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4-methylzymosterone and other intermediates of sterol biosynthesis from yeast mutants engineered in the *ERG27* gene encoding 3-ketosteroid reductase

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Abbreviations

OSC/Erg7p	yeast oxidosqualene cyclase
ERG7	gene encoding oxidosqualene cyclase
Erg27p	yeast 3-ketosteroid reductase
ERG27	gene encoding 3-ketosteroid reductase
HSD17B7	Hydroxysteroid-17β-dehydrogenase type 7
SC4MOL#	sterol-C-4 methyloxidase-like
NSDHL#######	NADH sterol dehydrogenase-like
C14ORF1	Chromosome 14 Open Reading Frame 1
MAS	meiosis-activating sterols
EGFR	Epidermal growth factor receptor
SAM	S-adenosyl methionine
TLC	Thin-layer chromatography
CI-MS##	Chemical ionization-mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
NMR##########	Nuclear magnetic resonance

Abstract

Studies in the post-squalene section of sterol biosynthesis may be hampered by the poor availability of authentic standards. The present study used different yeast strains engineered in 3-ketosteroid reductase (Erg27p) to obtain radioactive and non-radioactive intermediates of sterol biosynthesis hardly or not available commercially. Non-radioactive 3-keto 4-monomethyl sterones were purified from non-saponifiable lipids extracted from cells bearing point-mutated 3-ketosteroid reductase. Two strategies were adopted to prepare the radioactive compounds: i) incubation of cell homogenates of an *ERG27*-deletant strain with radioactive lanosterol; ii) incubation of growing cells of a strain expressing point-mutated 3-ketosteroid reductase with radioactive acetate. Chemical reduction of both radioactive and non-radioactive 3-keto sterones gave the physiological 3-β OH sterols, as well as the non-physiological 3-α OH isomers.

This combined biological and chemical preparation procedure provided otherwise unavailable or hardly available 4-mono-methyl intermediates of sterol biosynthesis, paving the way for research into their roles in physiological and pathological conditions.

Introduction

In non-photosynthetic eukaryotes (animals, yeasts and fungi), sterol biosynthesis is a metabolic pathway comprising two different sections: an “assembly line” section, from acetyl-CoA to oxidosqualene, and a “tailoring” section, from lanosterol to the end product cholesterol or ergosterol. These two sections are linked by a reaction that models the open triterpene oxidosqualene into the steroid-shaped triterpene lanosterol.

In recent years, the simple view of the post-squalene section as a tailoring process whose aim is to produce the end-product has changed radically, as novel roles have been recognized for some intermediates and enzymes involved in the section. For instance, 4,4-dimethyl sterols, resulting from 14-α demethylation of lanosterol, play a role in stimulating meiosis, and are thus known as meiosis-activating sterols (MAS) [1]. In mammals, the enzyme that reduces the 3-keto sterone intermediates, as an essential step of the double C-4 methyl removal in cholesterol synthesis, is a "moonlighting" protein, also playing the role of steroidogenic enzyme by forming estradiol from estrone (hence its name: Hydroxysteroid-17β-dehydrogenase type 7, HSD17B7) [2]. In yeasts, the same 3-ketosteroid reductase enzyme (Erg27p) acts as a chaperonine-like protein toward oxidosqualene cyclase (OSC) [3], suggesting the existence of a complex network of interactions among post-squalene enzymes.

The recent discovery that a number of severe inborn metabolic disorders are caused by mutations of the post-squalene enzymes of the sterol biosynthesis pathway [4] has raised interest in the last steps of sterol synthesis in humans, and in intermediates that may accumulate if these enzymes malfunction.

These malformation syndromes include sterol-C-4 methyloxidase-like (SC4MOL) deficiency and NADH sterol dehydrogenase-like (NSDHL) deficiency (causing the CHILD syndrome, Congenital Hemydysplasia with Icthiosiform Nevus and Limb Defects). These enzymes belong to the C-4 demethylase complex, which also includes 3-ketosteroid reductase (HSD17B7) and the non-enzymatic protein C14ORF1 [5].

Evidence from humans and from knock-out mouse models suggests that the malformation-inducing phenotypes developed during embryogenesis might depend not only on the reduced level of cholesterol, but also on the accumulation of precursors inhibiting the Hedgehog signaling, critical for proper development of the embryo in mammals [4, 6]. Recent observations of a marked accumulation of 4-methylzymosterone in the embryo brain, and a dramatic reduction of Sonic Hedgehog signaling in a HSD17B7-deficient mouse model, strongly suggest the possibility of a cause-effect relationship [7, 8]. In another mouse model, Hedgehog signaling appeared to be severely affected by mutation in the NSDHL gene, encoding for a 3-β-hydroxysterol dehydrogenase [9]. The sterol demethylase complex may also play a critical role in sensitizing some types of cancer cells to antitumor drugs: oncogenic EGF receptor (EGFR) signaling appears to be regulated by the post-squalene enzymes SC4MOL and NSDHL, which affect the signaling, vesicular trafficking, and degradation of EGFR [10].

