$\Delta^{8(9),22}$-Ergostadiene-3β-ol, an ergosterol precursor accumulated in wild-type and mutants of yeast

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Abstract Whereas wild-type strains of *Saccharomyces cerevisiae* can synthesize up to 7% dry weight of ergosterol, a polyene-resistant mutant has been obtained which produces no ergosterol. Instead, a C-28 methyl sterol is produced, and it has been identified as $\Delta^{8(9),22}$-ergostadiene-3β-ol. This sterol is converted to ergosterol by wild-type yeasts and is observed transiently in cells during aerobic adaption of anaerobically grown wild-type yeasts. The new sterol is proposed as an intermediate in ergosterol biosynthesis.

Supplementary key words ergosterol · sterol mutant · yeast sterol · polyene resistance

Ergosterol is the predominant sterol produced by *Saccharomyces cerevisiae* when that organism is grown under aerobic conditions (1). In the absence of oxygen, sterols are not formed and must be supplied in small amounts to sustain anaerobic growth (2). When the yeast is cultured anaerobically and the resulting population is vigorously aerated with a fermentable carbon source, sterol production is initiated and proceeds at a rapid rate (3). Simultaneous determination of ergosterol, fermenting capacity, and oxygen consumption reveals that the formation of ergosterol is a function of oxygen utilization, not dependent upon cell growth (4). Under anaerobic conditions, squalene is accumulated intracellularly by the organism; on exposure of the cells to oxygen, the squalene is converted rapidly to sterol (5). Molecular oxygen is essential for the enzymic cyclization of squalene to sterols (6). The requirement for ergosterol in the maintenance of promitochondrial structures during anerobic growth (7–9) and for mitochondrial development upon aeration of the cells has been well documented (10, 11).

Nuclear and cytoplasmic mutants of yeast lacking respiratory competency are fermentative under both aerobic and anaerobic conditions (12). Circumstances which temporarily limit ergosterol production in growing wild-type organisms promote a high frequency of respiratory deficiency in the progeny (13). Addition of ergosterol to those cultures can reduce greatly that high frequency. A wide variety of sterols can permit anaerobic growth of yeast (14), but little is known about the specificity of sterols in their respiratory function. Mutants lacking the capacity for ergosterol formation have been unavailable until recently. There has been reported a respirationally competent, polyene-resistant mutant of *S. cerevisiae* which did not form ergosterol (15). A study of sterol accumulation in that organism is the subject of this paper.

MATERIALS AND METHODS

Materials

Silica gel G was obtained from Brinkmann Instruments, Inc., Westbury, N.Y. [*Me*-^{14}C]Methionine was from Amersham/Searle, Northbrook, Ill. Zymosterol was a gift from Fleischmann Laboratories, Stamford, Conn. Ergosterol was obtained from Sigma Chemical Co., St. Louis, Mo., and was recrystallized from ethanol prior to use. Components of the media for culturing the yeast were products of Difco Laboratories, Detroit, Mich. The 2,5-diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)-benzene were purchased from Packard Instrument Co., La Grange, Ill. Digitonin was from Calbiochem, Los Angeles, Calif. Reagent grade dimethylsulfoxide was from J. T. Baker Chemical Co., Phillipsburg, N.J.

Yeasts and cultural conditions

Yeast strains used in the study were obtained as follows: nys-3 (nystatin-resistant haploid) from Dr. R.

Wood; 22B (methionine-requiring haploid) from Dr. R. Mortimer; 3701B (uracil-requiring haploid) from Dr. H. Roman; 1071-3b (tryptophan-requiring haploid) from Dr. D. C. Hawthorne; and MCC (a wild-type diploid) from the active culture collection at Oregon State University. Strain ET-2 was a diploid resulting from the mating of nys-3 to 1071-3b. 22B-RD, the respiratory deficient 22B, was obtained as a spontaneous mutant from plates of 22B. Procedures and media for the routine culturing of the organisms have been described (16).

