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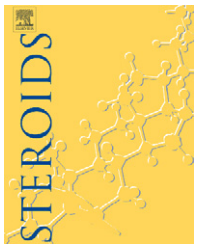

# Ergosterol biosynthesis pathway in *Aspergillus fumigatus*

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ABSTRACT

The sterol composition of *Aspergillus fumigatus* for the biosynthesis of ergosterol is of interest since this pathway is the target for many antifungal drugs in clinical use. The sterol composition of this fungal species was analyzed by gas chromatography–mass spectrometry in different strains (susceptible and resistant to azole drugs). Also, sterols were analyzed in several *A. fumigatus* mutant strains deficient in enzymatic steps of the ergosterol biosynthesis pathway such as 14-α sterol demethylases (Cyp51A and Cyp51B) and C-5 sterol desaturases (Erg3A, Erg3B and Erg3C). All sterols identified from azole-resistant *A. fumigatus* strains were qualitatively and quantitatively similar to the susceptible strain (CM-237). However, sterol composition of mutants strains were different depending on the lacking enzyme. The analysis of the sterol composition in these mutant strains led to a better understanding of the ergosterol biosynthesis pathway in this important fungus.



## 1. Introduction

*Aspergillus fumigatus* has become the most prevalent airborne fungal pathogen in developed countries, causing allergic diseases, fungal balls and fatal invasive aspergillosis (IA) [1]. Moreover, the incidence of aspergillosis has increased in recent years, due primarily to an upsurge in immunocompromized population, particularly those with acute leukemia and bone marrow or solid organ transplants [2]. Almost all antifungal agents currently in use in human mycoses and agricultural application use the ergosterol biosynthesis pathway as a target [3,4]. The majority of *A. fumigatus* isolates are susceptible in vitro to triazole drugs such as itraconazole and voriconazole [1,5]. However, resistant strains have already been well documented and recently, an increasing number of strains with cross-resistance to azoles have been reported [6–11].

Ergosterol, one of the most important components in fungal membranes, is involved in numerous biological func-

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tions, such as, membrane fluidity regulation, activity and distribution of integral proteins and control of the cellular cycle [12]. These facts make ergosterol and its biosynthetic pathway essential for fungal growth. The knowledge of the ergosterol biosynthesis route in *A. fumigatus*, may be useful in the design of new antifungal drugs and it could help in the study of antifungal drug resistance mechanisms.

The ergosterol biosynthesis pathway is a complex route in which about 20 enzymes are involved [13]. This route has been well studied and it is considered as the classical route in *Saccharomyces cerevisiae* [14]. Furthermore, complex networks involving different or alternative combinations in several steps of the classical ergosterol synthesis pathway have been studied in some other fungi and in plants [14–16].

In the ergosterol pathway, steps prior to squalene formation are important for pathway regulation and early intermediates are metabolized to produce other essential cellular components [14]. The functional deletion of the genes from squalene to lanosterol is essential in *S. cerevisiae*, since it completely prevents ergosterol production [17]. However, reaction sequences for ergosterol biosynthesis downstream in the pathway from lanosterol are specific to fungal taxa. For instance, in *S. cerevisiae* multiple routes have been reported for the sequence of steps leading from lanosterol to ergosterol depending on growth conditions [18].

In *A. fumigatus* very little is known about the genetics and biochemistry of this pathway. Previous studies have demonstrated the existence of multiple genes encoding some of the key enzymes. For example, two different 14-α sterol demethylases (Cyp51A and Cyp51B) [19] and three C-5 sterol desaturases (Erg3p) [13,20] have been identified. Therefore, the biosynthesis of ergosterol in *A. fumigatus* according to the classical *S. cerevisiae* route need to be reconsidered on the basis of the existence of multiple enzymes for the same reaction step.

In this work we describe the analysis of sterol intermediates participating in the ergosterol biosynthesis pathway in *A. fumigatus* strains susceptible and resistant to azole drugs. Moreover, the analysis of *A. fumigatus* knock-out strains defective in genes encoding enzymes acting in the ergosterol biosynthesis pathway have provided a very valuable information to deduce the sequence of reactions that conclude in the biosynthesis of ergosterol in this fungal species.

## 2. Experimental

### 2.1. Strains of A. fumigatus and culture media

The *A. fumigatus* strains used in the study were: (i) the azole susceptible CM-237 strain (CM refers to the fungal collection of the Mycology Reference Laboratory at the Centro Nacional de Microbiologia) [19] was used as reference strain for total ergosterol quantifications and the sterol composition analysis. (ii) Azole drugs resistant strains with different resistance mechanisms (CM-2159, CM-3269, CM-0796) [8,9] and (iii) ergosterol pathway enzyme defective strains: CM-A8 (*cyp51A*$^{-}$), CM-B7 (*cyp51B*$^{-}$), CM-A80 (*erg3A*$^{-}$), CM-B866 (*erg3B*$^{-}$) and CM-C65 (*erg3C*$^{-}$) [19–21].