Typically, studies of the different features of the post-squalene sterol synthesis pathway rely on the availability of sterol intermediates as standards, radioactive compounds for pulse experiments, or putatively bioactive molecules. A recent review summarized commercial and laboratory steroidal triterpene standards of cholesterol synthesis, pointing out the difficulty of obtaining some of the intermediates [11]; particular attention was paid to the availability of meiosis-activating sterols (MAS), although a fermentative procedure to produce meiosis-activating sterols (MAS) using engineered yeast strains [12] was not mentioned. The only compounds missing from the list given in the review [11] are 4-methylzymosterol and 4-methylzymosterone; the latter is hypothesized, in a cited study on a HSD17B7-deficient mouse model, to be toxic for Hedgehog proteins [7]. Very recently, 4-methylzymosterone became commercially available, even though extremely expensive and with a very long lead-time. The present study describes some fermentative procedures and purification methods that may be used to produce either significant amount of unlabeled 4-methylzymosterone (**1**, Fig.1) or its radioactive form, using engineered *Saccharomyces cerevisiae* strains bearing mutations at the *ERG27* gene encoding 3-ketosteroid reductase.

Because yeast contains a SAM-dependent sterol side chain methyl-transferase, a significant amount of the 24-methylene derivative of 4-methylzymosterone (4-methylfecosterone, **2**, Fig.1) was also obtained; the amount depended on the type of mutant and the culture conditions. From C-3-sterones, 3-α (**5**,**7**, Fig. 1) and 3-β (**6**,**8**, Fig. 1) hydroxyl-derivatives were readily obtained by chemical reduction.

Materials and methods

S. cerevisiae strains and growth conditions

The genotypes of strains used are listed in Table 1.

STY7 strain was obtained from STY2 strain [13] by deletion of the *ERG27* using one-step PCR deletion-disruption [13]. The plasmid pFA6-*KanMX4* [14] was used as the template for amplification of the *KanMX4* gene. The primers F-erg27kan (5'-GGAAAGTAGCTATCGTAACGGGTACTAATAGTAATCTTGGTCTGAACATTGTGTTCCGggcgtac gctgcaggtcgac-3') and R-erg27kan (5'-CGCGATAAGTGCAGAAGACACTATAGCAACGAGGATAGTTCGTTTCTCTGACAGAGGCgatcgat gaattcgagctcg-3'), containing approximately 60 bases of upstream and downstream *ERG27* sequence and 20 bases of the *KanMX4* gene, were used. Bases in the lowercase are conserved regions flanking the *KanMX4* gene in the pFA6-*KanMX4* vector, and bases in the uppercase refer to upstream and downstream *ERG27* sequence The *ERG27* knockout was confirmed by PCR using primer sequences up- and downstream of the *ERG27* deleted sequence.

Wild-type yeast strain SCY876 was grown to early stationary phase in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C. Mutants disrupted for either *ERG7* or *ERG27* (STY2, BTY6-5-3, SDG115 and STY7) were supplemented with ergosterol (0.02 mg/mL). Both *ERG27* mutants R40A and K206A were grown in synthetic complete medium without uracil (SC-UraD:

0.17% yeast nitrogen base, 0.2% amino acids, 0.5% ammonium sulphate, 2% glucose), supplemented with ergosterol (0.02 mg/mL). All mutant strains but SDG115 could be grown aerobically, with sterol supplementation, because of the *UPC2-1* allele that allows for aerobic sterol uptake [13]. SDG115, although lacking *UPC2-1* was also able to grow under aerobic conditions with sterol supplementation.

The different growing conditions used for optimizing 3-keto production are reported in the Results section.

Chemicals

Buffers, culture media, ergosterol and bovine albumin, used as a standard for protein determination, were obtained from Sigma-Aldrich (Italy). ATP, $NADP^+$, glucose-6-phosphate and G-6-P dehydrogenase were obtained from AlfaAesar (Germany).

