# Investigations on the Biosynthesis of Steroids and Terpenoids. Part II.† Role of 24-Methylene Derivatives in the Biosynthesis of Steroids and Terpenoids

By D. H. R. Barton,* D. M. Harrison, G. P. Moss, and D. A. Widdowson, Imperial College, London S.W.7

24,25-Dihydro-24-methylenelanosterol has been synthesised from lanosterol and investigated as a potential precursor of eburicoic acid and of ergosterol.

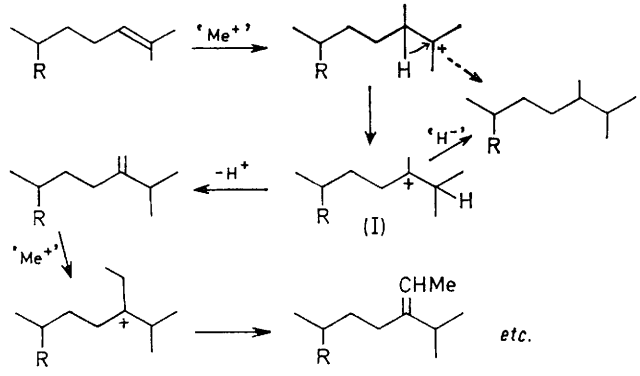
Two new sterols, 24,25-dihydro-4α-methyl-24-methylenezymosterol and 3β-hydroxy-4α-methylcholesta-8(14),24-diene have been isolated from yeast residues and their structures have been determined. The constitution of 24,25-dihydro-4α-methyl-24-methylenezymosterol has been confirmed by a partial synthesis from the more abundant 4α-methylzymosterol.

4α-Methylzymosterol, 24,25-dihydro-4α-methyl-24-methylenezymosterol and obtusifoliol have been shown to be incorporated into ergosterol in yeast cultures. 4,4-Dimethylergosterol is, however, not a precursor.

By a trapping experiment in which tritium-labelled squalene oxide and unlabelled 24,25-dihydro-24-methylenelanosterol were used it has been shown that the latter is almost certainly not on the biogenetically preferred route from lanosterol to ergosterol.

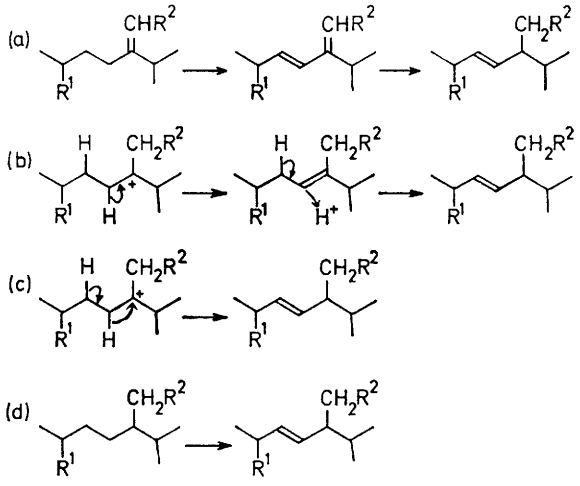


THE biogenetically 'extra' carbon atoms present in the side chain of numerous triterpenoids and plant steroids [1] have been shown to be introduced by a transmethylation process from S-adenosylmethionine (Scheme 1).[2]




SCHEME 1 R = Steroid nucleus

Variants on this Scheme can be envisaged for the formation of the 22(23)-double bond present in the ergosterol (Scheme 2; $R^2 = H$) and stigmasterol (Scheme 2; $R^2 = Me$) type side chains.[2f,g] Recently doubt has been cast on the Scheme 2c alternative since [23-$^3H_2$]lanosterol was incorporated into ergosterol by *Saccharomyces cerevisiae* without tritium migration to C-24.[3] In addition, the possibility of fully saturated side chains being involved (Scheme 2d) was suggested following the incorporation of 24,25-dihydro-24-methyllanosterol into ergosterol without loss of label from the 24,25- or 28-positions.[3] This shows that the 22(23)-double bond can be formed without involvement of a 24(28)-intermediate.




SCHEME 2 $R^1$ = Steroid nucleus

Clearly 24-methylene-sterols, or the derived carbonium ions of type (I), occupy a key position in phytosterol biosynthesis. It is conceivable that the introduction of the 'extra' carbon atoms could occur, in all cases, immediately after 2,3-epoxysqualene cyclisation. The finding of 24-alkylated triterpenoids in which no other modification of the basic triterpenoid skeleton has occurred [4] supports this contention.

Initially we chose to study the relatively simple case of eburicoic acid (II), the principle triterpenoid acid of the

† Part I, D. H. R. Barton, G. P. Moss, and J. A. Whittle, *J. Chem. Soc.* (*C*), 1968, 1813. Part of the work described in the present paper has already been summarised in preliminary form (D. H. R. Barton, D. M. Harrison, and G. P. Moss, *Chem. Comm.*, 1966, 595; D. H. R. Barton, D. M. Harrison, and D. A. Widdowson, *ibid.*, 1968, 17).

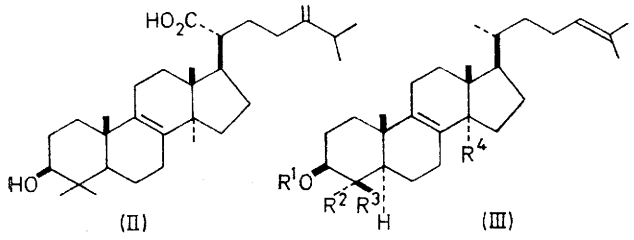
[1] L. J. Goad, 'Terpenoids in Plants,' ed. J. P. Pridham, Academic Press, New York, 1967, p. 159.

[2] (*a*) G. J. Alexander and E. Schwenk, *J. Biol. Chem.*, 1958, **232**, 611; (*b*) E. Lederer, *Experientia*, 1964, **20**, 473; (*c*) G. Jauréguiberry, J. H. Law, J. A. McCloskey, and E. Lederer, *Biochemistry*, 1965, **4**, 347; (*d*) M. Lenfant, E. Zissmann, and E. Lederer, *Tetrahedron Letters*, 1967, 1049; (*e*) M. Castle, C. A. Blondin, and W. R. Nes, *J. Biol. Chem.*, 1967, **242**, 5796, 5802; (*f*) A. R. H. Smith, L. J. Goad, T. W. Goodwin, and E. Lederer, *Biochem. J.*, 1967, **104**, 56c; (*g*) M. Lenfant, R. Ellouz, B. C. Das, E. Zissmann, and E. Lederer, *European J. Biochem.*, 1969, **7**, 159; (*h*) M. Devys, A. Alcaide, and M. Barbier, *Bull. Soc. Chim. biol.*, 1968, **50**, 1751.

[3] M. Akhtar, M. A. Parvez, and P. F. Hunt, *Biochem. J.*, 1968, **106**, 623.

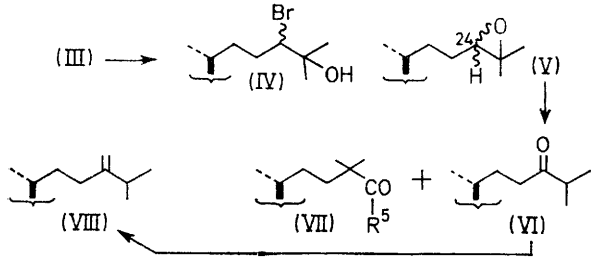
[4] (*a*) G. Ohta and M. Shimizu, *Chem. and Pharm. Bull. Japan*, 1958, **6**, 325; (*b*) G. Goulston, L. J. Goad, and T. W. Goodwin, *Biochem. J.*, 1967, **102**, 15c; (*c*) M. Devys, A. Alcaide, M. Barbier, and E. Lederer, *Phytochemistry*, 1968, **7**, 613; (*d*) A. Alcaide, M. Devys, J. Bottin, M. Fetizon, M. Barbier, and E. Lederer, *Phytochemistry*, 1968, **7**, 1773.

wood rotting fungus *Polyporus sulphureus* [5a] and other fungi.[5b] In (II), the only other modification of the lanosterol skeleton, besides the introduction of the 24-methylene group, is the oxidation of the 21-methyl group to a carboxylic acid function.




If side-chain alkylation was the initial modification process, then 24,25-dihydro-24-methylenelanosterol [6,7] would be the next discrete intermediate after lanosterol. We thus sought to synthesise this compound (Scheme 3).

Lanosteryl acetate (III; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me) was epoxidised in 70% yield with monoperphthalic acid in chloroform solution. The epimeric epoxides




SCHEME 3

were separable on alumina (grade III) to give epoxide A (V; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me, 24-H α or β), m.p. 144—146°, $[\alpha]_D$ +53°, and epoxide B (V; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me, 24-H β or α), m.p. 196—198°, $[\alpha]_D$ +60°, with almost identical n.m.r. (see Table 1) and i.r. spectra. The configurations at C-24 were not determined. Usually, the mixed epoxides were used without separation for subsequent transformations. Treatment with boron trifluoride–ether complex in benzene at room temperature for 5 min. gave the required 24-ketone (VI; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me), together with an isomeric aldehyde (VII; $R^1$ = Ac, $R^3$ = $R^3$ = $R^4$ = Me, $R^5$ = H). These were readily separated by oxidation of the aldehyde component of the mixture with chromic oxide in benzene–acetic acid–water and extraction of the derived acid with base. Chromatography of the resulting neutral fraction gave the pure ketone, m.p. 135—137°, $[\alpha]_D$ +58°. The acid fraction was methylated with diazomethane to give a crystalline methyl ester (VII; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me, $R^5$ = OMe), m.p. 155—158°, $[\alpha]_D$ +51°.

