Ergosterol biosynthesis in novel melanized fungi from hypersaline environments

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Abstract Halotolerant and halophilic melanized fungi were recently described in hypersaline waters. A close study of the sterol composition of such fungi, namely *Hortaea werneckii*, *Alternaria alternata*, *Cladosporium sphaerospermum*, *Cladosporium* sp., and *Aureobasidium pullulans* revealed the dominance of ergosterol and the presence of 29 intermediates of its biosynthesis pathway. The presence or absence of intermediates from distinct synthesis routes gave insight into the operative synthetic pathways from 4,4,14-trimethylcholesta-8,24-dien-3β-ol (lanosterol) to ergosterol in melanized fungi and in *Saccharomyces cerevisiae*, a reference yeast cultured in parallel. In all studied melanized fungi, initial methylation at C-24 took place before C-14 and C-4 demethylation, involving a different reaction sequence from that observed in *S. cerevisiae*. Further transformation was observed to occur through various routes. In *A. alternata*, isomerization at C-7 takes place prior to desaturation at C-5 and C-22, and methylene reduction at C-24. In addition to these pathways in *Cladosporium* spp., *H. werneckii*, and *A. pullulans*, ergosterol may also be synthesized through reduction of the C-24 methylene group before desaturation at C-5 and C-22 or vice versa. Moreover, in all studied melanized fungi except *A. alternata*, ergosterol biosynthesis may also proceed through C-24 methylene reduction prior to C-4 demethylation.—Méjanelle, L., J. F. Lòpez, N. Gunde-Cimerman, and J. O. Grimalt. **Ergosterol biosynthesis in novel melanized fungi from hypersaline environments. *J. Lipid Res.* 2001.** 42: **352–358.**

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Sterols are important constituents of biological membranes and the reaction sequence for their biosynthesis is well established for mammalian and plants. For most fungi, the end product is 24-methylcholesta-5,7,22-trien-3β-ol (ergosterol), and its synthesis has been worked out by extensive biochemical and genetic studies using the yeast *Saccharomyces cerevisiae* and species from other fungal taxa. Predominant reaction sequences for ergosterol biosynthesis downstream from 4,4,14-trimethylcholesta-8,24-dien-3β-ol (lanosterol) may vary according to fungal taxa and were reviewed by Weete (1, 2) and Mercer (3). Even though a main reaction sequence predominates in a given organism, multiple additional routes are made possible by the relatively broad substrate requirements of enzymes catalyzing the last steps of ergosterol biosynthesis (4–6).

A diverse fungal fauna was recently discovered in environments with salinities ranging between 15–32%, where it was so far assumed that bacteria only were able to grow. These fungi were first isolated in hypersaline waters of Secovlje salterns in Slovenia (7), and subsequently in the salterns of La Trinitat (Ebro Delta, Catalonia, Spain) and Bonmatí (Santa Pola, Valencian Community, Spain), with the occurrence of the same dominant species. The majority of species isolated belonged to melanized meristematic and yeast-like fungi, and a few different genera of filamentous fungi were also identified. Among the isolated halotolerant and halophilic mycobiota, dematiaceous Ascomycetales of the following genera were found: *Hortaea*, *Phaeotheca*, *Trimmatostroma*, *Aureobasidium*, *Alternaria*, and *Cladosporium* (8–10). Five melanized species could be isolated from La Trinitat salterns (Spain) and further enriched in cultures, as well as *S. cerevisiae*, a pertinent reference for Ascomycetes. Their sterols were identified by gas chromatography (GC) coupled to mass spectrometry (MS). A comparative study of intermediates of sterol biosynthesis from the newly described halophilic and halotolerant fungi is presented, with emphasis on the active pathways for ergosterol biosynthesis in melanized fungi.

MATERIAL AND METHODS

Description of the site and of the isolated fungal species

The solar saltern ponds of La Trinitat are located in the south wing of the Ebro Delta (5). As reported previously, the complete

Abbreviations: CFU, colony forming units; FID, flame ionization detector; GC, gas chromatography; MS, mass spectrometry.

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sequence of seawater evaporation including calcite, gypsum, and halite precipitation is represented in this system (11–14).

Aliquots of water (10–100 ml) from the diverse saltern ponds were filtered immediately after sampling on Millipore membrane filters (pore width, 0.45 μm) and placed on different selective agar media containing either high salt (17–32%) or high sugar (50–70% glucose or fructose) concentration. A drop of the original saline water was applied on the membrane and dispersed with a Drigalski spatula. For every medium, four aliquots were filtered in parallel and the average number of colony forming units (CFU) was calculated. Plates were incubated between 1–10 weeks at 25°C. CFU on enumeration media were counted every 3, 5, 7, 14, 30, and 60 days of incubation. Agar baits in dialysis tubings were left from June to November in diverse ponds. In autumn, they were collected, the agar blocks were pushed out, cut aseptically, and plated out on low-water activity media. An enrichment technique was applied by adding glucose and yeast extract to saline water from the salterns and incubating on a rotary shaker. Subsequently, the broth was diluted with 17% saline water several times.

All fungi isolated from selective media were inoculated in parallel on malt extract agar and on malt extract agar plus 3 M NaCl (17%). Only the fungal colonies able to grow in the presence of 17% salt were further determined taxonomically. Growth rate and morphological characteristics were determined on both media after 7 and 30 days. The agar medium used for growth yielded no significant content of sterols in the range of interest for the study.

