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Biosynthesis of Terpenes and Steroids

VII.[1] Unified Scheme for the Biosynthesis of Ergosterol in *Saccharomyces cerevisiae*

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Feeding experiments with labelled sterols support a proposed scheme for ergosterol biosynthesis. Some non-natural sterols have been found to be incorporated into ergosterol and the possible significance of these results is discussed.

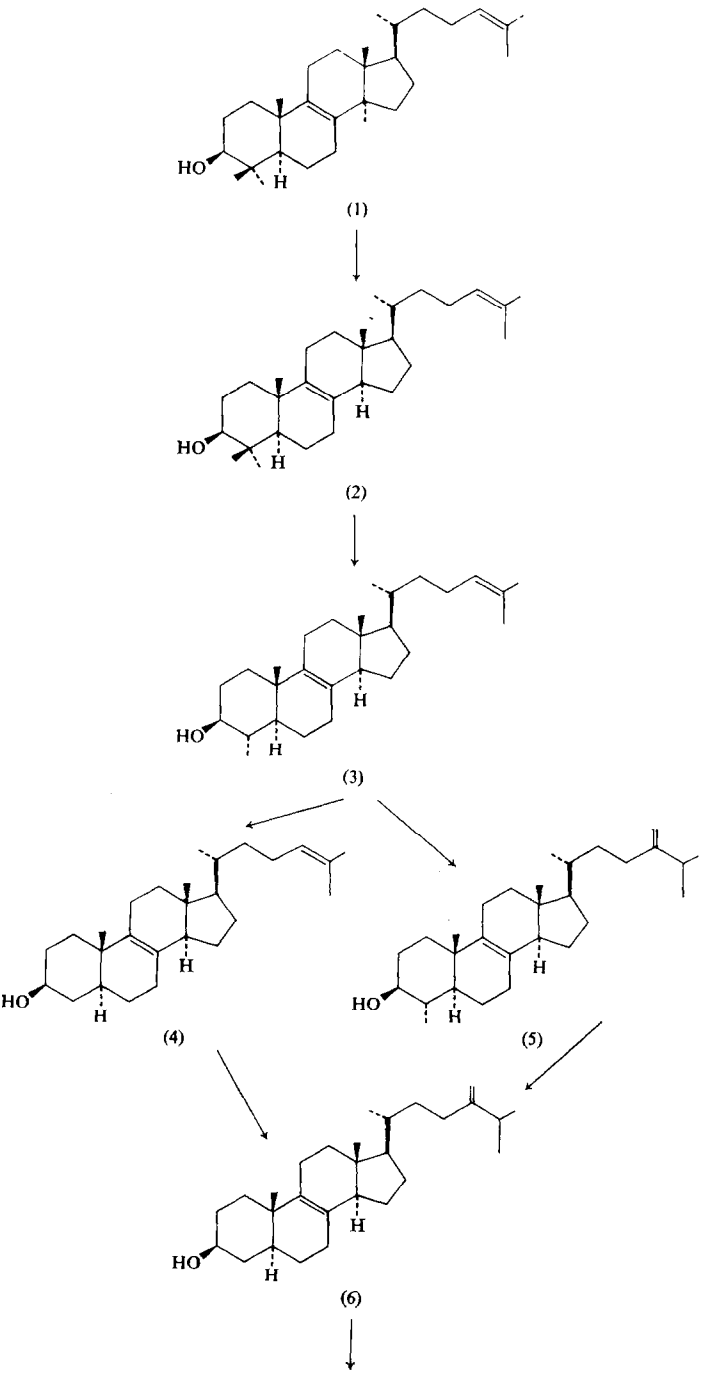
In Part VIII of this series, a tentative proposal for the late stages of ergosterol biosynthesis was defined (*1*). The complete hypothesis now depicted in Scheme I embraces the entire pathway between lanosterol, the first detectable steroidal precursor (*2*), and ergosterol. Only known yeast sterols (*3*)[2] have been involved but the relative importance of the various pathways remains undetermined. However, it is our experience that different strains of *Saccharomyces cerevisiae* vary widely in their relative proportions of sterols, so that any attempt to formulate a generalized major pathway on this basis seems likely to prove fruitless.