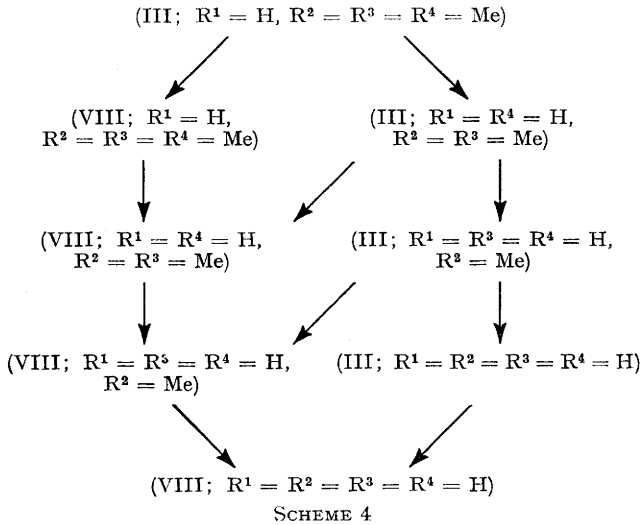
[5] (*a*) R. M. Gascoigne, J. S. E. Holker, A. D. G. Powell, A. Robertson, J. J. H. Simes, and R. S. Wright, *J. Chem. Soc.*, 1953, 2422; (*b*) G. Ourisson, P. Crabbé, and O. Rodig, 'Tetracyclic Triterpenes,' ed. E. Lederer, Holden-Day Inc., San Francisco, 1964, p. 147.

This had spectroscopic data fully consistent with the proposed structure (see Table 1 and Experimental section).

The 24-ketone (VI; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me) was converted into the required 24,25-dihydro-24-methylenelanosteryl acetate [4b] by a Wittig reaction with triphenylmethylenephosphorane in 75% yield. The i.r. spectrum showed the absorption at 890 $cm.^{-1}$ characteristic of a terminal methylene group, and the n.m.r. spectrum (Table 1) was fully consistent with structure (VIII; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me). The free sterol (VIII; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me),[4b] m.p. 158—159°, $[\alpha]_D$ +59° was obtained by saponification of the ester.

The Wittig stage was repeated with $[CH_2{}^3H_2]$triphenylmethylenephosphorane (conveniently prepared *via* base-catalysed exchange of the phosphonium salt with tritiated water) to give [24-$CH_2{}^3H_2$]-24,25-dihydro-24-methylenelanosterol of activity $1{\cdot}11 \times 10^5$ decomp. $sec.^{-1}$ $mg.^{-1}$. Similarly obtained was the [24-$CH_2{}^{14}C$]-labelled material of activity $1{\cdot}2 \times 10^5$ decomp. $sec.^{-1}$ $mg.^{-1}$, by use of [$^{14}$C]methyl iodide for the preparation of the Wittig reagent.

In order to check the radiopurity of these samples, a mixture having a $^3H$ : $^{14}C$ ratio of 22·7 : 1 was diluted 100 fold with inactive material and recrystallised six times. The ratio and specific activity remained constant. The specificity of the labelling was checked by ozonolysis and conversion of the derived formaldehyde into its dimedone derivative. This showed a $^3H$ : $^{14}C$ ratio of




SCHEME 4

only 15·4 : 1. The drop in tritium activity indicated that this label was partially scrambled (*ca.* 25%) between the 24-methylene group and (presumably) the 23- and 25-positions. The origin of this scrambling is not certain. The formation of the phosphorane by phenyl-lithium is irreversible and the benzene formed

[6] F. N. Lahey and P. H. A. Strasser, *J. Chem. Soc.*, 1951, 873.

[7] M. Akhtar, P. F. Hunt, and M. A. Parvez, *Biochem. J.*, 1966, **100**, 38c.

cannot exchange with the ketonic component. The ylide however could exchange with the ketone in a non-productive equilibrium process competing with betaine formation.[8] Also, if there is lithium hydroxide present in the mixture (its formation cannot be completely suppressed under our conditions) then this could mediate in the exchange process in a reaction competing with phenyl-lithium. We therefore studied the reaction at different temperatures, and found that if the phosphorane was added at 0°, then the scrambling was reduced to 6%. Exchange processes of this type have been previously observed.[9]

TABLE 1

N.m.r. spectra (τ values)

| | $CH_3CO_2$ | 4α-, 4β-, and 14α-$CH_3$ | 10β-$CH_3$ | 13β-$CH_3$ | 25-$CH_3$ | 3α-H | 24-H | 28-H |
|---|---|---|---|---|---|---|---|---|
| (III; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me) | 7·95 | 9·10 | 8·98 | 9·30 | 8·32, 8·40 | 5·48 (t, *J* 6 Hz) | 4·89 (t, *J* 7 Hz) | |
| (V; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me) | 7·96 | 9·12 | 9·00 | 9·30 | 8·70, 8·74 | 5·48 (t, *J* 7 Hz) | 7·31 (t, *J* 5 Hz) | |
| (VI; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me) | 7·96 | 9·12 | 9·00 | 9·31 | 8·86, 8·97 | 5·48 (t, *J* 7 Hz) | | |
| (VII; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me, $R^5$ = OMe) | 7·96 | 9·12 | 9·00 | 9·32 | | 5·51 (t, *J* 6·5 Hz) | | |
| (VI; R = Ac, $R^1$ = $R^2$ = Me) | 7·95 | 9·11 | 8·98 | 9·29 | 8·91, 9·02 | 5·45(m) | | 5·28br(s) |
| (VIII; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me) | | 9·01(4α) 9·19 (4β) 9·12 (14α) | 9·01 | 9·30 | 8·92, 9·01 | 6·77(m) | | 5·33br(s) |
| (III; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) | 7·96 | 9·15 (4α, d, *J* 6 Hz) | 9·01 | 9·39 | 8·32, 8·39 | 5·63(m) | 4·91 (t, *J* 5·5 Hz) | |
| (VIII; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) | 7·96 | 9·14 (4α, d, *J* 6 Hz) | 9·01 | 9·38 | 8·97 (d, *J* 6 Hz) | 5·65(m) | | 5·33br(s) |
| (IV; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) | 7·98 | 9·15 (4α, d, *J* 6 Hz) | 9·03 | 9·39 | 8·66, 8·66 | 5·67(m) | 6·07 (d, *J* 7·5 Hz) | |
| (V; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) | 7·95 | 9·14 (4α, d, *J* 6 Hz) | 9·01 | 9·38 | 8·70, 8·74 | 5·62(m) | 7·32 (t, *J* 5 Hz) | |
| (VI; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) | 7·96 | 9·14 (4α, d, *J* 6 Hz) | 9·02 | 9·39 | 8·92 (d, *J* 7 Hz) | 5·66(m) | | |
| (IX; R = H) | | (9·00) | 9·27 | 9·14 | 8·31, 8·38 | 6·90(m) | 4·92(m) | |

The labelled compounds, including [2-$^3$H]lanosterol (see later) were fed in solutions in acetone containing Tween 80 to a surface culture of *P. sulphureus* at 3-day intervals during 2 weeks. After a further 2 weeks the mycelia were harvested and dried, and the eburicoic acid was isolated. The acid was counted as the acetate, acetate methyl ester, and methyl ester. The observed incorporations are presented in Table 2.

If there is no alkylation of the C-30 skeleton before cyclisation of 2,3-epoxysqualene then lanosterol is an obligatory intermediate in eburicoic acid formation. A less elaborated precursor, farnesyl pyrophosphate, has been shown to be incorporated at an efficiency of 0·4%.[10] Our observed incorporations of lanosterol (0·053%) was low compared with this, but the discrepancy may be only an expression of the greater ease of assimilation of the pyrophosphate from aqueous medium.

The labelled 24,25-dihydro-24-methylenelanosterol was incorporated with efficiency comparable to that of the incorporation of the lanosterol. This result gives support to the hypothesis of alkylation at the lanosterol stage. Lederer and his co-workers[11] have reported the incorporation of [*Me*-$^2H_3$]methionine into the methylene groups of eburicoic and the related sulphurenic acids in *P. sulphureus*. They also noted the presence of an acid, $C_{30}H_{48}O_3$, which they did not fully characterise, but which contained no groups derived from methionine. This suggests that initial oxidation to a 21-carboxylic acid may occur in *P. sulphureus*. It is possible therefore that two (or more) biosynthetic routes to eburicoic acid operate. Alternatively the fungus may be able to assimilate unnatural precursors. These aspects of triterpenoid metabolism will be discussed in greater detail later in the paper.

