

STUDIES IN THE BIOSYNTHESIS

OF TRITERPENOIDS

a thesis presented by

DAVID MILES HARRISON

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Chemistry Department,
Imperial College,
London, S.W.7.

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ABSTRACT

The biosynthesis of sterols in mammals, plants and fungi is reviewed.

A new sterol, 4 α -methylergosta-8,24(28)-dien-3 β -ol has been isolated from yeast sterol residues and its partial synthesis is described.

Procedures for the feeding of sterols to the whole cells of Saccharomyces cerevisiae and Polyporus sulphureus are described. A number of ^{14}C and ^3H labelled sterols have been prepared and fed to these species in vivo in order to elucidate biosynthetic pathways to ergosterol and to eburicoic acid. The intermediacy of 24-methylene compounds in the biosynthesis of ergosterol has been demonstrated. The feeding results suggested the possible occurrence of several alternative biosynthetic pathways from lanosterol to ergosterol.

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REVIEW OF STEROL BIOSYNTHESIS

INTRODUCTION

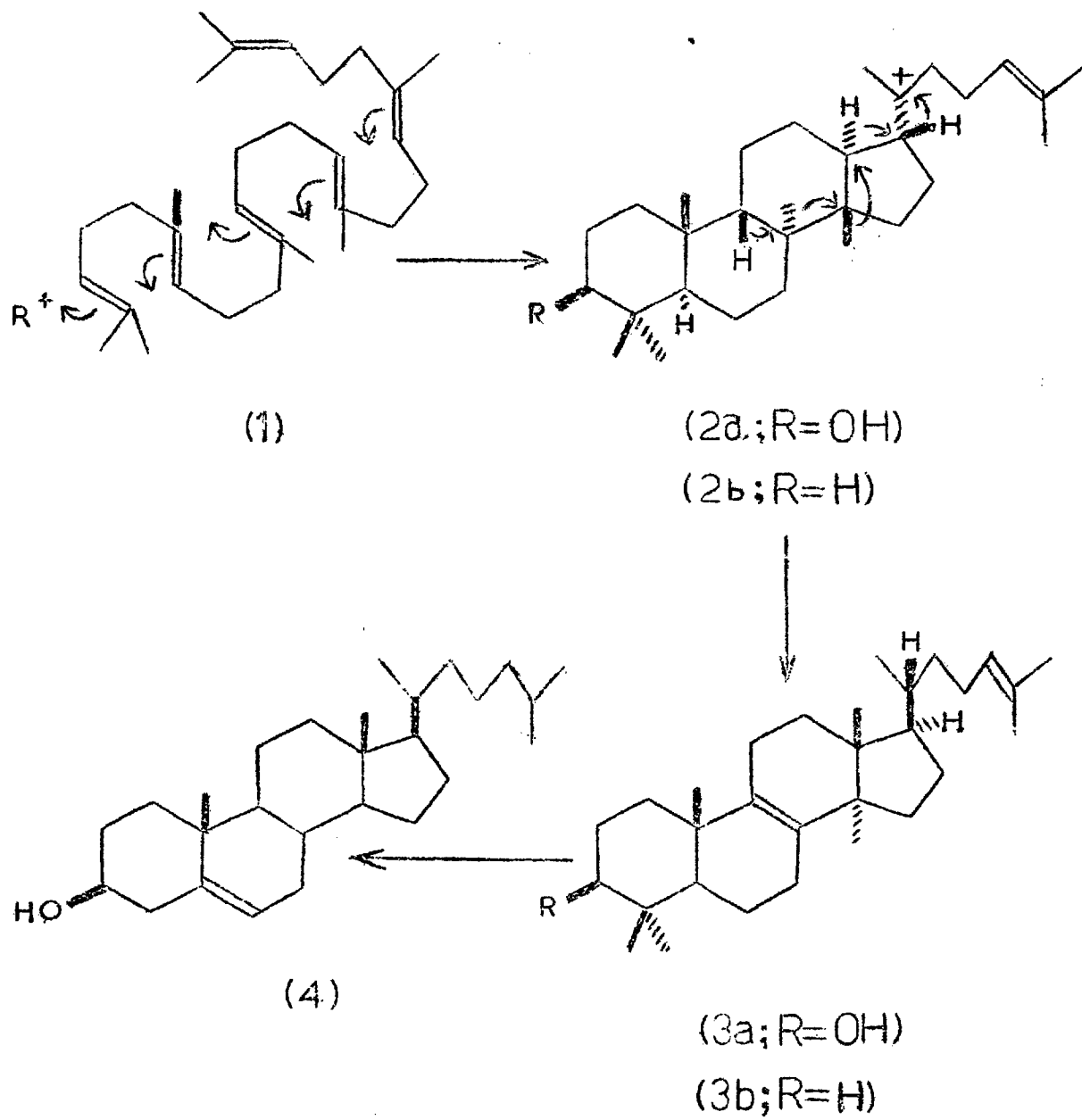
The work presented in this thesis is concerned mainly with the biosynthesis of the sterols of fungi. Particular emphasis is placed on the sequence of reactions and the mode of introduction of the biogenetically "extra" alkyl group at C-24 in some fungal sterols. In this connection published work on the biosynthesis of the sterols of higher plants will be discussed. An extensive account of the biosynthesis of cholesterol is inevitable since only in mammalian tissues has detailed work been reported concerning the nuclear transformations which occur during the later stages of sterol biosynthesis.

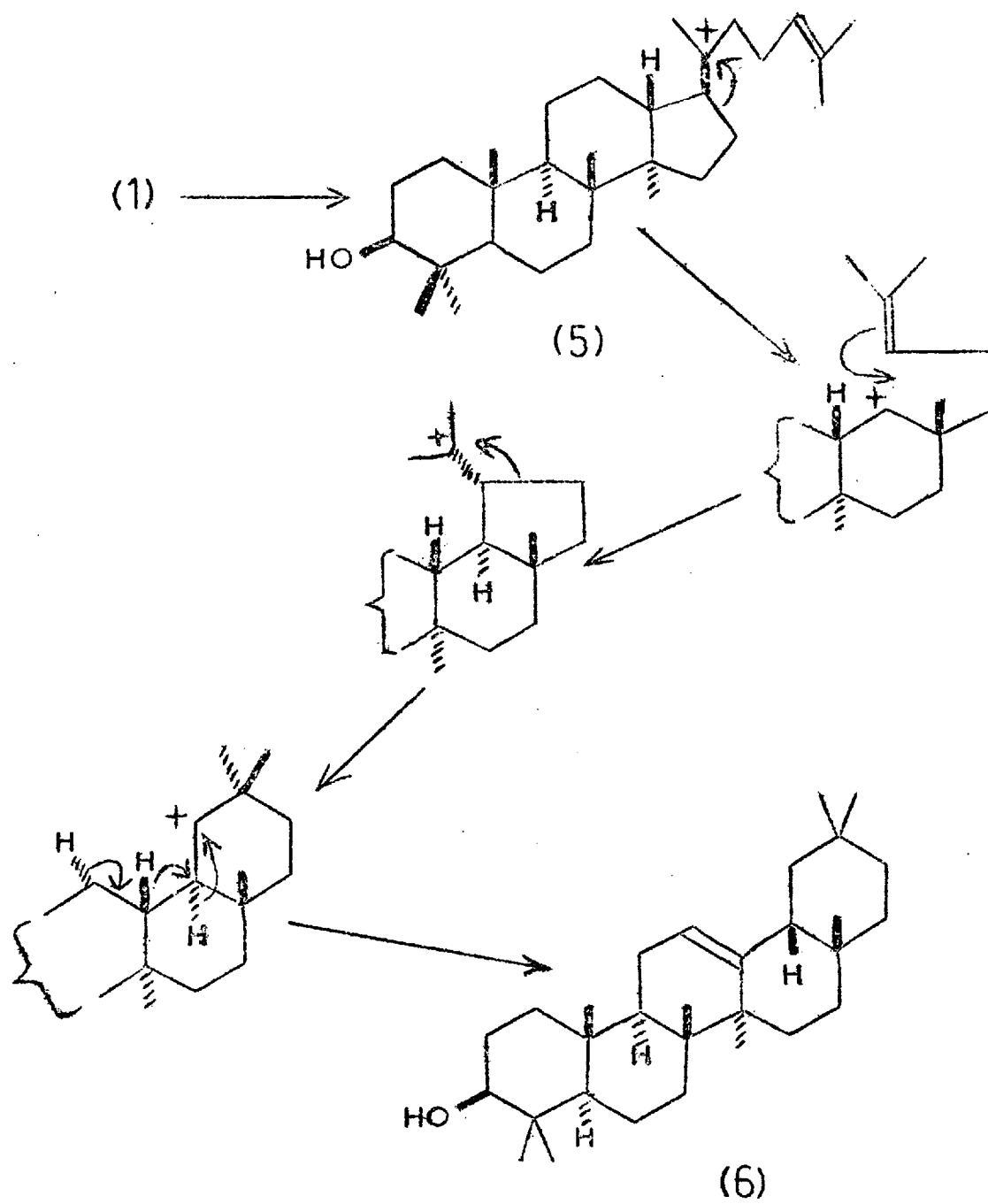
The early stages in sterol biosynthesis, from acetic acid through mevalonic acid to squalene, is of less relevance to the main theme, and will not be discussed explicitly. However, results from this field will be introduced when required throughout this review and subsequent discussion.

An excellent review on the stereochemical consequences of the biological conversion of mevalonic acid to squalene has recently appeared.¹

1. Conversion of squalene to lanosterol

The early theoretical treatment of Eschenmoser and his colleagues² envisaged cyclisation of squalene (1) by the hypothetical species OH^+ . The cyclising enzyme would hold the substrate in a chair-boat-chair-boat conformation which would define the stereochemistry of the intermediate cation (2a). A series of 1,2-hydride and methyl shifts (scheme 1; $\text{R}=\text{OH}$) culminating in loss of the C-9 proton would give lanosterol (3a). Cyclisation of squalene in a chair-chair-chair-boat conformation would lead to the cation (5) which after further cyclisation and rearrangements (scheme 2) would give β -amyrin (6). In like manner routes to all the then known triterpene skeletons were postulated.² Support for this scheme was advanced by Maudgal et al.³ and by Cornforth and his coworkers⁴ who demonstrated that the cyclisation of squalene to lanosterol occurs with two 1,2-methyl shifts from C-14 to C-13 and from C-8 to C-14 (scheme 1) rather than the alternative of one 1,3-methyl shift from C-8 to C-13. The tritium labelling patterns and $^3\text{H}/^{14}\text{C}$ ratios in cholesterol⁵ (4) and β -amyrin⁶ (6) biosynthesised from $[2\text{-}^{14}\text{C}, 4\text{R-}^3\text{H}]$ -mevalonic acid were in complete accord with the scheme of Eschenmoser et al.²

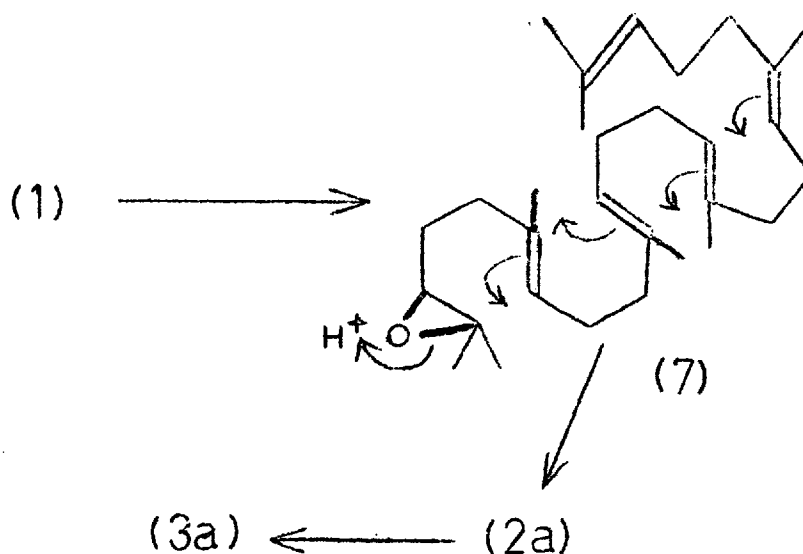
Scheme 1

Scheme 2

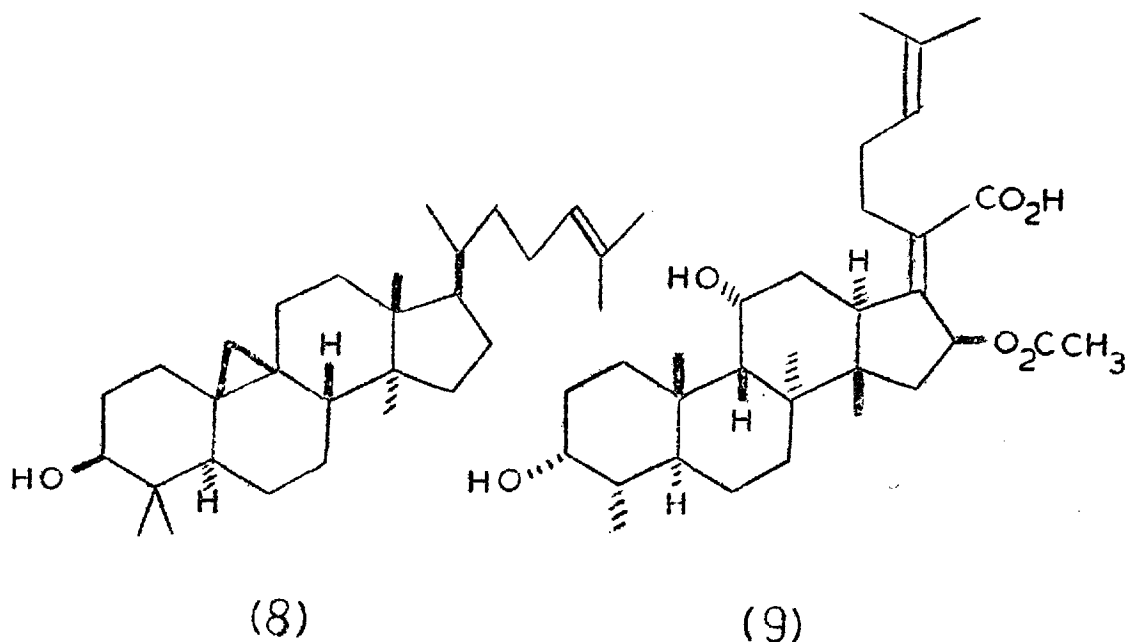
The mechanism of the hydroxylation at C-3 has been studied by Bloch and Tchen⁷ who demonstrated a requirement for NADPH and molecular oxygen. Also, label from $[^{18}\text{O}]$ -oxygen but not from $[^{18}\text{O}]\text{-H}_2\text{O}$ was incorporated into lanosterol by rat liver homogenates.⁸ Recently Barton and Moss⁹ have suggested that these data are consistent with a proton initiated cyclisation of squalene (scheme 1; R=H) followed by hydroxylation of the lanostadiene (3b) so formed to give lanosterol (3a). In support of this proposal, they have demonstrated⁹ a small incorporation of $[2\text{-}^3\text{H}]\text{-lanostadiene}$ into lanosterol in a yeast enzyme system. However, attempts to "trap" labelled lanostadiene after feeding $[2\text{-}^{14}\text{C}]\text{-mevalonic acid}$, were unsuccessful.

An alternative scheme, advanced simultaneously by Corey¹⁰ and van Tamelen¹¹ and their coworkers envisaged the oxidation of squalene to squalene-2,3-epoxide (7) followed by proton initiated cyclisation (scheme 3). Thus $[^{14}\text{C}]\text{-squalene-2,3-epoxide}$ was converted anaerobically to lanosterol (3a) and dihydrolanosterol (3a;24,25-dihydro) or aerobically to cholesterol (4) in excellent yield by a rat liver homogenate, and inactive squalene oxide efficiently trapped activity when $[^{14}\text{C}]\text{-squalene}$ was incubated

Scheme 3

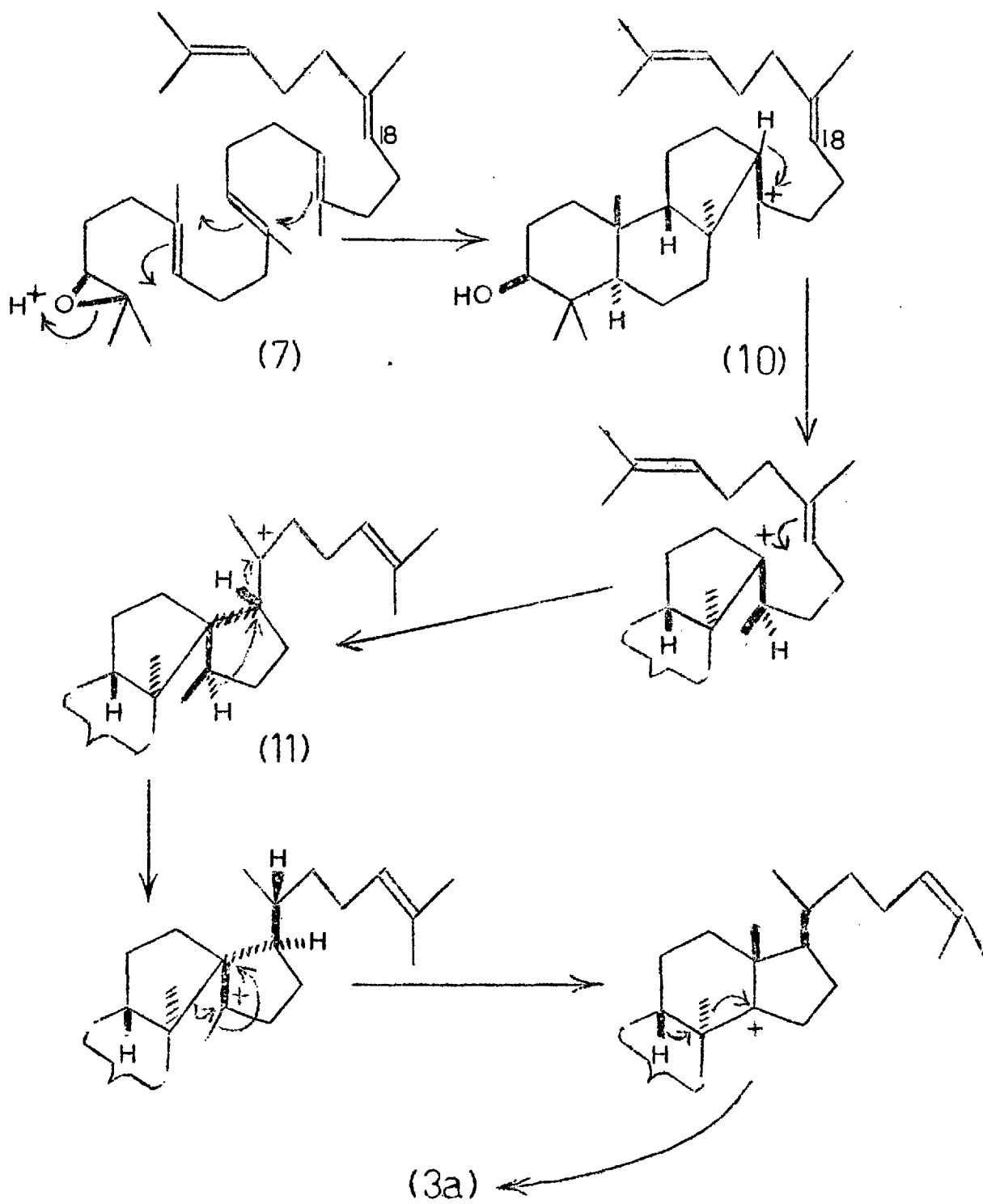


with this homogenate.^{10,11} Also [^3H , ^{18}O]-squalene-2,3-epoxide showed no loss of ^{18}O on enzymic conversion to lanosterol.¹² The generality of squalene epoxide as an intermediate between squalene and 3-hydroxy triterpenes has been demonstrated by the conversion of the oxide (7) to cycloartenol (8) in a bean leaf homogenate,¹³ to fusidic acid (9) by the fungus Fusidium ~~F. coccineum~~,¹⁴ and to β -amyrin (6) in a cell-free pea system.¹⁵ Also [$1\text{-}^{14}\text{C}$]-sodium acetate was converted inter alia to [^{14}C]-squalene-2,3-epoxide in the tobacco plant.^{16,17} The cyclising enzyme "2,3-oxidosqualene cyclase" has been isolated from hog liver¹⁸ and numerous

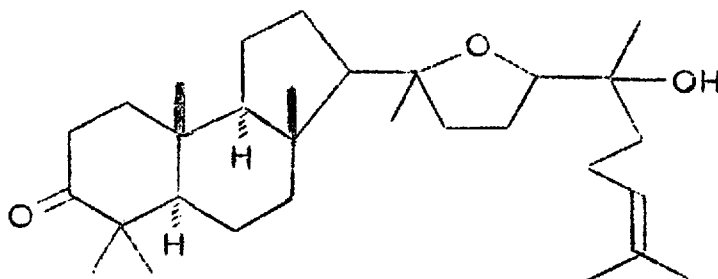


studies on the specificity of this enzyme have been reported.¹⁹ The enzyme has no cofactor requirements.^{18,20} Thus the requirement for oxygen and NADPH⁷ in the overall reaction squalene (1) $\longrightarrow$ lanosterol (3a) is concerned solely with the formation of the squalene oxide intermediate, as would be expected.

The enzymic cyclisation of squalene oxide to lanosterol can be considered as a concerted process (scheme 3) similar to that of Eschenmoser et al.² However, the non-enzymic cyclisation²¹ of squalene oxide led to tricyclic products derived from the cation (10; stereochemistry undefined). Also enzymic

Scheme 4

cyclisation of 18,19-dihydrosqualene-2,3-oxide (7;18,19-dihydro) gave similar tricyclic products which could have arisen from the intermediate cation (10;18,19-dihydro). On this basis, van Tamelen and his coworkers have suggested²² that enzymic cyclisation of squalene oxide may normally proceed via the cation (10) and the spiro cation (11;scheme 4). Some evidence for this speculation derives from the recent isolation by Chawla and Dev²³ of the interesting novel triterpene malabaricol (12) which must be derived biosynthetically by such a route.



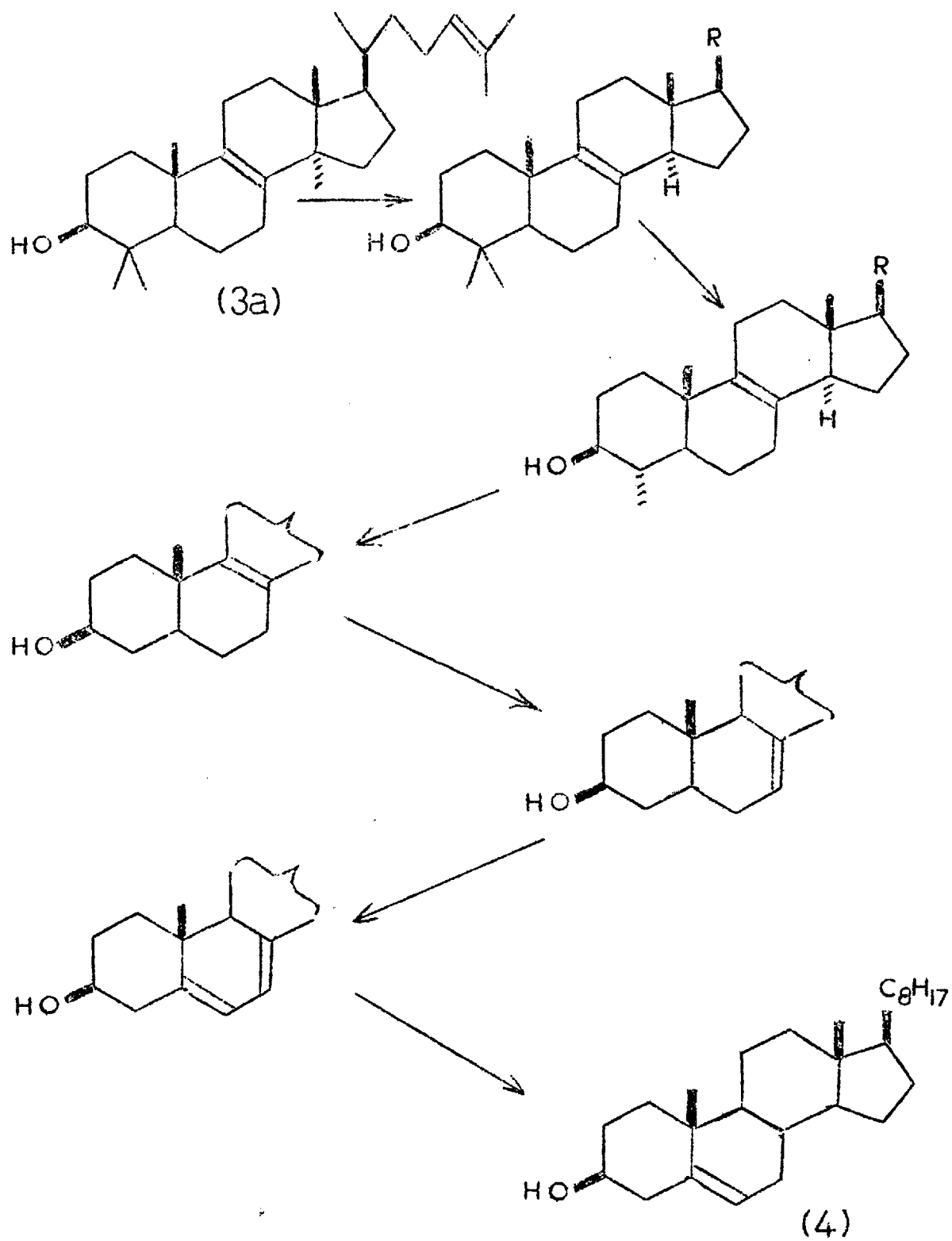
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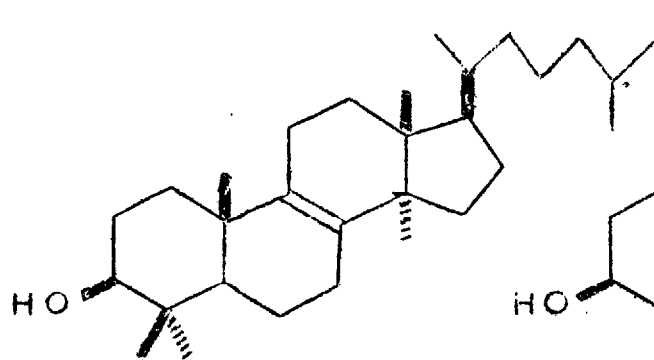
2. The conversion of lanosterol to cholesterol

The biosynthesis of cholesterol (4) from lanosterol (3a) has been the subject of extensive studies by many authors, and has recently been reviewed by Bloch,²⁴ Clayton²⁵ and Frantz and Schroepfer.²⁶ The conversion of lanosterol to cholesterol involves the reduction of the side chain double bond, removal of the C-14 and C-4 methyl groups and "isomerisation" of the nuclear double bond to the Δ^5 position. Scheme 5 shows the major route of cholesterol biosynthesis in rat livers, and will form the basis of the following discussion, which will be mainly concerned with recent mechanistic work.

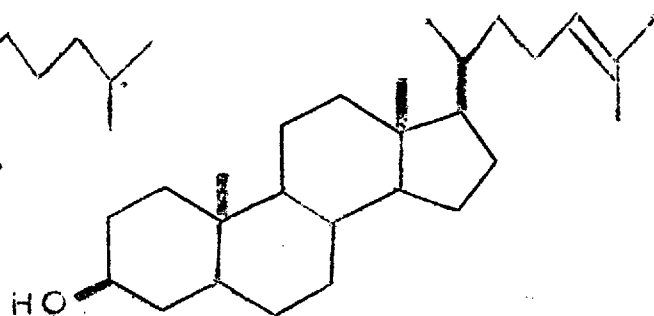
Reduction of the sterol side chain

Kandutsch and Russell²⁷ have shown that the first step in the conversion of lanosterol to cholesterol in a mouse preputial tumor is reduction of the Δ^{24} double bond. Dihydrolanosterol (13) is then further metabolised as shown (scheme 5; $R = C_8H_{17}$). However, in rat livers and most other mammalian tissues, the reduction of the side chain double bond may occur at almost any stage.²⁶ Lewis, Scallen and Frantz²⁸ have shown that the ⁿatural substrate, cholest-24-enol (14) is converted in 58% yield to cholestanol (15) in a rat liver preparation, so

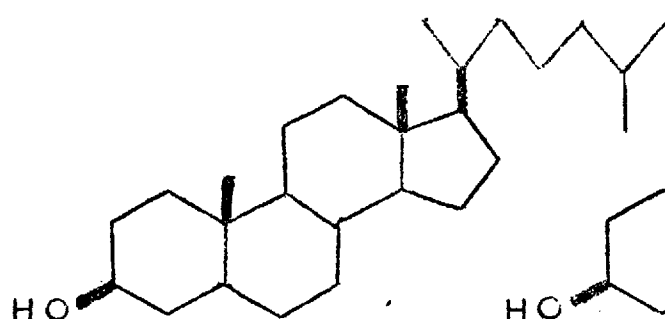
Scheme 5



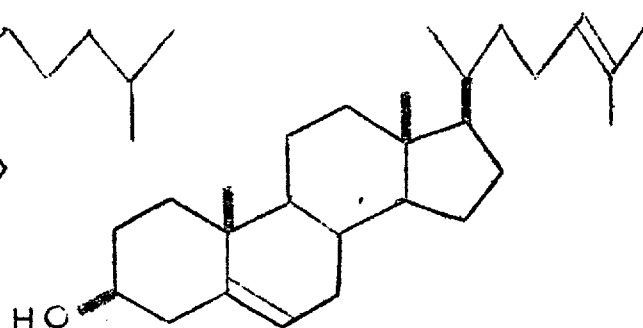
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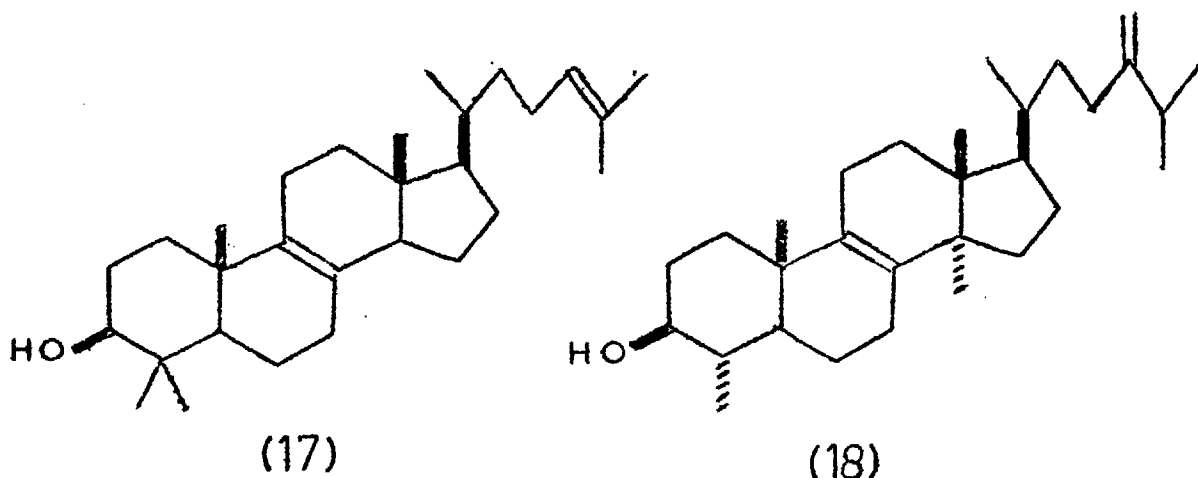
(14)



(15)



(16)



demonstrating that the side chain reduction occurs independently of minor alterations in the nucleus.

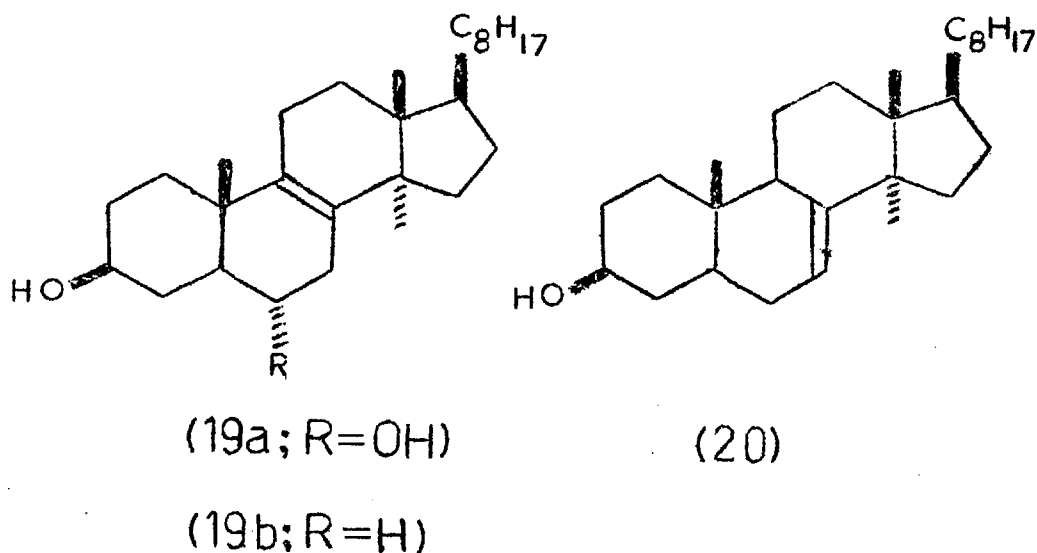
The irreversible conversion of lanosterol to dihydrolanosterol²⁹ or desmosterol (16) to cholesterol³⁰ occur anaerobically in rat liver homogenates.

