Biosynthesis of Terpenes and Steroids. Part IX.† The Sterols of Some Mutant Yeasts and their Relationship to the Biosynthesis of Ergosterol

By **Derek H. R. Barton, John E. T. Corrie, and David A. Widdowson,*** Department of Chemistry, Imperial College, London SW7 2AY
Martin Bard and Robin A. Woods,* Department of Genetics, University of Sheffield. Sheffield S10 2TN

The sterols of four mutant strains of *Saccharomyces cerevisiae* have been isolated and characterised and the mutations have been related to the pathway for ergosterol biosynthesis. The effects of the mutations are to block, respectively, the introduction of the side chain methyl group, the introduction of the 5,6- and 22,23-double bonds, and the $\Delta^8 \rightarrow \Delta^7$ isomerisation. The following sterols previously unreported in yeast have been isolated and characterised: cholesta-5,7,24-trien-3β-ol (2; R = H), ergost-8-en-3β-ol, ergosta-8,22-dien-3β-ol (5; R = H), ergosta-5,8,22-trien-3β-ol (6; R = H), and ergosta-5,7-dien-3β-ol. Cholesta-5,7,22,24-tetraen-3β-ol, ergosta-8,22,24(28)-trien-3β-ol, and ergosta-8,14,24(28)-trien-3β-ol, which were previously unknown, have also been isolated and characterised.

ONE of us has described the isolation of a number of nystatin-resistant mutants of *Saccharomyces cerevisiae* and has observed characteristic differences in the u.v. spectra of their sterols.[1] As part of our continuing interest in yeast sterol biosynthesis, we report the results of isolation and characterisation of these sterols and relate their structures to our suggested scheme for ergosterol biosynthesis.[2] In all cases the sterols were obtained by aerobic growth of the mutants to stationary phase (24 h); the cells were then harvested and saponified. The total ether-soluble fraction was treated with benzoyl chloride–pyridine. Preparative t.l.c. on silica gel afforded the mixed 4-demethylsteryl benzoates; this fraction was the only one examined since it was apparent from the earlier work that the chemical consequences of the mutations would be observed among the 4-demethylsterols. The mixed 4-demethyl benzoates were subjected to preparative t.l.c. on silver nitrate–silica gel[3] to afford a number of fractions which were further processed by standard techniques. The results of this analysis are shown in the Table, which should be considered with the provisos that (i) only those sterols which were isolable in workable quantities ($\geqslant$ *ca.* 20 mg) have been included and each mutant undoubtedly contains traces of other sterols, and (ii) entries shown grouped together represent mixtures that were not separated in this study (see later).

Sterols of *pol* mutants of *Saccharomyces cerevisiae*

Mutant	Sterols	Probable block
Wild-type A184D	Zymosterol (1a) Ergosterol (Ergosta-5,7,22-trien-3β-ol)	
pol 1	Zymosterol Cholesta-5,7,24-trien-3β-ol Cholesta-5,7,22,24-tetraen-3β-ol	Methyl transferase
pol 2	Ergost-8-en-3β-ol Ergosta-8,22-dien-3β-ol Ergosta-5,8,22-trien-3β-ol Fecosterol (7; R=H)	$\Delta^8 \rightarrow \Delta^7$ Isomerase
pol 3	Ergost-7-en-3β-ol } Ergost-8-en-3β-ol } Ergosta-7,22-dien-3β-ol } Ergosta-8,22-dien-3β-ol } Zymosterol Ergosta-7,22,24(28)-trien-3β-ol Fecosterol (7; R=H) Ergosterol (trace)	5,6-Dehydrogenase (partial)
pol 3 SM36 †	Ergosta-7,22-dien-3β-ol } Ergosta-8,22-dien-3β-ol } Ergosta-7,22,24(28)-trien-3β-ol } Ergosta-8,22,24(28)-trien-3β-ol } Episterol } Fecosterol }	5,6-Dehydrogenase
pol 5	Episterol (8; R=H) Ergosta-5,7-dien-3β-ol Ergosta-5,7,24(28)-trien-3β-ol Ergosta-8,14,24(28)-trien-3β-ol	22,23-Dehydrogenase

† Ref. 20.

As a preliminary to studies of the mutants, the wild-type strain from which they were derived was analysed. Only zymosteryl benzoate[3] and ergosteryl benzoate[4] were present in sufficient quantities to be isolated and characterised, although the u.v. spectrum of the crude

† Part VIII, D. H. R. Barton, P. J. Davies, U. M. Kempe, J. F. McGarrity, and D. A. Widdowson, *J.C.S. Perkin I*, 1972, 1231.

[1] S. W. Molzahn and R. A. Woods, *J. Gen. Microbiol.*, 1972, **72**, 339.

[2] D. H. R. Barton, J. E. T. Corrie, P. J. Marshall, and D. A. Widdowson, *Bioorg. Chem.*, 1973, **2**, 363; see also D. H. R. Barton, J. E. T. Corrie, D. A. Widdowson, M. Bard, and R. A. Woods, *J.C.S. Chem. Comm.*, 1973, 30.

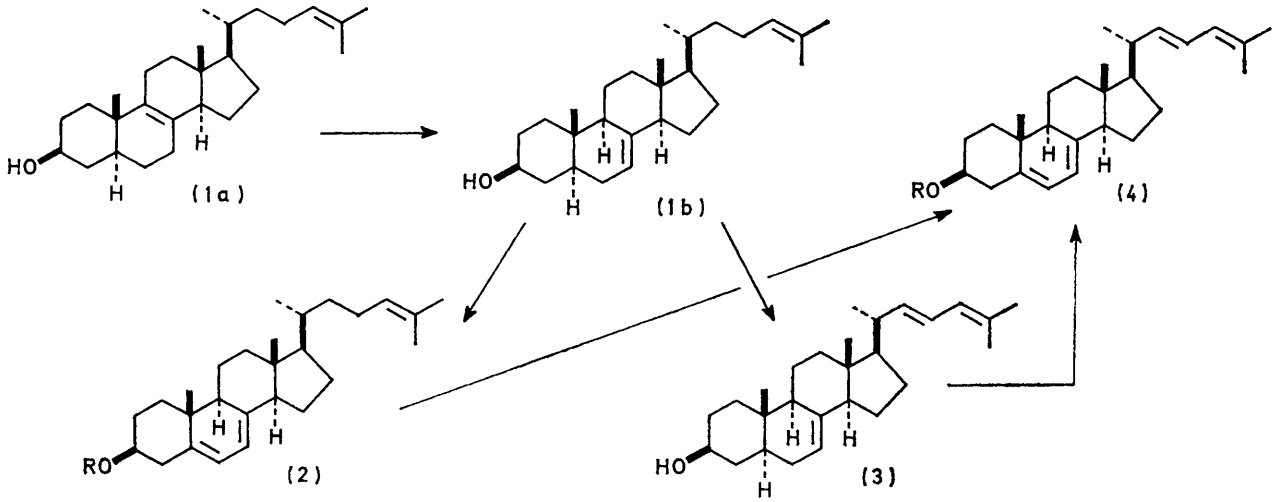
[3] D. H. R. Barton, U. M. Kempe, and D. A. Widdowson, *J.C.S. Perkin I*, 1972, 513 and references therein.

[4] D. H. R. Barton and J. D. Cox, *J. Chem. Soc.*, 1948, 783.

sterol extract showed the presence of low concentrations of sterols with side-chain diene systems.