The scheme has been devised by considering only single-step transformations of known yeast constituents. Although complete experimental substantiation is not yet available, we feel justified at the present time in making the proposal as a basis for further work. We note that Fryberg and co-workers have recently advanced a proposal (*6*) for late stages of ergosterol biosynthesis which is substantially similar to our hypothesis. One feature incorporated into the scheme is that, at least under normal growth conditions, there appears to be essentially no movement of metabolites backwards on the biosynthetic path.

The pronounced tendency towards cocrystallization exhibited by yeast sterols makes for some difficulty in the interpretation of feeding experiments and it is only for feedings to ergosterol, purified via its adduct with *N*-phenyl-1,2,4-triazoline-3,5-dione (*3*), that unambiguous results seem attainable. The results of incorporation of natural sterols into ergosterol are shown in Table 1, in which no corrections have been made for recovered activity in the precursor. All sterols used for the present feeding experiments were prepared by base-catalysed tritiation of the corresponding 3-ketones, followed by

[1] Part VI, *J. Chem. Soc.* (*Perkin I*), 513 (1972); Part VIII, *J. Chem. Soc.* (*Perkin I*), 1231 (1972). This paper is dedicated to Professor E. Lederer on his 65th birthday and to Professor F. Sörm on his 60th birthday.

[2] The occurrence of ergosta-5,7-dien-3β-ol (XII) as a known yeast sterol will be reported separately.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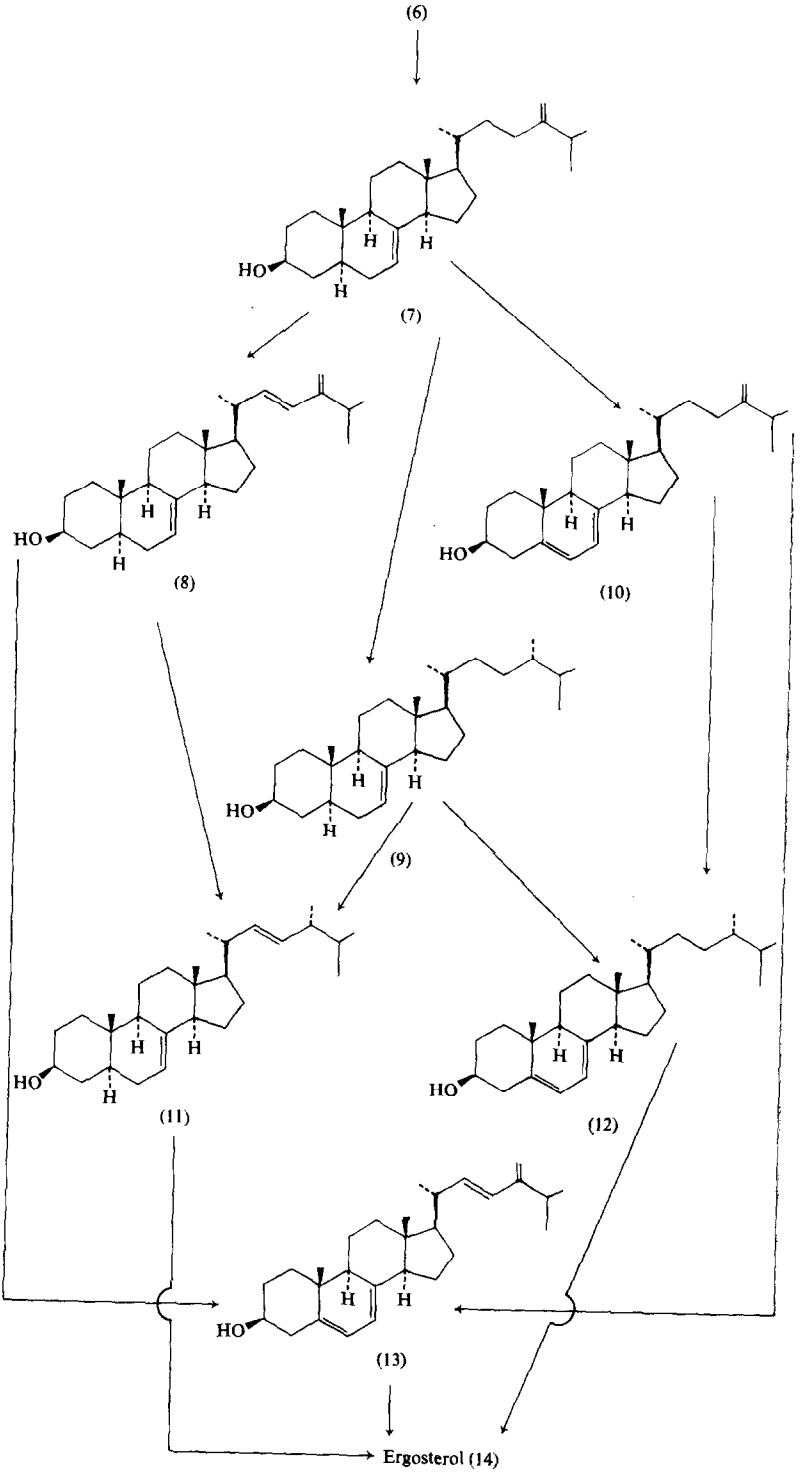
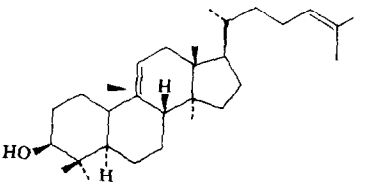