For the isolation of sterols, yeast cells were grown in a 1% tryptone, 0.5% yeast extract broth to which 1.9% ethanol was added after sterilization. A 15% inoculum, previously grown in the same medium with vigorous aeration, was added and the culture was allowed to reach the stationary growth phase. The cells were cultured in a New Brunswick Fermacell fermentor (CF-50) with stirring at 300 rpm and aeration at 2 l/min with a total culture volume of 35 l. The cells were harvested by transfer under positive pressure to a Sharples centrifuge. Approximately 400 g (wet wt) of cells were obtained from each batch operation.

Sterol isolation and separation

Saponification of the yeast cells was by the alkaline pyrogallol procedure (17) as modified for large volumes (18). The nonsaponifiable fraction was extracted into hexane (19) and recovered by evaporating the solvent under nitrogen. The sterols were dissolved in ethanol, precipitated with digitonin, recovered by dimethylsulfoxide cleavage (20), and recrystallized from ethanol. The partially purified sterols were then separated by thin-layer chromatography on $AgNO_3$–silica gel G (19). Transmethylation of [*Me*-^{14}C]methionine to sterols and their recovery have been described (21). In work reported here, we incorporated the whole cell modification of Adams and Parks (20).

To obtain large quantities of the sterols for detailed analyses of physical properties by various spectroscopic procedures, the sterols were recrystallized three times from alcohol after digitonin precipitation. The recovered product was streaked onto plates of silica gel G spread to a thickness of 1.0 mm, and the plates were developed in benzene–ethyl acetate 5:1 (v/v). Bands of the separated sterols were scraped from the plates and eluted with $CHCl_3$. The sterols recovered from many such plates were pooled.

Radioactive determinations

Scanning for radioactivity on thin-layer plates was performed with a Packard 7201B radiochromatogram scanner. Liquid samples were assayed for radioactivity in a Packard Tri-Carb 3214 scintillation spectrometer with external standardization.

Spectral determinations

Ultraviolet absorption spectra were determined in a Cary 11 recording spectrophotometer. The mass spectra were run on an LKB 9000, using an SE-30 column under standard conditions. The location of the double bonds and the nature of the side chain were determined by NMR spectroscopy on a Varian HR-220 (220 MHz) instrument. The spectrum was run on a 9.1-mg sample in 0.38 ml of deuterochloroform using tetramethylsilane as an internal reference.

RESULTS

The ultraviolet absorption spectra of the nonsaponifiable fractions dissolved in hexane for three of the yeast cultures are shown in Fig. 1. The diploid heterozygous

