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YEAST STEROL BIOSYNTHESIS:
AN INVESTIGATION OF 31-NORLANOSTEROL AND 4,4-DIMETHYLFECOSTEROL AS POSSIBLE INTERMEDIATES

by

Leonard Avruch

B.A., Queens College (C.U.N.Y.), 1970

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

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Yeast Sterol Biosynthesis: An Investigation of 31-Norlanosterol and 4,4-Dimethylfecosterol as Possible Intermediates

Author:

(signature)

Leonard Israel AVRUCH

(name)

August 27, 1974

(date)

Abstract

The role of 31-norlanosterol (5) and 4,4-dimethylfecosterol (4) as possible intermediates in the biosynthesis of ergosterol by the yeast, S. cerevisiae, has been investigated. Authentic samples of 4 and 5 were synthesized for use as both chromatographic standards and trapping agents. A search for 4 and 5 was conducted in three sources of total yeast sterol mixtures: yeast sterol concentrates, fresh laboratory grown yeast and fresh brewery grown yeast. Sterol 4 was not detected in any of these sources. Sterol 5 was found only in the fresh brewery yeast. An attempt to trap radioactivity from exogenously fed tritiated lanosterol into 4 and 5 gave inconclusive results. Based on the isolation of 5 from brewery yeast we have tentatively expanded the model of yeast sterol biosynthesis to include all possible routes through sterol 5.

DEDICATION

Dedicated to my parents, in whose honour I hereby bestow upon the hitherto unsynthesized sterol, 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol, the trivial name "rejuliol".

ACKNOWLEDGMENTS

The author wishes to acknowledge the assistance provided by the following persons and organizations:

Dr. A. C. Oehlschlager, for his continued guidance and support.

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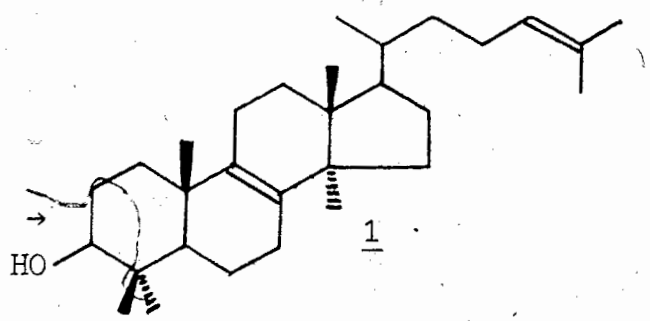
Introduction

The elaboration of sterols by the common yeast, Saccharamyces cerevisiae, encompasses a long sequence of biosynthetic conversions which can be traced from acetate to ergosterol (13)[1,2]. Based on the nature of the intermediates involved, this sequence may be divided into two phases, an isoprenoid phase and a steroid phase.

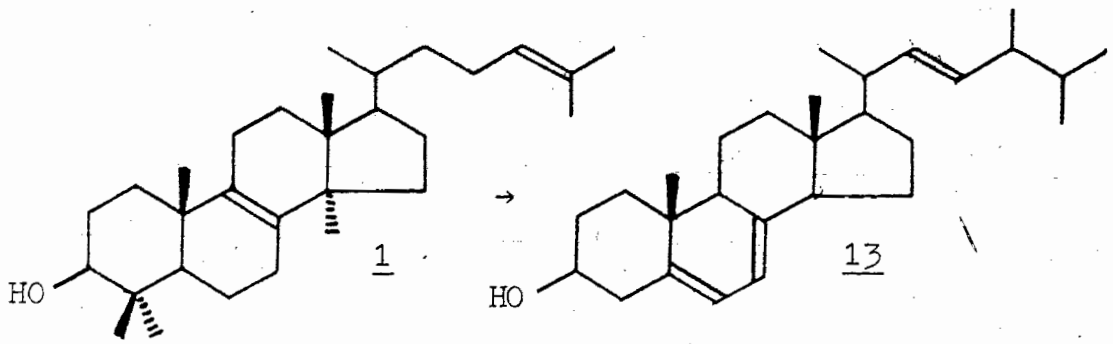
During the isoprenoid phase, the synthesis, elongation and cyclization of isoprenoid intermediates occurs[1]. It culminates in the formation of lanosterol (1).

Acetate → Mevalonate → Isoprenoids →



1

During the steroid phase, lanosterol (1) is converted to ergosterol (13) via seven essential structural modifications.



1 → 13

These modifications are:

1. removal of the 14α-methyl group (see appendix A for steroid numbering and nomenclature)
2. removal of the 4α-methyl group
3. removal of the 4β-methyl group
4. methylation at C_{24} (In actuality, this involves two steps. The introduction of a C_{24}-methylene group is followed at some later point by reduction of the $\Delta^{24(28)}$ double bond.)[1]
5. isomerization of the Δ^{8} double bond to Δ^{7}
6. introduction of the Δ^{22} double bond
7. introduction of the Δ^{5} double bond.

The order in which these seven modifications occur was initially open to speculation. It could be postulated that they occur randomly, in which case the number of alternate biosynthetic routes between 1 and 13 would be 7!/2! or 2520. On the other hand, it could also be postulated that one and only one route is followed. Evolutionary considerations argue against both these postulates. The former is energetically wasteful while the latter provides for no adaptive flexibility. We therefore opted for a median postulate, in which a single favoured route co-exists with a number of minor alternate pathways. It follows directly from this postulate that the enzyme or enzymes responsible for each particular

modification exhibit high, though not absolute, substrate specificity.

All evidence obtained thus far indicates that the structural transformations occurring between 1 and 13 do occur roughly in the order listed and that the concept depicted in Figure 1 is valid. This has been particularly well established for those events occurring between fecosterol (12) and ergosterol (13)[4,5]. Those modifications occurring prior to fecosterol (namely nuclear demethylation and side chain methylation) have, however, been given less careful consideration. It is these modifications, or more specifically the order in which they occur, toward which the present effort was directed.

The order of nuclear demethylation and side chain methylation is not the totally random process illustrated in Figure 2. Past experimental work involving three complementary approaches has verified some sections of this matrix and eliminated others. Figure 3 illustrates those routes and intermediates suggested by each of these three approaches.

The first and simplest approach involved the isolation and characterization of constituent yeast sterols. Implied in this approach is the assumption that most, if not all, of these sterols are true intermediates and not artefacts or side products. This approach has thus far netted the

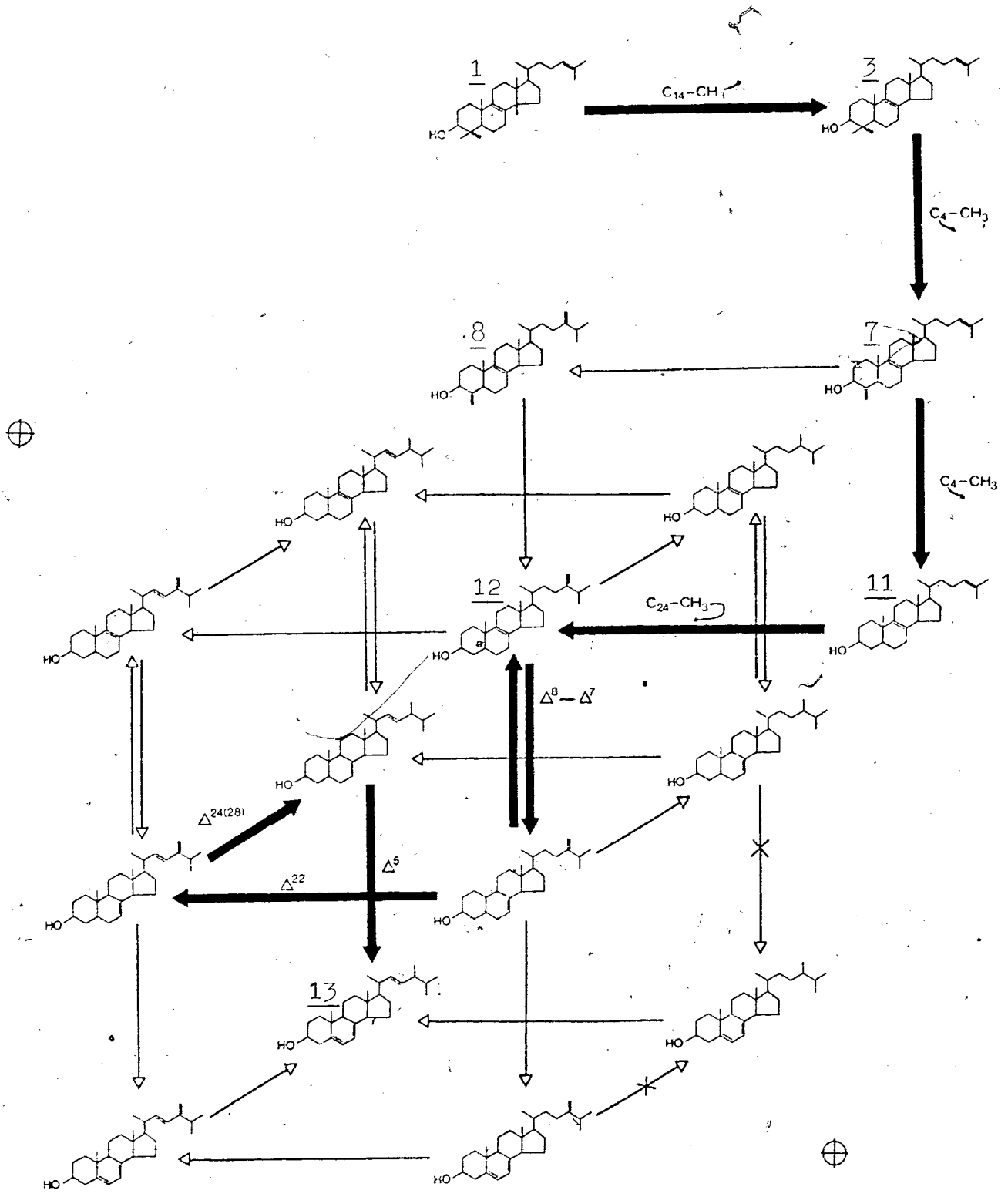


Figure 1 Sterol Biosynthesis in Nystatin Sensitive S. cerevisiae:[3]

Major Pathways Operative ▬
Minor Pathways Operative ——

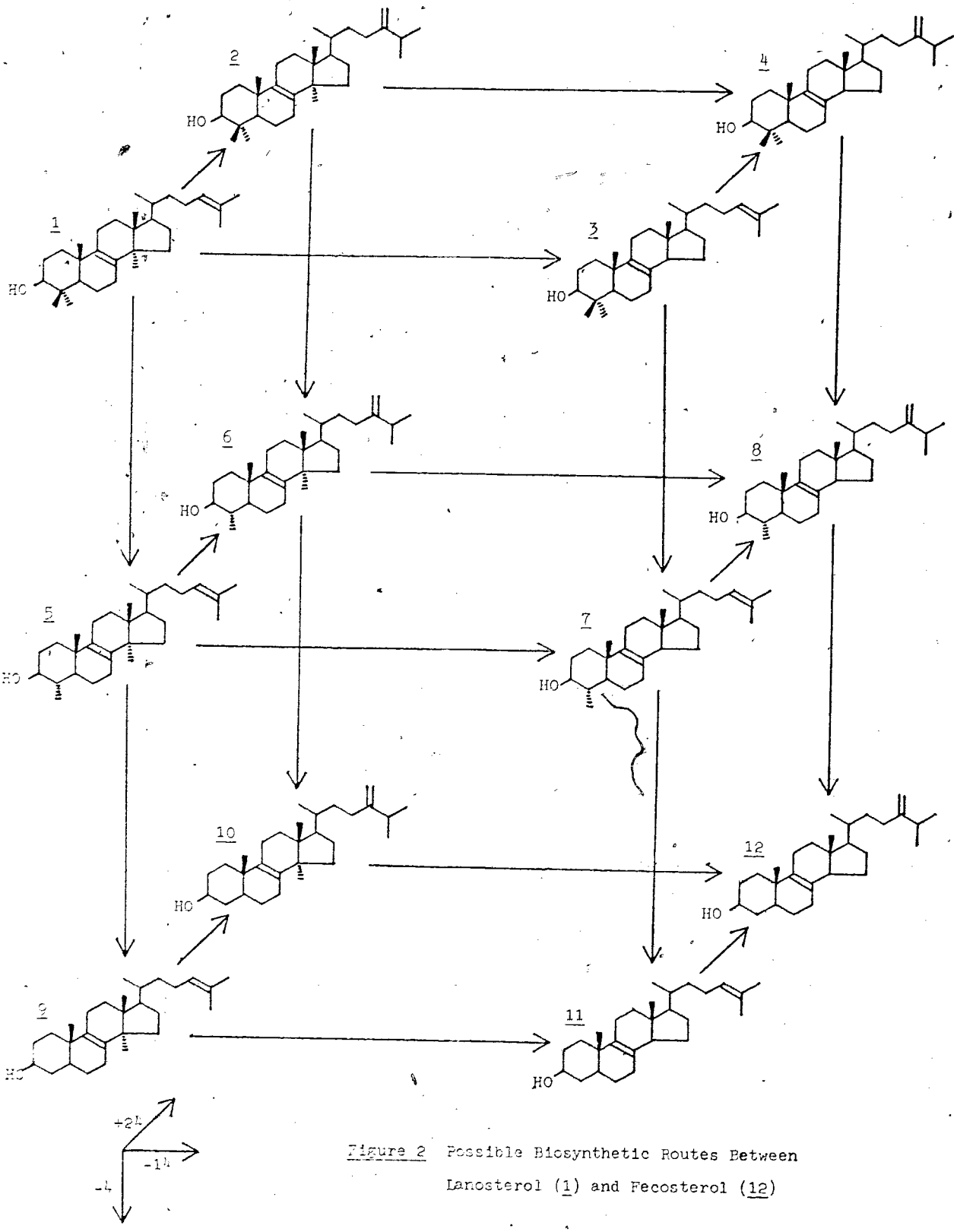
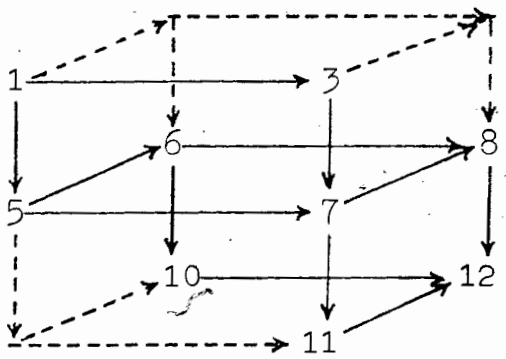
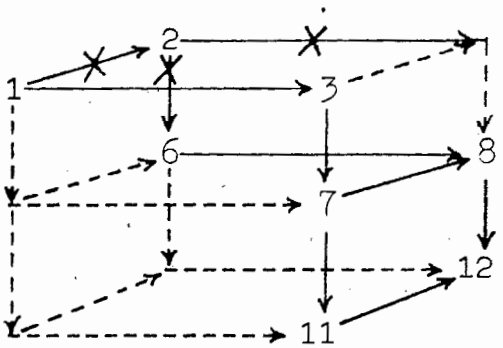


Figure 2 Possible Biosynthetic Routes Between Lanosterol (1) and Fecosterol (12)



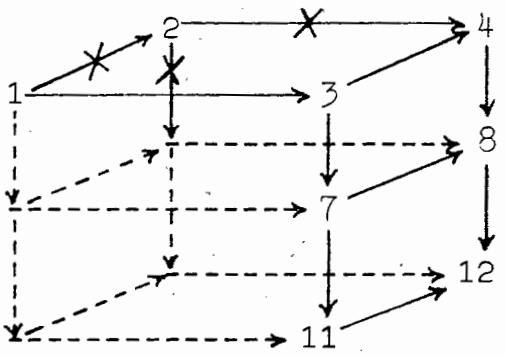
Approach 1

(Isolation of Constituent Sterols)



Approach 2

(Feeding and Trapping)



Approach 3

(Enzyme Characterization)

Key

→ conversion implied

-X→ conversion ruled out

--→ conversion untested

Figure 3

isolation of lanosterol (1), 32-norlanosterol (3), 4α-methylzymosterol (7), 4α-methylfecosterol (8), zymosterol (11) and fecosterol (12) from wild type yeast[6]. Their presence suggests the routes 1 → 3 → 7 → 11 → 12 and 1 → 3 → 7 → 8 → 12 to be operative.