Preparative t.l.c. of the *pol* 1 4-demethylsteryl benzoates on silver nitrate–silica gel gave three main compounds, of which the least polar was zymosteryl benzoate.[3] The second compound had m.p. 131—134°, $[\alpha]_D$ −55·0°, and its u.v. spectrum ($\lambda_{max.}$ 229, 262, 272, 282, and 294 nm) indicated a ring B diene; the mass spectrum (M^+ 486) showed peaks at *m/e* 375 and 373 indicative of a mono-unsaturated C_8 side-chain.[5] Loss of the elements of benzoic acid from the molecular ion gave a strong peak at *m/e* 364, which also showed losses of (side-chain) and (side-chain + 2) to give peaks at *m/e* 253 and 251. Microanalysis results agreed with the formula $C_{34}H_{46}O_2$. The n.m.r. spectrum showed an AB quartet at δ 5·54 (*J* 6 Hz) and two singlets at δ 0·65 (3H, 13β-Me) and 1·02 (3H, 10β-Me) (*cf.* ergosteryl benzoate: δ 5·55, 0·66, and 1·01, respectively), indicative of an ergosterol-type nucleus, together with a triplet at δ 5·1 (*J* 6 Hz) superimposed on the broad 3α-H signal. Two singlets at δ 1·65 (3H) and 1·69 (3H) and a doublet at δ 0·98 (3H) (*cf.* zymosteryl benzoate: δ 5·1, 1·64, 1·68, and 0·99, respectively) were indicative of a zymosterol side chain. The combination of these two structural elements suggested that the compound was cholesta-5,7,24-trien-3β-yl benzoate (2; R = PhCO) and this was confirmed by saponification to cholesta-5,7,24-trien-3β-ol (2; R = H). A reference sample was not available but comparison of the observed and published[6] physical constants of the free sterol established the identity.

The third compound had m.p. 165—167°, $[\alpha]_D$ −43·7°, and the u.v. spectrum again showed a typical ring B diene chromophore. An intense chromophore at shorter wavelength ($\lambda_{max.}$ 229, 232, and 247 nm) was suggestive of a side-chain diene chromophore overlaid on that of the benzoate. This absorption was different from that of the side-chain/benzoate chromophore of ergosta-5,7,22,24(28)-tetraen-3β-yl benzoate ($\lambda_{max.}$ 230 nm) and the free sterol showed low-wavelength absorption at $\lambda_{max.}$ 232sh, 238, and 247sh nm. Microanalytical and mass spectral data were consistent with the molecular formula $C_{34}H_{44}O_2$. The mass spectrum showed peaks at *m/e* 375 and 373 which corresponded to loss of (side-chain) and of (side-chain + 2) from the molecular ion at *m/e* 484 and suggested that the side-chain consisted of eight carbon atoms and was doubly unsaturated. The mass spectrum of the free sterol (M^+ 380) showed corresponding peaks at *m/e* 271 and 269. The n.m.r. spectrum showed two singlets at δ 0·67 (3H, 13β-Me) and 1·01 (3H, 10β-Me) (*cf.* ergosteryl benzoate, above), a doublet (*J* 7 Hz) at δ 1·07 (3H, 20-Me) [*cf.* ergosta-7,22,24(28)-trien-3β-yl benzoate,[3] doublet at δ 1·04], and a singlet at δ 1·76 (6H) which suggested the presence of two allylic methyl groups. The low-field region of the spectrum was complex but showed, *inter alia*, a broad resonance at δ 5·1 (1H, 3α-H) and an AB quartet at δ 5·53 (6- and 7-H). The total signal intensity in this region corresponded to six protons, of which three are accounted for by the assigned resonances. The remaining three protons must be accommodated by the side chain which therefore requires a $\Delta^{22,24}$-structure to encompass its other features. The compound was therefore identified as cholesta-5,7,22,24-tetraen-3β-yl benzoate (4; R = PhCO).



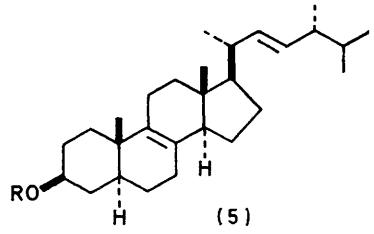
SCHEME

The isolation of these C_{27} sterols from *pol* 1 suggested that the mutant was unable to transfer from methionine the additional side-chain carbon atom of the ergostane skeleton. This was confirmed by isolation of the mixed 4-demethylsterols by chromatography of the free alcohols from a further culture of *pol* 1. The mass spectrum of this fraction showed molecular ions at *m/e* 380, 382, and 384 which corresponded to the formulae $C_{27}H_{40}O$, $C_{27}H_{42}O$, and $C_{27}H_{44}O$; no ions of higher mass were observed. By analogy with our suggested pathway for

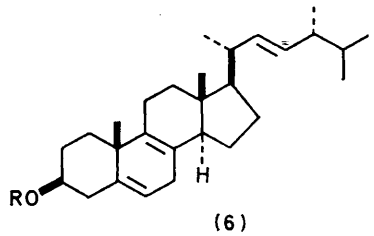
[5] S. G. Wyllie and C. Djerassi, *J. Org. Chem.*, 1968, **33**, 305.
[6] T. J. Scallen, *Biochem. Biophys. Res. Commun.*, 1965, **21**, 149.

ergosterol biosynthesis,[2] the tetraene (4; R = H) would be expected to be derived from zymosterol as shown in the Scheme, but the putative intermediates (1b) and (3) were not found in the present study.

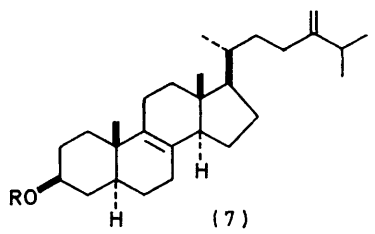
The mixed steryl benzoates of *pol* 2 were separated into four components when subjected to preparative t.l.c. on silver nitrate–silica gel. The spectral data of the least polar compound (see Experimental section) suggested that it was ergost-8-en-3β-yl benzoate [7] and this was supported by the identity of the observed and reported physical constants. The second compound had m.p. 152—153°, $[\alpha]_D$ +26·9°, and showed no u.v. chromophore other than that of the benzoate. Microanalytical and mass spectral data indicated the molecular formula $C_{35}H_{50}O_2$, which required that the steroid was doubly unsaturated. The mass spectrum showed that the side-chain contained one double bond, as it had peaks at *m/e* 377 and 375 which corresponded, respectively, to loss of side-chain (C_9H_{17}) and of (side-chain + 2) from the molecular ion at *m/e* 502. The n.m.r. spectrum located the double bond at the 22,23-position by showing a two-proton multiplet at δ 5·2 [*cf.* ergosteryl benzoate: δ 5·2 (22- and 23-H)]. The spectrum had no other olefinic proton signals but showed the 10β-Me and 13β-Me resonances as singlets at δ 1·01 and 0·63, respectively (*cf.* zymosteryl benzoate: δ 1·01 and 0·63, respectively) which suggested a Δ^8-sterol nucleus [7] and that the compound was ergosta-8,22-dien-3β-yl benzoate (5; R = PhCO). The corresponding free sterol has been reported by Parks and his co-workers [8] from the mutant *nys* 3, now renamed [1] *pol* 3 (the sterol was found in *pol* 3 in this work; see later), but the n.m.r. spectral data reported by these authors were inconsistent [9] with the proposed structure and it was therefore desirable to prepare an authentic sample. When 3β-acetoxy-22,23-dibromoergost-8-en-11-one [10] was heated under reflux in ether with lithium aluminium hydride for several hours, the major product was ergosta-8,22-dien-3β-ol.[11] Clearly the metal bromide, which was formed by reduction of the vicinal dibromide, acted on a Lewis acid to catalyse the further reduction of the initial 8-en-11β-ol product. Hydrogenolysis of allylic alcohol under such conditions has some precedent.[12] The n.m.r. spectrum of the alcohol showed singlets at δ 0·62 (3H, 13β-Me) and 0·97 (3H, 10β-Me) (*cf.* fecosterol: δ 0·61 and 0·96; zymosterol: δ 0·60 and 0·94, respectively), whereas Parks' reported values of δ 0·54 and 0·79 correspond much more closely to those for a Δ^7-sterol nucleus (*cf.* episterol: δ 0·56 and 0·82; ergost-7-en-3β-ol: δ 0·52 and 0·86, respectively). The authenticity of Parks' sterol must, therefore, remain uncertain. Benzoylation of our authentic ergosta-8,22-dien-3β-ol gave material identical with that isolated from *pol* 2.



