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Yeast Mutants Blocked in Removing the Methyl Group of Lanosterol at C-14. Separation of Sterols by High-Pressure Liquid Chromatography†

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ABSTRACT: Sterols of a nystatin resistant mutant and of the wild type parent of *Saccharomyces cerevisiae* were separated by a newly developed procedure involving high-pressure liquid chromatography and were identified. The mutant contained larger amounts of squalene and lanosterol (I) than the wild type, as well as 4,14-dimethylcholesta-8,24-dien-3β-ol (II), 4,14-dimethylergosta-8,24(28)-dien-3β-ol (III), and 14-methylergosta-8,24(28)-dien-3β-ol (IV), which were not hitherto found in yeast. These results indicated a block in removal of the methyl group at C-14 of lanosterol. An ergosterol requiring derivative of the mutant which carried in addition a mutation in heme biosynthesis had the same sterols as the parent, but at one-third the concentration. The low level of sterols may be due to a requirement for a heme or cytochrome in oxygenation reactions between lanosterol and ergosterol.

Yeast mutants deficient in ergosterol formation have received attention recently in several laboratories (Parks et al., 1972; Gollub et al., 1974; Fryberg et al., 1974; Barton et al., 1974). In most cases the mutants were isolated by selection for resistance to nystatin or other polyene antibiotics and were blocked in a step beyond lanosterol formation. In this report we describe a mutant deficient in removal of C-32 of lanosterol, i.e., the methyl group attached at C-14 of the sterol nucleus. This step is generally accepted as the first step in the transformation of lanosterol to ergosterol in yeast (Goad, 1970; Fryberg et al., 1973; Barton et al., 1973). We also describe a procedure for separating sterols by high-pressure liquid chromatography.[1] A preliminary communication of the results has appeared (Trocha et al., 1974).

Experimental Section

Strains of Yeast. The wild type strain D587-4B (*αhis1-1*) of *Saccharomyces cerevisiae* was from Dr. F. Sherman's collection and was a gift from Dr. L. Skogerson. Strain SG1 (*erg11-1*), a nystatin resistant derivative of strain D587, and strain SG100 (*erg11-1hem3-1*), a sterol requiring derivative of SG1, were isolated in this laboratory (Gollub et al., 1974). These strains were previously named *Nys1* and *Erg3*, respec-

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[1] Abbreviations used: LC, high-pressure liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; Eu(dpm)$_3$, tris(dipivalomethanato)europium; UV, ultraviolet.

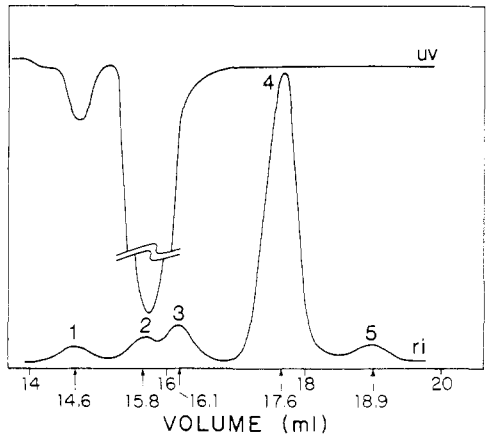

FIGURE 1: High-pressure liquid chromatography separation of TLC band with R_f 0.42–0.48 from sterols of wild type strain D587-4B. Abbreviations are: UV, ultraviolet absorption at 254 nm relative to air; ri, refractive index relative to solvent A. The composition of fractions 1–5 is explained in the section on Results. Absolute volumes of peak fractions from a typical experiment are indicated on the abscissa.

tively. Crosses were carried out between strain SG1 and wild type strain X2180 (obtained from Dr. S. Fogel). Tetrad analysis (12 asci were dissected and analyzed) of ascospores from these crosses gave 2:2 segregation of nystatin resistant and nystatin sensitive colonies, indicating a single mutation in sterol biosynthesis. Furthermore, sterol analysis by GLC of two sets of asci (8 ascospores) showed that the nystatin sensitive offspring contained ergosterol, whereas the nystatin resistant offspring had the normal sterol pattern of SG1 and did not have any ergosterol. Hence, strain SG1 carries a single mutation in sterol biosynthesis (E. G. Gollub, P. K. Liu, and D. B. Sprinson, unpublished results).