Extraction and fractionation

An aliquot of 1 to 1.5 g of fungal tissue, gently scrapped off the surface of the solid growth medium, was placed in 10 × 150 mm Pyrex tubes. Lipids were ultrasonically extracted in 5 ml of methanol for 20 min at 30°C. The procedure was repeated twice in methanol and two more times in dichloromethane. Extracts were recovered after centrifugation. Combined extracts were vacuum concentrated to 1 ml and hydrolyzed by addition of 50 ml of KOH in methanol (10%). The mixture was sonicated at room temperature for 10 min, flushed with nitrogen, and kept in the dark for 24 h. Neutral lipids were extracted with hexane (4 × 20 ml). The methanol solution was then acidified to pH 2 by addition of HCl (aq) and further extracted with hexane (4 × 20 ml) for isolation of the fatty acids.

The neutral lipids were fractionated by column chromatography using 1.5 g of 5% water-deactivated silica gel (40 mesh 70-230 Merck) in a 180 × 6 mm column. Three fractions were collected: F1 (hydrocarbons), 12 ml of hexane + 12 ml hexane–dichloromethane, 95:5 (v/v); F2, 24 ml dichloromethane; and F3 (alcohols), 24 ml of dichloromethane–methanol, 90:10 (v/v). Sterols (in F3) were converted into their trimethylsilyl ethers by reaction with bis-(trimethylsilyl)trifluoroacetamide (70°C, 30 min).

Instrumental analysis

Samples were dissolved in *iso*-octane and analyzed by GC in a Carlo Erba 5300 (Carlo Erba, Italy) in splitless mode at 300°C. A 25-m capillary column (0.25 mm id) coated with a 0.25-μm thick stationary phase of 5% phenyl-methyl polysiloxane (DB-5 J&W) was used. Hydrogen was the carrier gas. The oven was kept at 70°C for 1 min, heated to 150°C at 15°·min^{-1}, then to 310°C at 4°·min^{-1}, and finally held at 310°C for 30 min. The temperature of the flame ionization detector (FID) was 330°C, the flame was fed with air (300 ml·min^{-1}), and hydrogen (30 ml·min^{-1}). Nitrogen was used as make-up gas (30 ml·min^{-1}). The detector response was digitized by a Nelson 900 interface and processed with a Nelson 2600 software package (Perkin-Elmer). Relative concentrations of sterols were calculated from the GC-FID response areas.

Analyses by GC-MS were performed with a Fisons 8000 gas chromatograph coupled to a quadrupole mass analyzer Fisons MD-800. Samples were injected in splitless mode at 300°C into a 25-m capillary column (0.25 mm id) coated with a 0.25-μm thick stationary phase of 5% phenyl-methyl polysiloxane (HP-5, Hewlett Packard). Helium was the carrier gas and the temperature program was the same as for the GC analyses.

Mass spectra were recorded in electron impact at 70 eV by scanning between m/z 50–650 every second. Ion source and transfer line were kept at 300°C. Data were processed with Masslab software (THERMO). Sterol identification was based on mass spectral interpretation and comparison of mass spectra and retention time data with available standards.

RESULTS

Hortaea werneckii, *Alternaria alternata*, *Cladosporium cladosporioides*, *Aureobasidium pullulans*, and *Cladosporium* sp. were identified from waters of the solar salterns under study. *H. werneckii* was the prevailing melanized fungus, and is the most adapted species to life in hypersaline waters (10). This fungus is the ethiologic agent of the human hand infection tinea negra, but it is only a commensal species without pathogenecity (8). *C. sphaerospermum* and *Cladosporium* sp. are rather ubiquitous and were found in the calcite, gysum, and halite precipitation ponds, as well as in the magnesium-containing brines. *A. alternata* and *A. pullulans* were found in a more restricted salinity gradient. The same major species of halotolerant or halophilic melanized fungi have also been found in the Secovlje saltpans, an evaporitic saltern system situated in the delta of the Dragonja River on the Adriatic coast in Slovenia (7–10).

Neutral lipids of melanized fungi and of the reference *S. cerevisiae* were mostly composed of sterols and of squalene, the isoprenoid precursor of sterols. Squalene was present in all analyzed species.

In *S. cerevisiae* and in the melanized fungi, the observed sterols encompassed a mixture of 24-methyl homologues unsaturated at Δ^7, Δ^8, $\Delta^{7,22}$, $\Delta^{5,7}$, $\Delta^{7,24(28)}$, and $\Delta^{8,24(28)}$. The percentage of ergosterol in *S. cerevisiae* was comparable with that reported in the literature (15). Lanosterol was the only sterol with more than 28 carbon atoms detected in this species.

The major constituent of the sterol distribution of melanized fungi was ergosterol, the dominant sterol in Ascomycetous yeasts (2, 16). This compound is the end product of sterol biosynthesis in most fungi, whereas cholest-5-en-3β-ol is the major product of sterol synthesis in animals (2). Cholest-5-en-3β-ol was only detected at trace levels in melanized fungi and in the reference yeast. The other main sterols in the newly described melanized fungi consisted of compounds with 1 to 4 unsaturated bonds at positions Δ^5, Δ^7, Δ^8, Δ^{22}, $\Delta^{24(28)}$, or Δ^{24}. Their structures and relative composition are listed in **Table 1**.