A variant of this first approach came to light after the completion of our own work. A mutant of S. cerevisiae was investigated[7] and found to contain lanosterol (1), 31-norlanosterol (5), obtusifoliol (6), and 14-methylfecosterol (10). The accumulation of these sterols indicates that the mutant possesses a functional 4α-methyl demethylase and a functional Δ^{24}-methyltransferase but lacks a functional 14α-methyl demethylase. More importantly, it indicates that the 4α-methyl demethylase and the Δ^{24}-methyltransferase are capable of acting upon 14α-methyl sterols. It thus suggests that the routes 1 → 5 → 6 → 8 → 12, 1 → 5 → 6 → 10 → 12, 1 → 5 → 7 → 8 → 12 and 1 → 5 → 7 → 11 → 12 may possibly be operative in normal strains of S. cerevesiae.

Of the remaining three sterols in the matrix (2, 4 and 9) none has been reported found in S. cerevisiae, although 24-methylene-24,25-dihydrolanosterol (2) and 4,4-dimethylfecosterol (4) have been found in other fungi[8] and yeast[9].

The second, more sophisticated, approach involved

the use of feeding and trapping experiments. If a true precursor-product relationship exists between two sterols, the product will acquire radioactivity when the precursor, radiolabelled with ^{3}H or ^{14}C, is fed to growing yeast. If there is no transfer of radioactivity, then no precursor-product relationship exists. If the product sterol in question has not been isolated from yeast or if it is present only in small quantities, then a "trap" of unlabelled synthetic product sterol is added during the isolation process. Radioactivity in the recovered trap once again establishes a precursor-product relationship.

Feeding and trapping experiments can, if conducted properly, yield the most definitive information regarding sterol interconversions. There are pitfalls to be wary of, however. When trying to establish the intermediacy of a given sterol in a biosynthetic pathway, one must demonstrate input into it (from a known precursor) as well as output from it (to a known product). The establishment of output only (e.g., conversion to ergosterol) can lead to an initial false assumption of intermediacy, as was the case of 24-methylene-24,25-dihydrolanosterol (2)[10].

Care must also be exercised in isolating the product or trapping sterol. Difficulty is often encountered in separating sterols from one another and cross contamina-

tion can give ambiguous results (i.e., false radioactivity).

Feeding experiments have thus far demonstrated the conversion of the following sterols to ergosterol: lanosterol (1)[4b,5,10], 24-methylene-24,25-dihydrolanosterol (2)[10,11], 32-norlanosterol (3)[5], obtusifoliol (6)[11], 4α-methylzymosterol (7)[11], 4α-methylfecosterol (8)[11], zymosterol (11)[4b,5], and fecosterol (12)[4,5]. Within the matrix (of Figure 2) itself, the conversion of 11 to 12 has been shown to be operative[12], while that of 1 to 2 has been shown to be inoperative[11]. When considered collectively, these various conversions tend to support two of the routes already suggested by the first approach, namely 1 → 3 → 7 → 11 → 12 and 1 → 3 → 7 → 8 → 12.

The third approach involved the characterization of the enzyme or enzymes responsible for each particular structural modification. A determination of the preferred substrate of each enzyme would provide a good indication of the overall preferred route. In connection with this approach both the demethylase[13] and the Δ^{24}-methyltransferase[12,14] enzymes have been partially purified and studied.

The demethylase study yielded little information concerning substrate specificity. The only substrate employed was ^{14}C-lanosterol derived from 2-^{14}C-mevalonate.

This meant that of the 4α, 4β, and 14α-methyl groups, only the 4α was radiolabelled[2] and therefore only the 4α-methyl demethylase activity (assayed as $^{14}CO_2$ evolved) was measured. The activity of the 4α-methyl demethylase on the 4,4-dimethyl sterols (1, 2, 3 and 4) was not measured individually and therefore no relative substrate specificity could be deduced. The activity of the 4α-methyl demethylase on the 4α-methyl sterols (5, 6, 7 and 8) was not measured at all since the 4α-methyl group of these sterols is derived from the 4β-methyl group[2], which, as stated before, was unlabelled. However, one interesting fact did emerge from this study. The addition of S-adenosylmethionine (from whence the C_{24}-methylene group is derived[1]) increased the demethylase activity 2.5 to 3 fold. This seems to indicate that sterols 2 and/or 4 were better substrates than 1 and/or 3, although it was never established that C_{24}-methylation was actually occurring in the system used.

The Δ^{24}-methyltransferase experiments were somewhat better contrived. The relative activity of the transferase toward zymosterol (11), 4α-methylzymosterol (7), 32-norlanosterol (3) and lanosterol (1) was 100, 5, 2 and 0 respectively. This observation indicates three pathways of decreasing preference: 1 → 3 → 7 → 11 → 12, 1 → 3 → 7 → 8 → 12 and 1 → 3 → 4 → 8 → 12. Unfortunately,

neither 31-norlanosterol (5) nor 14-methylzymosterol (9) were tested as substrates. It thus remains unclear whether the preference shown by the transferase for 3 over 1 would be parallelled by a similar preference for 7 and 11 over 5 and 9 respectively.

In summary, then, the twelve possible routes between sterols 1 and 12 may be apportioned among four categories of decreasing probability:

A. operative; supported by all evidence

1 → 3 → 7 → 8 → 12 and 1 → 3 → 7 → 11 → 12

B. possibly operative; supported by some evidence

1 → 3 → 4 → 8 → 12; 1 → 5 → 6 → 8 → 12

1 → 5 → 6 → 10 → 12; 1 → 5 → 7 → 8 → 12

1 → 5 → 7 → 11 → 12

C. possibly operative; no supportive evidence; no counterindicative evidence

1 → 5 → 9 → 10 → 12 and 1 → 5 → 9 → 11 → 12

D. not operative; counterindicative evidence

1 → 2 → 4 → 8 → 12; 1 → 2 → 6 → 8 → 12

1 → 2 → 6 → 10 → 12

Fully seven of these routes (those in categories B and C) are in need of further verification. An inspection of Figure 2 reveals that this can best be done by determining whether the two key intermediates involved in these routes, namely 4 and 5, are in fact present in

wild type S. cerevisiae. We sought to do this by attempting to isolate 4 and/or 5 from yeast sterol mixtures and by attempting to trap radioactivity in 4 and/or 5 from exogenously supplied [2-^{3}H]-lanosterol.

Procedures and Results

Sterol Synthesis

Before any verification of biosynthetic pathways could be attempted, it was first necessary to synthesize authentic samples of 4 and 5. These samples would serve both as chromatographic standards and as trapping agents. A small quantity of tritiated lanosterol was also needed to serve as a radiolabelled precursor.

The synthesis of 4,4-dimethylfecosterol (4) presented no inordinate difficulties (Figure 4). Treatment of 32-norlanosteryl acetate (3 Ac), isolated from yeast sterol concentrates[15], with aqueous N-bromosuccinimide[16] yielded the bromohydrin, 14. Treatment of the bromohydrin with K_2CO_3[16] gave the 24,25-epoxide, 15. The epoxide was then rearranged[6] to the ketone, 16, using Kenner's reagent[17]. Finally, Wittig reaction of the ketone with triphenylmethylenephosphorane[6] afforded 4.

The synthesis of 31-norlanosterol (5) proved more challenging. The reported synthesis[18] of 5 was bypassed in favour of a more promising method developed by Cohen et al.[19]. Their procedure for removing a C-4 methyl group from lanosterol did have one drawback, however. At one stage of the reaction series, epoxidation of a seco-nitrile is followed by BF_3 induced ring closure. If this

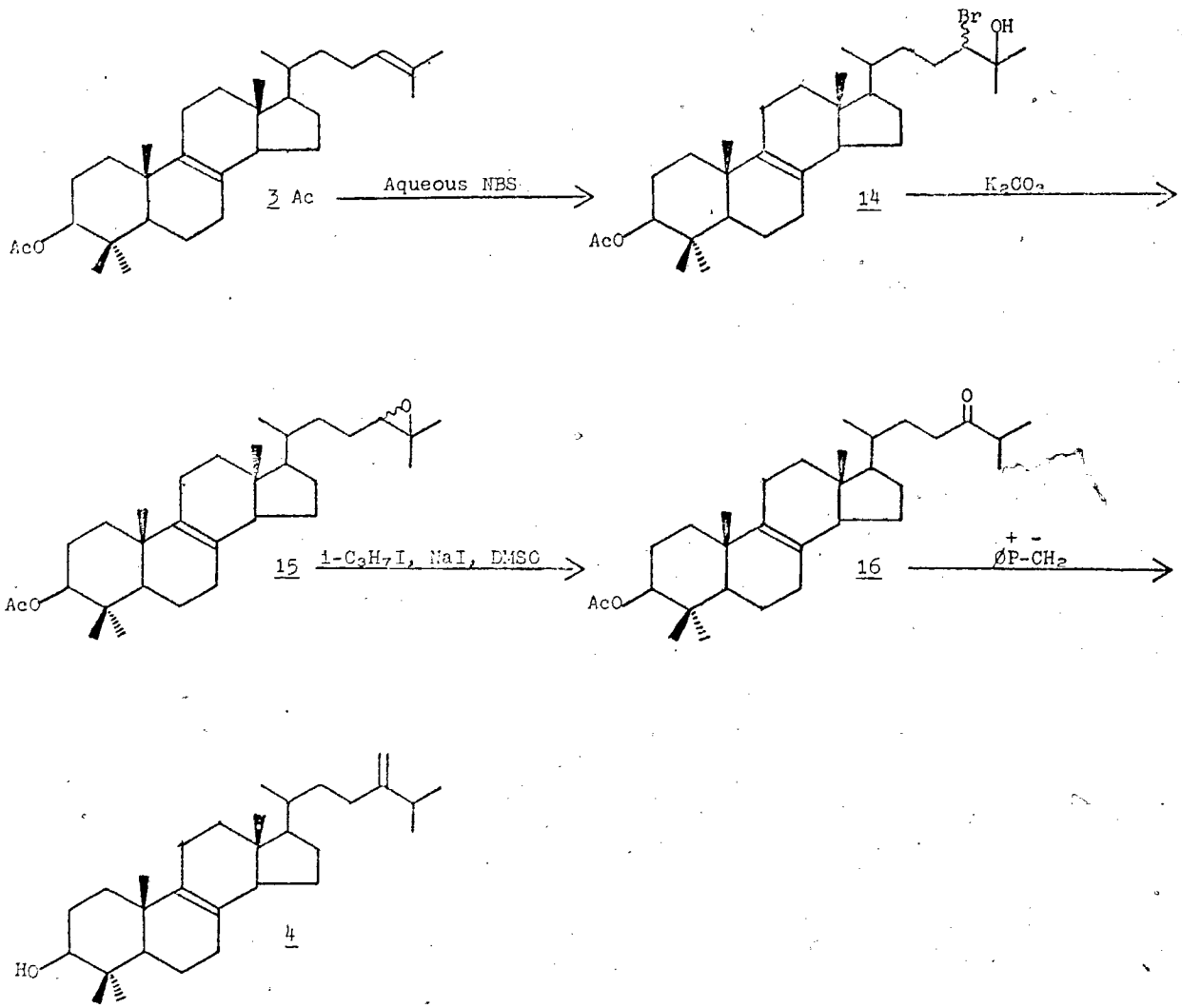
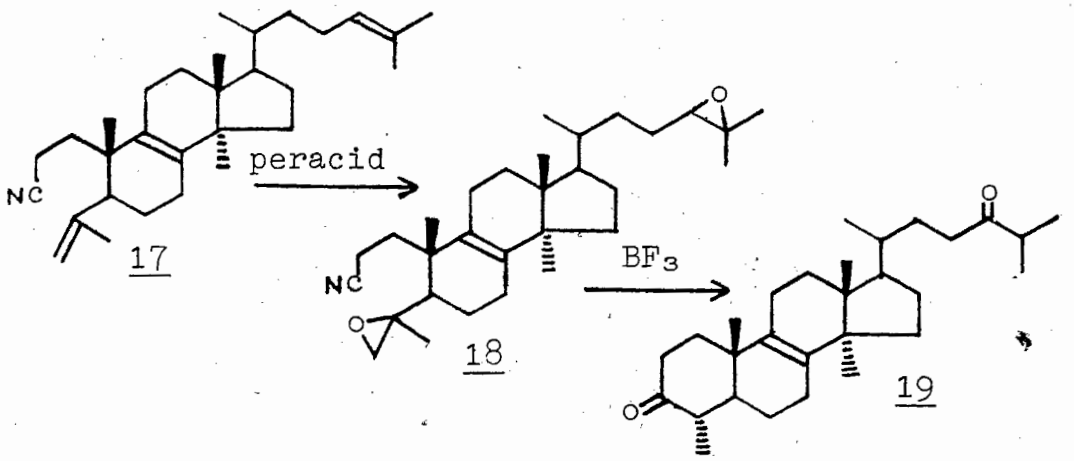

AcO
3 Ac
Aqueous NBS
Br
OH
14
K_2CO_3
15
$1\text{-}C_3H_7I$, NaI, DMSO
O
16
$\overset{+}{\text{Ø}}\text{P-}\overset{-}{\text{C}}H_2$
HO
4

Figure 4

sequence were performed on a Δ^{24} sterol, an unacceptable product (i.e., 19) would arise:



17 18 19

The two carbonyls of 19, because of their very similar steric environment, would be resistant to selective reduction or protection. Without such a selective reaction, 5 could not be obtained. Fortunately, two alternatives did avail themselves: selective oxidation of the terminal double bond of the seco-nitrile or protection of the Δ^{24} double bond prior to formation of the seco-nitrile.