Materials and Methods. Ergosta-7,22-dien-3β-ol, ergosta-8,22-dien-3β-ol, ergosta-7,24(28)-dien-3β-ol, ergosta-8,24(28)-dien-3β-ol, ergost-7-en-3β-ol, ergost-8-en-3β-ol, and 4,4-dimethylcholesta-8,24-dien-3β-ol were kind gifts from Dr. A. C. Oehlschlager. Obtusifoliol was a kind gift from Dr. L. J. Goad. Tetrahydrofuran was refluxed for 0.5 h over KOH and freshly distilled before use. Lanosterol (70–80%) was purchased from Sigma Chemical Co., St. Louis, Mo. L-[*methyl*-^{14}C]Methionine was from Amersham/Searle Corp., Arlington Heights, Ill. All other materials were obtained from commercial sources and were not further purified.

Silica gel G TLC plates (5 × 20 cm, 0.25 mm thick, from Analtech, Inc., Newark, Del.) were dipped briefly in a 10% solution of $AgNO_3$ in 50% aqueous methanol, dried overnight in the dark, heated for 25 min at 100 °C, and stored in a desiccator. LC absorbents and μBondapak C-18 columns were obtained from Waters Associates, Milford, Mass., and used with a Waters Associates Model ALC/202 liquid chromatograph. Neopentyl glycol succinate and Chromosorb W (AW/DMCS, 100–120 mesh) were purchased from Hewlett-Packard, Paramus, N.J. SE-30 and Gas-Chrom Q (AW/DMCS, 100–120 mesh) were from Applied Science Laboratories, Inc., University Park, Pa. GLC was carried out on a Hewlett-Packard Model 5700A gas chromatograph equipped with electronic integrator and splitter. Molecular weights of free and of trimethylsilylsterols were determined at 70 eV on a Jeolco mass spectrometer. When necessary perfluorokerosene was added as an internal standard. Computerized mass spectra for publication were taken on a Du Pont 492 mass spectrometer. NMR spectra were obtained in $CDCl_3$ with a Jeolco-100 NMR spectrometer equipped with spin decoupling capabilities. Deshielding was produced by adding 0.5 mol of Eu(dpm)$_3$ per mol of sterol. Chemical shifts are reported in parts per million downfield from an internal tetramethylsilane standard. Radioactivity was determined with an Intertechnique SL30 scintillation counter and detected on TLC plates with a Packard Model 7201 scanner.

Growth of Yeast and Isolation of Sterols. Strains D587 and SG1 were grown on 1% glucose, 2% bactopeptone, and 1% yeast extract at 30 °C for 18–30 h. Strain SG100 was grown in a similar manner except that a 0.2% solution of ergosterol in ethanol–Tween 80 (1:1) was added to a final concentration of 20 mg/L, or a 0.12% solution of hemin chloride (prepared from equal volumes of Tween 80 and a 0.24% solution of hemin chloride in 50% aqueous ethanol containing 0.01 N NaOH) was added to a final concentration of 12 mg/L. Cells were harvested and saponified with 20% KOH in 50% aqueous methanol (1 g wet weight/5 mL) at 85 °C for 60 min, and the mixture was extracted three times with petroleum ether (bp 30–60 °C).

The nonsaponifiable fraction remaining after removal of solvent was dissolved in a small volume of $CHCl_3$, spotted on thin-layer plates, and developed with benzene–ethyl acetate (4:1). Sterol bands, located by spraying a sample plate with Liebermann–Burchard reagent (Stadtman, 1957), were scraped off the plates and extracted three times with hot $CHCl_3$, and the solvent was removed at room temperature in a stream of N_2. An aliquot (2 mg) was dissolved in 50 μL of freshly distilled tetrahydrofuran and chromatographed on 60 cm (two 0.39 × 30 cm columns) of μBondapak C-18 with the following solvent systems: (A) tetrahydrofuran–acetonitrile–water (5:5:2) at a flow rate of 0.5 mL/min; (B) acetonitrile–water (10:1) at a flow rate of 1.2 mL/min. Commercial lanosterol was chromatographed with solvent system A at a flow rate of 0.5 mL/min. Areas under refractive index peaks obtained with 0.1 to 1.2 mg of known sterols were proportional to size of sample.