The fungi were grown at 37 °C in potato dextrose agar (Oxoid, Madrid, Spain) and liquid minimal medium (MM) [22]. Conidia stocks were preserved in sterile distilled water at 4 °C [8].

### 2.2. Total ergosterol extraction

Total ergosterol of *A. fumigatus* strains was extracted after 18 h of growth in liquid MM using the protocol described by Arthington-Skaggs [23]. Basically, mycelia were harvested by filtration and washed with sterile water, and then dried and weighted (fungal dry weight). A volume of 3 ml of 25% alcoholic potassium hydroxide solution (3:2 methanol:ethanol) was then added to dried mycelia and mixed by vortex for 1 min. The mixture was incubated in an 85 °C water bath for 1 h. Sterols were extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of pentane followed by vigorous vortex mixing for 3 min. The upper pentane layer was transferred to a clean glass tube and evaporated in a fume hood at room temperature.

The dried-down samples were re-dissolved again in 1 ml of methanol and filtered through 0.45-μm-pore-size polytetrafluoroethylene membranes (Acrodisc, Waters). Ergosterol content was analyzed using the system LC Module I HPLC Waters, including a W600 pump, a pneumatic W715 injection-valve, a μ-Bondapack C18 column (300 mm × 3.9 mm, 10 μm). A flow rate of methanol eluent (100% HPLC grade) of 1 ml min$^{-1}$ was established. Peak areas/heights recorded at a wavelength (282 nm) in a Millenium$^{32}$ photodiode array detector (PDA) (Waters Corporation) were used for quantification. The Millenium$^{32}$ software (Waters Corporation) was used for this purpose. Total ergosterol concentration was expressed as micrograms of ergosterol by milligrams of fungal dry weight.

Each experiment was repeated at least three times. The results of the repeated experiments were expressed as mean ± standard deviation. The significance of total ergosterol content was determined by Student's *t*-test (unpaired, unequal variance). A $p$-value <0.01 was considered significant. Statistical analysis was done with the SPSS Package (version 14.0, SPSS S.L., Madrid, Spain).

### 2.3. Sterol extraction

Aliquots of about 200 mg of fungal mats were essentially processed basically as previously reported [20,24]. However, neutral lipids were extracted twice with 1.5 ml hexane. Sterols were converted into their trimethylsilyl ethers by reaction with 1:1 solution of *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (85 °C, 60 min) in toluene and stored at −20 °C until analyzed.

### 2.4. Instrumental analysis

Neutral lipid fractions were analyzed by GC-MS with a Trace GC gas chromatograph coupled to a quadrupole mass analyzer Trace MS (Thermo, Manchester, UK). GC program and MS operation conditions were the same as described previously [20,24].

Sterol identification was based on mass spectral interpretation and comparison of mass spectra and retention time data with available standards.

## 3. Results

### 3.1. Total ergosterol content

The total ergosterol content of the wild type strain CM-237 and some mutants lacking single enzymes in the ergosterol pathway was previously analyzed in a recent study [20]. The results reported that, total ergosterol was $5.99 \pm 0.86\,\mu g\,mg^{-1}$ for wild type (CM-237), $6.58 \pm 0.29\,\mu g\,mg^{-1}$ for CM-A80 (*erg3A*$^-$) and $2.02 \pm 0.74\,\mu g\,mg^{-1}$ for CM-B866 (*erg3B*$^-$). In the present study three new single mutant strains were analyzed: CM-A8 (*cyp51A*$^-$), CM-B7 (*cyp51B*$^-$) and CM-C65 (*erg3C*$^-$) that gave $5.98 \pm 0.82\,\mu g\,mg^{-1}$, $4.25 \pm 0.64\,\mu g\,mg^{-1}$ and $5.64 \pm 2.02\,\mu g\,mg^{-1}$ of ergosterol, respectively.

The mutants CM-B866 (*erg3B*$^-$) and CM-B7 (*cyp51B*$^-$) showed a statistically significant lower amount of total ergosterol compared to their parental strain CM-237.

The HPLC results suggested that ergosterol precursors could have been accumulated as a result of the gene deletion, consequently, the wild type strain CM-237 and the mutant strains were subjected to GC–MS.