Isolation and characterization of sterones and sterols from yeast cultures

Saturated yeast cultures (approx. 2.4 L) were harvested by centrifugation at 3000 *g*. Cell pellet (approx. 20 g) was saponified in 45 mL of 10% (w/v) KOH in 90% aqueous ethanol (1 h, 80°C). Non-saponifiable lipids were extracted three times with petroleum ether (18, 9 and 9 mL). Extracts were evaporated to dryness using rotavapor at 35°C (15 min) and vacuum pump (1 h) (approx. 50 mg dry weight), then separated by TLC on silica gel glass plates (20×20 cm×0.25 mm) using cyclohexane/ethyl acetate (85:15, v/v) as the developing solvent. The two main chromatographic bands, visualized with berberine (1% ethanolic solution) or iodine stain and corresponding to 4-methylsterones (*Keto 1* fraction) and 4-desmethylsterones (*Keto 2* fraction) (Rf 0.81 and 0.62, respectively) were scraped off the plates separately and eluted first with 10 mL of diethyl ether/water (1:1; v/v) and then twice with 3 mL of diethyl ether. The two extracts were evaporated to dryness (approx. 8 mg of *Keto 1* and 4 mg of *Keto 2,* dry weight) and separated by $AgNO_3$-TLC on silica gel glass plates (20×20 cm×0.25 mm) in two development steps: a continuous development for 20 h with cyclohexane/chloroform (80:20; v/v), followed by chloroform/cyclohexane/ethyl acetate (17.5:77.5:5; v/v) until the solvent front had migrated about 18 cm. The $AgNO_3$-silica gel plates were prepared by immersing the plates into a $AgNO_3$ solution (25% w/v), then drying them in heater (110 °C, 3 h). The chromatographic bands were localized by comparison with a “reference lane”, consisting of co-chromatographed spots, resulting from the same mixture, visualized with sulfuric acid stain (MeOH 48.4% v/v, H_2O 48.4% v/v, H_2SO_4 3.3% v/v, $MnCl_2$ 0.5% w/v) at 120°C. Bands corresponding to 4-methylzymosterone, 4-methylfecosterone, zymosterone and episterone (**1**,**2**,**3**,**4**, Fig. 1) (Rf relative to lanosterol as internal standard 1.41, 1.19, 0.95 and 0.77, respectively) were scraped off the plates and eluted with diethyl ether as previously described. The identification and the purity of the compounds were checked by GC-MS and NMR (see Supplementary Material).

4-methylsterols (α and β) were prepared by chemical reduction with $NaBH_4$ of 4-methylsterones. In a current preparation, 4-methylsterones (approx. 4 mg) were incubated with $NaBH_4$ (5 mg) in 1 mL of ethanol for 30 min with magnetic stirring. The reaction was stopped by adding 1 mL of water.

The reaction mixture was extracted twice with 1.5 mL of petroleum ether and separated on silica gel glass plates (20×20 cm×0.25 mm) developed with cyclohexane/ethyl acetate (85:15; v/v). The transformation yield for the substrate was > 98%. The two chromatographic bands, visualized with berberine (1% ethanolic solution) or iodine stain and corresponding to 3-α-4-methylsterols (**5**,**7**, Fig. 1) and 3-β-4-methylsterols (**6**,**8**, Fig. 1) were scraped off the plates and eluted with diethyl ether as previously described. The identification of the compounds as 3-OH derivatives of the original sterones and the α/β-OH isomeric configuration were done by NMR The purity of the compounds were checked by GC-MS (see Supplementary Material).

GC and spectral methods

a) GC-MS and NMR analysis

Preparative TLC scraped fractions were analysed by GC-MS and NMR in order to identify compounds structures.

Samples were dissolved in chloroform and injected (splitless mode) on Agilent HP-5MS capillary column (30 m, i.d. of 0.25 mm, film thickness 0.25 μm) which was installed on a Agilent GC 6890 (Agilent Technologies, USA) equipped with a mass detector Agilent Network 5973, A splitless injection, 250°C injector temperature and helium as carrier gas (1.2 mL/min) were used. The gas chromatographic parameters were set up as follows: initial temperature 50°C, rate 20°C/min, final temperature 250°C, held for 10 min. The following parameters were set on mass spectrometer: low mass 40, high mass 800, MS quad 150°C, MS source 230°C.

NMR spectra (1H, 2D-COSY, 2D-1H-13C-HSQC and 2D-1H-13C-HMBC) were acquired at 7 T, on a Bruker Avance 300. Samples were dissolved in CDCl3 and the solvent residual peak was used as chemical shift internal reference.

b) CI-MS

Chemical ionization mass spectra were acquired on Finnigan MAT TSQ 700 using iso-butane as reagent gas (5500 mtorr).

Radiolabeled substrates

Radiolabeled (R,S)-[2-^{14}C]mevalonic acid (2.04 GBq/mmol) and [2-^{14}C]acetic acid (2.04 GBq/mmol) were purchased from Perkin Elmer (UK).

[^{14}C]lanosterol was prepared as previously reported [13] by a biological synthesis method, by incubating, 0.85 mL of a pig liver S10 preparation (25 mg of proteins) for 3 h at 37°C with (R,S)-[2-^{14}C]mevalonic acid (0.5 μCi, 2.04 GBq/mmol) in the presence of 0.1 mM ketoconazole, ATP (1 mM) and a NADPH generating system (1 mM $NADP^+$, 3 mM glucose-6-phosphate and 6 units of G-6-P dehydrogenase). The reaction was stopped by adding methanolic KOH (15% w/v, 1 mL) and lipids were saponified at 80°C for 30 min. The non -saponifiable lipids were then extracted three times with petroleum ether (1.5 mL) and separated by TLC on silica gel plates using cyclohexane/ethil acetate (85:15; v/v) as the developing system. The radioactive band (visualized with System 200 Imaging Scanner, Hewlett-Packard) corresponding to lanosterol was scraped off

and eluted twice with 3 mL of dichoromethane. Squalene, oxidosqualene, lanosterol, methylsterones and ergosterol were used as references.