We initially pursued the first alternative (Figure 5). The selective oxidation would be via hydroboration with disiamylborane[20]. This reagent rapidly attacks terminal double bonds[20], but is quite unreactive toward nitriles[21] and tri- and tetrasubstituted double bonds[20]. Surprisingly, the reaction of disiamylborane with 17 produced no isolable amount of 21. The terminal double bond of 17 is apparently sterically encumbered by a close proximity

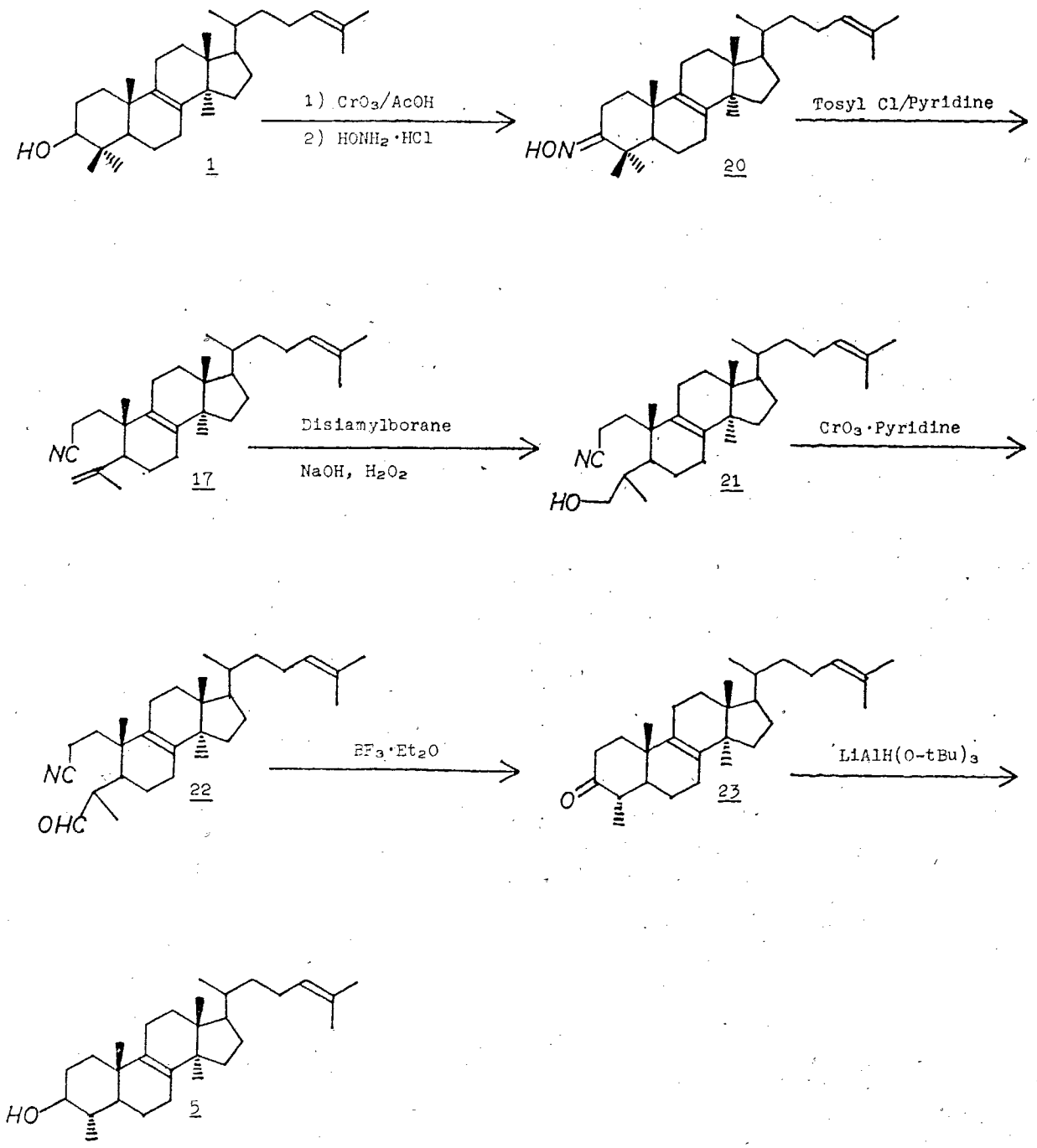

HO
1
1) CrO_3/AcOH
2) $HONH_2 \cdot HCl$
HON
20
Tosyl Cl/Pyridine
NC
17
Disiamylborane
$NaOH, H_2O_2$
NC
HO
21
$CrO_3 \cdot$Pyridine
NC
OHC
22
$BF_3 \cdot Et_2O$
O
23
$LiAlH(O\text{-}tBu)_3$
HO
5

Figure 5

to the nitrile, as evidenced by a downfield n.m.r. shift (by 0.24 p.p.m.) of one of the vinyl protons. This juxtaposition renders the terminal double bond inaccessible to attack by the bulky disiamylborane dimer.

The second option was therefore exercised (Figure 6). The usual method of double bond protection (i.e., bromination) was precluded by the harsh conditions to be encountered during some of the subsequent reactions. An indirect protection of the Δ^{24} double bond (i.e., degradation to the ester) had to be employed. Purified lanosterol* (1) was consequently treated successively with ozone, Jones reagent[25] and ethereal diazomethane[26] to give the keto ester, 25. Reaction of the oxime of 25 (i.e., 26), with p-toluenesulfonyl chloride in refluxing pyridine yielded

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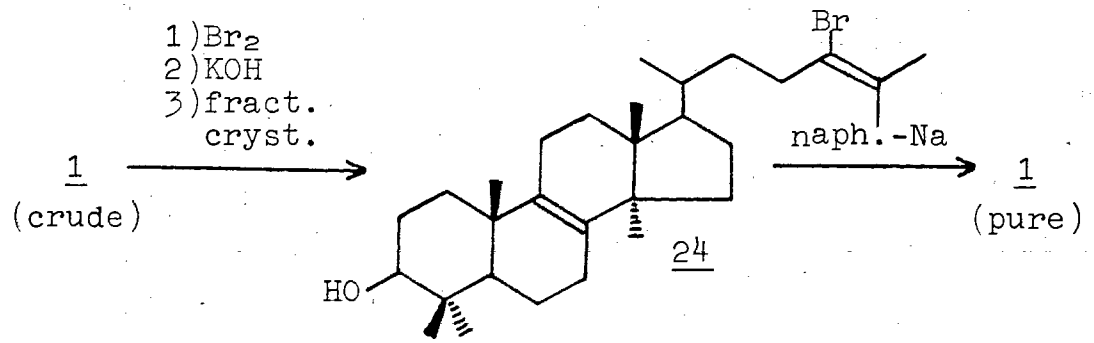
* Commercial lanosterol was purified via fractional crystallization[22] of its 24-bromide[23] (24). Purified lanosterol was regenerated from 24 by using naphthalene-sodium[24].



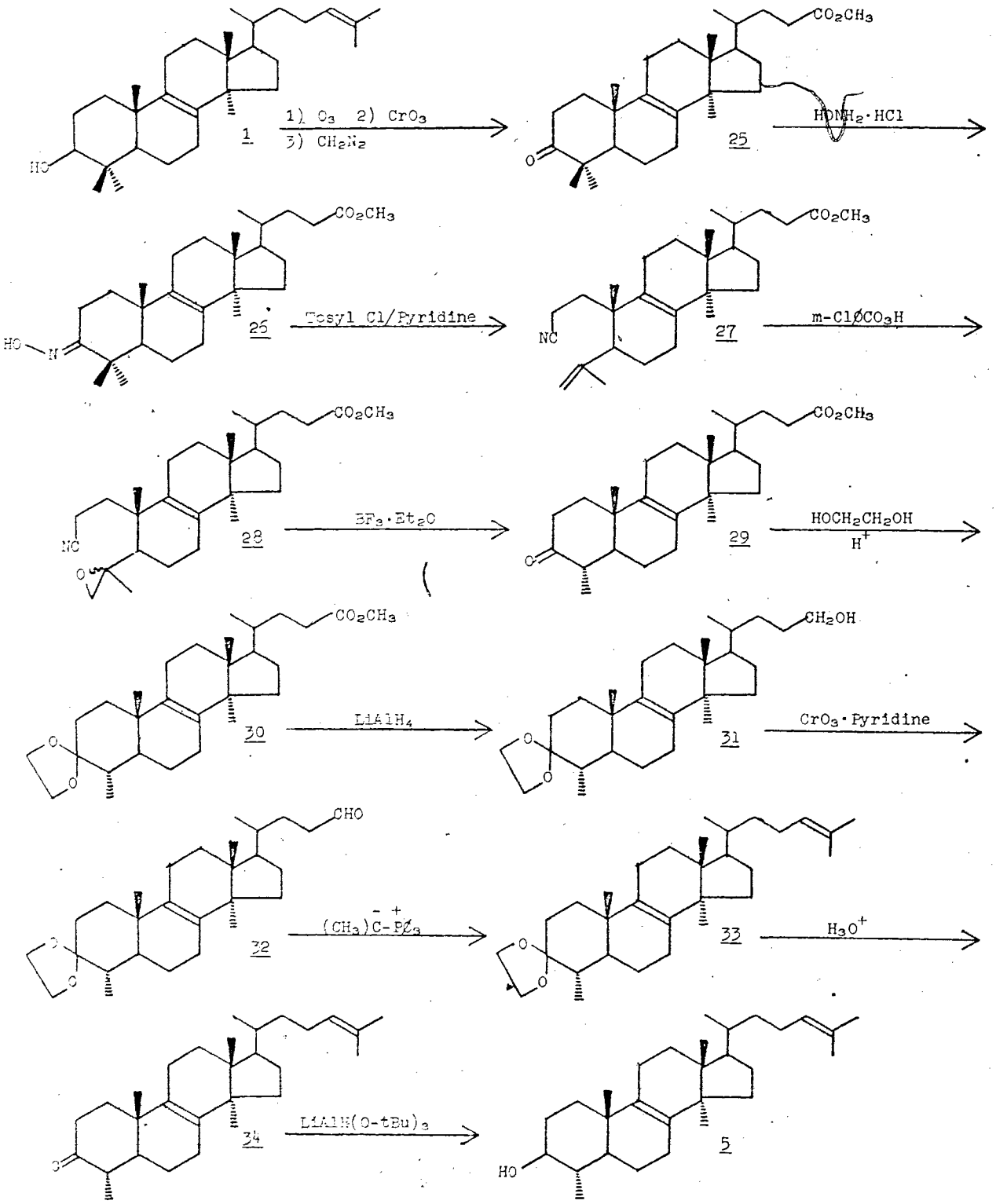

1) O3 2) CrO3
3) CH2N2
1
25
HONH2·HCl
26
Tosyl Cl/Pyridine
27
m-ClØCO3H
28
BF3·Et2O
29
HOCH2CH2OH
H+
30
LiAlH4
31
CrO3·Pyridine
32
(CH3)C-PØ3
33
H3O+
34
LiAlH(O-tBu)3
5

Figure 6

the seco-nitrile, 27. Epoxidation gave 28, and this, when treated with BF_3-etherate in refluxing toluene, gave the keto ester, 29. After protection of the C_3 ketone as the ethylene ketal (30), the side chain ester was first reduced to the alcohol (31) and then oxidized to the aldehyde (32). Reconstruction of the side chain isopropylidene group via a Wittig reaction with triphenylisopropylidenephosphorane gave 33. Finally, hydrolysis of the ketal to the ketone (34) and reduction of the ketone to the 3β alcohol with $LiAlH(O\text{-}tBu)_3$[27] gave the desired sterol, 5.

For the preparation of tritiated lanosterol, lanosterol (1) was first oxidized to lanosterone (35) (Figure 7). This was then passed through a column of tritiated basic alumina[28] during which enolic exchange at the C_2 position occurred. Reduction of the tritiated lanosterone (36) to the 3β alcohol gave [2-^{3}H]-lanosterol (37).

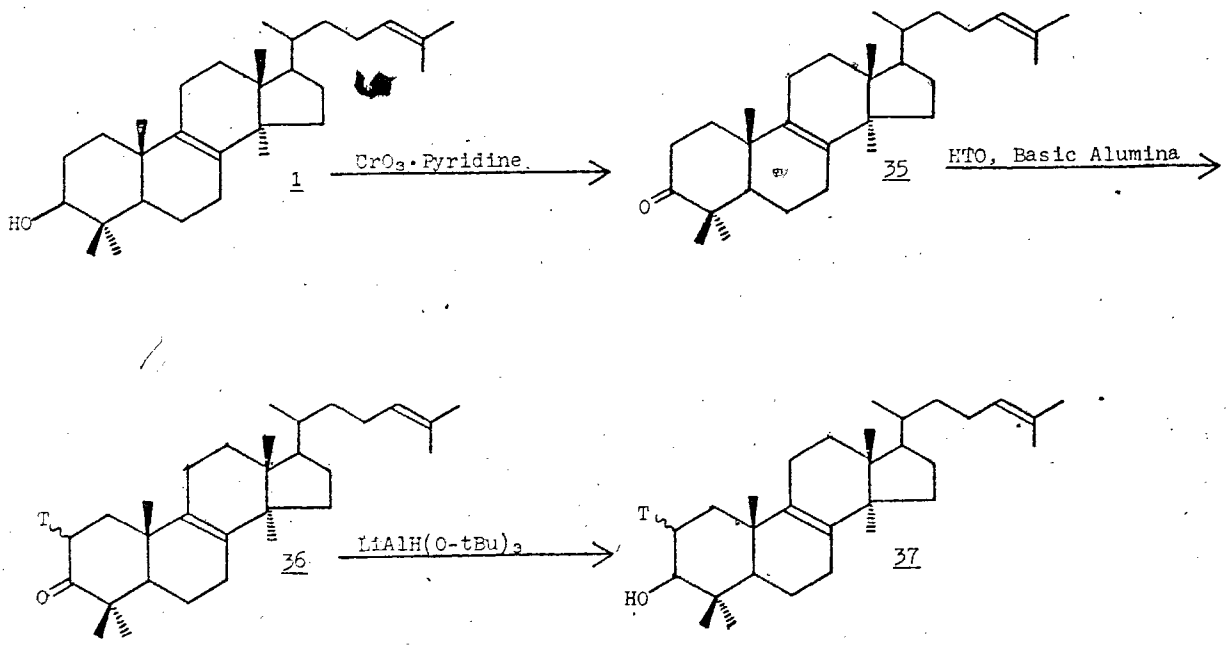

HO
1
CrO_3·Pyridine
O
35
HTO, Basic Alumina
T
O
36
$LiAlH(O\text{-}tBu)_3$
T
HO
37

Figure 7

Search for 4 and 5

If sterol 4 were indeed present in S. cerevisiae, it would be found in the 4,4-dimethyl sterol fraction along with lanosterol (1) and 32-norlanosterol (3) (see Table 1). Sterol 5 would similarly be found in the 4α-methyl sterol fraction along with 4α-methylzymosterol (7) and 4α-methylfecosterol (8). The search for 4 and 5 consequently began by separating these two fractions from the 4-desmethyl sterols and from one another[15] and then analyzing them (as TMS derivatives) on a 0.25 mm. X 30 m. OV-101 coated glass capillary column (see Table 1).

This preliminary separation and analysis was performed on three sources of yeast sterol mixtures: 1) yeast sterol concentrates*, 2) fresh laboratory grown yeast, and 3) fresh brewery grown yeast. Sterol 4 could not be detected in any of these sources. Equivocal evidence (i.e., peaks co-chromatographing with authentic sterol) for sterol 5 was found in the first and third sources.