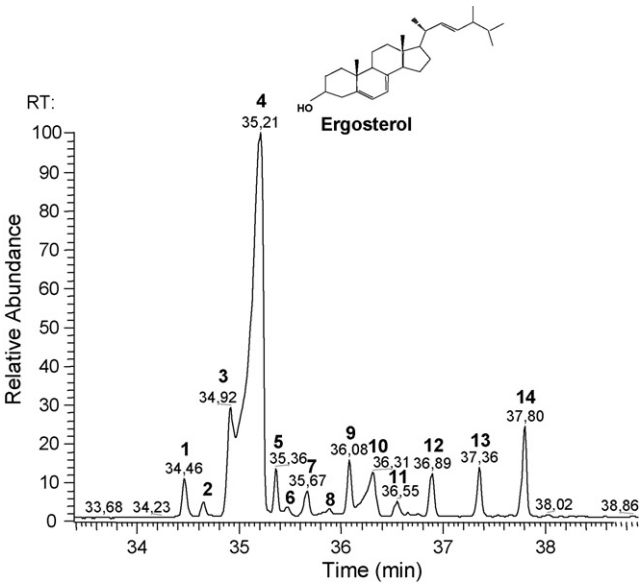


**Fig. 1 – Chromatogram of sterols from CM-237 strain. Numbers refer to peaks identification of sterols described in Tables 1 and 2.**

### 3.2. Ergosterol precursors of the A. fumigatus wild type strain CM-237

First, we used strain CM-237 as the type strain for the identification of the sterols involved in the biosynthesis of ergosterol in *A. fumigatus*. The GC–MS analysis of the neutral lipid fraction obtained from this strain yielded mainly squalene and sterols which were identified by comparison with known sterol spectra. Tables 1 and 2 show the relative sterol composition of the CM-237 strain in two different experiments. Each value represents the relative contribution of the GC–MS area of each compound to the sum of the total sterols quantified.

Fourteen different sterols where identified and their structures are listed in retention time order (Fig. 1): (**1**) 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol, (**2**) 24-methylcholesta-5,8,22-trien-3β-ol, (**3**) 24-methylcholesta-5,7,9,22-tetraen-3β-ol, (**4**) 24-methylcholesta-5,7,22-trien-3β-ol, (**5**) 24-methylcholesta-7,22-dien-3β-ol, (**6**) 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol, (**7**) 24-methylcholesta-7,22,24(28)-trien-3β-ol, (**8**) 24-methylcholesta-5,7,24(28)-trien-3β-ol, (**9**) 24-methylcholesta-7,24(28)-dien-3β-ol, (**10**) 24-ethylcholest-5,7,22-trien-3β-ol, (**11**) 4,4,14-trimethylcholesta-8,24-dien-3β-ol, (**12**) 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol, (**13**) 4,4,14,24-tetramethylcholesta-8,24(28)-dien-3β-ol and (**14**) 4,4,24-trimethylcholesta-8,24(28)-dien-3β-ol.

In the wild type strain CM-237, ergosterol content was 75.81% (geometric mean in Tables 1 and 2). The minor constituent sterols found were three tetraunsaturated sterols 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol (**1**), 24-methylcholesta-5,7,9,22-tetraen-3β-ol (**3**) and 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol (**6**) with abundances from 0.46% to 1.42%. Neoergosterol (**3**) and sterol (**1**) have been reported as final products biosynthetic routes in other fungi [18]. However, the presence of the former (**3**) is thought to arise as a consequence of ergosterol degradation. In this report, its relative abundance has been found to depend on laboratory handling, storage or GC injection conditions. They increased in sample vials re-analyzed after several days or weeks. Ergosterol is photochemically transformed into ergocalciferol [25] however, our GC–MS system cannot identify the presence of ergocalciferol to check for photochemical degradation. In a similar way, it is thought that reaction of the 5,7 conjugated diene system of some sterols, either marine or fungal, with radical singlet-oxygen ($O_2$) will produce an unstable cyclic 1,2-dioxane intermediate (forming a dioxygen bridge over sterol ring A) which after decomposition and methyl elimination reactions will give way to the various tetraunsaturated compounds that are found in GC–MS chromatograms, like sterol (**1**) (and other reported in the literature) as well as neoergosterol which involved the elimination of methyl 18 and full aromatization of ring B [26].

The only tetraunsaturated steroids known to be synthesized by common enzymatic reactions are sterol (**6**) and its structural analogues [14,15,24].

Three 24-methylcholestatrien-3β-ol isomers were identified: 24-methylcholesta-5,8,22-trien-3β-ol (**2**), 24-methylcholesta-7,22,24(28)-trien-3β-ol (**7**) and 24-methylcholesta-5,7,24(28)-trien-3β-ol (**8**) representing a total percentage of 3.5%. Two 24-methylcholestadien-3β-ols, 24-methylcholesta-7,22-dien-3β-ol (**5**) and 24-methylcholesta-7,24(28)-dien-3β-ol (**9**) representing 2.4% and 1.9%, respectively. Moreover, one sterol with an alkyl group at C-24: 24-ethylcholest-5,7,22-trien-3β-ol (**10**) [27] constituting 6.1% of total sterol content.