Sterol fractions obtained by LC and standard compounds were converted to trimethylsilyl ethers by heating with an excess of *N,O*-bis(trimethylsilyl)acetamide in pyridine at 60 °C for 30 min in a small vial stoppered with a serum bottle cap and chromatographed on a 96 × 0.25 in. glass column of 1% neopentyl glycol succinate on Chromosorb W with dry N_2 at 215 °C at a flow rate of 45 mL/min, and on a 72 × 0.25 in. glass column of 3% SE-30 on Gas-Chrom Q with dry N_2 at 245 °C at a flow rate of 45 mL/min. Squalene was used as internal standard. When necessary, individual GLC fractions were collected by the splitter and analyzed by mass spectrometry to determine molecular weight.

Incubation of Cells with [methyl-^{14}C]Methionine. Strain SG1 was grown as described above for 12–18 h, harvested, washed with water, and suspended in an equal volume of 0.2 N sodium phosphate buffer, pH 7.5. L-[*methyl*-^{14}C]Methionine (0.17 μmol, 10 μCi) in 1.5 mL of buffer was added to 0.20 mL of cell suspension, and the mixture was shaken at 30 °C for 3 h. Sterols were isolated and chromatographed on silica gel plates, and radioactive bands were located with a scanner, scraped off the plate, and extracted with $CHCl_3$. The residue after removal of solvent was counted in a scintillation counter.

Results

Separation and Identification of Yeast Sterols. The nonsaponifiable fractions were trimethylsilylated and analyzed quantitatively by GLC under standard conditions of material and sensitivity. The results were similar to those reported earlier (Figure 1 in Gollub et al., 1974). Ergosterol represented about 8% of total sterols in wild type strain D587 and was ab-

TABLE I: Relative Retention Values of Sterols.

Sterols	TLC (R_f)	LC[a]	GLC[b]	GLC[c]
Ergosta-5,7,22-trienol (ergosterol)[d,e]	0.25	1.90	4.12	2.84
Squalene[d-f]	0.30	2.19	1.00	1.00
Ergosta-7,22-dienol[d,e]	0.42	2.20	3.78	2.90
Ergosta-7,24(28)-dienol (episterol)[d,e]	0.42	2.02	4.96	3.30
Ergost-7-enol[d,e]	0.43	2.35	4.57	3.37
Ergosta-8,22-dienol[d,e]	0.44	2.19	3.22	2.69
Ergost-8-enol[d,e]	0.45	2.32	3.77	3.12
Ergosta-8,24(28)-dienol (fecosterol)[d,e]	0.45	2.00	4.22	3.05
Cholestanol[d]	0.46		2.72	2.36
Cholesterol[d]	0.47	2.20	2.82	2.35
14-Methylergosta-8,24(28)-dienol (14-methylfecosterol)[f]	0.47	1.94	4.00	2.95
Ergosta-8,22,24(28)-trienol[e,h]		1.83	4.28	2.84
Ergosta-7,22,24(28)-trienol[e,h]		1.83	5.05	3.04
4,14-Dimethylergosta-8,24(28)-dienol (obtusifoliol)[d,f]	0.57	1.96	4.28	3.13
4,14-Dimethylcholesta-8,24-dienol (4,14-dimethylzymosterol)[f]	0.56	2.07	4.78	3.61
4,4-Dimethylcholesta-8,24-dienol[d]	0.60	2.09	4.93	3.78
Lanosterol[d-g]	0.62	2.12	4.45	3.57
24,25-Dihydrolanosterol[g]	0.62	2.45	3.42	3.32
Lanosta-7,9,24-trienol[g]	0.62	1.94		
Lanosta-7,9-dienol[g]	0.62	2.21		
Unknown[f]	0.43	2.14		
Unknown[e,h]		1.96	3.64	