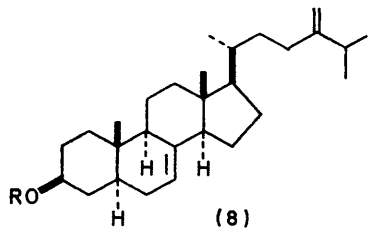
(5)



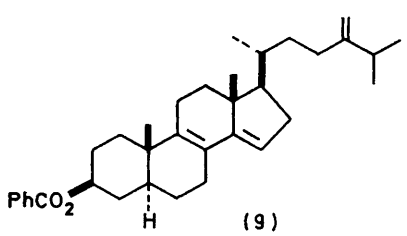
(6)



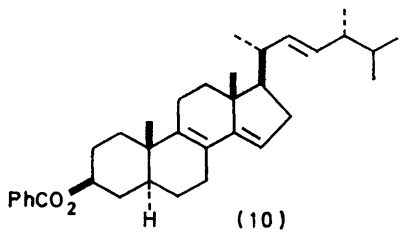
(7)



(8)



(9)



(10)

The third compound from *pol* 2 had m.p. 153—155°, $[\alpha]_D$ −6·1°, and microanalytical and mass spectral data were consistent with the molecular formula $C_{35}H_{48}O_2$. Its spectral properties (see Experimental section) suggested that the compound was ergosta-5,8,22-trien-3β-yl benzoate (6; R = PhCO), the parent sterol of which, trivially named lichesterol, was recently isolated from *Xanthoria parietina*.[13] Direct comparison of the two compounds (as the acetates) established their identity. The biosynthetic origin of lichesterol is of some interest, since it has been generally accepted that a 7,8-double bond is necessary for formation of Δ^5-structures.[14] *X. parietina* also contained ergosterol, and

[7] D. H. R. Barton and J. D. Cox, *J. Chem. Soc.*, 1949, 214.
[8] L. W. Parks, F. T. Bond, E. D. Thompson, and P. R. Starr, *J. Lipid Res.*, 1972, **13**, 311.
[9] N. S. Bhacca and D. H. Williams, 'Applications of N.M.R. Spectroscopy in Organic Chemistry,' Holden-Day, San Francisco, 1964.
[10] R. Budziarek, F. Johnson, and F. S. Spring, *J. Chem. Soc.*, 1952, 3410; J. Elks, R. M. Evans, C. H. Robinson, G. H. Thomas, and L. J. Wyman, *J. Chem. Soc.*, 1953, 2933.
[11] A. S. Hallsworth, H. B. Henbest, and T. J. Wrigley, *J. Chem. Soc.*, 1957, 1969.
[12] J. Broome, B. R. Brown, A. Roberts, and A. M. S. White, *J. Chem. Soc.*, 1957, 1406.
[13] J. R. Lenton, L. J. Goad, and T. W. Goodwin, *Phytochem.*, 1973, **12**, 1135.
[14] L. J. Goad in 'Natural Substances Formed Biologically from Mevalonic Acid,' ed. T. W. Goodwin, Academic Press, London, 1969, p. 45; L. J. Mulheirn and P. J. Ramm, *Chem. Soc. Rev.*, 1972, **1**, 259.

the possible intervention of a $\Delta^7 \longrightarrow \Delta^8$ isomerase was suggested,[13] but this pathway seems inadmissible in the present case, since *pol* 2 appears unable to form Δ^7-sterols. This objection was supported by a feeding experiment performed with [2,4-$^3H_2$]ergosta-7,22-dien-3β-ol, which when fed to a growing culture of *pol* 2 in the usual way [2] underwent an incorporation of 7·96% into ergosterol. Therefore if *pol* 2 had contained any endogenous Δ^7-sterols it would almost certainly also contain ergosterol.

The novel structure of lichesterol made it an attractive goal for synthesis and the dehydrobromination of 7α-bromocholesteryl benzoate to cholesta-5,8-dienyl benzoate [15] appeared to present a useful analogy. Since this report has been questioned [16] it was initially essential to establish its validity. When 7α-bromocholesteryl benzoate [17] was heated in pyridine [15b] no single product could be isolated from the complex reaction mixture, but treatment with ammonium thiocyanate in acetone solution [15a] gave two products which were separated by preparative t.l.c. During purification, however, the more polar compound was found to isomerise to the less polar, and the spectral and microanalytical data (see Experimental section) of the latter compound indicated that it was 7α-isothiocyanatocholesteryl benzoate. The two initial products may be explained as a thiocyanate/isothiocyanate pair, since thermal isomerisation of allyl thiocyanates to isothiocyanates is well known.[18] Formulation as the isothiocyanate was supported by the observation of a solvent shift of the NCS band in the i.r. spectrum [19] (see Experimental section). We have thus been unable to repeat the reported dehydrobromination and hence have not pursued the synthesis of lichesterol.

The fourth, most polar compound from *pol* 2 was shown by comparison to be fecosteryl benzoate (7).[3]

The *pol* 3 mutant was available in two forms,[20] one of which (*pol* 3 SM36) appeared to be totally blocked at the 5,6-dehydrogenase, while the other (*pol* 3) had a slight ' leakage ' at this point and therefore accumulated a very minor proportion of ergosterol. When the latter mutant was examined in the usual way a number of fractions were obtained, the first of which was identified as a mixture (*ca.* 1 : 1) of ergost-7-en-3β-yl benzoate and ergost-8-en-3β-yl benzoate. The mass spectrum of the mixture had a single molecular ion at *m/e* 504 and the n.m.r. spectrum showed singlet resonances of approximately equal intensity for the 13β-methyl group of the Δ^7- and Δ^8-isomers at δ 0·54 and 0·62, respectively); the intensity of the multiplet at δ 5·2 (7-H of the Δ^7-isomer) corresponded to the presence of *ca.* 50% of ergost-7-en-3β-yl benzoate.