NADPH is a necessary cofactor and label from $[4-^3\text{H}]\text{-NADPH}$ appears in cholesterol biosynthesised from desmosterol.³⁰

It is well known that the drug triparanol inhibits the enzyme or enzymes mediating this reduction, and in livers of triparanol-fed rats, cholesterol (4) is not formed, and desmosterol (16) accumulates.³¹

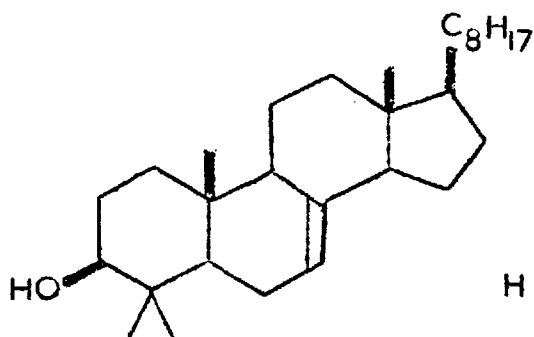
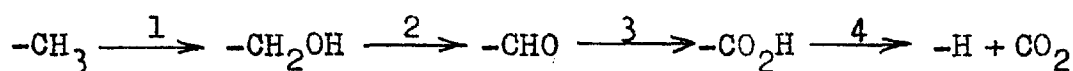
Loss of the C-4 and C-14 methyl groups

The isolation by Gautschi and Bloch of 4,4-dimethylcholesta-8,24-dienol (17) from rat tissues^{32,33} and its

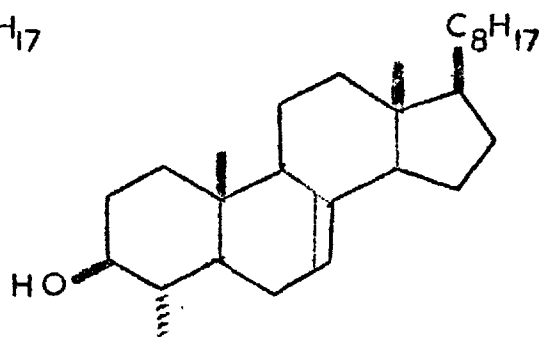


rapid conversion to cholesterol,³² demonstrates that the 14 α -methyl group is removed before those at C-4 in rats. However the existence of the plant sterols obtusifolior³⁴ (18) and macdougallin³⁵ (19a) demonstrates that at least in some plants, demethylation can occur at C-4 before loss of the 14 α -methyl group. It is interesting, therefore, that Knight and his colleagues have demonstrated the efficient incorporation of 14 α -methylcholest-8-enol³⁶ (19b) and 14 α -methylcholest-7-enol^{36,37} (20) into cholesterol by rat liver homogenates. However, no sterol with a 14 α -methyl group but lacking the 4,4-dimethyl substituents has yet been found in mammals, and hence the earlier conclusion³² that the C-14 methyl is the first removed in mammals remains sound.

It has long been known that the C-4 and C-14 methyl groups of lanosterol are lost as carbon dioxide during conversion to cholesterol.³⁸ Gaylor and his coworkers³⁹ have shown that 4,4-dimethylcholest-7-enol (21) labelled with ^{14}C in the C-4 methyl groups is converted to $[\text{}^{14}\text{C}]\text{-}4\alpha\text{-methylcholest-7-enol}$ (22) and one mole of $^{14}\text{CO}_2$ by a rat liver preparation. The methyl group removed was oxidised in discrete steps thus:

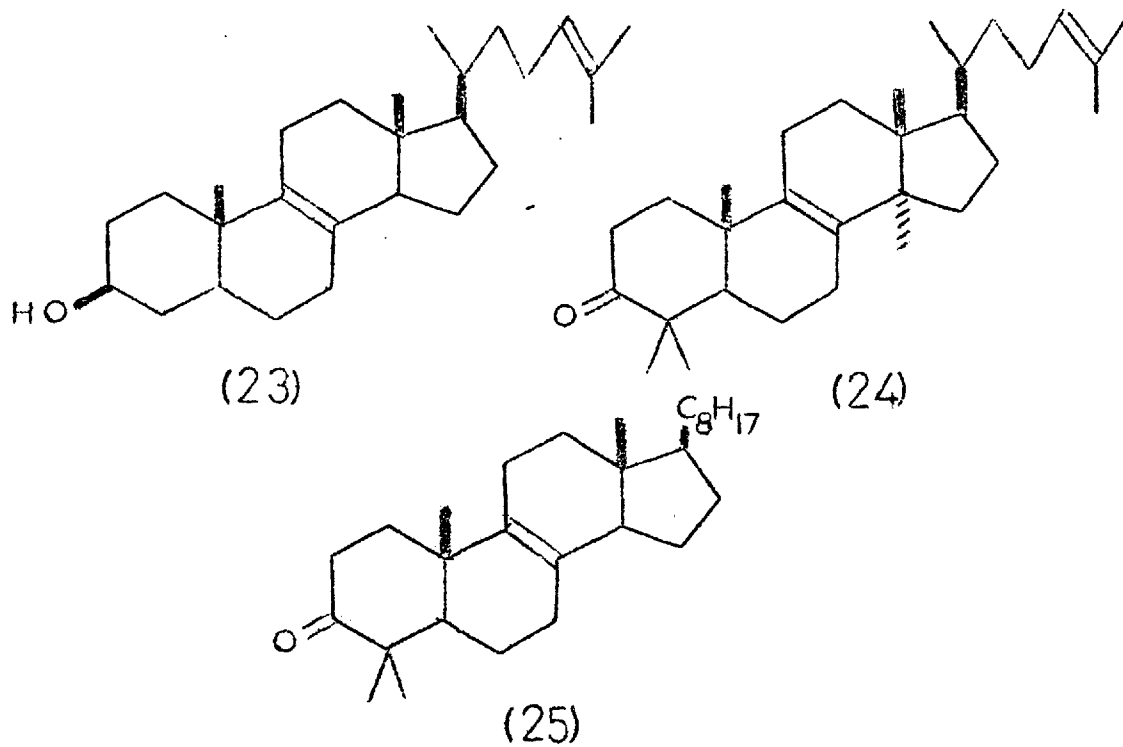


(21)

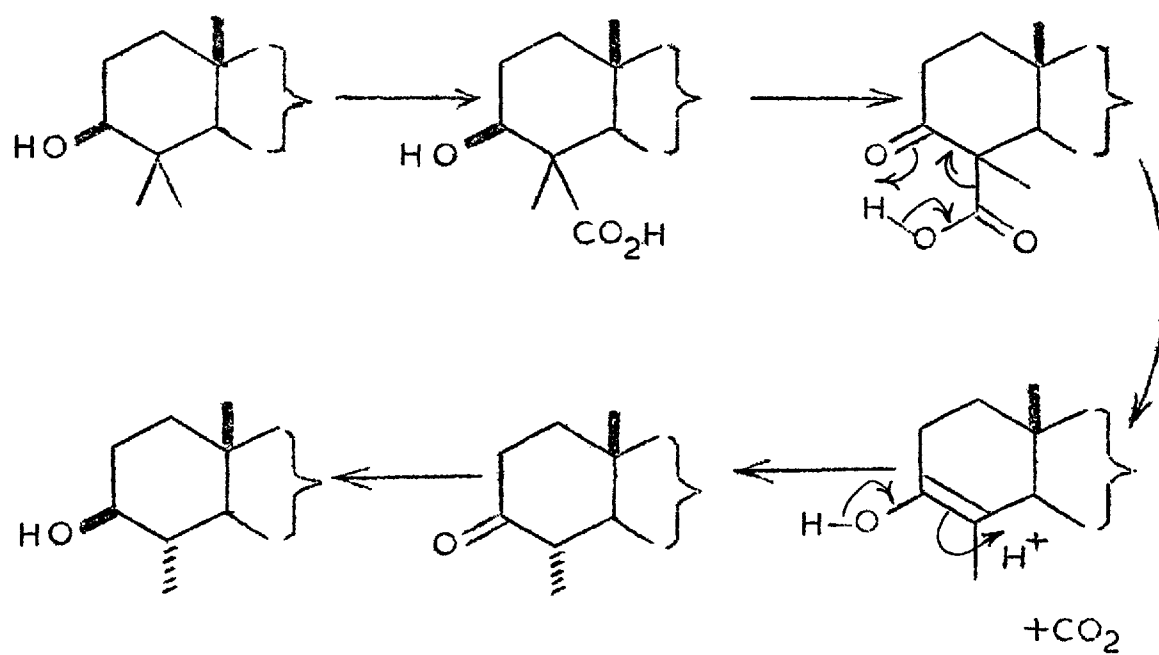


(22)

The first step required molecular oxygen and NADH while steps 2 and 3 required NAD^+ but not oxygen.³⁹ Decarboxylation at C-4 is believed to occur via a 3-oxo intermediate (scheme 6) and the almost quantitative loss of a

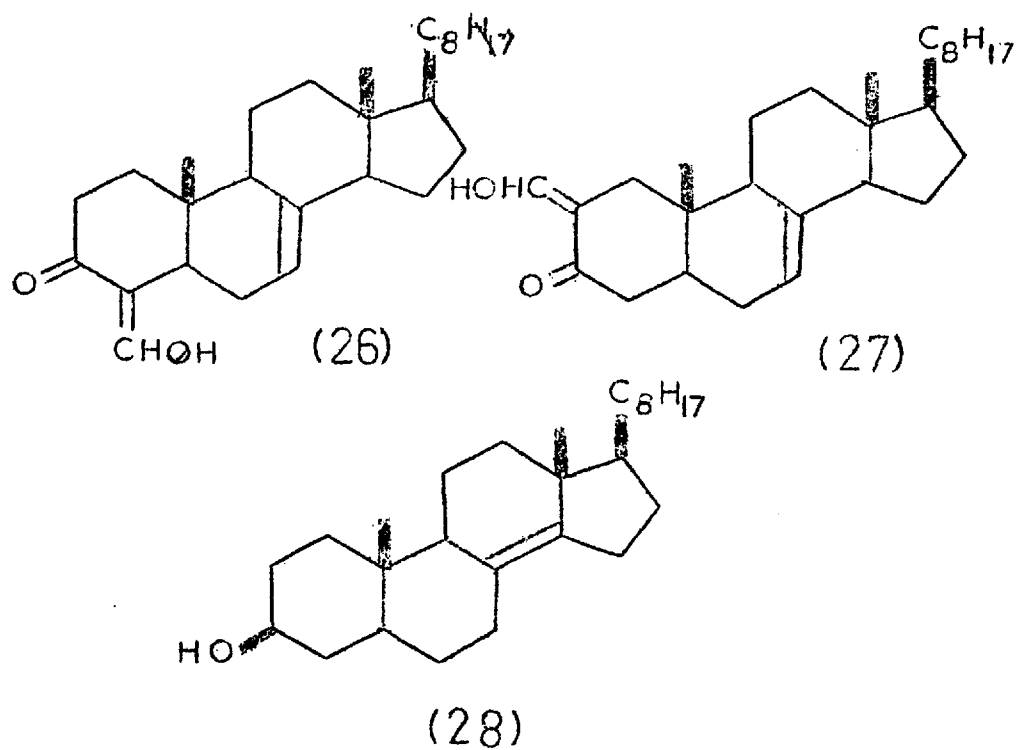


Scheme 6

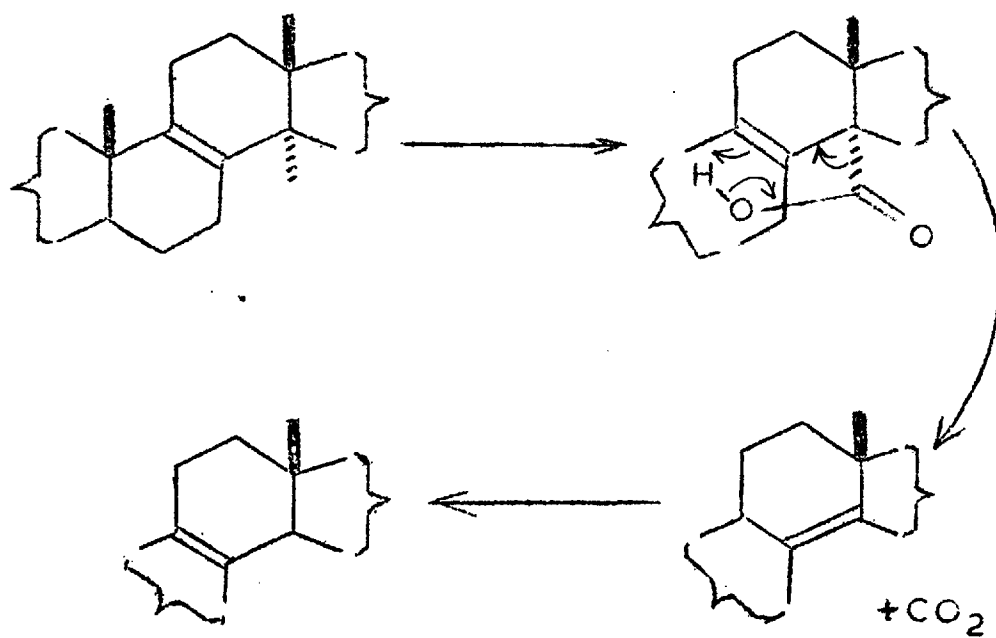


$[3\alpha-^3\text{H}]$ -label from 4,4-dimethyl or 4α -methyl sterols but not from zymosterol (23), on conversion⁴⁰ to cholesterol in vitro supports the postulate of a 3-oxo intermediate. The biological conversion of lanostadienone (24) and 4,4-dimethylcholest-8-enone (25) to cholesterol⁴⁰ gives further support to this proposal. Pudles and Bloch have reported⁴¹ the conversion of 4-hydroxymethylenecholest-7-en-3-one (26) labelled with ^3H at C-2, and with ^{14}C in the C-4 substituent to $[2-^3\text{H}]$ -cholesterol and $^{14}\text{CO}_2$ by rat liver homogenates in the presence of oxygen. Under anaerobic conditions cholest-7-enol and $^{14}\text{CO}_2$ were formed. This experiment seemed to further demonstrate the intermediacy of a 3-oxo derivative and the stepwise nature of the oxidation of the C-4 methyls. However, recent work by Morand, Stavrić and Godin has shown⁴² that the precursor of Pudles and Bloch may have been the 2-hydroxymethylene derivative (27) and the meaning of the earlier biosynthetic result is doubtful.

The 14α -methyl group of lanosterol is presumably³⁸ also oxidised to a carboxyl group before loss as carbon dioxide. The subsequent decarboxylation could conceivably be facilitated by the $\Delta^{8(9)}$ double bond (scheme 7) as suggested by Bloch.⁴³ However, $\Delta^{8(14)}$ sterols are not known in mammals and since cholest-8(14)-enol (28) was



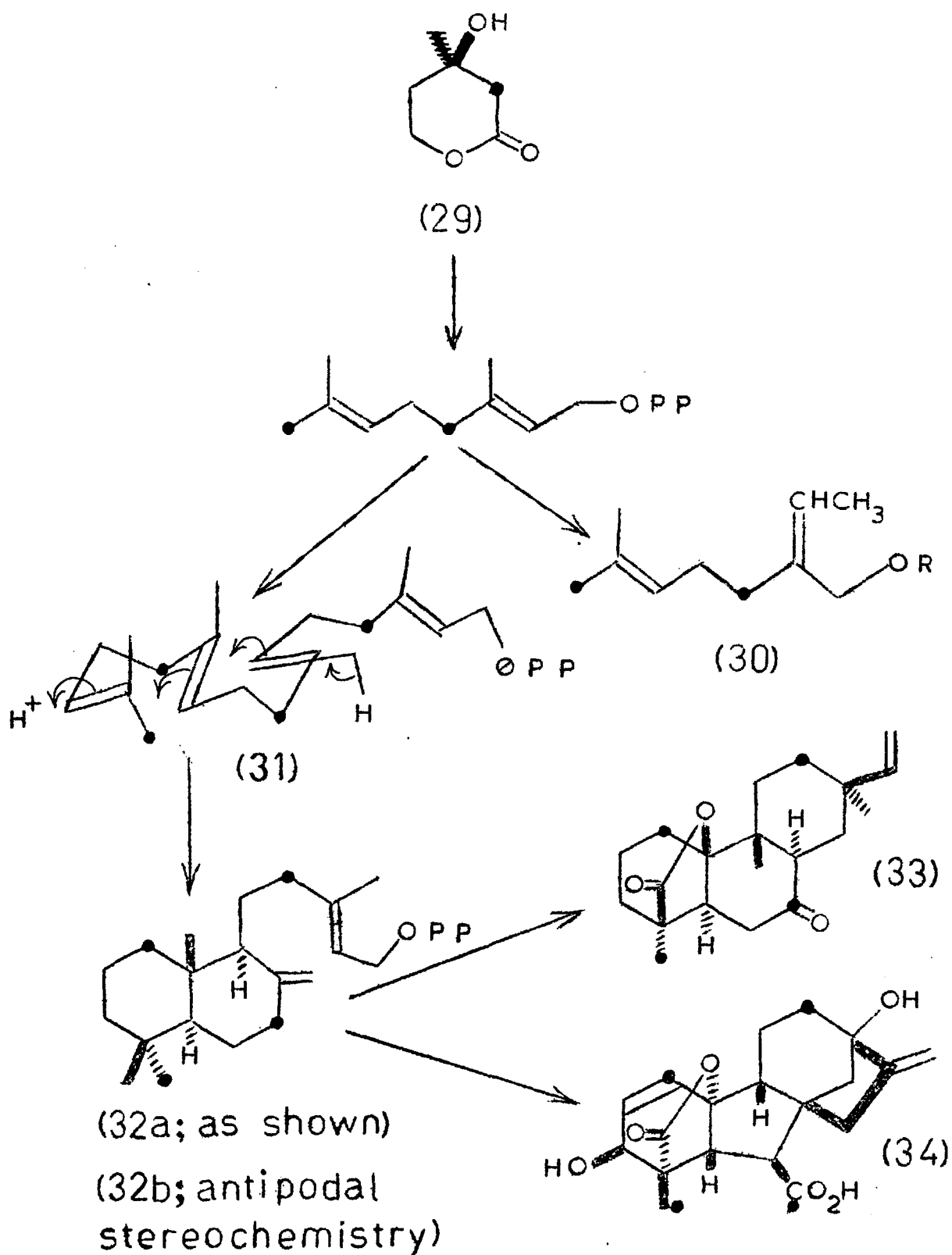
Scheme 7



not converted to cholesterol by a rat liver homogenate³⁶ the intermediacy of $\Delta^8(14)$ sterols seems unlikely.

The order of removal of the 4α and 4β methyl groups has been the subject of some discussion in the literature. Although the route $4,4$ -dimethyl sterol $\longrightarrow$ 4α -methyl sterol $\longrightarrow$ 4 -desmethyl sterol (scheme 5) is well established,²⁶ Popják and Cornforth have pointed out⁴⁴ that this does not constitute evidence for the prior removal of the 4β -methyl group from a $4,4$ -dimethyl precursor. The first demethylation involves an enol intermediate (scheme 6) and the remaining methyl substituent would be expected to adopt the more stable 4α (equatorial) configuration on reprotonation of the enol.⁴⁴ Gaylor and Delwiche⁴⁵ have prepared $[^{14}\text{C}]-24,25$ -dihydrolanosterol, labelled biosynthetically, inter alia, at one of the C-4 methyl groups, via $[2-^{14}\text{C}]-\text{mevalonate}$. On incubation of this precursor with a rat liver homogenate, one mole of inactive CO_2 was evolved to give a labelled 4α -methyl sterol fraction which released one mole $^{14}\text{CO}_2$ on further incubation to give C_{27} sterols. They interpreted this result to mean that the 4β -methyl was removed first and that the 4α -methyl of lanosterol is that labelled by $[2-^{14}\text{C}]-\text{mevalonate}$. However, as pointed out previously^{25,26} these results do not follow from the arguments presented, owing to the intermediacy of an enol in the demethylation as discussed above.

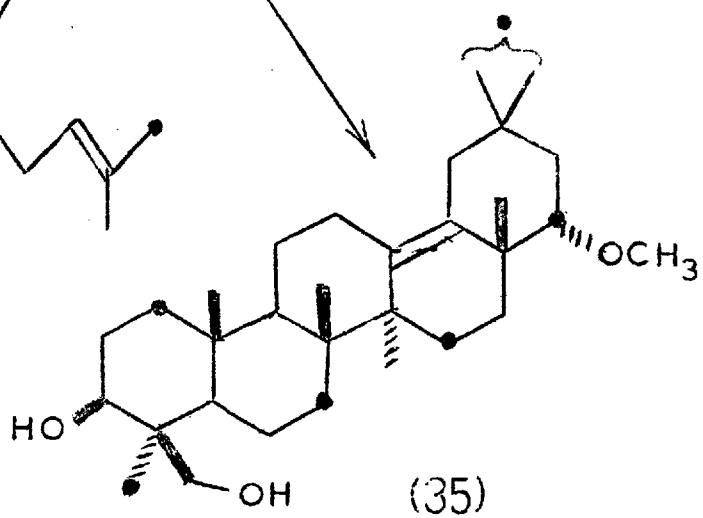
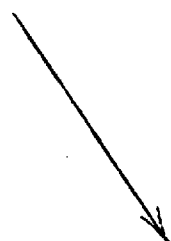
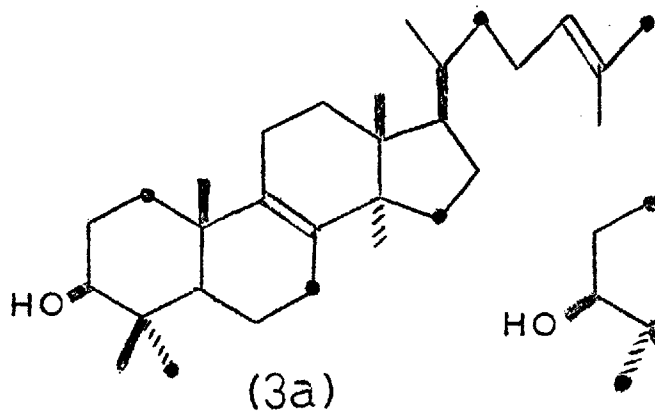
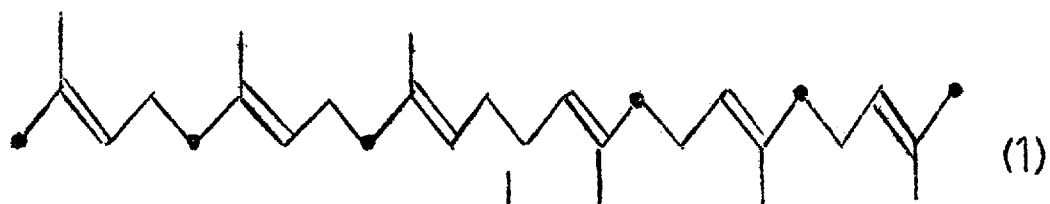
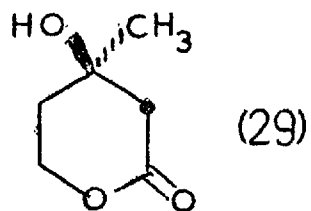
Scheme 8



There is no direct evidence concerning which of the C-4 methyl substituents in lanosterol is labelled by $[2-^{14}\text{C}]$ -mevalonate (29). However Birch et al.⁴⁶ have shown that this precursor labels the fungal product mycelianamide (30) in the trans terminal methyl group. Furthermore, rosenonolactone (33) and gibberellic acid (34) were labelled⁴⁷ by $[2-^{14}\text{C}]$ -mevalonic acid in the C-4-equatorial substituent and not in the C-4-axial substituent (steroid nomenclature). This supports the view that the trans terminal methyl group of geranylgeranylpyrophosphate (31) is labelled by $[2-^{14}\text{C}]$ -mevalonic acid, assuming that the cyclisation occurred in the sense depicted in scheme 8, to give the intermediate (32a) or (32b) respectively. By analogy, squalene should also be labelled by $[2-^{14}\text{C}]$ -mevalonate in the trans terminal methyl groups and formation of squalene epoxide followed by enzymic cyclisation should give an intermediate cation labelled in the 4α -methyl group (scheme 9). In support of this contention, Arigoni has shown⁴⁸ that soyasapogenol D (35) is labelled by $[2-^{14}\text{C}]$ -mevalonate in the 4α -methyl group but not in the 4β -hydroxymethyl group. Although the above arguments do not constitute a complete proof, it can be confidently predicted that lanosterol (3a) biosynthesised from $[2-^{14}\text{C}]$ -mevalonate will also be labelled in the 4α -methyl group (scheme 9). Hence,

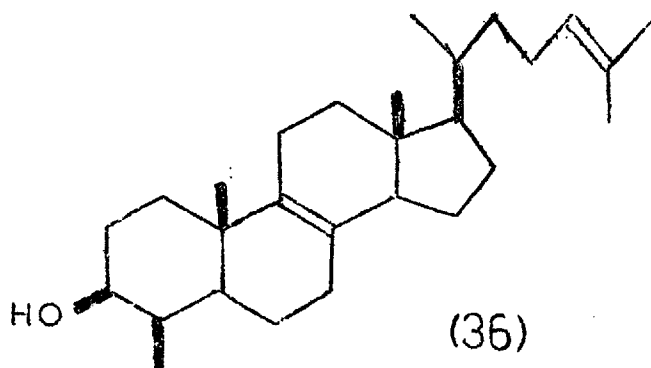
Scheme 9

27



the observation of Gaylor and Delwiche⁴⁵ that their $[^{14}\text{C}]$ -dihydrolanosterol released inactive carbon dioxide on enzymic conversion to 4α -methyl sterols demonstrates that the 4β -methyl group is removed before the 4α -methyl group in cholesterol biosynthesis.

Sanghvi and Frantz⁴⁹ have recently reported the isolation of 4β -methyl zymosterol (36) from the skin of triparanol treated rats and have shown⁵⁰ that this compound is converted to cholesterol in 92% yield by rat livers. This result is difficult to understand in view of the demonstrated intermediacy of 4α -methyl sterols in cholesterol biosynthesis, together with the fact that formation of a 4β -methyl sterol could only occur via protonation of the enol intermediate of scheme 6 in the thermodynamically less favoured sense. Accordingly, more rigorous evidence for the postulated structure (36) of this sterol would be desirable.



Isomerisation of the Δ^8 double bond

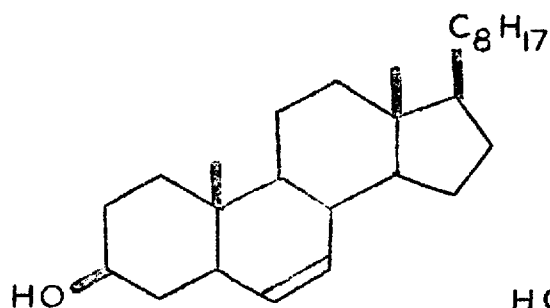
As outlined in scheme 5, the major route of cholesterol biosynthesis in rat livers involves complete removal of the C-4 and C-14 methyl groups of lanosterol, before isomerisation of the Δ^8 double bond to the Δ^7 position. However, the isolation of 4 α -methylcholest-7-en-3 β -ol (22) from rat feces⁵¹ and the high incorporation of this sterol into cholesterol by rats,⁵² demonstrates that this isomerisation can occur at an earlier stage. Gaylor and his coworkers have shown⁵³ that the presence of methyl substituents at C-4 in a sterol weakly inhibits the Δ^8 to Δ^7 isomerase of rat livers, while a 14 α -methyl group strongly inhibits this isomerisation. It has been suggested²⁶ that lanosta-7,24-dienol (~~21,24,25-dehydro~~) may be formed directly from squalene oxide in some tissues so explaining the minor Δ^7 sterol biosynthetic pathway. The mechanism of the Δ^8 to Δ^7 isomerisation has been studied by Akhtar and Rahimtula who showed⁵⁴ that $[7\text{-}^3\text{H}, 26, 27\text{-}^{14}\text{C}]$ -lanosterol lost tritium from C-7 during enzymic conversion to cholesterol, while label from a tritiated water medium was incorporated specifically at C-9 of cholesterol.

Formation of the $\Delta^{5,7}$ -diene

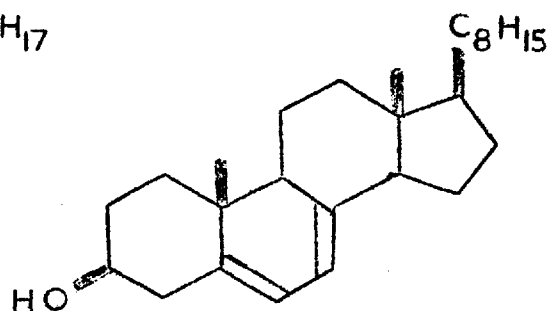
A stepwise isomerisation of the Δ^8 double bond of cholesterol precursors to the Δ^7 , Δ^6 and Δ^5 positions seems unlikely since $[4-^{14}\text{C}]$ -cholest-6-enol (37) was not converted to cholesterol by rats.⁵⁵ A considerable

amount of evidence has accumulated showing that the

$\Delta^7 \longrightarrow \Delta^5$ conversion occurs via a $\Delta^{5,7}$ diene intermediate^{56,57} and that the presence or absence of a Δ^{24} double bond in the Δ^7 precursor has no effect ^{the rate of} on this conversion. The conversion of $[^3\text{H}]$ -cholesta-7,24-dienol to the triene (38) by a liver enzyme system required molecular oxygen but not NADH or NADPH⁵⁶ while the conversion of cholesta-5,7-dienol (39) to cholesterol proceeded anaerobically but required NADPH.⁵⁸

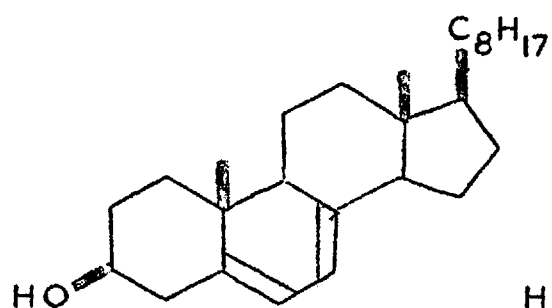


(37)

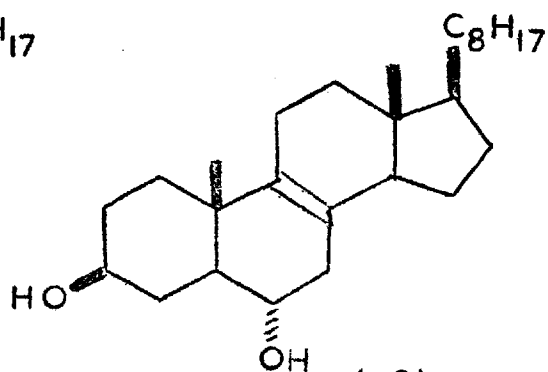


(38)

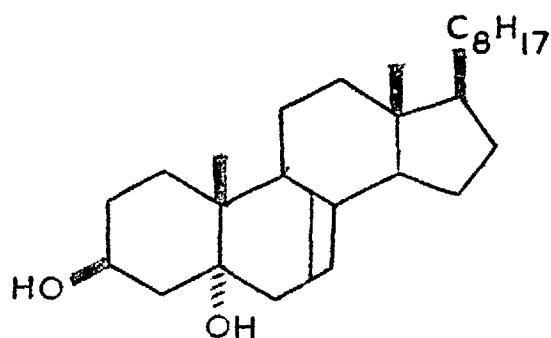
The mechanism of the formation of cholesta-5,7-dienol from its Δ^7 precursor has recently received much attention. Johnson and Bloch have suggested⁵⁹ that the enzymic conversion of cholest-7-enol (40) to cholesta-5,7-dienol (39) might proceed via the intermediacy of cholest-7-en-3 β ,6 β -diol (41a; scheme 10). Such an intermediate should be metabolised to the $\Delta^{5,7}$ -sterol (39) in the absence of oxygen. Slaytor and Bloch have tested⁶⁰ this proposal and found that cholest-7-en-3 β ,6 β -diol (41a), its C-6 epimer (41b) and peniocerol (42) were converted to cholesterol in the presence of oxygen. However, in the absence of oxygen, these compounds were converted to cholest-7-enol in good yields and not to the expected $\Delta^{5,7}$ -diene (39)! Macdougallin (19a) was not significantly metabolised aerobically or anaerobically by rat liver preparations.⁶⁰ Furthermore, Dewhurst and Akhtar have shown that cholest-7-en-3 β ,5 α -diol (43) was converted aerobically to cholesterol, but did not give cholesta-5,7-dienol on anaerobic incubation.⁶¹ The meaning of these results is not clear but it is evident from the unexpected course of the anaerobic incubations that none of these dihydroxy sterols are true intermediates in the biosynthesis of cholesterol.