[a] Retention time relative to solvent front (solvent system A) in liquid chromatography. [b] Retention time of trimethylsilyl ethers relative to squalene on 1% neopentyl glycol succinate in gas liquid chromatography. [c] As in footnote *b*, except of 3% SE-30. [d] Standard compounds. All sterols in this Table are 3-β-OH. [e] Sterols found in wild type strain D587. [f] Sterols found in strains SG1 and SG100. [g] Isolated from commercial lanosterol. [h] Found in diffuse band with R_f 0.42 to 0.48 from strain D587.

sent in mutants SG1 and SG100. The mutants, however, contained 20% squalene plus lanosterol compared with traces of these compounds in the wild type strain. The sterols of the ergosterol requiring mutant SG100 amounted to about one-third of the sterols in the prototrophic parent (SG1).

Sterols isolated from strain D587 were separated into three bands on silica gel G plates impregnated with $AgNO_3$ (Table I). Ergosterol migrated slowest (R_f 0.25), owing in part to B-ring conjugation, whereas lanosterol migrated fastest (R_f 0.62). The bulk of the sterols migrated as a diffuse band, R_f 0.42 to 0.48. Samples of this fraction (2 mg) were injected in 50 μL of tetrahydrofuran and separated by LC with solvent system A (Figure 1).

Although ergosterol was largely removed by TLC, small amounts of it occasionally contaminated the main TLC fraction and appeared in LC as the high UV-absorbing peak 2 (Figure 1). The sterol fraction under peak 4 represented approximately 75% of the total as estimated from areas under the refractive index peaks. GLC of fraction 4 on 1% neopentyl glycol succinate or 3% SE-30 (Table I) separated two sterol trimethylsilyl ethers of identical molecular weight (*m/e* 470). The major component, about 80% by GLC, was identified by GLC and mass spectra as ergosta-7,22,-dien-3β-ol by comparison with standard compound, and the minor component was similarly identified as ergosta-8,22-dien-3β-ol. Repeated fractionation by LC of the more rapidly eluted segments of fraction 4 afforded the 7,22-dienol as a single peak in GLC and LC. Its mass spectra showed the expected parent ion at *m/e* 398 (Figure 2).

Fraction 3 (Figure 1) was separated by GLC into two trimethylsilyl ethers with parent ions *m/e* 484 which were assigned the structures ergosta-7,24(28)-dien-3β-ol (episterol; 75%) and ergosta-8,24(28)-dien-3β-ol (fecosterol) by comparison with standard compounds in LC and GLC. In a similar manner fraction 5 (Figure 1) was found to be a mixture of the mono-enes ergost-7-enol (60%) and ergost-8-enol (40%).

Fraction 1 (Figure 1) had a significant absorbance at 254 nm, and its UV spectrum [240 nm (shoulder), 231 nm, 224 nm