The radioactivity was quantified by 2500 TR liquid scintillation analyzer (Packard). The average yield was 0.030 µCi of ^{14}C-lanosterol.

Biochemical preparation of [^{14}C]4-methylsterones and [^{14}C]4-methylsterols

The preparation of [^{14}C]4-methylsterones was carried out following two different procedures:

A) Incubation of yeast homogenates with [^{14}C]lanosterol

Cell-free homogenates were prepared as previously described [13]. After lysis of the yeast cell wall with lyticase, spheroplasts were homogenized with a Potter device. Proteins in the homogenate were quantified using the SIGMA Protein Assay Kit with bovine serum albumin as a standard. Cell-free homogenates (40-50 mg of proteins, 0.9 mL) were incubated with [^{14}C]lanosterol (0.5 µCi) in the presence of Tween 80 (0.1 mg/mL), ATP (1 mM) and a NADPH generating system (described above). The incubation lasted for 3 h at 30°C with vigorous shaking. Addition of the ATP and NADPH generating system was repeated at each hour. Non-saponifiable lipids were extracted and separated as described above. The radiochromatographic band corresponding to [^{14}C]4-methylsterones (*Keto 1* fraction) (Rf 0.81) was identified, scraped off the plates and eluted as described above. The radioactivity was quantified as described above.

B) Incubation of whole yeast cells with [2-^{14}C]acetate

As previously described [13], washed cells (1×10^9 cells) were resuspended in 1 mL of fresh media, incubated with [2-^{14}C]acetate (0.5 µCi) and shaken for 3 h at 30°C. Cells were then saponified in 1 mL of methanolic KOH (15% w/v) for 30 min at 80°C. Non-saponifiable lipids were extracted with petroleum ether and separated by TLC on silica gel plates as described above. The radiochromatographic band corresponding to [^{14}C]4-methylsterones (*Keto 1* fraction) (Rf 0.81) was identified, scraped off the plates and eluted as described above. The radioactivity was quantified as described above.

Keto 1 fraction, obtained from both procedures, was separated by $AgNO_3$-TLC as previously described, giving radioactive 4-methylzymosterone (**1**) and 4-methylfecosterone (**2**) (Fig. 2).

[^{14}C]4-methylzymosterol (3-β OH) and its 3-α isomer were prepared by chemical reduction of [^{14}C]4-methylzymosterone (0.014 µCi) with $NaBH_4$, following the procedure previously described. The transformation yield (judged by radiochromatografic analysis) was > 98%.

Results

Effective fermentative and purification procedures to obtain non-radioactive 4-methylzymosterone from the strain R40A

The *S. cerevisiae* strain R40A, a strain bearing a point mutation in *ERG27*, was used as a source of 4-methylzymosterone [15]. The flow diagram of the isolation and purification was reported in Fig. 1. Cells were cultured until the end of the log phase, harvested by centrifugation, and saponified as described in the Materials and Methods section. Separation of the non-saponifiable lipids on silica

gel plates developed with cyclohexane/ethyl acetate revealed the presence of two main chromatographic bands; these were denominated *Keto 1* fraction (Rf 0.81) and *Keto 2* fraction (Rf 0.62).

The chromatographic bands were scraped off and eluted with diethyl ether; they were analyzed by GC-MS, revealing two peaks for each fraction. The *Keto 1* fraction showed a peak at 24.15 min (m/z=396) and a second at 24.77 min (m/z=410), consistent with 4-methylzymosterone (**1**) and 4-methylfecosterone (**2**), respectively. The *Keto 2* fraction showed a peak at 23.61 min (m/z=382) and a second at 24.18 min (m/z=396), consistent with zymosterone (**3**) and episterone (**4**), respectively (see Supplementary Material).

$AgNO_3$ chromatography was used to separate the different components present in the *Keto 1* and *Keto 2* fractions; this is a powerful separation procedure capable of separating mixtures of lipid compounds differing only in the number and/or exposition of double bonds [16]. The *Keto 1* and *Keto 2* fractions each gave two bands, which were scraped off the plates. The resulting four TLC fractions were subjected to GC-MS analyses, revealing, each, a single chromatographic peak in fraction, confirming the presence of a single compound per fraction. Pure fractions were analyzed by NMR to clarify the compound structures (see Supplementary Material). The main diagnostic peaks in the 1H-NMR spectra were assigned exploiting COSY, 1H-13C-HSQC and 1H-13C-HMBC 2D experiments. The methyl (0.4 -1.8 ppm) and olefin (4.5-5.4 ppm) signals allowed the identification of the main differences in the four structures proposed. 4-Methylzymosterone was characterized by the presence of a triplet, due to the olefin proton in position 24 (between carbon 24 and carbon 25, at 5.10 ppm) and a doublet due to methyl protons in position 4 (1.02 ppm). 4-Methylfecosterone, analogously to its isomer 4-methylzymosterone, was characterized by the methyl in position 4 (doublet at 1.02 ppm), but differed in the position of the double bond, which in this case was between carbon 24 and 24'. The 1H NMR spectrum showed two doublets at 4.72 ppm and 4.66 ppm coupled by geminal coupling (J=0.8 HZ), confirmed by COSY and HSQC 2D spectra. The zymosterone and episterone 1H spectra were analogous to 4-methylzymosterone and 4-methylfecosterone, respectively, except for the absence of the signal relating to the protons of the C-4 methyl group. Bidimensional spectra confirmed these structures.