In addition to these compounds, four tetraunsaturated sterols were identified. They were generally found in minor amounts, although 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol, the major compound of this group, accounted

TABLE 1. Sterols identified in melanized fungi and in the reference yeast, *S. cerevisiae*. The biosynthesis intermediate numbers refer to sterol sequence on biosynthetic routes of Figs. 1, 2, and 3

Compound Number	Sterol	Biosynthesis Intermediate	Melanized Fungi					Reference Yeast
			H. werneckii	*A. alternata*	*Cladosporium* sp.	*C. sphaerospermum*	*A. pullulans*	*S. cerevisiae*
1	24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol	i17	2.2	3.1	4.9	3.2	2.1	1.9
2	24-methylcholesta-7,22,24(28)-trien-3β-ol	i22	0.5	3.9	5.2	0.5	2.0	—
3	Cholesta-8,24-dien-3β-ol	i5	—	—	—	—	—	3.8
4	24-methylcholesta-8,22-dien-3β-ol	i24	—	—	0.9	0.6	1.9	0.9
5	24-methylcholestatrien-3β-ol[a]	i21	tr	tr	tr	tr	1.1	—
6	24-methylcholesta-5,7,22-trien-3β-ol	i18	49.5	47.4	59.1	50.1	55.5	60.6
7	24-methylcholesta-7,22-dien-3β-ol	i25	2.5	1.3	3.4	6.4	4.4	4.5
8	24-methylcholestatetraen-3β-ol[a]		tr	0.8	1.3	0.9	tr	0.8
9	24-methylcholesta-8,24(28)-dien-3β-ol	i6	tr	5.7	0.6	tr	5.1	2.8
10	24-methylcholest-8-en-3β-ol	i23	0.8	—	1.3	2.1	7.1	2.8
11	24-methylcholesta-5,7,24(28)-trien-3β-ol		—	2.1	—	—	—	—
12	4α-methylcholestatrien-3β-ol[a]		—	2.2	—	—	—	—
13	24-methylcholestadien-3β-ol[a]		tr	—	tr	0.5	tr	—
14	24-methylcholesta-5,7-dien-3β-ol	i20	0.8	—	0.8	1.4	tr	6.2
15	24-methylcholesta-7,24(28)-dien-3β-ol	i15	0.9	28.4	2.6	0.7	5.3	4.4
16	24-methylcholest-7-en-3β-ol	i19	6.8	—	14.5	19.9	5.9	4.5
17	24-methylcholestatetraen-3β-ol[a]		tr	tr	tr	tr	0.6	tr
18	4α,4β,14-trimethylcholesta-8,24-dien-3β-ol	i1	tr	tr	tr	tr	tr	4.6
19	4α,24-dimethylcholest-5-en-3β-ol		tr	—	tr	tr	tr	tr
20	24-methylcholestatetraen-3β-ol[a]		tr	tr	0.9	0.9	0.6	tr
21	4α,24-dimethylcholesta-8,24(28)-dien-3β-ol	i9	tr	2.7	0.9	1.8	1.0	2.2
22	4α,24-dimethylcholest-8-en-3β-ol	i14	1.3	—	1.1	2.4	1.5	—
23	4α,24-dimethylcholesta-5,7-dien-3β-ol		tr	—	tr	tr	tr	—
24	4α,4β,14,24-tetramethylcholesta-8,24(28)-dien-3β-ol	i10	4.3	0.8	1.1	2.5	1.8	—
25	4α,24-dimethylcholesta-7,24(28)-dien-3β-ol		tr	0.8	tr	tr	tr	—
26	4α,24-dimethylcholest-7-en-3β-ol		29.3	—	0.8	3.8	3.4	—
27	4α,4β,14-trimethylcholestadien-3β-ol[a]		—	—	tr	tr	—	—
28	4α,4β,24-trimethylcholesta-8,24(28)-dien-3β-ol	i8	1.0	0.9	0.6	1.6	0.7	—
29	4α,4β,?-trimethylcholestadien-3β-ol		—	—	tr	0.6	—	—

tr, trace.
[a] Position of double bonds could not be determined from MS and retention time.

for 1.9–4.9% total sterols (Table 1). Four 24-methylcholestatrien-3β-ol isomers were identified. They have also been reported in other Ascomycetes (4).

Six 24-methylcholestadien-3β-ols with double bonds at positions $\Delta^{8,22}$, $\Delta^{7,22}$, $\Delta^{8,24(28)}$, $\Delta^{5,7}$, and $\Delta^{7,24(28)}$, and an unknown compound were found in the melanized fungi. 24-Methylcholesta-7,24(28)-dien-3β-ol was the most abundant, being the main diunsaturated sterol in *A. alternata* and *A. pullulans* (Table 1, compound number 15). Other 24-methyl sterols with double bonds at C-24(28), for example, $\Delta^{8,24(28)}$, were also very abundant in these two melanized fungi, representing up to 5% of total sterols. 24-Methylcholesta-7,22-dien-3β-ol was present in significant amounts in all studied melanized fungi (Table 1, compound number 7). Previous investigations have shown 24-methylcholesta-5,22-dien-3β-ol to be a dominant sterol in some fungi (2), but this compound was not identified in any of the melanized fungi considered here.

The monounsaturated 24-methylsterols encompassed two main compounds with double bonds at Δ^7 and Δ^8. 24-Methylcholest-7-en-3β-ol was the second principal sterol in both *Cladosporium* species studied (Table 1, compound number 16). These species also contained 24-methylcholesta-7,22-dien-3β-ol in significant abundance. In contrast, 24-methylcholest-8-en-3β-ol was the second major sterol in *A. pullulans* (Table 1, compound number 10), which also contained 24-methylcholesta-8,24(28)-dien-3β-ol in high abundance.

