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Production of Meiosis-Activating Sterols from Metabolically Engineered Yeast

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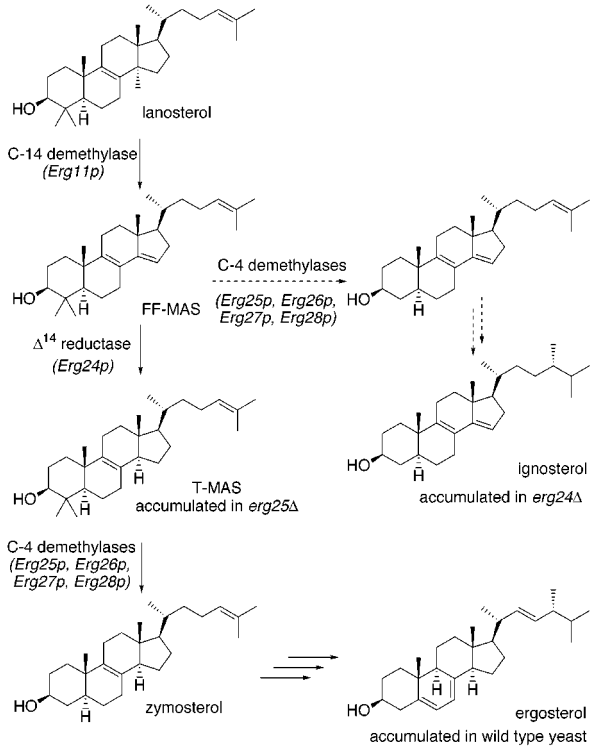
The recent discovery that meiosis can be activated by certain biosynthetic precursors of cholesterol has stimulated numerous investigations into the physiological and mechanistic underpinnings of this process.[1] The putative regulatory species, known as meiosis-activating sterols (MAS), are present at elevated levels in gonadal tissues[2] and accumulate in response to increased concentrations of specific progestins.[3] The exact role of MAS as native paracrine regulators remains controversial,[4] but their undisputed pharmacological activity has pointed to applications in the control of reproduction and in vitro fertilization.[5] The most abundant MAS are 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (FF-MAS), isolated from follicular fluid, and 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol (T-MAS), isolated from testis.[2a] These compounds are remarkably difficult to obtain, even in milligram amounts. Published syntheses of FF-MAS and T-MAS entail at least 12 steps from commercially available steroids and proceed in overall yields of well under 10%.[6] A more efficient route described in the patent literature is also lengthy.[7] Although natural sources can provide small amounts of MAS, the low concentrations[8] of these compounds make isolation impractical. Promising alternative approaches that have scarcely been explored are biosynthetic methods,[9] including the fermentative production of MAS using recombinant microorganisms.

Among many microbial candidates for metabolic engineering, the yeast *Saccharomyces cerevisiae* is especially amenable to sterol production.[10] Extensive genetic and biochemical investigations have established the intermediates, enzymes, and genes in yeast sterol biosynthesis. This pathway includes T-MAS and FF-MAS as transient intermediates (Scheme 1).[11] We have developed stable yeast strains in which these intermediates are formed but cannot be further metabolized to normal yeast sterols. Demonstrated herein is the use of yeast mutants for producing T-MAS or FF-MAS.

As shown in Scheme 1, FF-MAS is the biosynthetic product of lanosterol demethylase (Erg11p) and can be further metabolized to T-MAS by the sterol Δ^{14} reductase (Erg24p)[12] or to 4-desmethyl sterols by a series of enzymes beginning with C-4 methyl oxidase (Erg25p).[13] If sterol biosynthesis consisted of a linear sequence of reactions, then deleting the Δ^{14} reductase would force the accumulation of its precursor, FF-MAS. However, because many sterol biosynthetic enzymes have broad substrate specificity, blocking one reaction sequence causes metabolism via alternative sequences. For example, an *erg24*Δ deletion mutant[14] accumulated ergosta-8,14-dien-3β-ol (ignosterol, 65%), ergosta-8,14,24(28)-trien-3β-ol (21%), and lanosterol (6%). Any FF-MAS formed was completely consumed by C-4 demethylation and side-chain modification.

Deleting *ERG25* from the *erg24*Δ mutant would preclude FF-MAS demethylation at C-4. However, the *erg25*Δ mutant is nonviable under aerobic conditions because the resulting 4,4-dimethyl sterols are apparently ineffective ergosterol surrogates.[13a]

Scheme 1. Yeast Sterol Biosynthesis[a]



[a] Lanosterol is demethylated to FF-MAS, which is a substrate for both Δ^{14} reduction and C-4 demethylation. Both *ERG24* and *ERG25* must be deleted to accumulate FF-MAS.