(39)

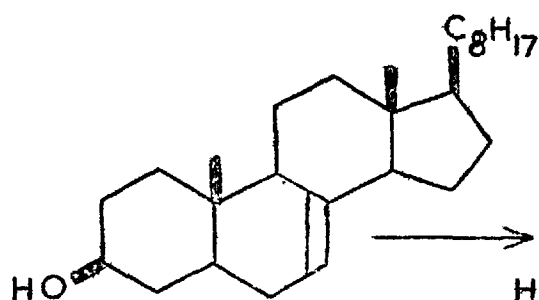


(42)

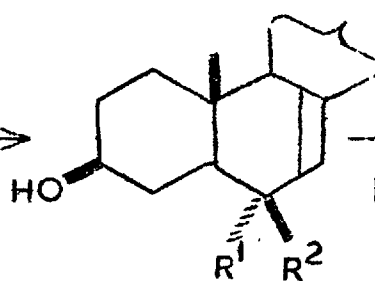


(43)

Scheme 10

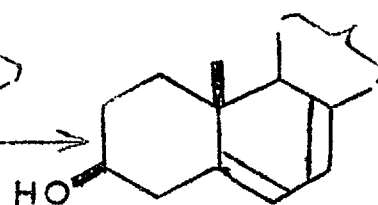


(40)



(41a; $R^1 = H, R^2 = OH$)

(41b; $R^1 = OH, R^2 = H$)



(39)

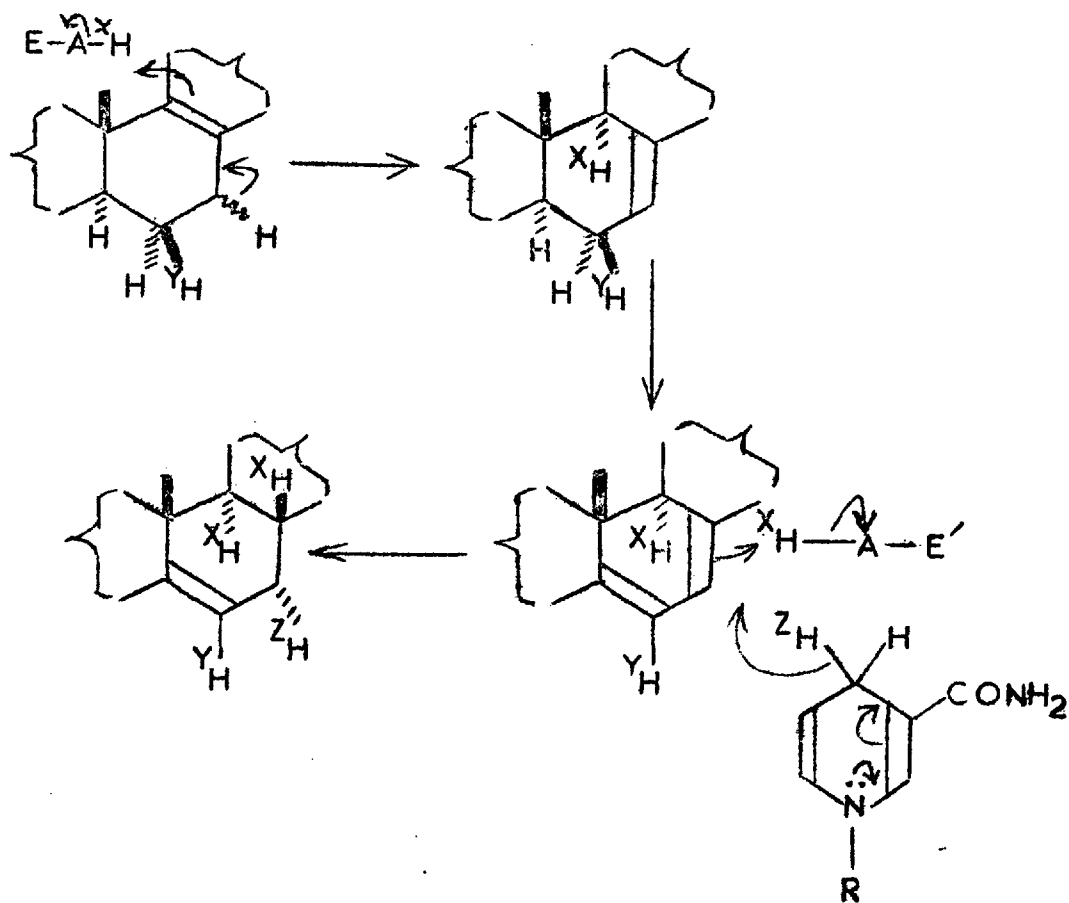
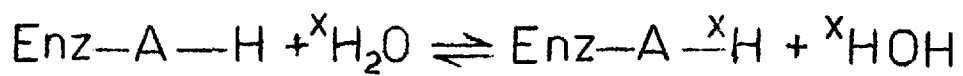
Paliokas and Schroepfer⁶² and Akhtar and Marsh⁶³ have shown that the 6α -proton of cholest-7-enol is lost on aerobic conversion to cholesterol, while the 6β -proton is retained.^{61,62} Furthermore, neither NADPH nor NADH seems⁵⁶ to be required for the enzymic conversion of Δ^7 precursors to $\Delta^{5,7}$ -sterols. Akhtar and Marsh have attempted to rationalise these results by suggesting⁶¹ that the $\Delta^7 \rightarrow \Delta^{5,7}$ conversion involves a cis dehydrogenation with molecular oxygen as an electron acceptor, but the unusual metabolism of the dihydroxy sterols ^{(41a), (41b),} ~~(19a), (19b),~~ (42) and (43) remains inadequately explained.

Reduction of the Δ^7 double bond

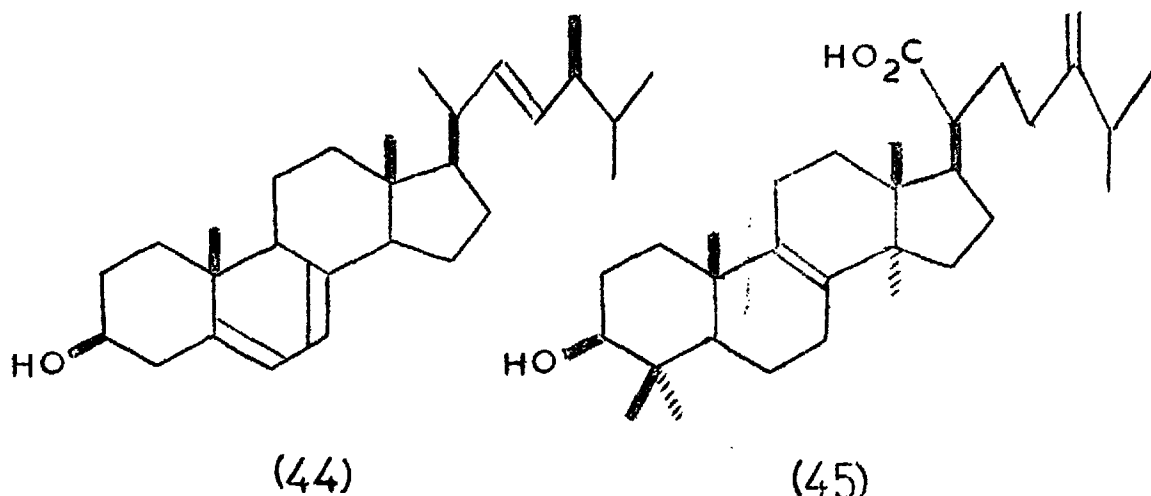
The mechanism of the reduction of the Δ^7 double bond of 7-dehydrocholesterol has been studied by Akhtar and his coworkers⁶⁴ in rat liver homogenates. Cholesterol was shown to incorporate activity from $[4-^3\text{H}]$ -NADPH solely in the 7α -proton when 7-dehydrocholesterol was fed, while activity from a tritiated medium appeared in the 8β -proton of the biosynthesised cholesterol.

The stereochemical fate of the various protons in the conversion of cholest-8-enol to cholesterol by rat liver enzymes is summarised in scheme 11, where protons are distinguished by prefixes X, Y and Z.

Scheme 11



The biosynthesis of the C-24 alkylated sterols

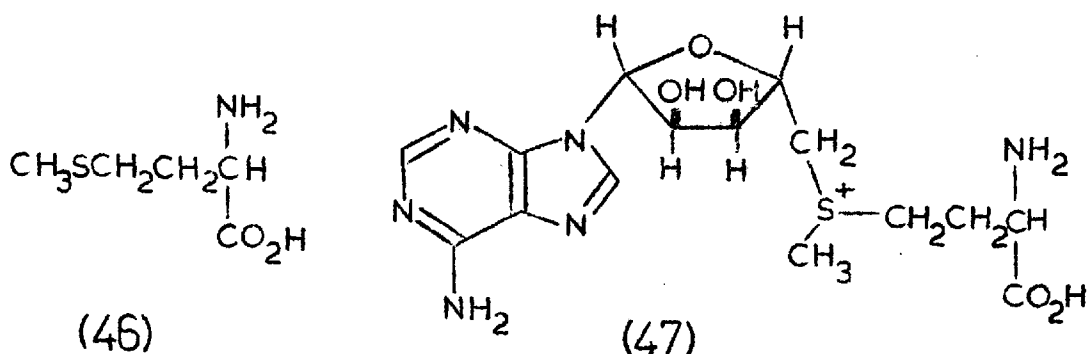


Introduction

The yeast sterol ergosterol (44) differs markedly from cholesterol in having an "extra" carbon atom (C-28) in the side chain, which cannot be derived from the presumed precursor, squalene. Likewise, eburicoic acid (45) and many other fungal sterols and tetracyclic triterpenes,⁶⁵ contain this interesting feature.

Hanahan and Wakil showed⁶⁶ that ergosterol, biosynthesised from $[1-^{14}\text{C}]$ -acetate by yeast, was labelled at C-23 and C-25, but not at C-24, 26, 27 or 28. Dauben et al.⁶⁷ extended these observations employing an "acetate-less" mutant of Neurospora crassa, and showed that $[1-^{14}\text{C}]$ -acetate labelled C-4, 11 and 12

but not C-3 of ergosterol. The results were in agreement with the labelling pattern found for cholesterol biosynthesised from $[1-^{14}\text{C}]$ -acetate.⁶⁸ Polyporus sulphureus was likewise grown in a medium containing $[1-^{14}\text{C}]$ -acetate⁶⁹ or $[2-^{14}\text{C}]$ -acetate⁷⁰ and again the labelling pattern in the biosynthesised eburicoic acid (45) corresponded to that found earlier in cholesterol. Significantly, the "extra" C-28 of eburicoic acid was not labelled by $[1-^{14}\text{C}]$ or by $[2-^{14}\text{C}]$ -acetate. Further work revealed that C-28 of ergosterol⁷¹ and eburicoic acid⁷² was labelled by $[^{14}\text{C}]$ -sodium formate and hence arose from a one-carbon pool. A more efficient source of C-28 of ergosterol was found to be $[\text{Me}-^{14}\text{C}]$ -methionine⁷³ (46), while Parks has shown⁷⁴ that $[\text{Me}-^{14}\text{C}]$ -S-adenosyl-methionine (47) is a still better one-carbon source of C-28 in ergosterol.



Pharbitis nil.⁸³ Goodwin and his collaborators have studied the incorporation of activity from $[2-^{14}\text{C}]$ -mevalonate into 4,4-dimethyl,4 α -methyl and 4-desmethyl sterols of peas^{84,85} and maize.⁸⁵

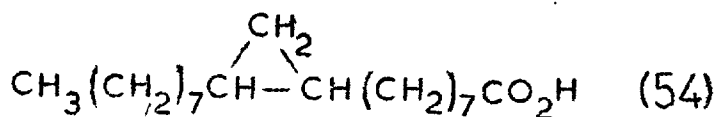
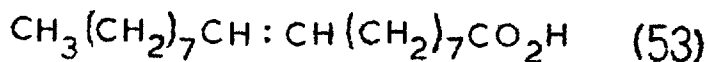
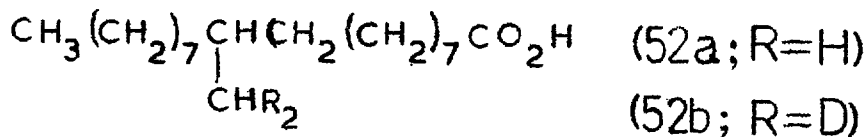
The origin of the "extra" alkyl group at C-24 in the phytosterols has been studied by many workers. $[1-^{14}\text{C}]$ -acetate did not label the 24-ethyl group of β -sitosterol⁸⁶ (48), but $[\text{Me}-^{14}\text{C}]$ -methionine specifically labelled the C-24 ethyl groups of β -sitosterol⁸⁷ and spinasterol⁸⁸ (50) and the ethylidene group of fucosterol⁸⁹ (51). Castle et al.⁸⁷ suggested that these results were consistent with a double methylation of a Δ^{24} sterol precursor as shown in scheme 12.

The mechanism of alkylation at C-24

Alexander and Schwenk have fed⁹⁰ $[\text{Me}-^{14}\text{C}, ^3\text{H}]$ - methionine to a cell-free yeast homogenate and isolated ergosterol with only a small change in its $^3\text{H}/^{14}\text{C}$ ratio. This result appeared to demonstrate the transfer of an intact methyl group from S-adenosyl-methionine to an ergosterol precursor. The transfer of an intact methyl group to an oxygen function⁹¹ and to nitrogen⁹² had been reported previously. However the use by Alexander et al. of tritiated methionine, in which most of the molecules which were labelled had only one tritium atom together with two hydrogen atoms in the methyl group, rendered

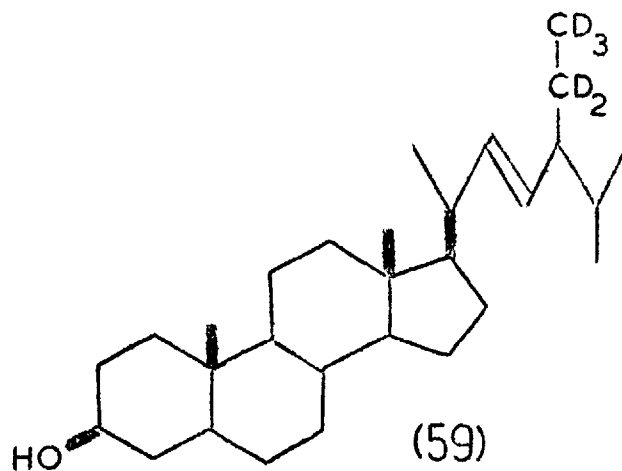
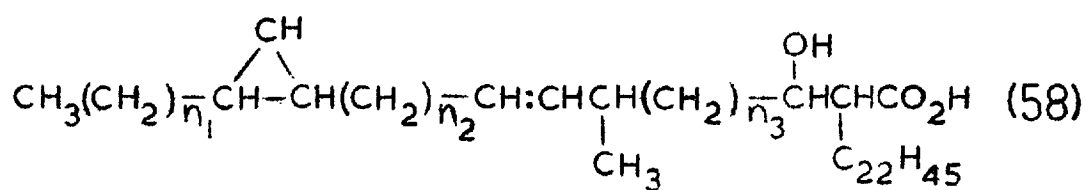
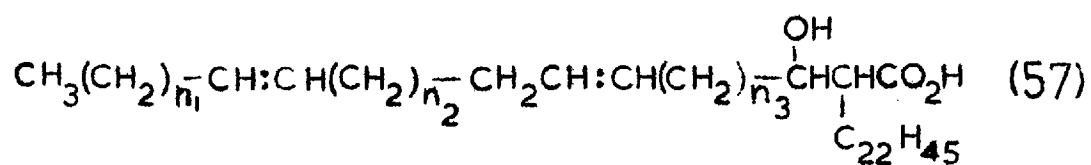
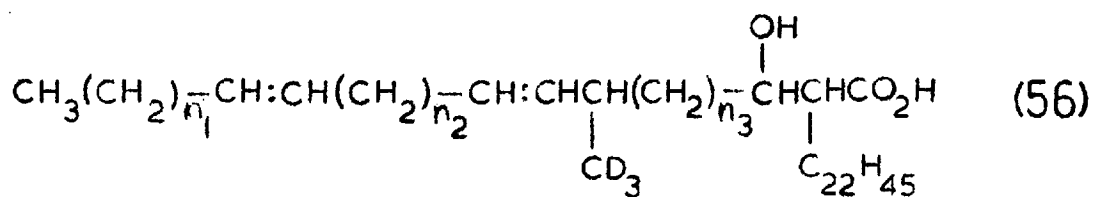
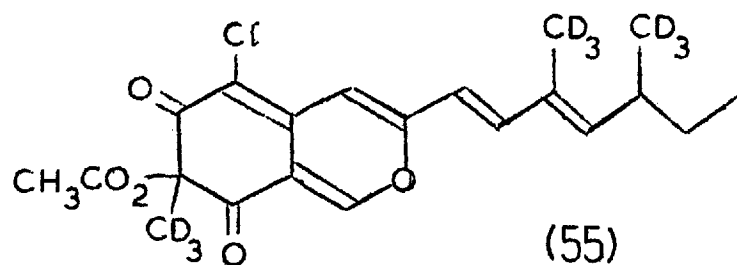
their experiment inconclusive, owing to the expected isotope effect if hydrogen were lost during the transfer of the methyl group.

Lederer and his coworkers have recently re-investigated this "transmethylation" by feeding^{93,94} $[\text{Me-D}_3]$ -methionine to Neurospora crassa, when $[\text{D}_2]$ -ergosterol was isolated. Also when the same trideuterated precursor was fed to Mycobacterium smegmatis, which produces tuberculostearic acid (52a), presumably from oleic acid (53), dideuterated tuberculostearic acid (52b) was formed. These results demonstrated quite clearly that one proton is lost during transfer of the labile methyl group from methionine to oleic acid or to an ergosterol precursor. The possible intermediacy of the



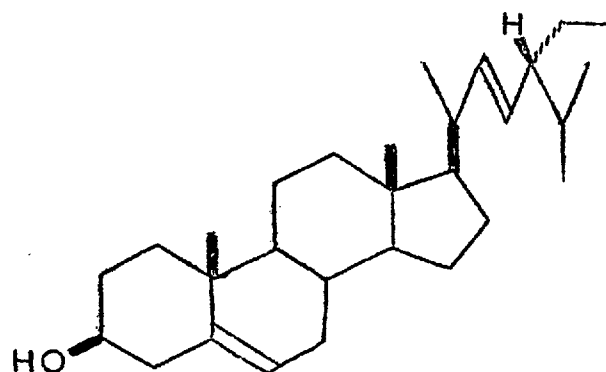
cyclopropanoic acid (54) in the biosynthesis of tuberculostearic acid was tested by feeding labelled (54) to M. phei, but no incorporation of activity into tuberculostearic acid (52a) was observed.⁹³ A number of possible interpretations of these results have been considered.^{93,95} Following normal practice, it will be assumed in this discussion that methyl transfer to carbon can occur only at a double bond and that S-adenosylmethionine is the active transmethylation agent though these points are not rigorously proven.

In contrast to the incorporation of only two deuterium atoms from $[CD_3]$ -methionine into ergosterol and tuberculostearic acid, Tropp et al. have demonstrated⁹⁶ the transfer of an intact methyl group to carbon in ribonucleic acids. This result was not unexpected, since the biological transmethylation occurred at an activated double bond as shown in scheme 13. However, Lederer and his coworkers have recently shown⁹⁷ that S-adenosylmethionine can also transfer an intact methyl group to an unactivated C=C double bond. Thus when penicillium sclerotiorum was grown in the presence of $[CD_3]$ -methionine, $[D_9]$ -sclerotiorine (55) was formed while in M. smegmatis $[D_3]$ - α -smegmycolic acids (56) were isolated.⁹⁷ Furthermore in a slime mould the same substrate gave $[D_5]$ -stigmast-22-en-3 β -ol (59) in

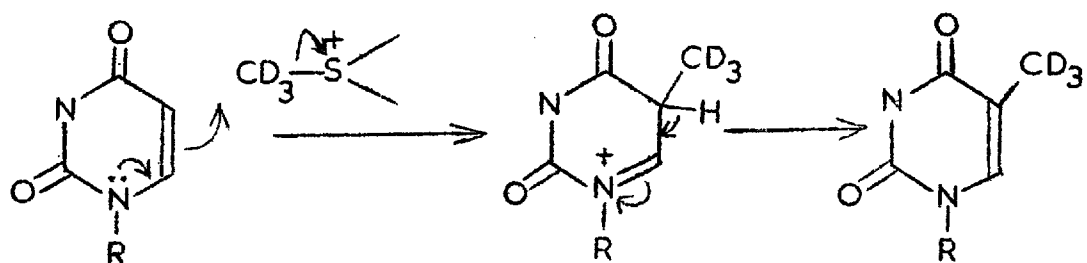


which it is clear that at least one intact methyl group has been transferred from methionine.⁹⁸

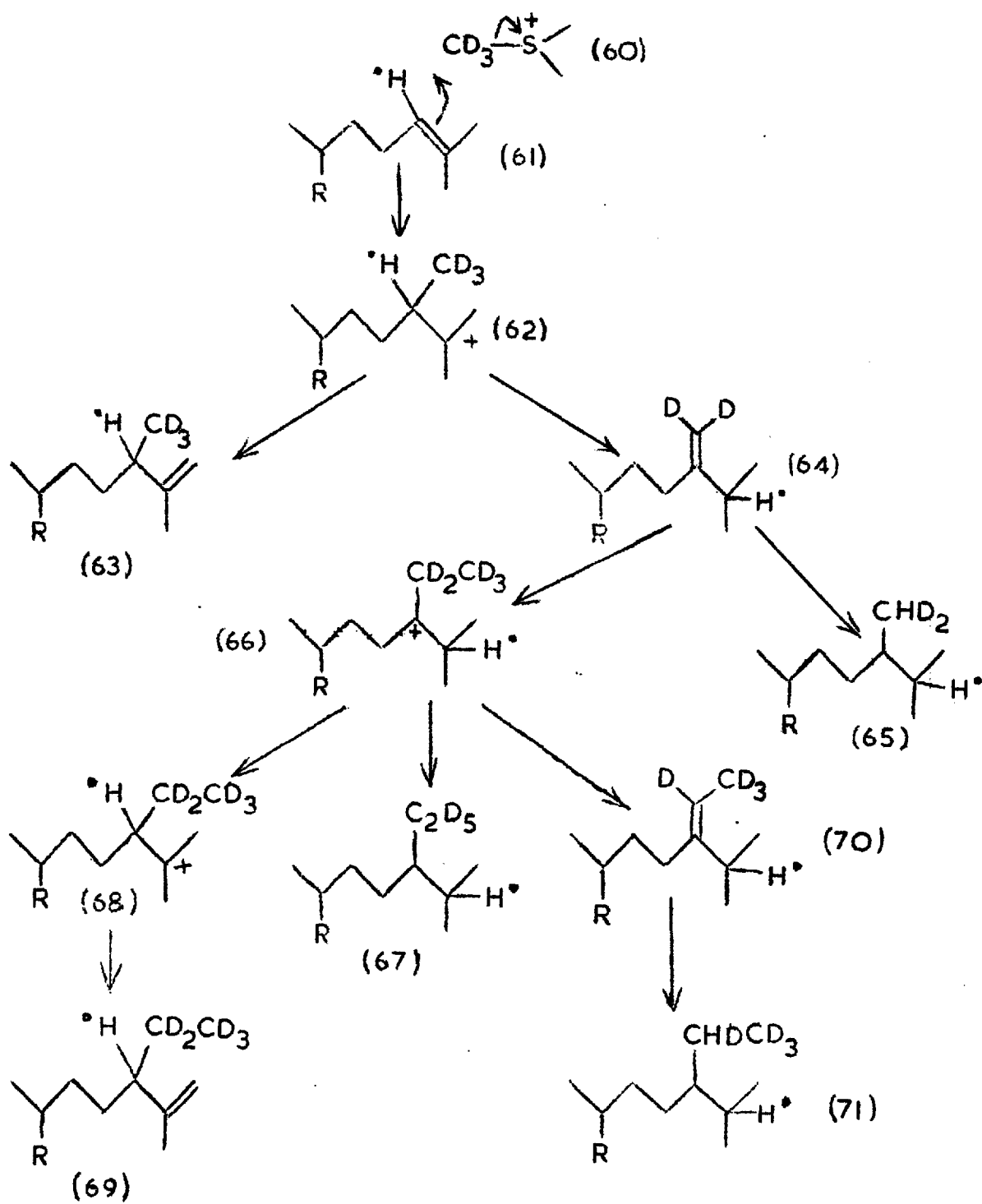
These results can only be rationalised in terms of scheme 14, in which $[D_3]$ -S-adenosylmethionine (60) transfers an intact methyl group to a sterol Δ^{24} double bond (61) to give the cation (62). Hydride migration from C-24 to the cationic centre and loss of a proton from C-28 would give the $[28-D_2]$ -24-methylene side chain (64), exemplified by the formation of dideuterated eburicoic acid (45) when $[CD_3]$ -methionine was fed to P.sulphureus.⁹⁹ Reduction of the 24-methylene compound (64) would give the $[28-D_2]$ -24-methyl side chain (65) as observed in the biosynthesis of ergosterol.⁹⁴ Transfer of a second methyl group from $[CD_3]$ -S-adenosyl methionine to the 24-methylene sterol (64) would give the intermediate cation (66) which on reduction would give the pentadeuterated side chain (67), as was found in the biosynthesis of stigmastenol⁹⁸ (59). Alternatively loss of a proton from C-28 of the cation (66) to give the 24-ethylidene sterol (70) followed by biological reduction would give the $[D_4]$ -24-ethyl sterol (71), as was found¹⁰⁰ in the biosynthesis of poriferasterol (72). Thus there appears to be at least two biosynthetic routes to the 24-ethyl sterols.



(72)

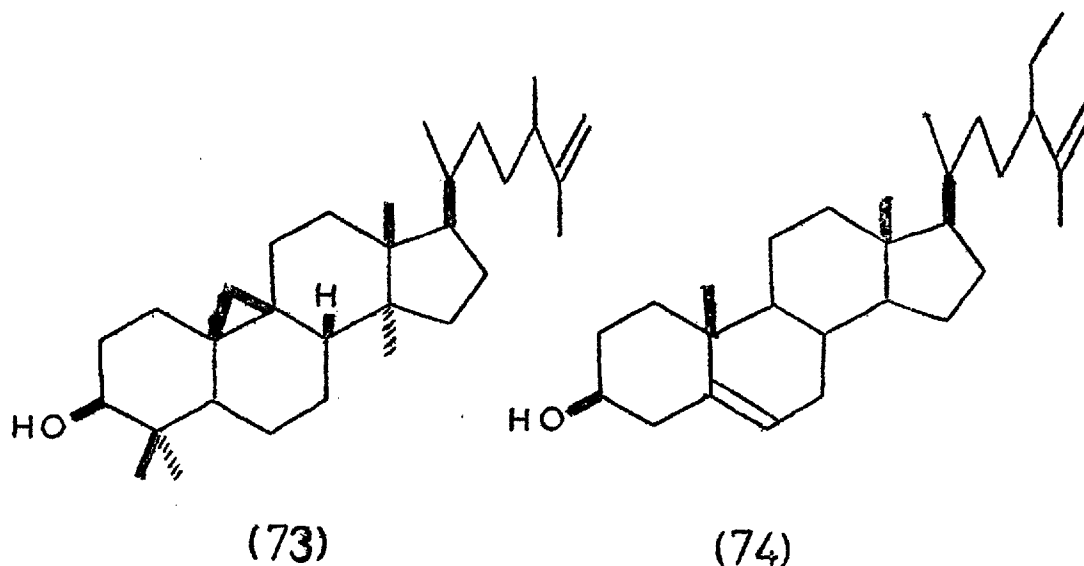
Scheme 13

Scheme 14



The biosynthesis of 24-ethylidene sterols has not yet been investigated with $[\text{CD}_3]$ -methionine as a precursor. However Goodwin and his collaborators have fed¹⁰¹ $[\text{Me-}^{14}\text{C}, ^3\text{H}]$ -methionine to Fucus spiralis and isolated $[^3\text{H}, ^{14}\text{C}]$ -fucosterol (51), with the same $^3\text{H}/^{14}\text{C}$ ratio as ergosterol (44) biosynthesised from the same doubly labelled precursor and already shown⁹⁴ to retain only two of the protons of the methionine methyl group. It was concluded that four hydrogen atoms in fucosterol are derived from $[\text{Me-}^{14}\text{C}, ^3\text{H}]$ -methionine in agreement with the route (61) $\rightarrow$ (64) $\rightarrow$ (70) (scheme 14), but this result is not entirely convincing owing to the isotope effect discussed above.

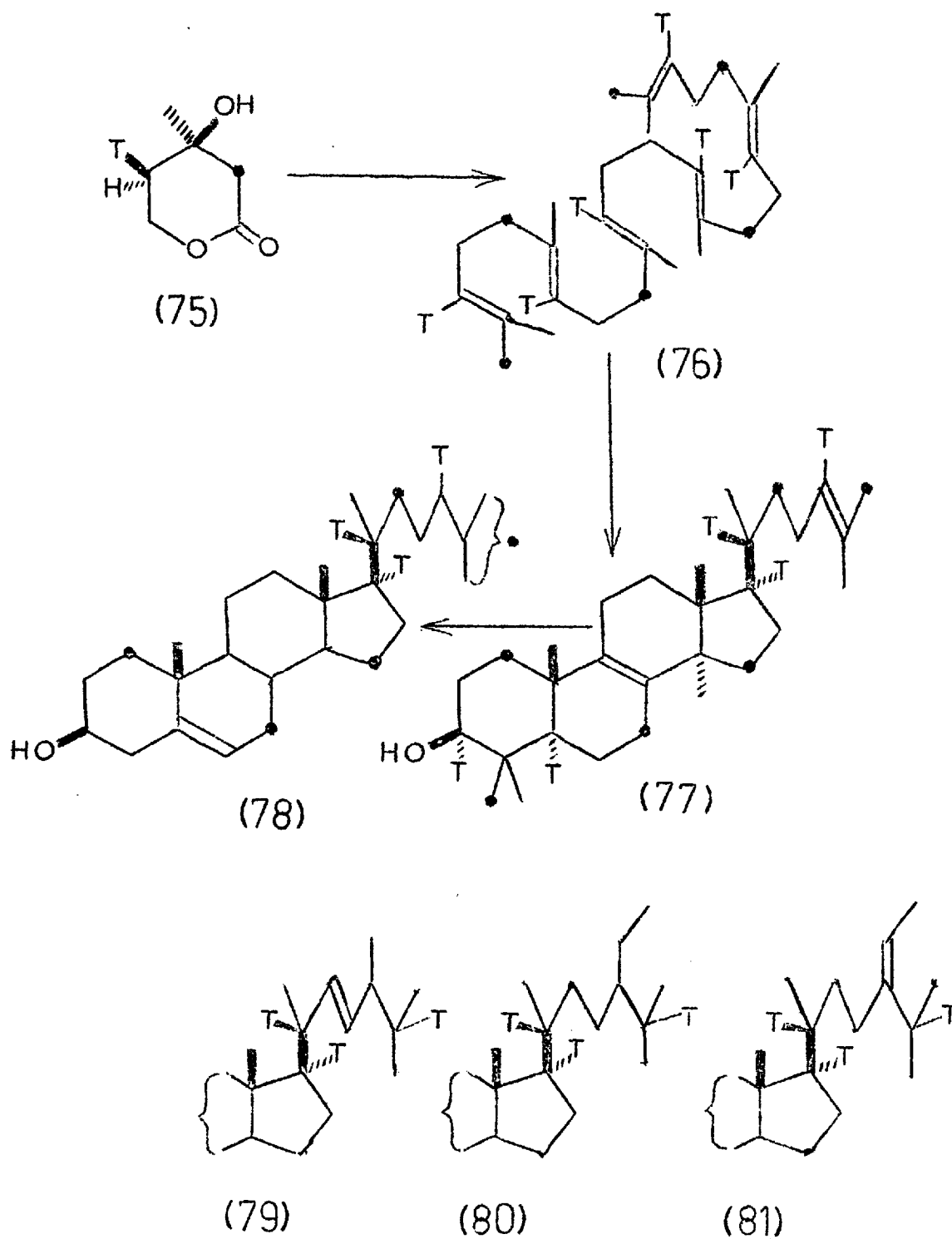
The structures of the sterols cyclolaudenol (73) and clerosterol¹⁰² (74) suggest further interesting possibilities. It can be predicted that $[\text{CD}_3]$ -methionine should give trideuterated cyclolaudenol by loss of a C-26 proton from the cation (62). Likewise, clerosterol derived from $[\text{CD}_3]$ -methionine should retain five deuterium atoms by rearrangement of the cation (66) to (68) followed by loss of a C-26 proton.



The fate of the C-24 proton of the Δ^{24} sterol (61) after alkylation by S-adenosylmethionine is worthy of comment. If scheme 14 is valid, this proton should remain at C-24 in cyclolanosterol (73) and clerosterol (74) but should migrate to C-25 during the biosynthesis of ergosterol (44), β -sitosterol (48) and fucosterol (51). It has been shown that $[4R-^3H, 2-^{14}C]$ -mevalonate (75) with a $^3H/^{14}C$ ratio of 1/1 is converted in rat livers to $[^{14}C, ^3H]$ -lanosterol (77) with $^3H/^{14}C$ ratio 5/6, via $[^{14}C, ^3H]$ -squalene (76). Further incubation⁵ gave cholesterol with a $^3H/^{14}C$ ratio of 3/5, and the probable tritium distribution shown in figure (78-scheme 15). Likewise, ergosterol,¹⁰³ β -sitosterol¹⁰⁴ and fucosterol¹⁰¹ biosynthesised from the same $[^{14}C, ^3H]$ -mevalonate had $^3H/^{14}C$ ratios of 3/5. Thus the tritium labelled C-24 proton present

Scheme 15

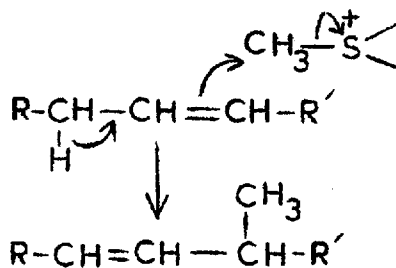
48



in the Δ^{24} precursor was still present in these sterols, and at least in fucosterol (51) is not at C-24. Mechanistic considerations (scheme 14) require that this proton be located at C-25 in ergosterol, β -sitosterol and fucosterol, as in figs. (79), (80) and (81) respectively. A recent direct proof of this hydride migration in ergosterol biosynthesis will be discussed in section 7.

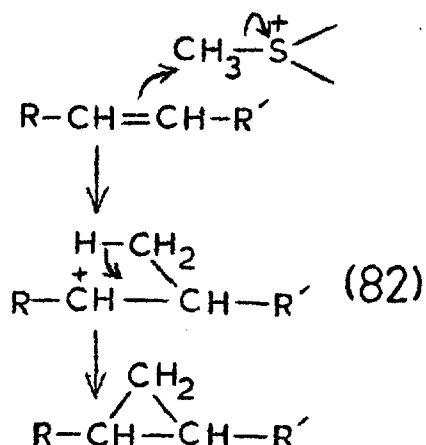
The branched chain fatty acids provide further interesting observations on the mechanism of the biological alkylation of C=C double bonds. It has been noted above that the α -smegmycolic acids (56; D=H) are biosynthesised⁹⁷ via transfer of an intact methyl group from S-adenosylmethionine. Lederer and his coworkers⁹⁷ have noted that the unmethylated acids (57) also present in M. smegmatis contain a double bond displaced allylically from that in the methylated acids (56, D=H) in accordance with scheme 16.

Scheme 16



Finally, Polacheck et al. have shown¹⁰⁵ that the vinyl protons of oleic acid (53) are retained in dihydrosterculic acid (54) formed biosynthetically. This is consistent with the intermediacy of the formal cation (82) in the biosynthesis of cyclopropano fatty acids from S-adenosylmethionine (scheme 17),

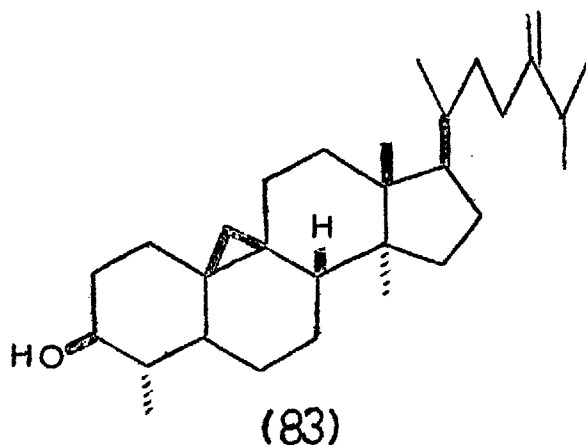
Scheme 17



Recent evidence for the intermediacy of 24-methylene sterols in the biosynthesis of the C-24 alkylated sterols will be discussed in section 7 together with the author's own contribution to this work.