The second fraction was similarly identified as a mixture of ergost-7,22-dien-3β-yl benzoate and ergosta-8,22-dien-3β-yl benzoate (proportions *ca.* 9 : 1). Thus the mass spectrum showed a single molecular ion at *m/e* 502 and the n.m.r. spectrum had singlet resonances at δ 0·55 and 0·63. In our experience it has not proved possible to separate, by silver nitrate silica gel t.l.c., steryl benzoates which are isomeric only with respect to the position of a double bond at C-7 or C-8. Nevertheless, as full spectral data for the individual compounds obtained here as mixtures were available, we are certain that our structural assignments are correct.

The next three fractions obtained were shown by comparison to be zymosteryl benzoate, ergosta-7,22,24(28)-trien-3β-yl benzoate,[3] and fecosteryl benzoate. Finally the presence of a trace of ergosterol was unequivocally shown by its isolation from a further culture as the adduct of its acetate with 4-phenyl-1,2,4-triazoline-3,5-dione.[21] The regenerated ergosterol was identified by comparison with authentic material.[22]

A more definitive identification of the metabolic block in *pol* 3 came from the variety SM36, of which the total crude sterol fraction showed no ring B diene chromophore. By application of the standard procedure there were obtained three fractions of which the least polar was identified as a mixture (*ca.* 6 : 1) of ergosta-7,22-dien-3β-yl and ergosta-8,22-dien-3β-yl benzoates. The second fraction was a mixture of ergosta-7,22,24(28)-trien-3β-yl benzoate and the hitherto unknown ergosta-8,22,24(28)-trien-3β-yl benzoate. The mass spectrum showed a single molecular ion at *m/e* 500 and the n.m.r. spectrum showed, for the Δ^7-isomer, singlets at δ 0·56 (13β-Me) and 0·86 (10β-Me) and, for the Δ^8-isomer, a singlet at δ 0·65 (13β-Me). The singlet at δ 1·02 (10β-Me) of the Δ^8-isomer coincided with the upfield part of the doublet centred at δ 1·04 (20-Me and 25-Me_2) but was revealed by an increase in the intensity of that signal. Finally, the complex olefinic region of the spectrum was identical with that of authentic ergosta-7,22,24(28)-trien-3β-yl benzoate [3] with the exception that the multiplet at δ 5·1 (7-H of the Δ^7-isomer) was of reduced intensity.

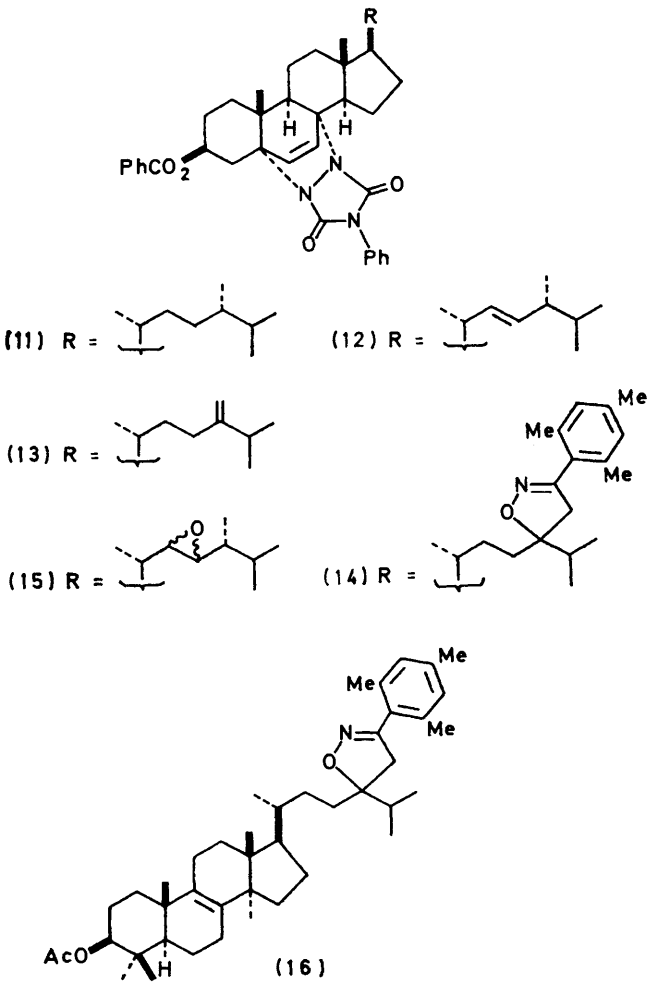
The third fraction was similarly shown to be a mixture of episteryl and fecosteryl benzoates. The range of sterols detected in *pol* 3 (SM36 demonstrates that all the normal enzyme functions in this mutant are operative, with the exception of the 5,6-dehydrogenase system. The presence in both types of *pol* 3 mutant of abnormal [3] Δ^8-sterols, despite the availability of a $\Delta^8 \longrightarrow \Delta^7$ isomerase, can be explained by increases in the normal pool sizes as a consequence of the genetic block. The solution of this problem is beyond the scope of the present work.

[15] (*a*) K. Arima, *Chem. and Pharm. Bull (Japan)*, 1953, **1**, 224; (*b*) K. Tsuda, K. Arima, and R. Hayatsu, *J. Amer. Chem. Soc.*, 1954, **76**, 2933.
[16] L. F. Fieser and M. Fieser, ' Steroids,' Reinhold, New York, 1959, p. 161 and references therein.
[17] H. Schaltegger, *Helv. Chim. Acta*, 1950, **33**, 2101.
[18] N. V. Sidgwick, ' The Organic Chemistry of Nitrogen,' eds. J. T. Millar and H. D. Springall, Clarendon Press, Oxford, 1966, 3rd edn., p. 468.
[19] L. J. Bellamy, ' Advances in Infrared Group Frequencies,' Methuen, London, 1968, p. 57.
[20] S. W. Molzahn and R. A. Woods, unpublished work.
[21] D. H. R. Barton, T. Shiori, and D. A. Widdowson, *J. Chem. Soc.* (*C*), 1971, 1968.
[22] D. H. R. Barton, P. J. Davies, U. M. Kempe, J. F. McGarrity, and D. A. Widdowson, *J.C.S. Perkin I*, 1972, 1231.