The 4α-methyl sterol fractions of these two sources were therefore acetylated, separated on $AgNO_3$ impregnated

---

* These concentrates are the residues (in both solid and solution form) remaining after the commercial extraction of ergosterol from brewery yeast. Their content of minor yeast sterols is thus enriched.

Table 1 - Chromatographic Properties of Sterols in Figure 2.

Sterol Name	Sterol Number	G.L.P.C. R.R.T.		T.L.C. R_f*	
		TMS	Acetate	Silica Gel (Free Sterol)	$AgNO_3$-Silica Gel (Acetates)
Cholestanol (standard)	---	1.00	1.00	---	---
Lanosterol	1	1.50	1.47	.58	.75
24-Methylene-24,25-dihydrolanosterol**	2	1.72	1.70	.58	.61
32-Norlanosterol	3	1.57	1.52	.58	.61
4,4-Dimethylfecosterol	4	1.82	1.77	.58	.39
31-Norlanosterol	5	1.31	1.24	.51	.69
4α-Methylzymosterol	7	1.37	1.27	.51	.51
4α-Methylfecosterol	8	1.59	1.47	.51	.20
Zymosterol**	11	1.12	1.12	.35	---
Fecosterol**	12	1.29	1.29	.35	---

* The values given are for analytical plates. The order of elution was the same for preparative plates.
** Supplied by M. Fryberg.

silica gel (see Table 1) and analyzed by G.L.P.C. and M.S. The presence of 5 acetate was confirmed in only one of these sources, the fresh brewery grown yeast. The isolated 5 acetate co-chromatographed with its synthetically prepared counterpart (both by T.L.C. and G.L.P.C.) and had an identical mass spectrum. It constituted approximately 1% of the 4α-methyl sterol fraction.

Trapping Experiment

An anaerobic-aerobic procedure[4] was employed for the trapping experiment. The procedure first depletes the yeast of endogenous sterols by culturing them under anaerobic conditions.* This is followed by a period of vigorous aeration and a simultaneous feeding of labelled precursor. The aeration initiates a rapid biosynthesis of endogenous sterols and consequently an enhanced uptake and incorporation of the exogenously supplied radiolabelled sterol.

Tritiated lanosterol (37) was accordingly fed to anaerobically pre-treated yeast. An uncertainty existed, however, as to what period of aeration would afford the maximum incorporation of the exogenously supplied 2-^{3}H-lanosterol into the endogenously produced 4α-methyl and 4,4-dimethyl sterols. Previous work indicated that the amount of 4 or 5 (if present at all) should peak two to four hours after the start of aeration[4], whereas the uptake of tritiated lanosterol should reach a maximum several hours later[4]. It was therefore decided to harvest the yeast in two equal batches, one batch after three hours of aeration and the other batch after six hours.

Extraction of the non-saponifiable fraction (N.S.F.) from each batch was followed by separation of each N.S.F.

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* Without molecular oxygen, sterol biosynthesis in yeast ceases at the squalene stage[1].

into its constituent 4,4-dimethyl, 4α-methyl and 4-desmethyl sterols. Since a higher incorporation into the 4,4-dimethyl and 4α-methyl fractions was found to occur after six hours (Tables 2 and 3), these fractions were chosen for further work.

Each of these two fractions was acetylated. To the 4α-methyl sterol acetates was added 100 mg of 31-norlanosteryl acetate (5 Ac). To the 4,4-dimethyl sterol acetates was added 100 mg of lanosteryl acetate (1 Ac), 100 mg of 32-norlanosteryl acetate (3 Ac) and 100 mg of 4,4-dimethylfecosteryl acetate (4 Ac). These four acetates were then re-isolated by preparative T.L.C. and purified by repeated crystallization and chromatography. The specific radioactivity of each sterol acetate was followed during this purification process, as delineated in Tables 4 to 7. The final total activity associated with each sterol and its percentage of the activity incorporated into the N.S.F. is shown in Table 8. The significance of these results is explored in the discussion section.

Table 2

Period of Aeration	Dry Wt. Cells	Supernatant Activity	N.S.F. Activity
3 hrs.	1.42 g	2.20×10^7 cpm	1.10×10^7 cpm
6 hrs.	1.31 g	1.74×10^7 cpm	1.64×10^7 cpm

Total activity fed = 8.48×10^7 cpm

Total activity recovered = 6.68×10^7 cpm = 78.8%

Table 3

	3 hr. N.S.F.		6 hr. N.S.F.	
Sterol Fraction	Total * Activity (cpm)	% of Total	Total * Activity (cpm)	% of Total
4,4-diMe	9.06×10^6	82.4	1.339×10^7	81.9
4α-Me	2.21×10^5	2.01	2.80×10^5	1.71
Remainder	1.71×10^6	15.5	2.67×10^6	16.3
Total	1.10×10^7	99.9	1.63×10^7	99.9

* Corrected for chromatographic losses.

Table 4

Labelled precursor: [2-^{3}H]-lanosterol (.669 mg @ 1.25×10^8 cpm/mg)

Trapping sterol: lanosteryl acetate (1 Ac) (100 mg)

Procedure	Amt. of Material Recovered (mg)	Specific Activity* (cpm/mg)
Chromatographic separation	96.8	9.24×10^4
1st crystallization	83.2	9.88×10^4
2nd crystallization	69.1	$9.96 \times 10^4 \pm 1100$†

* Figures given are an average of triplicate counts of duplicate assays (or single assays where quantities were limited). Each assay was performed on approximately 0.5 to 1.5 mg (weighing uncertainty = ±.004 mg) of sample. Each count was for 100 minutes or 10^6 counts, whichever occurred first. All counts were corrected for background but not corrected for counting efficiency. All assays had an external standard ratio of .70 ± 10%.

† Uncertainties were calculated from either the variation between duplicate assays or the statistical counting error,[37] whichever was greatest.

Table 5

Labelled precursor: [2-^{3}H]-lanosterol (.669 mg @ 1.25×10^8 cpm/mg)

Trapping sterol: 32-norlanosteryl acetate (3 Ac) (100mg)

Procedure	Amt. of Material Recovered (mg)	Specific Activity (cpm/mg)
Chromatographic separation	56.8	1.89×10^3
1st crystallization	51.3	1.18×10^3
2nd crystallization	45.1	8.85×10^2
3rd crystallization	39.7	8.07×10^2
4th crystallization	33.2	7.20×10^2
5th crystallization	27.3	9.55×10^2
6th crystallization	22.1	7.07×10^2
7th crystallization	17.4	7.02×10^2
Admixture with 20 mg 1 Ac, then chromatographic separation	14.6	7.14×10^2
Recrystallization	9.7	$7.00 \times 10^2 \pm 11$

Table 6

Labelled precursor: [2-^{3}H]-lanosterol (.669 mg @ 1.25×10^{8} cpm/mg).

Trapping agent: 4,4-Dimethylfecosteryl Acetate (4 Ac) (100 mg)

Procedure	Amt. of Material Recovered (mg)	Specific Activity (cpm/mg)
Chromatographic separation	76.8	1.44×10^{3}
1st crystallization	61.7	3.57×10^{2}
2nd crystallization	51.8	37.7
3rd crystallization	41.0	8.8
4th crystallization	34.2	8.5
5th crystallization	27.5	8.9
6th crystallization	20.9	10.8
Admixture with 20 mg 1Ac, then chromatographic separation	15.3	3.1
Recrystallization	7.9	4.9
Chromatographic separation	2.6*	8.8 ±3

* >99.9% pure by G.L.P.C.

Table 7

Labelled precursor: [2-^{3}H]-lanosterol (.669 mg @ 1.25×10^8 cpm/mg)

Trapping sterol: 31-norlanosteryl acetate (5 Ac) (100 mg),

Procedure	Amt. of Material Recovered (mg)	Specific Activity (cpm/mg)
Chromatographic separation	38.5	1.23×10^3
1st crystallization	26.2	5.69×10^2
2nd crystallization	21.1	3.77×10^2
3rd crystallization	16.0	2.87×10^2
4th crystallization	11.6	2.46×10^2
5th crystallization	6.3	2.18×10^2
6th crystallization	1.8	2.03×10^2 ±10
Admixture with ~5 mg 5 Ac and ~5 mg 1 Ac, followed by hydrolysis and chromatographic separation.	———	4.2×10^{1}* ±25

* The specific activity of the recovered 5 was 6.3 cpm/mg. Correcting for dilution and hydrolysis, this gives the 5 Ac a specific activity of:

$$\left(6.3 \text{ cpm/mg}\right)\left(\frac{413 \text{ g/mole}}{455 \text{ g/mole}}\right)\left(\frac{5.15 + .82 \text{ mg}}{.82 \text{ mg}}\right) = 42 \text{ cpm/mg}$$

Table 8

Sterol Acetate	Total Activity*	% of N.S.F. Activity
1 Ac	$9.93X10^6$ cpm	60.5
3 Ac	$7.00X10^4$ cpm	0.43
4 Ac	$8.8X10^2$ cpm	0.0053
5 Ac	$4.2X10^3$ cpm	0.026

* Not corrected for endogenous sterol content or losses prior to addition of trapping sterol acetate.

Discussion

In performing these experiments we sought an unequivocal determination of the involvement of sterols 4 and 5 in ergosterol biosynthesis. In view of the results obtained, this goal was perhaps too grandiose.

Search for 4 and 5

As was stated previously, sterol 4 was not found in any of the sources analyzed. Our failure to detect it, however does not insure its absolute absence and consequently its absolute non-involvement in sterol elaboration. It only insures that it constitutes less than 0.1% of the 4,4-dimethyl sterol fraction or 0.01%* of the total yeast sterols. (This is a conservative estimate, for even at this level a chromatographic peak for 4 would have been detected with the G.L.P.C. system used.)

Sterol 5 was found in only one source, the fresh brewery yeast, in which it constituted ~1% of the 4α-methyl sterol fraction. Although its identity could not be rigorously established because of the limited quanitity isolated (~200 μg), its chromatographic and mass spectral properties presented a good prima facie case. The T.L.C. properties of the isolated sterol (which were identical to

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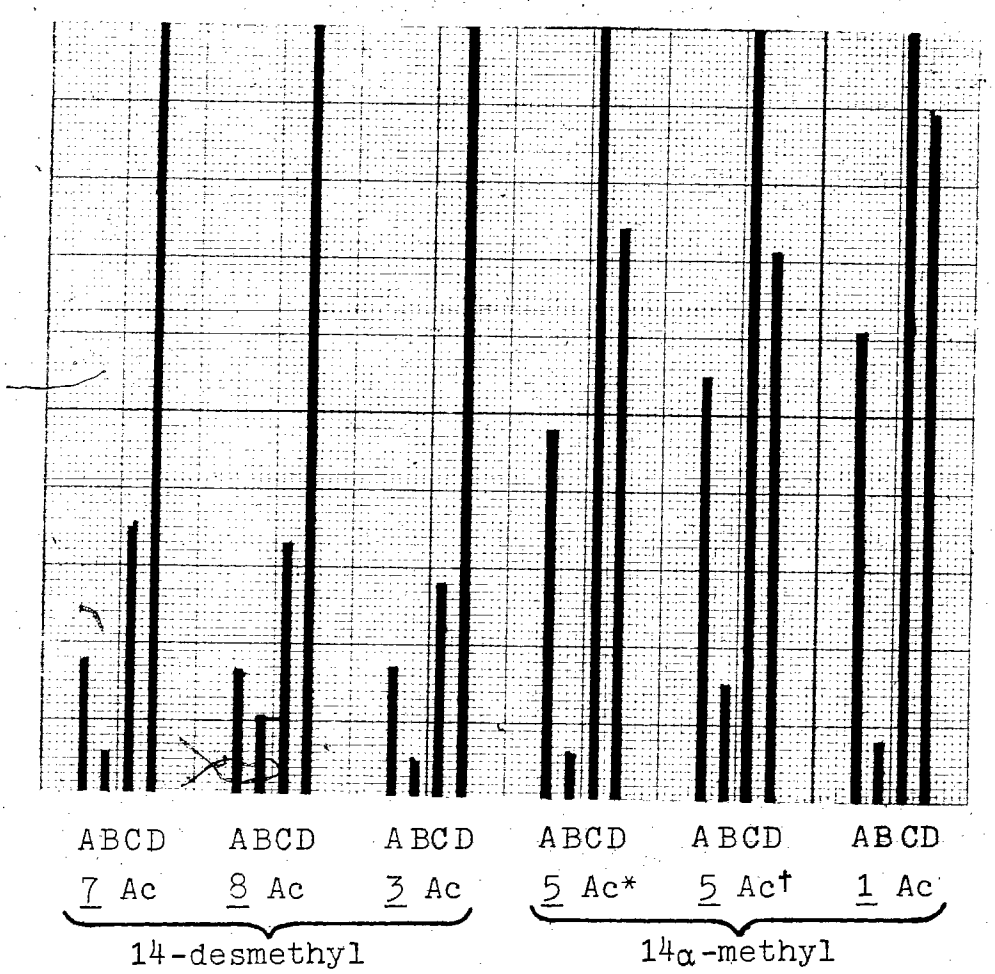
* The 4,4-dimethyl fraction accounts for no more than 10% of total yeast sterols.

those of synthetic 5) indicated that it possessed a 4α-methyl group, a 14α-methyl group and no 24-methylene group. Furthermore, it co-chromatographed with authentic 5 Ac on the G.L.P.C. system. Finally, its mass spectrum indicated a sterol acetate (of M. W. = 454) possessing a 14α-methyl group. (The 14α-methyl and 14-desmethyl sterol acetates display marked differences in relative peak intensities for the M^+, $M^+ - CH_3$ and $M^+ - CH_3 - AcOH$ peaks. See Figure 8.) Only sterol 5 would seem to possess all these properties.

One can only speculate as to why 5 was not detected in the other sources. Since the laboratory and brewery grown yeast were of the same strain, there can be no strain specific difference in biosynthetic pathways (as might be the case for the yeast from whence the sterol concentrates were obtained). Perhaps the absence of 5 in lab cultures can be attributed to a difference in culture conditions. The brewery yeast were grown under oxygen lean conditions, while the lab yeast were well aerated. Sterol 5 might tend to accumulate under the former conditions and diminish under the latter.

The low levels found for 5 and implied for 4 do not preclude them from being intermediates in major biosynthetic routes. If they were, however, they would be competing with much more abundant sterols. This would



*synthetic

†isolated

A=M^+-CH_3-AcOH

B=M^+-AcOH

C=M^+-CH_3

D=M^+

Figure 8

necessitate that they be highly favoured substrates of the demethylase and Δ^{24} methyltransferase enzymes. Such preference could be checked by experiments with the partially purified enzyme systems mentioned earlier, if one assumes, that is, that *in vitro* studies accurately reflect *in vivo* realities.

Trapping Experiment

The major problem which plagues all trapping experiments is that of contamination. One must establish beyond reasonable doubt that the radioactivity associated with the trapping sterol is intrinsic and not from contamination. The usual procedure is to recrystallize the trap until a constant specific activity is obtained, at which point the trap is assumed to be pure. Such an assumption is really only warranted when the trapping sterol contains the bulk of the incorporated activity, as was the case with 1 Ac (Table 8). In this case, a minor contaminant would not significantly alter the total activity associated with 1 Ac. If, on the other hand, the incorporation is minor (as was the case of 3 Ac, 4 Ac and 5 Ac), one must seriously question the purity of the trap. A minor contaminant (one below the level of detection) of high specific activity could impart a low level of radioactivity to an intrinsically "cold" trap.