In order to optimize the 3-keto sterone production, the following medium conditions were assayed: glucose concentrations of 2, 4 and 8%; fermentation time ranging from 24 h to 72 h. The highest yield was obtained when yeast cells were cultured for 24 h in medium containing 2% glucose. Further, for each set of conditions, the *Keto 1* mixture was analyzed by CI-MS, to determine the ratio of 4-methylzymosterone to 4-methylfecosterone. The results of this analysis (Fig. 3) confirmed that 4-methylzymosterone was obtained at highest yield in the above growth conditions.

Under the most favorable conditions, approximately 4 mg of highly-purified 4-methyl zymosterone (**1**) and 3 mg of highly-purified 4-methylfecosterone (**2**) were obtained from 20 g (wet weight) of yeast cells. Further, using the same procedure, approximately 1 mg of highly-purified zymosterone

(**3**) and 2 mg of highly-purified episterone (**4**) were obtained (Fig. 1). The purity, checked GC-MS analysis, was > 98% (see Supplementary material).

The stability of 4-methylzymosterone and 4-methylfecosterone was determined by CI-MS analysis over 43 days in different storage conditions (-20 °C, 4 °C, RT and 37 °C; dichloromethane solution, ethanol solution and powder). No trace of decomposition was observed (see Supplementary Material).

Chemical preparation of 3-hydroxyl-4-methyl sterols from the 4-methyl sterones

In order to extend the list of hardly or non-commercially available 4-methyl steroids [11], 3-hydroxy-4-methyl sterols were produced by reduction of both 4-methylzymosterone (**5**,**6** Fig.1) and 4-methylfecosterone (**7**,**8** Fig. 1) with $NaBH_4$ [13]. TLC produced two different bands (Rf 0.47 and 0.31), more polar than the starting compound. TLC mobilities of the two different bands resulting from reduction of 4-methylzymosterone are identical to that of the bands obtained from 4-methylfecosterone, being the original sterones undistinguishable on the usual (i.e. non-$AgNO_3$) silicagel TLC plates, used to separate their reduction products.

Because of their TLC mobilities, the bands were tentatively identified as the 3-α (**5**,**7**, Rf 0.47) and 3-β (**6**,**8**, Rf 0.31) OH isomers resulting from the original 3-keto group. The 3-hydroxy nature and the α/β-OH isomerism of the bands scraped off the TLC plate were confirmed by NMR. The purity, checked by GC-MS, was > 98%, except for the 3-α 4-methylfecosterol which is approx. 90%.

The 1H-NMR spectra of reduced sterols, when compared with relative sterones precursors, show the appearance of the characteristic peak due to the presence of the proton in position 3. The assignement of this signal was also confirmed by 2D-COSY spectrum. The chemical shift and shape of these segnals are different for the α and β isomers of a given sterols. More in detail, for one of the two isomers lower chemical shift and larger coupling constant is observed. The higher value of the coupling constant suggest that the protons in position 3 and 4 are in trans configuration which is typical of β isomer. The configuration assignement (α or β) obtained by NMR is fully consistent with all other observations previously described and so confirm the structure of the four sterols.

Production of radioactive 4-methylzymosterone and other 4-methylsteroids from *ERG27* yeast mutants

The primary goal of this part of the study was to produce radioactive 4-methylzymosterone and 4-methylzymosterol. The general strategy was first to obtain 4-methylzymosterone biologically, employing engineered yeast strains, and then reduce it chemically to 4-methylzymosterol, by treatment with $NaBH_4$. Labelled C-4 methylsterones can be obtained from *ERG27*-deletant strains by incubating a cell-free homogenate with radioactive lanosterol. A strain that has already been used for this purpose, the SDG115 strain [13], was compared with two other *ERG27*-deletant strains, BTY6-5-3 and STY7; the latter is a double mutant, in which both *ERG7* (oxidosqualene

cyclase) and *ERG27* (steroid-3-ketoreductase) genes have been deleted. All three strains proved to be effective in transforming radioactive lanosterol into a fraction (*Keto 1*) co-chromatographing (TLC) with 4-methyl zymosterone (Table 2), the most productive being STY7. With this strain, approximately 0.05#µCi of *Keto 1* fraction can be obtained by incubating cell-free homogenate with 0.5#µCi of radioactive lanosterol (Fig. 2A).