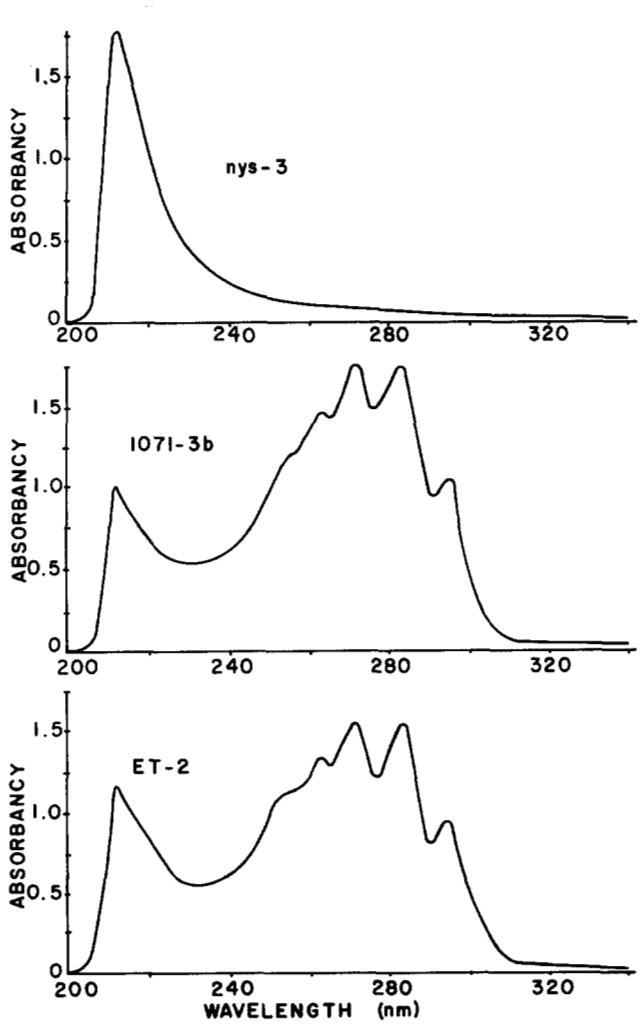

FIG. 1. Ultraviolet absorption spectra of the hexane-extracted nonsaponifiable fractions from *S. cerevisiae* strains nys-3, 1071-3b, and the heterozygotic diploid ET-2.

for sterol production (ET-2) has a spectrum identical with that of the wild-type haploid and with that of authentic ergosterol. The ergosterol-deficient clone (nys-3) contains material absorbing maximally at 212 nm and the spectrum appears to be similar to that obtained for zymosterol. Although not shown here, the spectra for MCC and 22B are identical for those where the predominant sterol is ergosterol. When the nonsaponifiable fraction of the nys-3 was concentrated approximately 100-fold, a weak but clearly discernible absorbancy characteristic of ergosterol was observed.

The sterols from each of the haploid organisms were purified by recrystallization and digitonin precipitation. The recovered sterols produced absorption spectra virtually identical with those identified above.

The nonsaponifiable fractions of each of the organisms were separated on $AgNO_3$-impregnated silica gel thin-layer plates spread to a thickness of 0.25 mm. The R_F values of the separated components for each of the organisms are shown in Table 1. With the exception of nys-3, the predominant sterol in these yeasts is ergosterol. The nys-3 produces a sterol which migrates coincidentally with zymosterol. Mixtures of zymosterol with the nys-3 component again migrated without separation. In the mutant, a very faint spot was observed at R_F 0.13. This spot was most obvious when the acetic anhydride–sulfuric acid-treated plates were examined under ultraviolet light. It was subsequently shown that the ultretaviol-fluorescing spot was not labeled in a transmethylation reaction, and was therefore ergosterol which had been accumulated from the culture medium.

TABLE 1. Mobility of major sterols of various yeast strains on thin-layer chromatography[a]

Yeast Strains and Sterol Standards	R_F Values
22B and 22B-RD	0.13
3701B	0.13
MCC	0.13
nys-3	0.32
Ergosterol	0.13
Zymosterol	0.33
Lanosterol	0.45

[a] The sterolic components of the nonsaponifiable fraction of the yeasts were prepared as described in the text. The sterols were applied to $AgNO_3$-impregnated silica gel G coated to a thickness of 0.25 mm. Development and visualization of the separated components are described in the text.

The incorporation of L-[*Me*-^{14}C]methionine into the nonsaponifiable fraction of both the nystatin-resistant mutant and wild-type cultures has been measured and reported (22). The kinetics of appearance of the label from [*Me*-^{14}C]methionine into the sterolic fraction of each of the organisms is shown in Fig. 2. In 22B, very short-term labeling reveals two radioactive sterolic components, ergosterol and the sterol accumulated by nys-3. Prolonged incubation permits migration of the label into the ergosterol peak of 22B, while there is continued production of the single component of nys-3.

