+ at m/e 570

$C_{37}H_{62}O_4$ requires C 77.84%

H 10.95%

M.W. 570

30. DIHYDROLANOSTERYL CARBOXYACETATE.

Treatment of dihydrolanosteryl carbo-t-butoxyacetate (250 mg.) with dry hydrogen chloride in the manner described in Section 28

yielded dihydrolanosteryl carboxyacetate (186 mg., 92%) which crystallised from methanol as needles, m.p. 158-160°C. R_f (10% MeOH in $CHCl_3$) 0.42.

ν_{max} (nujol) 3300 (br.), 1732, 1713, 1209, 1180, 1148, 1027 and 1006 cm^{-1}.

ν_{max} (CCl_4) 1755 cm^{-1} (ε 484), 1720 cm^{-1} (ε 578)

τ values 1.63 (1H, m), 5.48 (1H, m), 6.60 (2H, s)

Analysis Found C 76.93%

H 10.43%

$C_{33}H_{54}O_4$ requires C 77.00%

H 10.57%

31. TREATMENT OF THE CARBOXYACETATES WITH REFLUXING METHANOL.

a. Cholesteryl carboxyacetate (25 mg.) was refluxed in methanol (5 ml.) for 24 hours. After cooling, the solution was treated with excess ethereal diazomethane. Evaporation of the solvents and crystallisation from light petroleum/ethyl acetate yielded material (23 mg., 90%) which was identical (R_f., m.p., and mixed m.p.) with the cholesteryl carbomethoxyacetate prepared in Section 20.

b. Refluxing dihydrolanosteryl carboxyacetate (25 mg.) in methanol (5 ml.) for 24 hours and a similar treatment yielded

material (25 mg., 96%) which was identical (R_f., m.p., and mixed m.p.) to the dihydrolanosteryl carbomethoxyacetate prepared in Section 21.

32. BASE TREATMENT OF THE CARBOXYACETATES AND OF THE CARBOMETHOXYACETATES.

Cholesteryl carbomethoxyacetate (10 mg.) was allowed to stand at room temperature in 2N methanolic sodium hydroxide (4 ml.) for 30 minutes. 4N hydrochloric acid was added to neutrality and water (5 ml.) was added. After most of the methanol had been evaporated, the solution was ether extracted (5 ml.). T.l.c. in chloroform showed that the solid (6 mg.) recovered from this solution had the same R_f as cholesterol i.e. R_f ($CHCl_3$) 0.37. A similar treatment of cholesteryl carboxyacetate had the same result.

Dihydrolanosteryl carbomethoxyacetate or carboxyacetate under the same conditions yielded dihydrolanosterol. R_f ($CHCl_3$) 0.50. Treatment of dihydrolanosteryl acetate for the same length of time under similar conditions was shown by t.l.c. to effect only 50% hydrolysis.

PART II

THE METABOLITES OF LEPTOPORUS STIPTICUS

The most important result of the studies of the triterpene acids of P. betulinus and D. quercina was that they were, in the main, present as conjugated species rather than in the free state. Although the results of the survey carried out on other members of the Polyporaceae were disappointing, in that no other examples of the carbomethoxyacetate conjugating species could be detected, it was decided to examine one of these fungi in more detail.

The saprophytic organism Leptoporus stipticus (syn. Tyromyces albidus, Tyromyces stipticus, Polyporus stipticus) may be recognised by its characteristic white, hoof-shaped sporophores which appear on dead conifer timber. Fresh sporophores were collected from a fallen pine tree in the grounds of Dunkeld House Hotel in early September. After superficial cleaning, the tissue was extracted with cold and hot methanol. The combined extracts were treated with light petroleum to remove lipids (14% by weight). On evaporation of the solvent, a small quantity of crystalline acid was obtained. The major part of the material, a brown gum, was dissolved in methanol and treated with an excess of ethereal diazomethane. In the following discussion, the methyl esters of the five major metabolites are referred to as methyl tyromycate, methyl leptoporate and by the

TABLE II - 1. T.L.C. CHARACTERISTICS OF THE ESTERS OF L. STIPTICUS

	R_f		Kieselgel HF_{254}	Ceric ammonium nitrate/H_2SO_4
	100% $CHCl_3$	10% MeOH in $CHCl_3$		
methyl tyromycate	0.63	-	purple	brown
methyl leptoporate	0.31	-	purple	dark green
L.S.II	0.00	0.30	purple	green
L.S.III	0.00	0.44	purple	red
L.S.IV	0.00	0.37	purple	mauve

code names L.S.II, L.S.III and L.S.IV.

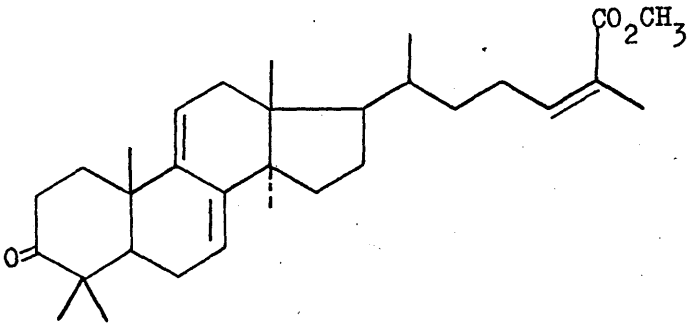
These compounds were defined chromatographically as shown in Table II - 1, i.e. by their R_f on Kieselgel H and on Kieselgel HF_{254}, by their characteristic light absorption when viewed in u.v. light (λ 254 mμ) and by their characteristic staining behaviour when sprayed with a solution of ceric ammonium nitrate/ sulphuric acid and heated at 140°C for 2 minutes.

Some difficulties were encountered in the separation of the five compounds. The two non-polar esters could be relatively easily separated by column chromatography of the mixture on silicic acid. This method was, however, extremely inefficient for the three polar esters since it incurred a serious loss of material and provided almost no separation. Preparative layer chromatography, although effecting a visible separation, led to even greater loss of material. A major step forward was the discovery that a partition system of light petroleum/ water/methanol (2:1:3) would completely separate the mixture into two parts, the light petroleum layer containing methyl tyromycate and methyl leptoporate, and the aqueous layer containing the polar esters L.S.II, L.S.III and L.S.IV. Methyl tyromycate and methyl leptoporate could then be completely separated by chromatography on silicic acid employing a gradient

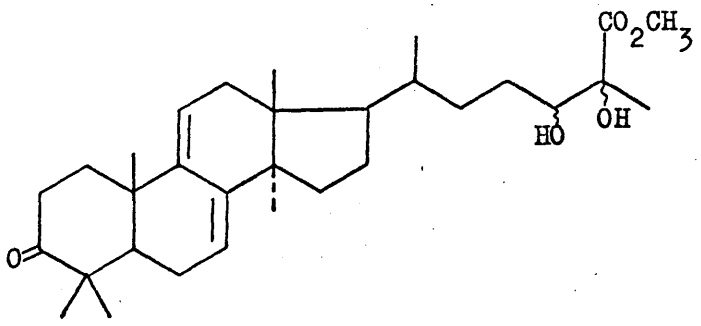
of light petroleum to 30% ethyl acetate in light petroleum. It is proposed to discuss the structures of these esters first.

The major component methyl tyromycate, $C_{31}H_{46}O_3$, was a crystalline solid, m.p. 154-156°C. General similarities in its physical data to those of previously encountered compounds indicated that it was a triterpenoid methyl ester. The u.v. spectrum showed the triple peaks at 235.5, 244 and 252 mμ which are characteristic of the lanosta-7,9(11)-diene skeleton. It was noticed, however, that the intensity of absorption below 235 mμ was higher than that expected for the diene system. This demonstrated the presence of another chromophore. That this was an α,β-unsaturated methyl ester was suggested by other spectroscopic properties. From the i.r. spectrum the presence of a double bond (1650 cm^{-1}), other than the diene system (1601 cm^{-1}), and two carbonyl groups, superimposed at 1706 cm^{-1} (ε 746), could be deduced. One of these was a ketone since methyl tyromycate formed a 2,4-dinitrophenylhydrazone, $C_{37}H_{50}O_6N_4$, m.p. 208-209°C. The other was thought to be an α,β-unsaturated methyl ester function in view of the methoxyl resonance appearing at 6.24τ.

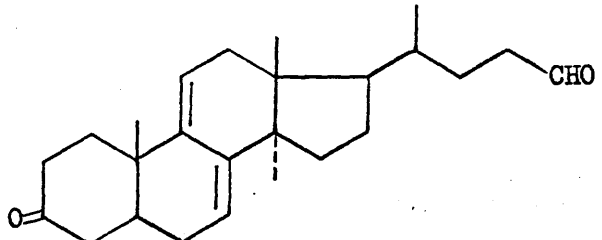
Confirmation of this conjugated system was obtained by hydrogenation to the dihydro compound, $C_{31}H_{48}O_3$. The u.v.



2.1



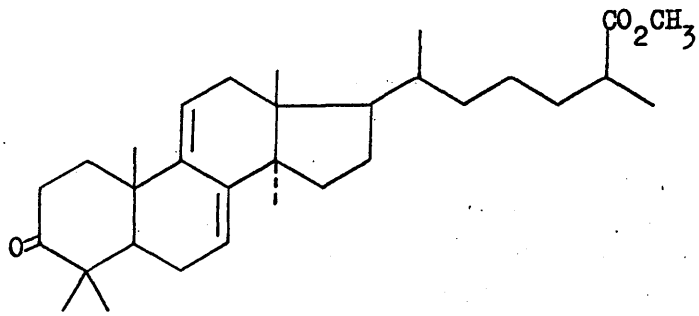
2.2



2.3

spectrum exhibited the triple maxima with the extinction coefficients expected for a simple lanosta-7,9(11)-diene system. The i.r. spectrum now showed a saturated methyl ester (1727 cm^{-1}) and the 6-membered ring ketone (1703 cm^{-1}) and no strong double bond absorption at 1650 cm^{-1}. By subtraction of the u.v. spectrum of methyl dihydrotyromycate from that of the parent compound, it was possible to obtain the absorption of the α,β-unsaturated ester (λ_{max} 222 mμ, ε 4140). The nature of the environment of this unsaturated ester could be determined from the n.m.r. spectra of methyl tyromycate and its hydrogenation product. A fine doublet in the former at 8.08τ (J=2 c/s) was assigned to a vinyl methyl group, while a broadened triplet at 4.00τ was thought to be due to a vinyl proton, the broadening being caused by allylic coupling with the methyl group. Both these features were absent in the spectrum of the dihydro compound whereas the multiplet at 4.50τ, caused by the vinyl protons of the diene system, was retained. On this evidence it was possible to postulate structure 2.1 for methyl tyromycate, since it would be expected that the third oxygen function would be at C-3.

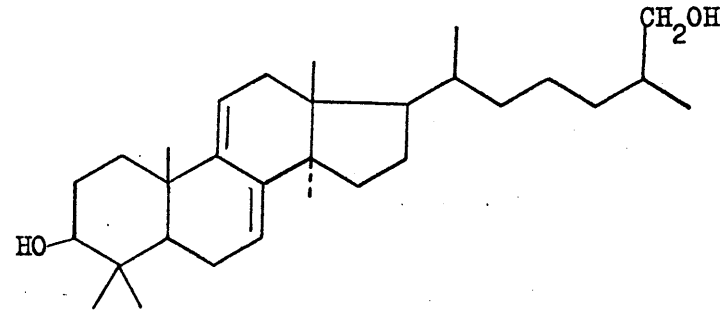
Confirmation of the position of the double bond was obtained as a result of the sequence 2.1 → 2.2 → 2.3. Treatment of methyl tyromycate with osmium tetroxide in ether, with a trace



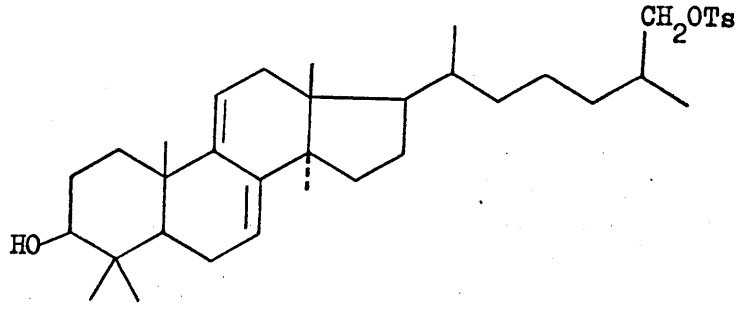
2.4

of pyridine, led to the black osmate ester. This was cleaved, without isolation, by treatment with gaseous hydrogen sulphide to give the glycol 2.2. The spectroscopic features of the α,β-unsaturated ester were no longer present. The i.r. spectrum showed two bonded alcohol groupings (3564 cm^{-1} and 3522 cm^{-1}) and two distinct carbonyl groups, the ester at 1736 cm^{-1} and the ketone at 1712 cm^{-1}. The deshielded methyl group, formerly a doublet at 8.08τ, was now a sharp singlet at 8.57τ, the expected value for such a grouping when α to an oxygen function. The ultra violet spectrum was again untrammelled lanosta-7,9(11)-diene absorption. Treatment of this compound with sodium metaperiodate led to the aldehyde 2.3, $C_{27}H_{40}O_2$. The aldehydic proton at 0.15τ and the diene 2H-multiplet at 4.51τ were the only low field signals in the n.m.r. spectrum. The i.r. spectrum showed a single carbonyl absorption at 1705 cm^{-1} (ε 840). On the scale of the experiment, attempts to isolate the C_4 fragment from the crude reaction mixture as its 2,4-dinitrophenylhydrazone were unsuccessful.

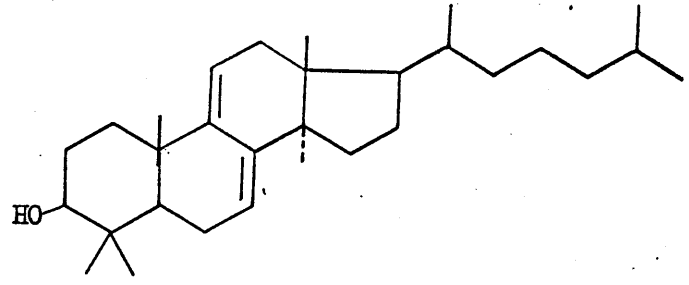
Final proof of the structure of methyl tyromycate was obtained by its degradation to ɣ-lanosterol (2.7) by the route 2.1 → 2.4 → 2.5 → 2.6 → 2.7. Reduction of the methyl dihydro-tyromycate (2.4) with lithium aluminium hydride yielded the diol



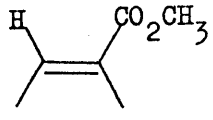
2.5



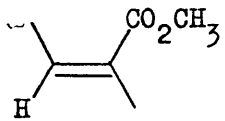
2.6



2.7



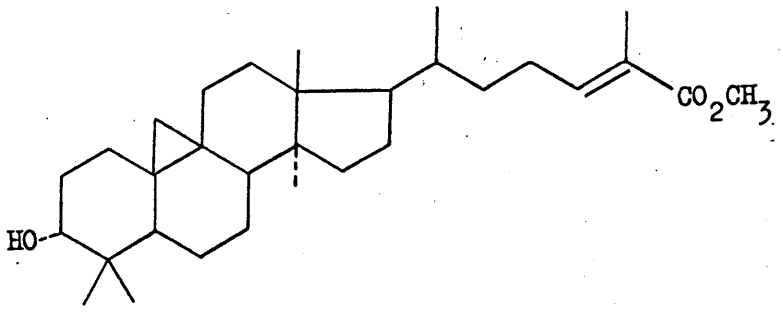
2.8



2.9

2.5, $C_{30}H_{50}O_2$. The i.r. spectrum confirmed the removal of the carbonyl functions and the presence of two hydroxyl groupings at 3615 cm^{-1} (ε 107). In the n.m.r. spectrum, the 3β-alcohol gave rise to a broad multiplet (half-band width 15 c/s) at 6.64τ due to the 3α-proton, while the C-26 methylene group of the primary alcohol appeared as a doublet at 6.42τ (J=6 c/s). Treatment of the diol 2.5 with a unimolar quantity of p-toluenesulphonyl chloride in pyridine with cooling gave mainly the monotosylate 2.6, $C_{37}H_{56}SO_4$, the resonance of the 3α-proton being unaffected while that due to the carbon-bound protons of the primary alcohol grouping was lowered to 6.00τ, thus establishing the position of the tosylate residue. Lithium aluminium hydride of this compound converted the primary tosylate to a methyl group and produced ϒ-lanosterol (2.7). This same substance was prepared from a sample of dihydrolanosterol[121] by acetylation, oxidation to the diene acetate with selenium dioxide and hydrolysis[122].