The other major sterol group was constituted by 4α,24-dimethylsterols. The 4α,24-dimethylsterol encompassed the same unsaturated compounds as the 24-methylsterols: Δ^7, Δ^8, $\Delta^{7,24(28)}$, $\Delta^{8,24(28)}$,and $\Delta^{5,7}$. In the case of *H. werneckii*, 4α,24-dimethylcholest-7-en-3β-ol was the second major sterol constituent after ergosterol (Table 1, compound number 26). This sterol was also abundant in *A. pullulans* and *C. sphaerospermum*. Another abundant sterol in this group was 4α,24-dimethylcholest-8-en-3β-ol, present in all melanized fungi except in *A. alternata* (Table 1, compound number 22). This last species contained 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol, which parallels the presence of 24-methylcholesta-8,24(28)-dien-3β-ol.

Three cholestadienols with 30 carbon atoms having methyl substitution at C4α, C4β, and C24 were found in trace amounts. In addition to these, lanosterol was detected in all studied species (Table 1, compound number 18). This compound constituted the single sterol with more than 28 carbon atoms in *S. cerevisiae*. In *C. sphaerospermum* and *H. werneckii*, 4α,4β,24-trimethylcholesta-8,24(28)-dien-3β-ol was also identified. One C_{31} sterol, 4α,4β,14,24-tetramethylcholesta-8,24(28)-dien-3β-ol, was also found in significant concentration in the five melanized fungi (Table 1, compound 24). *C. sphaerospermum* and *H. werneckii* are the species having this compound in higher abundance, which is consistent with the higher abundance of the C_{30} homologue.

DISCUSSION

In all melanized fungi, as well as in *S. cerevisiae*, ergosterol is the dominant steroid. However, biosynthesis of this compound may proceed through various routes (2, 3, 16). Most of the sterols detected in the newly isolated melanized fungi are intermediates of possible pathways documented in the literature. The presence of an intermediate of a given pathway indicates the feasibility of a specific reaction among all possible alternatives (5, 17). For clarity reasons, the discussion about the biosynthetic routes to ergosterol in these melanized fungi has been divided into three sections.

The transformation from lanosterol to 24-methylcholesta-8,24(28)-dien-3β-ol and to 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol

The first part of ergosterol biosynthesis involves alkylation at C-24 and demethylation at C-14 and C-4 (two carbon atoms at C-4). The order of the sequence of these reactions differentiates between true yeasts and other fungi (2, 3, 16).

In the Ascomycete, *Saccharomyces*, complete demethylation at C-14 and C-4 occurs prior to introduction of a methylene group to C-24. In this yeast, the enzyme responsible for methylation of the side chain shows affinity for cholesta-7,24-dien-3β-ol, cholesta-8,24-dien-3β-ol, and 4α-methylcholesta-8,24-dien-3β-ol (6, 18, 19), and it is generally agreed that the principal methyl acceptor is cholesta-8,24-dien-3β-ol (2, 3, 16, 20, 21). Concurrently, cholesta-8,24-dien-3β-ol and 24-methylcholesta-8,24(28)-dien-3β-ol (i5 and i6 in **Fig. 1**) were indeed detected in *S. cerevisiae* isolated from hypersaline waters of Spanish salterns, showing the operation of the sequence starting by demethylation (Fig. 1).

However, *S. cerevisiae* also contained 4α,24-dimethylcholesta-8,24-dien-3β-ol (i9), showing that C-24 methyl transferase may use substrates bearing at least one methyl at C-4 in this organism. The corresponding transformation (i4 → i9) has been already reported as a minor side-chain methylation process in *S. cerevisiae* and aerobically adapted yeasts (16–18, 22). No intermediates showing the methylation of cholesta-7,24-dien-3β-ol (5, 6) were evidenced in the present work.

In contrast to *S. cerevisiae*, melanized fungi considered in this study do not encompass cholesta-8,24-dien-3β-ol or any other intermediates of the pathway i1 → i2 → i3 → i4 → i5 → i6. The intermediates of C-24 methylation and A-ring demethylation of lanosterol comprised 4α,4β,14,24-tetramethylcholesta-8,24(28)-dien-3β-ol (i10), 4α,4β,24-trimethylcholesta-8,24(28)-dien-3β-ol (i8), and 4α,24-dimethylcholesta-8, 24(28)-dien-3β-ol (i9; **Fig. 2**). These sterols belong to a distinct operative pathway different from that in *S. cerevisiae*. This second sequence starts with methylation at C-24 prior to demethylation at C-14 and at C-4 (i1 → i10 → i7 → i8 → i9 → i6), as described in other species encompassing filamentous or yeast-like fungi and zygomycetes (2, 3, 21, 23–25). Now, this route is identified in hypersaline melanized fungi. The presence or absence of some particular intermediates of the ergosterol synthesis has been used to challenge the taxonomic position of fungi (26). The initial steps of ergosterol synthesis in the recently discovered melanized fungi show that they are biochemically different from the reference yeast, in agreement with their phylogenetic position as fungi in opposition to true yeasts.

