Stereochemical Specificity for Sterols in *Saccharomyces cerevisiae* 

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When sterol biosynthesis in oxygen-deprived wild type *Saccharomyces cerevisiae* was prevented by the presence of 2,3-iminosqualene, an inhibitor of 2,3-oxidosqualene cyclase, an absolute requirement for a sterol with a 24β-methyl group was found. Neither the configuration nor the size of the alkyl group at C-24 could be altered. For instance, while 24β-methylcholesterol (22-dihydrobrassicasterol) permitted good growth, contrary to earlier work without the inhibitor no growth at all resulted from the presence of cholesterol or its 24α-methyl-, 24α-ethyl-, or 24β-ethyl derivatives (campesterol, sitosterol, and chonasterol, respectively). The only sterol lacking a 24β-methyl group which allowed growth was desmosterol (24-dehydrocholesterol), but desmosterol was metabolized to 24β-methylcholesterol by C1-transfer and reduction. When cholesterol supported growth in the absence of the inhibitor, small amounts of endogenously synthesized 24β-methylsterols (ergosterol and 22-dihydroergosterol) were identified. This previously unrecognized absolute specificity for both chirality and bulk at C-24 suggests the involvement of protein binding in at least one of the roles which sterol plays in this single-celled eukaryote.

*Saccharomyces cerevisiae*, being a nonpathogenic, easily available, single-celled eukaryote which can be grown either with or without sexual reproduction, should be an excellent tool for research on the biochemical role of sterols. Although a great deal of research has actually been done with it, some serious anomalies still exist. Based on the structure of ergosterol (the dominant sterol of aerobic *S. cerevisiae*), one might expect that the stereochemical features of this molecule would be functionally important, perhaps even crucial. However, it has not been possible to prevent at least some growth from occurring when cholesterol is added to the medium of wild type strains (1–5) even though cholesterol lacks the 24β-methyl group (as well as two of the double bonds) found in ergosterol. In fact, cholesterol (as well as its methyl ether) has also permitted growth of mutants which are thought to lack the ability to synthesize sterols (6–10). Furthermore, 24α- and 24β-methylcholesterol (campesterol and 22-dihydrobrassicasterol, respectively), as well as 24α-ethylcholesterol (sitosterol), have supported various amounts of growth with supposedly anaerobic wild type yeast (1, 4, 5, 11). Although abnormalities (failure of budded cells to separate and premature death resulting in pseudomycelia and cell fragmentation) can be shown with cholesterol under certain (1, 2) but not all conditions (3–5), sitosterol (as well as its 22-dehydro derivative, stigmasterol) both in this (11) and other laboratories (4, 5) has always yielded effects which are virtually indistinguishable from those obtained with ergosterol. This is especially puzzling, since in sitosterol and stigmasterol not only is the configuration at C-24 inverted (compared to ergosterol), but the size of the substituent at C-24 has been doubled from C1 to C2.

These anomalies led us to reinvestigate the stereochemical specificity of this organism. On the assumption that a small amount of sterol was being formed by endogenous synthesis, we decided to remove this background in oxygen-deprived wild type yeast by inhibition of a crucial enzyme in the biosynthetic pathway. After development of the new conditions, the anomalies relating to C-24 disappeared and a very strict stereochemical requirement, coinciding with expectation based on the structure of ergosterol, became demonstrable. These results strongly implied that the reason why cholesterol permits growth in the absence of the inhibitor is that a background of sterol really does exist and this was then verified by an examination of the sterols present in cholesterol-grown cells. Our results complement and extend the recent findings of Rodriguez et al. (12) with yeast mutants. These authors showed that, while highly purified 5α-cholest-3-en-3β-ol would not support growth, growth would ensue if an otherwise subminimal amount of ergosterol was also added.