The stereochemistry of the 24(25)-double bond could be ascertained from the position of the C-24 vinyl proton by comparison with model systems. The vinyl proton of methyl tiglate (2.8), which is cis to the ester grouping and therefore deshielded by it, resonates at 3.27τ. The trans vinyl proton of methyl angelate (2.9) does not show this effect and appears at 4.12τ.



2.10

This comparative approach has been shown to be valid in the triterpene field with respect to methyl mangiferolate (2.10) (3.37τ) and the corresponding trans isomer methyl masticadienoate (4.19τ)[123]. Spin decoupling experiments determined the position of the C-23 methylene group as 7.67τ. This group was, as expected, deshielded. These results established the stereochemistry of the double bond in methyl tyromycate as that shown in 2.1.

The small amount of acidic material isolated before methylation of the extract was now examined. By crystallisation from methanol the pure acid $C_{30}H_{44}O_3$ could be isolated. The spectral properties of this acid (n.m.r. virtually identical to that of methyl tyromycate, save for the methoxyl resonance, identical u.v. and carbonyl absorption at 1711 and 1690 cm^{-1}) showed that it was tyromycic acid. This was confirmed by methylation with diazomethane to give the previously obtained ester.

The behaviour of tyromycic acid on melting is worthy of note. When crystallised from methanol, lustrous plates were obtained. On heating these crystals on the hot-stage it was found that, at 119-121°C, they completely liquified but, even with further heating the substance resolidified in the form of fine needles which remained intact until 176-178°C. The resulting liquid

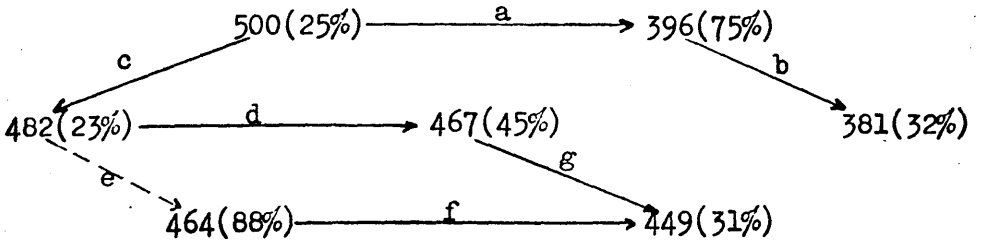
TABLE II - 2. PATTERN OF IONS AT LOW m/e.

187	173	159	147	135	123	109
185	171	157	145	133	121	107
183	169	155	143	131	119	105
				129		

was allowed to cool and its melting point redetermined and found to be 174-177°C. This could be explained by the first, low melting material containing water of crystallisation. The analytical figures tended to support this view since, despite drying under high vacuum at relatively high temperature (70°C), the results obtained could only be reconciled with the formula $C_{30}H_{44}O_3.H_2O$.

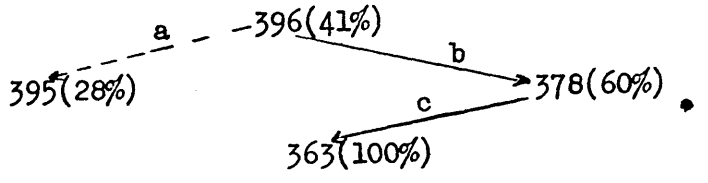
The results of a study of the mass spectral fragmentation of tyromycic acid, methyl tyromycate and its degradation products proved of great value in the structural elucidation of the other components of Leptoporus stipticus. The most notable feature was that all eight compounds showed an almost identical pattern below m/e 200, having the groups of peaks given in Table II - 2. It will be seen that these ions are very similar to those observed for the compounds of Daedalea quercina, collected in Table I - 5. Again no pathways leading to these fragment ions can be suggested, but it may be said that those groups of peaks appear to be characteristic of lanostane derivatives. A differentiation may be made in that the first four groups for lanost-8-ene compounds appear at two mass units higher than the corresponding groups for lanosta-7,9(11)-diene derivatives. For further discussion of the mass spectra it is convenient to divide the

TABLE II - 4 PROPOSED MASS SPECTRAL BREAKDOWN OF 2.2



Transition	Δ^m/e	Metastable ion
a	104	310.1
b	15	366.3
c	18	463.4
d	15	451.8
e	18	
f	15	434.3
g	18	429.9

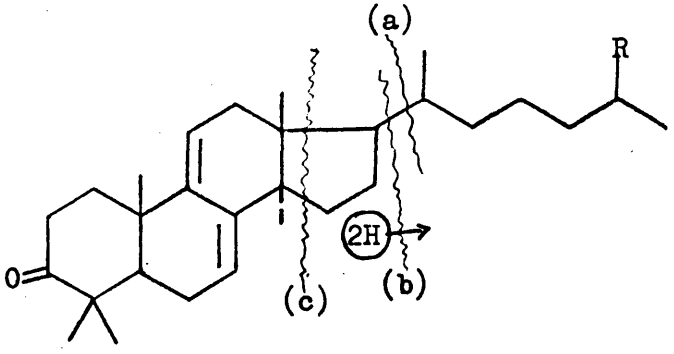
TABLE II - 5 PROPOSED MASS SPECTRAL BREAKDOWN OF 2.3



Transition	Δ^m/e	Metastable ion
a	1	
b	18	362.0
c	15	348.3

TABLE II - 3. PERCENTAGE ABUNDANCE OF THE MASS SPECTRAL FRAGMENTS IN THE TYROMYCIC ACID SERIES.

m/e	Methyl tyromycate	Tyromycic acid	Glycol 2.2	Aldehyde 2.3	Methyl dihydro-tyromycate
M^+	100	100	25	41	100
$M^+ -15$	17	12	7	15	18
311	24	17	52	26	30
309	28	12	30	98	9
269	50	37	100	86	65



2.11

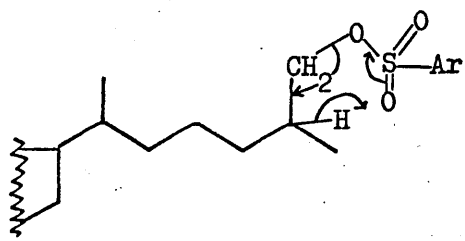
compounds into two groups, those having a 3-ketone function and those bearing a β-alcohol at C-3. The former group, tyromycic acid, its methyl ester and products 2.2, 2.3 and 2.4, all showed loss of a methyl radical and abundant ions at m/e 311, 309 and 269, as shown in Table II - 3. These may be explained by the cleavages shown in 2.11, involving loss of side chain (a), loss of side chain and two protons (b), and 'steroid-type' fission of the D ring (c).

These are the only important fragmentations of methyl tyromycate and its dihydro compound, but the α,β-unsaturated acid undergoes, in addition, decarboxylative loss of 44 mass units to give an ion at m/e 408 (20%). The functionality in the glycol 2.2 and the aldehyde 2.3 causes the additional patterns shown in Tables II - 4 and II - 5 respectively. The glycol 2.2, as might be predicted, undergoes two successive losses of 18 mass units. Also an important process is cleavage between the two alcohol groups, with hydrogen transfer, giving the rearranged ion at m/e 396.

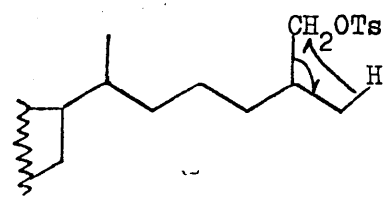
The 3β-hydroxy compounds formed in the degradation of methyl tyromycate behaved in a similar manner under ion impact, in that analogous cleavages to those shown in 2.11 occurred to produce ions at m/e 313, 311 and 271 (Table II - 6). It was also seen

TABLE II - 6. PERCENTAGE ABUNDANCE OF THE MASS SPECTRAL FRAGMENTS FOR COMPOUNDS WITH 3β-ALCOHOL.

m/e	Diol 2.5	Monotosylate 2.6	γ-Lanosterol
M^+	100	87	100
M^+ -15	26	97	26
313	5	14	17
311	4	10	18
271	8	33	70
M^+ -33	18	100	18
253	6	11	39



2.12



2.13

that in all three cases there was a loss of eighteen mass units (H_2O) from the M^+ -15 ion and from the ion at m/e 271. Other modes of breakdown of these substances depended on the functionality in the side chain. The ions in Table II - 6 are the only important peaks in the high mass region (>m/e 250) of the spectrum of γ-lanosterol (2.7), which has a completely saturated side chain. The diol 2.5, which has a primary alcohol function, undergoes an additional loss of eighteen mass units both from the parent ion and from M^+ -15. The additional ions in the mass spectrum of the monotosylate 2.6 at m/e 426 (18%) and 412 (30%) may be rationalised in terms of the rearrangements shown in 2.12 and 2.13, while the latter product loses a further eighteen mass units to give an ion at m/e 394 (20%).

A consideration of the saturated methyl region of the n.m.r. spectra of methyl tyromycate and its degradation products proved to be extremely useful in helping to assign structures to the other four compounds isolated from L. stipticus. By consultation with literature values [90] it was possible to assign all tertiary methyl resonances in the spectrum of methyl tyromycate, as shown in Table II - 7. As will be seen from the other data given in the table, these values remained almost constant in all compounds with a ketone at the 3-position. This was to be

TABLE II - 7. N.M.R. POSITIONS OF NUCLEAR METHYL GROUPS IN THE METHYL TYROMYCATE SERIES.

	C-18	C-19	C-32	C-30/C-31
Methyl tyromycate	9.42	8.81	9.12	8.89,8.92
Tyromycic acid	9.41	8.80	9.12	8.87,8.91
Glycol 2.2	9.40	8.80	9.13	8.88,8.92
Aldehyde 2.3	9.41	8.81	9.12	8.88,8.91
Methyl dihydrotyromycate	9.41	8.80	9.12	8.87,8.91

TABLE II - 8. N.M.R. POSITIONS OF NUCLEAR METHYL GROUPS IN COMPOUNDS WITH 3β-ALCOHOL.

	C-18	C-19	C-32	C-30/C-31
Diol 2.5	9.42	9.01	9.14	9.03,9.14
Monotosylate 2.6	9.42	8.98	9.16	9.03,9.16
ϒ-Lanosterol	9.41	9.01	9.18	9.03,9.12
Predicted[89,90]	9.37	9.01	9.18	9.04,9.20

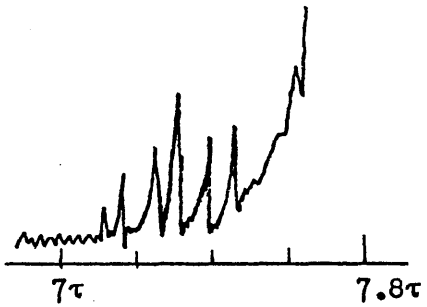
expected since alterations in the side chain would not be expected to affect nuclear angular methyl groups. However, as soon as the carbonyl group on C-3 was reduced, the deshielding effect associated with it disappeared, thus the resonances of the methyl groups at C-4 and C-10 moved upfield to the values given in Table II - 8. It was noted, with satisfaction, that the predicted values, which are included in the table, agreed with the observed signals.

Consideration of other features of the n.m.r. spectra yielded two other useful facts. Firstly, it was seen that the tertiary methyl peak at 9.12τ was, in all cases, broadened at the base, while the integration for this peak corresponded to six protons. Since in methyl tyromycate there was only one saturated secondary methyl, this broadening was ascribed to the C-21 secondary methyl group. On determination of the spectrum of methyl tyromycate at 100 Mc/s, one half of this doublet could be clearly seen at 9.07τ. Irradiation at 8.46τ caused this peak to disappear, while the single peak at 9.12τ increased in intensity. This was strong evidence in support of the C-20 methine being at 8.46τ and the C-21 methyl doublet being centred at 9.12τ.

The other interesting feature could only be detected at a sweep width of 100 Mc/s. In the region of 7.3τ in the n.m.r.

TABLE II - 9 LOW FIELD MULTIPLET IN THE N.M.R.

SPECTRUM OF METHYL TYROMYCATE



spectra of both methyl tyromycate and its dihydro derivative could be seen six minor peaks in three groups of two, as illustrated in Table II - 9. These were thought to be due to one of the protons at C-2, though no complete explanation of the splitting pattern could be advanced. It was noticed, however, that methyl polyporenate C (ketone at C-3) exhibited these same peaks, whereas methyl dehydrotumulosate (3β-alcohol) did not. This would have been in agreement with one of the C-2 protons, deshielded by the ketone at C-3, giving rise to the observed low field multiplet. A small quantity of methyl tyromycate was reduced to the corresponding 3β-alcohol. The 100 Mc/s spectrum of this substance showed that the multiplet under discussion had disappeared and further supported the conclusion that it was characteristic of a ketone function at C-3.

The second non-polar substance to be isolated from L. stipticus was then examined. Methyl leptoporate was a crystalline solid, m.p. 175-177°C. The formula $C_{33}H_{48}O_4$ was determined by high resolution mass spectrometry. The elemental analysis agreed with that of a monohydrate, even after high vacuum drying. A general consideration of the spectroscopic properties showed that this substance was closely related to methyl tyromycate, on the following evidence. The u.v. spectrum

TABLE II - 10. MASS SPECTRUM OF METHYL LEPTOPORATE.

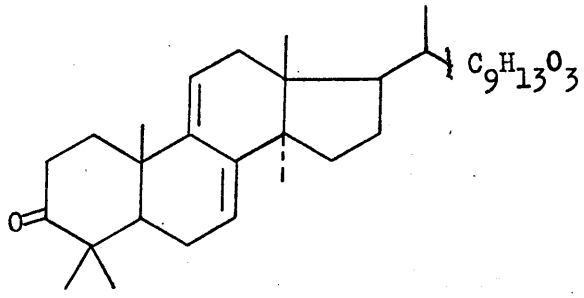
m/e	%
508	23
493	4
465	8
449	100
311	12
309	20
269	20
95	32

(228, 236, 244 and 252 mμ) agreed closely with that of methyl dihydrotyromycate i.e. a simple lanosta-7,9(11)-diene chromophore. An examination of the saturated methyl groups in the n.m.r. spectrum showed that they were almost superposable with those of methyl tyromycate. The values are shown below.

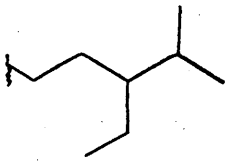
methyl tyromycate	8.81	8.89	8.92	9.12	9.42
methyl leptoporate	8.80	8.90	8.93	9.12	9.42

It was also noticed that the doublet due to the C-21 secondary methyl group was present at the same position. Again only one part of this doublet could be seen at 9.15τ. Irradiation at 8.41τ caused this signal to disappear and produced a single sharp peak at 9.12τ.