Let us initially consider the trapping of 3 Ac. The

recrystallization process (Table 5) seemed to indicate a genuine specific activity of 700 cpm/mg. This same specific activity could have been imparted, however, by a 0.7% contamination of 3 Ac by 1 Ac, its most likely contaminant. This possibility was checked by admixing additional cold 1 Ac with the recrystallized 3 Ac and then re-isolating the 3 Ac. Had the activity been from 1 Ac contamination, the admixture-re-isolation process would have decreased the specific activity of 3 Ac. There was, in fact, no decrease and the activity associated with 3 Ac was indeed genuine.

The incorporation into 4 Ac was so low as to hardly warrant serious consideration. The recrystallization process gave a specific activity of 9 cpm/mg (Table 6). Contamination by 1 Ac was ruled out by the admixture-re-isolation technique. Contamination by 3 Ac was ruled out by G.L.P.C. analysis which showed the 4 Ac to be >99.9% pure. (A 1.3% contamination by 3 Ac would be needed to impart 9 cpm/mg to 4 Ac.) Contamination by tritium exchange, though, was a definite possibility. Barton *et al*[5] noted a 0.025% incorporation of radioactivity into cholesterol (a sterol alien to yeast) when cholesterol and [2,4-$^3H_2$]-zymosterol were simultaneously fed to yeast. It is not clear whether this tritium exchange occurred during culturing or during isolation (i.e., on the silica gel

surface). It does, in any case, render suspect any incorporation at or below this level. A glance at Table 8 reveals that the total incorporation into 4 Ac was well below 0.025%.

The incorporation into 5 Ac at first seemed rather significant. The specific activity appeared to level off at 200 cpm/mg (Table 7). A control experiment performed at this point showed that when [2-^{3}H]-lanosteryl acetate (37 Ac) was introduced into "cold" 31-norlanosteryl acetate (5 Ac), it could not be removed by repeated recrystallization. Lanosteryl acetate will, in other words, co-crystallize with 31-norlanosteryl acetate. It thus seemed likely that at least part of this 200 cpm/mg was from 1 Ac contamination. By the admixture-re-isolation process we were able to determine that at least 42 cpm/mg of the 200 cpm/mg were not from 1 Ac contamination. This still left the possibility of contamination by 3 Ac and 7 Ac. Because of the manner in which 5 Ac was isolated, these two sterol acetates would be present as undiluted contaminants. Their specific activity would consequently be high. If either one had a specific activity of $5X10^4$ cpm/mg (which is not unreasonable, based on the intracellular specific activity of 3 - see p. 38) then a 0.1% level of contamination would impart 50 cpm/mg to 5 Ac. Such a level of contamination was not ruled out by G.L.P.C. analysis and

indeed it would be difficult to do so.

We have seen that the radioactivity associated with 4 Ac and 5 Ac is in some doubt. But what if we were to assume the activity to be genuine? What further information could we extract from the data?

One thing we could obtain is a rough estimate of the intracellular specific activity of the sterols involved. This can be obtained by dividing the quantity of intracellular sterol into its total activity (Figure 9). The 6 hr. N.S.F. contained approximately 3 mg of 4,4-dimethyl sterols, of which 1 to 2 mg was 1 and 1 to 2 mg was 3. This gives them an intracellular specific activity of 5 to $10X10^6$ cpm/mg (10^7 cpm/1-2 mg) and 3.5 to $7X10^4$ cpm/mg ($7X10^4$ cpm/1-2 mg) respectively. The amount of 4 can be no greater than 1/1000 of the 4,4-dimethyl sterols (see page 32) or 3 μg. This gives it a minimum intracellular specific activity of $\sim 3X10^5$ cpm/mg ($\sim$900 cpm/3 μg). The amount of 5 would be about 1% of the amount of 4α-methyl sterols (which was $\sim$1 mg). Its intracellular specific activity would therefore be $4X10^5$ cpm/mg ($\sim 4X10^3$ cpm/10 μg).

One conclusion that is immediately apparent from these specific activities is the discrepancy between 3 and 4. The specific activity of 4 is 5 to 10 times that of 3. This is an impossibility. The maximum specific activity a product intermediate can have is that of its immediate

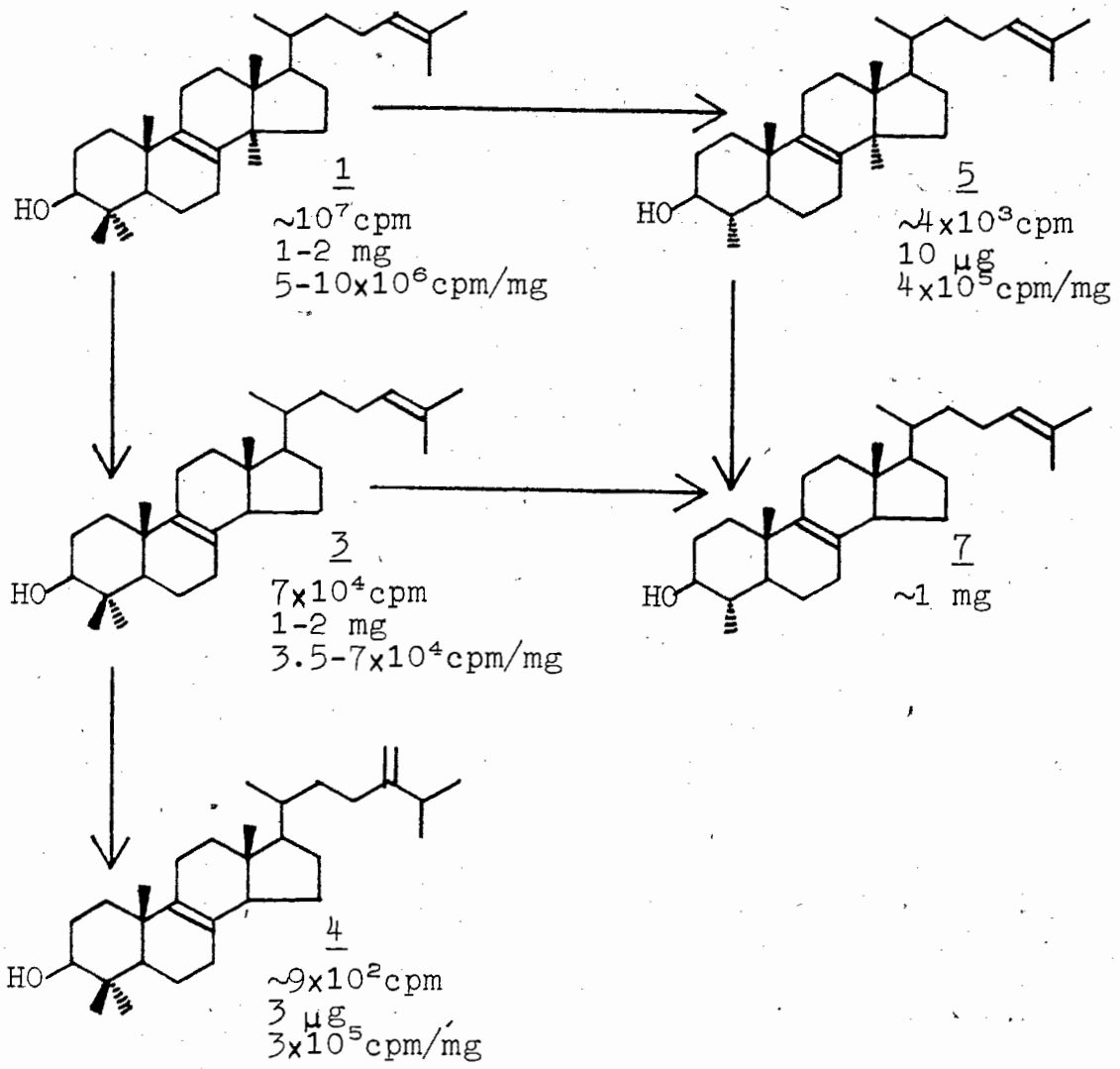

HO
1
~10⁷cpm
1-2 mg
5-10x10⁶cpm/mg
HO
5
~4x10³cpm
10 µg
4x10⁵cpm/mg
HO
3
7x10⁴cpm
1-2 mg
3.5-7x10⁴cpm/mg
HO
7
~1 mg
HO
4
~9x10²cpm
3 µg
3x10⁵cpm/mg

Figure 9

precursor[28,*]. This implies that either the activity found in 4 Ac was not genuine or the actual quantity of intracellular 4 was 5 to 10 times greater than estimated. The former alternative is the more likely.

One piece of information we had hoped to gain from a study of the incorporation of 1 into 3 and 5 was an estimation of the relative participation of the two routes 1 → 3 → 7 and 1 → 5 → 7. Through which route, in other words, does the bulk of sterol biosynthesis occur? An answer could be obtained via a kinetic analysis of the incorporation data. Such an analysis is impossible with the single data point obtained, unless the rate of incorporation has been linear throughout the experiment. In view of the difficulty in obtaining a single valid data point, the probability of obtaining a number of them over a period of time for use in a kinetic analysis is low indeed. We must be satisfied at the present time with a simple determination of the presence of 5 in yeast.

General Conclusion

A clear cut determination of the status of 4 and 5 in ergosterol production was not obtained, for our results remained more Delphic than definitive. The search for 4 and 5 did provide a single isolation of 5. Based upon this isolation, we may tentatively expand Figure 1 to include all routes through sterol 5 (see Figure 10).

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* See p. 42 for footnote.

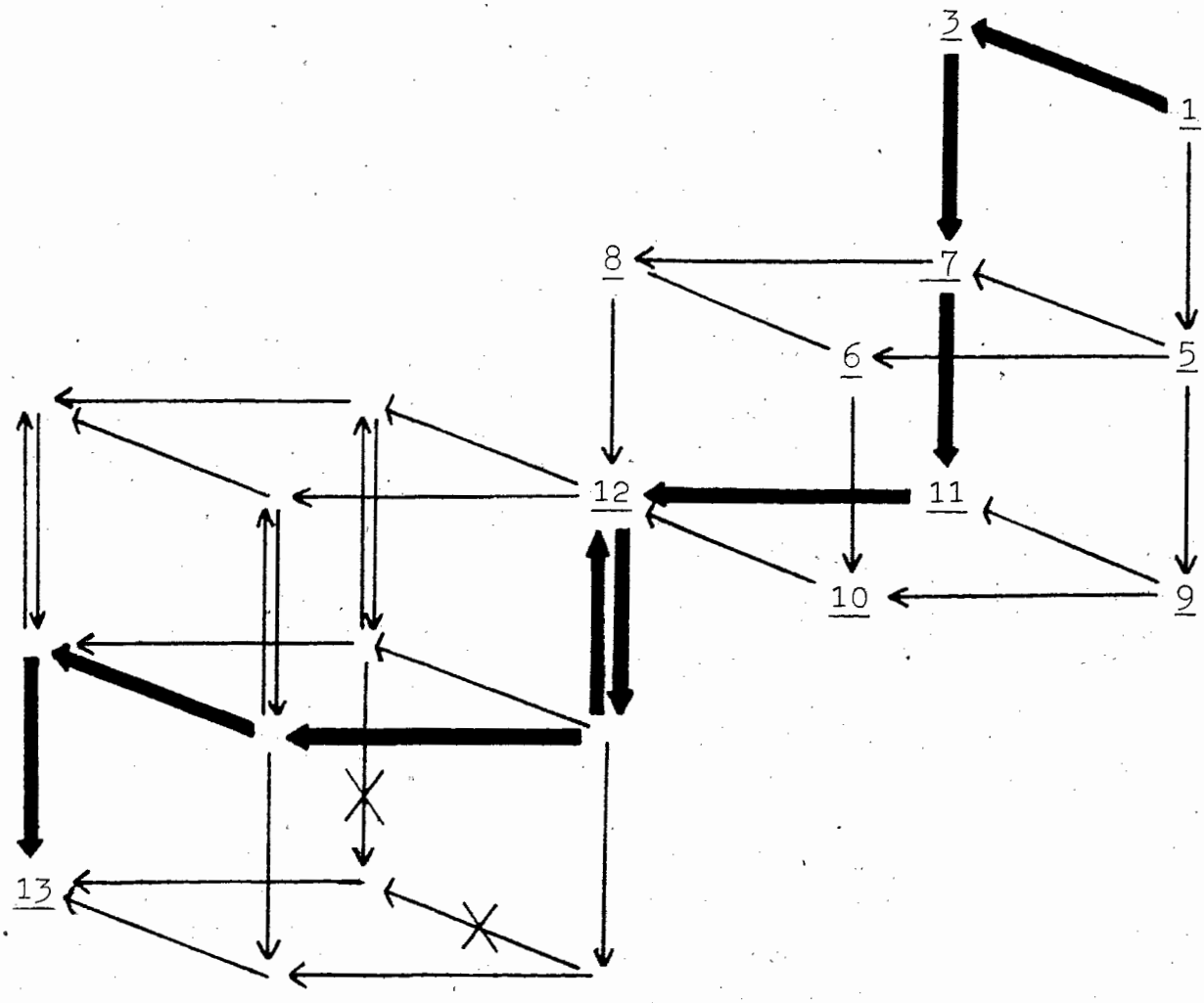

Figure 10

The trapping experiment provided no useful information because we could not unambiguously establish the significance of the incorporation we did obtain. Any effort at improving the trapping experiment must therefore be aimed at reducing this ambiguity. One might suggest the use of ^{14}C labelled rather than ^{3}H labelled lanosterol. This would eliminate the possibility of contamination by tritium exchange, which is an important factor in cases of low incorporation. One disadvantage of ^{14}C labelled sterols should be borne in mind. Their specific activity is usually on the order of a few million cpm/mg. This is considerably below the $1.25X10^{8}$ cpm/mg obtained for 37. The use of ^{14}C labelled lanosterol would thus reduce further the already low incorporations into sterols 3, 4 and 5.

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* We have assumed here that 3 has not yet reached maximum specific activity. A product can obtain a specific activity greater than its precursor only when the specific activity of the precursor is decreasing.

Experimental

Instrumentation

Infrared Spectrometer: Perkin-Elmer 457 using KBr dispersions.

Ultraviolet Spectrometer: Unicam SP-800 using standard quartz cells. Absolute ethanol was used as solvent.

Mass Spectrometer: Hitachi-Perkin-Elmer RMU-7 using an inlet temperature of 150-180° C and an ionization voltage of 80 ev.

Nuclear Magnetic Resonance Spectrometer: Varian A56/60 or XL-100 using $CDCl_3$ as solvent and TMS as internal standard. All chemical shifts are reported in δ values.

Scintillation Counter: Beckman LS-200B liquid scintillation system using a cocktail containing 4 g PPO and 50 mg POPOP per litre of toluene.

Fermenter: Virtis model 40-300.

Melting Point Apparatus: Fisher-Johns melting point apparatus. All mp.'s are corrected.

Culture Media

Complete Medium: 5 g Difco malt extract, 15 g B.B.L. yeast extract, 1 g NH_4Cl, 6.8 g KH_2PO_4, 11.4 g $K_2HPO_4.3H_2O$, and 25 g glucose per litre of medium.