For the isolation of sterols from *pol* 5 the general procedure was followed only to the preparation of the total crude benzoates. At this stage the mixture was treated with 4-phenyl-1,2,4-triazoline-3,5-dione.[21] Preparative t.l.c. then gave two fractions, namely unchanged 4-demethylsteryl benzoates and the adducts of ring B diene 4-demethylsteryl benzoates. Preparative t.l.c. on silver nitrate–silica gel of the first fraction gave two compounds, of which the less polar was shown by comparison to be episteryl benzoate (8; R = PhCO).[3] The more polar compound had m.p. 148—151°, $[\alpha]_D$ −4·1°, and was shown to be a conjugated diene by its u.v. spectrum ($\lambda_{max.}$ 230 and 251sh nm). Mass spectral and microanalytical data were consistent with the molecular formula $C_{35}H_{48}O_2$, and the presence of a mono-unsaturated side chain was revealed by ions at *m/e* 375 (M^+ — side chain) and 373 [M^+ — (side chain + 2)].[5] Similar losses from the ion (M — $PhCO_2H$)$^+$ gave peaks at *m/e* 253 and 251. A broad two-proton absorption at δ 4·7 in the n.m.r. spectrum suggested the presence of a 24,28-double bond (*cf.* fecosteryl benzoate: δ 4·7), which was confirmed by i.r. bands spectrum at 900 and 1640 cm^{-1}, characteristic of a 1,1-disubstituted ethylene. The n.m.r. spectrum also showed a broad one-proton absorption at δ 5·4, a broad signal at δ 4·95 (3α-H), a six-proton doublet at δ 1·04 (J 7 Hz, side-chain terminal methyl groups), a three-proton doublet (J 7 Hz) at δ 0·99 (20-Me), and two three-proton singlets at δ 1·07 and 0·84. Since the steroid nucleus contained a conjugated diene but only a single olefinic proton, it followed that one double bond was tetrasubstituted and on biogenetic grounds the most probable one would be that at positions 8 and 9. The trisubstituted double bond would then be that at positions 14 and 15, and the compound was tentatively identified as ergosta-8,14,24(28)-trien-3β-yl benzoate (9). This conjecture was fully confirmed by comparison with the isomeric ergosta-8,14,22-trien-3β-yl benzoate (10) obtained from ergosterol by a modification of known procedures[23] (see Experimental section). The u.v. spectrum of the 8,14,22-triene was identical with that of the isolated compound; its n.m.r. spectrum showed, *inter alia*, a broad, one-proton absorption at δ 5·4 (15-H), partly obscured by the two-proton multiplet at δ 5·3 (22- and 23-H), a broad multiplet at δ 4·95 (3α-H), and two three-proton singlets from the quaternary methyl group at δ 1·06 and 0·85.

The isolation of a $\Delta^{8,14}$-type sterol is in accord with the currently accepted hypothesis for the mechanism of C-14 demethylation during the modification of lanosterol, and complements the observed trapping of 4,4-dimethylcholesta-8,14-dien-3β-ol during cholesterol biosynthesis.[24] The presence in *pol* 5 of ergosta-8,14,24(28)-trien-3β-ol is nevertheless surprising, since the metabolic block in this mutant is at a much later stage (see later) and the compound may be best regarded as a metabolic 'left-over' (*cf.* ergosta-5,7,14,22-tetraen-3β-ol found in a strain of *Aspergillus niger*[25]).

The fraction which contained the ring B diene adducts presented some difficulties in analysis. A preliminary study of the *pol* 5 sterols had suggested that the major component was ergosta-5,7-dien-3β-ol, but the compound persistently retained a minor proportion of a didehydro-compound, indicated by the presence of an (M^+ − 2) ion in the mass spectrum of the sterol and its derivatives. The minor compound was most probably ergosterol and/or ergosta-5,7,24(28)-trien-3β-ol.[3] Thus the crude adducts showed a molecular ion for the diene adduct



(11) at *m/e* 677 and a much less intense ion (*ca.* 10%) at *m/e* 675 [for (12) or (13)]. The mixture was treated with 2,4,6-trimethylbenzonitrile oxide[26] in hot benzene. Under these conditions the nitrile oxide was shown (see Experimental section) to undergo cycloaddition to a 24,28-double bond but to be unreactive towards the 6,7- and 22,23-double bonds of the ergosteryl benzoate adduct (12). Chromatography of the reaction products afforded two fractions, of which the more polar was shown (n.m.r. spectroscopy) to be a 1 : 1 adduct of the

[23] M. Fieser, W. E. Rosen, and L. F. Fieser, *J. Amer. Chem. Soc.*, 1952, **74**, 5397.
[24] I. A. Watkinson and M. Akhtar, *Chem. Comm.*, 1969, 206.
[25] D. H. R. Barton and T. Bruun, *J. Chem. Soc.*, 1951, 2728.
[26] C. Grundmann and J. M. Dean, *J. Org. Chem.*, 1965, **30**, 2809.

nitrile oxide and a steroid. The compound could not be obtained crystalline and its structure was not rigorously established. Nevertheless there is a clear inference that it had been derived from ergosta-5,7,24(28)-trien-3β-ol and therefore had the structure (14).

We had previously shown [27] that *m*-chloroperbenzoic acid effected selective epoxidation of the ergosteryl benzoate adduct (12) to afford the epoxide (15) as a mixture of epimers. The less polar fraction from the reaction with the nitrile oxide was treated with peroxy-acid but only a trace of a new compound, which was chromatographically identical with the epoxide (15), was formed and it was not examined further. The quantity isolated could represent the presence of no more than 2% of ergosterol in the isolated mixed adducts. The recovered, homogeneous adduct (11) was reduced with lithium aluminium hydride [21] to give ergosta-5,7-dien-3β-ol, the physical constants of which (and those of its acetate) corresponded closely with the published values.[28] The *pol* 5 mutation is clearly associated with the inability to introduce the 22,23-double bond, although the possibility remains that this deficiency is not absolute.

The *pol* strains have been shown to be single mutants [1,29] and this is ultimately expressed in the loss (complete or partial) of activity of one enzyme in each case. Four such metabolic blocks have been found, namely, at C-24 methylation, at $\Delta^8 \longrightarrow \Delta^7$ isomerisation, at 5,6-dehydrogenation, and at 22,23-dehydrogenation. Since the blocks can be complete, only five enzymes (or enzyme complexes) must be involved in the terminal stages and the sterol distribution of the mutants shows that these can act independently of each other. Particularly noteworthy is the probability that the 5,6-dehydrogenase does not require a Δ^7-substrate.[13] A mutant which lacked the $\Delta^{24(28)}$ reductase was not found, possibly because such a mutant would not exhibit sufficient nystatin resistance to emerge from the initial screening procedure.[1]

Our earlier scheme for ergosterol biosynthesis [2] is thus confirmed in principle and may be expanded to include all the sterols reported herein. The concept of unit (enzymatic) transformations as revealed by blocked mutants is permissive of many routes to ergosterol. It allows, nevertheless, a precise definition of the significant operations in the biosynthesis. It is a far more satisfactory approach to the problem than the feeding of potential precursors.[2]

Many further biosynthetic and genetic questions have, however, been raised by this work and we are continuing our studies with mutants.

EXPERIMENTAL

M.p.s were determined on a Kofler hot-stage apparatus. Optical rotations were determined for solutions in $CHCl_3$. U.v. spectra were taken for solutions in EtOH, and n.m.r. spectra for solutions in $CDCl_3$ at 60 MHz, with tetramethylsilane as internal standard. T.l.c. refers to thin-layer chromatography on silica gel GF 254 (Merck). Silver nitrate plates refer to silica gel plates impregnated (10%) with silver nitrate. These plates were routinely developed three times with 35% benzene–light petroleum (b.p. 60—80°).

General Procedures.—Sterile Yeast Complete Medium (YCM) [1] (200 ml) was inoculated from a slope culture and shaken overnight at 30°. This culture was used to inoculate a fermenter which contained fresh YCM (7 l) maintained at 30°. The solution was stirred (350 rev. min^{-1}) and aerated (10 l min^{-1}) and the pH was maintained above 4·4 by controlled addition of 10% sodium hydroxide solution. After 24 h the cells were harvested by centrifugation and refluxed under nitrogen in methanolic 10% potassium hydroxide (700 ml) for 2 h. The methanol was removed *in vacuo* and the total ether-soluble fraction was obtained in the usual way and treated with excess of benzoyl chloride in pyridine overnight at 0°. After the usual work-up the mixed 4-demethylsteryl benzoates were separated by preparative t.l.c. in benzene, followed by preparative t.l.c. on silver nitrate plates. The bands obtained were assayed by silver nitrate t.l.c. and rechromatographed where necessary.