The biosynthesis of the phytosterols

Owing to the apparent absence of lanosterol (3a) in some higher plants, and the widespread occurrence of cycloartenol (8) in these species, it has been suggested¹⁰⁶ that cycloartenol may be the normal precursor of the phytosterols. The cyclopropane ring would be opened enzymatically at an appropriate stage, possibly¹⁰⁷ at the cycloeucaleanol (83) stage to give obtusifoliol (18) and the side chain and nuclear modifications would then proceed normally. In this connection, the biosynthesis of cycloartenol from $[4R,4-^3H,2-^{14}C]$ -mevalonate has recently been studied.¹⁰⁸ These suggestions do not, however, affect the biosynthesis of the fungal sterols and will not be further discussed.



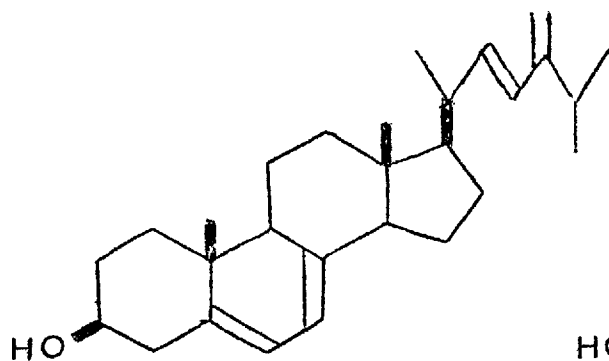
RESULTS AND DISCUSSION

4. An investigation of the 4 α -methyl sterols of yeast

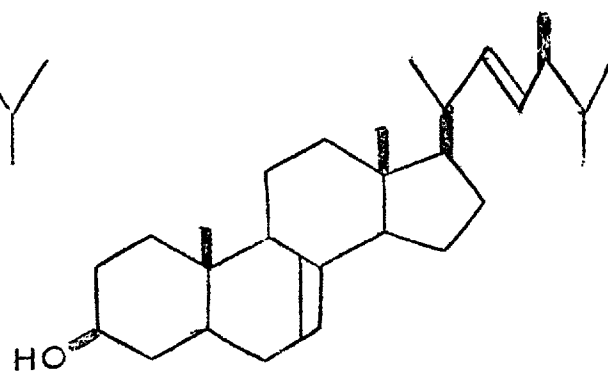
Introduction

Yeast has long been known as a source of many sterols¹⁰⁹ of which the most abundant is ergosterol (44). The occurrence of squalene¹¹⁰ and lanosterol¹¹¹ (3a) in yeast suggests that ergosterol is biosynthesised via the normal sterol pathway.²⁵ The second most abundant yeast sterol is zymosterol^{112,113} (23). An ergostatetraenol of yeast has been tentatively assigned¹¹⁴ the structure (84) and is an obvious claimant as the immediate precursor of ergosterol. However, 5,6-dihydroergosterol¹¹⁵ (85), also present in yeast, may be an alternative precursor. "Neosterol"¹¹⁶ has been shown^{115,117} to be a mixture of ergosterol and 5,6-dihydroergosterol. Cerevisterol¹¹⁸ (86) does not have an obvious biosynthetic role and is probably derived enzymatically from ergosterol.

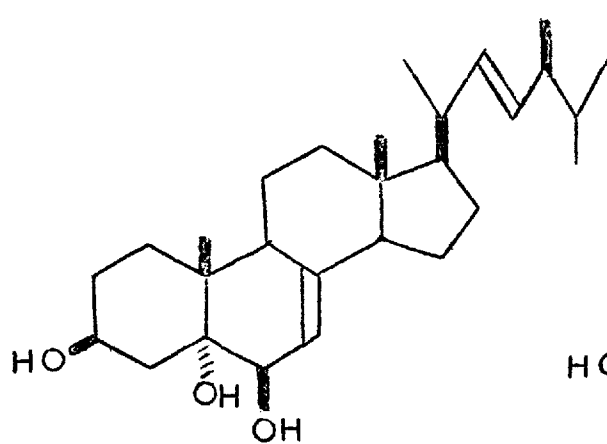
The yeast sterols¹¹⁶ ascosterol, episterol and fecosterol have been tentatively assigned¹⁰⁹ structures (87), (88) and (89) respectively, though the evidence remains very inadequate and reinvestigation is required. Recently Fürst¹¹⁹ claimed to have proven structure (87) for ascosterol but the evidence remains unconvincing. Other yeast sterols have been reported¹²⁰ but evidence of homogeneity and structure is lacking.



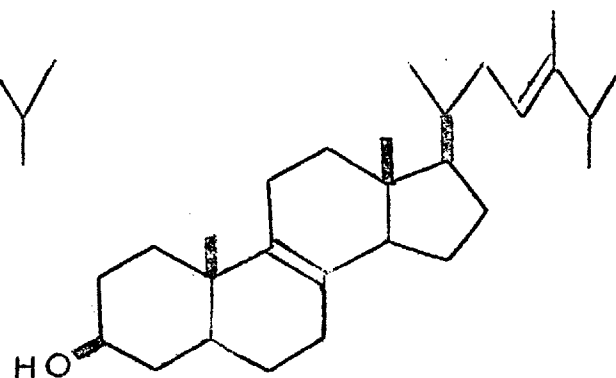
(84)



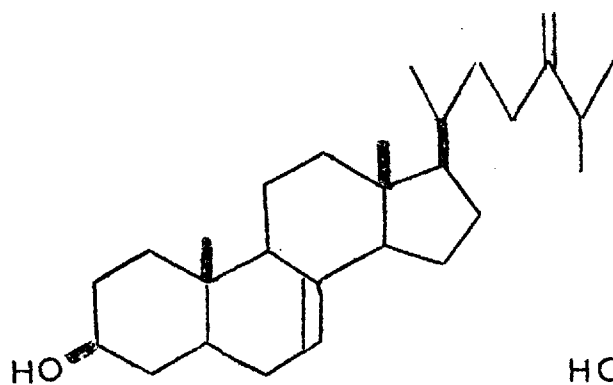
(85)



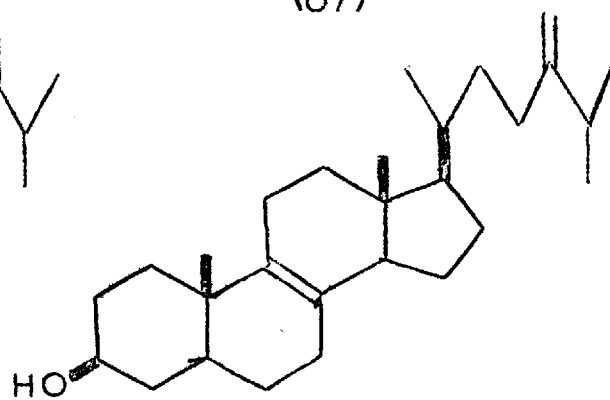
(86)



(87)



(88)

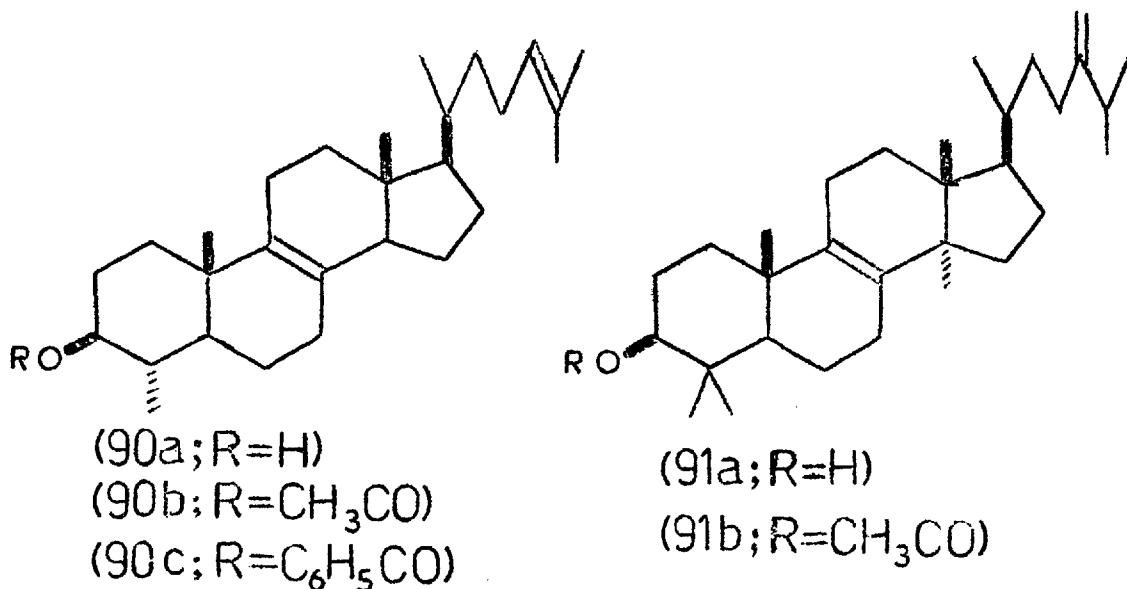


(89)

Recently Ponsinet and Ourisson have described¹²¹ the isolation of two new yeast sterols, 4,4-dimethylzymosterol (17) and 4 α -methylzymosterol (90a). The apparent absence of 4,4-dimethyl or 4 α -methyl sterols with a 24-methylene substituent was noted with some surprise by these workers.¹²¹

Examination of yeast residues

The incorporation of 24-methylenedi~~hydro-~~lanosterol (91a) into ergosterol by whole yeast cells, reported in section 7, led to a re-examination of the sterols of yeast.



Column chromatography of crude yeast residues on deactivated alumina, readily separated the

4,4-dimethyl sterols and the 4α -methyl sterols from the more abundant 4-desmethyl sterols. No evidence was found for the presence of 24-methylenedihydrolanosterol (91a) in the 4,4-dimethyl sterol fraction. However the 4α -methyl sterol fraction in carbon tetrachloride solution, had $\nu_{\max} 890 \text{ cm.}^{-1}$ possibly assignable to a 24-methylene substituent. The n.m.r. spectrum of this fraction could be interpreted in terms of a mixture of 4α -methylzymosterol¹²¹ (90a) and its unknown 24,25-dihydro-24-methylene homologue (92a) in the ratio 17:3. For example, a triplet at 4.9τ (J , 6c/s) was assigned to the C-24 proton in sterol (90a) and a broad singlet at 5.3τ could be assigned to the protons at C-28 in the new sterol (92a).

The crude 4α -methyl sterol fraction, homogeneous on silica gel thin-layer chromatography, was acetylated and the mixed acetates fractionally crystallised from acetone or acetone-methanol. The major acetate crystallised selectively, the composition of the various fractions being followed by n.m.r. and i.r. spectroscopy. In only nine crystallisations a 70% yield of the major acetate, homogeneous by silica gel thin-layer chromatography and n.m.r. spectroscopy, was obtained. The physical constants of the acetate, m.p. $143-145^{\circ}$, $[\alpha]_D +65^{\circ}$ and of the

derived sterol, m.p. $132-134^{\circ}$, resembled the reported¹²¹ values, m.p. $138-139^{\circ}$, $[\alpha]_D + 61^{\circ}$ and m.p. $130-131^{\circ}$ for 4α -methyl zymosteryl acetate (90b) and the free sterol (90a) respectively. The mass spectrum of the acetate showed the parent peak at m/e 440, and the n.m.r. spectra of the sterol and its acetate discussed below, supplied further support for this structure. Finally, comparison of the sterol and its acetate with authentic specimens established that the sterol was identical with 4α -methyl zymosterol¹²¹ (90a).

The mother liquors from the acetate fractional crystallisations were rich in an unknown acetate with $\nu_{\max} 890 \text{ cm.}^{-1}$ (carbon tetrachloride solution). Conversion of the mixture to the mixed benzoates and repeated crystallisation from chloroform-methanol increased the concentration of the unknown compound. However it was not possible to obtain a pure sample of the new benzoate by this procedure. Preparative thin-layer chromatography of the mixed benzoates on silica gel-silver nitrate plates allowed ready removal of residual 4α -methyl zymosteryl benzoate (90c) and permitted isolation of the new benzoate (92c) which on alkaline hydrolysis furnished the new sterol (92a). The same sterol was obtained by alkaline hydrolysis of the acetate (92b) which was obtained by silver

nitrate-silica gel thin-layer chromatography of the original acetate mother liquors. During the latter operation a band ran just ahead of the residual 4α -methyl zymosteryl acetate (90b).

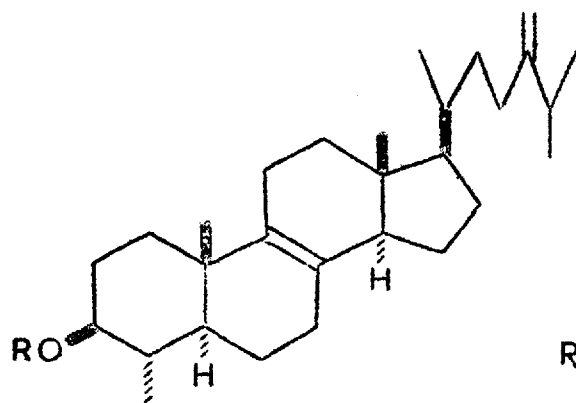
Very careful rechromatography of this band furnished a second new acetate (93b) which on hydrolysis gave a second new sterol (93a). Yields of the 4α -methyl sterols are listed in table 1.

The structural elucidation of the two new sterols is discussed below.

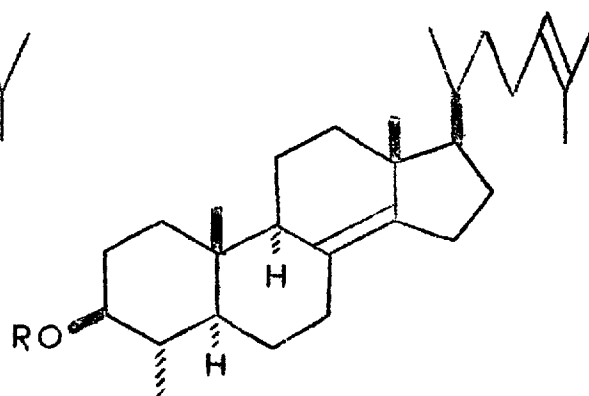
Structural elucidation of 4α -methylergosta-8,24(28)-dien-3 β -ol

The first new sterol (92a) and its acetate (92b) were transparent above 220 $m\mu$ in the ultra-violet region. Oxidation of the sterol in benzene with chromic oxide in aqueous acetic acid gave the ketone (94) demonstrating the secondary nature of the hydroxyl group. Microanalytical data on the sterol, acetate, benzoate and ketone indicated the molecular formula $C_{29}H_{48}O$ for the sterol (92a) in agreement with the parent peak at m/e 454 in the mass spectrum of its acetate. Thus if the 5α -cholestan-3-ol skeleton be assumed, two extra carbon atoms and two double bonds have to be accommodated.

<u>Sterols</u>	<u>Yields of sterols obtained</u>	
	<u>From 4α-Me fraction</u>	<u>From yeast residues</u>
(90a)	71%	0.92%
(92a)	3.5%	0.046%
(93a)	0.9%	0.012%



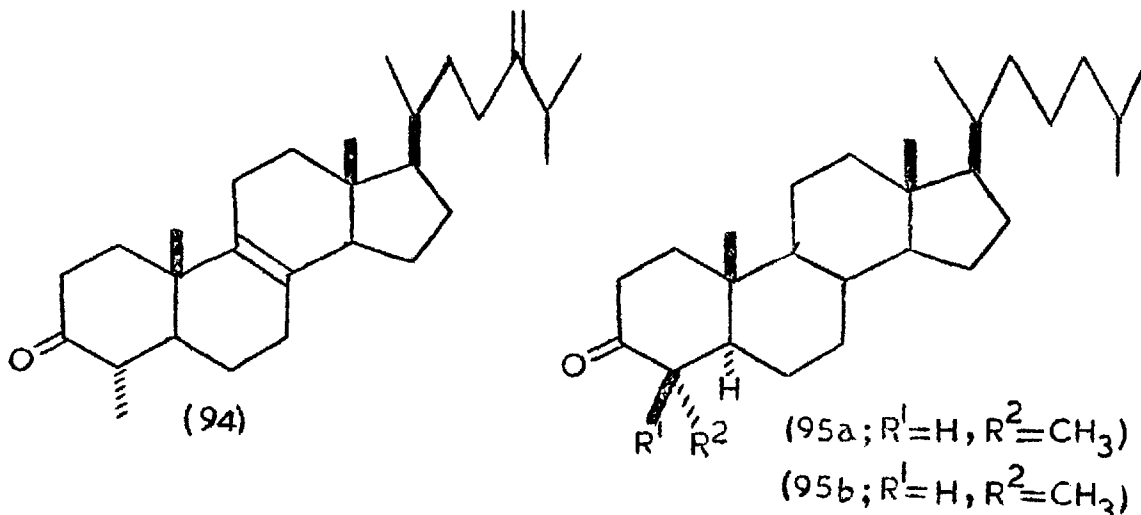
(92a; R=H)
(92b; R=CH₃CO)
(92c; R=C₆H₅CO)



(93a; R=H)
(93b; R=CH₃CO)

The infrared (nujol) spectra of the sterol (92a) and its derivatives showed strong absorption at 898 and medium absorption at 1647 cm.^{-1} , characteristic of a terminal methylene group. This conclusion was supported by the n.m.r. spectra of the sterol and its derivatives which showed a broad two-proton singlet at 5.31τ which was $0.4\overset{\text{p.p.m.}}{\times}$ upfield from the C-24 proton in 4α -methyl zymosteryl acetate (90b) but was identical to that observed in 24-methylene-dihydrolanosterol (91a). The absence of vinylic methyl absorption in these spectra preclude the possibility of a $\Delta^{25(26)}$ double bond. Hence a 24-methylene substituent, which is strongly favoured on biosynthetic grounds,⁹⁵ was indicated. In accord with this assignment the C-25 methyl groups in the sterol (92a) and its derivatives appeared at 8.91 and 9.02τ which were about 0.15 p.p.m. downfield from the C-25 methyls in the saturated side chain of cholesterol¹²² (4) but identical to those in 24-methylenedihydrolanosterol. There were no further vinylic resonances in the spectra of the sterol (92a) and its derivatives and hence the second double bond was tetrasubstituted. The absence of a vinylic methyl group allowed only two possibilities, $\Delta^{8(9)}$ or $\Delta^{8(14)}$.

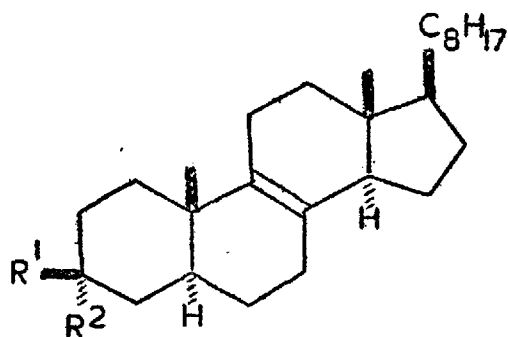
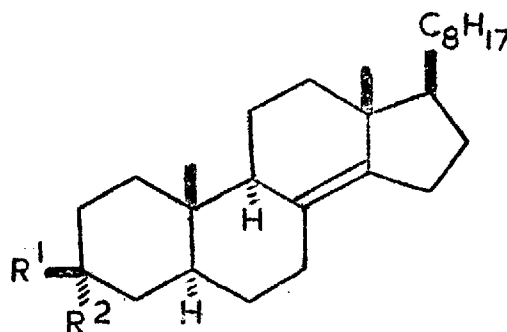
A doublet (J , 6 c/s) integrating for three protons appeared at 9.14τ in the acetate (92b). This was not assignable to the C-20 methyl group which was expected as a very broad doublet, difficult or impossible to recognise, between 9.0 and 9.1τ ,^{122, 123a} since in the sterol (92a) this signal had shifted downfield by 0.15 p.p.m. Hence the secondary methyl group must be present in the nucleus and on biosynthetic grounds²⁵ was assigned to C-4. The upfield shift of the C-4 methyl resonance on acetylation of the 3β -hydroxysterol (92a) suggests¹²⁴ the 4α -methyl configuration. The position (9.15τ , doublet, J , 6 c/s) of the 4α -methyl absorption in the known acetate (90b) agreed very well with that of the new acetate (92b), in support of the 4α -methyl assignment. Finally the stereochemistry at C-4 was proven by the o.r.d. curve of the ketone (94), which showed a positive Cotton effect at $286\text{ m}\mu$ of molecular amplitude¹²⁵ +43. By comparison, 4α -methyl cholestan-3-one (95a) had a positive Cotton effect at $288\text{ m}\mu$ of amplitude +54, while the 4β -methyl isomer (95b) had a positive Cotton effect at 282 m of amplitude +11.¹²⁶



A broad one-proton multiplet at 7.0τ in the sterol (92a) was assigned to the proton at C-3. In both the new acetate (92b) and the acetate (90b) the C-3 proton appeared at 5.65τ , implying the same stereochemistry^{123b} at C-3. Lithium aluminium hydride reduction of the ketone (94) regenerated the parent sterol (92a), confirming the 3β -hydroxy assignment for the sterol.

It remained only to distinguish between the $\Delta^{8(9)}$ and $\Delta^{8(14)}$ possibilities for the nuclear double bond. It was possible to calculate^{123c} the τ -values of the 10β - and 13β -methyls of cholest-8-en- 3β -ol (96a) and the derived acetate (96b) and ketone (96c). These resonances were likewise calculated for the corresponding derivatives of cholest-8(14)-en- 3β -ol (97a). The calculated values for the model compounds and the observed values for derivatives of the new sterol (92a) and for 4α -methyl zymosterol (90a) are listed in

table 2. The only important failing of the model compounds was that they lacked the 4α -methyl substituent of sterols (90a) and (92a). However, introduction of a 4α (and hence equatorial) methyl group into either of the model compounds should not greatly affect the conformation of the steroid nucleus and would therefore produce only a slight effect on the τ -values under consideration.

(96a; $R^1=OH, R^2=H$)(97a; $R^1=OH, R^2=H$)(96b; $R^1=O_2CCH_3, R^2=H$) (97b; $R^1=O_2CCH_3, R^2=H$)(96c; $R^1, R^2=O$)(97c; $R^1, R^2=O$)

The high field (9.38 τ) methyl group in the new sterol (92a) and its acetate was best assigned to the 13β -methyl of a $\Delta^{8(9)}$ steroid (calculated 9.43 τ) rather than to the 10β -methyl of a $\Delta^{8(14)}$ steroid (calculated 9.30 τ), though the agreement was inconclusive (see table 2). However, the absence in the new sterol (92a) or its acetate (92b) of a three-proton singlet at or near 9.17 τ , ruled out the $\Delta^{8(14)}$ structure. Furthermore in the ketone (94)

TABLE 2Calculated τ -values for 10β and 13β methyl groups

<u>C-3</u>	<u>$\Delta^{8(9)}$ double bond</u>			<u>$\Delta^{8(14)}$ double bond</u>		
	<u>Fig.</u>	<u>10β-Me</u>	<u>13β-Me</u>	<u>Fig.</u>	<u>10β-Me</u>	<u>13β-Me</u>
3β -OH	(96a)	9.07	9.43	(97a)	9.31	9.17
3β -OAc	(96b)	9.05	9.43	(97b)	9.29	9.17
3-oxo	(96c)	8.86	9.40	(97c)	9.10	9.14

Observed τ -values for 10β and 13β methyl groups

<u>C-3</u>	<u>4α-methyl-24-methylene - dihydrozymosterol</u>			<u>4α-methylzymosterol</u>		
	<u>Fig.</u>	<u>10β-Me</u>	<u>13β-Me</u>	<u>Fig.</u>	<u>10β-Me</u>	<u>13β-Me</u>
3β -OH	(92a)	9.02	9.38	(90a)	-	-
3β -OAc	(92b)	9.01	9.38	(90b)	9.01	9.39
3-oxo	(94)	8.82	9.36	-	8.81	9.36

the methyl group at highest field occurred at 9.36 τ in excellent agreement with the $\Delta^{8(9)}$ assignment (calculated 9.40 τ) while the methyl resonance at highest field in the $\Delta^{8(14)}$ ketone (97c) would occur at 9.14 τ . Finally a comparison of the n.m.r. spectra of the new acetate (92b) and the derived ketone (94) with those of the corresponding derivatives of 4 α -methyl zymosterol confirmed the $\Delta^{8(9)}$ assignment for the new sterol (92a).

Molecular rotation data¹²⁷ for the new sterol (92a) and its derivatives are presented in table 3, together with corresponding data for 4 α -methylcholesta-8,24-dienol¹²¹ (90a) and 4 α -methylcholest-8-enol¹²⁸ (90a; 24,25-dihydro). The close agreement in $[\text{M}]_D$ values for corresponding derivatives lends further support to the structure (92a) of the new sterol.

It has been noted by Djerassi and his coworkers¹²⁹ that the 4 α -methyl sterol lophenol (22) shows unusual molecular rotation differences¹¹³ for acetylation (Δ_1), benzoylation (Δ_2) and oxidation to the ketone (Δ_3). This was presumably due to the vicinal action of the 4 α -methyl group.¹²⁹ For the new 4 α -methyl sterol (92a) similar discrepancies were apparent. Thus $\Delta_1 = +51^\circ$ and $\Delta_2 = +192^\circ$ while the expected¹³⁰ increments for a Δ^8 sterol

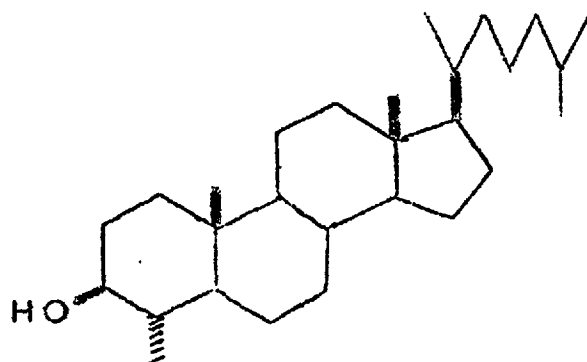
TABLE 3Molecular rotation data for 4 α -methyl- Δ^8 -sterols

	<u>3β-substituent</u>			
	<u>OH</u>	<u>OAc</u>	<u>OB$\bar{Z}$</u>	<u>oxo</u>
4 α -Me- Δ^8 -cholestenol ¹²⁸	+220°	+282°	+416°	-°
4 α -Me- $\Delta^{8,24}$ -cholesta- dienol* (90a)	+251	+288	+386	+198
4 α -Me-24-methylene- Δ^8 - cholestenol (92a)	+226	+277	+418	+193

* values of author except for OB $\bar{Z}$ for which see ref. 121.

are $\Delta_1 = -46^\circ$ and $\Delta_2 = +15^\circ$. The $[\bar{M}]_D$ values at C-3 for a number of 4α -methyl sterols are collected in table 4 for reference.

The mass spectrum of the new acetate (92b) showed the following major peaks in the high m/e region: m/e 454 (base peak) parent ion; 439 (26%) loss of CH_3 from 454, ($m^* 424.8$); 394 (18%) loss of $\text{CH}_3\text{CO}_2\text{H}$ from 454, ($m^* 342$); 379 (26%) loss of $\text{CH}_3\text{CO}_2\text{H}$ from 439 ($m^* 327.1$); 327 (30%) loss of (side chain + 2H) ($m^* 235.8$); and 243 (24%); 241 (44%); 227 (42%) etc. By comparison, 4α -methyl zymosteryl acetate (90b) showed prominent peaks at m/e 440 (base peak) parent ion; 425 (48%) loss of CH_3 from 440 ($m^* 410.6$); 380 (9%) loss of $\text{CH}_3\text{CO}_2\text{H}$ from 440 ($m^* 328.3$); 365 (22%) loss of $\text{CH}_3\text{CO}_2\text{H}$ from 425 ($m^* 313.4$); 327 (11%) loss of (side chain + 2) from 440 ($m^* 243$) etc. The two spectra could not be confidently interpreted for m/e values below 327. However the latter peak is of great significance since its appearance in both spectra confirms that the side chain of the new sterol (92a) carries the "extra" terminal methylene group, which was previously inferred largely on biosynthetic grounds. The loss of (side chain + 2H) has been observed¹³³ as a characteristic of steroids with unsaturation in the side chain. Steroids in general¹³⁴ lose (side chain + 42) as a major fragmenta-



(98)

<u>Subs. in (98)</u>	<u>Ref.</u>	<u>Δ_1</u>	<u>Δ_2</u>	<u>Δ_3</u>
None	129	+78°	-	-9°
24 ξ -ethyl	131	+64	-	-39
Δ^7	129	+104	+192°	+28
Δ^7 -24-methylene	132	+130	-	-
Δ^7 -24-ethylidene	131	+100	-	-38
Δ^7 -24-oxo	131	+102	-	-
Δ^8	128	+63	+193	-
$\Delta^{8,24}$	121	+37*	+135	-53*
Δ^8 -24-methylene	-	+51*	+192*	-33*
Δ^8 -14 α -Me-24-methylene	34	+59	-	-24*
$\Delta^8(14)$	129	+106	+126	-
$\Delta^8(14), 24$	-	+109*	-	-
Δ^{14}	129	+75	-	-

* author's values

tion. The latter peak is present in the spectra of the acetates (90b) and (92b) at m/e 287, but has a lower intensity (4% and 6% of the respective base peaks). It is significant that the two mass spectra are very similar below m/e 327, and are almost identical below m/e 161.

The evidence presented above provided powerful support for the postulated structure, 4 α -methylergosta-8,24(28)-dien-3 β -ol, for the new sterol. However, heavy reliance was placed on physical methods and further chemical proof was desirable. Accordingly, the new acetate (92b) was synthesised from 4 α -methylzymosteryl acetate (see below). The synthetic acetate was in every respect identical to the natural acetate thus confirming the structure of the new sterol.

The isolation from yeast of a sterol (92a), containing a 24-methylene substituent together with a 4 α -methyl group, has important biosynthetic implications. In particular, it demonstrates without doubt that yeast is capable of alkylating the sterol side chain at or before the 4 α -methyl stage.

Structural elucidation of 4 α -methylcholesta-8(14),
24-dien-3 β -ol

The sterol (93a) and its acetate (93b) were transparent above 220 m μ in the ultra-violet region. Microanalytical data on the sterol and its acetate indicated the molecular formula C₂₈H₄₆O for the sterol. In accordance with this formula the acetate showed its parent peak at m/e 440. The cracking pattern in the mass spectrum was very similar to that of 4 α -methyl zymosteryl acetate (90b).

On biosynthetic grounds, the sterol (93a) was assumed to possess the (5 α)-cholestane skeleton. The sterol showed a one-proton multiplet at 6.9 τ which on acetylation moved downfield to 5.6 τ . This resonance was assigned to the 3 α -proton in a 3 β -hydroxycholestane skeleton.

The derived acetate (93b) showed a three-proton doublet with J, 6 c/s, at 9.15 τ which moved downfield on conversion to the free sterol. This suggested a 4 α -methyl substituent by analogy with 4 α -methyl zymosteryl acetate (90b) and the 4 α -methyl steroid (92b). Furthermore, as in the sterol (90a), the second new sterol (93a) and its acetate (93b) showed two vinylic methyls at 8.32 and 8.39 τ , assigned to the C-25 methyl groups of a Δ^{24} side chain.

Finally the sterol and its acetate showed a triplet (J, 7 c/s) at 4.9τ assigned to the C-24 vinyl proton.

There were no further vinyl protons or vinylic methyls in the n.m.r. spectrum of sterol (93a), which restricted the second double bond to the $\Delta^{8(9)}$ or $\Delta^{8(14)}$ positions. If the stereochemical assignments in figure (93a) are accepted, the $\Delta^{8(9)}$ possibility is ruled out by the non-identity of the new sterol with 4α -methylzymosterol (90a). Hence the new sterol had a $\Delta^{8(14)}$ double bond. Support for this assignment was gained from the n.m.r. spectra. Thus the sterol (93a) had two three-proton singlets at 9.27 and 9.14τ assigned to the 10β and 13β methyl groups respectively (calculated^{123c} for cholest-8(14)-en- 3β -ol (97a) were 9.31 and 9.17τ respectively). Similarly, the acetate (93b) showed two three-proton singlets at 9.26 and 9.15τ which were assigned to the 10β and 13β methyls respectively (compare table 2). Further confirmation for the structure (93a) followed from the molecular rotations of the sterol and its acetate ($+76^\circ$ and $+185^\circ$ respectively) which were almost identical to those quoted by Djerassi and his co-workers¹²⁹ for 4α -methylcholest-8(14)-en- 3β -ol (93a; 24,25-dihydro) and its acetate, ($+76^\circ$ and $+181^\circ$ respectively).

The above arguments provide strong, though not conclusive, support for the structure (93a) assigned to the second new sterol. This sterol is probably best considered, at least until proven otherwise, to be an artefact derived from 4 α -methylzymosterol (90a). The ease of acid catalysed isomerisation of a $\Delta^{8(9)}$ double bond to the $\Delta^{8(14)}$ position, in the Δ^8 -cholestenol (96a) series is well known.¹³⁵

Although acid treatment was avoided during the present work, it is possible that the yeast residues may have been treated with mineral acid during their previous history, and hence no biosynthetic conclusions may be drawn from the presence of 4 α -methylcholesta-8(14),24-dien-3 β -ol (93a) in these yeast residues.