**EXPERIMENTAL PROCEDURES**

Yeast Culture—Wild type, diploid *Saccharomyces cerevisiae* (ATCC 18790) was grown in continuous culture at 27 °C on a synthetic nitrogen base medium (3) modified by Nes *et al.* (1). Cells adapted to oxygen deprivation by continuos culture semianaerobically (1, 13) for several months to several years were used for the experiments unless otherwise noted. Unadapted cells had been in continuous culture for not more than 4 weeks. For the analysis of sterol-induced growth, an inoculum (yielding 0.1 × 10⁶ cells/ml) from the continuous culture was taken at log phase (~24 h) and experiments were carried out for 68 h with 1 liter of medium as described earlier (1) in vessels which prior to inoculation were flushed with nitrogen scrubbed with CrCl₃. The headspace in the vessels was approximately 100 ml and, except for a gas outlet through 95% ethanol, the vessels were sealed. Test sterols, dissolved in 2 ml of absolute ethanol which was added to *Twen* 80 (15 ml per liter of final medium), were added through a septum to give a final concentration of 5.0 mg/liter of medium unless otherwise noted. 2,3-Iminosqualene was synthesized according to the procedure of Avruch and Oehlschlager (14). Appropriate aliquots of a 25 mm ethanolic stock solution of the inhibitor were used for the experiments. The inhibitor (at a final concentration in the medium of 50 μM unless otherwise noted) was added along with the test sterol to the *Twen* 80 solution. When air was added, 10 ml was injected through the septum to a point well below the surface of the medium.

Sterol Analysis—Yeast cells, collected after centrifugation, were saponified using 10% KOH in 95% ethanol at reflux temperature for 1 h. The neutral lipids obtained by dilution with water and extraction with ether were chromatographed on a thin layer of Silica Gel G in ether-etherbenzene (1:9). The 4-demethylsterols were eluted from the appropriate region with ether and analyzed by gas-liquid chromatography at 230 °C on a Hewlett-Packard gas chromatograph, Model...
5840A, equipped with a microprocessor, flame ionization detector, and a 6-foot coiled glass column packed with 1% XE-60. Cholesterol was the standard for determination of the RRT. Two HPLC systems were used. The instrumentation for one of them was assembled from component parts as recently described from this laboratory (15). A C18-Bondapak column from Waters Associates was used at 30°C with 100% acetonitrile as solvent. The instrumentation for the other system (DuPont C18 column, 45×20% isopropyl alcohol in acetonitrile) was from Perkin-Elmer, Series 3B, equipped with an LC-85 spectrophotometric detector capable of automatically giving a complete absorption spectrum by a “stop-flow” technique. The rate of movement is described in both systems as α, which is k for the test sterol/α for cholesterol (15). The 'H NMR spectra were obtained at 300 MHz and ambient temperature on a Bruker instrument, Model WH-360, in CDCl3 with tetramethylsilane as internal standard. Mass spectra were obtained by direct probe on a Finnegan Series 4021 GC-MS instrument with a series 6000 data system.

Materials—Ergosterol, cholesterol, 3-epicholesterol, 5α-cholestan-3β-ol, 5α-cholestan-3α-ol, cholest-4-en-3β-ol, cholest-5-en-3-one, 5α-cholestan-3-one, lathosterol, desmosterol, sitosterol, stigmasterol, cholest-5-ene, and cholesteryl methyl ether were recrystallized from cholesterol (19). The purity was checked by GLC and in many cases also by HPLC, 'H NMR, 13C NMR, and MS.

RESULTS AND DISCUSSION

In order to develop conditions in which the background of sterol could be brought to insignificant levels, we grew yeast in the presence of a small but known amount of added air at various concentrations of 2,3-iminosqualene. The latter compound is a competitive inhibitor (23) of the enzyme (2,3-oxidosqualene cyclase) which catalyzes the formation of lanosterol (Fig. 1). The extent of growth was dependent on the concentration of the inhibitor (Fig. 2) and the cell count fell to a minimal level (a few million cells/ml) at a concentration (of inhibitor) equal to or greater than 100 μM. At 50 μM growth was still not much above the minimal level, so to conserve material we used the latter concentration in the rest of the work. The amount of air we added (10 ml) to obtain the data of Fig. 2 was much more than should have been present inadvertently in our usual experiments. For this reason we expected the inhibitor at a concentration of 50 μM to be adequate to counteract the traces of air which might be present. In actual practice with neither added sterol nor added air, the inhibitor at 50 μM restricted growth to less than 0.5 million cells/ml (Fig. 2), while with added ergosterol (5 mg/liter) under the same conditions (inhibitor but no air) the cell count rose to 118 million cells/ml. Growth on ergosterol was independent of inhibitor concentration (Table I) and the cells appeared normal microscopically. Thus, in the presence of an appropriate sterol, the inhibitor had no deleterious affect.

