Biosynthesis of Terpenes and Steroids. Part X.† The Sterols of Some Yeast Mutants Doubly Defective in Ergosterol Biosynthesis

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The sterols of six double mutants of *Saccharomyces cerevisiae* have been isolated and characterised, and the sterol distribution has been related to the biosynthetic pathway from zymosterol to ergosterol. Hitherto unknown ergosta-5,8-dien-3β-ol and cholesta-7,24-dien-3β-ol have been isolated and characterised.

INVESTIGATION of single mutants (*pol* 1, 2, 3, and 5) of *Saccharomyces cerevisiae* for their sterols revealed that the mutations caused blocks in the introduction of the side chain methylene group, the $\Delta^8 \longrightarrow \Delta^7$ isomerisation, and the introduction of the 5,6-, and the 22,23-double bonds, respectively.[1] In an attempt to detect intermediates of biosynthetic interest and in order to relate their structures to our suggested scheme for ergosterol biosynthesis in yeast,[2] we have investigated the sterols of six double mutants of *Saccharomyces cerevisiae* derived from *pol* 1, 2, 3, and 5.[3]

The mutants were grown aerobically in Yeast Complete Medium (7 l) to stationary phase (24—53 h) after which the cells were harvested, saponified, and the total ether-soluble fraction was benzoylated in the usual way. Preparative t.l.c. as described previously[1] afforded the mixed 4-demethylsterol benzoates (Table 1). The mixed 4-demethylsterol benzoates were separated (with the exception of the Δ^8/Δ^7-double bond isomers) by standard methods.[4] The results of this analysis are in Table 2.

TABLE 1

4-Demethylsterol benzoates from double *pol* mutants of *Saccharomyces cerevisiae*

Double mutant	Growth period (h)	Yield of wet cells (g)	Total yield of sterol benzoates (g)	Yield (%)
pol 1–*pol* 2	53	63·5	0·125	0·20
pol 1–*pol* 3	27	81·5	0·352	0·43
pol 1–*pol* 5	24	249·0	0·210	0·08
pol 2–*pol* 3	32	72·5	0·436	0·60
pol 2–*pol* 5	27·5	96·0	0·608	0·63
pol 3–*pol* 5	24	184·0	0·293	0·16

† Part IX, ref. 1*a*.

[1] D. H. R. Barton, J. E. T. Corrie, D. A. Widdowson, M. Bard, and R. A. Woods, (*a*) *J.C.S. Perkin I*, 1974, 1326; (*b*) *J.C.S. Chem. Comm.*, 1974, 30.

[2] D. H. R. Barton, J. E. T. Corrie, P. J. Marshall, and D. A. Widdowson, *Bio-org. Chem.*, 1973, **2**, 363.

[3] (*a*) S. W. Molzahn and R. A. Woods, *J. Gen. Microbiol.*, 1972, **72**, 339; (*b*) M. Bard, R. A. Woods, D. H. R. Barton, J. E. T. Corrie, and D. A. Widdowson, *J. Gen. Microbiol.*, in the press.

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

G.l.c. analysis of the crude sterol extract of *pol* 1–*pol* 2 indicated the presence only of zymosterol (1; R = H). Preparative t.l.c. of the 4-demethylsterol benzoates on silver nitrate–silica gel afforded only one isolable compound which was identified (see Experimental section) as zymosteryl benzoate,[4] although the mass spectrum of the crude sterol extract indicated traces of a monodehydro- (*m/e* 382) and a didehydro- (*m/e* 380) species (<5%). The mass spectrum also showed the absence of any 24-methyl sterols. These results are in agreement with the anticipated metabolic blocks.[1] However, the accumulation of zymosterol in this mutant suggests either that it may be a poor substrate for the introduction of $\Delta^{5(6)}$- and $\Delta^{22(23)}$-unsaturation or that there is a requirement for a 24-methylene substituent for the efficient action of the enzymes responsible for further metabolism.