Partial synthesis of 3 β -acetoxy-4 α -methylergosta-8,24(28)-diene from 4 α -methylzymosteryl acetate

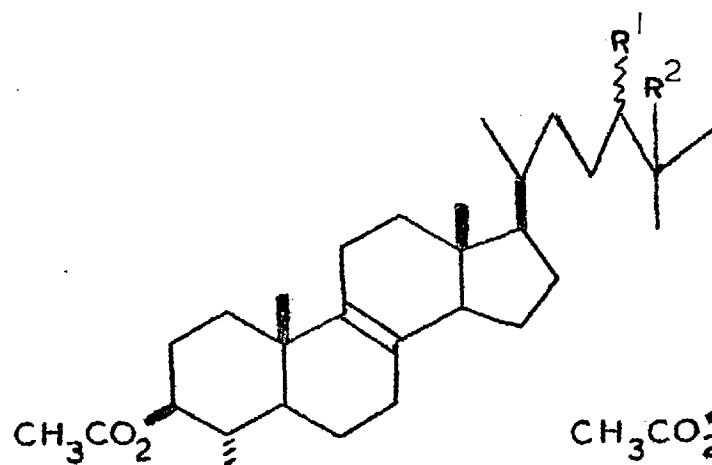
Treatment of 4 α -methylzymosteryl acetate (90b) with m-chloroperbenzoic acid led to a mixture of products observed by thin-layer chromatography. The mono-oxide fraction, isolated in 30% yield, was a mixture with τ 5.67 (3 α proton), 4.92 integrating for 0.6 proton (C-24 proton in Δ^{24} side chain) and showed vinylic methyls at τ 8.31 and 8.39 (C-25 methyl groups in Δ^{24} side chain). It was probable that epoxidation had occurred mainly on the Δ^8 double bond of the acetate (90b).

It is well known¹³⁶ that zymosterol (23) forms a 24,25-dibromide without attack at the $\Delta^{8(9)}$ double bond. Bromohydrin formation, at least formally, involves the same bromonium ion intermediate and so hypobromous acid also should not attack the nuclear double bond of zymosterol. Treatment of the acetate (90b) in aqueous glyme, with N-bromosuccinimide¹³⁷ at room temperature, gave an excellent yield of the mixed bromohydrins (99a). That reaction had occurred at the side chain double bond was evident from the unchanged τ -values for the nuclear methyl groups in the bromohydrin and the disappearance of the vinyl proton at $\tau 4.93$. The structure (99a) rather than the isomeric structure (99b) was assigned on the following basis: (i) the C-24 proton in the bromohydrin appeared at $\tau 6.07$ as a doublet with J , 7 c/sec., which compared favourably with the C-24 proton in lanosteryl acetate-24,25-dibromide (100) which had $\tau 5.83$, doublet with J , 10 c/s; on the other hand the C-24 proton in the 24,25-epoxide (101) had $\tau 7.32$ as a triplet with J , 5 c/s. This suggests a bromine atom at C-24 in the bromohydrin. (ii) The C-25 methyl resonance in the bromohydrin occurred as a singlet at $\tau 8.66$ comparing well with the corresponding resonances in the epoxide (101) which had $\tau 8.70$ and 8.75; in contrast, in the dibromide (100) the C-25 methyls

8.18.

occurred at τ 8.01 and ~~8.88~~. Thus the bromohydrin had an oxygen function at C-25 confirming the structure (99a). It is interesting that van Tamelen and his coworkers have assigned¹³⁷ the hydroxyl of squalene bromohydrin to the secondary (C-3) position but no evidence for this assignment was given and for farnesyl acetate bromohydrin they preferred¹³⁸ the alternative structure with a tertiary (C-2) hydroxyl.

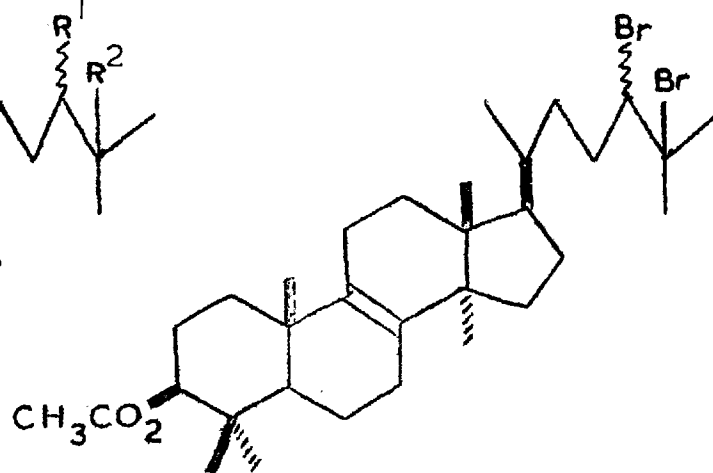
The bromohydrin (99a) was smoothly converted to the mixed epoxides (101) by chromatography on alumina. However, treatment of the epoxide (101) with boron trifluoride etherate in benzene at room temperature for five minutes gave four products: the required $\Delta^{8(9)}$ ketone (102a), its $\Delta^{8(14)}$ isomer (102b), the aldehyde (103a) and the $\Delta^{8(14)}$ aldehyde (103b). The isomeric aldehydes were readily separable from the ketone fraction but the two isomeric ketones were inseparable. The non-crystalline ketone mixture showed, for the required ketone (102a), methyl resonances at τ 8.86 and 8.98 (C-25 methyls), 9.02 (10 β -methyl), 9.15 as a doublet with J, 6 c/s (4 α -methyl) and 9.39 (13 β -methyl). The presence of about 20% of the $\Delta^{8(14)}$ isomer (102b) in the mixture was inferred from the sharp absorptions at τ 9.15 (13 β -methyl) and 9.26 (10 β -methyl - cf. Table 2). Treatment of the epoxide (101) with boron trifluoride



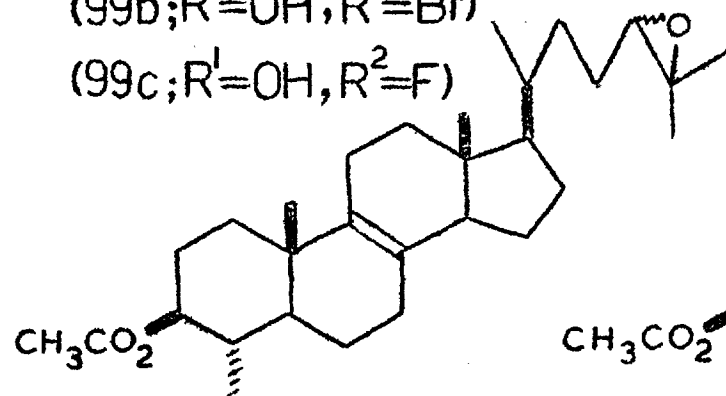
(99a; $\text{R}^1 = \text{Br}$, $\text{R}^2 = \text{OH}$)

(99b; $\text{R}^1 = \text{OH}$, $\text{R}^2 = \text{Br}$)

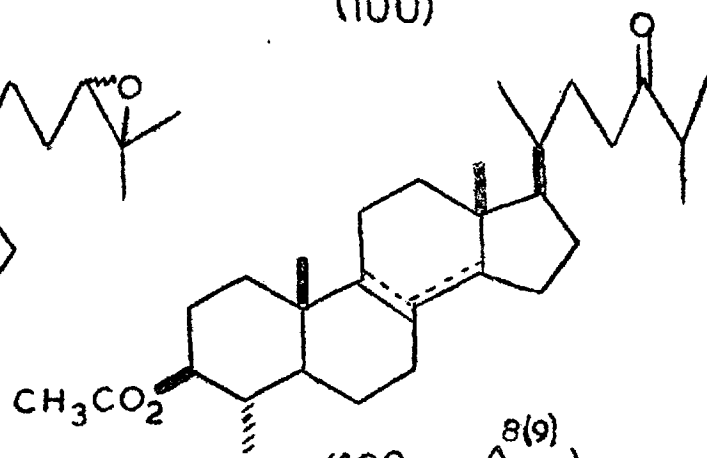
(99c; $\text{R}^1 = \text{OH}$, $\text{R}^2 = \text{F}$)



(100)

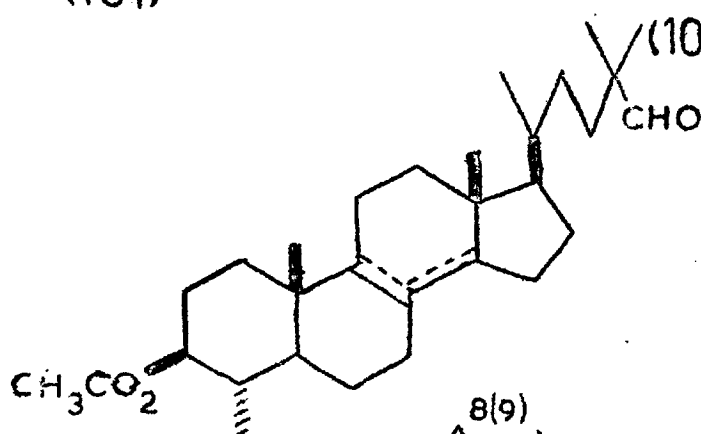


(101)



(102a; $\Delta^{8(9)}$)

(102b; $\Delta^{8(14)}$)



(103a; $\Delta^{8(9)}$)

(103b; $\Delta^{8(14)}$)

etherate in benzene at 5°C for 1.5 minutes or at room temperature for 15 seconds gave at least five products, of which the major product was more polar than the desired ketone (102a). Similar uncharacterised products were observed when lanosteryl acetate epoxide (104) was treated with boron trifluoride under either of these conditions, while the ketone (105) and aldehyde (106a) were the only products with a reaction time of five minutes at room temperature (see section 5). Thus conditions were not found such that the epoxide (101) gave the required ketone (102a) in major yield, without concomitant double bond migration, and a low yield of the ketone had to be accepted.

Boron trifluoride isomerisation of the epoxide (101) at room temperature for 15 seconds gave the crystalline ketone (102a) in 15% yield. This structure was assigned on the basis of the spectral data, $\nu_{\max}$ 1735 (CH_3CO_2^-) and 1708 (acyclic ketone) cm^{-1} and τ 8.92, six protons, doublet J, 7 c/s (C-25 methyls). The nuclear methyl resonances were as in 4 α -methylzymosterol. (90b). No trace of the $\Delta^{8(14)}$ isomer (102b) was evident either by n.m.r. or by thin-layer chromatography.

Two major by-products of the boron trifluoride rearrangement were examined by n.m.r. and i.r. spectroscopy, but not characterised. The least polar product had $\nu_{\max}$ 2692 (-CHO) and 1737 (-CHO and $\text{CH}_3\text{CO}_2\text{-}$) cm.^{-1} . The nuclear methyl groups had τ -values as in 4 α -methylzymosteryl acetate demonstrating an unisomerised Δ^8 nuclear double bond. The presence of a tertiary aldehyde function was confirmed by the one proton singlet at 0.57 τ . A six-proton singlet at 8.97 τ was assigned to two methyls alpha to the aldehyde carbonyl. The structure (103a) was assigned tentatively to this substance from mechanistic considerations and by comparison with the analogous boron trifluoride isomerisation of lanosteryl acetate oxide (104-see section 5).

A second by-product from the boron trifluoride rearrangement of the epoxide (101), was more polar than the ketone (102a) and contained a hydroxyl, $\nu_{\max}$ 3640 cm.^{-1} . Again, n.m.r. showed that no rearrangement of the Δ^8 double bond had occurred, but the C-25 methyl groups absorbed at τ 8.48 and 8.85. In addition the C-24 proton appeared at about 6.5 τ as a very broad multiplet. The formation of fluorohydrins has been reported^{139,140} from boron trifluoride rearrangement of epoxides though the mechanism¹³⁹ of their formation remains obscure.

The present uncharacterised substance was probably the fluorohydrin (99c) which might explain the unusual resonance of the C-25 methyls: τ 8.67 as a doublet with J, 22 c/s. This substance was not further investigated.

The final stage of the synthesis was accomplished by treatment of the ketone (102a) with methylene triphenyl phosphorane, according to Cocker, McMurry and Ntamila.¹⁴¹ After reacetylation, the acetate (92b) was isolated in 65% yield, with m.p. 110-112° (plates), $[\alpha]_D^{23} +61^\circ$ compared with m.p. 110-112° (plates), $[\alpha]_D^{29.5} +61^\circ$ for the natural acetate, and a mixed melting point determination showed no depression. (Dr. P.G. Sammes kindly redetermined these melting points and found natural acetate m.p. 110-111° synthetic acetate 109.5-110.5°, mixed melting point 109.5-110.5°). In addition the t.l.c. properties, the n.m.r. spectrum, mass spectrum and infra-red spectrum of the synthetic material were identical to those of the natural acetate. Hence the natural sterol had structure (92a).

It should be noted that 4 α -methylzymosterol (90a), the starting point of the synthesis described above, has not itself been synthesised. However, the recent synthesis¹⁴² of 4 α -methylcholest-8-en-3 β -ol (90a; 24,25-dihydro) confirms the structure assigned

by Ponsinet and Ourisson¹²¹ to this sterol.

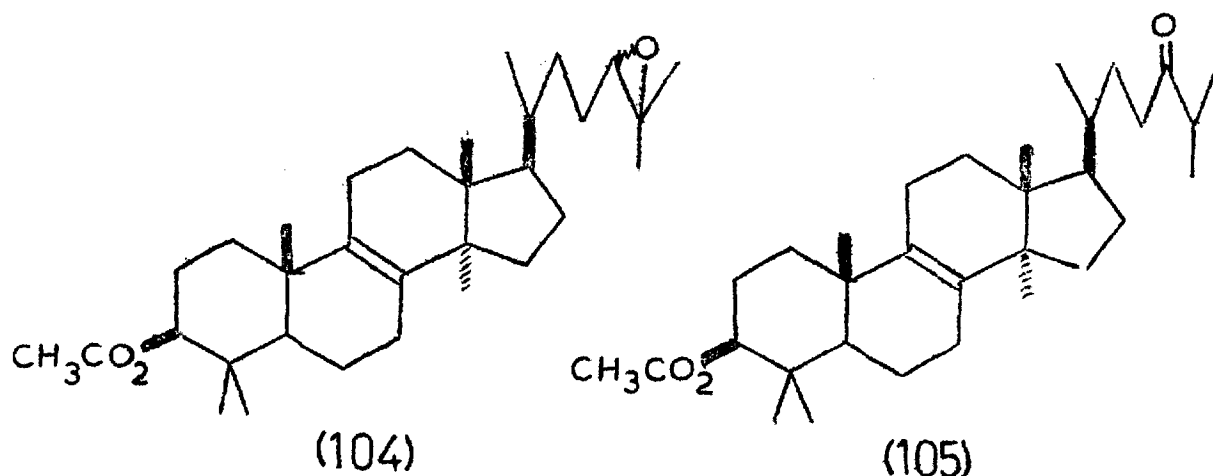
5. Preparation of labelled sterol precursors

24-methylenedihydrolanosterol

A supply of lanosteryl acetate dibromide (100) was available and it was necessary to convert this compound to the ketone (105). The preparation of ketones or aldehydes by hydrolysis of vic-dihalides with water and lead oxide^{143,144} or water alone¹⁴⁵ has long been known. In particular, 2,3-dibromo-2-methylbutane gave 3-methylbutan-2-one in 75% yield on refluxing with water.¹⁴⁶ The mechanism of the reaction has been discussed by Suter and Zook.¹⁴⁷ The reaction has however been demonstrated only with simple dihalides.

Lanosteryl acetate dibromide (100) was refluxed with lead oxide in dioxan-water (1:1) for 24 hours. 40% of the dibromide was recovered unchanged, and neither of the two products was the required ketone (105). However, the minor product (20%) was assigned the structure lanosteryl acetate-24,25-epoxide (104) on the basis of its n.m.r. spectrum, and the reported formation of epoxides in this reaction from 2,3-dibromo-2,3-dimethyl butane and 2,3-dibromo-2-methyl butane,¹⁴⁸ and ethylene dihalides.¹⁴³ The n.m.r. spectrum of the epoxide (104) showed a nine proton singlet at τ 9.12 (4 α , 4 β and 14 α methyls), and singlet methyls at τ 7.96 (acetate), 9.00 (10 β methyl) and 9.30 (13 β methyl) and a one-proton triplet with

J, 7 c/s at τ 5.48 (3α -proton), confirming that the nucleus was unaffected. A triplet at τ 7.31 with J, 5 c/s (C-24 proton) and singlet methyl absorptions at τ 8.70 and 8.74 (C-25 methyls) suggested a 24,25-epoxide. Further evidence for structure (104) was supplied by its parent peak at m/e 484 in the mass spectrometer. The epoxide mixture had m.p. 181° in agreement with literature values, m.p. 178° ¹⁴⁹ and 185° .¹⁵⁰



The same epoxide mixture (104), m.p. 196° after many crystallisations, was prepared in 60% overall yield by debromination of the dibromide (100) with sodium iodide in acetone and treatment of the resulting lanosteryl acetate with monoperphthalic acid in chloroform. On one occasion chromatography of the crude reaction mixture on alumina separated the two C-24 epimeric epoxides (104), which differed in

melting point and optical rotation, but had identical spectral properties. Cycloartenyl acetate epoxide¹⁴¹ has been similarly resolved.¹⁵¹

Treatment of the epoxide (104) with boron trifluoride etherate in benzene gave a mixture of the aldehyde (106a) and the desired ketone (105). Chromic oxide-acetic acid oxidation of the mixture in benzene converted the aldehyde to the acid (106b) which was extracted with base. The ketone (105) had $\nu_{\max}$ 1735 (CH_3CO_2^-) and 1708 cm^{-1} (acyclic ketone) and showed a six-proton doublet at τ 8.92 with J , 7 c/s (C-25 methyls). The C-23 and C-25 protons appeared as a multiplet at τ 7.5.

The acid by-product (106b) was treated with diazomethane and the crystalline methyl ester (106c) isolated. As usual the n.m.r. spectrum confirmed the unchanged nucleus and a six-proton singlet at τ 8.85 and a three-proton singlet at τ 6.36 were assigned to the C-24 methyls and the methoxycarbonyl methyl group respectively. The mass spectrum of the methyl ester (106c) had a parent peak at m/e 514 in agreement with its analytical figures. Finally the original aldehyde (106a) which was not isolated, showed a one-proton singlet at τ 0.61 confirming a tertiary aldehyde function, and supporting the structure assigned to the methyl ester. The corresponding

aldehyde has been obtained as a by-product in the boron trifluoride isomerisation of cycloartenyl acetate epoxide.¹⁵¹

Treatment of the ketone (105) with methylene triphenyl phosphorane in tetrahydrofuran¹⁴¹ gave a 75% yield of 24-methylenedihydrolanosteryl acetate (91b) which on alkaline hydrolysis gave the desired sterol (91a), both with the appropriate spectral properties. In particular the sterol and its acetate showed strong absorption at $\nu_{\max}$ 890 cm^{-1} and a broad two-proton singlet at τ 5.28 ($\text{C}=\text{CH}_2$). The physical constants of the sterol and acetate (m.p. 157.5-159°, $[\alpha]_D$ +59° and m.p. 147-150° respectively) were in agreement with those of "eburicol" (91a) and its acetate (m.p. 158-159°, $[\alpha]_D$ +66° and m.p. 138-139°) prepared¹⁵² as an unknown degradation product during the early work on the structure of eburicoic acid (45).

$[28\text{-}^{14}\text{C}]$ -24-methylenedihydrolanosterol was prepared in low yield from the ketone (105) utilising $[\text{CH}_3\text{-}^{14}\text{C}]$ -methyltriphenylphosphonium iodide in place of the usual phosphonium bromide. The ^{14}C labelled iodide was prepared by treatment of triphenyl phosphine in benzene with $[^{14}\text{C}]$ -methyl iodide in a sealed tube.

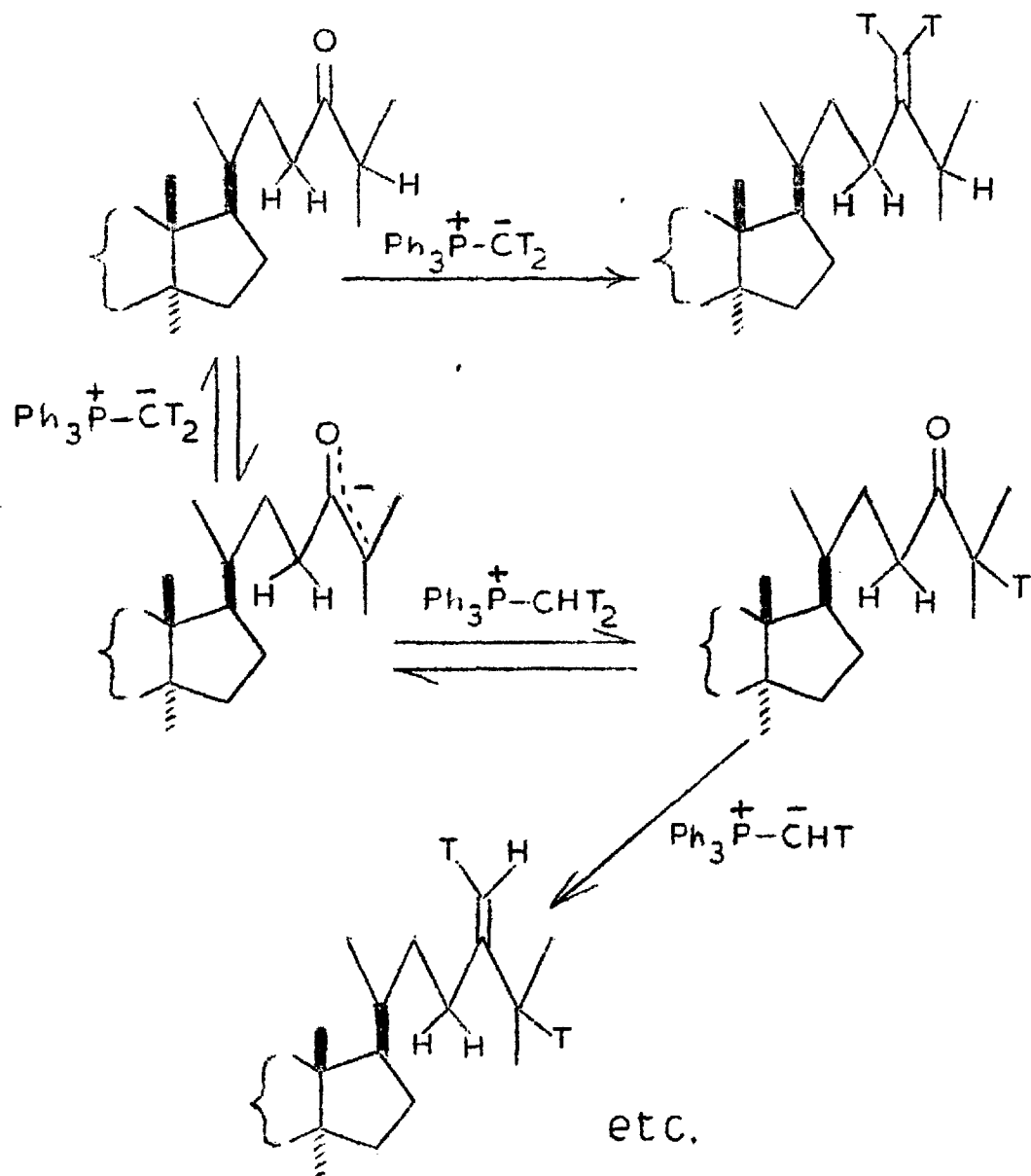
[28-³H]-24-methylenedihydrolanosterol was also required. As a preliminary experiment, methyl triphenyl phosphonium bromide was left overnight in deuterium oxide containing a catalytic amount of base. Removal of solvent gave the deuterated phosphonium salt, which was immediately utilised in a Wittig reaction with the ketone (105). The mass spectrum of the resulting 24-methylenedihydrolanosterol acetate had its parent ion at m/e 484 corresponding to a dideuterated molecule. A comparison of this spectrum with that of the undeuterated acetate, with parent peak at m/e 482, showed that the labelled acetate consisted of approximately 70% dideuterated, 20% monodeuterated and 10% undeuterated. A more exact analysis was not possible because of complications arising from the ease of loss of 2H from the parent ions together with slight differences in relative peak heights in successive scans. The deuterated acetate also showed only weak $\nu_{\max}$ 890 cm^{-1} , and the vinylic absorption at τ 5.28 corresponded to only 0.5 proton demonstrating that C-28 had been deuterated specifically.

[28-³H]-24-methylenedihydrolanosterol was prepared as for the deuterated sterol, starting from tritiated water. In order to assess the specificity of the tritium labelling, [¹⁴C, ³H]-24-methylene-

dihydrolanosterol with a $^3\text{H}/^{14}\text{C}$ ratio of 22.5, was ozonised in glacial acetic acid. After reductive work-up the formaldehyde isolated from C-28 was counted as its dimedone derivative, which had $^3\text{H}/^{14}\text{C}$ 16.8. The ^{14}C molar activity was identical to that in the doubly labelled sterol (91a) and hence the $^3\text{H}/^{14}\text{C}$ ratio in the formaldehyde demonstrated that only 75% of the tritium label in the sterol resided at C-28. Presumably scrambling of label occurred during the preparation of $[^3\text{H}]$ -24-methylenedihydrolanosterol. Reversible enolisation of the ketone (105) during addition to the ylid solution prior to formation of the betaine would lead to $[23,25,28\text{-}^3\text{H}]$ -24-methylene-dihydrolanosterol, (see scheme 18). Similar scrambling (of deuterium) was noted by Atkinson et al.¹⁵³ in Wittig reactions performed in dimethyl sulphoxide, and an analogous explanation was suggested. This result was somewhat surprising since the labelling in $[28\text{-}^2\text{H}_2]$ -24-methylenedihydrolanosteryl acetate discussed above was certainly not scrambled to a similar extent and the mass spectral peak at m/e 485 was entirely accounted for as the ^{13}C isotope peak of the parent (dideutero) peak at m/e 484. Conversely if significant exchange had occurred, some tri-deuterated acetate should have resulted. Fortunately in the tritium labelling procedure, addition of the ketone (105) to the tritiated ylid at 0°C ,

Scheme 18

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followed by a normal Wittig reaction, gave the tritiated 24-methylene sterol with 94% of its label in the terminal methylene group. This degree of specificity was adequate for the present work.

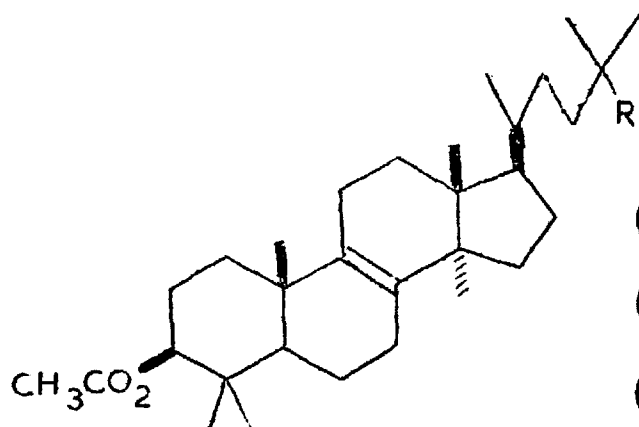
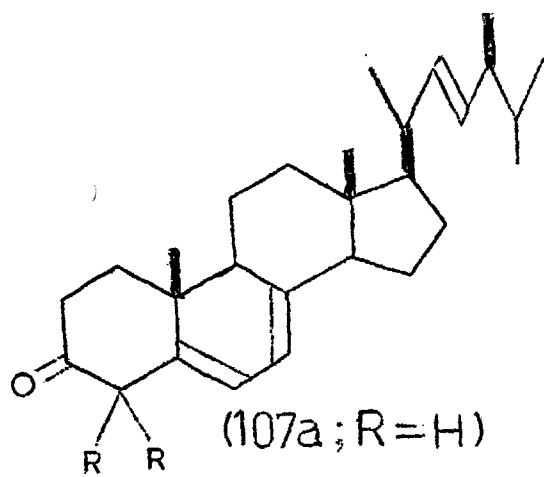
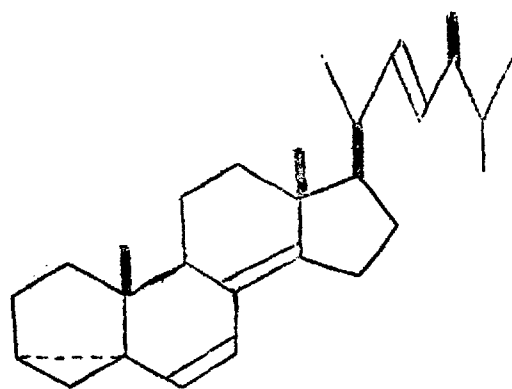
Preparation of $[2(4)-^3\text{H}]$ -labelled sterols

Jones and Wigfield have recently reported¹⁵⁴ the oxidation of androst-5-en-3 β -ol to the 3-oxo- Δ^5 derivative using dimethylsulphoxide and dicyclohexylcarbodiimide plus pyridinium trifluoroacetate. Ergosterol (44) was oxidised under the same conditions in the expectation of obtaining the $\Delta^{5,7}$ -3-oxo derivative (107a). In the event a complex mixture resulted containing two hydrocarbons as well as more polar material. The required ketone, if formed at all, was not a major product. The hydrocarbon fraction was obtained in 20% yield by chromatography of the crude reaction products. Repeated crystallisation furnished the major hydrocarbon, 3,5-cycloergosta-6,8(14),22-triene (108) with the appropriate n.m.r. spectrum, and which had the literature melting point¹⁵⁵ and showed no depression in mixed melting point with authentic material. The other products of the reaction were not investigated.

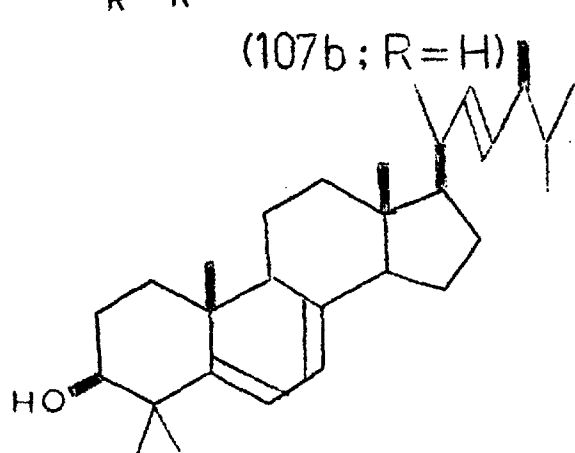
The required 4,4-dimethylergosterol (109) was finally prepared by a known procedure.¹⁵⁶ Oppenauer oxidation of ergosterol (44) gave the ketone (110) in 40% yield. Treatment of this ketone with potassium t-butoxide in t-butanol and addition of methyl iodide gave the 4,4-dimethyl ketone (107b). Lithium aluminium hydride reduction of the latter gave 4,4-dimethylergosterol (109). The ergosterol derivatives had the appropriate physical constants¹⁵⁶ and spectral properties.

Tritiated t-butanol was prepared by distilling a mixture of potassium t-butoxide and tritiated water. Exchange of the ketone (107b) with base in tritiated t-butanol gave $[2-^3\text{H}]-4,4\text{-dimethylergosta-5,7,22-trien-3-one}$ which on lithium aluminium hydride reduction gave $[2-^3\text{H}]-4,4\text{-dimethylergosterol}$.

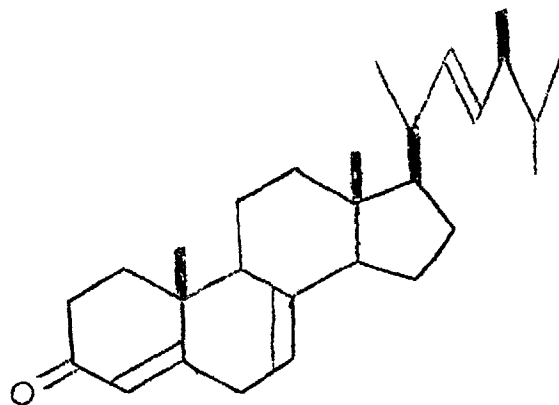
Lanosterol (3a), $4\alpha\text{-methylergosta-8,24(28)-dien-3}\beta\text{-ol}$ (92a), $4\alpha\text{-methylzymosterol}$ ¹²¹ (90a) and obtusifoliol³⁴ (18) were oxidised to the respective 3-oxo derivatives with chromic oxide-acetic acid-benzene. The ketones were labelled in the 2 or 2(4) positions in tritiated t-butanol as above. Lithium aluminium hydride reduction of the labelled ketones gave $[2-^3\text{H}]-\text{lanosterol}$, $[2,4-^3\text{H}]-4\alpha\text{-methylergosta-8,24(28)-dien-3}\beta\text{-ol}$, $[2,4-^3\text{H}]-4\alpha\text{-methylzymosterol}$ and $[2,4-^3\text{H}]-\text{obtusifoliol}$ respectively.