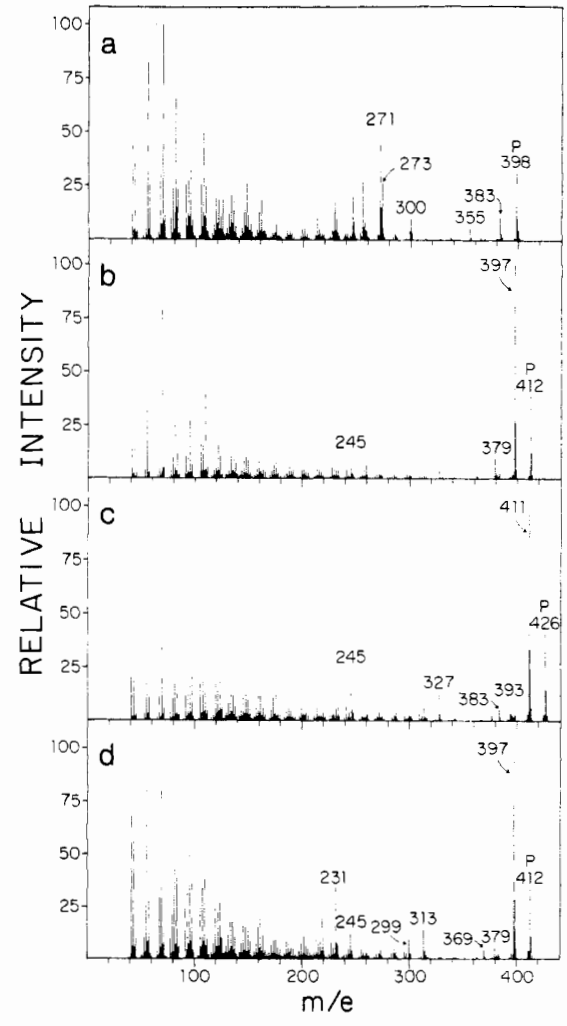

FIGURE 2: Mass spectra of (a) ergosta-7,22-dien-3β-ol; (b) 4,14-dimethylzymosterol (II); (c) obtusifoliol (III); (d) 14-methylfecosterol (IV). P, parent peak.

TABLE II: Sterol Composition of Wild Type and Sterol Deficient Mutants of Yeast.

Strain	Sterols[a]
D587-4B (wild type)	Ergosta-7,22-dien-3β-ol Ergosta-8,22-dien-3β-ol Episterol Ergosterol Ergost-7-en-3β-ol Fecosterol Ergosta-7,22,24(28)-trien-3β-ol Ergost-8-en-3β-ol Ergosta-8,22,24(28)-trien-3β-ol Unidentified sterol Lanosterol
SG1 and SG100[b]	14-Methylfecosterol Lanosterol Obtusifoliol 4,14-Dimethylzymosterol Unidentified sterol

[a] Listed in decreasing order of abundance (estimated from areas of peaks in Figure 1 and GLC analysis of the corresponding fractions). [b] Sterols were present at approximately one-third the concentration found in parent SG1 (Gollub et al., 1974). Ergosterol, also present in strain SG100, was derived from the growth medium.

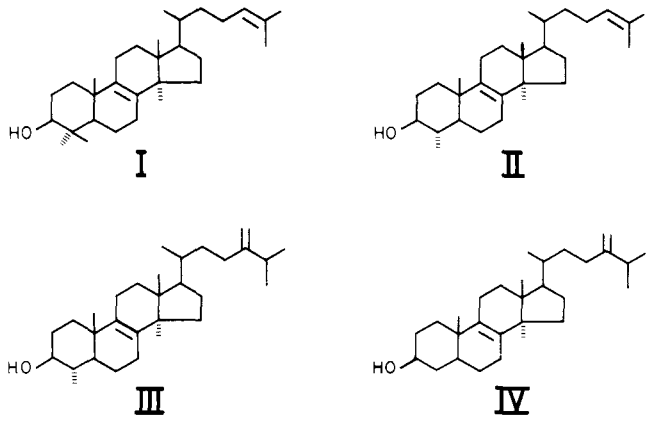

FIGURE 3: Sterols characterized from strains SG1 and SG100. I, lanosterol; II, 4,14-dimethylzymosterol; III, obtusifoliol; IV, 14-methylfecosterol.

(shoulder)] showed side chain conjugation, as reported for ergosta-7,22,24(28)-trien-β-ol (Barton et al., 1972). The ratio of relative retention times in GLC indicated a mixture of a Δ^7 and Δ^8 isomer (Patterson, 1971), and mass spectra of the sterol mixture showed a parent peak at *m/e* 396. Since standard compounds were not available for comparison, the components of LC peak 1 were tentatively assigned the structures of ergosta-7,22,24(28)-trien-β-ol (60% by GLC) and its Δ^8 isomer. A very small amount of a non-UV-absorbing material was isolated by LC from the main sterol fraction, but was not identified (Table I). Only traces of lanosterol (R_f 0.62) were observed in TLC of sterols from strain D587, as found previously by GLC. The relative abundance of sterols in this strain as derived from the above LC and GLC analysis is indicated in Table II.