Scheme I



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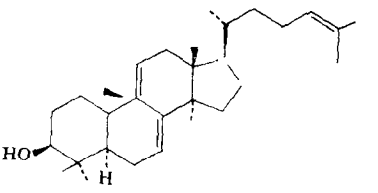

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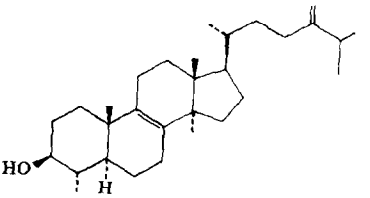

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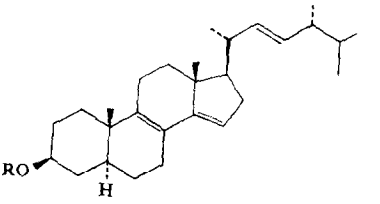

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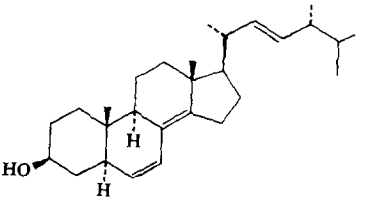

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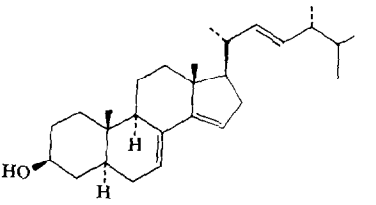

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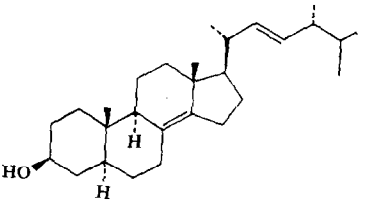

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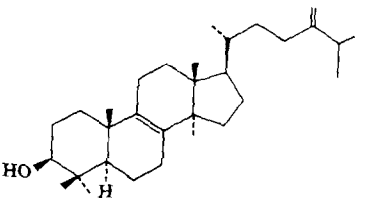

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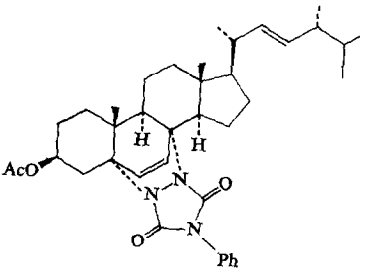