Saccharomyces cerevisiae

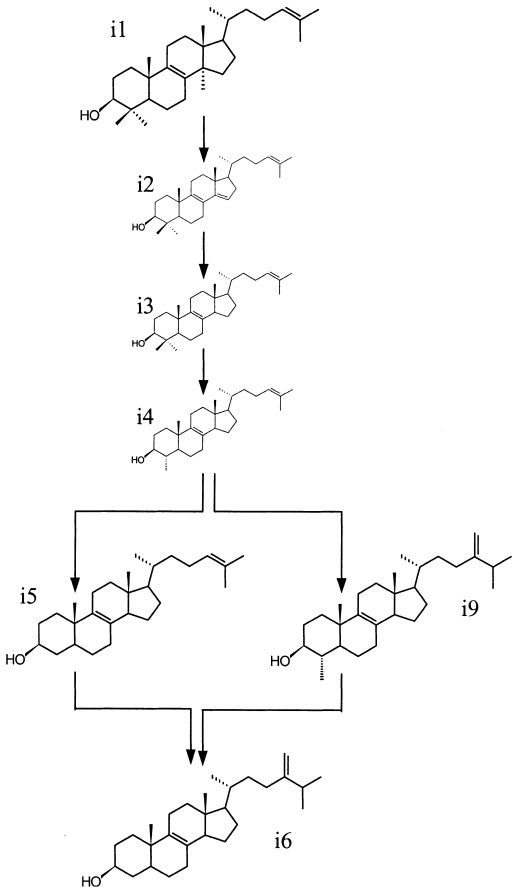

Fig. 1. Possible biosynthetic pathways from 4,4,14-trimethylcholesta-8,24-dien-3β-ol (lanosterol) to 24-methylcholesta-8,24(28)-dien-3β-ol in *S. cerevisiae*. Sterols identified during the present study are shown in bold and in larger size.

The transformation from 24-methylcholesta-8, 24(28)-dien-3β-ol to ergosterol

The second part of ergosterol synthesis consists of double-bond rearrangements in the steroid nucleus and in the side chain, isomerization of Δ^8 to Δ^7, dehydrogenation for unsaturation at C-5 and C-22, and reduction of the $\Delta^{24(28)}$ double bond. These transformations may occur through diverse alternative routes (**Fig. 3**). Several pathways may coexist in the same organism (2, 24, 27).

In many fungi including *S. cerevisiae*, the predominant sequence proceeds through isomerization of 24-methylcholesta-8,24(28)-dien-3β-ol (i6) to 24-methylcholesta-7, 24(28)-dien-3β-ol (i15; Fig. 3). The latter compound was present in all fungi studied including *S. cerevisiae*, being found in concentrations of 28% of the total sterols in *A. alternata* (Table 1). Δ^8-Δ^7 Isomerase may transform other Δ^8 sterols, as suggested here by the various pairs of Δ^8 and Δ^7

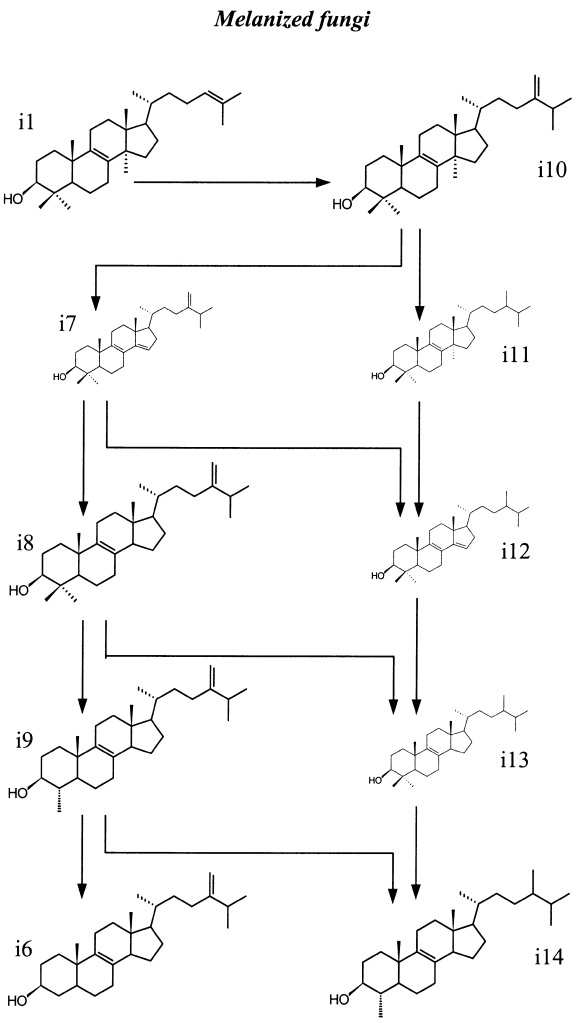

Fig. 2. Possible biosynthetic pathways from 4,4,14-trimethylcholesta-8,24-dien-3β-ol (lanosterol) to 24-methylcholesta-8,24(28)-dien-3β-ol and 4α,24-dimethylcholest-8-en-3β-ol in the studied melanized fungi. Sterols identified during the present study are shown in bold and in larger size.

sterols identified. Four of these pairs were identified in the present study: 24-methylcholesta-8,22-dien-3β-ol/24-methylcholesta-7,22-dien-3β-ol, 24-methylcholesta-8-en-3β-ol/24-methylcholesta-7-en-3β-ol, 4α,24-dimethylcholest-8-en-3β-ol/4α,24-dimethylcholest-7-en-3β-ol, and 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol/4α,24-dimethylcholesta-7,24(28)-dien-3β-ol.