Other structural similarities that could be deduced from the n.m.r. spectrum were the diene system (4.50τ) and a methyl ester (6.25τ). The group of peaks at 7.3τ which, it had been suggested, demonstrated the presence of a ketone at C-3, were again observed. The i.r. spectrum confirmed the presence of a saturated ester (1737 cm^{-1}, ε 360) and a ketone (1704 cm^{-1}, ε 355). The mass spectrum of methyl leptoporate (the important ions are given in Table II - 10) showed notable similarities to that of methyl tyromycate and its derivatives. The ions collected in Table II - 2 were again present. In addition the ions at m/e



2.14



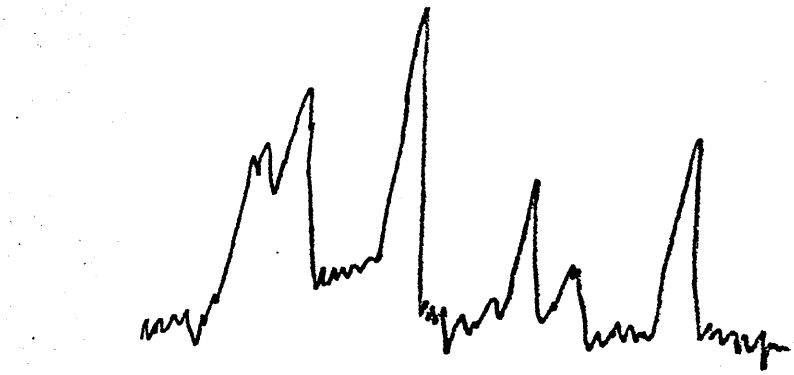
2.15

311, 309 and 269, which corresponded to loss of the side chain in the substances of the methyl tyromycate series bearing a ketone at C-3, were also detected.

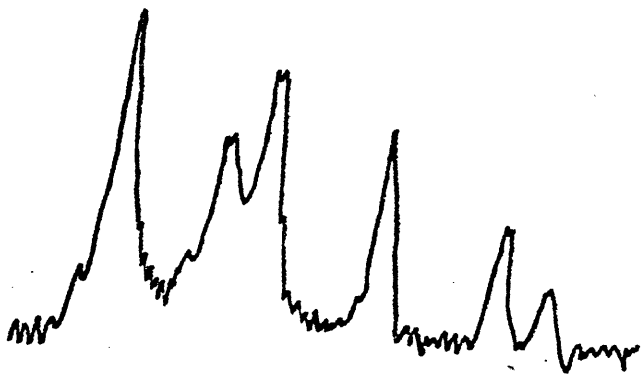
The spectroscopic evidence suggested that methyl leptoporate was similar to methyl tyromycate, at least up to C-20, and therefore had partial structure 2.14. Elaboration of the C_{30} lanostane structure to a C_{32} skeleton usually occurs, as discussed in the Introduction, by addition of a C_2 unit to C-24. Since methyl leptoporate was the methyl ester of a C_{32} compound it could be reasonably expected, on biogenetic grounds, that the fragment $C_9H_{13}O_3$ had the carbon skeleton shown in 2.15. The nature of this residue was then investigated. Two of the oxygen atoms had already been accounted for in the carbomethoxyl function. The i.r. spectrum showed that the third oxygen was not present as a hydroxyl grouping nor as a carbonyl function, since the former was absent and the position and intensity of the two peaks between 1650 - 1800 cm^{-1} corresponded to only a ketone and a methyl ester.

The conclusion that an ether grouping was present was confirmed by the n.m.r. spectrum. This showed, as illustrated in Table II - 11a, two sets of four peaks between 5 and 6τ, which could be assigned to a methylene grouping α to an oxygen

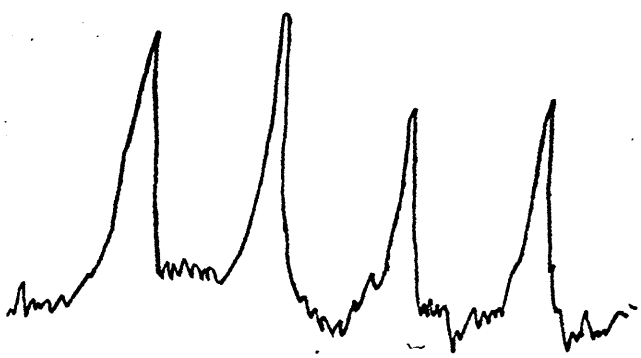
TABLE II - 12 N.M.R. SPIN TICKLING OF METHYL LEPTOPORATE



(c)

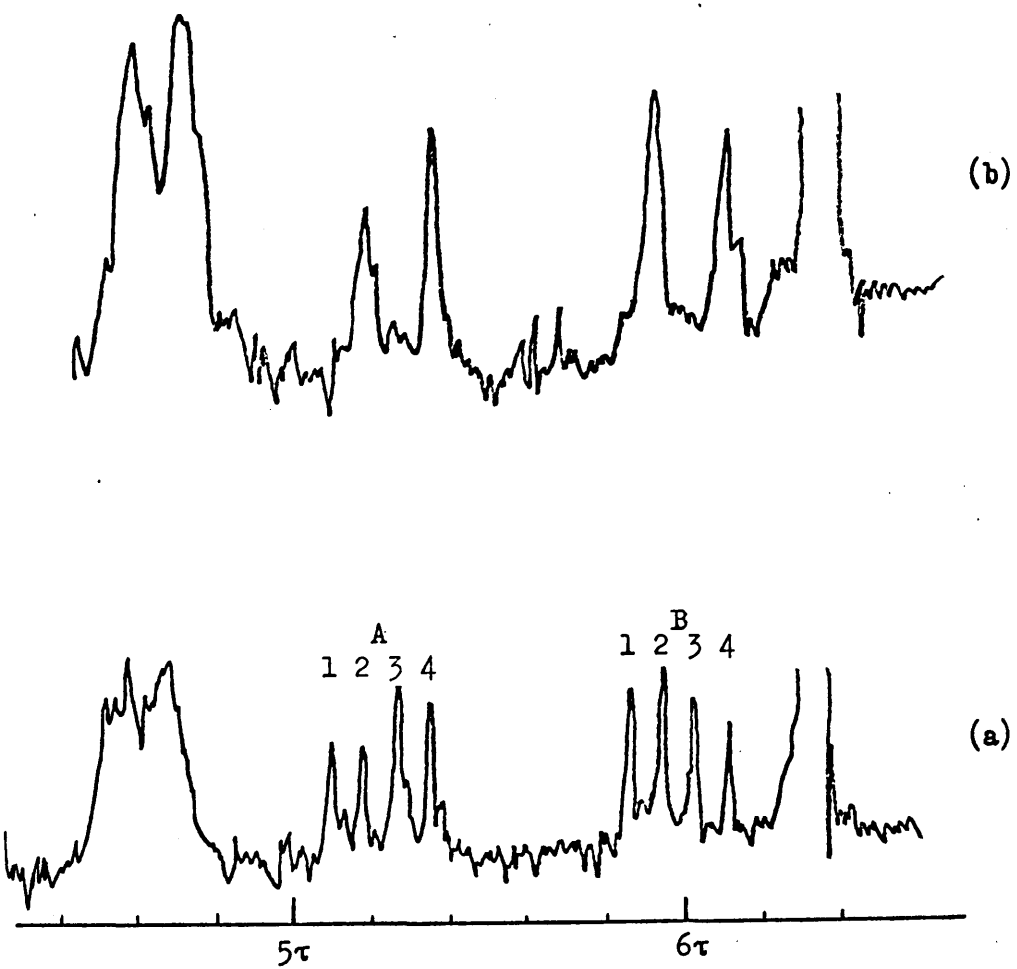


(b)



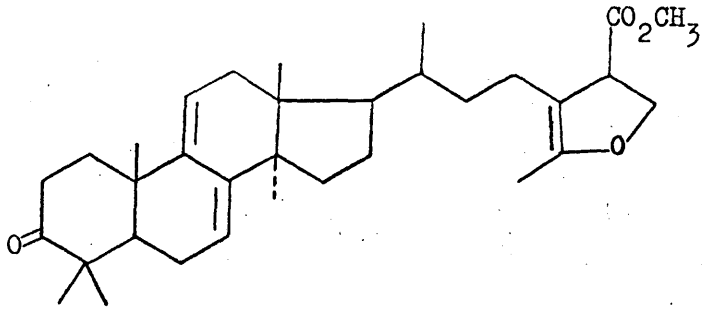
(a)

TABLE II - 11 N.M.R. DECOUPLING OF METHYL LEPTOPORATE

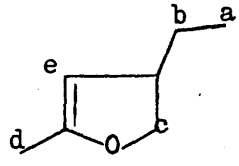


atom. These appeared as the AB part of an ABX system. N.m.r. tickling and decoupling studies established the part structure $O-CH_2-CH$ in the following manner. Irradiation at 8.09τ (the X proton) reduced the low field signals to the system shown in Table II - 11b. This was a simple AB system from which the coupling constant J=17 c/s was evaluated. It was also possible to deduce the chemical shift of each of the two protons as 5.20τ and 5.95τ , and, since δ/J was large, to obtain a value of $J_{AX}=J_{BX}=8$ c/s[124]. Table II - 12a shows the higher quartet of peaks (B) on a considerably expanded scale. II - 12b is the effect of applying a weak perturbing field to peak A_4, while II - 12c is the effect of a similar application to peak A_3. It has been established[125] that this result indicates that J_{AB} has a negative sign. Thus the large coupling constant and its negative sign were in agreement with a geminal AB system[126]. The shape of the system before irradiation indicated that only one proton (X) was splitting each part of the AB system. The τ values of the A and B protons were in the region expected for protons attached to a carbon α to an oxygen atom.

Another feature which had to be included in the side chain was the deshielded methyl group which appeared as a sharp singlet at 8.34τ. This suggested the presence of the system $CH_3-C=C$,



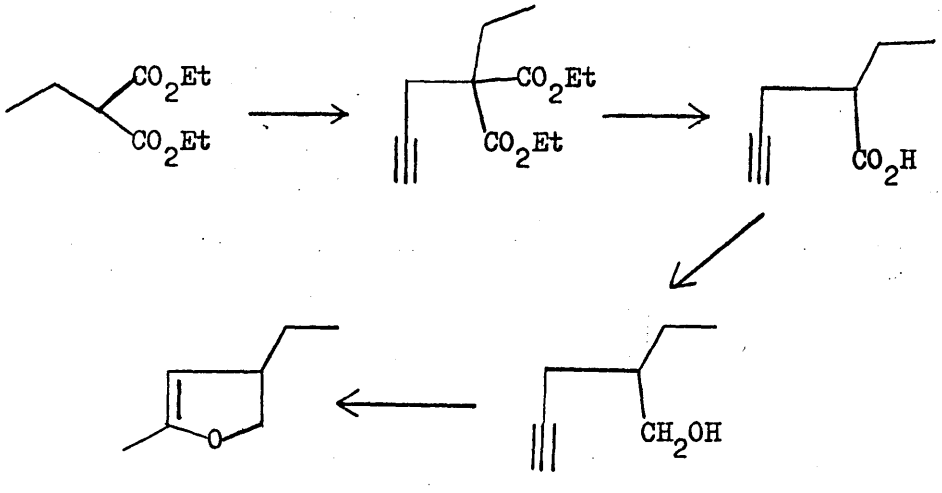
2.16



2.17

a	9.18 (t)
b	8.72 (q)
c	5.75,6.19 (AB of ABX)
d	8.32 (d)
e	5.58 (m)

TABLE II - 13 SYNTHETIC ROUTE TO 2.17



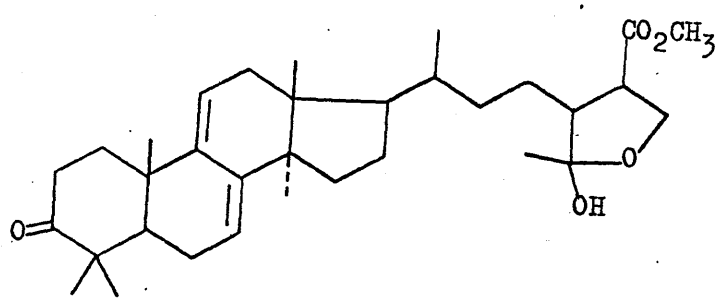
in which the double bond had to be tetrasubstituted, since no vinyl proton was visible in the n.m.r. spectrum. On incorporating the two part-structures discussed above and a saturated carbomethoxyl group into the fragment $C_9H_{13}O_3$ structure 2.16 was obtained as the unique solution for the structure of methyl leptoporate. It was noted that the equality of J_{AX} and J_{BX} was in agreement with the observation that J_{cis} and J_{trans} for coupling between the protons at C-2 and C-3 in 2,3-dihydrofuran are accidentally equal[128].

Since only a limited amount of the n.m.r. data was available for substituted 2,3-dihydrofurans[127], the synthesis of the model compound 2.17 was carried out. The literature route[129] which is shown in Table II - 13 was employed. The interpretation of its n.m.r. spectrum is shown in 2.17. The noteable features were that the value for the vinyl methyl group (8.32τ) lay close to that of methyl leptoporate (8.34τ) and that the two protons at C-2 appeared as the AB part of an ABX system, where A and B were quartets centred in the region of 5.75τ and 6.19τ. The greater separation between the A and B protons in methyl leptoporate was attributed to the effect of the carbonyl double bond in the carbomethoxyl grouping, since models showed that one of the protons under consideration was deshielded by it in both possible stereo-

isomers.

The base peak in the mass spectrum of methyl leptoporate at m/e 449 arose by loss of 59 mass units from the parent ion (metastable for this transition at m/e 396.0). Mass measurement of these two ions gave the formula $C_2H_3O_2$ for the fragment lost. This supported structure 2.16 since cleavage β to the dihydrofuran double bond to release $\dot{C}O_2Me$ would be expected to be a favoured fission.

It was expected that acid treatment would open the dihydrofuran ring to give a methyl ketone and a primary alcohol. No product could be isolated from an attempt to effect simultaneous hydration and oxidation using Jones reagent. Reaction with an ethanolic solution of 2,4-dinitrophenylhydrazine dihydrogensulphate gave an unstable product (possibly the bis derivative) in low yield. However, mild acid treatment in methanol at room temperature did produce a single product. This compound, $C_{33}H_{50}O_5$, of R_f ($CHCl_3$) 0.08, was a crystalline solid, m.p. 153-155°C. The n.m.r. spectrum of this substance indicated that the signals which had been associated with the dihydrofuran ring were considerably altered. The deshielded methyl group had moved upfield to 8.50τ while the AB part of the ABX system was now superimposed upon the methoxyl peak at 6.27τ. Otherwise the spectrum was



2.18

almost unchanged, the saturated methyl regions, 8.8 - 9.5τ, being superposable. The diene system was unaffected, giving rise to a multiplet at 4.51τ and absorbing at 236, 243.5 and 252 mμ in the u.v.. The i.r. spectrum showed the carbonyl peaks due to the ester (1731 cm^{-1}) and ketone (1703 cm^{-1}) as before, but also indicated the presence of a hydroxyl grouping (3624 cm^{-1}). The mass spectrum was identical to that of methyl leptoporate.