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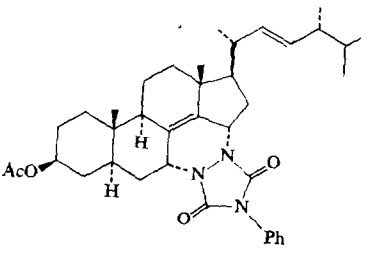
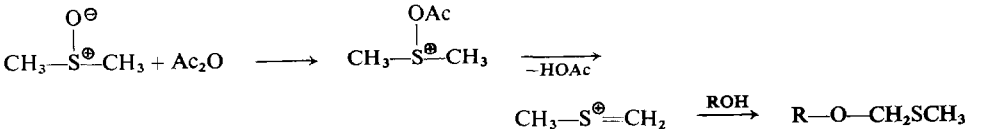


(24)

TABLE 1

Sterol fed	% activity recovered as ergosterol
Lanosterol (1)	0.12
14-Desmethyllanosterol (2)	7.2
4α-Methylzymosterol (3)	3.2
Zymosterol (4)	0.55
4α-Methylfecosterol (5)	0.24
Fecosterol (6)	1.2
Episterol (7)	4.8
Ergosta-7,22,24(28)-trien-3β-ol (8) (*1*)	0.79
Ergost-7-en-3β-ol (9)	5.0
Ergosta-7,22-dien-3β-ol (11)	13.0
Ergosta-5,7,22,24(28)-tetraen-3β-ol (13) (*5*)	24.9

reduction of the labelled ketones with lithium aluminium hydride (*4*). The ketones were obtained from the sterols by oxidation with chromium trioxide (*4*) or in the case of sterols with conjugated diene systems, by Oppenauer oxidation (*19*). Attempted oxidation of ergosterol B_1(18; R=H) with acetic anhydride-dimethyl sulphoxide (*20*) gave the corresponding methanethiomethyl ether (18; R=CH_3SCH_2) in good yield. This product must be formed by the interception of an intermediate during Pummerer rearrangement (*24*) of the sulphoxide (Scheme II)



Scheme II

The feeding and work-up procedures were the same as those previously described (*3*).

Apart from the establishment of these sterols as potential ergosterol precursors, the results obtained serve to emphasize some of the difficulties of interpretation in these experiments. For example, the incorporations obtained for 14-desmethyllanosterol (2) and 4α-methylzymosterol are significantly higher than those for lanosterol and zymosterol. However, since the metabolic pools of the two latter sterols are relatively large, much higher dilution within the cells of the labelled precursors would occur and contribute towards the low observed incorporations. The high incorporations of the two former sterols indicate a second pathway leading from 4α-methylzymosterol towards ergosterol but not proceeding via zymosterol. Although this interpretation is in agreement with our proposed scheme, there must always be entered the caveat of possible, unquantified differential permeability of the cell wall towards the various precursors. The solution of this problem must await the availability of cell-free enzyme preparations. The very high incorporations of ergosta-7,22-dien-3β-ol and ergosta-5,7,22,24(28)-tetraen-3β-ol suggest (but see above) that these sterols occupy penultimate positions

on separate branches of the biosynthetic pathway. We have not at this stage obtained any evidence for the position of ergosta-5,7-dien-3β-ol in the Scheme, but include it here for completeness. The recent report (*6*) of a high incorporation of this sterol into ergosterol in *S. cerevisiae* was accompanied by a failure to detect or trap the compound. Its status must therefore remain in doubt.

Our proposed scheme derives further support from the results of some trapping experiments shown in Tables 2 and 3.