Ergosterol, the main constituent of the non-saponifiable fraction of bakers yeast (*Saccharomyces cerevisiae*), is a more complex case where considerable modification of the steroid nucleus accompanies side-chain elaboration. Thus, introduction of a methylene group at C-24, which has been inferred in ergosterol biosynthesis by Lederer[2b,c] as a result of feeding [*Me*-$^2H_3$]methionine, could occur at any one of several stages of nuclear modification (Scheme 4: this represents two series; others are possible). The reported occurrence of zymosterol (III; $R^1$ = $R^2$ = $R^3$ = $R^4$ = H) and fecosterol (VIII; $R^1$ = $R^2$ = $R^3$ = $R^4$ = H) in yeast sterols[12] indicates that alkylation could occur at this stage, but does not preclude its occurrence at any other.

[2-$^3$H]Lanosterol and the labelled 24,25-dihydro-24-methylenelanosterol were fed to growing cultures of

[8] For a discussion of the mechanism of the Wittig reaction, see A. W. Johnson, 'Ylide Chemistry,' Academic Press, New York, 1966, p. 152.

[9] J. G. Atkinson, M. H. Fisher, D. Horley, A. T. Morse, R. S. Stuart, and E. Symes, *Canad. J. Chem.*, 1965, **43**, 1614.

[10] W. Lawrie, J. McLean, P. L. Pauson, and J. Watson, *J. Chem. Soc.*, 1962, 2002.

[11] V. R. Villaneuve, M. Barbier, and E. Lederer, *Bull. Soc., Chim. biol.*, 1967, **49**, 389.

[12] (*a*) I. Smedley-McLean, *Biochem. J.*, 1928, **22**, 22; (*b*) D. H. R. Barton and J. D. Cox, *J. Chem. Soc.*, 1949, 214; H. Wieland, F. Rath, and H. Hesse, *Annalen*, 1941, **548**, 34.

yeast in Tween 80–acetone solutions by a standard procedure (see Experimental section), and by the modified procedure of Akhtar.[7] In addition, a feeding to a cell-free system described by Turner and Parks [13] was carried out. The incorporations (Tables 3 and 4) show that the 24-methylene derivative was metabolised with the same order of efficiency as lanosterol. The cell-free system is reported not to produce ergosterol.[13] Our low yield (6 mg.; see Table 3) is essentially in accord with this, since a few whole cells may have been present in the homogenate.

These results prompted an examination of the yeast sterol mixture for 24-methylene derivatives as cited in Scheme 4. Crude yeast sterols from which most of the ergosterol had been removed by crystallisation * were separated on an alumina (grade III) column to give essentially three fractions: (i) the lanosterol (4,4-dimethylsterol) fraction, (ii) the 4α-methyl sterol fraction, and (iii) the zymosterol/ergosterol fraction. These were assayed for terminal methylene groups by measurement of the i.r. absorption at 890—900 cm.$^{-1}$. The lanosterol fraction was devoid of absorption in this region, but the 4α-methyl sterol fraction did show a weak absorption. This fraction was acetylated and the acetates were fractionally crystallised by a triangulation procedure. This yielded pure 4α-methylzymosterol, previously isolated by Ourisson from yeast,[14] and a mixture with enhanced absorption at 898 cm.$^{-1}$. This was separated on preparative 10% silver nitrate–silica gel plates into three components, 3β-acetoxy-4α-methylergosta-8,24(28)-diene (VIII; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H), 3β-acetoxy-4α-methylcholesta-8(14),24-diene (IX), and 4α-methylzymosteryl acetate (III; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H). The separation was more easily carried out with the corresponding benzoates. The free sterols and 3-ketones were obtained respectively by hydrolysis of the esters with 2% methanolic potassium hydroxide and oxidation of the sterols with chromium trioxide in acetic acid–benzene–water.

The structure for (VIII; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) was assigned in the light of the following evidence. Microanalytical data (confirmed by the mass spectrum of the derived acetate) for the free sterol and its derivatives indicated a molecular formula $C_{29}H_{48}O$ for the parent alcohol. The acetate (VIII; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) and free sterol showed only end absorption in their u.v. spectra. The hydroxy-group, initially assumed to be at C-3, was shown to have the β-configuration by regeneration of the original sterol on reduction of the 3-ketone with lithium aluminium hydride.[15]

Similarities in the mass spectra of the new sterol acetate and 4α-methylzymosterol acetate (III; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H) suggested that the second additional carbon atom was at C-4. The molecular rotation differences for the new sterol [16] ($\Delta_1$ +51°, $\Delta_2$ +192°, $\Delta_3$ −33°) were similar to those of 4α-methylzymosterol ($\Delta_1$ +37°, $\Delta_2$ +193°, $\Delta_3$ not recorded),[14] but dissimilar to those of a 4-unsubstituted sterol with a double bond at 7, 8, 8(14), or 14 ($\Delta_1$ negative, $\Delta_2$ low positive).[17] The possibility of a 5(6)-olefin was precluded by the rotation ($[\alpha]_D$ +55°) of the sterol.[18] The anomalous molecular rotation differences of 4-alkylated sterols have been noted previously.[19]

The o.r.d. curve of the derived ketone (VIII; 3-ketone, $R^2$ = Me, $R^3$ = $R^4$ = H) showed a positive Cotton effect at 286 nm., of amplitude +43°, which is in accordance with a 4α-configuration for the methyl group.[20]

Finally the n.m.r. spectrum (Table 1) showed a broad two-proton singlet at τ 5·31, 0·4 p.p.m. upfield from the signal for the C-24 proton of 4α-methylzymosterol but identical with that observed for 24-methylenedihydrolanosterol. This confirms the presence of the 1,1-disubstituted ethylene indicated by the i.r. spectrum ($\nu_{max.}$ 1642 and 898 cm.$^{-1}$). There were no other vinyl proton resonances; thus the additional double bond, which must be in the nucleus (see before) must be tetrasubstituted, *i.e.* at 8(9) or 8(14). The possibility of $\Delta^4$ unsaturation

TABLE 2

Feedings to *P. sulphureus*

| Precursor | Activity (decomp. sec.$^{-1}$) | Acetyleburicoic acid: Yield (mg.) | Acetyleburicoic acid: Activity (decomp.) sec.$^{-1}$ mg.$^{-1}$) | Methyl acetyleburicoate. Activity (decomp. sec.$^{-1}$ mg.$^{-1}$) | Methyl eburicoate. Activity (decomp. sec.$^{-1}$ mg.$^{-1}$) | Incorporation (%) |
|---|---|---|---|---|---|---|
| [2-$^3$H]Lanosterol (III; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me) | 8·25 × 10$^5$ | 205 | 1·97 | 2·07 | | 0·049 |
| [24-$CH_2$-$^3H_2$]-24,25-Dihydro-24-methylenelanosterol (VIII; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me) | 6·33 × 10$^5$ | 294 | 2·48 | 2·20 | 2·72 | 0·12 [a] |
| [24-$CH_2$-$^{14}$C]-24,25-Dihydro-24-methylenelanosterol (VIII; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me) | 6·15 × 10$^5$ | 224 | 0·98 | 0·94 | | 0·036 |

[a] 0·15% Incorporation allowing for a 22% recovery of precursor.

* Kindly provided by Koninklijke Nederlandsche Gist-en-Spiritus Fabriek N.V., Holland.

[13] J. R. Turner and L. W. Parks, *Biochim. Biophys. Acta*, 1965, **98**, 394.

[14] G. Ponsinet and G. Ourisson, *Bull. Soc. chim. France*, 1965, 3682.

[15] D. H. R. Barton, *J. Chem. Soc.*, 1953, 1027.

[16] D. H. R. Barton and W. Klyne, *Chem. and Ind.*, 1948, 755.

[17] W. Klyne, 'Determination of Organic Structures by Physical Methods,' ed. E. A. Braude and F. C. Nachod, 1955, p. 112.

[18] Ref. 17, p. 108.

[19] C. Djerassi, G. W. Krakower, A. J. Lemin, L. H. Liu, J. S. Mills, and R. Villotti, *J. Amer. Chem. Soc.*, 1958, **80**, 6284.

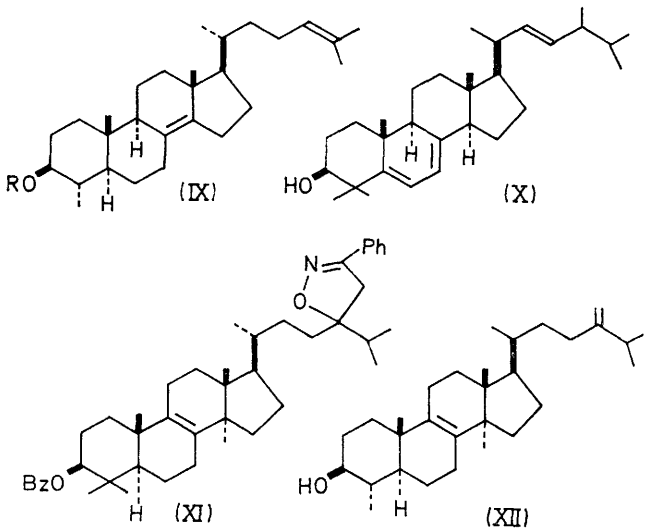
[20] C. Djerassi, O. Halpern, V. Halpern, and B. Riniker, *J. Amer. Chem. Soc.*, 1958, **80**, 4001.

was eliminated by the appearance of the 4-methyl proton signal as a doublet at $\tau$ 9·14 ($J$ 6 Hz). The 10- and 3-methyl resonances ($\tau$ 9·01 and 9·38 respectively) indicate 8(9)-unsaturation by comparison with 4α-methylzymosterol and with the calculated values [21] or $\tau$ 9·04 and 9·41 respectively for a $\Delta^{8(9)}$ nucleus and $\tau$ 9·28 and 9·16 respectively for the $\Delta^{8(14)}$ alternative.