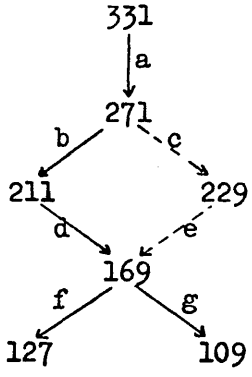
These facts were best interpreted by proposing structure 2.18 for the compound. This could very readily lose water, either thermally or by electron impact, to give the mass spectrum observed. 2.18 might also explain the changes in the n.m.r. spectrum, since the methyl group at 8.50τ was in the expected region for such a grouping when α to two oxygen atoms (cf. acetal 8.67τ)[130]. The upfield movement of the AB part of the ABX system of methyl leptoporate in its hydrated product was more difficult to explain, unless it is allowed that the double bond makes a definite contribution to the deshielding of these protons in methyl leptoporate.

Further structural work on methyl leptoporate was hampered by lack of material and the failure (q.v.) to produce it efficiently in culture.

The three polar compounds L.S.II, L.S.III and L.S.IV gave very characteristic t.l.c. colourations (green, red, mauve respectively) when sprayed with ceric ammonium nitrate in sulphuric acid solution, as described in Table II - 1. It was therefore easy to detect them and follow attempted separations. It was found, at a relatively early stage in the examination, that chromatography on silica or silicic acid was extremely costly in material. However, from the small amounts of fairly pure L.S.II and L.S.III which were isolated by this proceedure, it was possible to decide that they were polyhydroxy compounds based on the lanosta-7,9(11)-diene structure and this suggested possible methods of separation. Both compounds exhibited u.v. absorption at ca 230, 238, 246 and 254 mμ with extinction coefficients similar to those of methyl tyromycate. The hydroxyl functions were visible as intense broad absorptions at ca 3400 cm^{-1} and ca 1050 cm^{-1} in the i.r. spectrum, while the same groups gave rise to a broad hump in the region of 6.5τ in the n.m.r. spectrum.

L.S.II and L.S.III could be induced to solidify by precipitation from benzene solution on addition of light petroleum, but were obtained as amorphous solids and could not be obtained in crystalline form. Acetylation, it was hoped, would provide a solid derivative which could be purified by standard proceedures.

TABLE II - 14 LOW $^{m}/e$ FRAGMENTATION OF L.S.II PERACETATE OR L.S.III PERACETATE



Transition	Δ^{m}/e	Metastable ion
a	60	221.8
b	60	164.3
c	42	
d	42	135.5
e	60	
f	42	95.5
g	60	70.2

Treatment of the small quantities available did indeed convert both compounds into much less polar substances, which retained the characteristic t.l.c. colours. The determination of the mass spectra of these solids provided the first indication that a type of triterpene conjugate was involved. The base peak of both spectra was at m/e 43, while abundant ions at m/e 331 and below could be fitted into the breakdown patterns shown in Table II - 14. This is very similar to the fragmentation of pentaacetyl glucose[131] and strongly suggested that both compounds were triterpene glycoside tetraacetates. Recent work[132] has indicated that this pattern is characteristic of glucoside tetraacetates.

The probability that all three polar compounds were triterpene glycosides offered an alternative to separation as such, namely hydrolysis to the aglycones, which would be expected to be relatively non-polar and, therefore, separable by adsorption chromatography. A portion of the mixture, when treated under the mild acidic conditions normally used to hydrolyse aliphatic glycosides[133], showed that L.S.IV was completely hydrolysed, but that L.S.II and L.S.III were unaffected. Use of stronger acid, or the application of heat, did cleave these latter compounds, but examination, by t.l.c., of the re-esterified mixture showed a multitude of products. In one experiment, 18 distinct spots were counted when

1% methanol in chloroform was used as solvent.

Although acidic hydrolysis of the methyl esters was of little value in determining the aglycone portion, it could be used to determine the sugar moeity. Heating with 4N sulphuric acid in methanol for one hour completely hydrolysed all three glycosides. Treatment with an aqueous suspension of barium carbonate for 24 hours and then filtration gave a clear solution containing the sugars. Evaporation of the solvents, followed by descending paper chromatography against standard sugars with the solvent system ethyl acetate : pyridine : water (12:5:4), the spots being developed by treatment with methanolic sodium hydroxide and then silver nitrate in acetone[134], established that only glucose was present in the solution resulting from hydrolysis.

Since nearly all natural glucosides have the β-configuration[135], enzymic hydrolysis using β-glucosidase was attempted. It was hoped that cleavage in this manner would give fewer products. It was found that the main problem in this respect was the insolubility of the compounds in water. Shaking a suspension of the mixture in an aqueous solution of β-glucosidase for four days failed to achieve the desired hydrolysis. Base treatment of the mixture of methyl esters converted all three to the corresponding free acid. T.l.c. in 20% methanol in chloroform showed three spots

which stained in an identical manner to the esters. Since treatment with ethereal diazomethane regenerated the original methyl esters, none of the glucosides appeared to be base labile[136]. The free acids were only fractionally more soluble in water and again attempts at enzymic hydrolysis in aqueous suspension failed to cleave the glycoside bond. The mixture of acids was dissolved in a basic buffer solution (either borate buffer[137] or tris-(hydroxymethyl)-aminomethane buffer[138]). 0.1N hydrochloric acid was added until the pH (measured on a pH meter) was 7.2. On addition of a solution of β-glucosidase a homogeneous phase was obtained. The glucoside acids were unaffected even after two days at 30°C. Finally a mixture of dimethylsulphoxide/water was used to dissolve both the free acids and their methyl esters. T.l.c. showed that no reaction with β-glucosidase had taken place after 48 hours at 25°C. Cellobiose was converted to glucose under the same conditions. Attempts at enzymic hydrolysis of the mixture, under similar conditions to those described above, using emulsin (α-glucosidase) also failed.

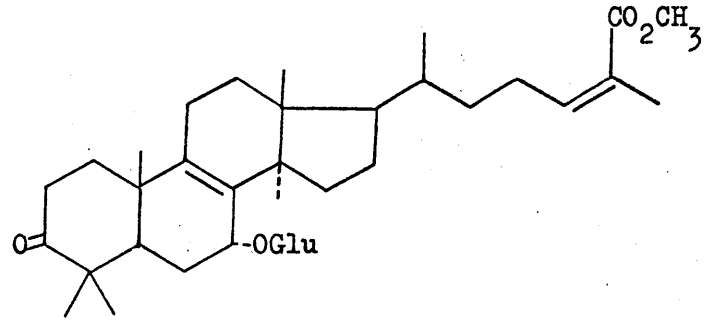
Separation of the three compounds as their pertrimethylsilyl ethers was then attempted. Overnight treatment of the ternary mixture of methyl esters with hexamethyldisilazane in pyridine[139] with a catalytic quantity of trimethylchlorosilane led to the recovery

of an oil which showed three compounds, R_fs 0.90, 0.33 and 0.31 in benzene. From the colour after spraying with ceric ammonium nitrate (green, red, mauve respectively), these were assigned to the pertrimethylsilyl ethers of L.S.II, L.S.III and L.S.IV respectively. P.l.c. permitted separation of the three components in low yield. Mild acid treatment in methanol regenerated L.S.II from the substance of R_f 0.90, and L.S.III from the substance of R_f 0.33. This confirmed the hypothesis that the colour reaction of these compounds on thin layers was retained in their derivatives. L.S.IV could not be isolated by a similar treatment of its derivative due to the succeptibilty of this compound to acid-catalysed elimination of glucose. Although the recovery from this separation was poor, it yielded sufficient quantities for acid hydrolyses and paper chromatographic identifications of the sugar residue of the three separated glycosides. Glucose was found to be the only sugar in all three cases.

The separation achieved as described above was of limited value since the recovery was low and L.S.IV could not be recovered from it. These factors, and the non-crystallinity of the L.S.II and L.S.III isolated, prompted further work on the acetates. Treatment of the mixture of methyl esters with acetic anhydride in pyridine yielded the peracetates of L.S.II, L.S.III and L.S.IV.

On thin layers in 25% light petroleum in ether these were visible at R_fs 0.35, 0.27 and 0.22 respectively, again the assignation being dependent on their t.l.c. colours (green, red, mauve respectively). Fractional crystallisation from methanol yielded needles which were shown, by t.l.c., to be the peracetate of L.S.IV. P.l.c. on Kieselgel HF_{254} permitted separation of L.S.II peracetate and L.S.III peracetate.

As has been discussed, it was found at an early stage in the work that L.S.IV could be easily hydrolysed by acid treatment. The aglycone obtained was identical in all respects to methyl tyromycate. Since this compound did not have a hydroxyl grouping, it followed that removal of the glucoside must give rise to one of the other functional groups. The isolation of L.S.IV peracetate allowed the distinction to be made between them. The u.v. spectrum of this material, $C_{45}H_{66}O_{13}$, m.p. 194-195.5°C., showed absorption at 216 mμ. Since the triple peak of the lanosta-7,9(11)-diene system was not visible, it was thought that this system must be generated on hydrolysis to the aglycone. Confirmation of this was obtained by addition of 6 drops of acid to the u.v. cells and rerunning the spectrum at 30 minute intervals, when the peaks at 236, 243 and 252 mμ became visible. Hydrolysis was complete after 3 hours, the u.v. spectrum then being super-



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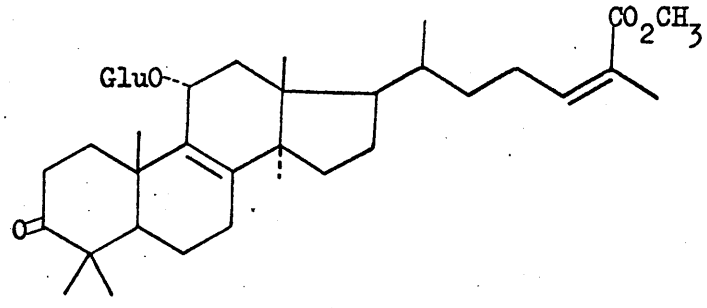
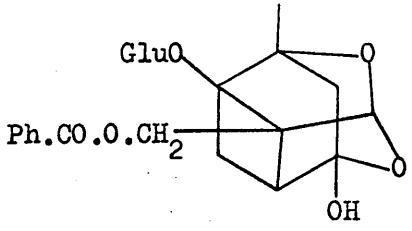


2.20

posable on that of methyl tyromycate.

Examination of the n.m.r. spectrum of the glucoside tetra-acetate confirmed that the diene system was absent and showed that the α-methyl α,β-unsaturated methyl ester was present, the vinyl proton appearing as a broadened triplet at 4.09τ while the vinyl methyl group gave rise to a fine doublet at 8.14τ. The methoxyl grouping resonated at 6.30τ. The four acetate groupings of the sugar residue appeared as two peaks at 7.96τ and 8.03τ (in the ratio of 1 : 3). The i.r. spectrum of L.S.IV peracetate showed two carbonyl peaks at 1761 cm^{-1} (ε 1670) and 1711 cm^{-1} (ε 830). These were assigned to the four acetate carbonyls and the ketone superimposed on the methyl ester respectively.

From the spectroscopic properties of the glucoside tetra-acetate, and the fact that methyl tyromycate was produced on hydrolysis, it was possible to write the two alternative structures 2.19 or 2.20 for L.S.IV. This follows from the fact that only one glucose residue was present and that the nuclear double bond had to be in the 8,9-position since no vinyl proton was visible in the n.m.r. spectrum. Either of the two structures could explain the ease of hydrolysis since the glycosidic linkage was in an allylic position. An alcohol group at C-7 in a lanost-8-ene system is known to be acid labile[140], giving rise to the



2.21

7,9(11)-diene system.

The configuration of the glucose residue may be determined from the n.m.r. spectrum. It has been shown[141] that the doublet due to an α-proton on C-1 of the glucopyranose residue resonates at higher field and has a larger coupling constant (J≃8 c/s) than a β-proton on the same position (J≃3 c/s). A doublet at 5.29τ (J=8 c/s) in the spectrum of L.S.IV peracetate was in good agreement with the value 5.45τ (J=8 c/s) for the β-glucoside tetra-acetate of β-sitosterol[142]. This established that the glucose residue had the β-configuration. The results of attempted enzymic hydrolyses were considered inconclusive since neither emulsin nor maltase effected hydrolysis. It was thought that this might be due to steric hinderance at the position to which the β-glucose residue was attached. Paeoniflorin (2.21), in which the sugar residue is bonded to a tertiary centre, was found to be resistant to enzymic hydrolysis[143].

There remained the problem of the point of attachment to the tetracyclic nucleus. It was found, by decoupling studies, that a low field broadened doublet at 5.76τ, integrating for one proton, collapsed to a singlet on irradiation at 8.17τ. This confirmed that the glucose was on a secondary allylic position and that it was most probably in the axial configuration. This

TABLE II - 15. EFFECT OF SUBSTITUENTS ON NUCLEAR ANGULAR METHYL GROUPS.

	C-19	C-18
7α-OH	-0.008	+0.008
7α-OAc	+0.008	-
11α-OH	+0.117	+0.025
11α-OAc	+0.092	+0.058
--	--	--
L.S.IV tetraacetate	+0.08	+0.12

latter conclusion was reached by a consideration of models, when it was found that the dihedral angles between (a) the 7β-proton and the 6α-proton, and (b) the 11β-proton and the 12α-proton were ca. 90°. Thus the coupling constants between these protons would be small. The angles between (a) the 7β-proton and the 6β-proton, and (b) the 11β-proton and the 12β-proton were ca. 0°C. The couplings between these protons would therefore be ca. 10 c/s. The total effect would be to give the observed broadened doublet. The tetraacetyl-β-glucose residue can therefore be assigned to the 7α- or the 11α- position.

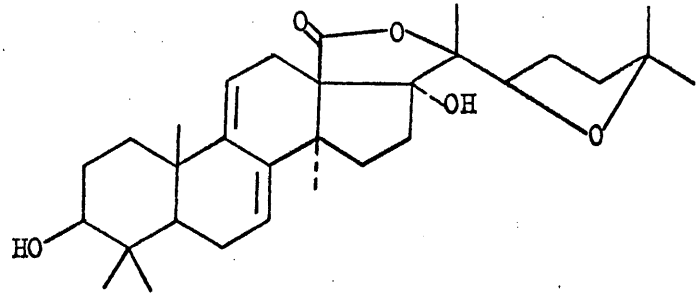
A consideration of the saturated methyl region favoured the 11α- position. The contour is shown below:

8.80 8.95,8.95 9.14 9.18

The predicted values for lanost-8-en- 3-one[89,90] are, with assignments,

8.88(C-19) 8.97,8.97(C-30,C-31) 9.13(C-32) 9.30(C-18)

It can be seen that the observed values are similar to the predicted values except for C-19 and C-18. This indicated that a deshielding influence must be operative on both these angular methyl groups. The effects of a 7α- or 12α-hydroxyl function and of a 7α- or 12α-acetate function on the angular methyls of 5α-steroids are shown in Table II - 15[144]. It can be seen that, in all cases, the effect of an 11α-substituent is larger than that



2.22

of a 7α-substituent. The size of the downfield shift in L.S.IV tetraacetate (included in Table II - 15) suggests C-11 as the point of attachment of the sugar residue. A mixture of glycosides, which on hydrolysis generate the lanosta-7,9(11)-diene system, have recently been reported. 22,25-oxidoholothurinogenin (2.22) has been identified, but the structure of the glucoside has not yet been determined[145].

Determination of the mass spectrum of L.S.IV tetraacetate showed that elimination of tetraacetylglucose occurred under electron impact. No parent ion was observed. The spectrum showed the ions at m/e 331 and below, collected in Table II - 14 and characteristic of the tetraacetylglucose portion. Otherwise the spectrum corresponded to that of methyl tyromycate, with the ions in Table II - 3 being clearly visible. This showed that elimination had occurred, rather than cleavage of the glycoside bonds to give ions at M^+ -331 (m/e 467) and M^+ -347 (m/e 451) as had been reported for a glucoside tetraacetate[146].