TABLE 2

Sterol fed	% activity recovered as ergost-7-en-3β-ol
Ergosta-7,22,24(28)-trien-3β-ol (8)	0.066
Episterol (7)	0.81
Ergosta-7,22-dien-3β-ol (11)	0.00

TABLE 3

Sterol fed	% activity recovered as ergosta-7,22-dien-3β-ol
Zymosterol (4)	0.042
Ergosta-7,22,24(28)-trien-3β-ol (8)	0.45
Ergost-7-en-3β-ol (9)	1.7
Episterol (7)	0.93

All these results, with the exception of an apparent, albeit low, incorporation of the 7,22,24(28)-triene (8) into ergost-7-en-3β-ol, are entirely in accord with the hypothesis. The anomalous result may be only a reflection of the experimental difficulty of ensuring complete purity of sterols other than ergosterol.

Another possible source of error might have arisen from lability of the label. Experiments were carried out to ensure that neither inter- nor intramolecular migration of tritium occurred. For the intermolecular case, [2,4-$^3H_2$]zymosterol was fed to yeast in the normal manner together with cholesterol, a sterol alien to yeast. After work-up, there was found to be an activity associated with the cholesterol to the extent of 0.025% of the activity fed, which disappeared upon oxidation of the cholesterol to cholest-4-en-3,6-dione (*7*). Even if the activity observed in the recovered cholesterol were "real," it was insignificant in relation to the incorporations generally observed in this study. In order to verify the intramolecular integrity of the label, [2-^{3}H]-14-desmethyllanosterol was fed in the normal way and the recovered ergosterol was converted by standard reactions to 4,4-dimethylergosterone (*8*). After two cycles of base-catalysed exchange with inactive *t*-butanol, no activity remained in the ketone.

A summary of the supporting evidence for the proposed scheme is as follows: lanosterol (1) is a well-established yeast component and 14-desmethyllanosterol (2), 4α-methylzymosterol (3) and zymosterol (4) have also been isolated (*3*) and are all

converted *in vivo* to ergosterol. Furthermore, zymosterol is known to act as a substrate for the C-24 methylenating enzyme (*9*), while a probable branch via 4α-methylfecosterol (5) has already been discussed above. The possibility of methylenation at C-24 prior to removal of the C-4 and C-14 methyl groups has been previously considered and shown to be unlikely in yeast (*4*, *10*), although 24-methylenelanost-8-en-3β-ol (22) has been isolated from other fungi (*11*).

The $\Delta^8 \rightarrow \Delta^7$ isomerization, which takes place with loss of 7α-H has been studied by two groups (*12*, *13*); since neither ergost-8-en-3β-ol nor ergosta-8,22,24(28)-trien-3β-ol has been detected in wild type yeast, it seems probable that the biosynthetic pathway converges at fecosterol (6) and follows a unique route to episterol (7) before diverging among the plethora of intermediates shown in the latter stages of the scheme. The most general view of these final steps encompasses at least three enzyme systems, apparently capable of accepting a range of substrates and acting essentially irreversibly, at least under normal growth conditions. The independent functions of these enzyme systems are the reduction of the $\Delta^{24(28)}$ olefin and introduction of the Δ^5 and Δ^{22} unsaturation. The precise mode of action and multiplicity of these systems remains unclear at the present.

Apart from the natural yeast sterols considered above, *S. cerevisiae* possesses the ability to metabolize a wide range of more or less related sterols into ergosterol. The results of these feeding experiments are shown in Table 4. Only one of these eight sterols,

TABLE 4

Sterol fed	% activity recovered as ergosterol
Parkeol (15)	0.00
Agnosterol (16)	0.00
Obtusifoliol (17)	1.30
Ergosterol B_1 (15)	4.1
Ergosterol B_2 (19)	0.38
Ergosterol B_3 (20)	3.86
Ergosta-8(14),22-dien-3β-ol (21)	0.29

parkeol (15), has been isolated from yeast (*3*) but its failure to incorporate into ergosterol suggests that it is alien to the main course of yeast sterol biosynthesis. Agnosterol (16), is a constituent of wool fat (*14*) and its non-incorporation is not unexpected, while the incorporation of obtusifoliol (17) is in accord with the previously discussed ability of yeast to incorporate 24-methylenelanost-8-en-3β-ol (22).