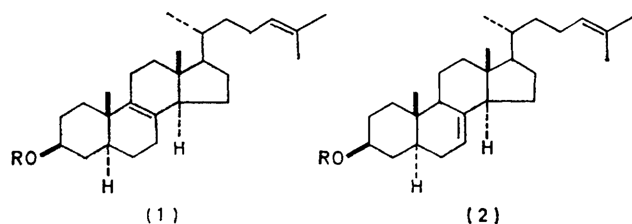
TABLE 2

Sterols of double *pol* mutants of *Saccharomyces cerevisiae*

Double mutant (*pol*/*pol*)	Sterols	Yield (%) *
1–2	Zymosterol (1; R = H)	0·20
1–3	Zymosterol; Cholesta-7,24-dien-3β-ol (2; R = H)	0·43
1–5	Zymosterol	0·069
	Cholesta-5,7,24-trien-3β-ol (9; R = H)	0·015
2–3	Fecosterol (3; R = H)	0·60
2–5	Ergost-8-en-3β-ol (7; R = H)	0·28
	Ergosta-5,8-dien-3β-ol (8; R = H)	0·075
	Fecosterol	0·24
3–5	Zymosterol	0·010
	Ergost-7-en-3β-ol (6; R = H)	0·008
	Fecosterol; Episterol (4; R = H)	0·141

* Percentage of benzoate of the wet weight.

The mixed 4-demethylsterol benzoates of *pol* 1–*pol* 3 on silver nitrate–silica gel t.l.c. separation gave a single band which co-chromatographed with zymosteryl benzoate. On 50% silver nitrate–silica gel plates [5] [hexane–benzene (7 : 3), double development] this appeared as two overlapping spots, but an attempted preparative scale separation was unsuccessful. The n.m.r. spectrum of this band showed singlet resonances



(1) (2)

for the 13β-methyl group of the Δ^7- and Δ^8-isomers at δ 0·53 and 0·63 respectively (*cf.* ergost-7-en-3β-yl benzoate, δ 0·54, and zymosterol benzoate, δ 0·63). These two signals were in the ratio of *ca.* 1 : 2. Furthermore, the intensity of the multiplet at δ 5·10 (7-H of the Δ^7-isomer; *cf.* ergost-7-en-3β-yl benzoate, δ 5·20) corresponded to the presence of *ca.* 30% of cholesta-7,24-dien-3β-yl benzoate (2; R = PhCO). The presence of a single molecular ion at *m/e* 384 in the mass spectrum of the total crude 4-demethyl sterol extract confirmed it to be a mixture of zymosterol (1; R = H) and cholesta-7,24-dien-3β-ol (2; R = H). The mass spectrum also showed the absence of any 24-methyl sterols. Whilst these results are in agreement with the expected blocks,[1] they suggest that as with zymosterol (see above), cholesta-7,24-dien-3β-ol is a poor substrate for the introduction of 22,23-double bond. Although cholesta-7,24-dien-3β-ol had been postulated to be an intermediate in the pathway leading to ergosterol [6a] it has only been noted once before in a yeast.[6b]

The mass spectrum of the crude 4-demethylsterol fraction from *pol* 1–*pol* 5 showed molecular ions at *m/e* 384 ($C_7H_{44}O$) and 382 (*ca.* 30% of *m/e* 384 peak) ($C_{27}H_{42}O$), thus confirming the non-occurrence of 24-methyl sterols in this mutant. The u.v. spectrum of the crude sterols showed the presence of a ring B conjugated diene chromophore ($\lambda_{max.}$ 294, 282, 272, and 262sh nm). Separation of the 4-demethylsterol benzoates as before gave zymosteryl benzoate and cholesta-5,7,24-trien-3β-yl benzoate (9; R = PhCO). The latter compound was identified by direct comparison (m.p., mixed m.p., $[\alpha]_D$, n.m.r., and u.v.) with an authentic specimen.[1] The absence of any detectable quantities of cholesta-7,24-dien-3β-ol (2; R = H) in this instance suggests that a Δ^7-sterol is a good substrate for the introduction of 5(6)-unsaturation.