Through this procedure, the untransformed radioactive lanosterol can be recovered from the TLC plates, thus reducing radioactive waste, and reducing the requirement for the radioactive lanosterol, which is difficult to produce.

As an alternative, the possibility of producing the 4-methylsterones by incubating *ERG27*-point mutant yeast cells (R40A or K206A, another point mutant similar to R40A [15]) with radioactive acetate was tested. With either strain, incubation of 10^9 cells with 0.5 µCi gave approximately 0.01 µCi of *Keto 1* fraction (Fig. 2B). This strategy was found to be an easier procedure, that gave good and reproducible results, although the low yield of radioactivity incorporation means that the cells must be cultured in the presence of high levels of radioactivity. Further, the initial radioactive acetate cannot be recovered.

Incubation of cell-free homogenates of an *ERG27*-deletant yeast strain with radioactive lanosterol thus appeared a more advantageous method for preparing radioactive 4-methylsterones.

Since the radioactive fraction *Keto 1*, obtained by the procedures described, was expected to be a mixture of 4-methylzymosterone and 4-methylfecosterone, it was further separated by $AgNO_3$-TLC chromatography, as for its non-radioactive counterpart. As shown in Fig. 4 the radiochromatographic TLC plate revealed the presence of two bands corresponding to the authentic samples of the two 4-methylsterones.

Separation of the radiolabeled *Keto 1* fraction produced with the two different methods revealed the relative amounts of 4-methylfecosterone and 4-methylzymosterone; the former was more abundant with method 1, while the latter was more abundant with method 2 (Table 3).

In short, both methods are comparable to produce 4-methylzymosterone; when the goal is to obtain 4-methylfecosterone the first procedure is preferred.

The success of the method to obtain radioactive 3-keto-4-monomethyl steroids suggested that a chemical step be added, consisting in treatment with $NaBH_4$, to produce 3-hydroxy derivatives. The procedure was refined in order to separate 4-monomethylzymosterol (3-β-hydroxy) from its α-isomer, both resulting from $NaBH_4$ treatment of 4-methylzymosterone. 3-β OH and 3-α OH isomers were separated on TLC silica gel plates, together with non-radioactive reference standards obtained as described above, giving a β/α ratio of approximately 2.5 (Fig. 5).

Discussion

The primary goal of this study being to obtain adequate quantities of 4-methylzymosterone, several *ERG27* mutants were examined; some were novel, others had already been characterized [13, 15]. In the yeast *S. cerevisiae*, the enzyme 3-ketosteroid reductase (Erg27p) is known to act as a

"moonlighting" protein; it is endowed with both catalytic activity and chaperonin-like action toward oxidosqualene cyclase, the latter being an upstream enzyme of ergosterol biosynthesis. *ERG27*-deletant strains are not only ergosterol auxotrophic (thus requiring ergosterol or cholesterol for growth), but are also unable to cyclize oxidosqualene; this lack is caused by the loss of oxidosqualene cyclase functionality in the absence of 3-ketosteroid reductase, the product of the *ERG27* gene [3]. As a consequence, *ERG27*-deletant strains cannot be used as a source of ketosteroids (substrates of Erg27p) as their cells accumulate the upstream acyclic sterol intermediates squalene, oxidosqualene and dioxidosqualene [17]. In these deletant strains it would only be possible to obtain 3-ketosteroids by culturing them in the presence of lanosterol, a procedure that might be expected to be poorly effective, due to the cells' low lanosterol uptake [18]. In a previous study aimed at separating the catalytic from the chaperonine-like activity of Erg27p, a series of *ERG27* mutants were developed in which the 3-ketosteroid reductase functionality was markedly reduced, while maintaining the protective action versus oxidosqualene cyclase in full [15]. These strains accumulate 3-ketosteroids in their cells, and were thus chosen for designing a fermentative procedure to produce 4-methyzymosterone. The main experimental difficult was to separate 4-methylzymosterone from the corresponding C-24 methylene derivative, 4-methylfecosterone, resulting from the activity of C-24-sterol SAM methyl transferase [17]. The presence of 4-methylfecosterone suggested that C-4 methylated steroids (as 4-methyl zymosterone), normally poor substrates for the enzyme owing to the presence of a methyl group at C-4 position [19], may effectively be alkylated in the side chain provided that the preferred substrate of transferase (zymosterol) is absent. Side-chain alkylated C-3 sterones were separated from non-alkylated ones by $AgNO_3$-TLC chromatography, a TLC procedure whereby lipid molecules differing in the number and/or exposure of double bonds can be separated [16]. The quest for the most appropriate conditions to lower the content of side-chain alkylated C-3 sterones showed that culture time must be as short as possible, so as to reduce the exposure time of the unalkylated side chain of (methyl)zymosterone to C-24 SAM methyl transferase.