The relatively high incorporations of ergosterol B_2 (19) and its dihydro-derivative (21) are surprising, especially as an earlier claim (*15*) for the involvement of a $\Delta^{8(14)}$ sterol in cholesterol biosynthesis has now been placed in doubt (*16*). However, it should be noted that ergosterol B_2 (and hence its dihydro-derivative (21) is difficult to separate from traces of ergosterols B_1 and B_3. When the incorporation of B_2 is compared with that of B_1 and of B_3 and that of (21) is compared with (11) it would seem reasonable to conclude that the B_2 and (21) incorporations are negligible. Good incorporations of ergosterol B_1 (18; R=H) and B_3 (20) are less unexpected since the involvement of both

7,14- and 8,14-diene systems in products formed during C-14 demethylation has recently been observed in cholesterol biosynthesis (*17*, *18*). Before it was possible to study the incorporations further, it was necessary to be able to separate mixtures of ergosterol, ergosterol B_1 and ergosterol B_3. All three sterols had similar physical properties, but separation could be achieved by treatment of their acetates with *N*-phenyl-1,2,4-triazoline-3,5-dione (*5*). The B_1 diene system was unaffected, but both ergosteryl acetate and ergosteryl B_3 acetate formed the expected 1:1 adducts (23) and (24), respectively. The adducts were formed at similar rates and had essentially identical chromatographic behaviour, but upon treatment with lithium aluminium hydride in tetrahydrofuran under reflux, the adduct (23) regenerated ergosterol (*5*), whereas (24) was converted to an undefined, highly polar substance which was easily separated from ergosterol by chromatography. As a control experiment a mixture of [2,4-$^3H_2$]ergosterol B_3 and inactive ergosterol was taken through this work-up procedure, after which the reisolated ergosterol contained no radioactivity. With these procedures in hand, we sought information on the occurrence of these sterols in yeast. [2,4-$^3H_2$]Zymosterol was fed to yeast according to the standard procedure and, at the termination of the growth period, inactive ergosterol B_1 was added as a carrier. After the normal work-up procedure, no activity was found with the ergosterol B_1. Similarly, when carrier ergosterol B_1 was added with the labelled zymosterol, the reisolated B_1 was inactive and the absence of any measurable level of ergosterol B_1 in *S. cerevisiae* seems certain. Finally, it was possible to show an *in vivo* conversion of B_3 to B_1 by feeding [2,4-$^3H_2$]ergosterol B_3 in the usual way. The culture was worked up after addition of carrier B_1 and after removal of the *cis*-dienes the recovered ergosterol B_1 contained 8.7% of the activity fed. Experimental difficulties, in particular the instability of the B_3 adduct (24), have made it as yet impossible to establish the reversal of this conversion, but such results as are available suggest that the $B_3 \rightarrow B_1$ transformation can occur. The efficiency of this step at present remains unquantified.

It seems unlikely that either ergosterol B_1 or B_3 is a true precursor of ergosterol in yeast. On the other hand, it is highly probable that either or both of a 7,14- or 8,14-diene species is involved in the removal of the C-14 methyl group, and that the observed incoporations of B_1 and B_3 are a consequence of the capacity of the enzymes involved in that transformation to accept a range of substrates. Akhtar has noted previously (*18*) that the analogous enzymes in cholesterol synthesis are relatively unselective with regard to the state of unsaturation of the side-chain.

In conclusion, it should be said that we are acutely aware that many aspects of our tentative scheme for yeast sterol biosynthesis rest largely upon inference. Our continuing work is directed towards the provision of experimental substantiation at an enzymic level.

EXPERIMENTAL

General details and feeding procedures have been given in Part VIII. *Saccharomyces cerevisiae* strain NRRL-y-2250 was used for all biosynthetic experiments.

Preparation of Ketones for Labelling

Most of the required ketones have been previously described (*3*, *4*, *23*).