Although substantially pure L.S.III could be isolated in small amounts, this compound was conveniently characterised as its more accessible tetraacetate, $C_{45}H_{64}O_{13}$, m.p. 169-171°C. Since it had been established, by hydrolysis and paper chromatography, that L.S.III was a glucoside, there remained the problems

of the configuration of the glucoside link and the nature of the aglycone. As in the case of L.S.IV, emulsin and maltase failed to cleave the glycoside bond. The n.m.r. spectrum was therefore employed to determine the configuration at C-1 of the glucose residue. In the free glucoside a doublet at 5.38τ (J=8 c/s) was observed, while in the tetraacetate this doublet occurred at 5.30τ (J=8 c/s). These values established that L.S.III was a β-glucoside. All attempts to hydrolyse either the free glucoside or its tetraacetate to the aglycone produced complex mixtures of products. However, it was possible to suggest a plausible structure for L.S.III, based on its spectroscopic properties and on those of its tetraacetate.

The data of L.S.III itself were very similar to those of methyl tyromycate. The u.v. spectrum (238, 245.5 and 254 mμ) with increased intensity ca. 225 mμ evidenced the presence of the lanosta-7,9(11)-diene system and an α,β-unsaturated ester. Further data in support of these proposals were obtained from the n.m.r. spectrum. Three vinyl protons were present, multiplets at 4.38 and 4.62τ representing the diene system, and a broadened triplet at 4.02τ being the signal due to the trans β-proton of the α,β-unsaturated ester. The methoxyl of this latter group appeared at 6.29τ, while the finely split doublet at 8.10τ was

the position of the vinyl methyl group. The i.r. spectrum showed that the peaks due to the ketone and the α,β-unsaturated ester were superimposed at 1706 cm^{-1} (ε 737). The crystalline tetraacetate showed spectroscopic data similar to those described above, and fully in keeping with its possessing a ketone function and a $-CH=C(CH_3)CO_2Me$ grouping.

In view of the close parallels between the data of L.S.III, its acetate and those of methyl tyromycate, it was considered that the basic skeleton was similar for these compounds. Like methyl tyromycate and the several other lanosta-7,9(11)-dien-3-ones which were examined, the n.m.r. spectra of L.S.III and its tetraacetate both showed the triplet of doublets, in the region of 7.3τ. In addition, a comparison of saturated methyl regions showed that the values, shown below, were almost the same as those encountered in methyl tyromycate.

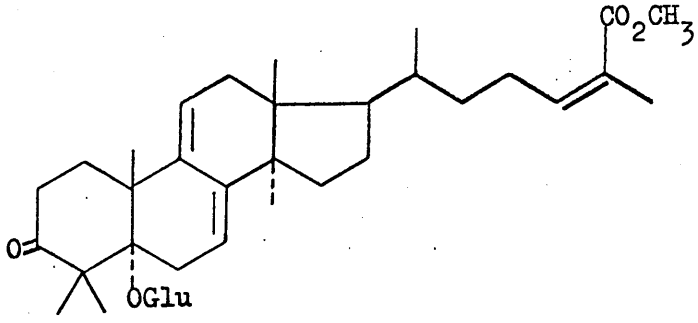
methyl tyromycate	8.81,	8.89,	8.92,	9.12,	9.42.
L.S.III	8.79,	8.88,	8.91,	9.09,	9.43.
L.S.III tetraacetate	8.79,	8.86,	8.89,	9.08,	9.46.

This not only substantiates that L.S.III is based on methyl tyromycate, but indicates that the position of the glucose residue is such that it has little effect on the resonances of the nuclear methyl groups. Careful examination of the n.m.r. spectra of L.S.III

and its acetate failed to reveal any signal which could be assigned to protons under the sugar residue. Because of this, it was considered that the glucoside unit was attached to a tertiary centre. This could perhaps account for the resistance of the free glucoside to enzymic hydrolyses.

The mass spectrum of L.S.III tetraacetate showed a similar pattern to that of L.S.IV tetraacetate at m/e 331 and below. Ions at m/e 481 (M^+ -331) and m/e 465 (M^+ -347) supported the postulate that the aglycone moiety was a hydroxylated analogue of methyl tyromycate and showed that cleavage of the glucoside bond was occurring under ion impact (cf. L.S.IV peracetate). The mass spectrum of L.S.III itself showed the highest ion at m/e 482. It has been reported[142] that, under mass spectral conditions, glucose is eliminated from a glucoside to give the free hydroxylated aglycone. The molecular weight of 482 agreed with methyl hydroxy-tyromycate. Loss of a methyl radical and of water accounted for most of the ions at high m/e.

Some indication as to the position of the sugar residue could be obtained from the mass spectra of L.S.III and its tetra-acetate. The free glucoside gave ions at m/e 325 and m/e 285. On comparison with the ions shown in Table II - 3, it will be seen that these former ions correspond to the fragmentations discussed



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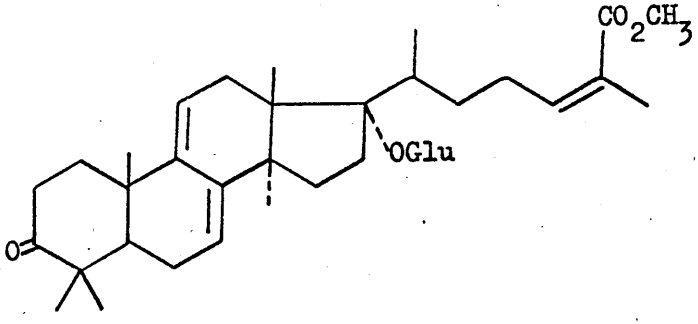


2.24

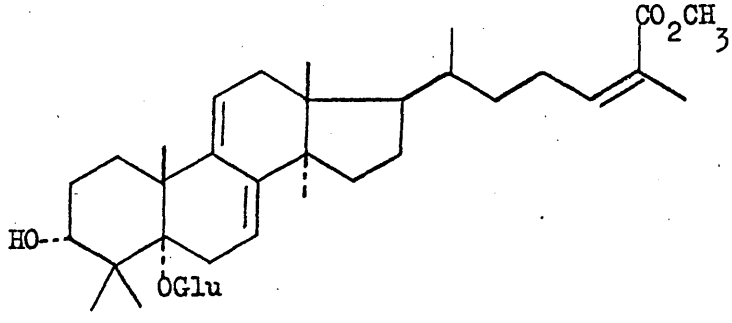
on page 92, if the tetracyclic nucleus bears an additional 16 mass units, i.e. a hydroxyl function. Both L.S.III and its tetra-acetate showed abundant ions at m/e 309. In the methyl tyro-mycate series, this ion corresponds to loss of the side chain with transfer of two hydrogen atoms. Since no ion at m/e 311 occurs in the spectra of L.S.III (or L.S.III tetraacetate) (cf. Table II - 3), it was thought that m/e 309 corresponds to the elimination of the elements of glucose (or its tetraacetate) followed by loss of the side chain. On the above evidence it is reasonable to suggest two possible structures for L.S.III, 2.23 and 2.24. An oxygen function at C-17 is more frequently encountered in natural steroids[147] than an oxygen function at C-5, but further work is required to distinguish between the two structures 2.23 and 2.24 for L.S.III.

Small quantities of substantially pure L.S.II could be isolated *via* its pertrimethylsilyl ether or by plate chromatography. It was found to be a low melting amorphous solid which could not be crystallised of further purified. However, this compound was conveniently characterised as its crystalline penta-acetate, $C_{47}H_{68}O_{14}$, m.p. 92-94°C.

From its spectroscopic data, it could be seen that L.S.II contained several of the structural features of L.S.III. The

ultra violet absorption and signals in the n.m.r. spectrum caused by the lanosta-7,9(11)-diene system and the α,β-unsaturated methyl ester were almost the same as in that substance. The α-proton at C-1 of the glucose residue could be seen at 5.45τ (J=8 c/s) in the n.m.r. spectrum of the free glucoside and at 5.32τ (J=8 c/s) in that of its pentaacetate, thus establishing the configuration of the glucoside linkage as β with respect to the glucose. As in the case of L.S.III, it was considered that the glucose was attached to a tertiary centre, since no proton under the glucose residue could be seen in the n.m.r. spectrum of the free glucoside or in that of its pentaacetate.

Some distinct differences between L.S.II and L.S.III were noted. The i.r. spectrum of L.S.II showed a single carbonyl peak at 1710 cm^{-1} (ε 444) which was attributed to an α,β-unsaturated methyl ester, in view of the methoxyl resonance at 6.29τ. There was therefore no ketone at C-3. It was seen that neither L.S.II nor its pentaacetate exhibited the complex of peaks ca. 7.3τ. The extra acetate grouping in this latter compound, which could be detected by integrating the 7.96 - 8.02τ region, could be seen as a shoulder, at ca. 1740 cm^{-1}, on the peak due to the other four acetates (1762 cm^{-1}). Since oxygenation might be expected at C-3, it was thought that this was the probable site for the fifth



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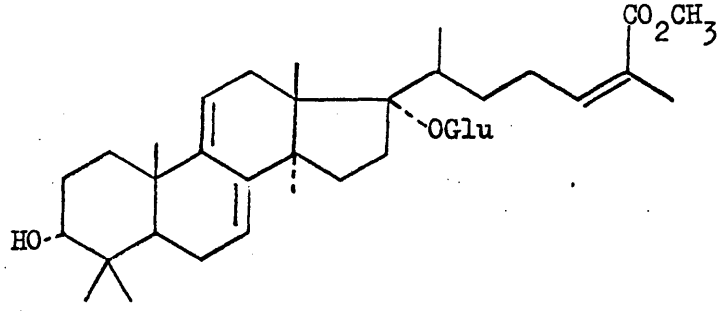


2.26

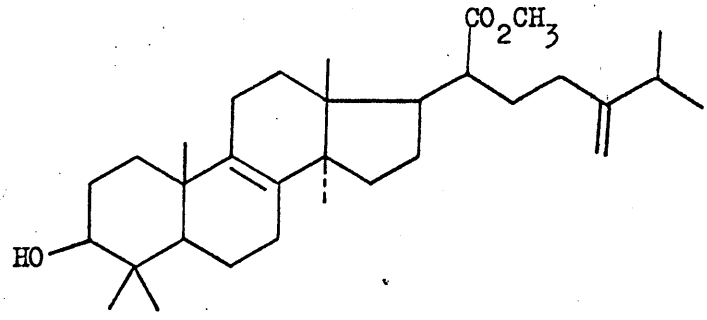
acetate grouping. A relatively sharp one-proton multiplet at 5.32τ, of half-band width 6 c/s, (superimposed on the glucose C-1 doublet), revealed that this acetate was secondary and strongly suggested that it was in the 3α-configuration (cf. configuration of D.Q.1 at C-3, page 21). It was therefore considered that L.S.II was based on the 3α-alcohol of methyl tyromycate.

The mass spectral data of L.S.II and its pentaacetate suggested that the sugar residue was attached to the tetracyclic nucleus. The free glucoside showed the highest ion at m/e 484, corresponding to the predicted mass of the aglycone. This ion underwent loss of the methyl radical followed by two successive losses of water. The ions at m/e 287 and m/e 311 indicated, as discussed for L.S.III, that the sugar residue was attached to the tetracyclic nucleus. Support for these ideas was obtained from the mass spectrum of L.S.II pentaacetate, in which ions at m/e 525 (M^+ -331) and m/e 509 (M^+ -347) corresponded to cleavage of the glucoside bonds. An abundant ion at m/e 353 indicated that loss of the sugar residue and cleavage of the side chain left an acetylated tetracyclic skeleton (311 + 42 cf. Table II - 5).

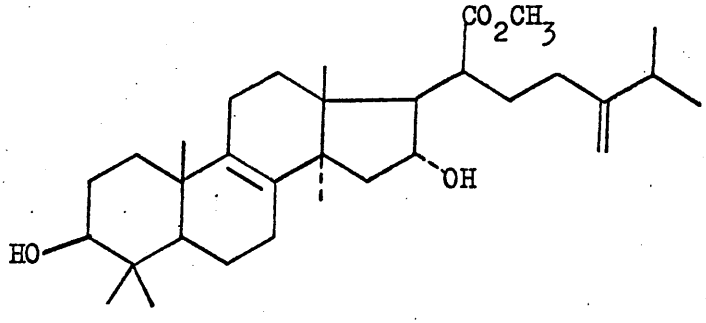
On the above evidence, it was possible to suggest structures 2.25 or 2.26 for L.S.II. As in the case of L.S.III, it is hoped that future study will distinguish between these two possibilities.

Since shortage of material was hindering structural elucidation of the methyl esters from the field collected sample of Leptoporus stipticus, it was decided to study the organism as grown in the laboratory. Accordingly, a stock culture of L. stipticus (Pers.) Quel. was obtained from Centraalbureau voor Schimmelcultures and grown on malt agar for 8 weeks in roux bottles. The harvested mycelium (61 g.) was extracted with hot and cold methanol as previously. The light petroleum soluble material (6.5 g.) was removed and the residue (22 g.) was dissolved in methanol and treated with an excess of ethereal diazomethane. T.l.c. analysis of the resulting mixture of methyl esters showed immediately that the five previously encountered metabolites had not been present, but that two new compounds were present, giving methyl esters visible at R_f 0.37 and R_f 0.19 in chloroform. Column chromatography failed to separate these substances but preparative layers of Kieselgel HF_{254} achieved a complete separation.

The compound of R_f 0.37 was a crystalline solid, $C_{32}H_{52}O_3$, m.p. 145-147°C. Its spectroscopic properties almost completely defined its structure. The i.r. spectrum showed one alcohol grouping (3621 cm^{-1}), a saturated methyl ester (1727 cm^{-1}) and a terminal methylene group (1647 cm^{-1} and 894 cm^{-1}). This latter function gave rise to a two-proton doublet at 5.24τ, while the



2.27



2.28

methoxyl of the methyl ester appeared at 6.32τ. The n.m.r. spectrum also showed that the alcohol grouping was secondary (1H multiplet at 6.77τ). The saturated methyl region was similar to that encountered in other tetracyclic triterpenes. The most obvious possibility was methyl eburicoate (2.27). On comparison with literature values for m.p., α_D[148] and the n.m.r. spectrum[89], it was found that these agreed well with the observed constants. An authentic sample of methyl eburicoate, obtained from an authentic sample[149] of the corresponding acetate, was identical (m.p., mixed m.p., R_f) with the isolated material. Similarly, acetylation of the isolated compound gave material identical to the authentic acetate.

The second methyl ester, R_f 0.19, obtained from the cultured Leptoporus stipticus (Pers.) Quel., was a crystalline solid, $C_{32}H_{52}O_4$, m.p. 175-177°C. The spectroscopic data obtained for this compound suggested that it was methyl tumulosate (2.28). The terminal methylene grouping was visible in the i.r. spectrum at 1641 cm^{-1} and 889 cm^{-1}, and in the n.m.r. spectrum as a two-proton doublet at 5.23τ (J=6 c/s). The two secondary alcohol functions absorbed together at 3626 cm^{-1} (ε 81) and gave rise to two one-proton multiplets at 5.74τ and 6.76τ respectively. Absorption at 1728 cm^{-1} and a singlet at 6.31τ confirmed the

presence of a saturated methyl ester function. The close similarity in these properties to those observed for methyl eburicoate and methyl dehydrotumulosate strongly suggested that the compound was methyl tumulosate. Comparison of the spectroscopic data of the substance under examination and those of the material isolated from Daedalea quercina, which had been shown to be methyl dehydrotumulosate contaminated with methyl tumulosate[56], showed only minor differences. Admixture of the ester from Leptoporus stipticus (Pers.) Quel. with some of this substance only slightly affected the melting point. It has been stated that there is an absence of melting point depression when a significant amount of 7,9(11)-diene is mixed with the parent 8-ene[150]. The above evidence was consistent with the compound of R_f 0.19 being methyl tumulosate.