The sterol composition of the nystatin-resistant strain SG1 was considerably different from that of the wild type (Table II). Strain SG100, a sterol requiring derivative of SG1, had the same sterols, but as mentioned earlier, at approximately one-third the concentration of SG1. TLC plates of the sterols from these strains showed four well defined bands, at R_f 0.62, 0.56–0.57, 0.47, and a very small UV-absorbing band at R_f 0.43 which was not identified. Lanosterol (R_f 0.62; Figure 3, I) was present in much larger quantities than the trace amounts found in wild type cells and was readily identified by comparison with a standard sample in GLC and LC relative retention times. The material with R_f 0.56–0.57 was resolved by LC into compounds II and III (Figure 3) with solvent system B. Owing to reduced solubility in this solvent sample size was reduced to about 0.5 mg per 50 μL of tetrahydrofuran. 4,14-Dimethylzymosterol (II) gave the expected parent ion at *m/e* 412 and showed the cleavages (Figure 2) reported for the synthetic compound by Fryberg et al. (1975). The trimethylsilyl ether had a parent ion at *m/e* 484: NMR δ 0.71 (s, 13-CH_3), 0.88 (s, 14-CH_3), 0.93 (20-CH_3), 0.96 (s, 10-CH_3), 0.99 (d, J = 6 Hz, 4α-CH_3), 1.60 and 1.68 (C=$C(CH_3)_2$, 5.12 (br t, C—CH=C). With Eu(dpm)$_3$, NMR had δ 2.08 (s, 10-CH_3, −1.12), 3.42 (d, J = 6 Hz, 4α-CH_3, −2.43), 4.84 (H-4). After irradiation at 4.85 the doublet of 4α-CH_3 collapsed to a singlet at 3.42.

Obtusifoliol (III) and its trimethylsilyl ether had the expected parent ions at *m/e* 426 (Figure 2) and 498, respectively. The mass spectrum of authentic obtusifoliol was identical with that of the isolated compound. NMR δ 0.72 (s, 13-CH_3), 0.93 (d, J = 7 Hz, 20-CH_3 and C—$C(CH_3)_2$), 0.99 (s, 14-CH_3), 0.99 (d, J = 6 Hz, 4α-CH_3), 1.06 (s, 10-CH_3), 4.67 and 4.72 (br s, C=CH_2). With Eu(dpm)$_3$, NMR had δ 1.98 (s, 10-CH_3, −0.92), 3.23 (d, J = 6 Hz, 4α-CH_3, −2.24), 4.60 (H-4). Upon irradiation at 4.60, the doublet of 4α-CH_3 collapsed to a singlet at 3.23. The structure assignment was supported by comparison with an authentic sample in LC and GLC retention times (Table I).

The major sterol of the mutants, 14-methylfecosterol (R_f 0.47; Figure 3, IV) had a parent peak at *m/e* 412 (Figure 2), and cleavages which were essentially identical with those reported by Ragsdale (1975). The most intense peak in the mass spectra of compounds II, III, and IV corresponded to the parent ion minus 15, as observed generally for 14-methylsterols (Ragsdale, 1975). The trimethylsilyl ether of compound IV had a parent ion at *m/e* 484: NMR δ 0.72 (s, 13-CH_3), 0.92 (d, 5–6 Hz, 20-CH_3 and C—$C(CH_3)_2$), 0.99 (s, 14-CH_3), 1.06 (s, 10-CH_3), 4.68 and 4.73 (br s, C=CH_2). With Eu(dpm)$_3$, NMR had δ 2.57 (10-CH_3, −1.65), whereas other methyl group protons were shifted only about 0.2 ppm. Deshielding by europium of the magnitude observed for 4α-CH_3 in II and IV was not observed.

Formation of Labeled Sterols from [methyl-^{14}C]Methionine. Sterols from strain SG1 incubated with [*methyl*-^{14}C]methionine showed two radioactive bands by TLC. A band of 14-methylfecosterol (R_f 0.47), further identified by GLC and LC, had 2050 dpm/g of wet weight yeast cells. The other band (R_f 0.56–0.57) was resolved by LC into inactive dimethylzymosterol and [^{14}C]obtusifoliol (2080 dpm/g of wet weight yeast cells). These findings lend support to the structures assigned to compounds II to IV.