Extraction of Wild-type A184D.—The standard procedure afforded two isolable compounds, which were identified as zymosteryl benzoate (114 mg), prisms from chloroform–methanol, m.p. 127—129°, $[\alpha]_D$ +45·9° (*c* 0·71) (lit.,[3] 126—128°, $[\alpha]_D$ +44·8°); and ergosteryl benzoate (268 mg), needles from chloroform–methanol, m.p. 164—165°, $[\alpha]_D$ −68·2° (*c* 0·83) (lit.,[4] m.p. 167—169°, $[\alpha]_D$ −69°).

Extraction of pol 1.—The general procedure gave three compounds, of which the least polar was zymosteryl benzoate (450 mg), m.p. 126—127°, $[\alpha]_D$ +45·7° (*c* 0·97).

Cholesta-5,7,24-trien-3β-yl benzoate (2; R = PhCO). The crude material (38 mg) gave the pure *ester* as plates, m.p. (from chloroform–methanol) 131—134°, $[\alpha]_D$ −55·0° (*c* 0·39), $\lambda_{max.}$ 229 (ε 18,200), 252sh (7400), 262sh (10,800), 272 (15,100), 282 (15,300), and 294 nm (8200) (Found: C, 84·0; H, 9·4. $C_{34}H_{46}O_2$ requires C, 83·9; H, 9·5%).

Cholesta-5,7,24-trien-3β-ol (2; R = H). The benzoate (2; R = PhCO) (12 mg) was refluxed for 2 h under nitrogen with potassium hydroxide (100 mg) in methanol (8 ml). Normal work-up gave the alcohol as needles, m.p. (from methanol) 103·5—105°, $[\alpha]_D$ −118·9° (*c* 0·35) (lit.,[6] m.p. 102—102·5°, $[\alpha]_D$ −111°).

Cholesta-5,7,22,24-tetraen-3β-yl benzoate (4; R = PhCO). Rechromatography on silver nitrate plates of the third band gave the *benzoate* (56 mg) as needles, m.p. (from chloroform–methanol) 165—167°, $[\alpha]_D$ −43·7° (*c* 0·46), $\lambda_{max.}$ 229 (ε 47,700), 232sh (46,300), 247sh (30,200), 262 (13,800), 272 (18,100), 282 (18,100), and 294 nm (9800) (Found: C, 84·1; H, 9·0. $C_{34}H_{44}O_2$ requires C, 84·2; H, 9·1%).

Cholesta-5,7,22,24-tetraen-3β-ol (4; R = H). The benzoate (4; R = PhCO) (11 mg) was saponified as above to afford the *alcohol* as needles, m.p. (from methanol) 141—143°, $[\alpha]_D$ −112·1° (*c* 0·31), $\lambda_{max.}$ 232sh (ε 36,100), 238 (39,000), 247sh (29,100), 261 (12,700), 271 (16,600), 282 (17,100), and 294 (9500) (Found: C, 83·5; H, 10·4. $C_{27}H_{40}O,0{\cdot}5H_2O$ requires C, 83·2; H, 10·6%).

Extraction of pol 2.—In this case the mutant was grown

[27] D. H. R. Barton, J. E. T. Corrie, and D. A. Widdowson, unpublished work; see also D. R. Crump, D. H. Williams, and B. Pelc, *J.C.S. Perkin I*, 1973, 2731.

[28] A. Windaus and R. Langer, *Annalen*, 1934, **508**, 105.

[29] K. A. Ahmed and R. A. Woods, *Genet. Res.*, 1967, **9**, 179.

in YCM (5 l) but all other conditions were unchanged. The general method gave four bands as shown below.

Ergost-8-en-3β-yl benzoate. This fraction (177 mg) crystallised as plates, m.p. (from chloroform–methanol) 152—155°, $[\alpha]_D$ +33·8° (*c* 0·60) (lit.,[7] m.p. 147—148°, $[\alpha]_D$ +34°), δ 0·63 (3H, s, 13β-Me), 1·01 (3H, s, 10β-Me), 4·9 (1H, m, 3α-H), M^+ 504 (100%) with significant peaks at *m/e* 489, 382, 377, 367, 335, 289, 255, 229, and 213 (Found: C, 83·3; H, 10·4. $C_{35}H_{52}O_2$ requires C, 83·3; H, 10·4%).

Ergosta-8,22,dien-3β-yl benzoate (5; R = PhCO). Rechromatography of the second band gave the *benzoate* (23 mg), which separated from chloroform–methanol as plates, m.p. 152—153°, $[\alpha]_D$ +26·9° (*c* 0·42) (Found: C, 83·6; H, 10·1. $C_{35}H_{50}O_2$ requires C, 83·6; H, 10·0%).

Synthesis of ergosta-8,22-dien-3β-benzoate. 3β-Acetoxy-22,23-dibromoergost-8-en-11-one [10] (0·61 g) was refluxed in anhydrous ether (75 ml) with lithium aluminium hydride (0·7 g). After 2·5 h the solution was cooled and the excess of hydride was destroyed with water and dilute sulphuric acid. The mixture was extracted with ether and the extract was washed with aqueous sodium hydrogen carbonate, water, and brine, dried, and evaporated. The residue was purified by preparative t.l.c. (solvent 30% ethyl acetate–benzene) to give ergosta-8,22-dien-3β-ol (90 mg) as laths, m.p. (from acetone) 165—168°, $[\alpha]_D$ +27·6° (*c* 0·51) (lit.,[11] m.p. 166—169, $[\alpha]_D$ +30°). The alcohol (35 mg) was kept overnight at 0° with benzoyl chloride (0·15 ml) and pyridine (2 ml) and after the usual work-up, the crude benzoate was purified by t.l.c. (solvent benzene) to give ergosta-8,22-dien-3β-yl benzoate as plates, m.p. (from chloroform–methanol) 152—154°, $[\alpha]_D$ +25·7° (*c* 0·63), identical (mixed m.p.) with the natural benzoate.

Ergosta-5,8,22-trien-3β-yl benzoate (6; R = PhCO). The third band was rechromatographed to afford the *ester* (50 mg) as needles, m.p. (from chloroform–methanol) 153—155°, $[\alpha]_D$ −6·1° (*c* 0·46), $\lambda_{max.}$ 229 nm (ε 18,200), δ 0·68 (3H, s, 13β-Me), 1·25 (3H, s, 10β-Me), 4·9 (1H, m, 3α-H), 5·2 (2H, m, 22- and 23-*H*), and 5·5 (1H, m, 6-H), M^+ 500, with significant peaks at *m/e* 378 (100%), 363, 337, 253, 251, and 211 (Found: C, 83·6; H, 9·75. $C_{35}H_{48}O_2$ requires C, 83·9; H, 9·7%).