The fact that only methyl eburicoate and methyl tumulosate could be isolated from the cultured strain suggested that the fungus was not the same as that which had been field collected. That this was not the case was demonstrated by subculture of the latter. After 8 weeks growth on malt agar it was seen, by t.l.c., that while the five previously encountered metabolites were still present, the fungus was now producing eburicoic acid and tumulosic acid. The converse of this, i.e. growing the purchased strain

on malt agar containing white pine wood for 16 weeks, showed that small quantities of tyromycic acid, leptoporic acid and the three glycosides were produced. These two experiments indicated that the two species were the same.

The discovery that incorporation of white pine wood into the culture medium induced the production of the five metabolites prompted an investigation of the variation of the metabolites of Leptoporus stipticus (Pers.) Quel. under different culture conditions. The aims of this work were three fold. It was hoped to find out if this effect was specific to white pine, if the agent causing this effect was an extractable compound and, finally, to point the way towards the optimum conditions for the production of the five requisite metabolites. Leptoporus stipticus (Pers.) Quel. was cultured for 8 weeks on malt agar with equal quantities of different woods incorporated into the medium. In other experiments carried out at the same time, after soxhlet extraction of white pine with light petroleum or methanol or water, firstly the extracts and, secondly the extracted wood, were incorporated into the malt agar medium.

The results of these experiments are shown in Table II - 16. The methanol extract of the mycelium was methylated with diazomethane and then assayed by two methods. T.l.c. in chloroform

TABLE II - 16. VARIATION OF CULTURE CONDITIONS FOR L. STIPTICUS (PERS.) QUEL.

	methyl tyromycate / methyl eburicoate	glucosides
Sitka spruce	0.5	+
White pine	0.1	-
Parana pine	0.1	-
Scots pine	0.1	-
Oak	0.02	-
Hemlock	0.02	-
Elm	0.03	-
MeOH extracted white pine	0.1	-
H_2O extracted white pine	0.2	-
Petroleum extracted white pine	0.1	-
MeOH extract of white pine	0.007	-
H_2O extract of white pine	0.00	-
Petroleum extract of white pine	0.003	-
Agar	0.00	-

and in 10% methanol in chloroform gave a qualitative picture, while g.l.c., after removal of the polar substances by p.l.c., allowed a comparison of the amounts of methyl eburicoate and methyl tyromycate. Table II - 16 shows the results of the assays with the various woods employed. Given in the table is the quantitative ratio of methyl tyromycate to methyl eburicoate and the result of the t.l.c. examination for the presence of the glucosides.

It can be seen, from the table, that the effect does not appear to be specific to white pine but that the four conifers used were all more efficient than the three desciduous woods. The factor of ten shows that the effect is not merely due to surface phenomena affecting the consistency of the culture medium, since in all seven cases this was the same. The table also shows that extraction of white pine wood had almost no effect on its property to induce the fungus to produce tyromycic acid and that the extract so obtained has only a very slight effect. This would appear to indicate that there is no extractable induction factor.

Previous qualitative studies appeared to show that, for culture on white pine powder, as the growing time was lengthened more tyromycic acid was produced, while the glucosides could be

detected after 16 weeks. It appears, therefore, that incorporation of Sitka spruce into the culture medium and a longer growing time offers the best alternative for future attempts to obtain tyromycic acid and its analogues from a culture of Leptoporus stipticus (Pers.) Quel..

EXPERIMENTAL

1. EXTRACTION AND METHYLATION.

Fresh sporophores of Leptoporus stipticus were collected in mid-Autumn from a fallen pine tree in the grounds of Dunkeld House Hotel. The cleaned, dried tissue (200 g.) was homogenised with methanol (2 l.). After standing for one week, the extracted tissue was filtered off and the filtrate evaporated to give a brown oil (19.1 g.) from which a crystalline white solid (400 mg.), m.p. 162-166°C, separated. Soxhlet extraction of the tissue with methanol (1 l.) for 24 hours yielded a further 7.4 g. of gummy material. Thin layer chromatography of the hot and cold extracts showed them to have similar composition. They were therefore combined and the whole triturated with light petroleum (b.p. 40-60°C, 200 ml.) to remove lipids. The petroleum insoluble material (23 g.) was dissolved in methanol (500 ml.) and treated with an excess of ethereal diazomethane. The metabolites of Leptoporus stipticus were thus obtained as a mixture of their methyl esters (22 g.).

2. T.L.C. REFERENCE CODE FOR L. STIPTICUS ESTERS.

The oil above was examined by thin layer chromatography using two solvent systems:

A 100% chloroform.

B 10% methanol in chloroform.

The characteristic R_f values of the five major esters of L. stipticus and their staining characteristics, when sprayed with ceric ammonium nitrate / sulphuric acid and heated for 2 minutes at 140°C, are shown in Table II - 1. Also shown is their appearance when run on layers of Kieselgel HF_{254} and viewed in u.v. light (λ 254 mμ). Throughout the text the names methyl tyromycate and methyl leptoporate, and the code names L.S.II, L.S.III and L.S.IV will be used to represent the compounds whose thin layer chromatographic properties are defined in Table II - 1.

3. CHROMATOGRAPHIC SEPARATION OF THE MIXTURE OF L. STIPTICUS ESTERS.

A portion (5 g.) of the mixture of methyl esters was adsorbed on to silicic acid (5 g.) from ether and placed on top of a column of silicic acid (300 g.). This was eluted with light petroleum (1 l.) to which ethyl acetate (2 l.) was gradually added. Fractions (20 ml.) were taken at a flow rate of 100 ml./hr. and were examined by t.l.c. for content. The following table summarises the separation achieved.

Fractions	Weight (mg.)	Content
24 - 38	163	methyl tyromycate
39 - 56	160	methyl tyromycate, methyl leptoporate
57 - 66	80	methyl leptoporate, ergosterol
114 - 160	2700	L.S.II, L.S.III, L.S.IV
Total	3100	i.e. 62% recovery

Methyl tyromycate was obtained from fractions 24 - 38 by crystallisation from methanol. Methyl leptoporate could be purified from fractions 57 - 66 by p.l.c. on Kieselgel HF_{254} layers using chloroform as eluent. Separation of the binary mixture in fractions 39 - 56 was also achieved by this method. An attempt to separate a portion (700 mg.) of the polar constituents of fractions 114 - 160 by a similar method using 10% methanol in chloroform as solvent permitted the isolation of pure samples of L.S.II and L.S.III, but in poor yield (ca. 50 mg. of each). Adsorption of the remainder of fractions 114 - 160 (2.4 g.) on to silicic acid (250 g.) and elution with chloroform (1 l.) to which ethyl acetate (1 l.) was slowly added gave only a partial separation of the three polar components with heavy loss of material, a total of 0.8 g. (33%) being recovered. Attempts to further separate these compounds by column or thin layer chromatography

met with the same problem of loss of material.

4. PARTITION OF THE METHYL ESTERS OF L. STIPTICUS.

A portion (18 g.) of the mixture of methyl esters was distributed between light petroleum (200 ml.) and methanol : water (3 : 1, 400 ml.) and allowed to come to equilibrium. The layers were separated and examined by t.l.c.. This showed that an almost complete separation of the non-polar light petroleum soluble compounds (methyl tyromycate, methyl leptoporate) from L.S.II, L.S.III and L.S.IV had been achieved. Shaking the aqueous layer with a further quantity of light petroleum (100 ml.) completed the separation. The combined petroleum layers were dried and the solvent removed to yield a pale yellow solid (2.3 g.). The aqueous phase was boiled to dryness to give a brown oil (13.8 g.).

5. CHROMATOGRAPHIC SEPARATION OF THE LIGHT PETROLEUM SOLUBLE ESTERS.

This fraction was crystallised from methanol to give a white solid (2.1 g.). This was adsorbed from light petroleum on to silicic acid (150 g.) and eluted with light petroleum (700 ml.) to which 30% ethyl acetate in light petroleum (500 ml.) was gradually added. Fractions (15 ml.) were collected at the rate of four per hour. The separation is shown in the table below.

Fractions	Weight (mg.)	Composition
40 - 55	1230	methyl tyromycate
56 - 60	396	methyl tyromycate, methyl leptoporate
61 - 73	92	methyl leptoporate
74 - 90	245	methyl leptoporate, ergosterol
Total	1963	i.e. 98% recovery

Methyl tyromycate could be obtained by crystallisation of fractions 40 - 55 from methanol. Methyl leptoporate was separated from the binary mixtures in fractions 56 - 60 and 74 - 90 by p.l.c.. Ergosterol was identified only by its thin layer properties (identical R_f and staining characteristics with authentic sample).

6. METHYL TYROMYCATE. METHYL 3-OXO-LANOSTA-7,9(11)-24-TRIEN-26-OATE.

This compound was isolated as detailed in Section 5. It crystallised from methanol as lustrous plates, m.p. 154-156°C. R_f ($CHCl_3$) 0.63. $[\alpha]_D$ +40 (c 0.9).

ν_{max} (KBr) 3035, 2960, 2918, 2875, 2823, 1714, 1650, 1601, 1449, 1434, 1384, 1373, 1360, 1350, 1278, 1263, 1220, 1215, 1200, 1152, 1126, 1110, 1075, 1002, 990, 902, 868, 851, 815 and 799 cm^{-1}.

ν_{max} ($CHCl_3$) 1706 cm^{-1} (ε 746)

τ values (60 Mc/s) 4.00 (1H, br.t, J=8 c/s), 4.49 (2H, m), 6.24 (3H, s), 8.08 (3H, d, J=2 c/s), methyl contour 8.79, 8.87, 8.90, 9.11 and 9.40.

τ values 4.07 (1H, br.t, J=8 c/s), 4.54 (2H, m), 6.29 (3H, s), 8.13 (3H, d, J=2 c/s), methyl contour 8.81, 8.89, 8.92, 9.12 and 9.42. λ_{max} 228 mμ (infl.) (log ε 4.12), 235.5 mμ (log ε 4.18), 244 mμ (log ε 4.18) and 252 mμ (log ε 4.12)

Analysis	Found	C 79.58%	M^+ at m/e 466
		H 9.85%	
$C_{31}H_{46}O_3$	requires	C 79.78%	M.W. 466
		H 9.93%	

Reaction of methyl tyromycate with an ethanolic solution of 2,4-dinitrophenylhydrazine hydrosulphate yielded the derivative which crystallised from methanol/chloroform as needles, m.p. 208-209°C.

Analysis	Found	C 68.41%
		H 7.63%
		N 8.95%
$C_{37}H_{50}O_6N_4$	requires	C 68.71%
		H 7.79%
		N 8.66%

7. HYDROGENATION OF METHYL TYROMYCATE. METHYL 3-OXO-LANOSTA-7,9(11)-DIEN-26-OATE.

Methyl tyromycate (500 mg.) in ethyl acetate (100 ml.) was hydrogenated over 10% palladium charcoal (50 mg.). When the uptake of hydrogen was complete (1 hour), the solution was filtered and

the solvent removed. The recovered methyl dihydrotyromycate (2.4) (474 mg., 93%) recrystallised from methanol as plates, m.p. 160-162°C. R_f ($CHCl_3$) 0.63.

ν_{max} (KBr) 2950, 2923, 2872, 2831, 1733, 1714, 1461, 1450, 1440, 1373, 1286, 1258, 1214, 1201, 1170, 1134, 1126, 1110, 1091, 1003, 990, 901, 848, 814 and 796 cm^{-1}.

ν_{max} ($CHCl_3$) 1703 cm^{-1} (ε 499), 1727 cm^{-1} (ε 354)

τ values 4.50 (2H, m), 6.30 (3H, s), methyl contour 8.80, 8.87, 8.91 9.08, 9.12 and 9.41.

λ_{max} 228 mμ (infl.) (log ε 3.98), 236 mμ (log ε 4.16), 244 mμ (log ε 4.21) and 252.5 mμ (log ε 4.05)

Analysis Found C 79.38% H 10.10% M^+ at m/e 468

$C_{31}H_{48}O_3$ requires C 79.44% H 10.32% M.W. 468

8. TREATMENT OF METHYL TYROMYCATE WITH OSMIUM TETROXIDE. METHYL 24,25-DIHYDROXY-3-OXO-LANOSTA-7,9(11)-DIEN-26-OATE.

Methyl tyromycate (200 mg.) and osmium tetroxide (140 mg.) were dissolved in ether (10 ml.) with a trace of pyridine (0.1 ml.) and left for 60 hours. Chloroform (30 ml.) was added and hydrogen sulphide passed through the solution for 15 mins.. The precipitated osmium sulphide was filtered off and the mother

liquor evaporated to give the glycol 2.2 (145 mg., 65%) which was treated with decolourising charcoal and crystallised from methanol as needles, m.p. 145-146.5°C. R_f ($CHCl_3$) 0.30.

ν_{max} (KBr) 3420 (br.), 2985, 2972, 2940, 2894, 1760, 1720, 1468, 1452, 1433, 1387, 1377, 1250, 1128, 1113, 1071, 1020, 1003, 994, 978, 900 and 810 cm^{-1}.

ν_{max} (CCl_4) 3564 cm^{-1} (ε 48), 3522 cm^{-1} (ε 52), 1736 cm^{-1} (ε 463) and 1712 cm^{-1} (ε 602)

τ values 4.53 (2H, m), 6.20 (3H, s), 6.43 (1H, m), methyl contour 8.57, 8.80, 8.88, 8.92, 9.13 and 9.40.

λ_{max} 227 mμ (infl.) (log ε 4.03), 236.5 mμ (log ε 4.16), 243.5 mμ (log ε 4.24) and 252 mμ (log ε 4.09)

Analysis	Found	C 74.06%	M^+ at m/e 500
		H 9.47%	
$C_{31}H_{48}O_5$	requires	C 74.36%	M.W. 500
		H 9.66%	

9. PERIODATE CLEAVAGE OF THE GLYCOL 2.2.

The glycol 2.2 (100 mg.) in methanol (4 ml.) was treated with sodium metaperiodate (55 mg.) in water (0.3 ml.) and the mixture was stirred at room temperature for 24 hours. The sodium iodide was filtered off and the solid (95 mg.), recovered after removal of the solvent, was purified by p.l.c. on one

20 x 20 x 0.1 cm. layer of Kieselgel HF_{254} with chloroform as solvent. Pure 2.3 (57 mg., 72%) crystallised from methanol as needles, m.p. 162-164°C. R_f ($CHCl_3$) 0.59.

ν_{max} (KBr) 2950, 2920, 2890, 2871, 2830, 1708, 1460 - 1454, 1446, 1429, 1378, 1370, 1282, 1258, 1241, 1187, 1130, 1119, 1056, 1047, 1018, 1009, 1001, 974, 901, 811 and 796 cm^{-1}.

ν_{max} ($CHCl_3$) 1705 cm^{-1} (ε 840)

τ values 0.15 (1H, t, J=2 c/s), 4.51 (2H, m), methyl contour 8.81, 8.88, 8.91, 9.12 and 9.41.