Purification of Lanosterol. The components of commercial lanosterol (Bloch and Urech, 1958) were readily separated by LC with solvent system A after injections of 2 mg per 10 μm of tetrahydrofuran. Lanosterol, about 70%, was eluted at 31.6 mL, and was well separated from 24,25-dihydrolanosterol at 36.5 mL. They had parent ions at *m/e* 426 and 428, respectively. Minor constituents absorbing in the ultraviolet were lanosta-7,9-dien-3β-ol at 32.9 min, and lanosta-7,9,24-trien-3β-ol at 28.9 min (parent ion at *m/e* 422). The lower retention time was assigned to the more highly unsaturated compound (Table I). A rerun of the center fractions of the major component gave pure lanosterol by GLC criteria.

Discussion

We were encouraged to attempt separation of sterols by LC owing to its success in separation of steroid hormones and related compounds. However, initial attempts failed to resolve sterol mixtures by normal phase absorption on Corasil, Porasil, Durapak OPN/Poracil C, and Durapak Carborax 400/Corasil with a wide variety of solvent mixtures. Efforts also failed to separate sterols on silver silicate columns by virtue of complex formation of nuclear and side chain double bonds with silver. On the other hand, reverse-phase LC on 60 cm of μBondapak C-18 afforded good separation of the sterols in commercial lanosterol. The more complex sterol mixtures encountered in yeast were also resolved by this procedure after preliminary separation according to polarity by TLC. Although solubility of sterols in tetrahydrofuran–water mixtures was generally high, partitioning of sterols between the solvents was not sufficient to allow separation. In acetonitrile–water mixtures some separation occurred, but retention times were too long owing to decreased solubility. Hence, solvent system A was developed.

Polarity, solubility, and molecular weight appear to be involved in separation of sterols by LC with the reverse phase μBondapak C-18 columns. In the ergostane sterols the more polar $\Delta^{24(28)}$ exomethylene sterols were eluted before similar compounds with Δ^5 instead of $\Delta^{24(28)}$ conjugation. Thus ergosta-7,22,24(28)-trien-β-ol was eluted before ergosterol, and the above trienes were eluted before the diene and monoene sterols. Lower molecular weight compounds were eluted before higher molecular weight sterols as shown by comparing obtusifoliol with 14-methylfecosterol.

As pointed out previously, Δ^7 and Δ^8 sterol mixtures were not resolved by LC with our solvent systems, but were readily separated by GLC of the trimethylsilyl ethers. On the other hand, the speed of operation (30 min per run for 2 mg of sterol in the present investigation) and separation of free sterols are distinct advantages of the LC method. Furthermore, LC may be able to resolve the Δ^7 and Δ^8 isomers by recycling on four 30-cm μBondapak C-18 columns.

Ergosterol constitutes only about 8% of total sterols in the wild type strain D587-4B. Although the sterol composition of yeast strains may vary widely, the low concentration of ergosterol and occurrence of four Δ^7 and Δ^8 isomer mixtures indicates a close resemblance to the nystatin resistant *pol-3* mutant recently described by Barton et al. (1974). Accumulation of Δ^7 and Δ^8 ergostanes was also observed by Fryberg et al. (1974) in cells grown with increasing levels of nystatin in the medium. Strain D587-4B may therefore be partially blocked in the 5,6-dehydrogenase system. In fact this strain is somewhat nystatin resistant (5 units) and complements with strain X2180 in which ergosterol is the predominant sterol (E. G. Gollub, personal communication).