**Table 1 – Relative composition (%) of sterols of the *A. fumigatus* wild type strain (CM-237) and of the azole drugs resistant strains**

| No.[a] | Compound | | Strains | | | |
|---|---|---|---|---|---|---|
| | Systematic name | Common name | CM-237[b] | CM-0796 | CM-3269 | CM-2159 |
| 1 | 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol | | 1.08 | 0.85 | 1.18 | 0.92 |
| 2 | 24-methylcholesta-5,8,22-trien-3β-ol | | 0.90 | 0.90 | 0.80 | 0.85 |
| 3 | 24-methylcholesta-5,7,9,22-tetraen-3β-ol | Neoergosterol | 1.42 | *ND* | *ND* | *ND* |
| 4 | 24-methylcholesta-5,7,22-trien-3ß-ol | Ergosterol | 75.81 | 84.08 | 83.70 | 88.44 |
| 5 | 24-methylcholesta-7,22-dien-3β-ol | | 2.37 | 1.51 | 1.67 | 1.09 |
| 6 | 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol | | 0.46 | 0.32 | 0.21 | 0.28 |
| 7 | 24-methylcholesta-7,22,24(28)-trien-3β-ol | | 0.53 | 0.82 | 0.51 | 0.20 |
| 8 | 24-methylcholesta-5,7,24(28)-trien-3β-ol | | 1.20 | 0.08 | 0.19 | 0.16 |
| 9 | 24-methylcholesta-7,24(28)-dien-3β-ol | Episterol | 1.86 | 3.59 | 1.84 | 1.08 |
| 10 | 24-Ethylcholesta-5,7,22-trien-3β-ol | | 6.07 | 2.43 | 6.46 | 5.10 |
| 11 | 4α,4β,14-trimethylcholesta-8,24-dien-3β-ol | Lanosterol | 1.63 | 3.63 | 0.29 | 0.18 |
| 12 | 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol | | 1.28 | 0.73 | 1.30 | 0.82 |
| 13 | 4α,4β,14,24-tetramethylcholesta-8,24(28)-dien-3β-ol | Eburicol | 1.93 | 0.41 | 0.62 | 0.23 |
| 14 | 4α,4β,24-trimethylcholesta-8,24(28)-dien-3β-ol | | 3.42 | 0.65 | 1.22 | 0.65 |

ND, not detected.

[a] Compound number.

[b] Geometric means from two different experiments.

Finally, the other major sterol group was constituted by di-tri-tetra-methylcholesta-8,24-dien-3β-ol compounds: 4,4,14-trimethylcholesta-8,24-dien-3β-ol (**11**), 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol (**12**), 4,4,14,24-tetramethylcholesta-8,24(28)-dien-3β-ol (**13**) and 4,4,24-trimethylcholesta-8,24(28)-dien-3β-ol (**14**). All of them are derived from lanosterol (**11**), followed by eburicol (**13**) and sequentially demethylated at C-14 and C-4. These compounds represented up to 8.3% of total sterols.

Lanosterol and eburicol were detected in all studied strains of *A. fumigatus* constituting the only sterols with more than 28 carbon atoms.

### 3.3. *Sterols in A. fumigatus itraconazole-resistant strains (CM-2159, CM-3269, CM-0796)*

Three *A. fumigatus* resistant strains with high MICs to azole drugs were chosen to compare their sterol composition with those of the CM-237 azole susceptible strain. The sterol identification was done by comparison with known sterols and with the sterol profile of the CM-237 wild type strain. In all strains the observed sterols encompassed a mixture of 24-methylcholesta-di-tri-tetraen-3β-ols and di-tri-tetramethylcolesta-8,24-dien-3β-ols. Their structures and relative composition are listed in Table 1. The identified sterols

**Table 2 – Relative composition (%) of sterols of *A. fumigatus* wild type strain (CM-237) and mutant strains**

| No.[a] | Compound | | Strains | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Systematic name | Common name | CM-237[b] | CM-A8 | CM-B7 | CM-A80 | CM-B866 | CM-C65 |
| 1 | 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol | | 1.08 | 3.69 | 2.74 | 3.27 | 1.20 | 1.16 |
| 2 | 24-methylcholesta-5,8,22-trien-3β-ol | | 0.90 | 0.87 | 0.77 | 0.84 | 0.06 | 0.79 |
| 3 | 24-methylcholesta-5,7,9,22-tetraen-3β-ol | Neoergosterol | 1.42 | 5.69 | 4.57 | 5.39 | 4.67 | *ND* |
| 4 | 24-methylcholesta-5,7,22-trien-3ß-ol | Ergosterol | 75.81 | 59.48 | 48.82 | 63.30 | 11.85 | 74.78 |
| 5 | 24-methylcholesta-7,22-dien-3β-ol | | 2.37 | 1.84 | 1.18 | 1.08 | 39.14 | 7.29 |
| 6 | 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol | | 0.46 | 0.41 | 0.25 | 0.48 | *ND* | 0.29 |
| 7 | 24-methylcholesta-7,22,24(28)-trien-3β-ol | | 0.53 | 1.44 | 0.71 | 0.88 | 19.76 | 1.76 |
| 8 | 24-methylcholesta-5,7,24(28)-trien-3β-ol | | 1.20 | 0.14 | 0.34 | 1.14 | *ND* | 0.21 |
| 9 | 24-methylcholesta-7,24(28)-dien-3β-ol | Episterol | 1.86 | 2.55 | 2.01 | 3.07 | 11.66 | 3.35 |
| 10 | 24-Ethylcholesta-5,7,22-trien-3β-ol | | 6.07 | 5.48 | 9.26 | 4.93 | 1.48 | 7.91 |
| 11 | 4α,4β,14-trimethylcholesta-8,24-dien-3β-ol | Lanosterol | 1.63 | 1.39 | 2.87 | 2.43 | 2.06 | 0.45 |
| 12 | 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol | | 1.28 | 2.03 | 0.98 | 2.57 | 1.59 | 0.74 |
| 13 | 4α,4β,14,24-tetramethylcholesta-8,24(28)-dien-3β-ol | Eburicol | 1.93 | 9.54 | 21.95 | 3.69 | 2.32 | 0.41 |
| 14 | 4α,4β,24-trimethylcholesta-8,24(28)-dien-3β-ol | | 3.42 | 5.44 | 3.56 | 6.90 | 4.20 | 0.86 |