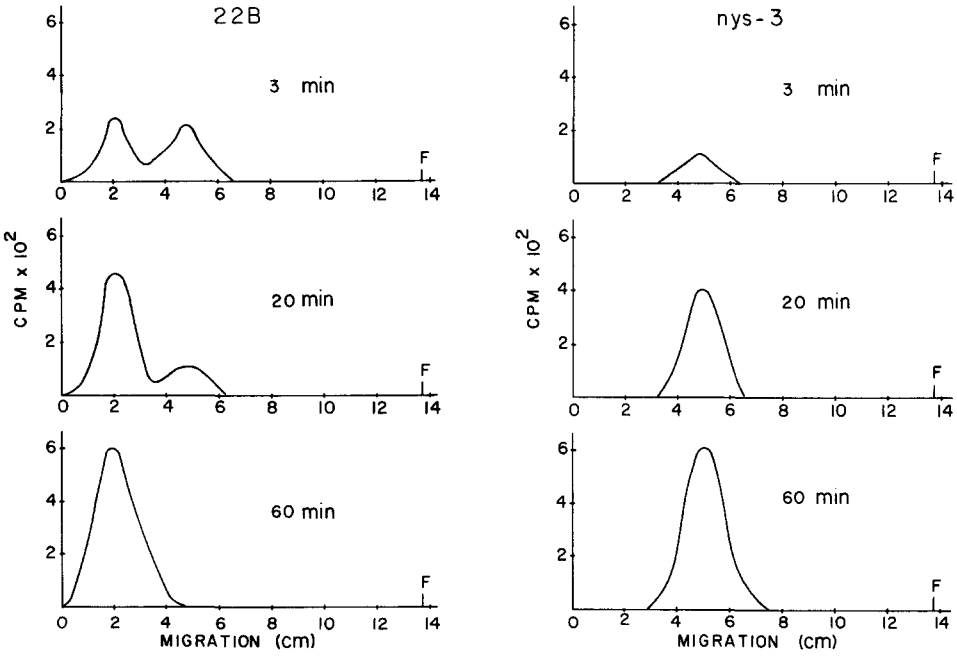

FIG. 2. Radiochromatographic scans of the appearance of label from [*Me*-^{14}C]methionine (0.025 μCi/ml final concentration) in the hexane-extracted nonsaponifiable fractions of cultures of *S. cerevisiae* strains nys-3 and 22B. Migration distances were measured from the point of sample application.

The labeled sterol of nys-3 was isolated by thin-layer chromatography and added to whole cells of strain 22B. Although the sterol appears to be poorly absorbed by 22B, accumulation of the labeled material does occur, and approximately half of it is converted to ergosterol (Fig. 3). Labeled ergosterol, when added to nys-3 cells, is taken up by the organism but is not converted to the unknown sterol. These data support previous conclusions that nys-3 will assimilate sterols from the medium, and they account for the ergosterol detected in that organism.

The mass spectrum of the free alcohol showed a molecular ion at m/e = 398 ($C_{28}H_{46}O$), with significant fragment peaks at m/e = 383 (M − CH_3), 380 (M − H_2O), 365 (M − CH_3 and H_2O), 273 (M − side chain, parent peak), and 255 (M − side chain and water). In particular, the intense peak at m/e = 273 ($C_{19}H_{29}O$) confirms that the side chain contains one double bond and the nucleus one double bond (cf. ergosterol, which has a corresponding peak at m/e = 271).

The location of the double bonds and the nature of the side chain were determined by NMR spectroscopy at 220 MHz. This spectrum showed the presence of two olefinic hydrogens (broadened triplet) at δ = 5.14 and a single hydrogen on carbon bearing oxygen as a broadened multiplet at δ = 3.56. In addition, there were clearly six distinct methyl resonances, with singlets at δ = 0.54 (C-18), and at δ = 0.79 (C-19). There were doublet methyls due to C-21, C-26, C-27, and C-28 at δ = 0.81 (J = 7), δ = 0.82 (J = 7), δ = 0.90 (J = 6), and δ = 1.00 (J = 6). In particular, the nonidentity of the C-26 and C-27 methyl groups requires the presence of an asymmetric center at C-24.