Under the conditions just described in which sterol biosynthesis was blocked both by oxygen deprivation and by enzyme inhibition, S. cerevisiae was unable to grow at all in the presence of cholesterol (Table I). The results (no growth) were the same regardless of whether the cells were or were not adapted to oxygen deprivation. By contrast, cholesterol permits good growth with adapted cells in the absence of the inhibitor (1) and significant growth occurs even with unadapted cells (2). This is the first time to our knowledge that S. cerevisiae has been unable to grow in the presence of cholesterol, although highly purified 5α-cholestan-3β-ol fails...
to support growth of certain mutants (12).

While cholesterol itself was biologically inactive, addition of a C₂₅-group, yielding 24β-methylcholesterol (22-dihydrobrassicasterol, Fig. 3), was all that had to be done in order to arrive at a molecule which would promote 83% of the level of growth achieved with ergosterol. With the further addition of a 22(trans)-double bond giving brassicasterol, 95% of the double bond and the conjugated 5,7-diene system which it creates in ring B appear to have only a marginal or at least an ill defined significance in the asexual, anaerobic growth of this organism. It is therefore not surprising, especially in view of the absence of a methyl group at C-24, that neither choleste-5-30-en-3β-ol (7-dehydrocholesterol) was active, although both have supported growth in the absence of the inhibitor (1).

The inability of sterols without a substituent at C-24 to allow growth was confirmed further by the inactivity of a number of other sterols as shown in Table II. For instance, (E)-17(20)-dehydrocholesterol, while quite active in the absence of the inhibitor (1), was totally inactive in its presence. In fact, the only unalkylated sterol that supported growth was 24-dehydrocholesterol (desmosterol), but, as shown earlier (24) by GLC and argentation chromatography, this sterol is metabolized by C₁-transfer to 24-methylene cholesterol which then proceeds by reduction to a 24-methylcholesterol presumed to have the 24β-configuration. We have not only confirmed conversion to a 24-methylcholesterol by HPLC, MS, and 1H NMR but have also proven that the configuration of the metabolite at C-24 really is β. The physical properties of the metabolite (24β-methylcholesterol) are given in Table III. They agree with expectation (16, 17, 22, 25, 26). The configuration was demonstrated by comparison of the 1H NMR spectrum with the spectra of authentic samples of the epimeric 24α- and 24β-methylcholesterol. The spectra of the epimers are quite different (17, 25, 26). There are sufficiently extensive shifts in the positions of methyl group doublets to cause partial overlap of peaks in the 24β-methyl case at 360 MHz. Therefore, in addition to quantitative differences in the 1H values, there is a gross qualitative difference in the intensities of the signals. The spectrum of the metabolite was found both quantitatively (Table III) and qualitatively (some peak overlap) to be the same as that of the authentic 24β-methylsterol.

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**Table I**

| Sterol (5.0 mg/liter) | Concentration of 2,3-iminosqualene | Visual cell counts* | µM | millions of cells/ml |
|-----------------------|----------------------------------|---------------------|-----|---------------------|
| Ergosterol            | 0                                | 116 (6)             |     |                     |
| Ergosterol 12.5       |                                  | 124 (2)             |     |                     |
| Ergosterol 25.0       |                                  | 115 (2)             |     |                     |
| Ergosterol 50.0       |                                  | 118 (5)             |     |                     |
| Ergosterol 100.0      |                                  | 111 (2)             |     |                     |
| Cholesterol 0         |                                  | 86 (4)              |     |                     |
| Cholesterol 50.0      |                                  | 0.1 (3)             |     |                     |

*a The count given is an average derived from the number of experiments enclosed by parentheses.