Minimal Medium: 6.8 g KH_2PO_4, 11.4 g $K_2HPO_4.3H_2O$ and 40 g glucose per litre of medium.

Gas Liquid Phase Chromatography

G.L.P.C. analyses were performed on a Varian 2100 gas chromatograph fitted with a 30m X 0.25 mm I.D. glass capillary column coated with OV-101. The column was prepared by static coating[30] a surface pre-treated[31] glass capillary column with 0.25% OV-101 in CH_2Cl_2. The coated column was adapted to the Varian 2100 by using an all glass injector-splitter on the injector end and an all glass zero dead volume detector adaptor on the detector end. Normal operating conditions employed an oven temperature of 245 or 250° C, a column flow rate of 1 ml/min. He, a make-up flow rate (through the F.I.D.) of 30 ml/min. N_2, and a splitter ratio of ~35/1. This column afforded approximately 50,000 theoretical plates.

Analytical T.L.C.

Free Sterols: Separations were performed on 1 X 3" silica gel T.L.C. plates developed once with hexane/EtOAc (3/1). For R_f's, see Table 1.

Sterol Acetates: Separations were performed on 1 X 3" 20% $AgNO_3$ impregnated silica gel T.L.C. plates developed once with CH_2Cl_2/Et_2O/EtOAc (92.5/5/2.5). For R_f.s, see Table 1.

All analytical plates were visualized by spraying with 50% aqueous sulfuric acid and charring.

Trapping Experiment

Anaerobic Pre-treatment

S. cerevisiae of unknown strain (local brewery ale yeast) was collected by centrifugation and washed three times with 0.1 M sterile phosphate buffer (6.8 g KH_2PO_4 and 11.4 g $K_2HPO_4 \cdot 3H_2O$ per litre of buffer; pH = 6.4). Approximately 40.0 g of this yeast was suspended in 1 litre of sterile complete medium and maintained under N_2 for 90 hrs.[4]. The yeast was then collected by centrifugation and washed twice with sterile phosphate buffer.

Incubation with [2-^{3}H]-Lanosterol

A 0.669 mg sample of [2-^{3}H]-lanosterol (37) and 50 mg Triton X-100 were made up to 10.0 ml with acetone. A 10 µl aliquot of this soln. was then taken for counting (gave 8.48×10^4 cpm; therefore total activity = 8.48×10^7 cpm). The remainder of this soln. was transferred quantitatively to the bottom of a four litre Virtis fermenting flask. The acetone was blown off and 1 litre of sterile minimal medium was added and stirred for 10 min. A 20 g portion of the anaerobically pre-treated yeast was suspended in this medium and fermented aerobically at 30° C (stirring rate = 400 rpm; air flow = 5 l/min.).

After 3 hrs. of aeration, 500 ml of the fermentation mixture was withdrawn and the yeast harvested (centrifugation) and washed three times with phosphate buffer.

The collected yeast was freeze-dried to give 1.42 g of dry yeast. After 6 hrs. the remainder of the fermentation mixture was similarly treated to give 1.31 g of dry yeast.

Radioactivity Recovered from Supernatants

Both the 3 hr. and 6 hr. supernatants (plus buffer washings) were individually treated as follows:

Each was made up to 1 l with H_2O. 100 ml of this was extracted into three 50 ml portions of Et_2O. The combined Et_2O extracts were washed once with 100 ml saturated NaCl soln., dried over $MgSO_4$ and made up to 200 ml with additional Et_2O. 5.0 ml of this ethereal soln. was taken for counting.

The 3 hr. aliquot gave $5.50X10^4$ cpm; therefore the total activity = $2.20X10^7$ cpm. The 6 hr. aliquot gave $4.35X10^4$ cpm; therefore the total activity = $1.74X10^7$ cpm.

Extraction of Non-saponifiable Fraction (N.S.F.) from Freeze-Dried Yeast

Both the 3 hr. and 6 hr. freeze-dried yeast were individually hydrolyzed as follows:

The yeast was suspended in a soln. contaihing 7.5 g KOH, 10 ml water, and 35 ml ethanol. This suspension was refluxed under N_2 for 3 hrs., cooled and diluted with 50 ml water and extracted into four 75 ml portions of hexane. The combined hexane extracts were washed thrice with 100 ml portions of water and dried ($MgSO_4$). The solvent was

then removed in vacuo.

The 3 hr. yeast yielded 50.4 mg N.S.F. and the 6 hr. yeast 40.6 mg N.S.F. Each N.S.F. was made up to 25.0 ml with benzene and 50 μl was taken for counting. The 3 hr. N.S.F. aliquot gave 2.20×10^4 cpm; therefore the total activity = 1.10×10^7 cpm. The 6 hr. N.S.F. aliquot gave 3.28×10^4 cpm; therefore the total activity = 1.64×10^7 cpm.

Isolation of 4,4-Dimethyl and 4α-Methyl Sterols

The 3 hr. N.S.F. and 6 hr. N.S.F. were individually treated as follows:

Each N.S.F. (dissolved in a minimum of cyclohexane) was applied to a single preparative T.L.C. plate (20 X 20 cm X 1 mm silica gel). The plate was developed one time with cyclohexane/ethyl acetate (3/1). The plate was then sprayed with a 0.1% solution of Rhodamine 6 G in acetone and visualized under UV light. The 4,4-dimethyl and 4α-methyl sterol bands (and also the remainder of the plate) were scraped off and eluted with ether. The ether was removed in vacuo. Each fraction was made up to 10.0 ml with benzene and a 25 μl aliquot of each was taken for counting. The counting results are given in Table 2.

Addition and Re-isolation of Trapping Sterols

The 4,4-dimethyl and 4α-methyl sterol fractions of the 6 hr. N.S.F. were acetylated by overnight treatment with acetic anhydride/pyridine (1/2).

A) 4,4-Dimethyl Sterols: To the 4,4-dimethyl sterol acetates was added 100 mg each of lanosteryl acetate (1 Ac), 32-norlanosteryl acetate (3 Ac) and 4,4-dimethylfecosteryl acetate (4 Ac). This mixture was applied to 8 preparative T.L.C. plates (20 X 20 cm X 1 mm 20% $AgNO_3$ impregnated silica gel) and developed once with CH_2Cl_2/EtOAc (97/3). The plates were sprayed with 0.1% Rhodamine 6 g in acetone and visualized under UV light. The appropriate bands were scraped off and eluted with ether. Each sterol acetate was then re-chromatographed as above on 5 preparative T.L.C. plates.

The recovered lanosterol acetate was recrystallized from 95% ethanol to constant specific activity (see Table 4).

The recovered 32-norlanosteryl acetate was recrystallized repeatedly from 95% ethanol. It was then mixed with 20.0 mg unlabelled lanosteryl acetate and chromatographed as above on 2 prep. T.L.C. plates. The 3 Ac recovered from these plates was again crystallized from 95% ethanol. The specific activity was essentially the same as before admixture (see Table 5).

The recovered 4,4-dimethylfecosteryl acetate was also recrystallized repeatedly from 95% ethanol. It was then mixed with 20.0 mg unlabelled lanosteryl acetate and chromatographed as above on 2 prep. T.L.C. plates. The 4 Ac

recovered from these plates was recrystallized from 95% ethanol and finally re-chromatographed on a single prep. T.L.C. plate. The specific activity was essentially the same as before admixture (see Table 6).

B) 4α-Methyl Sterols: To the 4α-methyl sterol acetates was added 100 mg of 31-norlanosteryl acetate (5 Ac). This was chromatographed on 4 prep. T.L.C. plates as above. The recovered 5 Ac was repeatedly recrystallized from methanol (see Table 7).

A 0.82 mg portion of 5 Ac was then mixed with 5.27 mg unlabelled 1 Ac and 5.15 mg unlabelled 5 Ac. The acetates in the mixture were reduced to the free alcohols (with $LiAlH_4$ in ether) and separated on 2 prep. T.L.C. plates (developed with hexane/EtOAc - 3/1). The lanosterol and 31-norlanosterol bands were scraped off and eluted with ether. Each eluted sterol was re-chromatographed as above on a single prep. T.L.C. plate and then assayed for radioactivity. The recovered lanosterol and 31-norlanosterol had specific activies of 25.9 cpm/mg and 6.3 cpm/mg respectively. This implies that the 0.82 mg sample of 5 Ac had 42 cpm/mg from intrinsic activity and 152 cpm/mg from 1 Ac contamination.

Search for 4,4-Dimethylfecosterol (4) and 31-Norlanosterol (5) in Total Yeast Sterols

Preparation of Total Yeast Sterols

Yeast Sterol Concentrate (Solid Residues): This concentrate was used as supplied by Mycofarm-Delft.

Yeast Sterol Concentrate (Solution Residues): A 500 ml portion of this material (supplied by Mycofarm-Selft) was concentrated further by removing most of the solvent on a rotary evaporator. The resulting resinous residue was applied to 150 g silica gel and eluted with 3 l benzene. Removal of the benzene from the eluent gave 17.6 g yellow semi-solid.

N.S.F. from Laboratory Grown Yeast: A 4 l Virtis fermenting flask containing 2 l sterile complete medium was inoculated with a 24 hr. test tube culture of S. cerevisae (brewery ale yeast). The yeast was grown aerobically for 48 hrs. (temp. = 30° C; aeration = 4 to 5 l of air/min.; stirring = 500 rpm), harvested (centrifugation), washed 3 times with water and freeze-dried. This gave 13.5 g dry wt. yeast.

A 13.0 g portion of this freeze-dried yeast was saponified by refluxing under argon for 3 hrs. in a mixture of 400 ml 95% ethanol, 100 ml water and 75 g KOH. After cooling, the mixture was diluted with 500 ml water and extracted into four 500 ml portions of hexane. The combined hexane

extracts were washed twice with water and dried ($MgSO_4$). Removal of the solvent in vacuo yielded 1.34 g yellow solid (N.S.F.).

N.S.F. from Brewery Grown Yeast: Fresh brewery ale yeast was harvested (centrifugation) and washed twice with water. Approximately 1 kg (wet wt.) of the yeast was saponified by refluxing under argon for 3 hrs. in a mixture of 1700 ml 95% ethanol, 300 ml water and 300 g KOH. The saponification mixture was then cooled, diluted with 1500 ml water and extracted with hexane. This gave 1.34 g yellow semi-solid (N.S.F.).

Isolation and Analysis of 4,4-Dimethyl and 4_α-Methyl Sterol Fractions

The sterol concentrate or N.S.F. was applied to 20 to 30 times its weight of neutral alumina (grade III) and eluted with hexane/benzene (1/1 - V/V). The 4,4-dimethyl sterols were eluted first, followed by the 4_α-methyl sterols and finally the 4-desmethyl sterols. Column development was followed by analytic T.L.C. of the eluent. Any column eluent of mixed composition was re-chromatographed on either columns or preparative T.L.C. plates (20 X 20 cm X 1 mm silica gel developed once with hexane/EtOAc - 3/1). Preparative plates were visualized by spraying with 0.1% Rhodamine 6 G in acetone and illuminating with UV light. The separated sterol fractions were analyzed (as TMS

derivatives) on the capillary G.L.P.C. column. Excessive quantities were often injected in an effort to detect minor components.

Separation and Analysis of Sterol Acetates

The sterols of the 4,4-dimethyl and 4α-methyl fractions were acetylated by overnight treatment with acetic anhydride/pyridine (1/2)[4].

The 4α-methyl sterol acetates were separated in an effort to isolate 31-norlanosteryl acetate (5 Ac). The acetates were applied to preparative T.L.C. plates (20 X 20 cm X 1 mm of 20% $AgNO_3$ impregnated silica gel) which were developed once with CH_2Cl_2/EtOAc (97/3). The plates were visualized by spraying with 0.1% Rhodamine 6 G in acetone and illuminating with UV light. The appropriate bands were scraped off, eluted with ether and analyzed on the capillary G.L.P.C. column.

The 4,4-dimethyl sterol acetates were separated in order to obtain sufficient 32-norlanosteryl acetate (3 Ac) for trapping and synthetic purposes. The acetates were applied to 50 times their weight of 20% $AgNO_3$ impregnated silica gel and eluted with hexane/CH_2Cl_2 (4/1 - V/V). Development was followed by analytic T.L.C. Lanosteryl acetate (1 Ac) was eluted first, followed by 32-norlanosteryl acetate (3 Ac).

Purification of Lanosterol (1)

24-bromolanosterol (24)

A solution of 7.5 ml bromine (140 mmole) in 100 ml acetic acid was added dropwise to a solution of 50.0 g crude lanosterol* in 700 ml ether maintained at 0° C. The reaction mixture was left stirring overnight at 4° C, after which it was washed sequentially with 500 ml water (containing sufficient $NaHSO_3$ to reduce any remaining free bromine), 500 ml 5% aqueous $NaHCO_3$ (twice), and 500 ml water (twice). Drying ($MgSO_4$) and removal of the ether *in vacuo* gave 60.8 g yellow solid (crude 24,25-dibromolanosterol).

This material was refluxed in 1400 ml 1 M ethanolic KOH for three hrs. and then poured into 1 l ice/water. The resulting suspension was neutralized with conc. HCl and extracted with ether. The ether extract was washed with saline and dried ($MgSO_4$). Removal of the ether gave 47.1 g yellow solid (crude 24-bromolanosterol).

The crude 24-bromolanosterol was fractionally crystallized from chloroform/methanol (1/1 - V/V) using the double withdrawal technique[22] to give 13.3 g purified 24-bromo-

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* The crude lanosterol was 58% lanosterol, 37% 24,25-dihydrolanosterol, 3% agnosterol and 2% 24,25-dihydroagnosterol by G.L.P.C. analysis.

lanosterol (24) as colourless needles, mp. 197-199° C (lit.[23] mp. 196-198° C).

M.S.: 506, 504 (M^+, 35) 491, 489 ($M^+ - CH_3$, 100), 473 471 ($M^+ - CH_3 - H_2O$, 51), 425 (M^+ - Br, 21)

Lanosterol (1)

A 1 M naphthalene-sodium solution was prepared by stirring 4.0 g (174 mmole) sodium and 12.8 g (100 mmole) naphthalene in 100 ml dry THF under N_2 for 1 hr.

The resulting dark green soln. was transferred via syringe to a soln. of the 24-bromolanosterol in 500 ml dry THF maintained under N_2. Decolouration of the napthalene-sodium soln. was instantaneous until an excess had been added, at which point addition was stopped.

The reaction mixture was worked up by pouring into 500 ml ice/water and extracting twice into 500 ml CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with water until neutral and dried ($MgSO_4$). Removal of the solvent in vacuo left a mixture of lanosterol and naphthalene. Removal of the naphthalene via sublimation afforded 11.4 g purified lanosterol (1) as colourless needles (from acetone/methanol - 2/1), mp. 137.5-139° C (lit.[32] mp. 140° C).