Final proof of structure was secured by a partial synthesis of the sterol acetate (VIII; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H), by use of the sequence employed for the preparation of 24,25-dihydro-24-methylenelanosterol. 4α-Methylzymosteryl acetate was converted into a mixture of bromohydrins (IV; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H), m.p. 146—149°, $[\alpha]_D$ +54°. The n.m.r. spectrum (Table 1) indicated the formation of the required side-chain bromohydrins by the shift of the C-24 proton signal to $\tau$ 6·07. Chromatography of these on alumina grade III gave the mixed epoxides (V; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H), m.p. 160—163°, $[\alpha]_D$ +55°, after repeated crystallisation from acetone. Treatment of the epoxides with boron trifluoride–ether complex at ambient temperatures for 30 sec. gave, after purification by thick-layer chromatography, the ketone (VI; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H), m.p. 121—125°, $[\alpha]_D$ +61°. Finally, reaction of the ketone with triphenylmethylenephosphorane yielded the 24-methylene sterol acetate (VIII; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H), m.p. 110—112°, not depressed on admixture with the acetate of the natural material, and with identical spectral properties.

The second new component of the 4α-methyl fraction was indicated to be an isomer of 4α-methylzymosterol by the microanalytical data and the similarities of the mass, i.r., and u.v. spectra. The n.m.r. spectrum, however, showed significant differences in the methyl region (see Table 1). In particular the singlets at $\tau$ 9·26 and 9·15 are assignable to the 10- and 13-methyl resonances respectively of an 8(14)-unsaturated nucleus (calculated [21] 9·28 and 9·16). Further confirmation of a 8(14)-unsaturated nucleus was obtained by a comparison of the molecular rotation of the sterol (+76°) and its acetate (+185°) with those of the known 24,25-dihydro-analogue (+76 and +181°, respectively).[19] These data strongly support the assigned formulation of this sterol as (IX). The origin however is not clear. Although acidic conditions, which could rearrange an 8(9)-olefin into an 8(14)-olefin were avoided during the separation process, the possibility of it being an artefact cannot be excluded. On the other hand 8(14)-unsaturated sterols have recently been implicated in the process of demethylation at C-14 in sterol biosynthesis.[22,23] The significance of an 8(14)-unsaturated sterol can only be assessed by the appropriate feeding experiments.

With these sterols on hand, attention was again turned to the biosynthesis of ergosterol. A series of preliminary feedings of 4α-methylzymosterol, obtusifoliol [24] (XII), 24,25-dihydro-4α-methyl-24-methylenezymosterol,




and 4,4-dimethylergosterol,[25] tritiated at C-2 (and/or C-4) indicated that yeast could assimilate to a significant extent all but 4,4-dimethylergosterol. More rigorous experimentation has since shown that the figures quoted in our preliminary communication for these incorporations may be significantly high. These new data will be reported in a subsequent paper. The results of the double-labelled feedings with 24,25-dihydro-24-methylenelanosterol (Table 4) indicate that this precursor was incorporated without scrambling.

These observations show that yeast can assimilate a series of 24-methylene sterols, even those which have not been detected in the organism. The incorporation of 24,25-dihydro-24-methylenelanosterol has also been observed by Akhtar.[26] The incorporation of 4α-methylergosta-8-24(28)-dienol, which cannot be interconvertible with zymosterol (see Scheme 4), a reported precursor of ergosterol,[27] means that there can be no unique biosynthetic route to ergosterol. Thus side-chain alkylation may occur at any one of at least two stages of steroid nucleus modification, the 4α-methyl and 4-desmethyl stages. Furthermore, the incorporation of obtusifoliol, a widely occurring [28] plant sterol which has never been detected in yeast, and the synthetic 24,25-dihydro-24-methylenelanosterol indicate that apparently unnatural precursors may be assimilable, provided that

[21] N. S. Bhacca and D. H. Williams, ' Applications of N.m.r. Spectroscopy in Organic Chemistry,' Holden-Day Inc., San Francisco, 1964, pp. 19—24.
[22] W. H. Lee and G. J. Schroepfer, *Biochem. Biophys. Res. Comm.*, 1968, **32**, 635.
[23] J. Fried, A. Dudowitz, and J. W. Brown, *Biochem. Biophys. Res. Comm.*, 1968, **32**, 568.
[24] J. Bermejo Barrera, J. L. Bretón, J. Delgado Martín, and A. González González, *Anales real Soc. espan. Fis., Quim.*, 1967, **63***B*, 191.
[25] G. Cooley, B. Ellis, and V. Petrow, *J. Chem. Soc.*, 1955, 2998.
[26] M. Akhtar, M. A. Parvez, and P. F. Hunt, *Biochem. J.*, 1966, **100**, 38c.
[27] H. Katsuki and K. Bloch, *J. Biol. Chem.*, 1957, **242**, 222.
[28] L. J. Goad, B. L. Williams, and T. W. Goodwin, *European J. Biochem.*, 1967, **3**, 232.

certain gross structural requirements are satisfied. These would appear to be: (1) a 24,25-double bond as the prerequisite for side-chain alkylation,[2] (2) an 8(9)-double bond for removal of the 14-methyl group,[29] and (3) the presence of a 3β-hydroxy-group for the removal of the 4-methyl groups.[30] Results so far indicate that there is a degree of latitude in the sequencing of these events. The non-incorporation of 4,4-dimethylergosterol indicates however that the loss of the methyl groups must occur at a relatively early stage.

cent of the active 2,3-epoxysqualene was recovered. The lanosterol produced contained 13·4% of the activity fed. 24,25-Dihydro-24-methylenelanosterol was conveniently isolated and characterised as the isoxazaline derivative (XI), formed by a 1,3-dipolar addition of benzonitrile oxide to the *exo*-methylene group. It was totally inactive. That the cell-free system will metabolise sterols beyond the lanosterol stage has been shown by the work of Block and his co-workers,[27] and therefore the lack of activity in the 24,25-dihydro-24-methylene-

TABLE 3

Feedings to yeast

| Precursor | Activity (decomp.) sec.$^{-1}$) | Recovered precursor (decomp. sec.$^{-1}$) From medium | Recovered precursor (decomp. sec.$^{-1}$) From cells | Ergosterol Yield (mg.) | Ergosterol Specific activity (decomp. sec.$^{-1}$ mmole$^{-1}$) | Ergosteryl acetate. Specific activity (decomp. sec.$^{-1}$ mmole$^{-1}$) | Dimedone derivative of 2,3-dimethylbutanal. Specific activity (decomp. sec.$^{-1}$ mmole$^{-1}$) | Incorporation (%) |
|---|---|---|---|---|---|---|---|---|
| [2-$^3$HLanosterol (III; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me) | 6·04 × 10$^5$ | 2·24 × 10$^5$ | 0·07 × 10$^5$ | 90 | 1·16 × 10$^4$ | 1·06 × 10$^4$ | | 0·71 [a] |
| [24-$CH_2$-$^3H_2$]-23,25-Dihydro-24-methylenelanosterol (VIII; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me) | 7·64 × 10$^5$ | 1·06 × 10$^5$ | 0·89 × 10$^5$ | 128 | 2·72 × 10$^4$ | 2·82 × 10$^4$ | 3·07 × 10$^4$ | 1·5 [a] |
| [24-$CH_2$-$^3H_2$]-24,25-Dihydro-24-methylenelanosterol (VIII; $R^1$ = H, $R^2$ × $R^3$ = $R^4$ = Me) [c] | 6·2 × 10$^5$ | 4·6 × 10$^5$ | | 6 [b] | 7·92 × 10$^2$ | | | ⩽0·12 [a] |

[a] Recovered precursor allowed for. [b] Diluted with inactive material (100 mg.). [c] Fed to the cell-free system of Turner and Parks.[13]