Ergosta-7,24(28)-dien-3-one (with U. M. Kempe)

Episterol (28 mg) in benzene (3 ml) was shaken with chromic oxide (32 mg) in water (0.3 ml) and glacial acetic acid (0.75 ml). After 2 hr, the mixture was diluted with ether and the organic phase was washed with water and brine, dried, and evaporated. The residue recrystallized twice from methanol to afford the ketone, mp 134–141°C, $[\alpha]_D$ +28.9°, ν_{max} 1710, 1640, 890 cm^{-1}, τ 4.76(b) (1H, s, 7-H), 5.29(b) (2H, s, 28-H), 8.98 (3H, s, 10-Me), 9.42 (3H, s, 13-Me) (Found: C, 84.7; H, 11.2. $C_{28}H_{44}O$ requires: C, 84.8; H, 11.2%).

Lanosta-9(11), 24-dien-3-one

Parkeol (*21*) (158 mg) was oxidized as above to afford the ketone (137 mg) as colourless needles, mp (from EtOH) 185°C, $[\alpha]_D$ +70.3°, ν_{max} 1710 cm^{-1}, τ 4.80(b) (1H, s, 11-H), 4.94 (1H, t, J, 6Hz, 24-H) (Found: C, 84.2; H, 11.5. $C_{30}H_{50}O$ requires: C, 84.5; H, 11.7%).

Ergosta-8(14),22-dien-3-one

Ergosta-8(14),22-dien-3β-ol (*22*) (300 mg) was oxidized as above to afford the ketone (160 mg) as colourless plates mp (from Me_2CO–MeOH) 117–119°C, $[\alpha]_D$ +2.2°, ν_{max} 1710 cm^{-1}, τ 5.18 (2H, m, 22 and 23-H) (Found: C, 84.9; H, 11.1. $C_{28}H_{44}O$ requires: C, 84.8; H, 11.2%).

Ergosta-8,14,22-trien 3β-yl Methanethiomethyl ether (*18*, $R{=}CH_3\ SCH_2$)

Ergosterol B_1 (*23*) (670 mg) in acetic anhydride (10 ml) and dimethyl sulphoxide (15 ml) was stirred overnight at room temperature, poured into ice water (300 ml) and the mixture basified with dilute sodium hydroxide. The precipitate was collected, washed with water, dried, and recrystallized to yield the ether as colourless plates (713 mg), mp (from EtOH) 87–88°C, $[\alpha]_D$ −50°, ν_{max} 1080 cm^{-1}, λ_{max} 251 nm (ε 14 900), *m/e* 456 (M^+), 409 $(M{-}CH_3S)^+$, τ 4.6–4.8 (3H, m, 15, 22 and 23-H), 5.3 (2H, s, $-SCH_2O-$), 7.9 (3H, s, CH_3S-) (Found: C, 78.9; H, 10.5; S, 6.9. $C_{30}H_{48}OS$ requires: C, 78.9; H, 10.6; S, 7.0%).

Ergosta-6,8(14),22-trien-3-one

A solution of cyclohexanone (1 ml) in toluene (150 ml) was heated under reflux in a Dean-Stark trap until no more water separated. Ergosterol B_2 (280 mg) and aluminium isopropoxide (75 mg) were added and the solution was refluxed for a further 3 hr then cooled and poured into cold dilute hydrochloric acid (20 ml). The organic phase was washed with water and sodium bicarbonate, dried and evaporated, and the residue was recrystallized from acetone/methanol to afford the ketone (200 mg) as colourless plates, mp 154–156°C, $[\alpha]_D$ −59°, ν_{max} 1710 cm^{-1}, λ_{max} 253 nm (ε 16 500), τ 3.74, (1H d, J 10 Hz, 7-H), 4.63–4.88 (3H, m, 6-, 22- and 23-H) (Found: C, 85.1; H, 10.7. $C_{28}H_{42}O$ requires: C, 85.2; H, 10.7%).

Control Experiment on Intermolecular Tritium Exchange

[2,4-$^3H_2$]Zymosterol (0.41 mg, 5.63×10^4 dps) was fed to a culture of yeast in the normal way, together with cholesterol (126 mg). After aerobic growth for 2 days, further cholesterol (77 mg) was added, and the culture was worked up in the usual way.