ND, not detected.

[a] Compound number.

[b] Geometric means from two different experiments.

were similar to those describe, for the CM-237 strain being ergosterol the main sterol with values ranging between 84% and 88% of total sterols depending on the strain.

### 3.4. *Sterols derivates in A. fumigatus enzyme defective strains*

Subsequently, the ergosterol biosynthesis pathway was analyzed in several *A. fumigatus* strains defective in different enzymes participating in the pathway. That is, CM-A8 (*cyp51A*$^{-}$), CM-B7 (*cyp51B*$^{-}$), CM-A80 (*erg3A*$^{-}$), CM-B866 (*erg3B*$^{-}$) and CM-C65 (*erg3C*$^{-}$) [19,20].

The qualitative sterol composition was in the same as that of the CM-237 strain although significant differences between some of them could be observed (Table 2).

Strains CM-A8 and CM-B7, blocked at Cyp51 level, have an increase of eburicol (**13**) 9.5% and 22%, respectively, which would correspond to the Cyp51 enzyme substrate. The eburicol increase in the CM-B7 strain was joined to a marked drop in ergosterol (as it was previously detected by HPLC).

The analysis of the mutant strains blocked at the C-5 sterol desaturases (Erg3) level, CM-A80, CM-B866 and CM-C65, provide further information on the ergosterol biosynthesis pathways. Ergosterol was the major sterol metabolite in both, the CM-A80 and the CM-C65 strains 63% and 75%, respectively. However, strain CM-B866 showed a remarkable increase in three sterol fractions: (**5**), (**7**) and (**9**) (39%, 20% and 12%), accumulation of these C-5 saturated sterols in the CM-B866 strain was coupled with a decrease of total ergosterol (11%).

## 4. Discussion

Sterols are isoprenoid-derived molecules that have essential functions in eukaryotes in general. Ergosterol is, by far, the major sterol component in fungal membranes and therefore, thought to be necessary for fungal development and survival [28,29]. For that reason, enzymes involved in the ergosterol pathway might be attractive targets for antifungal agents. One of the antifungal targets is the 14-α sterol demethylase (Cyp51) whose activity is inhibited by azole drugs causing the alteration of ergosterol biosynthesis in yeasts [12]. Resistance to azole agents in *A. fumigatus*, has been related to alterations in the 14-α sterol demethylase [8,11,21,30]. In addition, other resistance mechanisms related to modification of other enzymes involved in ergosterol biosynthesis have been described in yeasts. One such enzyme is the C-5 sterol desaturase encoded by *Erg3* [20,31,32].

In the present work we have identified fourteen different ergosterol precursors in *A. fumigatus* (Tables 1 and 2). Three azole-resistant strains with different azole resistance mechanisms [8,21,30] were selected for comparison of the sterol composition between azole susceptible (S) and azole-resistant (R) strains. However, no significant differences in sterol composition were found between the azole-S strain and the azole-R strains (Table 1). This result is in agreement with previous work [33] and it suggests that in *A. fumigatus* ITC resistance due to single amino acid substitutions in the azole target affects the azole drug affinity, but not the enzyme activity [31]. Therefore, the sterol composition in the biosynthesis pathway remains unaltered.

The biosynthesis of ergosterol in fungi has been reported to proceed through various routes depending on species and strains [14,18]. In order to assess the possible ergosterol biosynthesis pathways in *A. fumigatus* we compared the sterol composition of one strain CM-237 with various laboratory-engineered enzyme defective mutant strains in various key points along the ergosterol biosynthetic pathway. Two 14-α sterol demethylases defective strains (CM-A8 and CM-B7) and the three C-5 sterol desaturases strains with inactive Erg3s (CM-A80, CM-B866 and CM-C65) were used. The different ergosterol amount for some of the mutant strains detected by HPLC reflected a variable sterol composition as a consequence of the gene deletions.