(106a; $\text{R}=\text{CHO}$)(106b; $\text{R}=\text{CO}_2\text{H}$)(106c; $\text{R}=\text{CO}_2\text{CH}_3$)(107a; $\text{R}=\text{H}$)(107b; $\text{R}=\text{H}$)

(108)



(109)

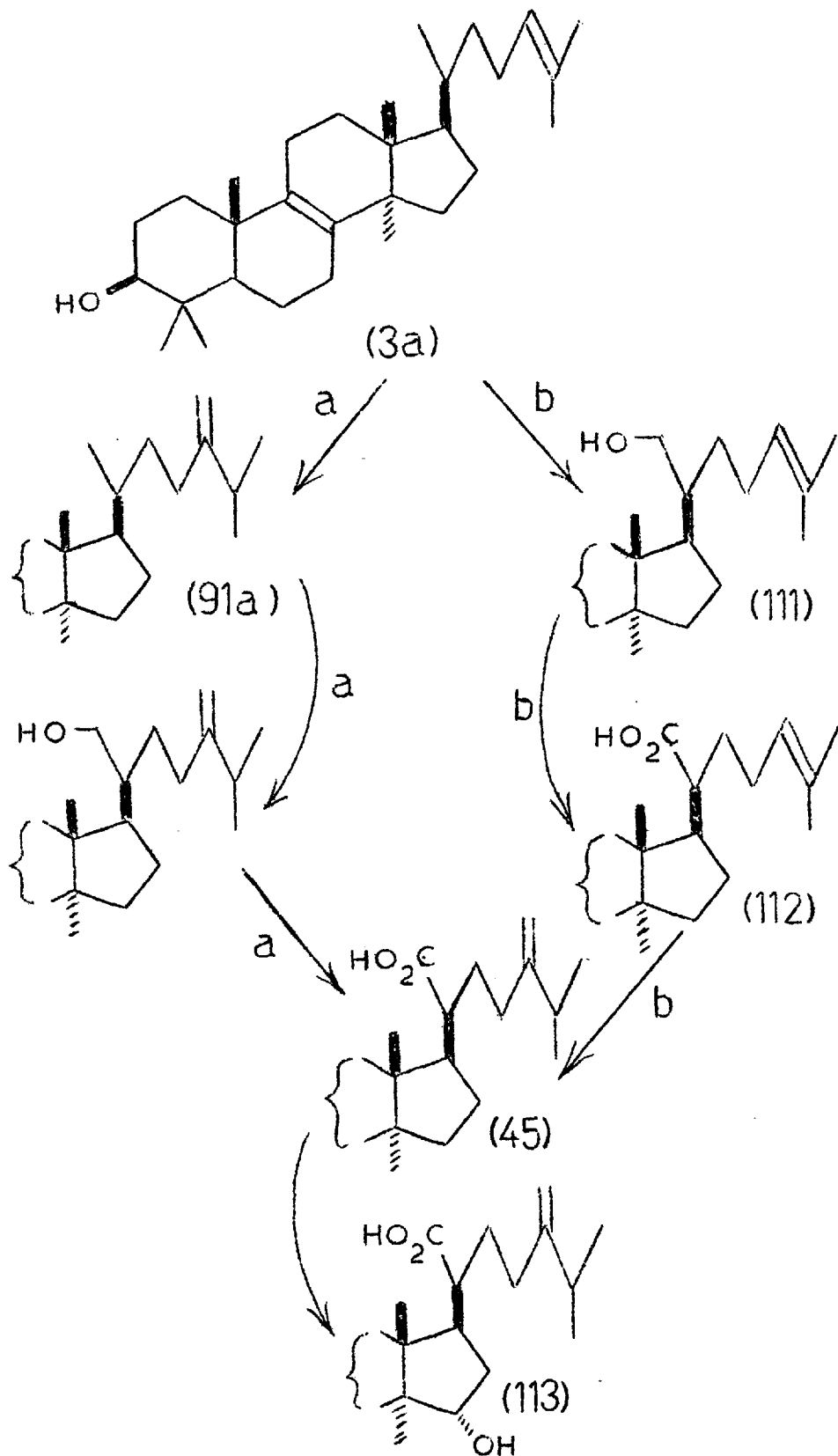


(110)

6. The biosynthesis of eburicoic acid

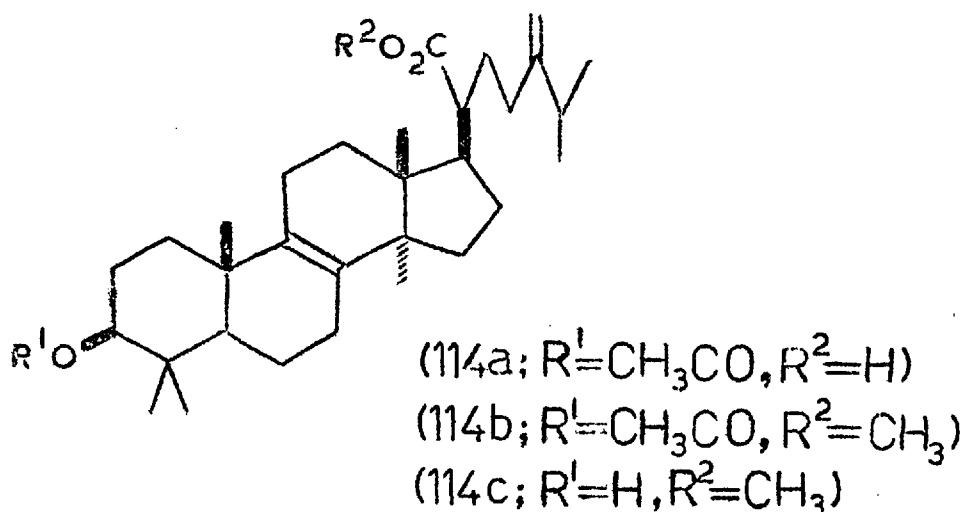
Two possible routes from lanosterol (3a) to eburicoic acid (45) were considered and are shown in scheme 19. Route (a) (scheme 19) visualised prior conversion of lanosterol to 24-methylene-dihydrolanosterol (91a) followed by oxidation of the C-21 methyl group to a carboxyl group. Route (b) visualised oxidation of lanosterol to trametenolic acid¹⁵⁷ (112) followed by introduction of the 24-methylene substituent. Since it had already been shown (see section 3) that in vivo cultures of Polyporus sulphureus were capable of utilising acetate and one-carbon donors as precursors of eburicoic acid, this species was used in the present work.

A surface culture of P.sulphureus was grown at 30° in the dark on a sterilised aqueous medium containing malt extract supplemented with glucose. [28-³H]-24-methylenedihydrolanosterol and Tween 80 were added to the medium in a small quantity of acetone. Addition of the precursor prior to inoculation of the medium with P.sulphureus led to unsatisfactory growth, and it was therefore necessary to feed the precursor to the vigorously growing culture at intervals over several weeks. The mycelium was harvested after six to eight weeks growth and



eburicoic acid and unutilised precursor recovered by standard procedures. The yield of eburicoic acid was about 15% of the dry weight of the mycelium. A second triterpene acid which was observed in small quantities had t.l.c. behaviour identical to authentic sulphurenic acid (113), a known¹⁵⁸ constituent of P.sulphureus, and was not further investigated.

The biosynthesised eburicoic acid was converted to 3-O-acetyl eburicoic acid (114a), methyl acetyl eburicoic acid (114b), and methyl eburicoate (114c), which were identified by comparison with authentic samples. Each of these derivatives was purified by thin-layer chromatography and rigorously crystallised to constant specific activity. The molar activities of the derivatives were equal within experimental error, and corresponded to an incorporation of 0.115%. Allowing for 22% recovery of precursor, the incorporation was 0.15%.



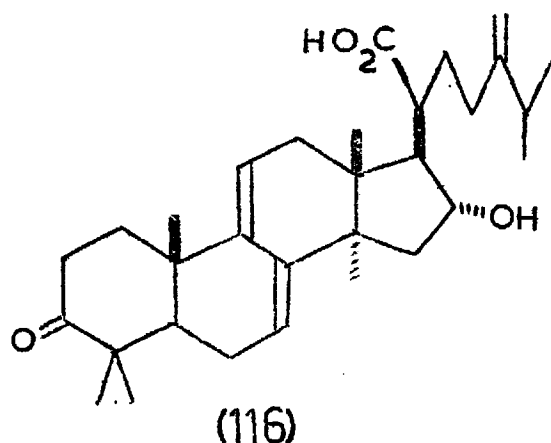
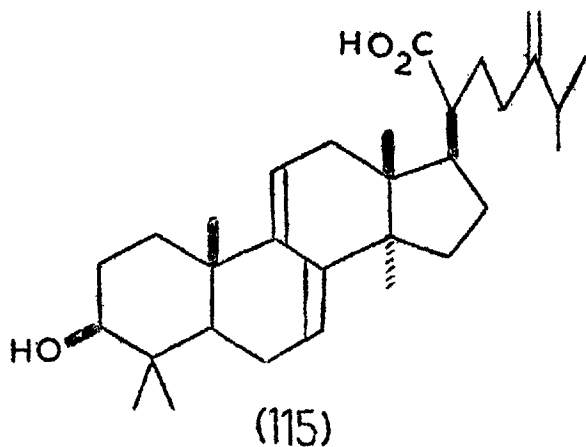
The feeding of 24-methylenedihydrolanosterol was repeated with the ^{14}C -labelled sterol, since ^{14}C could be counted at higher efficiency and lower background than ^3H , and an incorporation of 0.039% into eburicoic acid was observed. A parallel feeding of 2- ^3H -lanosterol showed an incorporation of 0.053% into eburicoic acid. The feeding results are summarised below:-

	<u>Incorporation into</u> <u>eburicoic acid</u>
[28- ^3H]-24-methylenedihydrolanosterol	0.115%
[28- ^{14}C]-24-methylenedihydrolanosterol	0.039%
[2- ^3H]-lanosterol	0.053%

The incorporations obtained in the present study were disappointingly low. Lawrie et al. have recently reported¹⁵⁹ the incorporation of farnesyl pyrophosphate and farnesol into eburicoic acid by P.sulphureus in vivo, with yields of 0.4%. In a cell-free extract farnesyl pyrophosphate was incorporated with an even higher efficiency.¹⁵⁹ However, as shown above, the incorporation of 24-methylenedihydrolanosterol was of the same order as that of lanosterol, which can be assumed to be an obligatory precursor of eburicoic acid. Hence the low incorporations recorded here are probably due to the low efficiency of transport of sterol

substrates from aqueous suspension, into the cells of the fungus.

The present study has demonstrated the first feedings of sterols to P.sulphureus and suggests that 24-methylenedihydrolanosterol may be a normal intermediate in the biosynthesis of eburicoic acid. This conclusion is reinforced by the recent isolation of 24-methylenedihydrolanosterol from Fomes officinalis, together with eburicoic acid, and dehydroeburicoic acid¹⁶⁰ (115). However, the occurrence¹⁵⁷ in Fomes pinicola of triterpenes related to (111) and (112), which have undergone oxidation at C-21, but not alkylation at C-24, suggests that biosynthesis of the 24-methylene triterpene acid, polyporenic acid C¹⁵⁷ (116) in this fungus may occur via a route analogous to route (b) in scheme 19.



7. The biosynthesis of ergosterol

At the outset of this work, Lederer and his coworkers had demonstrated^{93,94} that $[\text{CD}_3]$ -methionine fed to Neurospora crassa gave $[\text{D}_2]$ -ergosterol, suggesting the possibility of a 24-methylene intermediate in the biosynthesis of ergosterol (see section 3). Early experiments on the variation of sterol activity with time, in a yeast cell-free homogenate fed with $[\text{l-}^{14}\text{C}]$ -acetate, suggested¹⁶¹ that lanosterol (3a) but not zymosterol (23) were precursors of ergosterol (44). Also lanosterol, but not zymosterol, was incorporated into ergosterol by a cell-free homogenate.¹⁶² Although the identification of the radioactive sterols fed by the earlier workers was not adequate, the results suggested that side chain modification was an early step in the biosynthesis of ergosterol from lanosterol. In the light of the work of Lederer and his coworkers,^{93,94} 24-methylenedihydrolanosterol (91a) seemed a likely intermediate in the biosynthesis of ergosterol from lanosterol.

The early workers also established that yeast grown anaerobically was sterol deficient and that subsequent aeration gave a yeast culture which was very active in sterol synthesis.¹⁶³ Accordingly a sterilised solution of malt extract in water was inoculated with a little dried baker's yeast,

(Saccharomyces cerevisiae) and incubated anaerobically at 30° without shaking for 40 hours. The yeast cells were harvested by centrifugation and resuspended in an aqueous medium containing glucose and potassium dihydrogen phosphate. [28-³H]-24-methylenedihydrolanosterol was suspended in the medium with the aid of Tween 80, and the medium vigorously aerated for 16 hours at 30°. The yeast was again harvested by centrifugation, refluxed with dilute methanolic potassium hydroxide, and the non-saponifiable material extracted with benzene. The extract was diluted with inactive 24-methylenedihydrolanosterol, and precursor, and biosynthesised ergosterol recovered by preparative scale thin-layer chromatography. The incubation medium was also worked-up for unutilised precursor. The biosynthesised ergosterol was diluted with pure ergosterol and crystallised to a constant specific activity corresponding to an incorporation of 1.15% (or 1.5% allowing for 26% recovery of precursor). On acetylation, the ergosteryl acetate retained the radioactivity quantitatively after t.l.c. purification and several crystallisations. Finally ozonolysis of the biosynthesised ergosterol gave 2,3-dimethylbutanal which when counted as its dimedone derivative, retained all (111%) of the activity of the ergosterol, demonstrating that the ergosterol was not randomly labelled.

Following this encouraging result, $[28-^{14}\text{C}, (23), (25), 28-^3\text{H}]$ -24-methylenedihydro-lanosterol with ca. 75% of the tritium label at C-28, (see section 5) was fed to whole yeast cells as before. The biosynthesised ergosterol showed a 0.62% incorporation of tritium activity. The activity was quantitatively retained on conversion to ergosteryl acetate. The close similarity in the $^3\text{H}/^{14}\text{C}$ ratios in the precursor, 22.5, in the biosynthesised ergosterol, 22.6, and in ergosteryl acetate, 23.7 again demonstrated that the precursor was incorporated intact into ergosterol.

$[2-^3\text{H}]$ -lanosterol was fed to whole yeast cells under the same conditions and the biosynthesised ergosterol showed an incorporation of 0.44% (or 0.71% allowing for recovered precursor). Lanosterol is a normal constituent of yeast¹¹¹ and is presumably an obligatory precursor of ergosterol, as suggested by the earlier work employing cell-free homogenates.^{161,162} The incorporation of lanosterol thus provided a useful standard for comparison with other feeding results. A parallel feeding of $[2-^3\text{H}]$ -4,4-dimethylergosterol (109) gave no incorporation ($\leq 0.003\%$) into ergosterol demonstrating the high specificity of the enzymes mediating the removal of the C-4 methyl groups of lanosterol. It is noteworthy that the analogous sterol

4,4-dimethylcholesterol,³² was not converted to cholesterol by a rat liver homogenate.

Finally obtusifoliol (18), 4 α -methyl-
zymosterol (90a) and 4 α -methyl-24-methylenedihydro-
zymosterol (92a) were fed to cultures of whole yeast
cells, in parallel, and the biosynthesised ergosterol
showed incorporations of 8.3%, 15% and 12.5%
respectively. Allowing for recovered precursor,
these incorporations could be corrected to 26%,
26% and 13.8% respectively. These incorporations
were unexpectedly high compared with the results
obtained with lanosterol and 24-methylenedihydro-
lanosterol. The explanation probably lay in the
expected higher solubility of these 4 α -methyl
sterols in an aqueous medium and hence the greater
efficiency with which they could reach the yeast
cells. Also, it is clear that no unique route for
the biosynthesis of ergosterol in yeast could be
compatible with the intermediacy of both 4 α -methyl-
zymosterol and obtusifoliol. Alternative
explanations for these results will be discussed
below.

A possible objection to the feeding of
2-³H or 2,4-³H labelled sterols should be
mentioned. It has been shown in the biosynthesis
of cholesterol that the C-4 methyl group(s) of

cholesterol precursors are lost by oxidation to carboxyl group(s) followed by decarboxylation, assisted by a 3-oxo derivative (scheme 6, section 2). Presumably a similar sequence occurs during the conversion of 4,4-dimethyl and 4 α -methyl sterols to ergosterol in yeast, and the intermediacy of 3-oxo steroids might render protons at the C-2(4) position(s) labile, so conceivably losing part of the tritium label. However, it has been shown⁴⁰ that little exchange occurs at the C-2 position during conversion of 2-³H labelled sterols to cholesterol in rat liver homogenates, and so the results presented here should be at least qualitatively correct, though possibly slightly lower than the true values.

During the course of this work, Akhtar and his coworkers reported the incorporation of [23,25-³H]-24-methylenedihydrolanosterol¹⁶⁴ and [26,27-¹⁴C, 24-³H]-lanosterol¹⁶⁵ into ergosterol by whole yeast cells. The [¹⁴C,24-³H]-lanosterol incorporation gave [¹⁴C,³H]-ergosterol with all of its tritium label at C-25, so directly demonstrating the hydride migration from C-24 to C-25, discussed in section 3 of this thesis. The incorporations of activity into ergosterol achieved by these workers exceeded the incorporations of the same substrates in the present work by about ten times! Thus lanosterol gave a 15%

incorporation into ergosterol and 24-methylene-dihydrolanosterol gave an incorporation of 6.2%. However, the feeding procedure used by Akhtar et al.¹⁶⁴ was very different from that used in the present study. In particular a different source of yeast was used, the medium contained "peptone" and glucose as well as malt extract, the volume of medium inoculated was very much larger and perhaps most significantly, the substrate was fed during the anaerobic growth phase.¹⁶⁴

Accordingly, $[28-^3\text{H}, ^{14}\text{C}]$ -24-methylenedihydrolanosterol was fed to whole yeast cells during the anaerobic growth phase, employing the usual source of dried baker's yeast but otherwise under conditions approximating to those used by Akhtar et al.¹⁶⁶ The substrate now showed an incorporation of activity into ergosterol of 4.8%, based on tritium, which was a considerable improvement on the previous incorporations of this substrate (0.62% and 1.15%) recorded by the author, though still lower than that recorded by Akhtar et al.¹⁶⁴ However it was clear that the different source of yeast employed by the other workers was not the most important factor controlling the higher incorporations obtained in their work. In the present feeding, the precursor had a $^3\text{H}/^{14}\text{C}$ ratio of 15.6 while the biosynthesised ergosterol, and derived ergosteryl acetate had

ratios of 15.6 and 16.2 respectively. Ozonolysis of the biosynthesised ergosterol gave 2,3-dimethylbutanal with a $^3\text{H}/^{14}\text{C}$ ratio of 17.2.

Turner and Parks have recently described¹⁶⁷ the preparation of a cell-free homogenate which when incubated with $[\text{CH}_3\text{-}^{14}\text{C}]$ -methionine, gave rise to two unidentified ^{14}C -labelled sterols, although no ergosterol was formed. One of these sterols served as a precursor of ergosterol when reincubated with whole yeast cells.¹⁶⁷ Presumably one or more of the enzymes responsible for ergosterol biosynthesis, at some stage after transmethylation from S-adenosylmethionine, was destroyed during preparation of the cell-free homogenate.

$[28\text{-}^3\text{H}]$ -24-methylenedihydrolanosterol was incubated aerobically with a cell-free yeast homogenate prepared as described by Turner and Parks.¹⁶⁷ After 17 hours the medium was saponified in the presence of carrier ergosterol, and the sterols isolated. Thin-layer chromatography of the sterol mixture allowed recovery of 75% of the precursor fed. Approximately 1.6% of the activity fed was recovered in fractions more polar than ergosterol, while 1.8% of the activity fed was inseparable from authentic ergosterol by t.l.c. chromatography. However when the "ergosterol fraction" was diluted with inactive ergosterol and

crystallised four times, the ergosterol retained $\leq 0.03\%$ of the activity fed, demonstrating that ergosterol was not formed in this cell-free homogenate, in agreement with the earlier workers.¹⁶⁷

The feeding results with yeast, performed in the present study, are summarised in table 5. Full details of the feeding results are provided in the experimental. Part of the new work presented in this thesis has been the subject of preliminary publications.¹⁶⁸

During and since the completion of the work presented in this thesis, a number of relevant papers by other authors have appeared. Hamilton and Castrejon have reported¹⁶⁹ the isolation of an "ergosterol peroxide" from Saccharomyces cerevisiae fed with $[^{14}\text{C}]$ -acetate, and have claimed that this substance served as a precursor of ergosterol. Despite the reservations of these authors, this substance was probably $5\alpha, 8\alpha$ -epidioxyergosta-6,22-dien- 3β -ol (117a), which has been isolated previously from Aspergillus fumigatus.¹⁷⁰ The oxidation level of this compound suggests that it should be formed from ergosterol, and may be an artefact; the suggestion that it is a precursor of ergosterol is hard to understand. Paliokas and Schroepfer have recently demonstrated that the corresponding derivative (117b) is not converted to cholesterol by rat liver

TABLE 5

Summary of sterol incorporations into ergosterol
in *Saccharomyces cerevisiae*

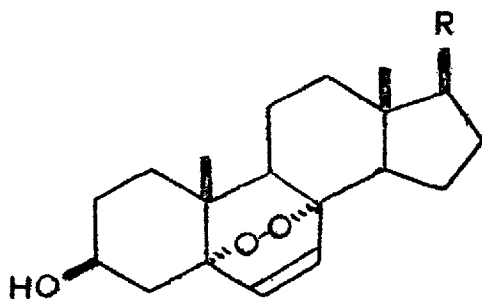
	<u>Incorporation (%)</u>	
[28- ³ H]-24-methylenedihydrolanosterol	1.15	(1.5)*
[28- ¹⁴ C, (23,25), 28- ³ H]-24-methylene- dihydrolanosterol	0.62	
[2- ³ H]-lanosterol	0.44	
[2- ³ H]-4,4-dimethylergosterol	≤0.003	≤0.005*
[2(4)- ³ H]-obtusifoliol	8.3	(26)*
[2(4)- ³ H]-4α-methylzymosterol	15.0	(26)*
[2(4)- ³ H]-4α-methylergosta-8,24(28)- dienol	12.5	(13.8)*
[28- ¹⁴ C, ³ H]-24-methylenedihydro- lanosterol	4.8†	
[28- ³ H]-24-methylenedihydrolanosterol	≤0.03‡	≤0.12)*

*Incorporation adjusted for recovered precursor

†Fed according to Akhtar et al.¹⁶⁶

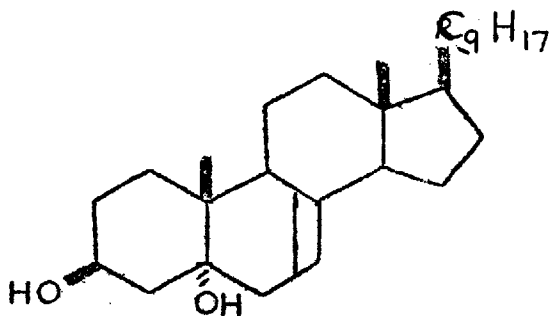
‡Fed to a yeast cell-free homogenate.¹⁶⁷

homogenates.



(117a; $R = C_9H_{17}$)

(117b; $R = C_8H_{17}$)

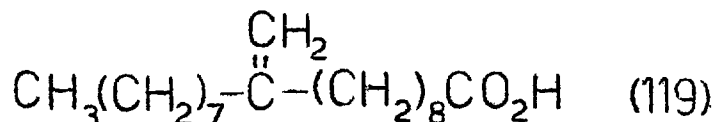


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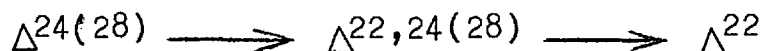
The terminal stage in the biosynthesis of the ergosterol nucleus has been studied by Topham and Gaylor, who showed that ergosta-7,22-dien- $3\beta,5\alpha$ -diol (118) was converted anaerobically to ergosterol by a cell-free homogenate of S.cerevisiae.¹⁷² As noted in Section 2, the corresponding cholestendiol (43) was not converted to cholesterol by a rat liver homogenate under anaerobic conditions.⁶¹ At least in this respect the biosynthesis of the ergosterol nucleus seems to differ from that of cholesterol.

The intermediacy of 24-methylene sterols in the biosynthesis of ergosterol has been amply demonstrated in the present study by the efficient incorporation of three 24-methylene sterols, one of which was shown to be present in yeast, into ergosterol. The independent work of Akhtar et al.¹⁶⁴ with 24-methylene-

dihydrolanosterol has provided useful confirmation of part of this work. Also Jauréguiberry et al. have demonstrated¹⁷³ the intermediacy of 10-methylene-stearic acid (119) in the biosynthesis of tuberculostearic acid (52a), the C-10 methyl group of which is also methionine-derived.



The conversion of the 24-methylene side chain to the Δ^{22} ergosterol side chain has been studied recently. Katsuki and Bloch¹⁷⁴ have reinvestigated the work of Turner and Parks¹⁶⁷ and have carried out some elegant degradations on one of the unidentified labelled sterols found by these workers and shown to be a precursor of ergosterol. The results suggested that this sterol was ergosta-5,7,22,24(28)-tetraenol (84), and the probable occurrence¹¹⁴ of this sterol in whole yeast cells provides strong evidence for the biosynthetic route:-



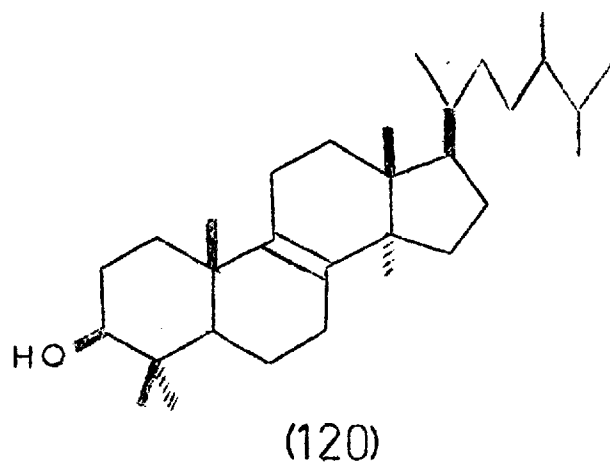
However, Akhtar et al. have recently shown¹⁷⁵ that [23,25-³H]-24-methyl^{dihydro}lanosterol (120) is converted in 8% yield to ergosterol by whole yeast cells.

Also [24,28-³H]-24-methyl^{dihydro}lanosterol was converted to ergosterol with retention of most of the tritium label at C-24, demonstrating that introduction of the

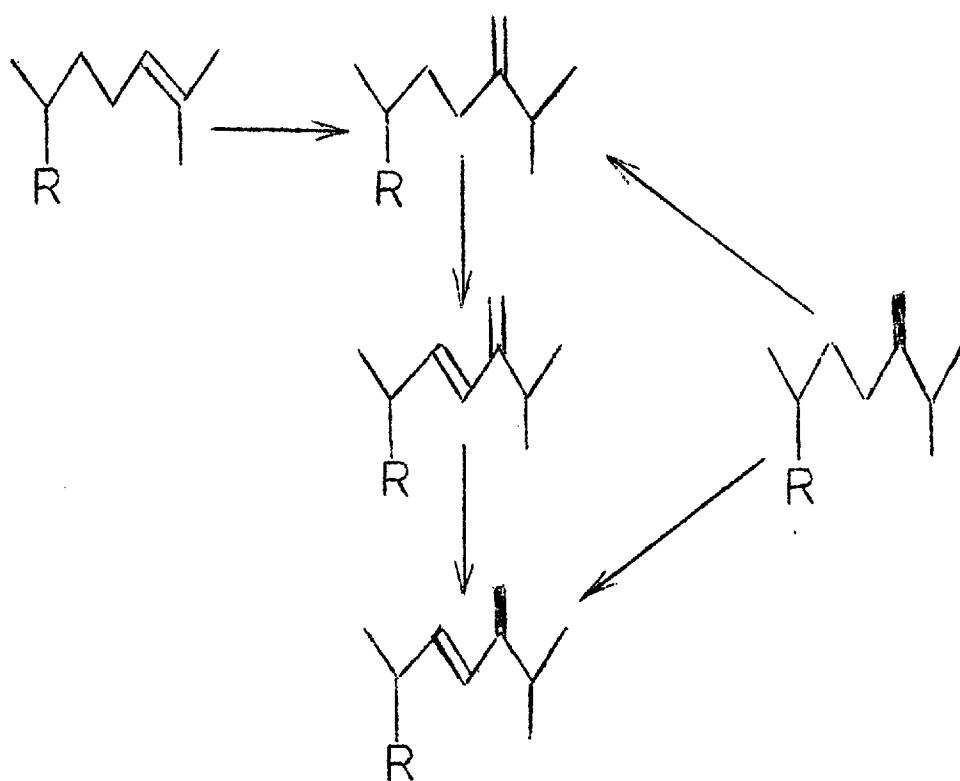
Δ^{22} double bond does not require a $\Delta^{24(28)}$ double bond.¹⁷⁵ Conversely, the loss of some label from C-24 suggests that at least some of this precursor was converted to a 24-methylene side chain before introduction of the Δ^{22} double bond. These results are consistent with the routes shown in scheme 20. The possible occurrence of 24-methyl sterols in yeast remains to be investigated, and these results probably demonstrate the non-specificity of the yeast sterol Δ^{22} dehydrogenase rather than the intermediacy of 24-methyl sterols in ergosterol biosynthesis.

Frantz and Schroepfer have listed²⁶ a useful set of criteria for the occurrence of a specific biosynthetic route. The most elementary requirements are that the proposed intermediate must be synthesised in the organism under normal conditions, and must be convertible to the end product in the organism. If both of these requirements are satisfied, it is clear that the compound is an intermediate on a biosynthetic route, however important or unimportant. To demonstrate that a compound lies on a major or unique biosynthetic route requires more conclusive evidence.

The present study has shown that lanosterol (3a) and 4 α -methylzymosterol (90a) are both converted to ergosterol and hence their known presence in yeast



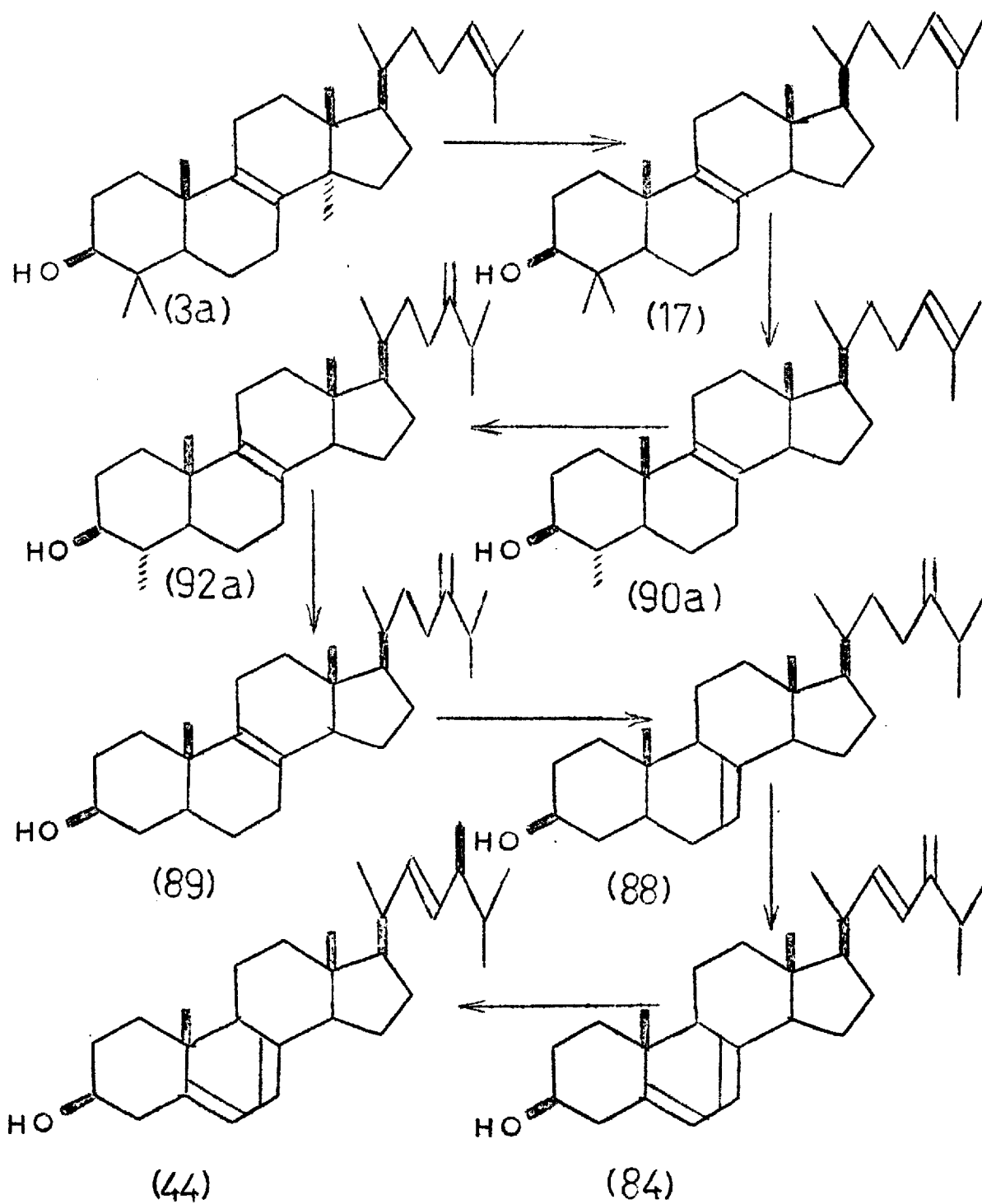
Scheme 20



implicates them as precursors of ergosterol. Furthermore the isolation of 4 α -methyl-24-methylenedihydrozymosterol (92a) and its efficient conversion to ergosterol in yeast demonstrates that this sterol lies on a biosynthetic pathway to ergosterol.

An attractive route in which all the intermediates have been isolated from yeast, is presented in scheme 21, in which side chain alkylation occurs at the 4 α -methyl sterol stage. Such a route would contradict the work of Katsuki and Bloch¹⁷⁴ who showed that zymosterol was a precursor of ergosterol, in disagreement with the earlier workers,^{161,162} and who showed that side chain alkylation occurred only at the zymosterol stage, in disagreement with the isolation of the 4 α -methyl-24-methylene sterol (92a) from yeast in the present study.

The incorporations of 24-methylenedihydrolanosterol (91a) and obtusifoliol (18) into ergosterol present further difficulties. Neither of these sterols was detected in the yeast sterol residues investigated by the author and their possible contribution to other biosynthetic routes to ergosterol cannot be assessed. 24-methylenedihydrolanosterol is however present in Aspergillus fumigatus¹⁷⁶ and Fomes officinalis¹⁶⁰ both of which produce ergosterol. The incorporation of obtusifoliol into ergosterol is analogous to the

Scheme 21

incorporation of 14 α -methyl sterols which have lost methyl groups from C-4,³⁶ into cholesterol in rat liver systems. In mammals and yeast, these sterols are probably unⁿ~~a~~tural substrates metabolised by non-specific enzymes.

In summary, no unique biosynthetic route to ergosterol can be presented at this stage. The present evidence suggests either that there are many possible biosynthetic pathways to ergosterol in yeast, or that the enzymes may accept unⁿ~~a~~tural substrates. Finally, the conflicting evidence from different laboratories suggests that ergosterol biosynthesis may vary from one strain of S.cerevisiae to another.

EXPERIMENTAL

"Petrol" refers to the fraction of petroleum ether boiling in the range 60-80°. Benzene extracts were dried over sodium sulphate and evaporated with gentle heating in vacuo. Solvent mixtures used are described in ratios of volumes.

T.l.c. refers to thin-layer chromatography performed, unless otherwise stated, on silica gel G plates. Preparative scale t.l.c. separations were performed on thin silica gel G plates with a loading of 10-25 mg. of mixture to 8 g. of silica gel. Silver nitrate-silica gel plates contained 10% silver nitrate byweight.