Ergosta-5,8,22-trien-3β-yl acetate (6; R = Ac). The benzoate (6; R = PhCO) (14 mg) was saponified as before and the crude product was kept with an excess of acetic anhydride–pyridine overnight at 0°. After the usual work-up the residue was recrystallised from acetone–methanol to afford the acetate as plates, m.p. 138—140°, $[\alpha]_D$ −40·2° (*c* 0·39), M^+ 438 with significant peaks at *m/e* 378, 363, 253, and 251 (Found: C, 82·0; H, 10·5. Calc. for $C_{30}H_{46}O_2$: C, 82·1; H, 10·6%). In our hands an authentic sample of lichesteryl acetate [13] had m.p. (from acetone–methanol) 138—140°, which was not depressed on admixture with our material.

7α-Isothiocyanatocholesteryl benzoate. 7α-Bromocholesteryl benzoate [17] (0·2 g) and anhydrous ammonium thiocyanate (0·5 g) in dry acetone (30 ml) were stirred at room temperature for 24 h. The solvent was evaporated off and the residue extracted into ether. The extract was washed with water and brine, dried, and evaporated and the residue was separated by preparative t.l.c. (solvent toluene) into a less polar (82 mg) and a more polar fraction (56 mg), both of which crystallised upon trituration with warm methanol. T.l.c. and i.r. and n.m.r. analysis at this stage showed that the more polar compound had isomerised completely to the *less polar*, which crystallised as plates, m.p. (from chloroform–methanol) 151—153°, $[\alpha]_D$ −234° (*c* 2·01), $\nu_{max.}$ (CCl_4) 2150sh, 2100 (NCS), and 1709 cm^{-1}, $\nu_{max.}$ ($CHCl_3$) 2155, 2100sh, and 1704 cm^{-1}, δ 5·6 (1H, d, *J* 5 Hz, 5-H), 4·9 (1H, m, 3α-H), 4·0 (1H, m, 7β-H), 1·07 (3H, s, 10β-Me), 0·90 (9H, d, *J* 6 Hz, 20-Me and 25-Me_2), and 0·69 (3H, s, 13β-Me) (Found: C, 76·5; H, 8·9; N, 2·35; S, 5·8. $C_{35}H_{49}NO_2S$ requires C, 76·7; H, 9·0; N, 2·55; S, 5·85%).

Fecosteryl benzoate. The fourth band was rechromatographed to give the pure benzoate as plates, m.p. (from chloroform–methanol) 130—132°, $[\alpha]_D$ +42·5° (*c* 0·65) (lit.,[3] m.p. 127—129°, $[\alpha]_D$ +39·9°).

Feeding of [2,4-$^3H_2$]ergosta-7,22-dien-3β-ol to pol 2. The labelled precursor [2] (0·67 mg; 7·58 × 10^5 counts s^{-1}) was fed to a culture of *pol* 2 in the manner previously described,[22] with the exception that Yeast Complete Medium [1] was used instead of the previous malt–peptone medium. At the end of the growth period carrier ergosterol (263 mg) was added and the culture was worked up in the usual way.[22] The recovered ergosterol was crystallised to a constant activity of 229·3 counts s^{-1} mg^{-1}, equivalent to a total activity of 6·035 × 10^4 counts s^{-1}, equivalent to 7·96% of the activity fed.

Extraction of pol 3.—Application of the general procedure gave the following fractions.

Fraction I. This material (19 mg) appeared to be homogeneous when examined by analytical t.l.c., and co-chromatographed with ergost-7-en-3β-yl or ergost-8-en-3β-yl benzoate, but its n.m.r. spectrum (see before) showed it to be a mixture (*ca.* 1 : 1) of these two compounds.

Fraction II. Analytical t.l.c. of this fraction (154 mg) on silver nitrate plates gave a single spot, chromatographically identical with ergost-7,22-dien-3β-yl or ergosta-8,22-dien-3β-yl benzoate. The mass spectrum had M^+ at *m/e* 502 and the n.m.r. spectrum showed δ 5·2 (<3H, m, 22- and 23-H, and 7-H of Δ^7-isomer), 4·9 (1H, m, 3α-H), 0·63 (≪3H, s, 13β-Me of Δ^8-isomer), and 0·56 (<3H, s, 13β-Me of Δ^7-isomer). The relative intensities of the last two signals showed that the $\Delta^{7,22}$- and $\Delta^{8,22}$-benzoates were present in the proportions *ca.* 9 : 1.

Fraction III. Spectroscopic and chromatographic data for this fraction (24 mg) were identical with those of authentic zymosteryl benzoate. After several recrystallisations, the material had m.p. (from chloroform–methanol) 125—127°.

Fraction IV. Rechromatography on silver nitrate plates gave two bands. The less polar fraction (7 mg) had m.p. (from chloroform–methanol) 137—139°, $[\alpha]_D$ +15·0° and was identical (chromatography and mass spectroscopy) with authentic ergosta-7,22,24(28)-trien-3β-yl benzoate (lit.,[3] m.p. 139—141°, $[\alpha]_D$ +13·5°).

The more polar fraction (7 mg) was fecosteryl benzoate (7), m.p. (from chloroform–methanol) 125—128°, $[\alpha]_D$ +39·2° (*c* 0·29).

Fraction V. This fraction (72 mg) was mainly fecosteryl benzoate (n.m.r. spectroscopy) but the u.v. spectrum showed some ring B diene chromophore ($\lambda_{max.}$ 281 nm). A fresh culture (7 l) was grown and worked up for ergosterol in the usual way,[22] to afford the crude, crystalline adduct (23 mg) of ergosteryl acetate with 4-phenyl-1,2,4-triazoline-3,5-dione. The recovered [22] ergosterol had $\lambda_{max.}$ 281 nm (ε 12,300), M^+ 396.

Extraction of pol 3 SM36.—The general procedure afforded three fractions as follows.

Fraction I. This material (149 mg) was shown as

before to be a mixture (*ca.* 6 : 1) of ergosta-7,22-dien-3β-yl and ergosta-8,22-dien-3β-yl benzoate.

Fraction II. Further chromatography gave subfractions (a) and (b). Subfraction (a) (58 mg) was shown (see before) to be a mixture (*ca.* 2 : 1) of ergosta-7,22,24(28)-trien-3β-yl and ergosta-8,22,24(28)-3β-yl benzoate. Subfraction (b) was combined with fraction III.

Fraction III. Together with subfraction II(b), this was rechromatographed on silver nitrate plates to afford material (81 mg) which was chromatographically identical with fecosteryl and episteryl benzoates, δ 5·2 (<1H, m, 7-H of Δ^7-isomer), 4·7 (2H, m, 28-H_2), 1·01 (<3H, s, 10β-Me of Δ^8-isomer), 0·87 (<3H, s, 10β-Me of Δ^7-isomer), 0·62 (<3H, s, 13β-Me of Δ^8-isomer), and 0·56 (<3H, s, 13β-Me of Δ^7-isomer). The relative intensities of the last two signals were *ca.* 1 : 2.

Extraction of pol 5.—The crude total benzoylated extract, in $CHCl_3$ (40 ml), was added at −70° to 4-phenyltriazoline-3,5-dione (1·5 g) in dry acetone (60 ml). The solution was stirred at −70° for 3 h, treated with alumina (*ca.* 10 g) and allowed to warm to 25° over 1·5 h. The solution was filtered and evaporated and the residue dissolved in benzene and filtered through a column of alumina (60 g). The eluate was subjected to preparative t.l.c. (solvent 1% ethyl acetate–benzene) to afford two major fractions: (a) unchanged 4-demethylsteryl benzoates and (b) 4-demethylsteryl benzoate adducts. Fraction (a) was chromatographed on silver nitrate plates in the usual way to give two bands, as follows.