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**Table II**

| Sterol (5.0 mg/liter) | Exp. 1 | Exp. 2 | Exp. 3 | Average | Cell counts | millions of cells/ml |
|-----------------------|--------|--------|--------|---------|-------------|---------------------|
| A. 24-Alkylsterols    |        |        |        |         |             |                     |
| Ergosterol            | 112    | 118    | 112    | 114     |             |                     |
| Brasicasterol         | 108    | 110    | 106    | 108     |             |                     |
| 24β-Methylcholesterol (22-dihydrobrassicasterol) | 90 | 96 | 98 | 95 |         |                     |
| 24α-Methylcholesterol (campostenol) | 0.2 | 0.3 | 0.2 | 0.2 |             |                     |
| 24β-Ethylcholesterol (clionasterol) | 0.2 | 0.1 | 0.3 | 0.2 |             |                     |
| 24α-Ethylcholesterol (sitosterol) | 0.3 | 0.2 | 0.2 | 0.2 |             |                     |
| Stigmasterol          | 0.2    | 0.1    | 0.2    | 0.2     |             |                     |
| B. 24-Desalkylsterols |        |        |        |         |             |                     |
| Desmosterol           | 104    | 95     | 99     | 99      |             |                     |
| 7-Dehydrocholesterol  | 0.5    | 0.5    | 0.5    | 0.5     |             |                     |
| Lathosterol           | 0.2    | 0.1    | 0.4    | 0.2     |             |                     |
| Cholesterol           | 0.1    | 0.1    | 0.2    | 0.2     |             |                     |
| Cholest-5-en-3α-ol    | 0.2    | 0.1    | 0.1    | 0.1     |             |                     |
| Cholest-5-en-3β-ol    | 0.2    | 0.1    | 0.1    | 0.1     |             |                     |
| 5α-Cholest-3β-ol      | 0.2    | 0.1    | 0.1    | 0.1     |             |                     |
| 5α-Cholest-3α-ol      | 0.2    | 0.1    | 0.2    | 0.2     |             |                     |
| 21-Norcholesterol     | 0.2    | 0.1    | 0.2    | 0.2     |             |                     |
| Cholest-4-en-3β-ol    | 0.2    | 0.2    | 0.2    | 0.2     |             |                     |
| Lanosterol            | 0.1    | 0.1    | 0.1    | 0.1     |             |                     |
| 24-Dihydrolanosterol  | 0.2    | 0.2    | 0.1    | 0.2     |             |                     |
| Cycloartenol          | 0.1    | 0.1    | 0.1    | 0.1     |             |                     |
| C. Related steroids   |        |        |        |         |             |                     |
| Cholest-5-en         | 0.1    | 0.1    | 0.1    | 0.1     |             |                     |
| Cholest-5-en-3-one    | 0.8    | 0.1    | 0.2    | 0.4     |             |                     |
| Cholestan-3-one       | 0.1    | 0.2    | 0.2    | 0.2     |             |                     |
| Cholesteryl methyl ether | 0.4    | 0.2    | 0.1    | 0.2     |             |                     |

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**Fig. 3.** Sterols with varying stereochemistry at C-24.
epimer. The cell counts of cultures grown with desmosterol and the inhibitor (Table II) were much the same as obtained earlier (24) with this sterol in the absence of the inhibitor.

Although both lanosterol and cycloartenol have a double bond at C-24, neither of these sterols was able to induce growth either in the presence (Table II) or the absence (1) of the inhibitor. This is not surprising, since, owing to the specificities of enzymes in the yeast pathway (27–30), neither of the sterols would themselves be expected to undergo methylation at C-24. Furthermore, the presence of the nuclear methyl groups would probably prevent use of these sterols as membrane components (1, 6). However, anomalies exist on this point, since lanosterol as well as cycloartenol and cycloartenol have recently been reported to support anaerobic growth of the GI-7 yeast mutant (31).

The 24-alkylsterols produced by yeast all have the β-configuration and have a single carbon atom added to C-24 (32). We therefore examined the significance both of the chirality at C-24 and of the size of the added group (Table II). As already mentioned, 24β-methylcholesterol supports growth very well. By contrast, when the configuration at C-24 was inverted to give 24α-methylcholesterol (campesterol), no growth at all was observed. Similarly, an increase in the size of the alkyl group to C₃ gave either 24α- or 24β-ethylcholesterol (sitosterol or clionasterol, respectively) failed to be consonant with growth of the GL-7 yeast mutant (31).