IR.: 3300cm^{-1} (O-H), 1030 cm^{-1} (C-O)

U.V.: <0.5% 7,9 diene (from agnosterol and 24,25-dihydroagnosterol)

G.L.P.C.: <0.5% agnosterol + 24,25-dihydroagnosterol

~ 1% 24,25-dihydrolanosterol

M.S.: 426 (M^+, 57), 411 ($M^+ - CH_3$, 100), 393 ($M^+ - CH_3 - H_2O$, 37)

N.M.R.: 0.70 (s, 3H, 13-C<u>H</u>$_3$), 0.82 (s, 3H, 4β-C<u>H</u>$_3$), 0.89 (s, 3H, 14-C<u>H</u>$_3$), 0.94 (d, J=4 cps, 3H, 20-C<u>H</u>$_3$), 1.00 (s, 6H, 4α-C<u>H</u>$_3$ and 10-C<u>H</u>$_3$), 1.61 and 1.68 (br s and br s, 3H and 3H, C=C(C<u>H</u>$_3$)$_2$)

Synthesis of 4,4-Dimethylfecosterol (4)

4,4-Dimethyl-24ξ-bromo-25-hydroxy-5α-cholest-8-en-3β-yl Acetate (14)

To 600.9 mg 32-norlanosteryl acetate (3 Ac) (1.32 mmole), dissolved in 10.5 ml THF and 2.5 ml water at 0° C, was added with stirring 270 mg N-bromosuccinimide (1.52 mmole). After stirring an additional 2 hrs. at 0° C, the reaction mixture was poured into 25 ml water and extracted into two 25 ml portions of ether. The combined ether extracts were washed once with 50 ml water and dried ($MgSO_4$). Removal of the ether in vacuo gave 0.745 g tan solid (14), mp. 160-165° C.

IR.: 3500 cm^{-1} (O-H), 1725 cm^{-1} (C=O, ester), 1255 cm^{-1} (C-O, ester)

M.S.: 550 and 552 (M^+, 100), 535 and 537 ($M^+ - CH_3$, 14)

4,4-Dimethyl-24ξ,25-epoxy-5α-cholest-8-en-3β-yl Acetate (15)

To the hydrobromide, 14, dissolved in a mixture of 15 ml acetone and 5 ml methanol, was added 0.5 g powdered anhydrous K_2CO_3. This was stirred at room temp. for 1½ hrs. The reaction mixture was then poured into 25 ml water and extracted twice into 25 ml CH_2Cl_2. The combined CH_2Cl_2 extracts were washed once with 50 ml water and dried ($MgSO_4$). Removal of the CH_2Cl_2 in vacuo gave 0.615 g light yellow solid (15), mp. 113-118° C.

IR.: 1730 cm^{-1} (C=O, ester), 1250 cm^{-1} (C-O, ester and epoxide)

M.S.: 470 (M^+, 100), 455 ($M^+ - CH_3$, 27), 452 (27), 410 (M^+ - AcOH, 63), 395 ($M^+ - CH_3$ - AcOH, 59)

4,4-Dimethyl-24-oxo-5α-cholest-8-en-3β-yl Acetate (16)

To the epoxide, 15, (1.30 mmole), dissolved in 25 ml DMSO was added 1.12 g NaI and 1.28 g 1-iodopropane. The reaction mixture was stirred at 80° C for 3 hrs. The resulting dark red soln was poured into 50 ml salted ice/water and extracted into four 50 ml portions of CH_2Cl_2. The combined CH_2Cl_2 extracts were washed once with 200 ml water containing 4.5 g $Na_2S_2O_3.5H_2O$ and twice with 200 ml water. Drying ($MgSO_4$) and removal of the solvent in vacuo gave 0.614 g yellow solid (crude 16).

This crude material was chromatographed on 25 g silica gel developed with benzene/ethyl acetate.)98/2-V/V). Collection of the appropriate fractions gave 0.335 g of 16 as yellow needles, mp. 115-120° C.

IR.: 1730 cm^{-1} (C=O, ester), 1715 cm^{-1} (C=O, ketone), 1250 cm^{-1} (C-O, ester)

M.S.: 470 (M^+, 100), 455 ($M^+ - CH_3$, 16), 410 (M^+ - AcOH, 91), 395 ($M^+ - CH_3$ - AcOH, 58), 367 (36)

4,4-Dimethylfecosterol (4)

A) Phosphonium salt: A solution of 10.1 g bromomethane (105 mmole) and 30.0 g triphenylphosphine (114

mmole) in 200 ml benzene was stirred at room temp. for four days. The salt which precipitated was collected by filtration, washed with benzene and dried in vacuo over P_2O_5 to give 38.3 g methyltriphenylphosphonium bromide, as a white solid, mp. 330-333° C (lit.[33] mp. 327-331° C).

B) Wittig reaction: A 1.803 g (5.05 mmole) portion of the phosphonium salt was suspended in 20 ml dry THF under N_2. An equimolar amount of butyllithium in hexane (23.1 wt. %) was added via syringe through a septum. The resulting orange ylide soln was stirred under N_2 for an additional 2 hrs.

The keto ester, 16, (0.71 mmole), dissolved in 5 ml dry THF, was added via syringe to the ylide soln and the reaction mixture was refluxed under N_2 for 24 hrs. after cooling, it was poured into 25 ml water and extracted three times into 25 ml benzene. The combined benzene extracts were washed twice with 50 ml water and dried ($MgSO_4$). Removal of the benzene in vacuo gave 0.906 g orange solid (crude 4 plus triphenylphosphine oxide).

This crude sterol was purified by chromatography on 20 g silica gel developed with benzene. Collection of the appropriate fractions gave 0.256 g of 4,4-dimethylfecosterol (4), as colourless plates, mp. (from methanol) 167-170° C.

M.S.: 426 (M^+, 100), 411 ($M^+ - CH_3$, 33), 393 $M^+ - CH_3 - H_2O$, 17)

4,4-Dimethylfecosterol Acetate (4 Ac)

A portion of 4 was converted to its acetate by overnight treatment with acetic anhydride/pyridine (1/2). This gave colourless plates (from 95% ethanol), mp. 134-135.5° C (lit.[8] mp. 152-154° C).

IR.: 3080 cm^{-1} (C=CH_2), 1730 cm^{-1} (C=O, ester), 1640 cm^{-1} (C=C), 1245 cm^{-1} (C-O, ester), 890 cm^{-1} (C=CH_2)

M.S.: 468 (M^+, 100), 453 ($M^+ - CH_3$, 22), 408 (M^+ - AcOH, 33), 393 ($M^+ - CH_3$ - AcOH, 31)

N.M.R.: 0.61 (s, 3H, 13-CH_3), 0.88 (s, 6H, 4α-CH_3 and 4β-CH_3), 0.99 (d, J=2.5 cps, 6H, 25-($C\underline{H}_3$)$_2$, 1.08 (s, 3H, 10-CH_3), 2.04 (s, 3H, $C\underline{H}_3CO_2$-), 4.70 (br s, 2H, C=$C\underline{H}_2$)

Synthesis of 31-Norlanosterol (5)

Methyl 3-Oxo-4,4,14-trimethyl-5α-chol-8-en-24-oate (25)

Two 6.0 g (14 mmole) batches of purified lanosterol (1) were individually treated as follows. Each was dissolved in 1 l CH_2Cl_2/EtOAc (1/1) containing 1% pyridine and cooled to -78° C (acetone/dry ice). A stream of O_3/O_2 was passed through the soln. (1 l/min.) until a pale blue colour developed, indicating a excess of ozone. After warming to room temperature the solvent was removed *in vacuo* and replaced by 250 ml ice cold acetone. Jones reagent[25] was added dropwise to the ozonide soln. (maintained at 0° C) until an orange-brown colour persisted. This was then poured into 1 l ice/H_2O and extracted twice into 500 ml ether. The combined ether extracts were shaken with 1 l 5% aqueous NaOH. The aqueous layer and interphase solid (emulsions were broken via centrifugation) were drawn off, acidified with conc. HCl and extracted twice into 800 ml ether.

The combined ethereal extracts of both batches were washed with saline and dried ($MgSO_4$). Removal of the ether *in vacuo* gave 10.0 g light yellow solid (crude keto acid).

Methylation of the crude keto acid with ethereal diazomethane[26] and purification via column chromatography (350 g silica gel developed with benzene/EtOAc - 95/5)

gave 4.61 g white solid (purified 25), colourless needles (from methanol), mp. 141-143° C, (lit.[34] mp. 141-143° C).

IR.: 1740 cm^{-1} (C=O, ester), 1710 cm^{-1} (C=O, ketone), 1170 cm^{-1} (C-O, ester)

M.S.: 428 (M^+, 25), 413 ($M^+ - CH_3$, 100), 399 (16), 395 (10), 381 (11)

N.M.R.: 0.73 (s, 3H, 13-$C\underline{H}_3$), 0.90 (br s, 6H, 14-$C\underline{H}_3$ and 20-$C\underline{H}_3$), 1.07 (s, 3H, 4α or 4β or 10-$C\underline{H}_3$), 1.09 (s, 3H, 4α or 4β or 10-$C\underline{H}_3$), 1.11 (s, 3H, 4α or 4β or 10-$C\underline{H}_3$), 3.68 (s, 3H, $-CO_2C\underline{H}_3$)

Methyl 3-Hydroxyimino-4,4,14-trimethyl-5α-chol-8-en-24-oate (26)

To 4.47 g (10.4 mmole) 25 suspended in 100 ml absolute ethanol was added 2.0 g hydroxylamine hydrochloride (29 mmole) and 5 ml triethylamine. This was refluxed for ½ hr. After cooling, the ethanol was removed in vacuo and the residue extracted twice into 50 ml CH_2Cl_2 against 50 ml water. The combined CH_2Cl_2 extracts were washed once with 100 ml water and dried ($MgSO_4$). Removal of the CH_2Cl_2 in vacuo gave 4.66 g white solid (26), colourless needles (from methanol), mp. 168-170° C.

IR.: 3470 cm^{-1} (O-H), 1740 cm^{-1} (C=O, ester), 1715 cm^{-1} (C=N), 1170 cm^{-1} (C-O, ester), 930 cm^{-1} (N-O)

M.S.: 443 (M^+, 35), 428 ($M^+ - CH_3$, 100), 412 (41),

410 (45), 330 (34)

N.M.R.: 0.73 (s, 3H, 13-$C\underline{H}_3$), 0.88 (br s, 6H, 14-$C\underline{H}_3$ and 20-$C\underline{H}_3$), 1.10 (s, 6H, 4α-$C\underline{H}_3$ and 4β-$C\underline{H}_3$), 1.17 (s, 3H, 10-$C\underline{H}_3$), 3.69 (s, 3H, -$CO_2C\underline{H}_3$)

Methyl 2-Cyano-3,4-seco-4,14-dimethyl-4-methylene-3-nor-5α-chol-8-en-24-oate (27)

A solution of 4.65 g (10.4 mmole) 26 and 9.0 g (46 mmole) freshly recrystallized p-toluenesulfonyl chloride in 60 ml dry pyridine was refluxed under N_2 for 3 hrs. This was then neutralized with cold 3 N HCl and extracted twice into 150 ml ether. The combined ether extracts were washed sequentially with 200 ml saline, 200 ml 5% $NaHCO_3$ soln. and 200 ml saline. Drying of the extract ($MgSO_4$) and removal of the ether in vacuo gave 4.21 g red brown gum.

This gum was chromatographed on 200 g neutral alumina (grade III) developed with benzene. Collection of the appropriate fractions gave 2.22 g pale yellow oil which slowly crystallized as colourless needles (27), mp. (from methanol) 82-83° C.

IR.: 3080 cm^{-1} (C-H of $C=CH_2$), 2250 cm^{-1} (C≡N), 1735 cm^{-1} (C=O of ester), 1635 cm^{-1} C=C of $C=CH_2$), 1165 cm^{-1} C-O, ester), 895 cm^{-1} (C-H of $C=CH_2$)

M.S.: 425 (M^+, 100), 410 ($M^+ - CH_3$, 80), 382 (13), 377 (14), 371 (64), 357 (17)

N.M.R.: 0.73 (s, 3H, 13-$C\underline{H}_3$), 0.93 (br s, 6H, 14-$C\underline{H}_3$ and 20-$C\underline{H}_3$), 0.97 (s, 3H, 10-$C\underline{H}_3$), 1.77 (br s, 3H 4-$C\underline{H}_3$), 3.69 (s, 3H, -$CO_2C\underline{H}_3$), 4.70 and 4.94 (m and m, 1H and 1H, C=$C\underline{H}_2$)

Methyl 2-Cyano-3,4-seco-4ξ,14-dimethyl-4,4-methyleneoxy-3-nor-5α-chol-8-en-24-oate (28)

To 2.20 g of the seco-nitrile, 27, (5.15 mmole) dissolved in 50 ml ice cold $CHCl_3$, was added, over a period of 15 minutes, 1.04 g 85% technical m-chloroperbenzoic acid (5.15 mmole). After an additional 2 hrs. of stirring at 0° C the $CHCl_3$ was removed in vacuo and replaced with 100 ml benzene. This was washed once with 50 ml saturated $NaHCO_3$ soln., twice with 50 ml water and dried ($MgSO_4$). Removal of the benzene in vacuo gave 2.48 g colourless needles (28), mp. (from methanol), 123-126° C.

IR.: 2250 cm^{-1} (C≡N), 1740 cm^{-1} (C=O, ester), 1275 cm^{-1} (epoxide), 1170 cm^{-1} (C-O, ester)

M.S.: 441 (M^+, 34), 426 ($M^+ - CH_3$, 100), 425 (20), 423 (12), 410 (26), 408 (52), 398 (17), 394 (32), 387 (31), 376 (23), 369 (53), 358 (42), 326 (19), 308 (12)

N.M.R.: 0.74 (s, 3H, 13-$C\underline{H}_3$), 0.91 (br s, 6H, 14-$C\underline{H}_3$ and 20-$C\underline{H}_3$), 1.08 (s, 3H, 10-$C\underline{H}_3$), 1.33 (s, 3H, 4-$C\underline{H}_3$), 2.70 (s, 2H, 30-$\underline{H}_2$), 3.69 (s, 3H, -$CO_2C\underline{H}_3$)

Methyl 3-Oxo-4α,14-dimethyl-5α-chol-8-en-24-oate (29)

The epoxide, 28, and 0.5 ml freshly distilled BF_3 etherate were refluxed in 50 ml dry toluene under N_2 for 1½ hrs. After cooling, an additional 50 ml benzene was added, and the organic layer was washed sequentially with 100 ml saline, 100 ml 5% $NaHCO_3$ and 50 ml saline. Drying ($MgSO_4$) and removal of the solvent in vacuo gave 2.21 g red-brown gum.

This gum was chromatographed on 100 g silica gel using benzene and then benzene/$CHCl_3$ (3/1) as development solvent. This gave 0.557 g of the keto-ester, 29, as colourless needles, mp. 131-136° C.