The methyl group at C-14 of lanosterol was conserved in the sterols of strains SG1 and SG100, whereas either one or both of the methyl groups at C-4 were removed. Hence the mutation in these strains resulted in a block in removal of the methyl group at C-14, the first step in the conversion of lanosterol to ergosterol (Goad, 1970). Our finding of 4,14-dimethylzymosterol in the mutants is analogous to the occurrence of 4-α-sterols in wild type yeast (Barton et al., 1972; Fryberg et al., 1973). The absence of detectable amounts of $\Delta^{7,8}$ isomers of compounds II to IV indicates that the $\Delta^{7,8}$ isomerase is inhibited by a methyl group at C-14, as reported previously in cholesterol biosynthesis (Slayton and Bloch, 1965; Gaylor et al., 1966). Transmethylation occurred at C-24 of 4,14-dimethylzymosterol (II), presumably in accord with the analogous reaction of 4α-methylzymosterol and 4,4-dimethylzymosterol (5% and 2%, respectively, of zymosterol) reported for purified *S*-adenosylmethionine: Δ^{24}-sterol methyltransferase (Moore and Gaylor, 1970). On the other hand, the presence of a methyl group at C-14 seems to have blocked further transformation of the side chain since, in wild type yeast, obtusifoliol (III) is converted to ergosterol (Barton et al., 1973). The most reasonable sequence for formation of 14-methylfecosterol (IV) is by way of I → II → III → IV (Figure 3). Compounds III and IV have not so far been found in yeast. Obtusifoliol (III) is widely distributed in plants, and 4,14-dimethylzymosterol (II) had been isolated previously only from potato leaves (Rees et al., 1968). It has also been prepared from lanosterol (Barton and Kumari, 1970; Fryberg et al., 1975) and has been found more recently in very small amounts in yeast (Fryberg et al., 1975). 14-Methylfecosterol has not been reported previously in yeast but has been isolated from *Ustilago maydis* grown in the presence of triarimol (Ragsdale, 1975), and from *Chlorella* grown in the presence of triparanol (Doyle et al., 1971). Strain D587-4B readily afforded several nystatin resistant mutants with the phenotype of SG1. On the other hand, a strain with a different genetic background provided polyene resistant mutants with four distinct gene alterations, but not with the block found in strain SG1 (Woods and Molzahn, 1972; Barton et al., 1974).

In cholesterol biosynthesis the methyl groups at C-4 of lanosterol are oxygenated to carboxylic acids which are decarboxylated (Miller et al., 1971). The methyl group at C-14 is similarly converted to an aldehyde which is removed as formic acid (Alexander et al., 1972), and the resulting double bond is reduced (Goad, 1970). If the same oxygenase system is used in both types of demethylation in yeast, the enzyme activity apparently lacking in strain SG1 may normally function to eliminate formate or reduce the Δ^{14} intermediate. These possibilities are subject to direct test and are being explored.

4,14-Dimethylzymosterol (II) has been added as carrier to the 4-α-methylsterol fraction from yeast which had been maintained anaerobically and then allowed to ferment aerobically with [2-^{3}H]lanosterol (Fryberg et al., 1975). Since essentially no radioactivity was detected in reisolated compound II, it was concluded that it was not a significant intermediate in wild type yeast. This finding is not surprising owing to the rapid rate at which the methyl group at C-14 is normally removed. Thus, 4,4-dimethylsterols and 4-α-methylsterols are found in yeast, but not 14-methylsterols (Fryberg et al., 1973). When demethylation at C-14 is blocked as in strain SG1, the methyl groups at C-4 can apparently be removed. However, this may occur relatively slowly, since appreciable amounts of lanosterol and squalene are accumulated in this strain. Further work is required to show whether the C-4 demethylase system has a wider specificity or a new enzyme is induced in the mutant.

In sterol requiring strain SG100 the same sterols were present, and in essentially the same relative abundance as in the parent SG1, but at approximately one-third the concentration. Thus, when strain SG100 was grown on limiting ergosterol (0.1 mg per L), squalene was the predominant intermediate (Gollub et al., 1974). Presumably the requirement of ergosterol for growth was due to the production of insufficient sterols. In addition to the sterol mutation which results in retention of the methyl group at C-14, strain SG100 has a mutation in porphyrin biosynthesis (Gollub et al., 1976). The requirement of ergosterol for growth may be attributed to the function of a heme or a cytochrome in several oxygenation reactions which normally participate in converting lanosterol to ergosterol, or in the case of strain SG1 to compounds II, III,

and IV. When strain SG100 is grown on heme, ergosterol is not required and the level of sterols is the same as in strain SG1 (not shown). The presence of significant quantities of sterols in strain SG100 grown on ergosterol may therefore be due to a leaky heme mutation.

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