TABLE 4

Feeding of [24-$CH_2$-$^{14}$C, (23,25),24-$CH_2$-$^3H_2$]-24,25-dihydro-24-methylenelanosterol to yeast

| | Precursor activity $^3$H (decomp. sec.$^{-1}$ mmole$^{-1}$) | Precursor activity $^{14}$C (decomp. sec.$^{-1}$ mmole$^{-1}$) | Atomic ratio | Dimedone derivative of formaldehyde activity $^3$H (decomp. sec.$^{-1}$ mmole$^{-1}$) | Dimedone derivative of formaldehyde activity $^{14}$C (decomp. sec.$^{-1}$ mmole$^{-1}$) | Atomic ratio | Ergosterol Yield (mg.) | Ergosterol $^3$H (decomp. sec.$^{-1}$ mmole$^{-1}$) | Ergosterol $^{14}$C (decomp. sec.$^{-1}$ mmole$^{-1}$) | Atomic ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| (1) | 8·14 × 10$^7$ [a] | 3·58 × 10$^6$ | 22·7 | 5·72 × 10$^7$ | 3·71 × 10$^6$ | 15·4 | 112 [c] | 9·15 × 10$^3$ | 4·04 × 10 | 22·6 |
| (2) | 1·21 × 10$^8$ [b] | 7·83 × 10$^6$ | 15·5 | | | 14·6 | 1020 | 5·15 × 10$^3$ | 3·29 × 10$^2$ | 15·7 |

| | Ergosterol acetate $^3$H (decomp. sec.$^{-1}$ mmole$^{-1}$) | Ergosterol acetate $^{14}$C (decomp. sec.$^{-1}$ mmole$^{-1}$) | Atomic ratio | Dimedone derivative of 2,3-dimethylbutanal $^3$H (decomp. sec.$^{-1}$ mmole$^{-1}$) | Dimedone derivative of 2,3-dimethylbutanal $^{14}$C (decomp. sec.$^{-1}$ mmole$^{-1}$) | Atomic ratio | Incorporation (%) |
|---|---|---|---|---|---|---|---|
| (1) | 8·41 × 10$^3$ | 3·55 × 10$^2$ | 23·7 | | | | 0·62 |
| (2) | 4·95 × 10$^3$ | 3·02 × 10$^2$ | 16·2 | 3·77 × 10$^3$ | 2·19 × 10$^2$ | 17·2 | 4·8 |

[a] Fed by our standard method. [b] Fed by the method of Akhtar.[12] [c] Diluted to 302 mg. with inactive ergosterol.

The apparent non-specificity of the enzyme systems concerned means that no feeding experiment alone can provide conclusive evidence for the intermediacy of a supposed precursor. It is necessary for the precursor to be detected in the organism, either by isolation or by trapping experiments to obtain such proof. Accordingly, in experiments carried out by Mr. G. Mellows, [1,24,25,30-$^3H_4$]-2,3-epoxysqualene [31] and inactive 24,25-dihydro-24-methylenelanosterol were fed to a cell-free yeast system [13] during 14 hr. Seventy-seven per-

lanosterol cannot be due to a metabolic block in the system used. It thus appears that 24,25-dihydro-24-methylenelanosterol can, in fact, be excluded as a precursor of ergosterol in *Saccharomyces cerevisiae*.

## EXPERIMENTAL

Radioactivity was measured with an Isotopes Development Limited type 6012 Liquid Scintillation Head at

[29] *Cf.* M. Akhtar, I. A. Watkinson, A. D. Rakimtula, D. C. Wilton, and K. A. Munday, *Chem. Comm.*, 1968, 1406.

[30] W. L. Miller, M. E. Kalafer, J. L. Gaylor, and C. V. Delwich, *Biochemistry*, 1967, **6**, 2673; A. C. Swindell and J. L. Gaylor, *Fed. Proc.*, 1967, **26**, 341.

[31] J. D. Willet, K. B. Sharpless, K. E. Lord, E. E. van Tamelen, and R. B. Clayton, *J. Biol. Chem.*, 1967, **242**, 4182.

efficiencies of 25% for $^3H$ and 80% for $^{14}C$, for single isotope counting. Doubly labelled samples were counted at $^3H$ and $^{14}C$ efficiencies of 20 and 20% at the lower gate voltages and 2·5 and 65% respectively at the higher gate voltages.

Preparative t.l.c. was performed on 0·2 mm. layers at a loading of 10—25 mg. of mixture to 8 g. of silica gel. Silver nitrate–silica gel plates contained 10% silver nitrate by weight.

Unless otherwise stated, i.r. spectra were run for Nujol mulls, optical rotations determined for solutions in AnalaR chloroform, u.v. spectra obtained for solutions in absolute ethanol, and n.m.r. spectra obtained for solutions in deuteriochloroform at 60 MHz.

Dried bakers yeast (*Sacchoromyces cerevisiae*) was supplied by Heath and Heather Limited, St. Albans, Herts., and *P. sulphureus* by the Centraalbureau voor Schimmelcultur, Baarn, Netherlands.

3β-*Acetoxy*-24,25-*epoxylanost*-8-*ene* (V; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me).—Monoperphthalic acid in chloroform (0·18M; 46 ml.) was added dropwise during 0·5 hr. to a stirred solution of lanosteryl acetate (3·47 g.) in chloroform (5 ml.) at 0°. After 18 hr. the solvent was removed under reduced pressure and the residue was suspended in benzene and washed with aqueous sodium carbonate, followed by water. The crude product was chromatographed on grade III alumina (220 g.). Elution with benzene–petroleum (1 : 4) removed unchanged lanosteryl acetate; benzene–petroleum (3 : 7) eluted the required epoxide (70%), m.p. 170—182° (from acetone–methanol) (m.p. 194° after repeated crystallisation) (lit.,[32] 185°).

*Separation of the Epimeric Epoxides*.—Careful chromatography of the crude epoxides from lanosteryl acetate (840 mg.) on grade III alumina gave two maxima in the wt./ml. ratios of epoxide fractions eluted. The faster running epoxide (A) (210 mg.) gave needles from acetone, m.p. 138—141° (resolidifying immediately to needles, m.p. 144—146°), $[\alpha]_D^{25}$ +53° (*c* 3) (lit.,[32b] m.p. 137·5—138·5°, $[\alpha]_D$ 42·5°). The more slowly eluted epoxide (B) (270 mg.) gave plates from acetone, m.p. 196—198°, $[\alpha]_D^{25}$ +60° (*c* 3) (lit.,[32b] m.p. 190—191°, $[\alpha]_D$ +53·3°). The n.m.r. (Table 1) and i.r. (carbon tetrachloride) spectra of the isomers were almost identical.

*Rearrangement of the Mixed Epoxides* (V; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me).—Epimeric lanosteryl acetate monoepoxides (3·83 g.) in dry benzene (250 ml.) were treated with boron trifluoride–ether complex (5 ml.) for 5 min. at room temperature. The solution was washed with aqueous sodium carbonate followed by water. The product consisted of the required ketone (VI; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me) together with an isomeric aldehyde (VII; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me, $R^5$ = H) [τ 0·61 (sharp s); t.l.c. (benzene) $R_F$ 0·2 (ketone) and 0·25 (aldehyde)]. The benzene extract was shaken with chromic oxide (4 g.) in water (20 ml.) and acetic acid (50 ml.) for 3 days at room temperature. The benzene phase was washed with water, thoroughly extracted with saturated aqueous sodium carbonate, and again washed with water. Removal of the solvent under reduced pressure gave the crude ketone (2·62 g.). Chromatography on grade V alumina (90 g.) with benzene–light petroleum (3 : 17) as eluant gave pure 3β-*acetoxy*-2-*lanost*-8-*en*-24-*one*, m.p. 135—137° (from acetone–methanol), $[\alpha]_D^{23}$ +58° (*c* 3), $\nu_{max.}$ 1732s, 1712s, and 1250s cm.$^{-1}$; only end absorption in the u.v. region (Found: C, 79·3; H, 10·6. $C_{32}H_{52}O_3$ requires C, 79·3; H, 10·8%).

The aqueous sodium carbonate extract from the oxidation of the crude boron trifluoride rearrangement products was neutralised and extracted with benzene. The extract yielded a gel (1·21 g.). Treatment with diazomethane in ether at ambient temperature gave *methyl* 3β-*acetoxy*-24,24-*dimethyl*-26,27-*bisnorlanost*-8-*en*-25-*oate* (VII; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me, $R^5$ = OMe), m.p. 155—158°, needles from acetone–methanol, $[\alpha]_D^{26}$ +51° (*c* 2), $\nu_{max.}$ 1735s and 1245s, cm.$^{-1}$ (Found: C, 76·8; H, 10·4. $C_{33}H_{54}O_4$ requires C, 77·0; H, 10·55%).

3β-*Acetoxy*-24-*methylenelanost*-8-*ene* (VIII; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me).— Methyltriphenylphosphonium bromide (3·51 g.) was shaken for 2 hr. with phenyl-lithium in ether (1·4M; 8 ml.) under dry nitrogen. The solution was filtered into a solution of 24-oxolanostenyl acetate (402 mg.) in dry ether (80 ml.) and the mixture was stirred overnight. Ether was replaced by dry tetrahydrofuran and the solution was refluxed overnight. Most of the tetrahydrofuran was removed *in vacuo*, water (100 ml.) was added, and the mixture was extracted with ether. The extract was washed rapidly with N-sulphuric acid, followed by water. The crude product was reactylated with pyridine–acetic anhydride at 100° for 2 hr., rechromatographed on a thick (2 mm.) plate and eluted with benzene. The band $R_F$ 0·5 was eluted with acetone to yield 3β-acetoxy-24-methylenelanost-8-ene (VIII; $R^1$ = Ac, $R^2$ = $R^3$ = $R^4$ = Me) (75%), m.p. 147—150° (plates from acetone–methanol) (lit.,[4b] m.p. 138—139°), $\nu_{max.}$ 3100, 1645, 890 (C=$CH_2$), 1735, and 1245 ($MeCO_2$) cm.$^{-1}$; only end absorption in the u.v. spectrum (Found: C, 82·4; H, 11·1. Calc. for $C_{33}H_{54}O_2$: C, 82·1; H, 11·25%).