Episteryl benzoate. The less polar band (21 mg) gave the ester as prisms, m.p. (from chloroform–methanol) 165—166°, $[\alpha]_D$ +8·8 (*c* 0·72) (lit.,[3] m.p. 175—177°, $[\alpha]_D$ +7·35°), δ 5·2 (1H, m, 7-H), 4·75 (2H, m, 28-H), 1·04 (9H, d, *J* 7 Hz, 20-Me and 25-Me_2), 0·87 (3H, s, 10β-Me), and 0·56 (3H, s, 13β-Me), M^+ 502.

Ergosta-8,14,24(28)-trien-3β-yl benzoate (9). The more polar band (24 mg) gave the *ester* as plates, m.p. (from chloroform–methanol) 148—151°, $[\alpha]_D$ −4·1° (*c* 0·38), $\lambda_{max.}$ 230 (ε 25,800) and 251sh nm (21,400), M^+ 500 (100%) with significant peaks at *m/e* 485, 457, 441, 378, 375, 373, 363, 335, 279, 271, 253, 251, 239, 238, and 237 (Found: C, 83·8; H, 9·5. $C_{35}H_{48}O_2$ requires C, 83·9; H, 9·7%).

Ergosta-8,14,22-trien-3β-yl benzoate (10). A solution of ergosterol (3 g) in 95% ethanol (260 ml) and 10M-hydrochloric acid (15 ml) was refluxed for 3 h and concentrated *in vacuo*. The residue was diluted with water and extracted with ether and the extract was washed with water and aqueous sodium hydrogen carbonate, dried, and evaporated. The residue was left overnight with pyridine (30 ml) and acetic anhydride (10 ml) and worked up in the usual way. The crude acetate in chloroform (25 ml) was added at −70° to 4-phenyltriazoline-3,5-dione (0·4 g) in dry acetone (50 ml) with stirring. After 3 h the solution was treated with alumina (5 g) and allowed to warm to 25° over 1·5 h. After work-up, the unchanged acetates were recovered by chromatography on alumina (110 g) in benzene–petroleum (1 : 4). Recrystallisation from ethyl acetate–methanol gave plates (0·57 g), m.p. 136—138°, $[\alpha]_D$ −65° (*c* 0·78). The n.m.r. spectrum clearly showed the presence of impurities, notably ergosta-6,8(14),22-trién-3β-yl acetate. The mixture was refluxed for 1 h with potassium hydroxide (1 g) in methanol (30 ml). After the usual work-up the residue was left overnight with benzoyl chloride (2 ml) and pyridine (15 ml) and the recovered benzoates were purified by preparative t.l.c. on silver nitrate plates (solvent 40% benzene–petroleum). Recovery of the major band (210 mg) gave the *benzoate* (10) as plates, m.p. 140—142°, $[\alpha]_D$ −30·3 (*c* 0·89), $\lambda_{max.}$ 230 (ε 26,200) and 252sh nm (22,000), M^+ 500 (Found: C, 84·1; H, 9·6. $C_{35}H_{48}O_2$ requires C, 83·9; H, 9·7%).

Fraction (b). Trituration with ether afforded the mixed adducts as a colourless powder (0·44 g), which was heated for 3 h under reflux in dry benzene (8 ml) with 2,4,6-trimethylbenzonitrile oxide[26] (0·2 g). The solvent was removed and the residue was separated by preparative t.l.c. (solvent 10% ethyl acetate–benzene) to afford unchanged material and the epimeric isoxazoline adduct (14) (47 mg) as a gum, δ 8·05 (2H, m, *o*-H of PhCO), 7·4 (7H, m, *m*- and *p*-H of PhCO, and Ph), 6·89 (2H, s, ArH), 6·36 (4H, ABq, 6- and 7-H), 5·7 (1H, m, 4α-H), and 2·24 (9H, s, $ArMe_3$). The recovered material was kept with *m*-chloroperbenzoic acid (0·14 g) in dry methylene chloride (28 ml) for 100 h at 0°; the solution was then shaken with aqueous potassium iodide, sodium thiosulphate, sodium hydrogen carbonate, and water, dried, and evaporated. The residue was purified by preparative t.l.c. (solvent 5% ethyl acetate–benzene) to afford the ergosta-5,7-dien-3β-yl benzoate adduct (11) (300 mg) and a more polar compound (8 mg) which co-chromatographed with the authentic epoxide[27] (15).

Ergosta-5,7-dien-3β-ol. The adduct (11) formed *needles*, m.p. (from chloroform–methanol) 203—204°, $[\alpha]_D$ −73·6° (*c* 0·51) (Found: C, 76·1; H, 8·0; N, 6·3. $C_{43}H_{55}N_3O_4$ requires C, 76·2; H, 8·2; N, 6·2%). This adduct (200 mg) in tetrahydrofuran (10 ml) was refluxed for 24 h with lithium aluminium hydride (150 mg) and the mixture was worked up in the usual way. The residue was crystallised from methanol and divided into two portions. One was identified as ergosta-5,7-dien-3β-ol, m.p. (from chloroform–methanol) 148—150°, $[\alpha]_D$ −128·7° (*c* 0·41) (lit.,[28] m.p. 152—153°, $[\alpha]_D$ −109°), $\lambda_{max.}$ 262 (8900), 272 (12,800), 282 (13,600), and 294 nm (7700), M^+ 398.

The second portion was converted into ergosta-5,7-dien-3β-yl acetate, m.p. (from acetone–methanol) 153—155°, $[\alpha]_D$ −87·6° (*c* 0·45) (lit.,[28] m.p. 157—158°, $[\alpha]_D$ −74·8°), $\lambda_{max.}$ 262 (ε 8400), 272 (12,400), 282 (12,800), and 294 nm (7400), M^+ 440.

Cycloaddition with 2,4,6-Trimethylbenzonitrile Oxide.—24,25-Dihydro-24-methylenelanosteryl acetate (48 mg) and 2,4,6-trimethylbenzonitrile oxide (160 mg) in dry benzene (5 ml) were refluxed for 3 h; t.l.c. then showed the disappearance of the starting material. The solvent was removed and the residue was purified by preparative t.l.c. (solvent 2% ethyl acetate–benzene) to afford the epimeric *isoxazolines* (16), m.p. (from acetone–methanol) 171—181°, $[\alpha]_D$ +51·6° (*c* 0·31), δ 6·88 (2H, s, ArH), 4·5 (1H, m, 3α-H), 2·26 (9H, s, ArMe), and 2·04 (3H, s, COMe), M^+ 643 (Found: C, 80·4; H, 10·2; N, 1·9. $C_{43}H_{65}NO_3$ requires C, 80·2; H, 10·2; N, 2·2%).

When the ergosteryl benzoate adduct (12) (68 mg) and the nitrile oxide (161 mg) in dry benzene (5 ml) were refluxed for 3 h, t.l.c. showed no change in the reaction mixture. The adduct (12) was recovered by preparative t.l.c. (solvent 1% ethyl acetate–benzene).

We thank Professor T. W. Goodwin, F.R.S., and Dr. L. J. Goad for an authentic specimen of lichesteryl acetate. This work was supported by the S.R.C., the Nuffield Foundation, and the Medical Research Council.

[4/148 *Received*, 25*th January*, 1974]