λ_{max} 228 mμ (infl.) (log ε 3.97), 235.5 mμ (log ε 4.17), 242.5 mμ (log ε 4.23) and 251 mμ (log ε 4.06)

Analysis	Found	C 81.77%	M^+ at m/e 396
		H 10.02%	
$C_{27}H_{40}O_2$	requires	C 81.77%	M.W. 396
		H 10.17%	

10. LITHIUM ALUMINIUM HYDRIDE REDUCTION OF METHYL DIHYDROTYROMYCATE. LANOSTA-7,9(11)-DIENE-3β,26-DIOL (2.5).

Methyl dihydrotyromycate (2.4) (400 mg.) in dry ether (10 ml.) was added to a suspension of $LiAlH_4$ (400 mg.) in dry ether (50 ml.) and stirred at room temperature for 12 hours. The excess reagent was neutralised by dropwise addition of 1N hydrochloric acid. The solution was washed with 6N hydrochloric acid (2 x 10 ml.),

saturated sodium bicarbonate (10 ml.) and brine (2 x 10 ml.) and then dried. Evaporation of the solvent gave the diol 2.5 (320 mg., 74%) which crystallised from methanol as needles, m.p. 161-162°C. R_f ($CHCl_3$) 0.10.

ν_{max} (KBr) 3350 (br.), 3035, 2923, 2870, 1461 - 1443, 1370, 1256, 1135, 1096, 1074, 1031, 996, 987, 905, 811 and 798 cm^{-1}.

ν_{max} ($CHCl_3$) 3615 cm^{-1} (ε 107)

λ_{max} 228 mμ (infl.) (log ε 3.99), 237 mμ (log ε 4.20), 244 mμ (log ε 4.27) and 252.5 mμ (log ε 4.11)

τ values 4.56 (2H, m), 6.42 (2H, d, J=6 c/s), 6.64 (1H, m), methyl contour 9.01, 9.03, 9.14 and 9.42.

Analysis	Found	C 81.14%	M^+ at m/e 442
		H 11.62%	
$C_{30}H_{50}O_2$	requires	C 81.39%	M.W. 442
		H 11.38%	

11. 26-TOSYLOXY-LANOSTA-7,9(11)-DIEN-3β-OL (2.6).

The diol 2.5 (200 mg.) was added to p-toluenesulphonyl chloride (600 mg.) in dry pyridine (2 ml.), cooled in ice. The mixture was stirred for 30 minutes at 0°C. Water (0.5 ml.) was added and the reaction stirred for a further 15 minutes. It was then taken up in ether (20 ml.), washed with 6N sulphuric acid (2 x 15 ml.), saturated sodium bicarbonate (2 x 5 ml.), brine

(10 ml.) and dried. Evaporation of the solvent gave a clear oil which, after p.l.c. on two 20 x 20 x 0.1 cm. layers of Kieselgel HF_{254} using chloroform as eluent, yielded the monotosylate 2.6 (177 mg., 65%) which crystallised from methanol as needles, m.p. 107-108.5°C. R_f ($CHCl_3$) 0.26.

ν_{max} (KBr) 3400 (br.), 3024, 2959, 2918, 2842, 1596, 1495, 1482, 1455, 1367, 1358, 1290, 1187, 1175, 1096, 1067, 1033, 973 - 967, 947 - 942, 844, 813 and 774 cm^{-1}.

ν_{max} ($CHCl_3$) 3625 cm^{-1} (ε 41)

τ values 1.83, 1.98, 2.32, 2.47 (2H, AB system, J=10 c/s), 4.47 (2H, m), 6.00 (2H, d, J=7 c/s), 6.64 (1H, m), 7.47 (3H, s) methyl contour 8.97, 8.98, 9.03, 9.09, 9.16, 9.29 and 9.42.

λ_{max} 227 mμ (log ε 4.49), 244 mμ (log ε 4.22) and 252 mμ (log ε 4.04)

Analysis Found C 74.39% H 9.50% M^+ at m/e 596

$C_{37}H_{56}SO_4$ requires C 74.56% H 9.47% M.W. 596

12. Δ-LANOSTEROL. (LANOSTA-7,9(11)-DIEN-3β-OL)

(i) By Lithium Aluminium Hydride Reduction of 2.6.

The monotosylate (60 mg.) in anhydrous ether (2 ml.) was added to a suspension of $LiAlH_4$ (50 mg.) in dry ether (15 ml.). The reaction mixture was stirred at room temperature for 12 hours,

and then neutralised with 1N hydrochloric acid (4 drops). The ether solution was washed with 6N hydrochloric acid (2 x 5 ml.), saturated sodium bicarbonate (5 ml.) and brine (5 ml.). After drying and evaporation of the solvent γ-lanosterol was obtained (36 mg., 86%) which crystallised from aqueous methanol as needles, m.p. 155-157°C. R_f ($CHCl_3$) 0.40.

ν_{max} (KBr) 3350 (br.), 2955, 2927, 2880 - 2870, 2845, 1465, 1444, 1373, 1098, 1067, 1034, 1000, 988, 906, 813 and 801 cm^{-1}.

ν_{max} ($CHCl_3$) 3618 cm^{-1} (ε 53)

τ values 4.55 (2H, m), 6.68 (1H, m), methyl contour 9.01, 9.03 9.10, 9.12, 9.18 and 9.41.

λ_{max} 228 mμ (infl.) (log ε 3.96), 236 mμ (log ε 4.15), 243.5 mμ (log ε 4.24) and 252 mμ (log ε 4.04)

M^+ at m/e 426.

This material was identical in all respects with that prepared as below (n.m.r., i.r., mass spectrum, R_f, m.p. and mixed m.p.).

(ii) <u>From Dihydrolanosterol</u>.[122]

Acetylation of dihydrolanosterol (1 g.) with refluxing acetic anhydride (20 ml.) yielded dihydrolanosteryl acetate (0.97 g., 89%) which crystallised from methanol as needles, m.p. 118.5-120°C. (Literature m.p. 120-121°C). Treatment of dihydrolanosteryl acetate (500 mg.) in acetic acid (50 ml.)

with selenium dioxide (500 mg.) under reflux for 1 hour, yielded lanosta-7,9(11)-dien-3β-acetate (350 mg., 70%) which crystallised from acetone as needles, m.p. 167.5-168.5°C. (Literature m.p. 167°C). Hydrolysis of a portion of this material (200 mg.) in 4N methanolic sodium hydroxide (20 ml.) for 12 hours at room temperature led to ɣ-lanosterol (181 mg., 93%) which crystallised from methanol/acetone as needles, m.p. 156-158°C. (Literature m.p. 158-159°C).

13. TYROMYCIC ACID.

This material was obtained by crystallisation of the small quantity of solid obtained on evaporation of the methanol extract of the sporophores, before methylation. It crystallised from methanol as needles, double m.p. 119-121°C and 176-178°C.

R_f (10% MeOH in $CHCl_3$) 0.68. $[\alpha]_D$ +45 (c 0.8).

ν_{max} (KBr) 3300 - 2500 (br.), 3025, 2980, 2890, 2877, 1716, 1695, 1649, 1600, 1458, 1430, 1374, 1307, 1270 - 1250, 1192, 1160, 1131, 1114, 1080, 1058, 1002, 992, 903, 811, 796 and 784 cm^{-1}.

ν_{max} (CCl_4) 3300 - 2800 (br.), 1711 cm^{-1} (ε 660), 1690 cm^{-1} (ε 646)

τ values 3.88 (1H, t, J=7 c/s), 4.50 (2H, m), 8.06 (3H, d, J=2 c/s), methyl contour 8.80, 8.87, 8.91, 9.12 and 9.41.

λ_{max} 228 mμ (infl.) (log ε 4.16), 235 mμ (log ε 4.19), 244 mμ (log ε 4.19) and 252 mμ (log ε 4.02)

Analysis	Found	C 76.56%	M^+ at m/e 452
		H 9.68%	
$C_{30}H_{44}O_3.H_2O$	requires	C 76.55%	M.W. 452
		H 9.85%	

14. METHYL LEPTOPORATE.

This compound was isolated as described in Section 5. It crystallised from methanol as plates, m.p. 175-177°C. R_f ($CHCl_3$) 0.31. $[\alpha]_D$ +105 (c 0.6).

ν_{max} (KBr) 3018, 2957, 2943, 2924, 2880, 1741, 1710, 1600, 1454 - 1448, 1434 - 1430, 1373, 1348, 1303, 1291, 1284, 1267, 1256, 1244, 1235, 1214, 1204, 1191, 1184, 1167, 1136, 1113, 1102, 1003, 991, 987, 901, 814 and 798 cm^{-1}.

ν_{max} ($CHCl_3$) 1737 cm^{-1} (ε 360), 1704 cm^{-1} (ε 355)

τ values 4.50 (2H, m), 5.06, 5.13, 5.24, 5.32; 5.83, 5.91, 6.00, 6.08 (2H, AB part of ABX system, J_{AB}=17 c/s), 6.25 (3H, s), 8.34 (3H, s), methyl contour 8.80, 8.90, 8.93, 9.12 and 9.42.

λ_{max} 228 mμ (infl.) (log ε 3.96), 236 mμ (log ε 4.17), 244 mμ (log ε 4.23), 252 mμ (log ε 4.05)

Analysis	Found	C 75.61%	M^+ at m/e 508≡$C_{33}H_{48}O_4$
		H 9.68%	
$C_{33}H_{48}O_4.H_2O$	requires	C 75.25%	M.W. 508
		H 9.57%	

15. PREPARATION OF 2,3-DIHYDRO-2-ETHYL-4-METHYLFURAN[129].

Redistilled diethyl ethyl malonate (23.5 g.) was condensed with redistilled propargyl bromide (15 g.) in the presence of sodium methoxide (2.87 g. sodium in 4 ml. methanol). After work up, distillation gave diethyl 2-ethyl-2-propargyl malonate (23.3 g., 82%) b.p. (1mm.) 96-98°C. (Literature b.p. (17 mm.) 125°C). This substance was hydrolysed with 1N methanolic potassium hydroxide to give the corresponding diacid (15 g., 80%) as a gum. (Literature m.p. 118°C). Decarboxylation was achieved by heating at 200°C for 2 minutes. Distillation gave 2-ethyl-2-propargylacetic acid (7.6 g., 68%), b.p. (0.02 mm.) 65-68°C. (Literature b.p. (18 mm.) 117°C). Treatment with ethanol in the presence of sulphuric acid yielded the corresponding ethyl ester (5.9 g., 75%), b.p. (20 mm.) 78-80°C. (Literature b.p. (22 mm.) 77°C). $LiAlH_4$ reduction gave 2-ethyl-pent-4-yne-1-ol (4 g., 78%), b.p. 172-174°C. (Literature b.p. 174°C). Slow distillation of this substance from sodamide (200 mg.) produced 2,3-dihydro-2-ethyl-4-methylfuran (2.6 g., 61%), b.p. 110-112°C. n_D 1.4384. (Literature b.p. 113°C. n_D 1.4345).

16. HYDRATION OF METHYL LEPTOPORATE IN ACID SOLUTION.

Methyl leptoporate (50 mg.) was dissolved in methanol (7 ml.), to which 36N hydrochloric acid (1 drop) had been added, and was

allowed to stand at room temperature for 30 minutes. Saturated sodium carbonate was added to neutrality and a further 10 ml. of water was added. After boiling off the methanol, the solution was extracted with ethyl acetate (2 x 5 ml.). Drying and evaporation of the solvent yielded a white solid (44 mg., 85%) which crystallised from aqueous methanol as needles, m.p. 153-155°C. R_f ($CHCl_3$) 0.08.

ν_{max} (KBr) 3400 (br.), 3045, 2972, 2960, 2937, 2890, 1738, 1716, 1600, 1470 - 1462, 1458, 1439, 1381, 1290, 1275, 1230, 1215, 1201, 1144 - 1136, 1013, 999, 989, 907, 820 and 815 cm^{-1}.

ν_{max} ($CHCl_3$) 3624 cm^{-1} (ε 41), 1731 cm^{-1} (ε 404), 1703 cm^{-1} (ε 419)

τ values 4.51 (2H, m), 6.25 (2H, m), 6.27 (3H, s), 8.50 (3H, s), methyl contour 8.81, 8.89, 8.92, 9.12 and 9.42.

λ_{max} 228 mμ (infl.) (log ε 3.96), 236 mμ (log ε 4.15), 243.5 mμ (log ε 4.24), 252 mμ (log ε 4.04)

Analysis	Found	C 75.31%	Highest ion at m/e 508
		H 9.30%	
$C_{33}H_{50}O_5$	requires	C 75.25%	M.W. 526
		H 9.57%	

17. SEPARATION OF THE POLAR ESTERS L.S.II, L.S.III AND L.S.IV.

(a) Formation of trimethylsilyl ethers.

A portion of the mixture of methyl esters (500 mg.) was dissolved in dry pyridine (1 ml.) and treated with hexamethyldisilazane (1 ml.) and trimethylchlorosilane (3 drops) at room temperature for 12 hours. The solvents were removed under vacuum and the resulting gummy solid was ether extracted. After drying, the ether was removed to yield a gum (400 mg.). P.l.c. on four 20 x 20 x 0.1 cm. layers of Kieselgel HF_{254}, which had been dried at 140°C for 30 min., using benzene as eluent, separated the three derivatives: L.S.II pertrimethylsilyl ether (40 mg.), R_f 0.90; L.S.III pertrimethylsilyl ether (30 mg.), R_f 0.33 and L.S.IV pertrimethylsilyl ether (18 mg.), R_f 0.31. Pure L.S.II and L.S.III were obtained by treatment of their derivatives in methanol (2 ml.) with 4N sulphuric acid (3 drops).

(b) Acetylation.

Treatment of the mixture of methyl esters (1.5 g.) with an excess of acetic anhydride and pyridine at room temperature for 24 hours completely converted the three compounds into their peracetates. Removal of the solvents yielded a gummy solid (1.6 g.). This was treated with hot methanol and allowed to cool. The crystalline solid (180 mg.) which separated was shown, by t.l.c.,

to be L.S.IV peracetate. P.l.c. on 50 x 20 x 0.1 cm. layers of Kieselgel HF_{254}, using benzene as eluent, separated the peracetate of L.S.II (235 mg.) and the peracetate of L.S.III (220 mg.).

18. ACID HYDROLYSES OF THE GLYCOSIDES.

A portion of the ternary mixture of methyl esters (500 mg.) was dissolved in 4N methanolic sulphuric acid (10 ml.) and left at room temperature for 12 hours. The solid (37 mg.), which had precipitated, was filtered off and, after crystallisation from methanol, was shown to be methyl tyromycate (i.r., n.m.r., mass spectrum, m.p., mixed m.p. and R_f). T.l.c. of the filtrate showed that L.S.IV had been hydrolysed, but that L.S.II and L.S.III were unaffected. The mixture was refluxed until t.l.c. showed that these two glycosides had been hydrolysed (1 hour). An aqueous suspension of barium carbonate was added and the mixture was left for 24 hours. Filtration through glass paper gave a clear, aqueous solution. This was reduced to 0.5 ml. and then examined by descending paper chromatography using Whatman No. 1 paper in ethyl acetate : pyridine : water (12:5:4). The spots were developed by passing them through (a) a solution of acetone (99.5 ml.) and saturated aqueous silver nitrate (0.5 ml.), then (b) 5N ethanolic sodium hydroxide. After the spots had appeared, the excess reagent was removed by passing the strip through (c) 6N

aqueous ammonium hydroxide. By comparison with the standards D-glucose, D-galactose, D-mannose, D-arabinose and L-fucose, it was seen that only glucose was present in the hydrolyses filtrate.