The successful preparation of significant amount of highly-purified 4-methylzymosterone starting from engineered yeast strains makes now possible extensive studies of its biological properties. For example, the supposed toxicity of 4-methylzymosterone on the Hedgehog protein cascade directly [7], or its putative teratogenic effect on the development of mouse embryos [8] (an experimental assay requiring high amount of compound) can be directly assayed. In short, the question often mentioned in the conclusion of studies on post-squalene cholesterol disorders, i.e. whether the malformative phenotypes depend on the accumulation of C-4 methyl intermediates of cholesterol biosynthesis [4], can now be addressed directly.

The second aim of this study was to develop a procedure to obtain radioactive 4-methylzymosterone and 4-methyl zymosterol. These compounds are substrates, respectively, of 3-ketosteroid reductase (Erg27p in yeast, HSD17B7 in mammals) and steroid-C4-methyl oxidase (Erg25p in yeast, SC4MOL enzyme in mammals), two enzymes of the C-4 demethylase complex

of sterol biosynthesis. Using these radioactive substrates, it would then be possible to assay the functionality of the above enzymes directly, e.g. in projects addressed at designing novel inhibitors of the enzymes, or in studies of known and novel cholesterol disorders caused by their deficiency (human 3-ketosteroid reductase-deficiency has been hypothesized, but never actually detected [2].
As in the preparation of non-radioactive intermediates, the problem of alkylation of the side chain by C-24-sterol SAM methyl transferase had to be overcome; this reaction changes the intermediates (of interest here) of cholesterol biosynthesis to intermediates of ergosterol biosynthesis.
It was attempted to determine the most appropriate conditions that would increase the ratio of C-24 non-alkylated/C-24 alkylated intermediates: the incubation of highly concentrated cell cultures of the point mutant K206A or R40A with radioactive acetate was found to be successful. This procedure gave a mixture of 4-methyl sterones in which 4-methylzymosterone widely predominated over 4-methylfecosterone (> 70%); the undesired outcome was the low specific radioactivity of the compound extracted and purified from the non-saponifiable yeast lipids, due to the high amount of non-radioactive 4-methylsterones accumulated in the cells. The alternative procedure, consisting in incubating cell homogenates of an *ERG27*-deletant strain (a strain that accumulates squalenoid molecules rather than sterones [2]) with radioactive lanosterol, gave 4-methyl sterones with higher specific radioactivity; however, the sterone mixture predominately contained the side-chain-alkylated 4-methylfecosterone (> 80%). This might be caused by the unusual conditions in which the SAM-dependent sterol side-chain methyl transferase must act, that is a cell homogenate from an *ERG27*-deletant yeast strain incubated with radioactive lanosterol: in this environment, the starting precursor lanosterol, as well as all the radioactive derivatives resulting from its demethylation in C-14 (FF-MAS, T-MAS) and in C-4 (4-methylzymosterone), are possible substrates for the SAM-dependent sterol side-chain methyl transferase, thus putative precursors of 4-methylfecosterone.
In short, the two procedures described above provide two different mixtures of radioactive 4-methyl sterones, one richer in 4-methylzymosterone, the other in 4-methylfecosterone. The two mixtures can be used as such in preliminary enzymatic assays, respectively with mammalian or with yeast 3-ketosteroid reductase. Obviously, when one of the two radiolabeled sterones is required as a highly-purified substrate, an additional purification $AgNO_3$-TLC chromatographic step is needed.
3-hydroxy derivatives were readily obtained from 4-methylzymosterone, by treatment with $NaBH_4$. Reduction of a 3-keto steroid with $NaBH_4$ gave the two α- and β- epimeric isomers, the latter being the physiological intermediate of sterol biosynthesis. The availability of the α- and β- OH isomers will allow to explore the isomeric substrate specificity of the C-4 demethylase complex.
To conclude, the study developed a fermentative/chemical procedure whereby intermediates of post-squalene cholesterol biosynthesis 4-methylzymosterone and 4-methylzymosterol can be effectively produced, together with the non-physiological sterol 3-α-hydroxy-4-methylzymosterol. The corresponding intermediates of ergosterol biosynthesis, bearing an alkylated side chain, were

also produced. These molecules, prepared in both radioactive and non-radioactive versions, can be used to characterize the enzymes of the sterol C-4 demethylase apparatus, an enzymatic complex supposedly involved in certain cholesterol disorders, and in resistance to EGF receptor inhibitors [9].