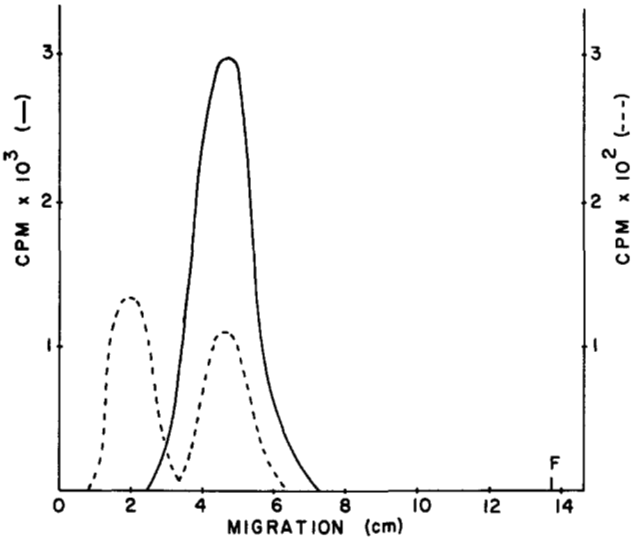

FIG. 3. Experiments illustrating the conversion of $\Delta^{8(9),22}$-ergostadiene-3β-ol to ergosterol by strain 22B. The $\Delta^{8(9),22}$-ergostadiene-3β-ol was isolated from cultures of nys-3 grown aerobically in the presence of [Me-^{14}C]methionine (0.025 μCi/ml final concentration) and separated by thin-layer chromatography (——). The radioactive sterol was removed from the plate and added to anaerobically growing cells of 22B. The culture was grown anaerobically for 48 hr followed by vigorous aeration for 4 hr. The nonsaponifiable fraction was extracted and rechromatographed under the same conditions (– – –).

Significant parts of the spectrum were similar to that of ergosterol determined at the same time. In particular, ergosterol shows its 22-23 olefinic hydrogens as a broadened triplet at δ = 5.15, and four secondary C-methyl groups as doublets centered at δ = 0.82, 0.83, 0.90, and 1.01. This agreement clearly defines the nature of the side chain as identical with that of ergosterol.

Based on the above observations, the structure of the sterol formed by nys-3 was assigned the structure $\Delta^{8(9),22}$-ergostadiene-3β-ol. The purified material has a melting point of 165–167°C (lit. mp 166–169°C) and α_D = +32° (lit. α_D = +30°) corresponding closely to the published values for that compound (23).

DISCUSSION

During the past fifty years, the sterols have been one of the most intensively investigated groups of biochemicals, due primarily to their availability in both quantity and high purity. Extensive research has been conducted on the chemical and physical properties of sterols and their cellular and phylogenic distribution. Although such a vast literature exists on the isolated compounds, virtually nothing is known of the precise role of sterols in the metabolism of the cell. Although an intensive search has been made in several laboratories for mutants which have defects in ergosterol production, none has been isolated by the usual mutant inductive procedures followed by sterol feeding. That certain nystatin-resistant mutants are coincidentally defective in ergosterol production should be a valuable tool in studying sterol metabolism.

If the sterol defect is responsible for the nystatin resistance, it is clear that the resistance must be due to the changes in unsaturation of the B ring. The sterol reported here is similar to ergosterol in the side chain, but lacks the conjugated diene of the nucleus. It is possible that the sterol defect and nystatin resistance are fortuitous. In cell-free reactions, transmethylation is not followed by completion of the ergosterol ring structure. This may be a function reserved for the membrane during localization of the sterol and could consist of identical enzymes or cofactors functioning separately in nystatin sensitivity and sterol formation. The compound $\Delta^{8(9),22}$-ergostadiene-3β-ol is of interest not only because it has never been isolated in quantity from a natural source before (it has been synthesized [23]), but also because it leads to a better understanding of the terminal reactions in ergosterol biosynthesis. The mutation appears to be recessive since it is not expressed in a heterozygotic