Three main pathways have been reported for double-bond arrangements downstream 24-methylcholesta-7,24(28)-dien-3β-ol (i15) in fungi (2, 3, 19, 21). A first sequence is that reported in *S. cerevisiae*, involving introduction of the Δ^{22} double bond prior to Δ^{5} and final C-24 methylene reduction: i15 → i22 → i17 (or i25) → i18. Accordingly, the intermediates, 24-methylcholesta-7,22,24(28)-trien-3β-ol (i22), 24-methylcholesta-7,22-tetraen-3β-ol (i25), and 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol (i17) were present in minor amounts in the melanized fungi. In *S. cerevisiae*, 24-methylcholesta-7,22,24(28)-trien-3β-ol (i22) was not observed (Table 1). The newly described melanized fungi have, therefore, the capability to synthesize ergosterol through this route, similarly to that observed in the reference yeast.

In the second pathway, the double bond is introduced at C-5 before unsaturation at C-22 and subsequent reduction of the C-24 methylene group: i15 → i16 → i17 → i18. 24-Methylcholesta-5,7,22,24(28)-tatraen-3β-ol was detected in the melanized fungi considered in this study and in *S. cerevisiae* (Table 1), showing that the sequence i6 → i15 → i16 → i17 → i18 is also active in all strains. This sequence is the dominant one in most filamentous fungi and has occasionally been reported in *S. cerevisiae* (17).

The third sequence is initiated by reduction of the C-24 methylene group and subsequent desaturation at either C-5 or C-22, ending in further desaturation to obtain ergosterol (i15 → i19 → i20 (or i25) → i18). This mechanism is the preferred route in *Candida* sp. and zygomycetes (2, 26). 24-Methylcholest-7-en-3β-ol (i19) and 24-methylcholesta-5,7-dien-3β-ol (i20) were also reported in some fungi (25). All the melanized fungi except *A. alternata* contain these compounds (Table 1), showing the feasibility of this reaction sequence in these organisms. As mentioned above, 24-methylcholesta-7,22-dien-3β-ol (i25) was detected in all melanized fungi. In *Cladosporium* spp., *H. wernekii*, and *A. pullulans*, 24-methylcholesta-7,22-dien-3β-ol (i25) could have been produced by both the aforementioned and present pathways and its presence may reflect the activation of all these mechanisms. In the case of *A. alternata*, the lack of 24-methylcholest-7-en-3β-ol (i19) suggests that the presence of the $\Delta^{7,22}$ sterol was due to the aforementioned route, starting with initial C-22 desaturation and not C-24 methylene reduction.

Two other steroids could be evidenced in melanized fungi: 24-methylcholest-8-en-3β-ol (i23) in all of them except *A. alternata*, and 24-methylcholesta-8,22-dien-3β-ol (i24) in *Cladosporium* spp. and *A. pullulans* (Table 1). They show that reduction of the $\Delta^{24(28)}$ double bond may occur very early in the reaction sequence downstream from 24-methylcholesta-8,24(28)-dien-3β-ol (i6). Given the detected intermediates in melanized fungi and in *S. cerevisiae*, 24-methylcholest-8-en-3β-ol (i23) could have been produced either from C-24(28) reduction of 24-methylcholest-8,24(28)-dien-3β-ol (i6) or from C-4 demethylation of 4α,24-dimethylcholest-8-en-3β-ol (i14). The latter pathway will be discussed in the next section. The transformation of 24-methylcholest-8-en-3β-ol (i23) into ergosterol may operate through many possible combined isomerization and unsaturation reactions. However, the intermediates detected in melanized fungi and in *S. cerevisiae* suggest that among these possibilities, three sequences are possible: i23 → i24 → i25 → i18, and i23 → i19 → i20 (or i25) → i18 (Fig. 3). The multiple routes converging into these intermediates do not allow us to ascertain which pathways are active.

The transformation from 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol to ergosterol

Both melanized fungi and *S. cerevisiae* contained 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol (i9 in Figs. 1 and 2). In the reference yeast, no other C_{29} sterols were detected,