The foregoing results imply that the reason why cholesterol, sitosterol, etc., have supported growth in the absence of the inhibitor (1–11) is that small amounts of endogenously synthesized 24β-methylsterols are formed due to the presence of traces of air. This would also account for occasional growth of the yeast culture when no sterol (and no inhibitor) was added (11). We therefore examined the sterols of cells grown with cholesterol but with no inhibitor. Good growth (86 million cells/ml) was observed (1). After saponification and TLC, the 4-demethylsterol fraction showed four peaks in GLC and five in HPLC (Table IV). In both chromatographic systems, the principal peak had the value expected of cholesterol. The mass spectrum was similarly dominated by cholesterol giving major peaks at m/z (assignment) 386 (M⁺), 371 (M⁺ - CH₃), 368 (M⁺ - H₂O), 353 (M⁺ - CH₃ - H₂O), 301 (M⁺ - C₃H₅O), 275 (M⁺ - C₃H₉O), 255 (M⁺ - side chain - H₂O), and 213 (M⁺ - side chain - C₃H₅ - H₂) ppm. The five peaks found in HPLC were obtained with a monitoring wavelength of 205 nm. However, when 282 nm was used, the peak for cholesterol (α, 1.00) and one other (α, 0.74) disappeared. The remaining three (α, 0.60, 0.72, and 0.85) showed base line resolution which permitted us to obtain a complete UV absorption spectrum on each one without interference from others by the stop-flow technique. In all three cases, a typical spectrum for the Δ⁵,7-diene chromophore was found with λₚₕ 272, 292, and 294 nm, λₚₖ 262 nm, and λₚₜ 230 nm. Based on known or calculated (15) rates of movement in HPLC, the three sterols were, respectively, increasing α, values, the double bond isomer of ergosterol (24-methylene-7-dehydrocholesterol, 24-methylcholesta-5,7,24(28)-triene-3β-ol), ergosterol, and 22-dihydroergosterol (Fig. 1). The MS verified the presence of ergosterol. Not only was its M⁺ at m/z 398 quite clear, but we could easily observe fragments at, for instance, m/z 363 (M⁺ - H₂O - CH₃), 357 (M⁺ - H₂O - C₃H₂), and 271 (M⁺ - side chain). The other two sterols were in too low a concentration to detect. One of them also has the same molecular weight as ergosterol. The results of GLC agreed with these various assignments. Cholesterol and the three Δ⁵,7-sterols should have given (33) only three peaks at the RRT values observed, since the Δ⁵,7-diene isomer of ergosterol and 22-dihydroergosterol would have nearly the same retention time (33, 34). There remains one peak in HPLC (α, 0.74) and GLC (RRT 1.20) unaccounted for. On the assumption that the Δ⁵ and Δ⁶-bonds have similar contributions to the rates of movement in both chromatographic systems which has some experimental basis (35), the remaining substance was zymosterol. Further evidence for this was afforded by a peak definitely above background at m/z 384 which corresponds to M⁺ for zymosterol. Endogenous synthesis in the
cholesterol-grown cells was also shown by the presence of lanosterol which was the only sterol in the 4,4-dimethylsterol fraction. In addition to the expected properties in GLC and HPLC (Table IV), a characteristic mass spectrum was obtained. The principal peaks in MS were: m/z (assignment), 426 (M'), 411 (M' - CH3), and 393 (M' - CH3 - H2O). The various sterols were present in the following order of amounts: cholesterol > lanosterol > ergosterol > zymosterol > 22-dihydroergosterol > 24-methylene-7-dehydrocholesterol.

The ability of cholesterol to support growth in the presence of a small amount of endogenously synthesized 24β-methylsterol complements work discussed elsewhere (36, 37) in which the medium contains a small (otherwise subminimal) amount of ergosterol (12) and lanosterol and cholesterol act synergistically only if the medium contains a small (otherwise subminimal) amount of ergosterol (12) and lanosterol and cholesterol act synergistically in the prokaryotic Mycoplasma capricolum (38, 39). These various pieces of information indicate (12, 36, 39), as previously known in multicellular systems (40), that sterol can play more than one role in single-celled organisms.

One of the functions, thought to be regulation of unsaturated fatty acid uptake in M. capricolum, appears to be quantitatively minor, because it requires the least sterol. In S. cerevisiae the biochemical nature of the minor function is not yet known, but our results indirectly suggest that binding to a receptor molecule might be involved. This follows from the high degree of specificity which we found with regard to the stereochemistry of the substrate at C-24. It seems unlikely that the three-dimensional characteristics of this group, being in the acyclic, nonrigid side chain, would be as critical as we observed if the only function of the sterol were to interact in the membrane with the conformationally fluid fatty acyl chains of phospholipids. On the other hand, interaction with phospholipids and the resulting modulation of the properties of the lipid leaflet presumably do constitute the quantitatively major role and the one which does not have an absolute requirement for a 24β-methyl group.

It is perhaps worth mentioning in conclusion that our studies were not done under conditions in which sexual reproduction was occurring or in which the mitochondrion was functioning significantly in a respiratory capacity. This may account for our inability to find an absolute requirement for the double bonds at C-7 and C-22 in ergosterol. That is, the reason why these double bonds are added to precursor molecules biosynthetically could conceivably find an explanation in one or both of these (aerobic and sexual) aspects of the life history of yeast.

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W J Pinto and W R Nes

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