The biosynthetic routes to ergosterol in *A. fumigatus* can be evaluated following two sections according to the position of the enzymes in the synthetic route.

### 4.1. *Transformation from lanosterol to 24-methylcholesta-8,24(28)-dien-3β-ol*

Lanosterol (**11**), the first sterol formed after squalene oxide cyclization (Fig. 2), represents a branching point of the pathway. One branch leads towards zymosterol by sequential demethylation at C-14, C-4α and C-4β. The other starts with a methylation at C-24 producing eburicol (**13**) and continues with a sequence of demethylations at C-14 and both C-4 (two methyls) like those described above. Both sub-pathways converge at 24-methylcholesta-8,24(28)-dien-3β-ol (fecosterol) level (Fig. 2).

The choice of each branch of the sequence has been found to be specie dependent on both yeasts and moulds [14,16,34]. For example, in the most studied fungus, the ascomycete *S. cerevisiae*, the first of the two branches described above is observed (Fig. 2) [14,16] while in *Schizosaccharomyces pombe*, both routes have been described to occur [35].

Although it cannot be fully ascertained, in *A. fumigatus*, the lack of detection of any of the intermediates of the classical branch, through zymosterol, and the presence of all the intermediates sterols (**13**), (**14**) and (**12**) strongly suggests that the biosynthesis to fecosterol proceeds along eburicol (**13**) (Fig. 2B). Although it is not shown in the tables, trace amounts of fecosterol were detected in some of the strains.

Additionally, when the 14-α sterol demethylase enzymes are lacking (Cyp51A or Cyp51B) the sterol accumulates its substrate eburicol (**13**) (Table 2). This fact would indicate that in *A. fumigatus*, lanosterol (**11**) is methylated in the C-24 to give a sterol of 31 carbons, eburicol, on which the 14-α sterol demethylase would act and the derived product would be demethylated in the C-4 afterwards (black arrow, Fig. 2B).

Accordingly, the initial steps of ergosterol synthesis in *A. fumigatus* are biochemically different from those of *S. cerevisiae* that could explain the differences in the performance to antifungals among these fungi.

### 4.2. *Transformation from fecosterol to ergosterol*

The second part of ergosterol biosynthesis pathway (Fig. 3) consists of double-bond rearrangements in the steroid