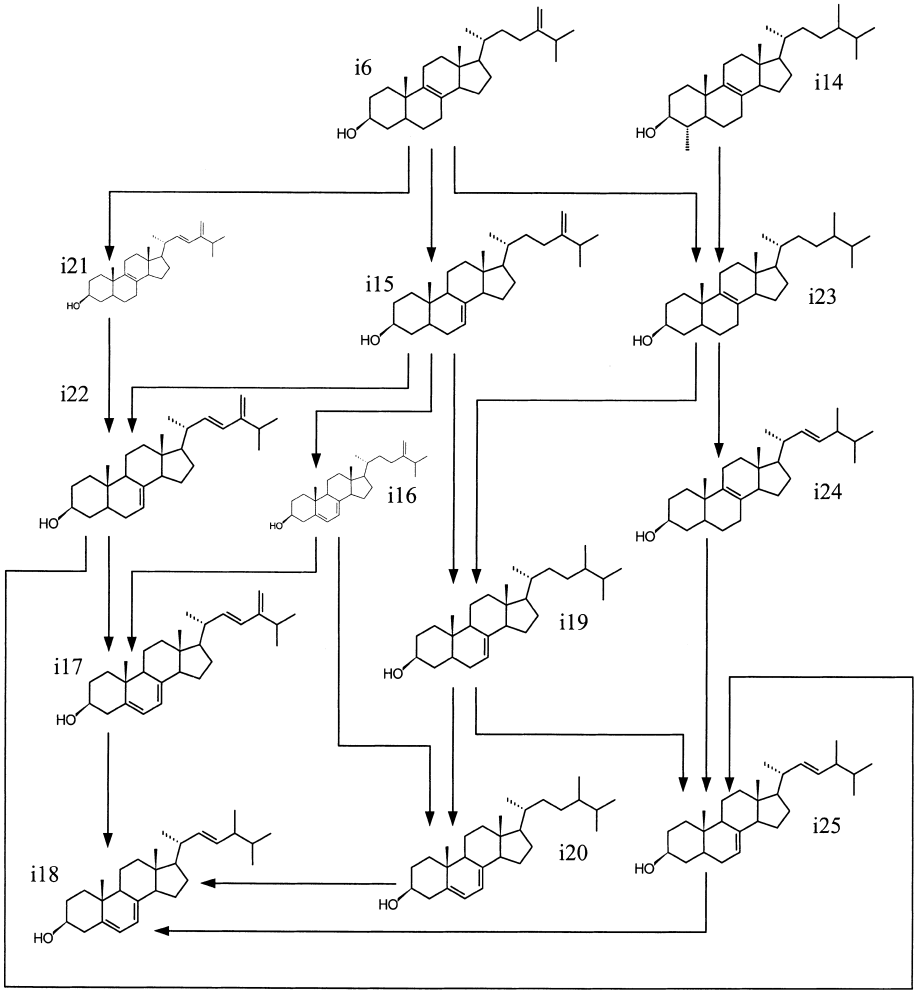

Fig. 3. Possible biosynthetic pathways from 24-methylcholesta-8,24(28)-dien-3β-ol and 4α,24-dimethylcholest-8-en-3β-ol to ergosterol in the studied melanized fungi and in *S. cerevisiae*. The intermediate i14 was not detected in *S. cerevisiae*. Sterols identified during the present study are shown in bold and in larger size.

suggesting that C-4 demethylation occurs prior to double-bond transformations. In contrast, melanized fungi contain other C_{29} sterols. 4α,24-Dimethylcholest-8-en-3β-ol (i14) was identified in all melanized fungi except *A. alternata* (Table 1), showing that ergosterol synthesis may proceed through 4α,24-dimethylcholest-8-en-3β-ol (i14), as well as through 24-methylcholesta-8,24(28)-dien-3β-ol (i6), in these organisms. According to the biosynthetic scheme of Fig. 2, the most immediate precursors of 4α,24-dimethylcholest-8-en-3β-ol (i14) could be 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol (i9) or 4α,4β,24-trimethylcholest-8-en-3β-ol (i13). Both alternatives involve the ability of the $\Delta^{24(28)}$ desaturase for the transformation of intermediates bearing at least one methyl substituent at C_4. A possible pathway involving 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol (i9) and 4α,24-dimethylcholest-8-en-3β-ol (i14) has been proposed in the literature (3): i1 → i9 → i14 → i23 → i24 → i25 → i18 (Figs. 2 and 3). Two intermediates of this pathway could be found: 24-methylcholest-8-en-3β-ol (i23) in all melanized fungi except *A. alternata*, and 24-methylcholesta-8,22-dien-3β-ol (i24) in *Cladosporium* spp. and *A. pullulans* (Table 1).

In addition to other C_{29} sterols, 4α,24-dimethylcholesta-7,24(28)-dien-3β-ol, 4α,24-dimethylcholesta-5,7-dien-3β-ol, and 4α,24-dimethylcholest-7-en-3β-ol were detected in trace amounts in all melanized fungi (the latter not in *A. alternata*; Table 1). These sterols are not intermediates of the pathways documented in the literature (Figs. 1–3); their presence evidences that double-bond transformations occur prior to C-4 demethylation. Therefore, isomerization of Δ^8 to Δ^7 may have converted 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol (i9) into 4α,24-dimethylcholesta-7, 24(28)-dien-3β-ol. Double-bond isomerization in the steroid nucleus usually follows the sequence $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$ (3). Accordingly, 4α,24-dimethylcholesta-5,7-dien-3β-ol could also have been produced from this sequence of reactions with 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol (i9) being the initial substrate. 4α-Methylsterols with double bonds in position Δ^5 and Δ^7 have been principally evidenced in algae from the class of Dinophyceae.

In melanized fungi, several postlanosterol biosynthetic pathways converge into ergosterol; their relative importance still needs to be determined. The possibility of growing melanized fungi in culture provides a good alternative

for using them as novel model organisms for genetic or enzymologic characterization of ergosterol biosynthesis, especially in the sequences that differ from those in the well-studied *S. cerevisiae*. The influence of salt stress on sterol biosynthesis and regulation (28, 29) is another important issue whose study can be undertaken with these organisms.