Unless otherwise stated, melting points were determined on a Kofler block and are uncorrected; infra-red (i.r.) spectra were run in nujol mulls; ultra-violet spectra were run in Analar ethanol; optical rotations were determined in Analar chloroform. Nuclear magnetic resonance (n.m.r.) spectra were run at 60 Mc/sec. in deuteriochloroform.

All appropriate stages of Wittig reactions were performed under dry, oxygen-free nitrogen. T.H.F. and L.A.H. refer to tetrahydrofuran and lithium aluminium hydride respectively.

Tritiated water had activity ca. $\frac{0.2}{0.02}$ curies ml.⁻¹. Radiocounting was performed in an I.D.L. type 6012 liquid scintillation head. Tritium was counted at ca. 25% efficiency and ¹⁴C at ca. 80%

efficiency, with background counts of about 0.9 and 0.5 counts sec.⁻¹ respectively. Doubly labelled steroids, containing ³H and ¹⁴C were counted at efficiencies of 20% and 20% at the first setting and 2.5% and 65% respectively at the second setting. Eburicoic acid derivatives from two of the feedings were counted in a Beckmann liquid scintillation system, by kind permission of Dr. Challis, with ³H and ¹⁴C counting efficiencies of ca. 49% and ca. 89% and backgrounds of about 0.5 and 0.2 counts sec.⁻¹ respectively.

Derivatives of biosynthesised ergosterol or eburicoic acid were crystallised repeatedly until constant activity was maintained over at least four crystallisations. Variations from the average activity for each derivative are quoted in terms of the "probable error", which is 0.675x standard deviation.¹⁷⁷

Dried baker's yeast was purchased from Heath and Heather Ltd., St. Albans, Herts., and P.sulphureus from the Centraalbureau voor Schimmelcultures, Baarn.

Isolation of 4 α -methyl sterols from yeast residues

Silica gel thin-layer chromatography of crude yeast residues* with ethyl acetate-benzene (1:3) as eluant, showed three sterol fractions, R_F 0.30, 0.42 and 0.50; the ergosterol, 4 α -methyl sterol and lanosterol fractions respectively.

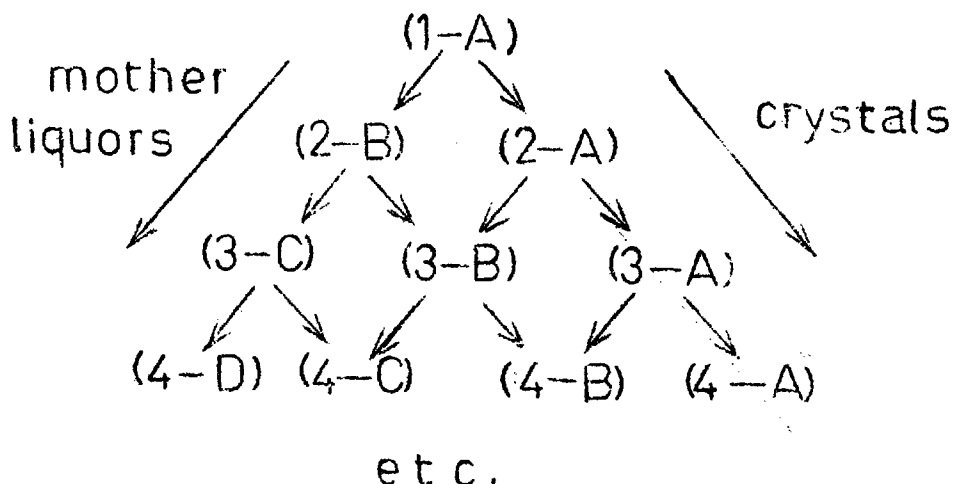
The yeast residues (75 g.) were eluted on a column of grade 5 alumina (1.75 kg.) with benzene-petrol (30:70, 3 litres) and benzene-petrol (35:65, 3 litres). Two litres of benzene-petrol (40:60) then removed the lanosterol fraction and a further one litre eluted the 4 α -methyl sterols and some of the 4-desmethyl sterols. This fraction (2.6 g.) was carefully rechromatographed on grade 5 alumina (180 g.) and the 4 α -methyl sterol fraction (600 mg.), homogeneous on silica gel thin-layer chromatography was isolated. The process was repeated from crude yeast residues until 2 g. of the 4 α -methyl sterol mixture was available.

Isolation of 4 α -methyl zymosterol (90a) from yeast residues

The 4 α -methyl sterol mixture (1.92 g.) was treated with pyridine and acetic anhydride at room temperature overnight, and the crude acetate mixture

* (kindly supplied by Koninklijke Nederlandsche Gist en Spiritus Fabriek N.V., Holland)

(1-A) fractionally crystallised from acetone-methanol by the triangulation procedure:-



Fractions 6-A (1.05 g.) and 9-B (0.38 g.) were pure 4 α -methyl zymosteryl acetate, m.p. 143-145.5° (plates from acetone), $[\alpha]_D^{25} +65.4^\circ$ (C,8), (lit.¹²¹ m.p. 138-139°, $[\alpha]_D +61^\circ$), undepressed by authentic material in mixed melting point.*

Hydrolysis of the acetate with potassium hydroxide-methanol gave 4 α -methylzymosterol m.p. 132-134° (needles from acetone-methanol), undepressed by authentic sterol*, $[\alpha]_D^{25} +63^\circ$ (C,2), (lit.¹²¹ m.p. 130-131°, $[\alpha]_D$ not quoted).

* (kindly performed by Professor G. Ourisson and Dr. G. Ponsinet).

Preparation of 3-oxo-4 α -methylcholesta-8,24-diene

4 α -methylzymosterol (108 mg.) in benzene (7 ml.) was shaken at room temperature for 0.5 hour with chromic oxide (130 mg.) in water (1.3 ml.) and acetic acid (3 ml.) The crude product was eluted with benzene on silica gel t.l.c. plates and the band with R_F 0.5 crystallised from acetone-methanol to give the required ketone (88 mg.), m.p. 105-107° (flat needles), $[\alpha]_D^{25} +50^\circ$ (C, 0.7).

Isolation of 4 α -methylergosta-8,24(28)-dien-3 β -ol (92a) from yeast residues

Fractions (5-E) to (9-E) from the fractional crystallisation of the 4 α -methyl sterol acetates (above) were combined (204 mg.) and eluted three times with benzene-petrol (1:3) on preparative silica gel-silver nitrate t.l.c. plates. Two bands, A (R_F 0.1 to 0.3) and B (R_F 0.35 to 0.5) were faintly visible in daylight (but not in U.V. light). Band A was removed with chloroform to give 3 β -acetoxy-4 α -methylergosta-8,24(28)-diene, (81 mg.), m.p. 110-112° (plates from acetone-methanol), $[\alpha]_D^{29.5} +61^\circ$ (C,1), $\nu_{\max}$ 3086 (w), 1645 (m), 898 (s) (>C=CH₂), 1744 (s), 1250 (s) (CH₃CO₂-), cm.⁻¹, $\lambda_{\max}$ only end absorption, (Found: C, 81.7; H, 11.1. C₃₁H₅₀O₂ requires C, 81.88; H, 11.08%).

Alternatively the acetate fractions rich in the 24-methylene compound were hydrolysed with methanolic potassium hydroxide and the sterol mixture treated with pyridine-benzoyl chloride overnight. The mixed benzoates (409 mg.) were eluted four times with benzene-petrol (1:4) on silica gel silver-nitrate preparative t.l.c. Two bands were visible under ultra-violet light, with R_F s 0.4 to 0.5 and 0.2 to 0.35. The latter band was extracted with chloroform, and the extracted benzoate filtered through grade 3 alumina in benzene-petrol (2:3) to give 3 β -benzoyloxy-4 α -methylergosta-8,24(28)-diene (285 mg.), m.p. 142-144° (thin rods from chloroform-methanol), $[\alpha]_D^{27} +81^\circ$ (C, 2), $\nu_{\max}$ 3100 (w), 1645 (m), 898 (s) (C=CH₂), 1715 (s), 1605 (m), 1588 (w), 1500 (w), 1275 (s) (C₆H₅CO₂-) cm.⁻¹ $\lambda_{\max}$ 228 (ϵ 14,000), 273 (ϵ 1,000), 281 (ϵ 700) m μ in chloroform-ethanol (1:10). (Found: C, 83.3; H, 10.1. C₃₆H₅₂O₂ requires C, 83.66 H, 10.14%).

Hydrolysis of the acetate or benzoate with 2% methanolic potassium hydroxide gave 4 α -methylergosta-8,24(28)-dien-3 β -ol, m.p. 143-145° (needles from acetone-methanol), $[\alpha]_D^{28} +55^\circ$ (C, 3), $\nu_{\max}$ 3400, 1647, 888 cm.⁻¹, $\lambda_{\max}$ only end absorption. (Found: C, 84.5; H, 11.8. C₂₉H₄₈O requires C, 84.40; H, 11.72%).

Preparation of 3-oxo-4 α -methylergosta-8,24(28)-diene (94)

The sterol (100 mg.) was oxidised with chromic oxide, benzene and aqueous acetic acid as before. Purification on silica gel t.l.c. plates gave 3-oxo-4 α -methylergosta-8,24(28)-diene (70 mg.), m.p. 123-125° (needles from acetone-methanol), $[\alpha]_D^{26} +47^\circ$ (C, 0.9), $\nu_{\max}$ 1710, 1644, 890 cm^{-1} (CCl_4), $\lambda_{\max}$ only end absorption, o.r.d.* in dioxan (C, 0.01), $[\phi]_{227} -4650$, $[\phi]_{232} -5900$, $[\phi]_{250} -1860$, $[\phi]_{272} -1860$, $[\phi]_{306} +2480$, $[\phi]_{400} +775^\circ$. (Found: C, 84.7; H, 11.25. $\text{C}_{29}\text{H}_{46}\text{O}$ requires C, 84.81; H, 11.29%).

Isolation of 4 α -methylcholesta-8(14), 24-dien-3 β -ol (93a) from yeast residues

As described above, preparative t.l.c. on silica gel impregnated with silver nitrate separated fractions (5-E) to (9-E), from the 4 α -methyl sterol acetates, into two bands, A and B. Band A was homogeneous and afforded the acetate of the new methylergostadienol.

Band B had partially separated into two zones B₁ and B₂. B₁, when eluted three times on silica gel silver-nitrate t.l.c. with benzene-petrol (2:3),

* (kindly determined by Dr. P.M. Scopes, Westfield College, London).

had R_F 0.44 and was inseparable from 4 α -methyl-zymosteryl acetate. B_2 under these conditions had R_F 0.50.

Repeated t.l.c. purification of B_2 as above gave 3 β -acetoxy-4 α -methylcholesta-8(14),24-diene, (20 mg.), m.p. 78-80°, $[\alpha]_D^{25} +42^\circ$ (C, 0.2), $\nu_{\max}$ 1742, 1245 cm^{-1} (Found: C, 81.6; H, 11.2. $C_{30}H_{48}O_2$ requires C, 81.76; H, 10.98%).

Hydrolysis of the acetate with 2% methanolic potassium hydroxide gave 4 α -methylcholesta-8(14),24-dien-3 β -ol, m.p. 131.5-136°, $[\alpha]_D + 19$ (C, 0.5), $\nu_{\max}$ 3300 cm^{-1} (Found: C, 84.2; H, 11.9. $C_{28}H_{46}O$ requires C, 84.35; H, 11.63%).

Attempted preparation of 3 β -acetoxy-4 α -methyl-24,25-epoxycholest-8-ene (101)

4 α -methylzymosteryl acetate (100 mg.) was treated in chloroform (10 ml.) with m-chloroperbenzoic acid (51.7 mg., 80% pure) in chloroform (10 ml.) at 0°. Reaction was essentially instantaneous. The solvent was removed in vacuo and the product treated with dilute aqueous sodium bicarbonate and extracted with benzene. The benzene extract was washed with water until neutral. Thin-layer chromatography (acetone-petrol, 1:25), showed spots with R_Fs 0.63 (starting material), 0.58 (strongly fluorescent under U.V. light), 0.34, 0.25 (strongly fluorescent in U.V. light), 0.1 and 0.0. Preparative t.l.c. allowed removal of the mono-epoxide fraction with R_F 0.34 (30 mg.) m.p. ca. 140° (wide range), whose n.m.r. spectrum showed a mixture which was not further investigated.

Preparation of 3 β -acetoxy-24 ξ -bromo-25-hydroxy-4 α -methylcholest-8-ene (99a)

4 α -methylzymosteryl acetate (820 mg.) was dissolved in glyme (360 ml.) and water (60 ml.). Recrystallised N-bromosuccinimide (362 mg.) was added in portions to the vigorously stirred solution over 45 min. at room temperature. The solution was stirred for a further 15 min., evaporated to a small volume in vacuo and water added. The mixture

was extracted with ether and the extract washed with sodium sulphite solution followed by water, and evaporated to a solid. The product was crystallised from ethanol-water to give the crude bromohydrin (95%) m.p. 138-143° (needles from ethanol-water).

A small sample of bromohydrin was eluted on silica gel t.l.c. plates with acetone-petrol (1:9). The band with R_F 0.5 was repeatedly crystallised from acetone to give 3 β -acetoxy-24 ξ -bromo-25-hydroxy-4 α -methylcholest-8-ene, m.p. 146-149° (needles from acetone) unchanged on further crystallisation, $[\alpha]_D^{23} +54^\circ$ (C, 1.9), $\nu_{\max}$ 3590(m)(OH) 1735(s), 1250(s) (CH₃CO₂-), 1665(w) ($>C=C<$) cm.⁻¹ (in CCl₄). (Found: C, 66.7; H, 9.3; Br, 14.7. C₃₀H₄₉BrO₃ requires C, 67.02; H, 9.19; Br, 14.87%).

Preparation of 3 β -acetoxy-24 ξ ,25-epoxy-4 α -methylcholest-8-ene (101)

The crude 4 α -methylzymosteryl acetate bromohydrin (767 mg.) was eluted on a column of grade 3 alumina (50 g.) successively with petrol-benzene (90:10, 200 ml.), (80:20, 200 ml.) and (70:30, 500 ml.), which removed traces of 4 α -methylzymosteryl acetate. Petrol-benzene (60:40, 3 litres) eluted the required mixed epoxides (86%), m.p. 123-131°.

Repeated crystallisation of the mixed epoxides from acetone gave 3 β -acetoxy-24 ξ ,25-epoxy-4 α -methylcholest-8-ene, m.p. 160-163° (plates from acetone,

unchanged on further crystallisation), $[\alpha]_D^{24} + 55^\circ$
 (C, 0.9). (Found: C, 78.9; H, 10.6.
 $C_{30}H_{48}O_3$ requires C, 78.89; H, 10.59%).

Preparation of 3 β -acetoxy-4 α -methyl-24-oxocholest-8-ene (102a)

4 α -methylzymosteryl acetate oxide (137 mg.) in benzene (3.5 ml.) was treated with boron trifluoride etherate in benzene (0.7 ml. of 1:20) for 30 seconds at room temperature. The reaction was quenched by shaking with aqueous sodium bicarbonate solution. The benzene phase was washed with water and solvent removed to leave an oil. Thin-layer chromatography on silica gel, eluted three times with benzene, showed five components with R_F s: 0.45 (A), 0.35 (B), 0.30-traces (C), 0.20 (D) and 0.15 (E). The oil was separated into its components by preparative silica gel t.l.c., eluting three times with benzene:-

The band with R_F 0.5 to 0.6 provided a crystalline substance A, (23 mg.), $\nu_{\max}$ 2690(m), 1735(s), 1245(s) cm^{-1} (in CCl_4), τ 0.57(1H), 5.62 (1H-multiplet), 7.97 (CH_3CO_2^-), 8.96 (2 CH_3), 9.02 (CH_3), 9.16 (doublet, τ 6 c/s, CH_3), 9.40 (CH_3).

The band with R_F 0.2 to 0.3 provided a substance D (20 mg.), $\nu_{\max}$ 3650(m), 1730(s), 1250(s), 680(s) cm^{-1} (in CCl_4), τ 5.6 (1H multiplet), 6.55 (1H multiplet), 7.95 (CH_3CO_2^-), 8.49 (CH_3), 8.86 (CH_3), 9.02 (CH_3),

9.14 (doublet, $\int 6 \text{ c/s}$, CH_3), 9.38 (CH_3).

The band with R_F 0.05 to 0.2 gave a substance E (47 mg.) ν_{max} 3600(m), 1730(s), 1250(s) cm^{-1} (in CCl_4), which was not further investigated.

Finally the band with R_F 0.3 to 0.5 provided the required ketone (B) and the trace component (C). This mixture (23 mg.) was eluted ten times with benzene and petrol (1:1) on silica gel t.l.c. plates. The band with R_F 0.3 to 0.5 provided pure 3β -acetoxy- 4α -methyl-24-oxocholest-8-ene, m.p. 102.5-104.5° (needles from acetone-methanol, $[\alpha]_{\text{D}}^{25} + 61^\circ$ (C,1), ν_{max} 1735, 1245 (CH_3CO_2^-), 1708 ($>\text{C}=\text{O}$) cm^{-1} (in CCl_4). (Found: C, 78.9; H, 10.4. $\text{C}_{30}\text{H}_{48}\text{O}_3$ requires C, 78.89; H, 10.59%).

Preparation of 3β -acetoxy- 4α -methylergosta-8,24(28)-diene (92b)

Methyl triphenyl phosphonium bromide¹⁷⁸ (210 mg.) was stirred in ether (2 ml.) with ethereal phenyl lithium¹⁷⁹ (0.4 ml. of 1.57M) for 2 hours.

3β -acetoxy- 4α -methyl-24-oxocholest-8-ene (15 mg.) was added in ether (4 ml.) and stirred for 20 hours. The ether was replaced by tetrahydrofuran freshly distilled over lithium aluminium hydride and the solution refluxed for 20 hours. All operations were carried out in an inert atmosphere.

The solution was treated with water, extracted with ether, and the product reacylated with pyridine-acetic anhydride (1:1). The crude product was eluted twice with benzene-petrol (1:1) on silica gel t.l.c. plates. The band with R_F 0.37 to 0.46 furnished 3 β -acetoxy-4 α -methylergosta-8,24(28)-diene (10 mg.) m.p. 110-112° (plates from acetone-methanol) undepressed in mixed melting point^{*} by the acetate of the natural product, $[\alpha]_D^{23} +61^\circ$ (c, 0.5), infra-red, n.m.r. and mass spectra superposable on those of the acetate of the natural product. (Found: C, 81.65; H, 10.8. $C_{31}H_{50}O_2$ requires C, 81.88; H, 11.08%).

^{*} (kindly performed by Dr. P.G. Sammes).

Attempted conversion of lanosteryl acetate dibromide to 3 β -acetoxy-24-oxolanost-8-ene (105)

The dibromide (510 mg.) and lead monoxide (500 mg.) were refluxed for 20 hours in dioxan and water (1:1), (50 ml.). The cooled mixture was filtered, the solid and filtrate extracted with benzene, and the crude extract chromatographed on grade 3 alumina (20 g.). Elution with benzene-petrol (20:80, 300 ml.) gave unreacted dibromide (210 mg.); benzene-petrol (30:70, 180 ml.) gave lanosteryl acetate oxide (80 mg.) m.p. 181° (from acetone-methanol, lit. mps. 178°, 149 185°, 150) undepressed by authentic material; benzene-petrol (70:30) eluted a substance (130 mg.), $\nu_{\max}$ 1735 cm.⁻¹ (acetate), but no $\nu_{\max}$ ca. 1715 cm.⁻¹, which was not further investigated.

Preparation of lanosteryl acetate

Lanosteryl acetate dibromide (6.13 g.) and sodium iodide (16 g.) were refluxed for 4 hours in acetone (250 ml.). Solvent was removed in vacuo, the residue treated with water, and the benzene extract washed with 20% aqueous sodium thiosulphate, followed by water. The product was crystallised from acetone-methanol to yield lanosteryl acetate (90%), m.p. 129-132° (needles, lit.¹⁵⁷ m.p. 129-130°).

Preparation of 3~~8~~-acetoxy-24,25-epoxylanost-8-ene (104)

Monoperphthalic acid¹⁸⁰ in chloroform (46 ml. of 0.18M) was added dropwise during 0.5 hour to a stirred solution of lanosteryl acetate (3.47 g.) in chloroform (5 ml.) at 0°. After 18 hours solvent was removed and the residue suspended in benzene and washed with aqueous sodium carbonate followed by water. The crude product was chromatographed on grade 3 alumina (220 g.). Elution with benzene-petrol (20:80) removed unreacted lanosteryl acetate while benzene-petrol (30:70) removed the required epoxide (70%), m.p. 170-182° (microcrystalline) from acetone-methanol, rising to 194° (plates) on repeated crystallisation, (lit.¹⁵⁰ m.p. 185°).

Resolution of the two epimeric epoxides (104)

On one occasion ~~chromatography~~ of the crude epoxide from lanosteryl acetate (840 mg.) as described above, gave two maxima in the wt./ml. of epoxide eluted. The faster running epoxide (210 mg.) gave needles from acetone with m.p. 138-141° resolidifying immediately in needles, m.p. 144-146°, $[\alpha]_D^{25} + 53^\circ$ (C, 3). The more slowly eluted epoxide (270 mg.) gave plates from acetone, m.p. 196-198°, $[\alpha]_D^{25} + 60^\circ$ (C, 3). The two epoxides were not separated by t.l.c. and their n.m.r. and i.r. (carbon tetrachloride) spectra were almost identical.

Preparation of 3 β -acetoxy-24-oxolanost-8-ene (105)

Lanosteryl acetate monoepoxide (3.83 g.) in dry benzene (250 ml.) was treated with boron trifluoride etherate (5 ml.) for five minutes at room temperature. The solution was washed with aqueous sodium carbonate followed by water. The product consisted of the required ketone and an isomeric aldehyde, (0.61 τ , sharp singlet (CHO); t.l.c. with benzene eluant, R_F 0.2 ketone, R_F 0.25 aldehyde). The benzene extract was shaken with chromic oxide (4 g.) in water (20 ml.) and acetic acid (50 ml.) for three days at room temperature. The benzene phase was washed with water, thoroughly extracted with strong aqueous sodium carbonate and again washed with water. Removal of solvent gave the ketone (2.62 g.). Trace impurities were removed by chromatography on grade 5 alumina (90 g.) commencing with petrol, and benzene-petrol (15:85) eluted 3 β -acetoxy-24-oxo-lanost-8-ene, m.p. 135-137°, (plates from acetone-methanol); $[\alpha]_D^{23} + 58^\circ$ (C, 3); $\nu_{\max}$ 1732(s), 1250(s) (CH₃CO₂-), 1712 (>C=O) cm.⁻¹; $\lambda_{\max}$ only end absorption. (Found: C, 79.3; H, 10.6. C₃₂H₅₂O₃ requires C, 79.28; H, 10.81%).

A by-product (106c) from the boron trifluoride rearrangement of lanosteryl acetate oxide

The aqueous sodium carbonate extract from the oxidation of the crude BF_3 rearrangement products (see above) was neutralised and extracted with benzene. The extract yielded a gel (1.21 g.). Treatment with diazomethane in ether at room temperature gave methyl-3 β -acetoxy-24,24-dimethyl-26,27-bisnorlanost-8-en-25-oate, m.p. 155-158° (needles from acetone-methanol), $[\alpha]_D^{26} +51^\circ$, ν_{max} 1735(s), 1245(s) cm^{-1} . (Found: C, 76.8; H, 10.4. $\text{C}_{33}\text{H}_{54}\text{O}_4$ requires C, 76.99; H, 10.57%).

Preparation of 3 β -acetoxy-24-methylenelanost-8-ene (91b)

Methyl triphenyl phosphonium bromide (3.51 g.) was shaken for 2 hours with phenyl lithium in ether (8 ml. of 1.4M solution) under dry nitrogen. The solution was filtered into a solution of 24-oxolanostenyl acetate (402 mg.) in dry ether (80 ml.) and the mixture stirred overnight. Ether was replaced by dry tetrahydrofuran (freshly distilled over lithium aluminium hydride) and the solution refluxed overnight. Most of the THF. was removed in vacuo, water (100 ml.) added and the mixture extracted with ether. The extract was washed rapidly with 1N sulphuric acid, followed by water. The crude product was reacetylated with pyridine-acetic

anhydride at 100° for 2 hours, and chromatographed on a thick (2 mm.) silica gel t.l.c. plate eluted with benzene. The band with R_F 0.5 was removed with acetone to yield 3β -acetoxy-24-methylenelanost-8-ene (75%), m.p. $147-150^{\circ}$ (plates from acetone-methanol, lit.¹⁵² m.p. $138-139^{\circ}$), $\nu_{\max}$ 3100 (v.w.), 1645(m), 890(s) ($>C=CH_2$), 1735(s), 1245(s) (CH_3CO_2-) cm^{-1}
 $\lambda_{\max}$ only end absorption. (Found: C, 82.4; H, 11.1. Calc. for $C_{33}H_{54}O_2$: C, 82.09; H, 11.27%).

Preparation of 24-methylenedihydrolanosterol (91a)

The acetate (260 mg.) was refluxed for 2 hours with 2% methanolic potassium hydroxide (20 ml.) then treated with water and extracted with benzene to give 24-methylenedihydrolanosterol (90%)
 m.p. $157.5-159^{\circ}$ (needles from acetone-methanol), $[\alpha]_D^{25} +59^{\circ}$ (c, 2), (lit.¹⁵² m.p. $158-159^{\circ}$, $[\alpha]_D^{17} +66^{\circ}$),
 $\nu_{\max}$ 3300(s), 1645(m), 894(s).

Preparation of $[28-^3H]$ -24-methylenedihydrolanosterol

Methyl triphenyl phosphonium bromide (205 mg.) was dissolved in tritiated water (0.6 ml.) in the presence of potassium t-butoxide (14 mg.). The solution was left at room temperature overnight and the solvent removed in vacuo. The residue was crystallised from tritiated water (0.1 ml.) and dried for two days over phosphorous pentoxide in vacuo to give $[CH_3-^3H]$ -methyl triphenyl phosphonium

bromide (78 mg.). The tritiated bromide was finely powdered and shaken with phenyl lithium in ether (0.18 ml. of 1.34M.) for one hour. The phosphorane solution was transferred to a Carius tube and 24-oxolanosteryl acetate (59 mg.) added in ether (4 ml.) at 0°. The tube was sealed under nitrogen and maintained at 64° for 24 hours. After the usual work-up and reacetylation the product was chromatographed on thin silica gel plates to give [28-³H]-24-methylenedihydrolanosteryl acetate (20 mg.). Hydrolysis gave the tritiated sterol (18.5 mg.), activity 1.11×10^5 d.sec.⁻¹mg.⁻¹

Preparation of [CH₃-¹⁴C]-methyl triphenyl phosphonium iodide

[¹⁴C]-methyl iodide (ca. 1 mg., total activity 0.5 mC.) was distilled under vacuum into triphenylphosphine (44 mg.) in dry benzene (0.3 ml.) in a Carius tube cooled in liquid nitrogen. The Carius tube was isolated and allowed to stand at room temperature for 2 hours. Inactive methyl iodide (35 mg.) was distilled into the [¹⁴C]-methyl iodide container and thence into the Carius tube as before. The tube was sealed in vacuo and allowed to warm to room temperature. After 24 hours the precipitate was filtered and washed with benzene to leave [CH₃-¹⁴C]-methyl triphenyl phosphonium iodide (52 mg.) m.p. 186° (lit.¹⁸¹ 180°) of activity ca.

1.2×10^5 d.sec. $^{-1}$ mg. $^{-1}$

Preparation of [28- ^{14}C]-24-methylenedihydrolanosterol

[Me- ^{14}C]-methyl triphenyl phosphonium iodide (21.5 mg.) was stirred for 2 hours, under nitrogen, with phenyl lithium (0.04 ml. of 1.33N) in ether (4 ml.). 24-oxo-lanosteryl acetate (69.5 mg.) in dry ether (4 ml.) was added dropwise and the mixture stirred overnight. Ether was then replaced by dry THF and the solution refluxed overnight. The usual work-up gave [28- ^{14}C]-24-methylenedihydrolanosterol (6 mg.) of activity 1.5×10^5 d.sec. $^{-1}$ mg. $^{-1}$

Radiopurity and ozonolysis of [28- ^{14}C , ^3H]-24-methylenedihydrolanosterol

^{14}C and ^3H labelled sterols were cocrystallised. A sample of the ^{14}C , ^3H labelled sterol was diluted 100-fold with inactive 24-methylene-dihydrolanosterol and crystallised 6 times to demonstrate the radiopurity.

The doubly labelled sterol (42 mg.) was suspended in glacial acetic acid (2 ml.) and ozonised oxygen bubbled through the liquid until a clear solution resulted. Zinc dust (2 g.) was added and the solution allowed to stand for 30 min. Water (2 ml.) was added and the material steam-distilled. 15 ml. of distillate was collected.

The distillate was neutralised with respect to phenolphthalein with conc. sodium hydroxide solution and the solution rendered just colourless by addition of dilute acetic acid solution. A 10% solution of dimedone in ethanol (1 ml.) was added and after 12 hours the precipitate of the dimedone derivative was collected and recrystallised from ethanol-water (yield 4 mg.).

Ratio $^3\text{H}/^{14}\text{C}$

Sterol 15.6 ± 0.4

H_2CO -dimedone 14.6 ± 0.1

Thus, if all the ^{14}C was at C-28 in the sterol,

$\frac{14.6}{15.6} \times 100\% = 94\%$ of the ^3H was at C-28. An earlier

preparation of [$^{14}\text{C}, ^3\text{H}$]-24-methylenedihydrolanosterol and used for one of the feedings described below gave the following activities:-

	<u>d.sec.⁻¹mg.⁻¹</u>		<u>d.sec.⁻¹mm.⁻¹</u>		
	<u>$^{14}\text{C} \times 10^{-3}$</u>	<u>$^3\text{H} \times 10^{-5}$</u>	<u>$^{14}\text{C} \times 10^{-6}$</u>	<u>$^3\text{H} \times 10^{-7}$</u>	<u>$^3\text{H}/^{14}\text{C}$</u>
Sterol	8.13 ± 0.12	1.85 ± 0.03	3.63	8.14	22.5 ± 0.6
H_2CO - dimedone	1.27 ± 0.05	1.96 ± 0.08	3.72	5.73	16.8 ± 0.3

Thus only $\frac{16.8}{22.5} \times 100\% = 75\%$ of the ^3H label was at C-28. This result is discussed fully in the text.

Attempted DMSO oxidation¹⁵⁴ of ergosterol

Dimethyl sulphoxide was dried by stirring over calcium hydride at 50° for four hours, and then distilled at 70° at 9.5 mm. of mercury pressure.

Ergosterol (1.16 g.), dicyclohexylcarbodiimide (2.85 g.) and pyridinium trifluoroacetate (0.39 g.) were dissolved in benzene (10 ml.) and dimethyl sulphoxide (10 ml.), and maintained at 55° for one hour. The mixture was poured into ethyl acetate (40 ml.), allowed to stand for 10 minutes and filtered. The filtrate was extracted with pH 6.5 phosphate buffer and water. The organic layer was dried over magnesium sulphate and solvent removed to leave an oil. Silica gel thin-layer chromatography eluted with benzene showed a number of components with R_F less than 0.2, a weak spot R_F 0.45 and a hydrocarbon fraction R_F 0.7. The crude mixture was filtered through grade 5 alumina in benzene to provide the hydrocarbon fraction (20% yield). Silica gel t.l.c. eluted with petrol showed the major component, R_F 0.5, and a minor impurity, R_F 0.46. The mixture was crystallised repeatedly from acetone to provide the major component, 3,5-cycloergosta-6,8(14),22-triene, (165 mg.) m.p. 99-102° (needles from acetone), (lit.¹⁵⁵ m.p. 102-103°) undepressed in mixed melting point with the authentic material; i.r. spectrum

identical to that of authentic material.

Preparation of 4,4-dimethylergosta-5,7,22-trien-3-one

Ergosterone, m.p. 128-132° (plates from acetone), $\lambda_{\max}$ 239 m μ (ϵ , 14,300), (lit. m.p. 132°, $\lambda_{\max}$ 242 m μ (ϵ , 14,800)) was prepared from ergosterol by Oppenauer oxidation as described by Shepherd et al.¹⁸²

Treatment of ergosterone with potassium t-butoxide and methyl iodide in t-butanol according to Cooley et al.¹⁵⁶ gave 4,4-dimethylergosta-5,7,22-trien-3-one, m.p. 161-165° (from acetone), (lit.¹⁵⁶ 167-168°).

Preparation of Obtusifolione

Obtusifoliol* (87 mg.) in benzene (5 ml.) was shaken with chromic oxide (130 mg.) in water (1 ml.) and acetic acid (2.5 ml.) for 0.75 hour. The usual work-up and silica gel t.l.c. purification furnished 3-oxo-4 α ,14 α -dimethylergosta-8,24(28)-diene (54 mg.) m.p. 109-111°, $[\alpha]_D^{26}$ +67°, $\nu_{\max}$ 1710, 1645, 890 cm.⁻¹ (nujol and CCl₄), $\lambda_{\max}$ only end absorption, M.Wt. 424 (mass spec.).

*(Obtusifoliol was a kind gift from Professor González González).

Preparation of [2-³H]-lanosterol

Lanosterol (1.5 g.) in benzene (100 ml.) was shaken for 5 hours with chromic oxide (2 g.) in water (20 ml.) and acetic acid (50 ml.). The benzene layer was washed with aqueous sodium carbonate and water. Removal of solvent and crystallisation from acetone-methanol gave lanostadienone (1.25 g.) m.p. 80° (needles, lit.¹⁵⁷ 80-81°).

Tritiated water (0.5 ml.) and potassium t-butoxide (3.47 g.) were gently heated in a sealed distillation apparatus. The distillate was collected in a U-tube cooled in cardice, and redistilled into a second U-tube to give [OH-³H]-t-butyl alcohol.