24,25-*Dihydro*-24-*methylenelanosterol* (VIII; $R^1$ = H, $R^2$ = $R^3$ = $R^4$ = Me).—The acetate (260 mg.) was refluxed for 2 hr. with 2% methanolic potassium hydroxide (20 ml.), then treated with water and extracted with benzene to give 24,25-dihydro-24-methylenelanosterol (90%), m.p. 157·5—159° (needles from acetone–methanol), $[\alpha]_D^{25}$ +59° (*c* 2) (lit.,[4b] m.p. 158—159°, $[\alpha]_D^{17}$ +66°), $\nu_{max.}$ 3300s, 1645m, and 894s cm.$^{-1}$.

[24-$CH_2$-$^3H_2$]-24,25-*Dihydro*-24-*methylenelanosterol*.—Methyltriphenylphosphonium bromide (205 mg.) was dissolved in tritiated water (0·6 ml.; 0·2 c/ml.) in the presence of potassium t-butoxide (14 mg.). The solution was left at room temperature overnight and the solvent was removed *in vacuo*. The residue was crystallised from tritiated water (0·1 ml.) and dried for 2 days ($P_2O_5$ *in vacuo*) to give [*Me*-$^3H_3$]-methyltriphenylphosphonium bromide (78 mg.). The tritiated bromide was finely powdered and shaken with phenyl-lithium in ether (1·34M; 0·18 ml.) for 1 hr. The phosphorane solution was transferred to a Carius tube and 24-oxolanosteryl acetate (59 mg.) was added in ether (4 ml.) at −20°. The tube was sealed under nitrogen and maintained at 64° for 24 hr. After the usual work-up and reacetylation the product was chromatographed on thin silica gel plates to give [24-*CH*$_2$-$^3H_2$]-24,25-dihydro-24-methylenelanosteryl acetate (20 mg.). Hydrolysis gave the tritiated sterol (18·5 mg.), activity 1·11 × $10^5$ decomp. sec.$^{-1}$ mg.$^{-1}$.

[Me-$^{14}C$]*Methyltriphenylphosphonium Iodide*.— [$^{14}C$]-Methyl iodide (*ca.* 1 mg.; total activity 0·5 mc) was distilled under vacuum into triphenylphosphine (44 mg.) in

[32] (*a*) L. J. Bellamy and C. Dorée, *J. Chem. Soc.*, 1941, 176; (*b*) D. A. Lewis and J. F. McGhie, unpublished results.

dry benzene (0·3 ml.) in a Carius tube cooled in liquid nitrogen. The Carius tube was isolated and set aside at room temperature for 2 hr. Inactive methyl iodide (35 mg.) was distilled into the [$^{14}$C]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 hr. the precipitate was filtered off and washed with benzene to leave [*Me*-$^{14}$C]methyltriphenylphosphonium iodide (52 mg.), m.p. 186° (lit.,[33] 180°), of activity *ca.* $1·2 \times 10^5$ decomp. sec.$^{-1}$ mg.$^{-1}$.

[24-$CH_2$-$^{14}C$]-24,25-*Dihydro*-24-*methylenelanosterol*.—[*Me*-$^{14}$C]Methyltriphenylphosphonium iodide (21·5 mg.) was stirred for 2 hr., under nitrogen, while phenyl-lithium (1·33N; 0·04 ml.) in ether (4 ml.) was added dropwise; the mixture was stirred overnight. Ether was then replaced by dry tetrahydrofuran and the solution was refluxed overnight. The usual work-up gave [24-$CH_2$-$^{14}$C]-24,25-dihydro-24-methylenelanosterol (6 mg.) of activity $1·5 \times 10^5$ decomp. sec.$^{-1}$ mg.$^{-1}$.

*Radiopurity and Ozonolysis of* [24-$CH_2$-$^{14}C$,$^3H_2$]-24,25-*dihydro*-24-*methylenelanosterol*.—A mixture of [$^{14}$C]- and [$^3$H]-labelled sterols was cocrystallised. A sample of the doubly labelled sterol was diluted 100-fold with inactive 24,25-dihydro-24-methylenelanosterol and crystallised 6 times without change in specific activity to demonstrate the radiopurity.

The doubly labelled sterol (42 mg.) was suspended in glacial acetic acid (2 ml.) and ozonised oxygen was bubbled through the liquid until a clear solution resulted. Zinc dust (2 g.) was added and the solution was set aside for 30 min. Water (2 ml.) was added and the material was steam-distilled until 15 ml. of distillate had collected. The distillate was neutralised (phenolphthalein) with 4N-sodium hydroxide and the solution was rendered just colourless by addition of dilute acetic acid. A 10% solution of dimedone in ethanol (1 ml.) was added and after 12 hr. the precipitate of the dimedone derivative was collected and recrystallised from ethanol–water (yield 4 mg.).

The activity ratios ($^3$H : $^{14}$C) were 22·7 (for the sterol) and 15·4 (for the dimedone derivative of formaldehyde).

A second preparation, in which the initial phase of the Wittig reaction was carried out at 0° gave ratios of 15·5 and 14·6 respectively.

*Feeding of Labelled Precursors to* P. sulphureus.—Malt extract (30 g.) and glucose (30 g.) were dissolved in hot distilled water (1 l.) and the cooled solution was filtered through Celite. The filtrate was divided equally between six conical flasks (each 1 l.) which were loosely plugged with cotton wool and sterilised at 100° for 15 min. After 1 day at room temperature, the flasks were resterilised, and when cool 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. [24-$CH_2$-$^3H_2$]-24,25-Dihydro-24-methylenelanosterol 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 during 2 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 2 weeks then the mycelium was filtered off.

* Checked by Dr. G. Ponsinet.

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 1 hr. 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 hr. 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.,[6] 293°), and a fraction consisting mainly of eburicoic acid and some sulphurenic acid [34] (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 was crystallised from ethanol–water to give acetyleburicoic acid, m.p. 229—233° (lit.,[6] 259°), as 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 acetyleburicoate as needles, m.p. 159·5—161° (from acetone–methanol) (lit.,[6] 157—158°).

The methyl acetoxy-derivative was refluxed with 2% methanolic potassium hydroxide for 2 hr. to give methyl eburicoate, m.p. 147—149° (lit.,[6] 146—147°).

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 m.p.s. of the isolated acid and the 3-*O*-acetyl derivative.

The observed incorporations are presented in Table 3 together with those of [24-$CH_2$-$^{14}$C]-24,25-dihydro-24-methylenelanosterol and [2-$^3$H]lanosterol which were fed according to the same procedure.

*Isolation of* 4α-*Methyl Sterols from Yeast Residues*.—Yeast sterol residues (75 g.) were absorbed on a column of grade 5 alumina (1·75 kg.) and elution was commenced with benzene–petroleum (3 : 7; 3 l.) and benzene–petroleum (7 : 13; 3 l.). Benzene–petroleum (2 : 3; 2 l.) eluted the lanosterol fraction and a further 1 l. eluted the 4α-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 4α-methyl sterol fraction (600 mg.), homogeneous on silica gel t.l.c., was isolated. The process was repeated from crude yeast residues until 2 g. of the 4α-methyl sterol mixture was available.

*Isolation of* 4α-*Methylzymosterol from Yeast Residues*.—The 4α-methyl sterol mixture (1·92 g.) was treated with pyridine–acetic anhydride at room temperature overnight, and the crude acetate mixture was fractionally crystallised from acetone–methanol by the triangulation procedure.

This yielded pure 4α-methylzymosteryl acetate (1·43 g.), m.p. and mixed m.p. 143—145·5° (plates from acetone), $[\alpha]_D^{25}$ +65·4° (*c* 8) (lit.,[14] m.p. 138—139°, $[\alpha]_D$ +61°).

Hydrolysis of the acetate with potassium hydroxide–methanol gave 4α-methylzymosterol, m.p. and mixed m.p.*

[33] C. H. Collins and G. S. Hammond, *J. Org. Chem.*, 1960, **25**, 1434.

[34] J. Fried, P. Grabowich, E. F. Sabo, and A. I. Cohen, *Tetrahedron*, 1964, **20**, 2297.

132—134° (needles from acetone–methanol), $[\alpha]_D^{25}$ +63° (*c* 2) (lit.,[14] m.p. 130—131°, $[\alpha]_D$ not quoted).

4α-*Methylcholesta*-8,24-*dien*-3-*one*.— 4α-Methylzymosterol (108 mg.) in benzene (7 ml.) was shaken at room temperature for 0·5 hr. with chromic oxide (130 mg.) in water (1·3 ml.) and acetic acid (3 ml.). The crude product was purified on silica gel t.l.c. plates and material from the band with $R_F$ 0·5 was crystallised from acetone–methanol to give the required *ketone* (88 mg.), m.p. 105—107° (flat needles), $[\alpha]_D^{25}$ +50° (*c* 0·7).