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Fig. 1 Flow diagram of the isolation and purification of (**1**) 4-methylzymosterone, (**2**) 4-methylfecosterone, (**3**) zymosterone, (**4**) episterone, (**5**) 4-methylzymoster(3α)ol, (**6**) 4-methylzymoster(3β)ol, (**7**) 4-methylfecoster(3α)ol and (**8**) 4-methylfecoster(3β)ol from R40A yeast cells. Conditions: (i) saponification with ethanolic KOH and extraction with petroleum ether, (ii) TLC separation on silica gel plates using cyclohexane/ethyl acetate (85:15, v/v), elution from silica with diethyl ether, (iii) $AgNO_3$-TLC separation on silica gel plates in two development steps [continuous for 20 h with cyclohexane/chloroform (80:20; v/v), followed by chloroform/cyclohexane/ethyl acetate (17.5:77.5:5; v/v) until the solvent front had migrated about 18 cm], elution from silica with diethyl ether, (iv) $NaBH_4$ reduction, TLC separation on silica gel plates using cyclohexane/ethyl acetate (85:15, v/v) and elution from silica with diethyl ether

Fig. 2 Flow diagram of the isolation and purification of both radiolabeled (**1**) 4-methylzymosterone and (**2**) 4-methylfecosterone by (A) incubation of STY7 cell-free homogenates with [^{14}C]lanosterol, (B) incubation of whole K206A yeast cells with [^{14}C]acetate. Conditions: (i) saponification with methanolic KOH and extraction with petroleum ether, (ii) TLC separation on silica gel plates using cyclohexane/ethyl acetate (85:15, v/v), elution from silica with dichloromethane, (iii) $AgNO_3$-TLC separation on silica gel plates in two development steps [continuous for 20 h with cyclohexane/chloroform (80:20; v/v), followed by chloroform/cyclohexane/ethyl acetate (17.5:77.5:5; v/v) until the solvent front had migrated about 18 cm], elution from silica with diethyl ether

Fig. 3 Ratio of 4-methylzymosterone and 4-methylfecosterone during 72 hours of growth in different culture conditions. The ratio was calculated from the relative abundances of the two compounds in the CI-MS spectra, after growing cells in the presence of three different glucose (D) concentrations. Insert: the record obtained after 24 h of growth in a medium containing 2% of glucose, as an example of the mass spectra used to calculate the ratio of 4-methylzymosterone (a) to 4-methylfecosterone (b) (Mean and S.D. of three separate experiments, each one carried out in duplicate)

Fig. 4 Radiochromatogram of radiolabeled *Keto 1* obtained from the yeast strain K206A separated by $AgNO_3$ – TLC. The separation was performed using two development steps: a continuous development for 20 h with cyclohexane/chloroform (80:20; v/v), followed by chloroform/cyclohexane/ethyl acetate (17.5:77.5:5; v/v) until the solvent front had migrated about 18 cm. Comparable results were obtained with the strain R40A. Peak a, 4-methylfecosterone; peak b, 4-methylzymosterone

Fig. 5 Radiochromatogram of compounds resulting from the reduction of [^{14}C]methylzymosterone with excess $NaBH_4$ and separated by TLC. [^{14}C]methylzymosterone was incubated with $NaBH_4$ for 30 min, reduction products were extracted using petroleum ether and separated using

cyclohexane/ethyl acetate (85:15; v/v) as developing solvents. Peak a, β-hydroxy derivative; peak b, α-hydroxy derivative. [^{14}C]methylzymosterone position is shown by "c"

Table 1. *S. cerevisiae* strains used in this study

Strains	Genotype	cited in the text as
SCY876[a]	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1*	SCY876
STY7 (*erg7*Δ, *erg27*Δ)	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg7Δ::HIS3 erg27Δ::KanMX4*	STY7
STY2 (*erg7*Δ)[a]	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg7Δ::HIS3*	STY2
SDG115 (*erg27*Δ)[a]	*MATa, ade5, his7, leu2-3,112 ura3-52, erg27△::URA3*	SDG115
BTY6-5-3 (*erg27*Δ)[b]	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg27Δ::LEU2*	BTY6-5-3
BTY6-5-3 (Erg27p-K206A)[c]	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg27-K206A*	K206A
BTY6-5-3 (Erg27p-R40A)[c]	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg27-R40A*	R40A

[a] [13], [b] [20], [c] [15]

Table 2. Incubation of homogenates from yeast strains with [^{14}C]lanosterol.

Strain	Radioactivity incorporated into non-saponifiable lipids (%)		
	Lanosterol	*Keto1*[a]	*Keto 2*[b]
SDG115	47.52	40.50	11.98
BTY6-5-3	44.72	37.48	17.80
STY7	27.99	59.32	12.69

[a] *Keto 1* is a mixture of 4-methylzymosterone and 4-methylfecosterone.
[b] *Keto 2* is a mixture of zymosterone and episterone.
(Values are the means of three separate experiments, each one carried out in duplicate. The maximum deviations from the mean were less than 10%)

Table 3. $AgNO_3$-TLC separation of radiolabeled components of the *Keto 1* fraction obtained from different strains.

Strain	Radioactivity (%)	
	4-methylzymosterone	4-methylfecosterone
STY7	16.15	83.85
K206A	73.05	26.95

(Values are the means of three separate experiments, each one carried out in duplicate. The maximum deviations from the mean were less than 10%. Procedures adopted to obtain radioactive compounds in the two strains were different (see text).