Application of the standard procedure to *pol* 2–*pol* 3 gave a single compound which was shown to be pure fecosteryl benzoate.[4] The absence of any ergosta-8,22,24(28)-trien-3β-ol (5; R = H), ergosta-8,22-dien-3β-ol (10; R = H), or ergost-8-en-3β-ol (7; R = H) in this mutant would indicate that the enzyme (or enzyme complex) responsible for the introduction of Δ^{22}-unsaturation is inactive or of low activity on fecosterol. However, isolation of compounds (10; R = H) and (7; R = H) from *pol* 3 SM 36 [1] suggests that this process can occur on the Δ^7-isomer [*i.e.* episterol (4; R = H)].

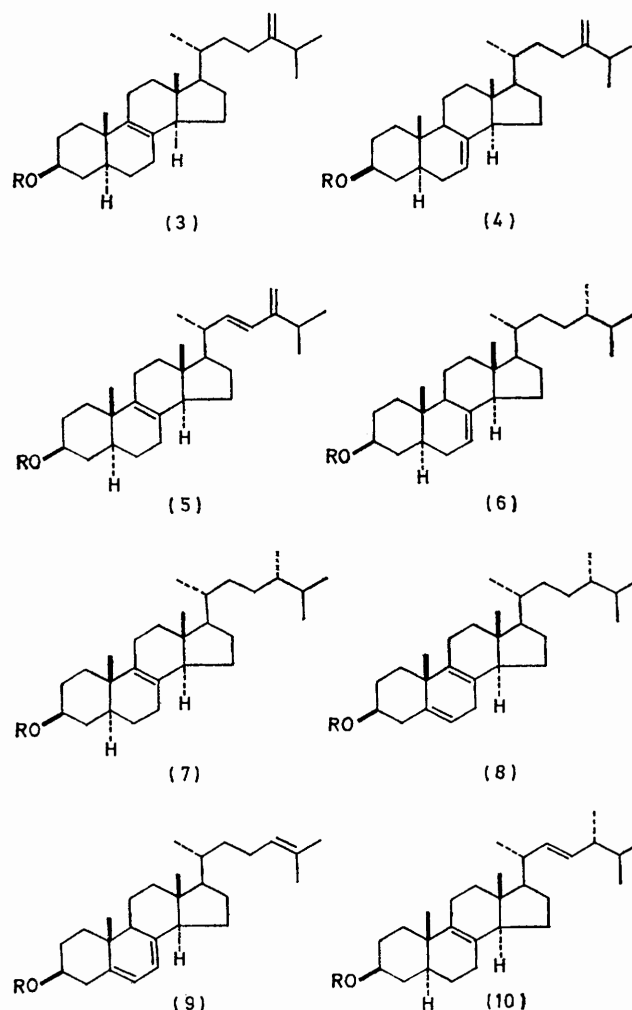
The mixed 4-demethylsterol benzoates of the *pol* 2–*pol* 5 mutant on silver nitrate–silica gel chromatography afforded three fractions. The physical data of the compound isolated from the least polar fraction (see Experimental section) identified it as ergost-8-en-3β-yl benzoate (7; R = PhCO) and this assignment was supported by direct comparison with an authentic sample.[1]

The middle fraction was a mixture of two compounds, and repeated chromatography afforded ergost-8-en-3β-yl benzoate (as above) and a second compound with

[5] M. Fryberg, A. C. Oehlschlager, and A. M. Unrau, *J. Amer. Chem. Soc.*, 1973, **95**, 4747.