IR.: 1740 cm^{-1} (C=O, ester), 1715 cm^{-1} (C=O, ketone)

M.S.: 414 (M^+, 17), 399 ($M^+ - CH_3$, 100), 385 (15), 367 (12)

N.M.R.: 0.74 (s, 3H, 13-CH_3), 0.89 (br s, 6H, 14-CH_3 and 20-CH_3), 1.02 (d, J=6.5 cps, 3H, 4α-CH_3), 1.20 (s, 3H, 10-CH_3), 3.68 (s, 3H, $-CO_2CH_3$)

3-Ethylene Ketal of Methyl 3-Oxo-4α,14-dimethyl-5α-chol-8-en-24-oate (30)

The keto ester, 29, (1.35 mmole), together with 3 ml ethylene glycol (54 mmole) and 100 mg p-toluenesulfonic acid were dissolved in 50 ml benzene and refluxed for 4½ hrs. in a flask fitted with a Dean-Stark trap. After cooling, an additional 50 ml benzene was added and the organic layer was washed once with 50 ml saturated $NaHCO_3$

soln. and twice with 50 ml water. Drying ($MgSO_4$) and removal of the benzene in vacuo gave 0.606 g of the ketal ester, 30, as off-white needles, mp. 146-150° C.

IR.: 1740 cm^{-1} (C=O, ester), 1170 cm^{-1} (C-O, ester)

M.S.: 458 (M^+, 35), 443 ($M^+ - CH_3$, 100), 429 (20), 381 (11), 359 (21)

3-Ethylene Ketal of 4α,14-Dimethyl-24-hydroxy-5α-chol-8-en-3-one (31)

To the ketal ester, 31, (1.32 mmole) dissolved in 50 ml ice cold dry ether, was added 100 mg $LiAlH_4$ (2.64 mmole). After stirring this for 5½ hrs. at room temp. the excess $LiAlH_4$ was destroyed by adding 1 ml of 10% aqueous NaOH. The ethereal soln. was then filtered and dried ($MgSO_4$). Removal of the ether in vacuo gave 0.567 g 31 as colourless needles, mp. 174-177° C.

IR.: 3490 cm^{-1} (O-H)

M.S.: 430 (M^+, 28), 415 ($M^+ - CH_3$, 100), 401 (18), 353 (13), 331 (26)

3-Ethylene Ketal of 3-Oxo-4α,14-dimethyl-5α-chol-8-en-24-al (32)

A soln. of chromium trioxide-pyridine complex in CH_2Cl_2 was produced by adding 800 mg dry CrO_3 (8 mmole) to a soln. of 1.29 ml dry pyridine (16 mmole) in 20 ml dry spectro grade CH_2Cl_2 and stirring under N_2 at room temp. for ½ hr.

The ketal alcohol, 31, (1.32 mmole) was dissolved in 10 ml dry CH_2Cl_2 and added to the above soln. A black sludge immediately precipitated. The reaction mixture was stirred at room temp. under N_2 for ½ hr., after which 100 ml benzene was added. The organic layer was then washed three times with 50 ml 5% aqueous NaOH, twice with 50 ml saline and dried ($MgSO_4$). Removal of the solvent in vacuo gave 0.556 g 32, as a pale yellow resin. The material hardened but did not crystallize.

IR.: 2720 cm^{-1} (C-H, aldehyde), 1725 cm^{-1} (C=O, aldehyde)

M.S.: 428 (M^+, 25), 413 ($M^+ - CH_3$, 100), 399 (11), 351 (11), 329 (21)

4α,14-Dimethyl-5α-cholesta-8,24-dien-3-one (34)

A) Phosphonium salt: A soln. of 13.0 g triphenylphosphine (50 mmole) and 6.0 ml 2-bromopropane (63.5 mmole) in 75 ml m-xylene was refluxed for two weeks. The salt which precipitated was collected from the cooled soln. by filtration and then washed with benzene and dried in vacuo over P_2O_5. This gave 3.20 g isopropyltriphenylphosphonium bromide as colourless crystals, mp. 238-240° C (lit.[35] mp. 238-239° C).

B) Wittig reaction: A 3.19 g (8.25 mmole) portion of the phosphonium salt was suspended in 40 ml dry THF under N_2. An equimolar amount of butyllithium in hexane

(23.1 wt. %) was added via syringe through a septum. The resulting red orange ylide soln. was stirred under N_2 for an additional 1½ hrs.

The ketal aldehyde, 32, (1.30 mmole) dissolved in 5 ml dry THF was added via syringe to the ylide soln. and the reaction mixture was refluxed for 24 hrs. under N_2. After cooling, it was poured into 75 ml water and extracted twice into 75 ml benzene. The combined benzene extracts were washed twice with 75 ml water and dried ($MgSO_4$). Removal of the benzene in vacuo gave 1.59 g off-white solid (ketal, 33, plus triphenylphosphine oxide).

C) Hydrolysis of ketal, 33: The crude ketal was dissolved in a mixture of 30 ml THF and 10 ml 0.5% aqueous H_2SO_4 and stirred for 13 hrs. This was then poured into 50 ml saturated $NaHCO_3$ soln. and extracted into 100 ml benzene. The benzene layer was washed twice with 50 ml water and dried ($MgSO_4$). Removal of the benzene in vacuo gave 1.52 g light yellow solid (crude ketone, 34, plus triphenylphosphine oxide).

The ketone was purified on 75 g silica gel developed with benzene to give 0.418 g colourless resinous material, (34).

IR.: 1710 cm^{-1} (C=O, ketone)

M.S.: 410 (M^+, 35), 395 ($M^+ - CH_3$, 100), 381 (14)

N.M.R.: 0.73 (s, 3H, 13-C$\underline{H}_3$), 0.89 (br s, 6H, 14C$\underline{H}_3$

and 20-$C\underline{H}_3$), 1.02 (d, J=6.5 cps, 3H, 4α-$C\underline{H}_3$), 1.19 (s, 3H, 10-$C\underline{H}_3$), 1.60 and 1.69 (br s and br s, 3H and 3H, $C{=}C(C\underline{H}_3)_2$)

31-Norlanosterol (5)

To the ketone, 34, (1.02 mmole) dissolved in 50 ml dry ether was added 1.27 g $LiAlH(O\text{-}tBu)_3$ (5.0 mmole). After stirring this for 2 hrs. at room temp. the excess hydride reagent was destroyed by adding 1 ml of 10% aqueous NaOH. The ethereal soln. was then dried with $MgSO_4$ and filtered. Removal of the ether in vacuo gave 0.341 g white solid (5), colourless needles (from acetone/methanol), mp. 110-112° C.

M.S.: 412 (M^+, 50), 397 ($M^+ - CH_3$, 100), 383 (13), 379 ($M^+ - CH_3 - H_2O$, 11)

31-Norlanosteryl Acetate (5 Ac)

A portion of 5 was converted to its acetate by overnight treatment with acetic anhydride/pyridine (1/2). This gave colourless needles (from methanol), mp. 105-106.5° C (lit.[18] mp. 103-104° C).

IR.: 1740 cm^{-1} (C=O, ester), 1255 cm^{-1} (C-O, ester)

M.S.: 454 (M^+, 74), 439 ($M^+ - CH_3$, 100), 425 (11), 379 ($M^+ - CH_3 - AcOH$, 49)

N.M.R.: 0.73 (s, 3H, 13-$C\underline{H}_3$), 0.87 (d, J=6 cps, 3H, 4α-$C\underline{H}_3$), 0.90 (s, 3H, 14-$C\underline{H}_3$), 0.94 (d, J=5 cps, 3H, 20-$C\underline{H}_3$), 1.00 (s, 3H, 10-$C\underline{H}_3$),

1.61 and 1.69 (br s and br s, 3H and 3H, $C{=}C(C\underline{H}_3)_2$), 2.05 (s, 3H, $C\underline{H}_3CO_2$-)

Synthesis of [2-^{3}H]-Lanosterol (37)

Lanosterone (35)

To a soln. of 0.95 g dry pyridine (12 mmole) in 20 ml dry spectro grade CH_2Cl_2 was added 0.60 g dry CrO_3 (6 mmole). A 213 mg portion of purified lanosterol (1) (0.5 mmole) was added to the resulting red soln. and stirred for 15 minutes. The organic layer was then decanted from the black precipitate, diluted with 50 ml ether and washed successively with 25 ml 5% aqueous NaOH (thrice), 25 ml 0.25 N HCl, 25 ml saturated $NaHCO_3$ soln. and 25 ml water. Drying ($MgSO_4$) and removal of the solvent in vacuo gave 199.8 mg clear oil. Recrystallization of this oil from acetone/methanol gave 89.2 mg of lanosterone, 35, as colourless needles, mp. 62-64° C (lit.[6] mp. 80° C).

M.S.: 424 (M^+, 50), 409 ($M^+ - CH_3$, 100)

N.M.R.: 0.72 (s, 3H, 13-C$\underline{H}_3$), 0.90 (s, 3H, 14-C$\underline{H}_3$), 0.93 (d, J=5 cps, 3H, 20-C$\underline{H}_3$), 1.07 (s, 3H, 4α or 4β or 10-C$\underline{H}_3$), 1.10 (s, 3H, 4α or 4β or 10-C$\underline{H}_3$), 1.12 (s, 3H, 4α or 4β or 10-C$\underline{H}_3$), 1.61 and 1.68 (br s and br s, 3H and 3H, C=C(C$\underline{H}_3$)$_2$), 5.10 (br t, J=7 cps, 24-H)

[2-^{3}H]-Lanosterone (36)

A) Tritiated Alumina Column: A 6.6 g sample of basic alumina was thoroughly dehydrated by maintaining it under high vacuum at 350° C for three hrs. After cooling, 0.2 ml

of HTO (5 Ci/ml) was added to the alumina and equilibrated by shaking for three hrs. in a sealed flask. The tritiated alumina was packed in dry pentane/benzene (1/1 - V/V) in a small glass column. (A 10 ml burette fitted with a teflon stopcock was used.)

B) Tritium Exchange of Lanosterone: A 50.0 mg portion of the lanosterone was dissolved in a small volume of dry pentane/benzene (1/1) and applied to the column. The column was then developed with 200 ml dry pentane/benzene (1/1) at a flow rate of 0.5 ml/minute, collecting all of the 200 ml eluent in one fraction. Removal of the solvent from this eluent gave 51.6 mg clear oil ([2-^{3}H]-lanosterone) (36).

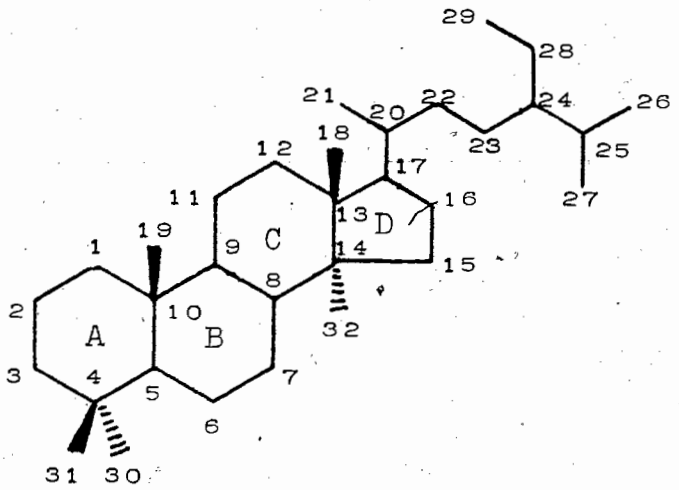
[2-^{3}H]-Lanosterol (37)

To the tritiated lanosterone (36) (0.118 mmole), dissolved in 10 ml dry ether, was added 300 mg (1.18 mole) of $LiAlH(O\text{-}tBu)_3$. The reaction flask, fitted with a drying tube, was left stirring at room temp. for two hrs. Excess hydride reagent was then destroyed by adding 0.25 ml 10% aqueous NaOH and stirring for 15 minutes. Drying of the ethereal soln. ($MgSO_4$), followed by removal of the ether (N_2 stream and then vacuum), gave 49.0 mg crude [2-^{3}H]-lanosterol as a white solid. This was recrystallized from acetone/methanol (1/1 - V/V) to give 35.8 mg purified [2-^{3}H]-lanosterol (37), as colourless needles, mp. 135-136.5° C (lit.[32] mp. 140°), with a specific activity of 1.25×10^8

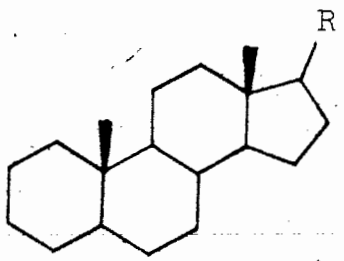
cpm/mg (=24.2 Ci/mole). The [2-^{3}H]-lanosterol was pure by T.L.C. and G.L.P.C. (>99% purity) and in both cases co-chromatographed with authentic lanosterol.

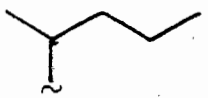
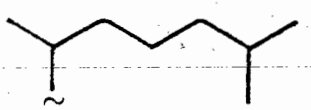
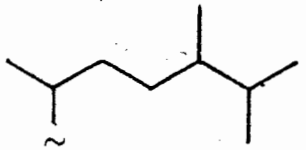
Appendix A - Relevent IUPAC Steroid Nomenclature[36]

1) Numbering



2) Carbon Skeletons



R	Name
	Cholane
	Cholestane
	Ergostane

3) Symbols and Prefixes

α - Substituent below plane of paper

β - Substituent above plane of paper

ξ - Stereochemistry unknown

Seco - Ring is broken between carbons designated

Nor - Carbon designated is lacking

Appendix B - IUPAC Nomenclature of Trivial Names Used

Trivial Name	IUPAC Name
1) Agnosterol	4,4,14-Trimethyl-5α-cholesta-7,9(11),24-trien-3β-ol
2) 24-Bromolanosterol	4,4,14-Trimethyl-24-bromo-5α-cholesta-8,24-dien-3β-ol
3) Cholestanol	5α-Cholestan-3β-ol
4) Cholesterol	Cholesta-5-en-3βol
5) 24,25-Dibromolanosterol	4,4,14-Trimethyl-24ξ,25-dibromo-5α-cholesta-8-en-3β-ol
6) 24,25-Dihydroagnosterol	4,4,14-Trimethyl-5α-cholesta-7,9(11)-dien-3β-ol
7) 24,25-Dihydrolanosterol	4,4,14-Trimethyl-5α-cholest-8-en-3β-ol
8) 4,4-Dimethylfecosterol	4,4-Dimethyl-5α-ergosta-8,24(28)-dien-3β-ol
9) Ergosterol	Ergosta-5,7,22-trien-3β-ol
10) Fecosterol	5α-Ergosta-8,24(28)-dien-3β-ol

11) Lanosterol	4,4,14-Trimethyl-5α-cholesta-8,24-dien-3β-ol
12) Lanosterone	4,4,14-Trimethyl-5α-cholesta-8,24-dien-3-one
13) 24-Methylene-24,25-dihydrolanosterol	4,4,14-Trimethyl-5α-ergosta-8,24(28)-dien-3β-ol
14) 4α-Methylfecosterol	4α-Methyl-5α-ergosta-8,24(28)-dien-3β-ol
15) 14-Methylfecosterol	14-Methyl-5α-ergosta-8,24(28)-dien-3β-ol
16) 4α-Methylzymosterol	4α-Methyl-5α-cholesta-8,24-dien-3β-ol
17) 14-Methylzymosterol	14-Methyl-5α-cholesta-8,24-dien-3β-ol
18) 31-Norlanosterol	4α,14-Dimethyl-5α-cholesta-8,24-dien-3β-ol
19) 32-Norlanosterol	4,4-Dimethyl-5α-cholesta-8,24-dien-3β-ol
20) Zymosterol	5α-Cholesta-8,24-dien-3β-ol

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