*Isolation of* 4α-*Methylergosta*-8,24(28)-*dien*-3β-*ol* (VIII; $R^1$ = OH, $R^2$ = Me, $R^3$ = $R^4$ = H) *from Yeast Residues*.— The residues from the fractional crystallisation of the 4α-methyl sterol acetates (see before) were combined (204 mg.) and fractionated with benzene–petroleum (1 : 3) on preparative silica gel–silver nitrate t.l.c. plates. Two bands, A ($R_F$ 0·1—0·3) and B ($R_F$ 0·35—0·5) were faintly visible in daylight (but not under u.v. light). Band A was removed with chloroform to give 3β-*acetoxy*-4α-*methylergosta*-8,24-(28)-*diene* (81 mg.), m.p. 110—112° (plates from acetone–methanol), $[\alpha]_D^{29\cdot5}$ +61° (*c* 1), $\nu_{max.}$ 3086w, 1645m, 898s, (C=$CH_2$), 1744s, and 1250s ($MeCO_2$) cm.$^{-1}$; only end absorption in the u.v. spectrum (Found: C, 81·7; H, 11·1. $C_{31}H_{50}O_2$ requires C, 81·9; H, 11·1%).

Alternatively the acetate fractions rich in the 24-methylene compound were hydrolysed with methanolic potassium hydroxide and the sterol mixture was treated with pyridine–benzoyl chloride overnight. The mixed benzoates (409 mg.) were eluted four times with benzene–petroleum (1 : 4) on silica gel silver–nitrate preparative t.l.c. Two bands were visible under u.v. light, $R_F$ 0·4—0·5 and 0·2—0·35. The latter band was extracted with chloroform, and the extracted benzoate was filtered through grade III alumina in benzene–petroleum (2 : 3) to give 3β-*benzoyloxy*-4α-*methylergosta*-8,24(28)-*diene* (258 mg.), m.p. 142—144° (thin rods from chloroform–methanol), $[\alpha]_D^{27}$ +81° (*c* 2), $\nu_{max.}$ 3100w, 1645m, 898s (C=$CH_2$), 1715s, 1605m, 1588w, 1500w, and 1275s ($PhCO_2$) cm.$^{-1}$, $\lambda_{max.}$ 228 (ε 14,000), 273 (1000), and 281 (700) mμ in chloroform–ethanol (1 : 10) (Found: C, 83·3; H, 10·1. $C_{36}H_{52}O_2$ requires C, 83·65; H, 10·15%).

Hydrolysis of the acetate or benzoate with 2% methanolic potassium hydroxide gave 4α-*methylergosta*-8,24(28)-*dien*-3β-*ol*, m.p. 143—145° (needles from acetone–methanol), $[\alpha]_D^{28}$ +55° (*c* 3), $\nu_{max.}$ 3400, 1647, and 888 cm.$^{-1}$ (Found: C, 84·5; H, 11·8. $C_{29}H_{48}O$ requires C, 84·4; H, 11·7%).

4α-*Methylergosta*-8,24(28)-*dien*-3-*one*.—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 4α-*methylergosta*-8,24(28)-*dien*-3-*one* (70 mg.), m.p. 123—125° (needles from acetone–methanol), $[\alpha]_D^{26}$ +47° (*c* 0·9), $\nu_{max.}$ ($CCl_4$) 1710, 1644, and 890 cm.$^{-1}$, 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° (Found: C, 84·7; H, 11·25. $C_{29}H_{46}O$ requires C, 84·8; H, 11·3%).

*Isolation of* 4α-*methylcholesta*-8(14),24-*dien*-3β-*ol* (IX) *from Yeast Residues*.—As already described, preparative t.l.c. on silica gel impregnated with silver nitrate separated the fractional crystallisation residues from the 4α-methyl sterol acetates into two bands, A and B. Band A was homogeneous and afforded the acetate of the new 4α-methylergostadienol.

Band B had partially separated into two zones $B_1$ and $B_2$. Zone $B_1$, when eluted three times on silica gel–silver nitrate t.l.c. with benzene–petroleum (2 : 3), had $R_F$ 0·44 and was inseparable from 4α-methylzymosteryl acetate. Zone $B_2$ under these conditions had $R_F$ 0·50.

Repeated t.l.c. purification of $B_2$ as just described gave 3β-*acetoxy*-4α-*methylcholesta*-8(14),24-*diene* (20 mg.), m.p. 78—80°, $[\alpha]_D^{25}$ +42° (*c* 0·2), $\nu_{max.}$ 1742 and 1245 cm.$^{-1}$ (Found: C, 81·6; H, 11·2. $C_{30}H_{48}O_2$ requires C, 81·75; H, 11·0%)

Hydrolysis of the acetate with 2% methanolic potassium hydroxide gave 4α-*methylcholesta*-8(14),24-*dien*-3β-*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, 11·65%).

3β-*Acetoxy*-24ξ-*bromo*-25-*hydroxy*-4α-*methylcholest*-8-*ene* (IV; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H).—4α-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 during 45 min. at room temperature. The solution was stirred for a further 15 min. then evaporated to a small volume *in vacuo*, and water was added. The mixture was extracted with ether and the extract was 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 purified on silica gel t.l.c. plates with acetone–light petroleum (1 : 9). The material from the band with $R_F$ 0·5 was repeatedly crystallised from acetone to give 3β-*acetoxy*-24ξ-*bromo*-25-*hydroxy*-4α-*methylcholest*-8-*ene*, m.p. 146—149° $[\alpha]_D^{23}$ +54° (*c* 1·9), $\nu_{max.}$ 3590m (OH), 1735s, 1250s ($MeCO_2$), and 1665w (C=C) cm.$^{-1}$ (Found: C, 66·7; H, 9·3; Br, 14·7. $C_{30}H_{49}BrO_3$ requires C, 67·0; H, 9·2; Br, 14·85%).

3β-*Acetoxy*-24ξ,25-*epoxy*-4α-*methylcholest*-8-*ene* (V; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H).—The crude 4α-methylzymosteryl acetate bromohydrin (767 mg.) was eluted on a column of grade III alumina (50 g.) successively with petroleum–benzene (9 : 1; 200 ml.), (4 : 1; 200 ml.), and (7 : 3; 500 ml.), which removed traces of 4α-methylzymosteryl acetate. Petroleum–benzene (3 : 2; 3 l.) eluted the required mixed epoxides (86%), m.p. 123—131°.

Repeated crystallisation of the mixed epoxides from acetone gave 3β-*acetoxy*-24ξ,25-*epoxy*-4α-*methylcholest*-8-*ene*, m.p. 160—163° (plates from acetone) $[\alpha]_D^{24}$ +55° (*c* 0·9) (Found: C, 78·9; H, 10·6. $C_{30}H_{48}O_3$ requires C, 78·9; H, 10·6%).

3β-*Acetoxy*-4β-*methylcholest*-8-*en*-24-*one* (VI; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H).—4α-Methylzymosteryl acetate epoxide (137 mg.) in benzene (3·5 ml.) was treated with boron trifluoride–ether complex in benzene (1 : 20; 0·7 ml.) for 30 sec. at room temperature. The reaction was quenched by shaking with aqueous sodium hydrogen carbonate. The benzene phase was washed with water and solvent was removed to leave an oil. T.l.c. on silica gel, eluted three times with benzene, showed five components, $R_F$ 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., with elution three times with benzene.

The band $R_F$ 0·5—0·6 provided a crystalline substance A (23 mg.), $\nu_{max.}$ ($CCl_4$) 2690m, 1735s, and 1245s cm.$^{-1}$, τ 0·57 (1H), 5·62 (1H, m), 7·97 ($MeCO_2$), 8·96 (2 × Me), 9·02 (Me), 9·16 (d, *J* 6 Hz, Me), and 9·40 Me).

The band $R_F$ 0·2—0·3 provided a substance D (20 mg.), $\nu_{max.}$ ($CCl_4$) 3650m, 1730s, 1250s, and 680s cm.$^{-1}$, τ 5·6

(1H, m), 6·55 (1H, m), 7·95 ($MeCO_2$), 8·49 (Me), 9·02 (Me), 9·11 (d, $J$ 6 Hz, Me), and 9·38 (Me).

The band $R_F$ 0·05—0·2 gave a substance E (47 mg.), $\nu_{max.}$ ($CCl_4$) 3600m, 1730s, and 1250s cm.$^{-1}$, which was not further investigated.

Finally the band $R_F$ 0·3—0·5 provided the required ketone (B) and the trace component (C). This mixture (23 mg.) was eluted ten times with benzene–petroleum (1 : 1) on silica gel t.l.c. plates. The band $R_F$ 0·3—0·5 provided pure 3β-*acetoxy*-4α-*methylcholest*-8-*en*-20-*one*, m.p. 102·5—104·5° (needles from acetone–methanol), $[\alpha]_D^{25}$ +61° (*c* 1), $\nu_{max.}$ ($CCl_4$) 1735, 1245 ($MeCO_2$), and 1708 (C=O) cm.$^{-1}$ (Found: C, 78·9; H, 10·4. $C_{30}H_{48}O_3$ requires C, 78·9; H, 10·6%).