[6] (*a*) J. D. Weete, *Phytochemistry*, 1973, **12**, 1843; (*b*) L. W. Parks, C. Anding, and G. Ourisson, *European J. Biochem.*, 1974, **43**, 451.

m.p. 136—139° and $[\alpha]_D$ +0·50° which did not compare with any of the known sterols. This compound 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. Loss



of the elements of benzoic acid from the molecular ion (*m/e* 502) gave the base peak at *m/e* 380, which also showed losses of (side chain, C_9H_{19}) (*m/e* 253) and (side chain + 2) (*m/e* 251). These data also suggested the presence of a fully saturated side chain. The n.m.r. spectrum indicated a 5,6-double bond [one-proton multiplet at δ 5·45 (*cf.* ergosta-5,8,22-trien-3β-yl benzoate, δ 5·50)]. The spectrum had no other olefinic protons but showed the 10β- and 13β-methyl resonances as singlets at δ 1·25 and 0·67 respectively (*cf.* ergosta-5,8,22-trien-3β-yl benzoate, δ 1·25 and 0·68 respectively),[1] thus indicating a 5,8-diene nucleus. The low-field multiplet at δ 2·51 (*cf.*[7] lichesteryl acetate, δ 2·52) due to the C-7 methylene group sandwiched by the two double bonds further confirmed this assignment. The compound must therefore be ergosta-5,8-dien-3β-yl benzoate (8; R = PhCO). The presence of this 5,8-diene sterol in this mutant is of some biosynthetic interest and it further supports the idea[1] that a Δ^7-double bond is not essential for the introduction of Δ^5-unsaturation.

The third and the most polar compound from *pol* 2–*pol* 5 was shown by comparison to be fecosteryl benzoate (3; R = PhCO).[4]

Application of the general procedure (as above) to the 4-demethylsterol benzoates of *pol* 3–*pol* 5 gave three components on silver nitrate–silica gel plates. The physical data of the least polar compound (see Experimental section) indicated it to be ergost-7-en-3β-yl benzoate (6; R = PhCO) and its identity was confirmed by direct comparison with an authentic sample prepared by the catalytic hydrogenation[8] of ergosterol.

The second compound was zymosteryl benzoate.[4] The third, most polar band co-chromatographed with episteryl and fecosteryl benzoates on silver nitrate–silica gel analytical plates, and was identified as a mixture of episteryl and fecosteryl benzoates (relative proportions by n.m.r. *ca.* 95 : 5). Thus the mass spectrum showed a single molecular ion at *m/e* 502 and the n.m.r. spectrum had singlet resonances at δ 0·53 and 0·63 due to 13β-methyl groups, in addition to the olefinic region which was identical with the spectrum of authentic episteryl benzoate.

On the basis of the defined metabolic blocks of the single mutants, the double mutants produced from them by conjugation should have predictable sterol patterns. These are presented in Table 3. *pol* 1–*pol* 2, blocked at the methyl transferase and $\Delta^8 \longrightarrow \Delta^7$ isomerase steps, should produce a cholesta-5,8,22,24-tetraen-3β-ol. In practice, the most developed sterol observed was cholesta-8,24-dien-3β-ol. Similarly *pol* 1–*pol* 3 and *pol* 2–*pol* 3 did not produce detectable quantities of the sterol predictable from the random pathway hypothesis.[1] Conversely the mutants *pol* 1–*pol* 5, *pol* 2–*pol* 5, and *pol* 3–*pol* 5 did produce the predictable ultimate sterols. In no case did a double mutant effect a chemical step which would be genetically forbidden.

It was shown in the wild type and the single mutants that the enzymes involved in the final transformations of ergosterol biosynthesis were not substrate-specific.[1,2] The viability of these yeasts lessens with increasing mutation and the double mutants are less vigorous in growth and have a lower sterol content than their progenitors. In parallel, the transformations of the sterol intermediates are becoming more apparently selective, presumably owing either to a simple lowering of the substrate and hence product concentrations below detectable levels, or to the more significant assertion of other control mechanisms.

However, the additional limitations on the spectrum of

[7] J. R. Lenton, L. J. Goad, and T. W. Goodwin, *Phytochemistry*, 1973, **12**, 1135.

[8] D. H. R. Barton and J. D. Cox, *J. Chem. Soc.*, 1948, 1345.

sterols found in these mutants is compensated for by their enhanced usefulness as sources of pure, normally inaccessible sterols. Notably *pol* 2-*pol* 3 produced fecosterol as the sole detectable product. This sterol could be considered as a starting material for the synthesis of steroid hormones.