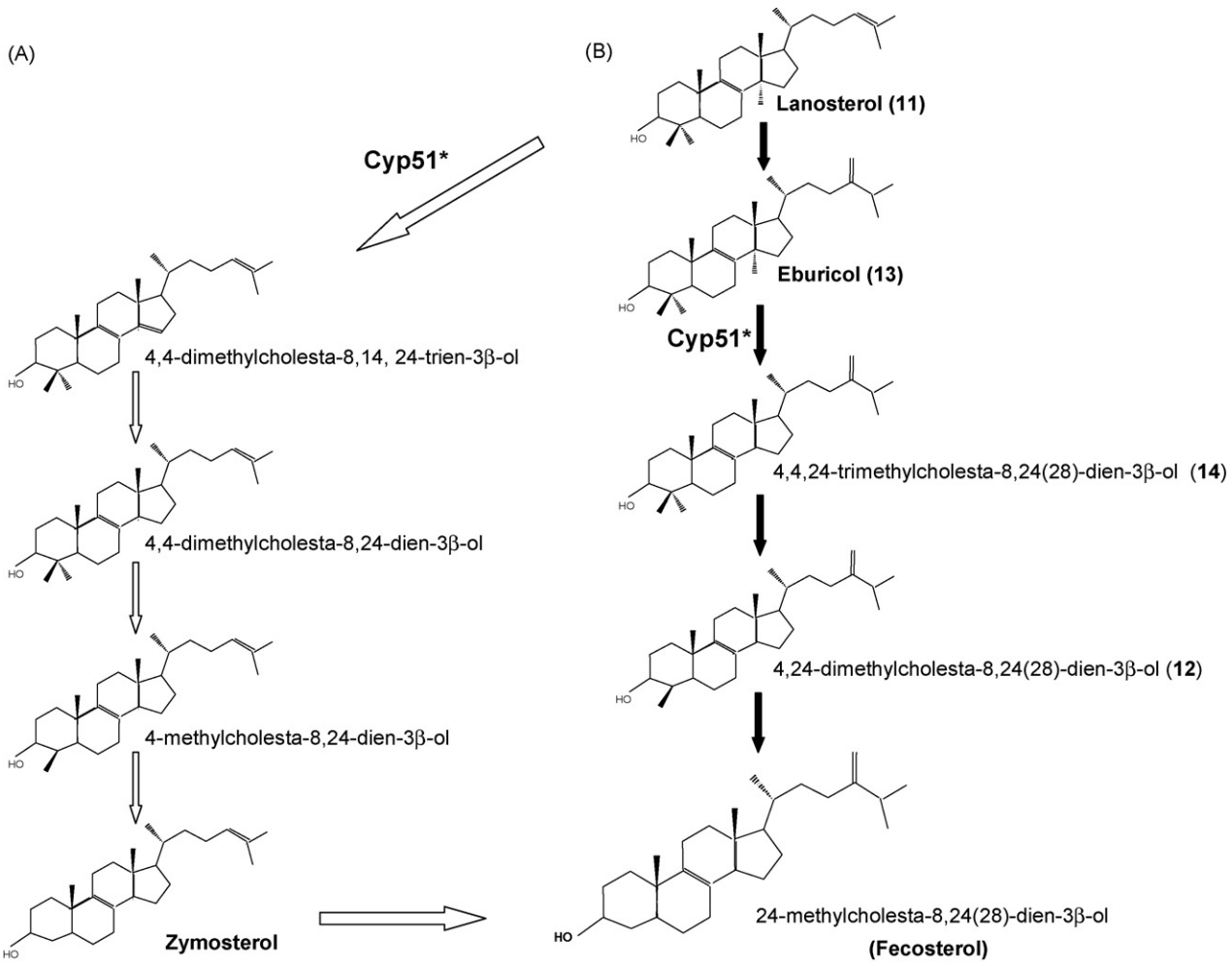


**Fig. 2 – Alternative pathways from lanosterol to fecosterol. (A) The described pathway for *Saccharomyces cerevisiae* (white arrows) [14]. (B) The proposed pathway for *Aspergillus fumigatus* (black arrows).*14-α sterol demethylase (Cyp51) enzyme. Numbers refer to sterols in Tables 1 and 2.**

nucleus and in the side chain, isomerization of the double connection in the C-8 to the C-7 followed by the desaturation of the C-5 and C-22, and the reduction of the C-24.

An important finding in *A. fumigatus* is the identification of 24-ethylsterols (**10**) that represents the only addition reaction found downstream from fecosterol. The C-24 alkylation is catalyzed by S-adenosylmethionine-sterol-C-methyltransferases (SAMs). In *S. cerevisiae* SAM converts zymosterol into fecosterol, but few reports exist on the detection of 24-ethyl sterols [27] in fungi. In higher plants different methyltransferases are involved in the two consecutive methylation reactions leading to 24-ethyl sterols (methylation at C-24 and at C-28) [36]. According to the present results, in *A. fumigatus* one, or perhaps two, SAM would transform lanosterol (**11**) to eburicol (**13**) and after fecosterol–episterol transformation, another SAM would continue the process towards sterol (**10**).

Fecosterol is converted to episterol (**9**) by the C-8 sterol isomerase [37]. This reaction is the unique reversible reaction of the ergosterol biosynthesis pathway. Kinetic studies have demonstrated that equilibrium for double-bond isomerization is shifted to the forward reaction [38]. This could explain why fecosterol has been found in very low concentrations or even absent in *A. fumigatus* as in other fungi [39].

The presence of low amounts of 24-methylcholesta-5,8,22-trien-3β-ol (**2**) in the present dataset cannot be explained, its seems to derive from fecosterol by desaturation at C-5. This compound has been seldom reported in fungal analysis [18,40].

Once episterol is synthesized, transformation from episterol to ergosterol may occur through diverse alternative routes (Fig. 3) and some of them may even coexist in the same organism [16,24,28,41,42].

In *A. fumigatus*, three possible sequences of reactions from episterol to ergosterol could be possible. The three pathways are shown in Fig. 3 marked with white, gray and solid arrows. In the first route (solid) episterol (**9**) is desaturated at C-22 to produce 24-methylcholesta-7,22,24(28)-trien-3β-ol (**7**). At this point two synthesis may be generated, one involves hydrogenation of the methylene at C-24 and leads to 24-methylcholesta-7,22-dien-3β-ol (**5**) and through desaturation at C-5 into ergosterol, the other proceeds through desaturation at C-5 to 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol (**6**) and