Cholesteryl acetate was separated by plc and converted to the benzoate, which was crystallized to a constant activity of 0.439 dps/mg, equivalent to a total activity of 13.85 dps or 0.025% of the activity fed. The benzoate was saponified, and the cholesterol (23 mg) and sodium dichromate (60 mg) were mixed in glacial acetic acid (1 ml) and benzene (2 ml). The mixture was left at 0°C for 24 hr, then diluted with water (1 ml), and extracted with ether. The organic phase was washed with water, sodium carbonate and brine, dried and evaporated. The solid was digested with boiling hexane, and the solution filtered. Evaporation of the filtrate and recrystallization from acetone/methanol yielded cholest-4-ene-3,6-dione as pale yellow crystals, mp 123–125°C, $[\alpha]_D$ −39° (lit. (*7*) mp 124–125°C, $[\alpha]_D$ −40°). The enedione was inactive.

Control Experiment on Intramolecular Tritium Exchange

[2-^{3}H]-14-Desmethyllanosterol (0.27 mg, 4.22×10^4 dps) was fed to yeast in the usual way and ergosterol (254 mg) was isolated after the addition of inactive carrier. The sterol was oxidized as above to ergosterone (206 mg), mp 130°C, $[\alpha]_D$ −10.2 (lit. (*19*) mp 129–132°C, $[\alpha]_D$ −10°). The ketone was converted by the published method (*8*) to 4,4-dimethylergosterone (169 mg), mp 164–166°C, $[\alpha]_D$ −35.9° (lit. (*8*) mp 167–168°C, $[\alpha]_D$ −37°) and had specific activity of 3.82 dps/mg. It was refluxed overnight under nitrogen with potassium *t*-butoxide (500 mg) in *t*-butanol (25 ml), recovered in the usual way and the procedure repeated. After recrystallization of the recovered 4,4-dimethylergosterone no activity was detectable.

Separation of Ergosterol and Ergosterol B_3

A mixture of [2,4-$^3H_2$]ergosterol B_3 (0.55 mg, 1.365×10^5 dps) and ergosterol (63 mg) was acetylated and the mixed acetates were treated with excess *N*-phenyl-1,2,4-triazoline-3,5,-dione (*5*) for 1 hr at −70°C. After the usual work-up the mixed adducts were purified by plc and treated in the usual way with excess lithium aluminium hydride in refluxing tetrahydrofuran. Ergosterol was recovered from the reaction products by plc and after recrystallization (chloroform/methanol) showed no detectable activity.

Search for Ergosterol B_1 as a Yeast Metabolite

(a) [2,4-$^3H_2$]Zymosterol (0.89 mg, 1.16×10^5 dps) was fed to yeast in the usual manner, and the culture was grown aerobically for 2 days. After the normal work-up, carrier ergosterol B_1 (98.4 mg) was added. The sterol mixture was acetylated, and *cis*-dienes were removed with phenyltriazolinedione. Ergosterol B_1 acetate was separated by plc and recrystallized from ether/ethanol. No activity was found. (b) The same procedure was followed, except that carrier ergosterol B_1 (125 mg) was fed together with [2,4-$^3H_2$]zymosterol (0.48 mg, 6.89×10^4 dps). After the same work-up procedure, the recovered ergosterol B_1 acetate was again inactive.

Conversion of Ergosterol B_3 to Ergosterol B_1

[2,4-$^3H_2$]Ergosterol B_3 (0.21 mg, 4.66×10^4 dps) was fed to yeast in the normal manner, and the culture was grown aerobically for 1 day. After the normal work-up, carrier ergosterol B_1 (57 mg) was added to the crude sterol mixture, which was then acetylated and treated with excess phenyltriazolinedione at −70°C. The remaining ergosterol B_1 acetate was isolated by plc and recrystallized to a constant activity of 64.1

dps/mg, equivalent to a total activity of 4.05×10^3 dps, equivalent to 8.7% of the activity fed.

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