EXPERIMENTAL

M.p.s were determined on a Kofler hot stage apparatus, optical rotations in AnalaR chloroform, u.v. spectra in ethanol, and n.m.r. spectra in deuteriochloroform at 60 MHz. Mass spectra were run on an A.E.I. MS9 or a Perkin-Elmer 270 mass spectrometer. T.l.c. was carried out on silica gel GF_{254} plates. For preparative work these were 1 mm thick. Silver nitrate plates refer to silica gel plates impregnated (10%) with silver nitrate.

General Procedures.—These were identical with those previously reported [1] except that the growth period in the fermenter varied from 24 to 53 h (see Table 1), according to the characteristic growth rates of the mutants.

Extraction of pol 1–pol 2.—The standard procedure afforded only one isolable compound, identified as zymosteryl benzoate (125 mg), m.p. 126—127° (from chloroform–methanol), $[\alpha]_D$ +46·0° (*c* 0·4) (lit.,[4] m.p. 126—128°, $[\alpha]_D$ +44·8°).

Extraction of pol 1–pol 3.—Silver nitrate chromatography of the crude sterol benzoates gave a single band (352 mg) which was shown (see above) to be a mixture (*ca.* 2 : 1) of zymosteryl benzoate and cholesta-7,24-dien-3β-yl benzoate (2; R = PhCO).

Extraction of pol 1–pol 5.—The general procedure afforded the two fractions detailed below.

Fraction I. Further purification of this fraction gave zymosteryl benzoate (172 mg) as cubes, m.p. 125—126° (from chloroform–methanol), $[\alpha]_D$ +45·6° (*c* 0·39) (lit.,[4] m.p. 126—128°, $[\alpha]_D$ +44·8°).

Fraction II. This fraction yielded cholesta-5,7,24-trien-3β-yl benzoate (9; R = PhCO) (37·5 mg) as needles, m.p. and mixed m.p. 129—132° (from chloroform–methanol), $[\alpha]_D$ −52° (*c* 0·2) (lit.,[1] m.p. 131—134°, $[\alpha]_D$ −55·0°), $\lambda_{max.}$ 230 (ε 18,150), 252sh (7400), 262sh (10,900), 272 (15,000), 282 (15,200), and 294 nm (8100).

Extraction of pol 2–pol 3.—The standard procedure afforded fecosteryl benzoate (3; R = PhCO) (436 mg) as plates, m.p. 129—130° (from acetone–methanol), $[\alpha]_D$ +43·5° (*c* 0·30) (lit.,[4] m.p. 127—129°, $[\alpha]_D$ +39·9°).

Extraction of pol 2–pol 5.—The general method afforded three bands as shown below.

(i) *Fecosteryl benzoate.* This was obtained from the most polar band as plates (234 mg), m.p. 127—128° (from acetone–methanol), $[\alpha]_D$ +43·2° (*c.* 0·40) (lit.,[4] m.p. 127—129°, $[\alpha]_D$ +39·9°).

(ii) *Ergosta*-5,8-*dien*-3β-*yl benzoate* (8; R = PhCO). Analytical t.l.c. of the second band on silver nitrate plates showed two spots, and rechromatography afforded two sub-fractions. The more polar sub-fraction (72 mg), on repeated recrystallisation from acetone–methanol, gave *ergosta*-5,8-*dien*-3β-*yl benzoate* as plates, m.p. 136—139°, $[\alpha]_D$ +0·50° (*c* 0·25) (calc.[9] $[\alpha]_D$ +2·0°), $\lambda_{max.}$ 230 nm (ε 10,630), δ 0·67 (3H, s, 13β-Me), 1·25 (3H, s, 10β-Me), 4·90 (1H, m, 3α-H), and 5·45 (1H, m, 6-H), *m/e* 502 (M^+), 380 (100%), 365, 253, and 251 (Found: C, 83·8; H, 10·1. $C_{35}H_{50}O_2$ requires C, 83·6; H, 10·0%).