3β-*Acetoxy*-4α-*methylergosta*-8,24(28)-*diene* (VIII; $R^1$ = Ac, $R^2$ = Me, $R^3$ = $R^4$ = H).— Methyltriphenylphosphonium bromide (210 mg.) was stirred in ether (2 ml.) with ethereal phenyl-lithium (1·57M; 0·4 ml.) for 2 hr., under dry nitrogen. 3β-Acetoxy-4α-methylcholest-8-en-24-one (15 mg.) was added in ether (4 ml.) and the mixture was stirred for 20 hr. The ether was replaced by tetrahydrofuran freshly distilled from lithium aluminium hydride, and the solution was refluxed for 20 hr.

It was then treated with water and extracted with ether, and the product was reacetylated with pyridine–acetic anhydride (1 : 1). The crude product was purified by developing twice with benzene–petroleum (1 : 1) on silica gel t.l.c. plates. The band $R_F$ 0·37—0·46 furnished 3β-*acetoxy*-4α-*methylergosta*-8,24(28)-*diene* (10 mg.), m.p. and mixed m.p. with the acetate of the natural product 110—112° (plates from acetone–methanol), $[\alpha]_D^{23}$ +61° (*c* 0·5). The i.r., n.m.r., and mass spectra were identical with those of the acetate of the natural product (Found: C, 81·65; H, 10·8. $C_{31}H_{50}O_2$ requires C, 81·9; H, 11·1%).

4,4-*Dimethylergosta*-5,7,22-*trien*-3-*one*.—Ergosterone, m.p. 128—132° (plates from acetone), $\lambda_{max.}$ 239 mμ (ε 14,300) [lit.,[35] m.p. 132°, $\lambda_{max.}$ 242 mμ (ε 14,800)] was prepared from ergosterol by Oppenauer oxidation as described by Shepherd and his co-workers.[35]

Treatment of ergosterone with potassium t-butoxide and methyl iodide in t-butyl alcohol[25] gave 4,4-dimethylergosta-5,7,22-trien-3-one, m.p. 161—165° (from acetone) (lit.,[25] 167—168°).

*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 hr. The usual work-up and silica gel t.l.c. purification furnished 4α,14α-*dimethylergosta*-8,24(28)-*dien*-3-*one* (54 mg.), m.p. 109—111°, $[\alpha]_D^{26}$ +67° (*c* 0·4), $\nu_{max.}$ (Nujol and $CCl_4$); 1710, 1645, and 890 cm.$^{-1}$; only end absorption in the u.v. spectrum, *M*, 424 (mass spec.).

[2-$^3$H]*Lanosterol*.—Lanosterol (1·5 g.) in benzene (100 ml.) was shaken for 5 hr. 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 as needles (1·25 g.), m.p. 80° (lit.,[6] 80—81°).

Tritiated water (0·5 ml.; 0·2 c/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 t-butyl [$^3$H]alcohol.

* Supplied by Professor A. González González.

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 N-hydrochloric acid (2 ml.) and extracted with benzene; the extract was washed with water and evaporated. Crystallisation from acetone–methanol gave [2-$^3$H]lanostadienone (97 mg.), activity 3 × $10^5$ decomp. sec.$^{-1}$ mg.$^{-1}$.

The ketone (95 mg.) in dry ether (40 ml.) was added to lithium aluminium hydride (283 mg.) in ether (10 ml.) and refluxed for 2 hr. After addition of water the ethereal layer was washed rapidly with dilute hydrochloric acid, then water, and the solvent was removed. The residue was eluted on silica gel t.l.c. plates with ethyl acetate–benzene (1 : 9). The band $R_F$ 0·45 was eluted, giving [2-$^3$H]-lanosterol, m.p. and mixed m.p. 136° (lit.,[6] 140°), activity 2·69 × $10^5$ decomp. sec.$^{-1}$ mg.$^{-1}$.

*Feedings of Labelled Precursors to Yeast* (Saccharomyces cerevisae).—(a) *Anaerobic growth*. 'Oxoid' malt extract (60 g.) was shaken with boiling water (2 l.) and the cooled solution was filtered through Celite. The filtrate was divided between two conical flasks (1 l.) which were plugged with cotton wool. Both flasks were sterilised at 100° for 15 min., set aside at room temperature for 16 hr., and then 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. The cultures were incubated at 30° for 40 hr., without shaking. Yeast was harvested by centrifugation for 30 min. The precipitate was washed twice by centrifugation with 0·1M-phosphate buffer (pH 6·5).

(b) *Aerobic growth*. The yeast (16 ml.) was resuspended in a solution of glucose (10 mg.) and potassium dihydrogen phosphate (13·6 g.) in water (1 l.). [28-$^3H_2$]-24,25-Dihydro-24-methylenelanosterol (2·67 mg.) and Tween 80 (60 mg.) in acetone (2 ml.) was divided between three flasks (each 1 l.) and the solvent was 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 hr. 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, $R_F$ 0·4, was removed and counted.

The yeast was refluxed with 10% methanolic potassium hydroxide (300 ml.) for 4 hr. Water (300 ml.) was added and the non-saponifiable material was 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; 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 was crystallised from acetone to constant specific activity. Finally, ozonolysis of the biosynthesised ergosterol, according to the procedure

[35] D. A. Shepherd, R. A. Donia, J. A. Campbell, B. A. Johnson, R. P. Holysz, G. Slomp, J. E. Stafford, R. L. Pederson, and A. C. Ott, *J. Amer. Chem. Soc.*, 1955, **77**, 1212.

of Hannahan and Wakil,[36] gave 2,3-dimethylbutanal, which was counted as its dimedone derivative, m.p. 158—159° (from ethanol–water).

Similarly, [24-$CH_2$-$^{14}$C, (23,25),24-$CH_2$-$^3H_2$]-24,25-dihydro-24-methylenelanosterol was fed under aerobic conditions. The incorporations are presented in Tables 3 and 4.

In addition, [24-$CH_2$-$^3H_2$]-24,25-dihydro-24-methylenelanosterol (2·1 mg.) in Tween 80 (45 mg.) and acetone (1 ml.) were added to the cell-free system prepared according to the method of Turner and Parks[13] (60 ml.). After 17 hr. at 30°, potassium hydroxide (20 mg.) in methanol (200 ml.) was added, together with inactive ergosterol (4 mg.), and the mixture was refluxed for 4 hr. The nonsaponifiable material was extracted with benzene and fractionated by t.l.c. to give recovered precursor, and the ergosterol fraction. This was diluted with a further 200 mg. of inactive ergosterol and crystallised to constant activity (see Table 3).

A modified method of feeding to whole cells, described by Akhtar and his co-workers[7] was used, in addition to the whole cell procedure for the doubly labelled precursor. The results are compared in Table 4.

*Attempted Trapping of 24,25-Dihydro-24-methylenelanosterol in a Cell-free Yeast System* (with Mr. G. Mellows). —An aqueous emulsion of [1,24,25,30-$^3H_4$]-2,3-epoxysqualene (2·213 mg., $4·31 \times 10^7$ decomp./min.), inactive 24,25-dihydro-24-methylenelanosterol (30 mg.) and Tween 80 (100 mg.) were incubated at 29° with a cell-free system[13] of *Saccharomyces cerevisae* (100 mg.) for 14 hr. Inactive 2,3-epoxysqualene (115·4 mg.), lanosterol (101·1 mg.), and 24,25-dihydro-24-methylenelanosterol (93·8 mg.) were added and the mixture was saponified under nitrogen with 17% potassium hydroxide in methanol (150 mg.), for 2 hr. The non-saponifiable ether extract was separated by preparative t.l.c., with ethyl acetate–petroleum (6 : 94) as eluant. Removal of band $R_F$ 0·45—0·55 gave 2,3-epoxysqualene (79·0 mg.), activity $2·88 \times 10^5$ decomp. min.$^{-1}$ mg.$^{-1}$ (77% recovery). The sterols ($R_F$ 0·00—0·15) were extracted with acetone and benzoylated (benzoyl chloride–pyridine). The mixed benzoates were separated on preparative 20% silver nitrate–silica gel plates, by elution four times with benzene–petroleum (1 : 4). The band $R_F$ 0·45—0·55 gave lanosterol benzoate (102·8 mg.), which was crystallised to a constant activity of $5·22 \times 10^4$ decomp. min.$^{-1}$ mg.$^{-1}$ (13·4% incorporation). The band $R_F$ 0·30—0·40 gave 24,25-dihydro-24-methylenelanosterol benzoate (81·6 mg.), which was further purified by reaction of a sample (69 mg.) with benzohydroxamic acid chloride (85 mg.) and triethylamine (90 mg.) in ether (7 ml.) under reflux for 16 hr.. The solution was filtered and evaporated. The residue was purified by preparative t.l.c., with 10% chloroform–benzene as eluant. The band $R_F$ 0·42 gave the epimeric *isoxazoline* derivatives of 24,25-dihydro-24-methylenelanosteryl benzoate (XI), m.p. 160° (resolidifying; second m.p. 201—204°), $[\alpha]_D^{26}$ +33° (*c* 0·1), $\nu_{max.}$ 1710, 1690, and 1600 cm.$^{-1}$ (Found: C, 81·45; H, 9·15; N, 2·1. $C_{45}H_{61}NO_3$ requires C, 81·4; H, 9·3: N, 2·1%).

After four crystallisations, the activity had dropped to zero.

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[36] D. J. Hanahan and S. J. Wakil, *J. Amer. Chem. Soc.*, 1953, **75**, 273.