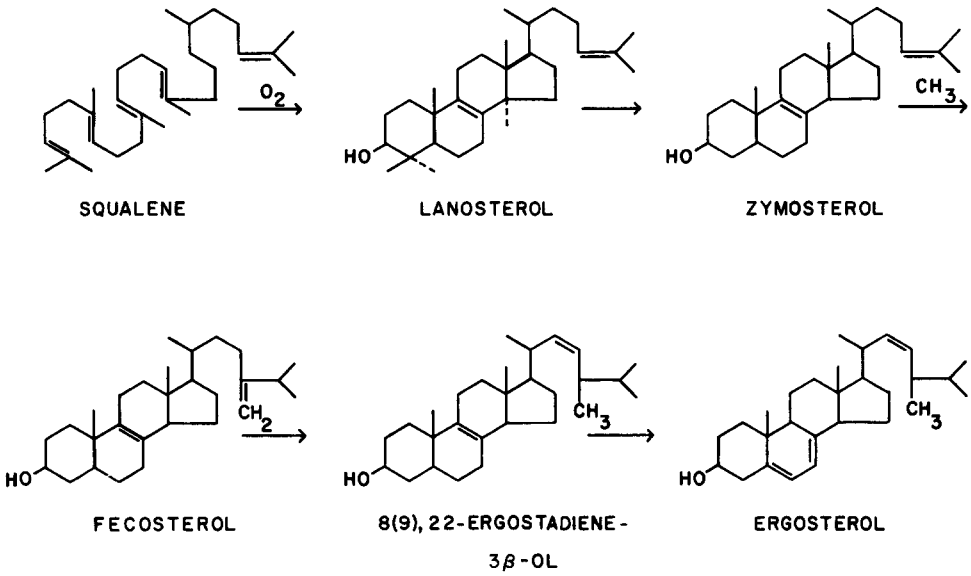

FIG. 4. Relative positions of the proposed sterolic intermediates in ergosterol formation.

state. Also, the conversion of $\Delta^{8(9),22}$-ergostadiene-3β-ol to ergosterol by whole cells of 22B suggests that the compound is an intermediate in the biosynthesis of ergosterol and accumulates because of the loss of an enzyme in that pathway.

Transmethylation of zymosterol to fecosterol ($\Delta^{8(9),24(28)}$-ergostadiene-3β-ol) has been demonstrated and suggested as the product of the transmethylation event. The relative positions of the proposed intermediates in ergosterol formation are shown in Fig. 4. This schema is consistent with the known data and the proposed mechanism for side-chain completion in C-28 methyl sterol synthesis. Our results from the whole cells reported here support the cell-free data indicating that transmethylation and maturation of the side chain can proceed independently of the nuclear transformations (24).

The mutant studied here appears to respire normally, although small differences may exist that were not detectable by our procedures.[1] Although a variety of sterols can support anaerobic growth of yeast (14), ergosterol has been presumed to be essential for acquisition of respiration. This now seems unlikely with the $\Delta^{8,22}$-sterol reported here apparently serving that function. As additional mutants are isolated, it will be of interest to determine which sterols are capable of supporting respiratory development.

In assigning the structure of the sterol reported here, it is important to note that the nuclear double bond, which must be tetrasubstituted, has not been shown definitely to be 8(9). An 8(14) alternative exists. We favor the 8(9) assignment because of close agreement between the published values and ours for melting point and rotation. Additionally, the molecular rotation contribution of an 8(14) double bond should be significantly different from an 8(9) isomer (Ref. 25, p. 178). It is important to note that the chemical shift of the C-19 methyl (0.79) is not in agreement with calculations for either the 8(9) or 8(14) isomer. Reasonable agreement of the C-18 methyl between the observed (0.54) and that calculated (0.58) is seen, however. Because of the discrepancy in the chemical shift in the C-19 methyl group, our assignment of structure in the sterol must be somewhat qualified.

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[1] Starr, P. R., and L. W. Parks. Unpublished results.

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