Tritiated t-butyl alcohol (1 ml.), potassium t-butoxide (22 mg.) and lanostadienone (142 mg.) were sealed under nitrogen in a Carius tube and maintained at 100° for 2 days. The cooled mixture was poured into ice-cold 1N hydrochloric acid (2 ml.), extracted with benzene, and washed with water. Removal of solvent and crystallisation from acetone-methanol gave [2-³H]-lanostadienone (97 mg.), activity ca. 3×10^5 d.sec.⁻¹mg.⁻¹

The ketone (95 mg.) in dry ether (40 ml.) was added to LAH. (283 mg.) in ether (10 ml.) and refluxed for 2 hours. After addition of water the ethereal layer was washed rapidly with dilute hydrochloric acid, then water, and the solvent removed. The residue was eluted on silica t.l.c. plates with ethyl acetate-benzene (1:9). The band with R_F 0.45 was removed giving $[2-^3H]$ -lanosterol, m.p. 136° (lit.¹⁵⁷ m.p. 140°), undepressed by authentic lanosterol; activity 2.69×10^5 d.sec.⁻¹mg.⁻¹. The radiopurity was demonstrated by co-crystallisation with inactive material.

Preparation of some further labelled precursors

The following labelled sterols were prepared from the corresponding ketones exactly as described for $[2-^3H]$ -lanosterol:-

	<u>d.sec.⁻¹ mg.⁻¹</u>	<u>m.p.</u>	<u>Lit.m.p.</u>
$[2-^3H]$ -4,4-dimethyl-ergosterol	2.60×10^5	181°	181° 156
$[2,4-^3H]$ -obtusifoliol	3.56×10^5	$138-141^\circ$	$138-140^\circ$ 183
$[2,4-^3H]$ -4 α -methyl-zymosterol	4.41×10^5	$130-132^\circ$	$130-131^\circ$ 121
$[2,4-^3H]$ -4 α -methyl-ergosta-8,24(28)-dien-3 β -ol	3.24×10^5	$142.5-144.5^\circ$	

Feeding of [28-³H]-24-methylenedihydrolanosterol to Yeast in vivo

Anaerobic growth

"Oxoid" malt extract (60 g.) was shaken with boiling water (2 litre) and the cooled solution filtered through celite. The filtrate was divided between two one-litre conical flasks which were plugged with cotton wool. Both flasks were sterilised at 100° for 15 min., allowed to stand at room temperature for 16 hours, and resterilised. The cooled flasks were each inoculated with dried baker's yeast (100 mg.) and tightly replugged with cotton wool such that only a small air gap remained in the flasks. The cultures were incubated at 30° for 40 hours, without shaking. Yeast was harvested by centrifugation at 800 r.p.m. for 30 min. The precipitate was washed twice by centrifugation with pH 6.5, 0.1M phosphate buffer.

Aerobic growth

The yeast (16 ml.) was resuspended in a solution of glucose (10 g.) and potassium dihydrogen phosphate (13.6 g.) in water (1 litre). [28-³H]-24-methylenedihydrolanosterol (2.67 mg.) and Tween 80 (60 mg.) in acetone (2 ml.) was divided between three one-litre flasks and solvent removed under a stream of nitrogen. The yeast suspension was divided equally between the three flasks, which were loosely

plugged with cotton wool, and vigorously shaken at 30° for 16 hours in air. The yeast (22 ml.) was harvested and washed as before, the washings being added to the supernatant which was extracted with benzene to leave an oil (55 mg.). The oil was chromatographed on thin silica gel plates, with ethyl acetate-benzene (1:9) eluant, and the precursor, with R_F 0.4, removed and counted.

The yeast was refluxed with 10% methanolic potassium hydroxide (300 ml.) for four hours. Water (300 ml.) was added and the non-saponifiable material extracted with benzene. The extract was diluted with inactive precursor and eluted twice with ethyl acetate-benzene (1:9) on thin silica gel plates. Ergosterol (R_F 0.5) and the precursor (R_F 0.7) were removed. The ergosterol band was diluted with inactive precursor and rechromatographed, when the ergosterol retained all of its activity. The biosynthesised ergosterol (128 mg.) was crystallised from acetone-methanol to constant specific activity. Mother liquors from these crystallisations were acetylated with acetic anhydride-pyridine and the ergosteryl acetate crystallised from acetone to constant specific activity. Finally, ozonolysis of the biosynthesised ergosterol, according to Hannahan and Wakil,⁶⁶ gave 2,3-dimethylbutanal which was counted as

its dimedone derivative, m.p. 158-159°, from
ethanol-water.

Summary:-Aerobic incubation

Volume of medium = 1000 ml.

Volume of yeast before = 16 ml.

Volume of yeast after = 22 ml.

Precursor

Fed to yeast 2.67 mg. = 7.64×10^5 d.sec.⁻¹

Recovered from medium = 1.06×10^5 d.sec.⁻¹ = 14%

Recovered from cells = 0.89×10^5 d.sec.⁻¹ = 12%

Total recovery of precursor = 26%

Activity recovered not associated with precursor

or ergosterol = 2.8×10^3 d.sec.⁻¹ or 0.37%

Activity of ergosterol isolated

Total weight = 128 mg.

	<u>d.sec.⁻¹mg.⁻¹</u>	<u>d.sec.⁻¹mm.⁻¹</u>	<u>Molar ratio</u>
Ergosterol	68.8 ± 1.6	2.72×10^4	1.00
Ergosteryl acetate	64.0 ± 0.6	2.82×10^4	1.04
Dimedone of 2,3-dimethyl- butanal	83.1 ± 1.0	3.07×10^4	1.11

$$\text{Incorporation into ergosterol} = \frac{128 \times 68.8}{7.64 \times 10^5} \times 100\%$$

$$= 1.15\%$$

or allowing for 26% recovered precursor,

$$\text{incorporation} = 1.5\%$$

Feeding of [28-¹⁴C; (23,25)28-³H]-24-methylene-
dihydrolanosterol to yeast in vivo

Aerobic incubation

Volume of medium 700 ml.

Volume of anaerobically grown yeast 11 ml.

Precursor fed 6.09 mg

$$= 1.13 \times 10^6 \text{ d.sec.}^{-1} \text{ of } ^3\text{H}$$

Ergosterol isolated

Total ergosterol = 112 mg.

Diluted with inactive ergosterol to 302 mg.

	$\frac{^3\text{H d.sec.}^{-1}\text{mg.}^{-1}}$	$\frac{^{14}\text{C d.sec.}^{-1}\text{mg.}^{-1}}$	$\frac{^3\text{H}/^{14}\text{C}}$
Precursor	1.85×10^5	8.13×10^3	22.5 ± 0.6
H ₂ CO dimedone (C-28) from precursor	1.96×10^5	1.27×10^3	16.8 ± 0.3
Ergosterol	23.1 ± 1.2	1.02 ± 0.06	22.6 ± 1.2
Ergosteryl acetate	19.2 ± 0.6	0.81 ± 0.05	23.7 ± 1.0

	$\frac{^3\text{H d.sec.}^{-1}\text{mm.}^{-1}}$	$\frac{^{14}\text{C d.sec.}^{-1}\text{mm.}^{-1}}$
Ergosterol	9.15×10^3	4.04×10^2
Ergosteryl acetate	8.41×10^3	3.55×10^2

Incorporation based on ³H

$$= \frac{302 \times 23.1 \times 100}{1.13 \times 10^6} \%$$

$$= 0.62\%$$

Feeding of $[2-^3\text{H}]-4,4\text{-dimethylergosterol}$ to yeast in vivo

Aerobic incubation

Carried out under oxygen for 14 hours at 30° with shaking.

Volume of anaerobically grown yeast = 9 ml.

Volume of yeast after aerobic growth = 10 ml.

Medium - 500 ml. in 3 one-litre flasks

Precursor fed 2.34 mg.

$$= 5.98 \times 10^5 \text{ d.sec.}^{-1}$$

Recovered from medium $2.37 \times 10^5 \text{ d.sec.}^{-1} = 39.6\%$

Recovered from cells $0.26 \times 10^5 \text{ d.sec.}^{-1} = 4.3\%$

Total recovered precursor $2.63 \times 10^5 \text{ d.sec.}^{-1} = 43.9\%$

Ergosterol isolated

Total isolated = 91 mg.

Activity = $0.17 \text{ d.sec.}^{-1} \text{mg.}^{-1}$ after five crystallisations

Incorporation $\leq 0.003\%$

(or allowing for 44% recovered precursor, incorporation $\leq 0.005\%$).

Feeding of [2-³H]-lanosterol to yeast in vivoAerobic incubation

Carried out in oxygen atmosphere in parallel with the feeding of 4,4-dimethylergosterol.

Volume of anaerobically grown yeast = 9 ml.

Volume of yeast after aerobic growth = 10.5 ml.

Medium - 500 ml. in 3 one-litre flasks.

Precursor fed 2.34 mg.

$$= 6.04 \times 10^5 \text{ d.sec.}^{-1}$$

Recovered from medium $2.24 \times 10^5 \text{ d.sec.}^{-1} = 37.1\%$

Recovered from cells $0.07 \times 10^5 \text{ d.sec.}^{-1} = 1.2\%$

Total precursor recovered $2.31 \times 10^5 \text{ d.sec.}^{-1} = 38.3\%$

Ergosterol isolated

Total isolated = 90 mg.

	<u>d.sec.⁻¹mg.⁻¹</u>	<u>d.sec.⁻¹mm.⁻¹</u>	<u>Molar ratio</u>
Ergosterol	29.4 ± 1.6	1.16 × 10 ⁴	1.00
ergosteryl acetate	24.1 ± 0.4	1.06 × 10 ⁴	0.92

$$\text{Incorporation} = \frac{90 \times 29.4 \times 100}{6.04 \times 10^5} \%$$

$$= 0.44\%$$

(or allowing for 38% recovery of precursor,
incorporation = 0.71%).

Feeding of [2,4-³H]-obtusifoliol to yeast in vivo

Aerobic incubation

Carried out in oxygen atmosphere in parallel with the other 4 α -methyl sterol feedings.

Volume of medium was 500 ml., in 3 one-litre flasks

Volume of yeast after anaerobic growth = 4 ml.

Volume of yeast after aerobic growth = 5 ml.

Precursor fed 2.39 mg.

$$= 8.51 \times 10^5 \text{ d.sec.}^{-1}$$

Recovered from medium $4.60 \times 10^5 \text{ d.sec.}^{-1} = 54.1\%$

Recovered from cells $1.2 \times 10^5 \text{ d.sec.}^{-1} = 14.1\%$

Total recovery of precursor $5.8 \times 10^5 \text{ d.sec.}^{-1} = 68\%$

Ergosterol

Isolated = 49 mg.

Activity = $1,430 \pm 60 \text{ d.sec.}^{-1} \text{ mg.}^{-1}$

Incorporation $\frac{1,430 \times 49 \times 100}{8.51 \times 10^5} \%$

$$= 8.3\%$$

(or allowing for 68% recovery of precursor, incorporation = 26%).

Feeding of [2,4-³H]-4 α -methylcholesta-8,24-dienol
to yeast in vivo

Aerobic incubation was exactly as described for
obtusifoliol feeding.

Precursor fed 2.33 mg.

$$= 1.03 \times 10^6 \text{ d.sec.}^{-1}$$

Recovered from medium $3.98 \times 10^5 \text{ d.sec.}^{-1} = 39\%$

Recovered from cells $3.07 \times 10^4 \text{ d.sec.}^{-1} = 3\%$

Total recovered precursor $4.29 \times 10^5 \text{ d.sec.}^{-1} = 42\%$

Ergosterol

Isolated = 54 mg.

Activity = $2,860 \pm 30 \text{ d.sec.}^{-1} \text{ mg.}^{-1}$

Incorporation $\frac{2,860 \times 54 \times 100}{1.03 \times 10^6} \%$

$$= 15\%$$

(or allowing for 42% recovery of precursor,
incorporation = 26%).

Feeding of [2,4-³H]-4 α -methylergosta-8,24(28)-dienol
to yeast in vivo

Aerobic incubation exactly as described for obtusifolios.

Precursor fed 2.14 mg.

$$= 6.93 \times 10^5 \text{ d.sec.}^{-1}$$

Recovered from medium $3.23 \times 10^4 \text{ d.sec.}^{-1} = 4.7\%$

Recovered from cells $2.87 \times 10^4 \text{ d.sec.}^{-1} = 4.1\%$

Total recovery of precursor $6.1 \times 10^4 \text{ d.sec.}^{-1} = 8.8\%$

Ergosterol

Isolated = 52 mg.

Activity = $1670 \pm 90 \text{ d.sec.}^{-1} \text{ mg.}^{-1}$

Incorporation $\frac{52 \times 1670 \times 100}{6.93 \times 10^5} \%$

$$= 12.5\%$$

(or allowing for 9% recovery of precursor,
incorporation = 13.8%).

Feeding of $[28-^{14}\text{C}, ^3\text{H}]-24\text{-methylenedihydrolanosterol}$ to yeast in vivo according to Ahktar et al.¹⁶⁶

Glucose (40 g.), peptone (40 g.) and yeast extract (20 g.) were boiled with water (2 litre), filtered through celite, and the filtrate sterilised. The precursor (0.99 mg.) and Tween 80 (10 mg.) were dissolved in acetone, the solution divided between 8 one-litre flasks, and acetone removed under a stream of nitrogen. The medium was divided between the flasks and dried baker's yeast (100 mg.) added to each. The flasks were shaken under nitrogen for 40 hours at 30° followed by shaking under oxygen for 20 hours at 30° . Centrifugation at 800 r.p.m. for 30 min. followed by washing with phosphate buffer gave 45 ml. of yeast cells, which were saponified as usual. The non-saponifiable material (357 mg.) consisting mainly of ergosterol (t.l.c.) was diluted with pure ergosterol (1020 mg.) and inactive precursor (10 mg.). The mixture was chromatographed on grade 3 alumina (50 g.). Chloroform-benzene (1:9) removed the precursor and pure ergosterol was eluted with chloroform-benzene (1:3, 600 ml.).

Summary of results

	^3H d.sec. $^{-1}\text{mg.}^{-1}$	^{14}C d.sec. $^{-1}\text{mg.}^{-1}$	$\frac{^3\text{H}}{^{14}\text{C}}$
Precursor	2.76×10^5	1.78×10^4	15.6 ± 0.4
H_2CO dimedone (C-28 of precursor)			14.6 ± 0.1
Ergosterol	13.0 ± 0.7	0.83 ± 0.05	15.6 ± 0.6
Ergosteryl acetate	11.3 ± 0.6	0.70 ± 0.03	16.2 ± 0.8
2,3-dimethyl- butanal dimedone	12.9 ± 0.5	0.75 ± 0.04	17.2 ± 0.8

Molar activities (^3H)

	d.sec. $^{-1}\text{mm.}^{-1}$	Ratio
ergosterol	5.17×10^3	1.00
ergosteryl acetate	4.97×10^3	0.96
2,3-dimethylbutanal dimedone	4.78×10^3	0.92

Incorporation

Wt. ergosterol = 1.02 g.

Precursor fed = 0.99 mg.

= 2.74×10^5 d.sec. $^{-1}$ of ^3H

Incorporation of ^3H $\frac{1.02 \times 13.0 \times 10^3 \times 100}{2.74 \times 10^5} \%$

= 4.8%

dihydro

Feeding of [28-³H]-24-methylene~~lanosterol~~ to a cell-free yeast homogenate prepared according to Turner and Parks.¹⁶⁷

Anaerobically grown yeast cells (20 ml.) were suspended in a solution of potassium dihydrogen phosphate (13.6 g.) and glucose (10 g.) in water (1 litre) and the suspension divided between four one-litre flasks. The flasks were loosely plugged with cotton wool and shaken vigorously at 30° in air for two hours. The yeast was harvested and washed twice with pH 6.5 phosphate buffer, by centrifugation.

A mortar and pestle had been left in crushed cardice for several hours. The mortar was emptied and a layer of the wet yeast applied to the surface of the pestle. The pestle was held in cardice until the yeast was frozen, and the yeast scraped into the mortar. The process was repeated until all the yeast was transferred. The frozen yeast was vigorously ground with glass beads (100 g. of 80 mesh) for 20 minutes. The paste was allowed to thaw (3 hours) and the volume made up to 80 ml. with phosphate buffer (0.1M, pH 6.5). Cell debris were removed by four 10-minute centrifugations (600 r.p.m.), to give a slightly cloudy liquid (60 ml.). Manganese^{II} chloride (6 mg.), potassium carbonate (78 mg.), ATP. (64 mg.), NAD.⁺ (30 mg.) and ascorbic acid (39mg.)

were added. The homogenate was made up to 100 ml. volume with pH 6.5 phosphate buffer.

[28-³H]-24-methylenedihydrolanosterol (2.1 mg.) and Tween 80 (45 mg.) in acetone (1 ml.) were added to the yeast homogenate in a one-litre flask which was loosely plugged with cotton wool. The suspension was shaken vigorously at 30° in air for 17 hours. The medium was refluxed with potassium hydroxide (20 g.) and methanol (200 ml.) for four hours in the presence of carrier ergosterol (4 mg.), and the non-saponifiable material extracted with benzene. This mixture was fractionated by t.l.c. The precursor fraction retained 75% of the activity fed while the ergosterol fraction retained 1.8% inseparable from ergosterol on repeated t.l.c. chromatography. 1.6% of the activity was recovered in fractions more polar than ergosterol. The ergosterol fraction (6 mg.) was diluted with inactive ergosterol (100 mg.) and crystallised to activity 2.0 d.sec.⁻¹mg.⁻¹

$$\text{Precursor fed} = 6.2 \times 10^5 \text{ d.sec.}^{-1}$$

Thus incorporation into ergosterol

$$\leq \frac{2.0 \times 100 \times 100}{6.2 \times 10^5} \%$$

$$\leq 0.03\%$$

Subculturing technique for Polyporus sulphureus

Malt extract (2.51 g.), sucrose (1.51 g.) and 10 czapek dox tablets were heated with water (25 ml.) on a steam bath until homogeneous. Water (15 ml.) was added and 5 ml. aliquots were poured into 5 x $\frac{5}{8}$ th inch test-tubes which were plugged with cotton wool. The tubes were sterilised in a steam autoclave at 15 lb.in.² for 20 minutes and allowed to cool, tilted almost horizontally. The agar slopes were stored at 0° until used.

P.sulphureus was subcultured every two months as follows:- An inoculum from the previous slope culture was removed, with a sterilised nichrome wire, and introduced into a fresh agar slope, which was immediately replugged with cotton wool. Slope cultures were stored at room temperature in the dark.

Feeding of [28-³H]-24-methylenedihydrolanosterol to *P.sulphureus*

Malt extract (30 g.) and glucose (30 g.) were dissolved in hot distilled water (1 litre) and the cooled solution filtered through celite. The filtrate was divided equally between six one-litre conical flasks which were loosely plugged with cotton wool and sterilised at 100° for 15 min. After standing at room temperature for one day, the flasks were resterilised, and on cooling each flask was inoculated with a growth of *P.sulphureus*. The flasks were then maintained at 30° in the dark without shaking.

After 12 days a vigorous surface culture of the fungus was established. [28-³H]-24-methylene-dihydrolanosterol and Tween 80 (15 mg.) in acetone (0.3 ml.) were introduced into the medium at this stage. Feeding was repeated every 3 or 4 days over a period of two weeks. When the mycelium had completely covered the medium, feeding was effected by pushing a thin pipette through the mycelium into the medium. After the last feeding the fungus was allowed to grow further for two weeks then the mycelium filtered off.

The filtrate was extracted with benzene to yield an oil (12 mg.). Inactive precursor was added and recovered after t.l.c. purification.

The mycelium was dried in vacuo to constant weight (2.46 g.) crushed to a fine powder, and extracted with refluxing light petroleum, in a soxhlet apparatus, for one hour. The oily extract (40 mg.) contained the sterols including most of the recovered precursor and some eburicoic acid and was worked up for precursor.

The mycelium was then extracted with refluxing chloroform for 24 hours to yield the crude triterpene acids. Further extraction with chloroform afforded little more material. Several crystallisations of the crude triterpene acids from ethanol gave eburicoic acid m.p. 261-263°, (lit.¹⁵⁷ m.p. 293°) and a fraction consisting mainly of eburicoic acid and some sulphurenic acid (t.l.c. comparison with authentic samples). A third fraction was rich in sulphurenic acid. The best sample of eburicoic acid was acetylated with acetic anhydride-pyridine overnight at room temperature, and the product crystallised from ethanol-water to give acetyl eburicoic acid, m.p. 229-233° (lit.¹⁵⁷ 259°) in needles. The acetate was purified by preparative t.l.c. on silica gel with chloroform eluant, and crystallised to constant activity. Treatment of the acetate in methanol with diazomethane in ether gave methyl acetyl eburicoate as needles, m.p. 159.5-161° from acetone-methanol (lit.¹⁵⁷ 157-158°).

The methyl acetoxy derivative was refluxed with 2% methanolic potassium hydroxide for 2 hours to give methyl eburicoate, m.p. 147-149° (lit.¹⁵⁷ m.p. 146-147°).

Satisfactory mixed melting points, superposable i.r. spectra and identical t.l.c. behaviour were observed for all eburicoic acid derivatives when compared with the authentic derivatives, despite the low mps. of the isolated acid and the 3-O-acetyl derivative.

Summary

Volume of medium, 1 litre, divided between six 1-litre conical flasks.

Feedings

	<u>Wt. of precursor fed</u>	<u>Time</u>
Inoculation of medium	0 mg.	1st day
1st feeding	0.30 mg.	12th day
2nd feeding	0.66 mg.	15th day
3rd feeding	0.63 mg.	18th day
4th feeding	0.52 mg.	23rd day
Work-up	-	38th day

Total precursor fed = 2.11 mg.

$$= 6.33 \times 10^5 \text{ d.sec.}^{-1}$$

Precursor recovered

from medium $3.26 \times 10^3 \text{ d.sec.}^{-1} = 0.5\%$

from cells $1.37 \times 10^5 \text{ d.sec.}^{-1} = 21.6\%$

Total precursor recovered $= 22\%$

Eburicoic acid

Wt. of dried mycelium $= 2.46 \text{ g.}$

Pure acetyl eburicoic acid $= 294 \text{ mg.}$

	<u>$\text{d.sec.}^{-1} \text{mg.}^{-1}$</u>	<u>$\text{d.sec.}^{-1} \text{mm.}^{-1}$</u>
acetyl eburicoic acid	2.48	1160 ± 60
methyl acetyl eburicoate	2.20	1160 ± 40
methyl eburicoate	2.72	1310 ± 100

$$\text{Incorporation} = \frac{294 \times 2.48 \times 100\%}{6.33 \times 10^5}$$

$$= 0.115\%$$

or allowing for 22% recovery of precursor,

$$\text{incorporation} = 0.15\%$$

Feeding of $[28-^{14}\text{C}]$ -24-methylenedihydrolanosterol to *P. sulphureus*

Volume of medium 500 ml. in three one-litre conical flasks.

Feedings

	<u>Activity fed</u>	<u>Time</u>
Inoculation of medium	-	1st day
1st feeding	$1.23 \times 10^5 \text{ d. sec.}^{-1}$	13th day
2nd feeding	$1.23 \times 10^5 \text{ d. sec.}^{-1}$	23rd day
3rd feeding	$1.23 \times 10^5 \text{ d. sec.}^{-1}$	31st day
4th feeding	$2.46 \times 10^5 \text{ d. sec.}^{-1}$	37th day
Work-up	-	45th day

Total precursor fed = $6.15 \times 10^5 \text{ d. sec.}^{-1}$

Eburicoic acid

Wt. of dried mycelium = 1.90 g.

Crude triterpene acids = 352 mg.

Wt. of t.l.c. pure eburicoic acid = 224 mg.

Material rich in sulphuric acid = 123 mg.

	<u>$\text{d. min.}^{-1} \text{ mg.}^{-1}$</u>	<u>$\text{d. min.}^{-1} \text{ mm.}^{-1}$</u>
Acetyl eburicoic acid	58.9 ± 1.0	30,200
Methyl acetyl eburicoate	56.6 ± 2.0	29,800

Calculated weight of acetyl eburicoic acid = 244 mg.

$$\begin{aligned} \text{Thus incorporation} & \quad \frac{244 \times 58.9 \times 100}{6.15 \times 60 \times 10^5} \% \\ & = 0.039\% \end{aligned}$$

Feeding of [2-³H]-lanosterol to P.sulphureus

Volume of medium 500 ml. in three one-litre conical flasks, and feeding carried out in parallel with that of [¹⁴C]-24-methylenedihydrolanosterol.

Feedings

	<u>Precursor fed</u>	<u>Time</u>
Inoculation of medium	-	1st day
1st feeding	0.51 mg.	14th day
2nd feeding	0.54 mg.	23rd day
3rd feeding	1.44 mg.	31st day
4th feeding	0.71 mg.	37th day
Work-up	-	47th day

Total precursor fed = 3.20 mg. = 8.25×10^5 d.sec.⁻¹

Eburicoic acid

Wt. of dried mycelium = 1.76 g.

Crude triterpene acids = 380 mg.

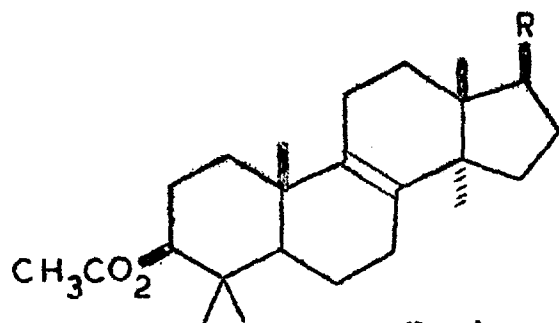
Wt. of t.l.c. pure eburicoic acid = 205 mg.

	<u>d.min.⁻¹mg.⁻¹</u>	<u>d.min.⁻¹mm.⁻¹</u>
Acetyl eburicoic acid	118 ± 24	60,400
Methyl acetyl eburicoate	124 ± 5	65,200

Calculated weight of acetyl eburicoic acid = 224 mg.

$$\text{Incorporation} = \frac{224 \times 118 \times 100}{8.25 \times 60 \times 10^5} \%$$

$$= 0.053\%$$

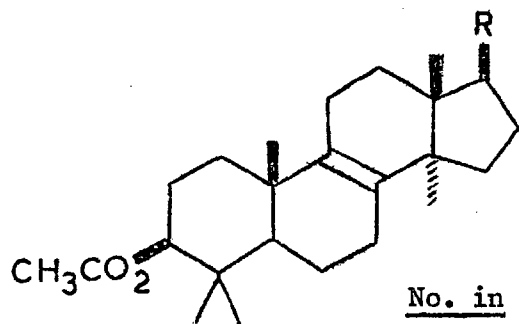


N.m.r. table I *

	<u>No. in</u>	<u>CH₃CO₂-</u>	<u>4α, 4β &</u> <u>14α-CH₃</u>	<u>10β-CH₃</u>	<u>13β-CH₃</u>	<u>25-CH₃</u>	<u>3α-H</u>	<u>24-H</u>
	(100)	7.96	9.11	8.98	9.29	8.01 8.18	5.47 (t, J, 7)	5.83 (d, J, 10)
	-	7.95	9.10	8.98	9.30	8.32 8.40	5.48 (t, J, 6)	4.89 (t, J, 7)
	(104)	7.96	9.12	9.00	9.30	8.70 8.74	5.48 (t, J, 7)	7.31 (t, J, 5)
	(105)	7.96	9.12	9.00	9.31	8.86 8.97	5.48 (t, J, 7)	-
	-	7.97	9.14	9.00	9.31	8.66	5.47 (t, J, 6.5)	6.07 (d, J, 7)

† Uncharacterised

* Resonances in τ-values, coupling constants in c/s.
d, t and m refer to doublet, triplet and multiplet
respectively.



N.m.r. table II

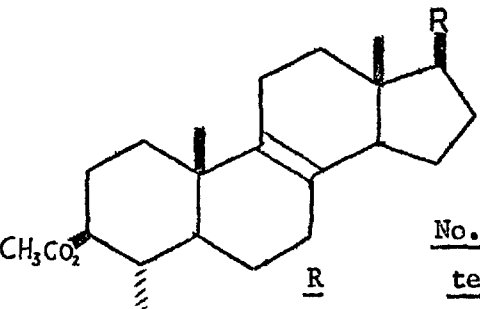
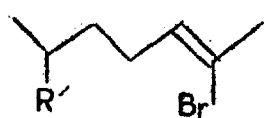
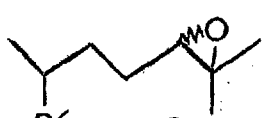
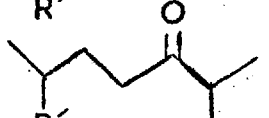
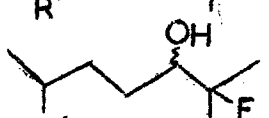
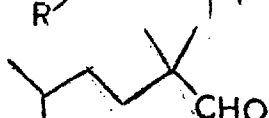
<u>R</u>	<u>No. in</u> <u>text</u>	<u>CH₃CO₂-</u>	<u>4α,4β &</u> <u>14α-CH₃</u>	<u>10β-CH₃</u>	<u>13β-CH₃</u>	<u>25-CH₃</u>	<u>3α-H</u>	<u>28-H*</u>
	(91b)	7.95	9.11	8.98	9.29	8.91 9.02	5.45 (m)	5.28
	-	7.96	9.11	8.99	9.30	8.92 9.03	5.47 (t,J,8)	-
†	(106c)	7.96	9.12	9.00	9.32	-	5.51 (t,J,6.5)	-
	(91a)	-	9.01(4 α) 9.19(4 β) 9.12(14 α)	9.01	9.30	8.92 9.01	6.77 (m)	5.33

† 24-CH₃ groups at 8.85 τ

† 24-CO₂CH₃ group at 6.36 τ

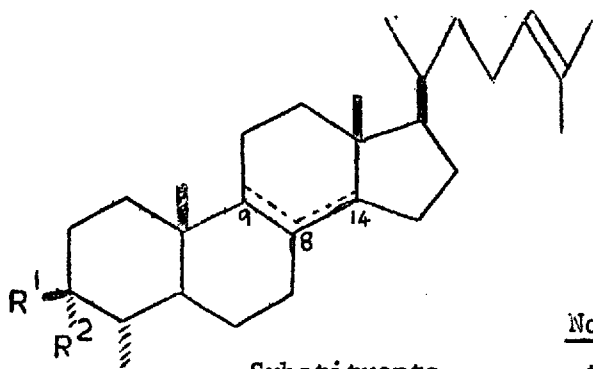
* broad singlet

N.m.r. table III

	No. in text	N.m.r. table III						
		CH_3CO_2	$\frac{4\alpha\text{-CH}_3}{(\text{d}, \text{J}, 6)}$	$\frac{10\beta\text{-CH}_3}{}$	$\frac{13\beta\text{-CH}_3}{}$	$\frac{25\text{-CH}_3}{}$	$\frac{3\alpha\text{-H}}{(\text{m})}$	$\frac{24\text{-H}}{}$
	(90b)	7.96	9.15	9.01	9.39	8.32 8.39	5.63	4.91 (t, J, 5.5)
	(99a)	7.98	9.15	9.03	9.39	8.66	5.67	6.07 (d, J, 7.5)
	(101)	7.95	9.14	9.01	9.38	8.70 8.74	5.62	7.32 (t, J, 5)
	(102a)	7.96	9.15	9.02	9.39	8.92 (d, J, 7)	5.66	-
	(99c)	7.96	9.14	9.02	9.38	8.67 (d, J, 22)	5.65	6.5 (broad m)
	(103a)	7.97	9.16	9.01	9.40	-	5.6	-

† Uncharacterised

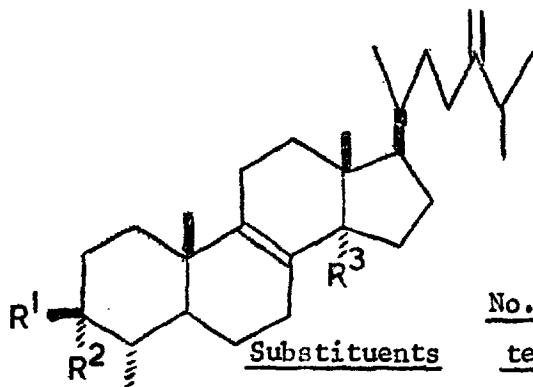
* CHO at 0.57 τ , C-24 CH_3 groups at 8.97 τ .



N.m.r. table IV

<u>Substituents</u>	<u>No. in</u> <u>text</u>	<u>4α-CH₃</u> <u>(d, J, 6)</u>	<u>10β-CH₃</u>	<u>13β-CH₃</u>	<u>25-CH₃</u>	<u>3α-H</u> <u>(m)</u>	<u>24-H</u>
$\left\{ \begin{array}{l} \Delta^{8(9)} \\ R^1, R^2 = 0 \end{array} \right.$	-	(8.99)	8.81	9.36	8.33; 8.39	-	4.91 (t, J, 6.5)
$\left\{ \begin{array}{l} \Delta^{8(14)} \\ R^1 = OH, R^2 = H \end{array} \right.$	(93a)	(9.00)	9.27	9.14	8.31; 8.38	6.90	4.92 (m)
$\left\{ \begin{array}{l} \Delta^{8(14)} \\ R^1 = CH_3CO_2, R^2 = H \end{array} \right.$	(93b)	9.15	9.26	9.15	8.32; 8.39	5.58	4.88 (t, J, 7)

* CH₃CO₂ at 7.96 τ



N.m.r. table V

<u>Substituents</u>	<u>No. in</u> <u>text</u>	<u>CH₃CO₂</u>	<u>4α-CH₃</u> <u>(d, J, 6)</u>	<u>10β-CH₃</u>	<u>13β-CH₃</u>	<u>14α-CH₃</u>	<u>25-CH₃</u>	<u>3α-H</u> <u>(m)</u>	<u>28-H</u> <u>(broad s)</u>
R ¹ = OH R ² , R ³ = H	(92a)	-	8.99	9.02	9.38	-	8.96 (d, J, 7)	7.0	5.31
R ¹ , R ² = O R ³ = H	(94)	-	(9.00)	8.82	9.36	-	8.99 (d, J, 7)	-	5.32
R ¹ = CH ₃ CO ₂ R ² , R ³ = H	(92b)	7.96	9.14	9.01	9.38	-	8.97 (d, J, 6)	5.65	5.33
R ¹ = PhCO ₂ R ² , R ³ = H	(92c)	-	9.07	9.04	9.38	-	8.98 (d, J, 7)	5.5	5.34
R ¹ = OH, R ² = H R ³ = CH ₃	(18)	-	9.01	9.04	9.29	9.12	8.98 (d, J, 7)	6.9	5.30
R ¹ , R ² = O R ³ = CH ₃	-	-	(8.96)	8.80	9.25	9.10	8.97 (d, J, 7)	-	5.31

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