When the separated pertrimethylsilyl ethers of L.S.II, L.S.III and L.S.IV were similarly treated under acid conditions, and then the water soluble hydrolyses products examined by parer chromatography under the same conditions, it was found that only glucose was present in all three cases. It was seen also that L.S.IV, on hydrolysis, gave methyl tyromycate.

19. L.S.IV TETRAACETATE. (7α- OR 11α- TETRAACETYLGLUCOSYLOXY-LANOSTA-8,24-DIEN-26-OATE).

This compound was isolated as detailed in Section 17(b). It crystallised from methanol as needles, m.p. 194-195.5°C. R_f (25% light petroleum in ether) 0.22. $[\alpha]_D$ +22 (c 1.0).

ν_{max} (KBr) 2960, 2893, 1765, 1754, 1714, 1654, 1460, 1446 - 1440, 1380, 1328, 1245 - 1230, 1160, 1139, 1120, 1096, 1042, 990 and 813 cm^{-1}.

ν_{max} (CCl_4) 1761 cm^{-1} (ε 1760), 1711 cm^{-1} (ε 830)

τ values 4.09 (1H, br.t, J=7 c/s), 4.88 (3H, m), 5.29 (1H, d, J=8 c/s), 5.73 (1H, d, J=6 c/s), 5.80 (3H, m), 6.30 (3H, s), 7.96 (3H, s), 8.03 (3 x 3H), 8.14 (3H, d, J=1.5 c/s), methyl contour 8.80, 8.95, 9.14, and 9.18.

λ_{max} 216 mμ (log ε 4.00)

After addition of 6 drops of 6N hydrochloric acid to both cells, and standing for 3 hours:

λ_{max} 228 mμ (infl.) (log ε 4.10), 236 mμ (log ε 4.13), 243 mμ (log ε 4.11), 252 mμ (log ε 3.90)

Analysis Found C 66.07%
H 8.20%
Highest ion at m/e 466

$C_{45}H_{66}O_{13}$ requires C 66.32%
H 8.16%
M.W. 814

20. L.S.III. (METHYL 5α- OR 17α- GLUCOSYLOXYTYROMYCATE).

This compound was isolated as described in Section 17(a). It could be obtained as an amorphous solid, m.p. 90-97°C, by addition of light petroleum to its benzene solution. R_f (10% MeOH in $CHCl_3$) 0.44.

ν_{max} (KBr) 3400 (br.), 2950. 2928, 1710, 1647, 1454, 1436, 1377, 1310, 1250, 1203, 1148, 1077 - 1073, 1043, 1016, 997, 908, 891 and 818 cm^{-1}.

ν_{max} ($CHCl_3$) 3450 cm^{-1} (br.), 1706 cm^{-1} (ε 737)

τ values 4.02 (1H, t, J=7 c/s), 4.38,4.62 (2H, m), 5.38 (1H, d, J= 8.5 c/s), 5.64 (1H, m), 6.29 (3H, s), 6.0 - 6.8 (br., m), 8.10 (3H, d, J=1.5 c/s), methyl contour 8.79, 8.88, 8.91, 9.09 and 9.43.

λ_{max} 230 mμ (infl.) (log ε 4.14), 238 mμ (log ε 4.27), 245.5 mμ

(log ε 4.28), 254 mμ (log ε 4.12)

Highest ion at m/e 482.

21 L.S.III TETRAACETATE. (METHYL 5a- OR 17a- TETRAACETYL-GLUCOSYLOXYTYROMYCATE).

This compound was isolated as detailed in Section 17(b). It crystallised from methanol as needles, m.p. 169-171°C. R_f (25% light petroleum in ether) 0.27. $[\alpha]_D$ -9.2 (c 0.7).

ν_{max} (KBr) 2954, 2880, 1744, 1707, 1640, 1452, 1432, 1370, 1243, 1230 - 1220, 1050 - 1040, 1036, 997 and 906 cm^{-1}.

ν_{max} (CCl_4) 1763 cm^{-1} (ε 1730), 1714 cm^{-1} (ε 782)

τ values 4.02 (1H, br.t, J=7 c/s), 4.44,4.66 (2H, m), 4.88 (3H, m), 5.30 (1H, d, J=8 c/s), 5.80 (3H, m), 6.28 (3H, s), 7.97 (4 x 3H), 8.10 (3H, d, J=1.5 c/s), methyl contour 8.79, 8.86, 8.89, 8.98, 9.08 and 9.46.

λ_{max} 229 mμ (infl.) (log ε 4.17), 237 mμ (log ε 4.22), 245 mμ (log ε 4.23), 254 mμ (log ε 4.09).

Analysis	Found	C 65.79%	Highest ion at m/e 481
		H 7.61%	
$C_{45}H_{64}O_{13}$	requires	C 66.15%	M.W. 812
		H 7.89%	

22. L.S.II. (METHYL 5α- OR 17α- GLUCOSYLOXY-3α-HYDROXYLANOSTA-7,9(11),24-TRIEN-26-OATE).

This compound was isolated as described in Section 17(a). Addition of light petroleum to its benzene solution precipitated it as an amorphous solid, m.p. 86-92°C. R_f (10% MeOH in $CHCl_3$) 0.30.

ν_{max} (KBr) 3400 (br.), 2946, 2873, 1710, 1646, 1456, 1425, 1374, 1250, 1205, 1152 - 1149, 1074, 1071, 1043, 1017, 993, 944, 905 and 820 cm^{-1}.

ν_{max} ($CHCl_3$) 3450 cm^{-1} (br.), 1710 cm^{-1} (ε 444)

τ values 4.05 (1H, br.t, J=7 c/s), 4.46,4.67 (2H, m), 5.45 (1H, d, J=8 c/s), 5.64 (1H, m), 6.29 (3H, s), 6.10 - 6.80 (br., m). 8.10 (3H, d, J=1.5 c/s), methyl contour 8.94, 9.04, 9.09, 9.11, 9.16 and 9.39.

λ_{max} 230 mμ (infl.) (log ε 4.06), 238 mμ (log ε 4.13), 246 mμ (log ε 4.14), 254.5 mμ (log ε 3.94)

Highest ion at m/e 484

23. L.S.II TETRAACETATE. (METHYL 3α-ACETOXY-5α- OR 17α- TETRA-ACETYLGLUCOSYLOXYLANOSTA-7,9(11),24-TRIEN-26-OATE).

This substance was isolated as described in Section 17(b). It crystallised from aqueous methanol as needles, m.p. 92-94°C. R_f (25% light petroleum in ether) 0.35. $[\alpha]_D$ +4.8 (c 0.3).

ν_{max} (KBr) 2935, 2916, 2840, 1755, 1742, 1710, 1642, 1451, 1433, 1372, 1243 - 1239, 1220 - 1216, 1150, 1130, 1090, 1038 - 1033, 1001, 990, 954 and 906 cm^{-1}.

ν_{max} (CCl_4) 1762 cm^{-1}, 1740 cm^{-1} (sh.) (ε 1720), 1713 cm^{-1} (ε 443)

τ values 4.02 (1H, br.t, J=7 c/s), 4.45, 4.68 (2H, m), 4.88 (3H, m), 5.32 (2H, m), 5.80 (3H, m), 6.28 (3H, s), 7.99 (5 x 3H), 8.10 (3H, d, J=1.5 c/s), methyl contour 8.94, 9.00, 9.05, 9.12, 9.17 and 9.49.

λ_{max} 228 mμ (infl.) (log ε 4.09), 237 mμ (log ε 4.15), 245 mμ (log ε 4.16), 253.5 mμ (log ε 3.97)

Analysis	**Found**	C 66.21%	Highest ion at m/e 525
		H 8.15%	
$C_{47}H_{68}O_{14}$	**requires**	C 65.87%	M.W. 856
		H 7.99%	

24. CULTURE, EXTRACTION AND METHYLATION.

A stock culture of Leptoporus stipticus (Pers.) Quel. was obtained from Centraalbureau voor Schimmelcultures, Baarn, Netherlands, and grown on 5% malt agar slopes for 3 weeks. Further culture on malt extract for 3 weeks provided mycelium which was blended and used to inoculate 5% malt agar in 100 roux bottles. This medium had previously been autoclaved at 12 p.s.i. for 30 minutes (twice, at an interval of 24 hours). Cultures were allowed to grow, at 25°C and 70% relative humidity with artificial illumination being provided for 12 hours per day, for 8 weeks. The mycelium was separated and dried at 40°C.

The dried mycelium (61 g.) was extracted with cold methanol (1 l.) and then by soxhlet treatment with methanol. The combined extracts (29 g.) were treated with light petroleum to remove lipids (20%) and then allowed to react with excess ethereal diazomethane overnight. Evaporation of the solvents provided a brown gum (22 g.). T.l.c. in chloroform indicated that this material contained two main components at R_f 0.37 and R_f 0.19.

25. SEPARATION OF THE TWO MAJOR COMPONENTS.

A portion (5 g.) was treated with benzene (100 ml.). This efficiently separated the two major components (2.9 g.) from the considerably more polar insoluble residue. The benzene soluble

fraction was adsorbed on to silicic acid (750 g.) and eluted successively with benzene (3 x 250 ml.), chloroform (5 x 250 ml.) and finally with ethyl acetate (2 x 250 ml.). T.l.c. in chloroform showed that the substances of R_f 0.37 and 0.19 had been eluted together by chloroform and were present in all five fractions. These were therefore combined and the resulting yellow solid (1.3 g.) was chromatographed on silicic acid (75 g.), using as eluent benzene (500 ml.) to which chloroform (500 ml.) was gradually added. No distinct separation was achieved, the two components again being co-eluted. The material recovered from this column (1.2 g.) was separated into its two components by p.l.c. on four 50 x 20 x 0.1 cm. layers of Kieselgel HF_{254} with chloroform as eluent. This allowed complete separation of the compound of R_f 0.37 (360 mg.) from the other component of R_f 0.19 (500 mg.). Total yields of each of these compounds from 61 g. of mycelium: 1.9 g. and 2.2 g. respectively.

26. <u>THE COMPOUND OF R_f 0.37. (METHYL EBURICOATE.)</u>

This compound was isolated as described in Section 25. It crystallised from aqueous methanol as fine needles, m.p. 145-147°C. $[\alpha]_D$ +46 (c 0.4).

ν_{max} (KBr) 3300 (br.), 2967, 2895, 2849, 1733, 1647, 1469, 1458, 1440, 1379, 1368, 1281, 1260, 1164, 1152, 1102, 1087, 1070, 1042,

1023, 1008 and 894 cm^{-1}.

ν_{max} ($CHCl_3$) 3621 cm^{-1} (ε 41), 1727 cm^{-1} (ε 403)

τ values (60 Mc/s) 5.24 (2H, d, J=5 c/s), 6.32 (3H, s), 6.77 (1H, m), methyl contour 8.95, 9.01, 9.04, 9.11, 9.20 and 9.27.

Analysis	Found	C 78.99%	M^+ at m/e 484
		H 10.73%	
$C_{32}H_{52}O_3$	requires	C 79.29%	M.W. 484
		H 10.81%	

This material was identical (m.p., mixed m.p., R_f) with material obtained by hydrolysis of authentic methyl acetyleburicoate, generously gifted by Dr. J. McLean (University of Strathclyde). Acetylation with acetic anhydride/pyridine yielded the acetate, which crystallised from methanol as needles, m.p. 157-159°C. This was identical (m.p., mixed m.p., R_f) with the authentic sample.

27. THE COMPOUND OF R_f 0.19. (METHYL TUMULOSATE.)

This compound was isolated as described in Section 25. It crystallised from aqueous methanol as needles, m.p. 175-177°C.

$[\alpha]_D$ +34 (c 0.9)

ν_{max} (KBr) 3400 (br.), 3946 - 3932, 3871, 1737, 1716, 1641, 1452, 1440 - 1433, 1373, 1275, 1202, 1160 - 1154, 1096, 1052, 1041, 1029, 997 and 889 cm^{-1}.

ν_{max} ($CHCl_3$) 3626 cm^{-1} (ε 81), 1728 cm^{-1} (ε 473)

τ values (60 Mc/s) 5.23 (2H, d, J=6 c/s), 5.74 (1H, m), 6.31 (3H, s), 6.50 (1H, s), 6.76 (1H, m), methyl contour 8.96, 9.01 9.04, 9.07, 9.21 and 9.24.

λ_{max} 237 mμ (ε 2100), 244 mμ (ε 2500), 244 mμ (ε 1900)

Analysis	Found	C 73.96%	M^+ at m/e 500
		H 10.37%	
$C_{32}H_{52}O_4.H_2O$ requires		C 74.09%	M.W. 500
		H 10.49%	

28. CULTURE OF LEPTOPORUS STIPTICUS (PERS.) QUEL. UNDER DIFFERENT CONDITIONS.

(a) Variation of the type of wood included.

Equal quantities of seven different types of wood: Sitka spruce, white pine, Parana pine, Scots pine, oak, hemlock and elm, in fine powder form (2%) were added to 5% malt in 2% agar, and the whole was sterilised (twice). Each was inoculated with Leptoporus stipticus (Pers.) Quel. and allowed to grow, under the same conditions as previously, for 8 weeks. The methanol extract of the harvested mycelium was methylated with an excess of ethereal diazomethane. T.l.c. of this material in 10% methanol in chloroform allowed detection of the glucosides, if present. P.l.c. on Kieselgel H in chloroform and removal of all

material between R_f 0.1 and 0.8 (relative to the standard dye spots) gave, after extraction with ethyl acetate, the mixture which was examined by g.l.c. on 1% Q.F.1 at 225°C with a flow rate of 55 ml./ min.

A mixture containing equal quantities of methyl tyromycate and methyl eburicoate was found, under these conditions, to give well separated peaks of equal height, R_T 17.5 min. and 26.5 min. respectively.

(b) Extracts of white pine.

Finely powered white pine wood was soxhlet extracted for 12 hours with light petroleum. The residue obtained by evaporation of the light petroleum extract and the extracted powder were added separately to malt agar culture medium. The whole proceedure was repeated using water and then again using methanol. The six media so prepared were inoculated with Leptoporus stipticus (Pers.) Quel.. Further treatment was identical to that described above (part (a))

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SUMMARY.

Structural studies on the methyl esters of the triterpene acids from the polypore Daedalea quercina have established the structure of the major component, methyl carbomethoxyacetyl-quercinate. In common with this compound, three other esters from this fungus are 23-keto-27-carboxylic esters. The first and second of these are methyl 16-hydroxycarbomethoxyacetylquercinate and methyl 16-hydroxyquercinate respectively. The third ester, methyl carbomethoxyacetyldaedaleate, which is the first recorded triterpene based on a C_{32} skeleton, is highly reactive and has not, as yet, yielded recognisable transformation products. The structure advanced for this compound is based on spectroscopic examination. A minor constituent of Daedalea quercina is thought to be methyl 25-hydroxypolyporenate C.

The synthesis and properties of carbomethoxyacetyl and carboxyacetyl derivatives of triterpenes have been studied.

Examination of the methyl esters of the triterpene acids from Leptoporus stipticus has established that the major component, methyl tyromycate, is methyl 3-oxolanosta-7,9(11),24-trien-26-oate. Four other compounds from this fungus have been studied. Three of these have been shown to be triterpene glucosides, the first from a fungal source, whose basic nuclei are similar to

that of methyl tyromycate. The last ester from Leptoporus stipticus, methyl leptoporate, is the second example of a C_{32} triterpene.

Leptoporus stipticus (Pers.) Quel., when cultured on malt agar medium, produces eburicoic acid and tumulosic acid. Attempts to influence the metabolite production of this latter fungus are described.