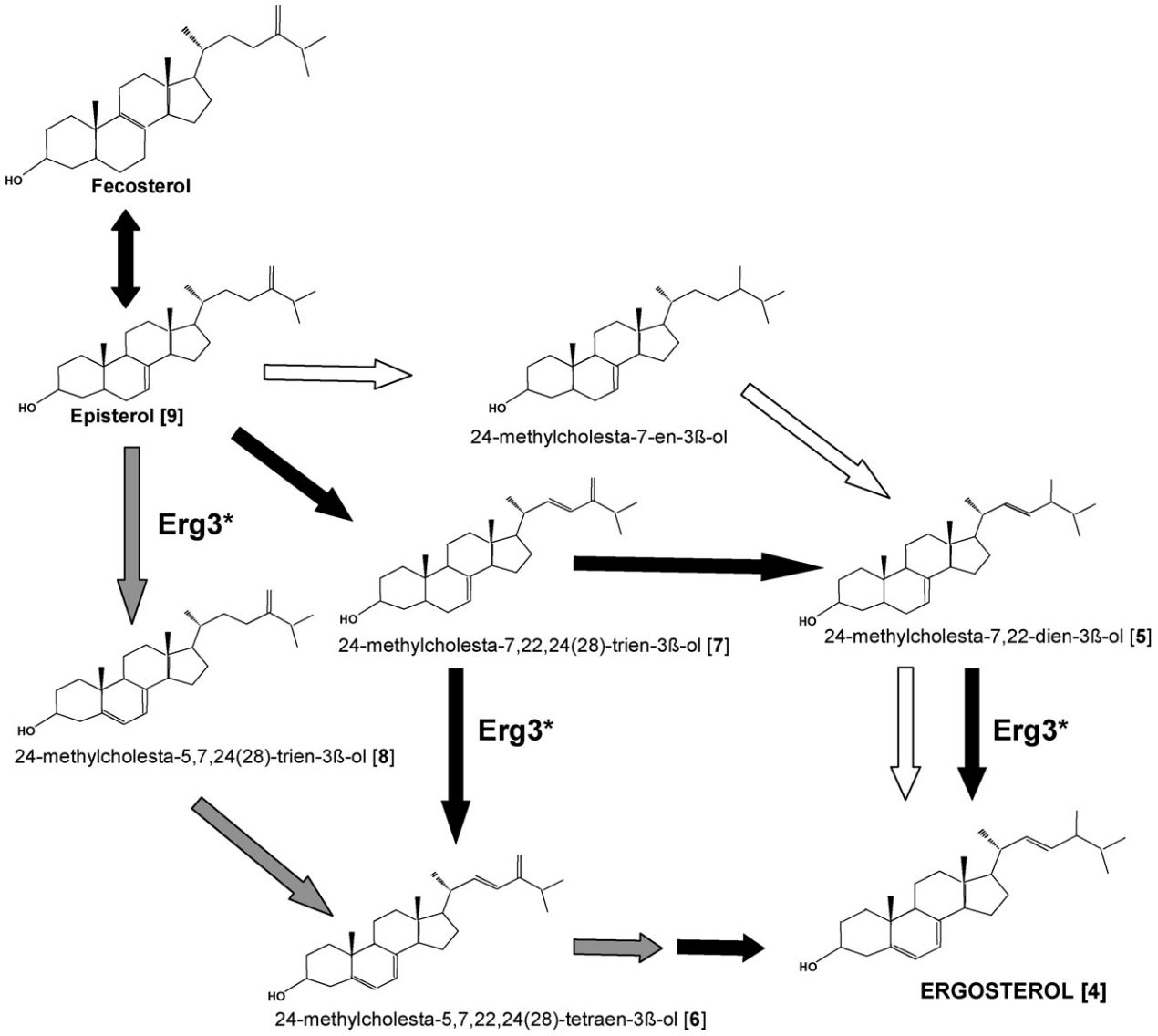


**Fig. 3 – Possible alternative sterol biosynthetic pathways from fecosterol to ergosterol in *A. fumigatus*. *C-5 sterol desaturases enzyme (Erg3). Numbers refer to sterols in Tables 1 and 2.**

continues into ergosterol by hydrogenation of the methylene bond at C-24.

In the second route (gray arrows, Fig. 3) episterol is desaturated at C-5 and C-22 to produce sterol (**6**) which, as described above, continues to ergosterol by reduction of the C-24 methylene group. In these two routes the intermediate compound is (**6**) being the dominant in most filamentous fungi and being occasionally reported in *S. cerevisiae* [14,15,24].

In the third alternative route (white arrows, Fig. 3) a reduction of the methylene group at C-24 leads to 24-methylcholesta-7-en-3β-ol, followed by two consecutive desaturations at C-22 and C-5 to generate ergosterol. This is the preferred mechanism reported in *Candida* spp. and zygomycetes [16,24,28].

According to the sterol composition found in *A. fumigatus*, there is no evidence of the existence of the third mechanism in this fungal species. In contrast, the other two pathways are possible since all the intermediates from episterol to ergosterol were identified in significant amounts.

Thus, interruption of the ergosterol pathway at Erg3 level in CM-B866 strain caused the accumulation of three different non-C-5 desaturated sterols (**9**), (**7**) and (**5**) which it suggests that the pathway described in Fig. 3 in solid arrows, is the dominant route in *A. fumigatus*. Therefore, blocking at Erg3B level may induce a greater accumulation of its direct substrate (**5**) and its preceding substrates (**7** and **9**).

In summary, *A. fumigatus*, is able to synthesize ergosterol, even with suppression of several enzymes in the ergosterol pathway. This fact, once more, reflects that this fungi have alternatives to overcome severe drawbacks. The precise knowledge of this complex route could facilitate the future development of novel and more selective antifungal drugs.

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