Ergosta-5,8-*dien*-3β-*ol* (8; R = H). The benzoate (8; R = PhCO) (20 mg) was refluxed for 2 h under nitrogen with potassium hydroxide (100 mg) in methanol (2 ml). The usual work-up afforded the *alcohol* (8; R = H) as plates, m.p. 119—122° (from acetone–methanol), $[\alpha]_D$ −0·40° (*c* 0·2), *m/e* 398 (M^+) 383, 380, 365 (100%), and 271 (M^+ − side chain) (Found: C, 84·0; H, 11·5. $C_{28}H_{46}O$ requires C, 84·3; H, 11·6%).

(iii) *Ergost*-8-*en*-3β-*yl benzoate* (7; R = PhCO). Rechromatography of the third band combined with the foregoing less polar sub-fraction on silver nitrate plates gave the ester (264 mg) as plates, m.p. 149—152° (from acetone–methanol), mixed m.p. 150—152°, $[\alpha]_D$ +33·5° (*c* 0·42) (lit.,[10] m.p. 147—148°, $[\alpha]_D$ +34°), δ 0·63 (3H, s, 13β-Me), 1·00 (3H, s, 10β-Me), and 4·90 (1H, m, 3α-H).

Extraction of pol 3–pol 5.—Application of the general procedure afforded the following three fractions.

Fraction I. Spectroscopic and chromatographic data for this fraction (14 mg) were identical with those of authentic ergost-7-en-3β-yl benzoate (6; R = PhCO)

TABLE 3

Enzyme blocks of first and second generation mutants

First generation mutant (*pol*)	Block	Second generation double mutant (*pol*/*pol*)	Transmitted blocks expected	Ultimate skeletal pattern: Predicted	Ultimate skeletal pattern: Found
1	Methyl transferase	1–2	Methyl transferase $\Delta^8 \longrightarrow \Delta^7$Isomerase	Cholesta-5,8,22,24-tetraene	Cholesta-8,24-diene
		1–3	Methyl transferase 5,6-Dehydrogenase	Cholesta-7,22,24-triene	Cholesta-7,24-diene
		1–5	Methyl transferase 22,23-dehydrogenase	Cholesta-5,7,24-triene	Cholesta-5,7-24-triene
2	$\Delta^8 \longrightarrow \Delta^7$ Isomerase	2–3	$\Delta^8 \longrightarrow \Delta^7$ Isomerase 5,6-Dehydrogenase	Ergosta-8,22-diene	Ergosta-8,24(28)-diene
		2–5	$\Delta^8 \longrightarrow \Delta^7$ Isomerase 22,23-Dehydrogenase	Ergosta-5,8-diene	Ergosta-5,8-diene
3	5,6-Dehydrogenase	3–5	5,6-Dehydrogenase 22,23-Dehydrogenase	Ergost-7-ene	Ergost-7-ene
5	22,23-Dehydrogenase	See above			

[9] D. H. R. Barton, *J. Chem. Soc.*, 1945, 813; 1946, 512.
[10] D. H. R. Barton and J. D. Cox, *J. Chem. Soc.*, 1949, 214.

prepared from ergosta-5,7,22-trien-3β-ol as reported.[8] After three recrystallisations, the material had m.p. 175—177° (from chloroform–methanol), mixed m.p. 173—176°, $[\alpha]_D$ −1·3° (*c* 0·23) (lit.,[4] m.p. 174—175°, $[\alpha]_D$ −1°), *m/e* 504 (M^+), 489, 382, 377, 375, and 255.

Fraction II. This fraction (19 mg) on repeated recrystallisation gave zymosteryl benzoate as plates, m.p. 126—127° (from chloroform–methanol), $[\alpha]_D$ +45·0° (*c* 0·20) (lit.,[4] m.p. 126—128°, $[\alpha]_D$ +44·8°).

Fraction III. This material (260 mg) appeared to be homogeneous when examined by analytical t.l.c. on silver nitrate plates, and co-chromatographed with episteryl and fecosteryl benzoates. N.m.r. data (see above) showed the ratio to be 35 : 5 respectively.

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