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REFERENCES

1. Weete, J. D. 1980. Lipid Biochemistry of Fungi and Other Organisms. Plenum Press, New York.
2. Weete, J. D. 1989. Fungal sterols. *Adv. Lipid Res.* **23:** 115–167.
3. Mercer, E. I. 1984. The biosynthesis of ergosterol. *Pestic. Sci.* **15:** 133–155.
4. Parks, L. W. 1978. Metabolism of sterols in yeast. *CRC Crit. Rev. Microbiol.* **6:** 301–341.
5. Nishino, T., S. Hata, T. Osumi, and H. Katsuki. 1980. Biosynthesis of ergosterol in cell-free system of yeast. *J. Biochem.* **88:** 247–254.
6. Yabusaki, Y., T. Nishino, N. Ariga, and H. Katsuki. 1979. Studies on Δ^8-Δ^7 isomerization and methyl transfer of sterols in ergosterol biosynthesis of yeast. *J. Biochem.* **85:** 1531–1537.
7. Gunde-Cimerman, N., P. Zalar, G. S. de Hoog, A. Plemenitas, and D. A. Caron. 2000. Hypersaline waters in salterns—natural ecological niche for halophilic black yeasts. *FEMS Microbiol. Ecol.* **32:** 235–240.
8. Zalar, P., G. S. de Hoog, and N. Gunde-Cimerman. 1999. Taxonomy of the endoconidial black yeast genera *Phaeotheca* and *Hyphospora*. *Stud. Mycol.* **43:** 49–56.
9. Zalar, P., G. S. de Hoog, and N. Gunde-Cimerman. 1999. *Trimmatostroma salinum*, a new species from hypersaline water. *Stud. Mycol.* **43:** 57–62.
10. Zalar, P., G. S. de Hoog, and N. Gunde-Cimerman. 1999. Ecology of halotolerant dothideaceous black yeasts. *Stud. Mycol.* **43:** 41–52.
11. Villanueva, J., J. O. Grimalt, R. de Wit, B. J. Keely, and J. R. Maxwell. 1994. Chlorophyll and carotenoid pigments in solar saltern microbial mats. *Geochim. Cosmochim. Acta.* **58:** 4703–4715.
12. de Wit, R., and J. O. Grimalt. 1992. Microbial ecosystems in Spanish coastal salinas: an ecological and geochemical study of biomarkers. *Limnetica.* **8:** 205–212.
13. Grimalt, J. O., R. de Wit, P. Teixidor, and J. Albaiges. 1992. Lipid biogeochemistry of *Phormidium* and *Microcoleus* mats. *Org. Geochem.* **19:** 509–530.
14. Hartgers, W. A., J. F. Lopez, J. S. Sinninghe Damsté, C. Reiss, J. R. Maxwell, and J. O. Grimalt. 1997. Sulphur-binding in recent environments: II. Speciation of sulfur and iron and implications for the occurrence of organo-sulfur compounds. *Geochim. Cosmochim. Acta.* **61:** 4769–4788.
15. Chambon, C., V. Ladeveze, M. Servouse, L. Blanchard, C. Javelot, B. Vladescu, and F. Karst. 1991. Sterol pathway in yeast. Identification and properties of mutant strains defective in mevalonate diphosphate decarboxylase and farnesyl diphosphate synthetase. *Lipids.* **26:** 633–636.
16. Parks, L. W., and W. M. Casey. 1995. Physiological implications of sterol biosynthesis in yeast. *Annu. Rev. Microbiol.* **45:** 95–116.
17. Osumi, T., S. Taketani, H. Katsuki, T. Kuhara, and I. Matsumoto. 1978. Ergosterol biosynthesis in yeast. *J. Biochem.* **83:** 681–691.
18. Fryberg, M., A. C. Oehlschlager, and A. M. Unrau. 1972. Biosynthetic routes to ergosterol in yeast. *Biochem. Biophys. Res. Commun.* **48:** 593–597.
19. Rattray, J. B. M., A. Schibeci, and D. K. Kidby. 1975. Lipids of yeasts. *Bacteriol. Rev.* **39:** 197–231.
20. McCammon, M. T., M-A. Hartmann, C. D. K. Bottema, and L. W. Parks. 1984. Sterol methylation in *Saccharomyces cerevisiae*. *J. Bacteriol.* **157:** 475–483.
21. Weete, J. D., and S. R. Gandhi. 1996. Biochemistry and molecular biology of fungal sterols. *In* The Mycota III. Biochemistry and Molecular Biology. R. Brambl and G. A. Marzluf, editors. Springer Verlag, Berlin Heildelberg. 421–437.
22. Parks, L. W., C. Anding, and G. Ourisson. 1974. Sterol transmethylation during aerobic adaptation in yeast. *Eur. J. Biochem.* **43:** 451–458.
23. Goulston, G., L. J. Goad, and T. W. Goodwin. 1967. Sterol biosynthesis in fungi. *Biochem. J.* **102:** 15c–17c.
24. Goosey, M. W., and D. J. Moore. 1991. Molecular aspects of fungal sterol biosynthesis and inhibition. *Biochem. Soc. Trans.* **19:** 769–774.
25. Herber, R., J. Villoutreix, P. Granger, and S. Chapelle. 1983. Influence de l'anaérobiose sur la composition en stérols de *Mucor hiemalis*. *Can. J. Microbiol.* **29:** 606–611.
26. Weete, J. D., and S. R. Gandhi. 1997. Sterols of the phylum Zygomycota: Phylogenetic implications. *Lipids.* **32:** 1309–1316.
27. Nes, W. D., and P. H. Le. 1990. Evidence for separate intermediates in the biosynthesis of 4β-methylsterol end products by *Gibberella fujikuroi*. *Biochim. Biophys. Acta.* **1042:** 119–125.
28. Petrovic, U., N. Gunde-Cimerman, and A. Plemenitas. 1999. Salt stress affects sterol biosynthesis in the halophilic black yeast *Hortaea werneckii*. *FEMS Microbiol. Lett.* **180:** 325–330.
29. Turk, M., L. Méjanelle, J. O. Grimalt, N. Gunde-Cimerman, and A. Plemenitas. 1999. Comparison of the sterol portion and composition in halophilic yeasts grown on different NaCl concentrations. 3rd Meeting of the Slovenian Biochemical Society with International Participation in Portoroz, Slovenia. 1999.