Exogenous ergosterol cannot rescue the *erg25*Δ mutant because yeast does not import sterols under aerobic conditions. This dilemma was addressed by abolishing heme biosynthesis. Heme mutations simulate anaerobiosis and thereby facilitate aerobic sterol import.[15] We constructed an *erg24*Δ *erg25*Δ *hem1*Δ triple mutant (RXY4.3),[16] which was viable when supplemented with 20 μg/mL ergosterol and 13 μg/mL heme. This heme concentration was adequate for respiration and lanosterol demethylation but low enough to allow aerobic sterol import. RXY4.3 grown under these conditions produced 1.3 μg of FF-MAS/mL of culture.[17]

We next optimized RXY4.3 fermentation conditions (Figure S1, Supporting Information). FF-MAS production was maximal after 3 days in 4% glucose. Although lowering the concentration of exogenous ergosterol dramatically decreased cell density, the resultant ergosterol depletion induced sterol biosynthesis and improved the FF-MAS yield to 3.2 μg/mL (Figure 1A). Substituting cholesterol for ergosterol[18] at 20 μg/mL further increased the FF-MAS yield to 5.4 μg/mL (Figure 1B).[19] The reaction was scaled to 1 L with comparable yield and product purity (Figure S2B, Supporting Information).[20] However, attempts to stimulate sterol synthesis further by lowering the exogenous cholesterol concentra-

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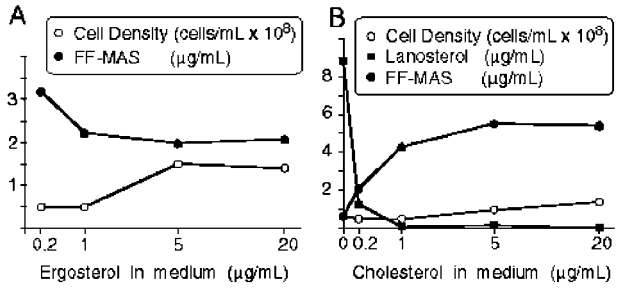

Figure 1. Effects of supplementing the culture medium with ergosterol (A) or cholesterol (B). RXY4.3 cultures were fermented for 3 days in synthetic complete medium lacking histidine and containing 4% dextrose, 13 μg/mL heme, and the indicated concentrations of ergosterol or cholesterol.

tion led to increased lanosterol levels at the expense of FF-MAS. In the many optimization experiments, RXY4.3 showed little or no tendency to undergo spontaneous mutations affecting sterol biosynthesis.[21]

We also investigated the *erg25*Δ *hem1*Δ double mutant RXY4.2,[16] which has a functional Δ^{14} reductase. When RXY4.2 was grown in synthetic complete medium lacking histidine and containing 2% dextrose, 13 μg/mL heme, and 20 μg/mL ergosterol, T-MAS (1.3 μg/mL culture) was isolated together with medium-derived ergosterol (1.0 μg/mL culture) (Figure S5, Supporting Information).[22] T-MAS accumulated despite the presence of the Δ^8-Δ^7 isomerase, which does not efficiently process 4,4-dimethyl sterols.

The sterol biosynthetic pathways of these mutants generated the desired products cleanly. Except for media-derived cholesterol or ergosterol, the MAS comprised ~90% of total sterols as judged by GC and ^{1}H NMR. The heme supplementation conditions allowed nearly complete C-14 demethylation, and RXY4.3 accumulated very little lanosterol. Further metabolism of FF-MAS by *S*-adenosylmethionine:Δ^{24}-methyltransferase (Erg6p) was minimal, and no other ring or side-chain modifications were observed. This result indicates that FF-MAS accumulation can be achieved by abolishing only two enzymes: Δ^{14} reductase and C-4 demethylase (Scheme 1).

In summary, we have illustrated the use of metabolic engineering to provide valuable sterols that are otherwise difficult to prepare. Some effort is needed to construct the yeast strains and to optimize culture conditions, but once established, these organisms provide an inexhaustible source of MAS requiring little expenditure of labor or materials. In vivo MAS production needs no added substrate and is technically much simpler than chemical synthesis, especially for laboratory-scale preparations. Further genetic manipulation is in progress to improve MAS yields and to obtain related sterols.

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Supporting Information Available: Details of yeast strain construction, optimization of fermentation conditions, NMR characterization of FF-MAS and T-MAS, and Figures S1−S5 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) RXY4.2 was generated from the *hem1*Δ strain SMY4 by replacing the *ERG25* gene with the *S. cerevisiae HIS3* gene using homologous recombination. RXY4.3 was then constructed from RXY4.2 similarly by replacing the *ERG24* gene with the Geneticin resistance marker gene *KanMX4*.

(17) After saponification, 7.8 μg of FF-MAS was isolated from a 6-mL culture and was quantitated by GC−FID and identified by GC−MS and ^{1}H NMR.

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(19) Saponifying yeast cells from a 2-mL saturated culture (1.4×10^8 cells/mL) of RXY4.3 grown in 20 μg/mL cholesterol and 0.2 μg/mL sparking ergosterol provided 21 μg total of free sterols, including 10.9 μg of FF-MAS (5.4 μg/mL) that was biosynthesized in vivo and 7.8 μg of cholesterol imported from the medium.

(20) Fermentation of RXY4.3 in 1 L of synthetic complete medium lacking histidine and containing 4% dextrose, 13 μg/mL heme, 20 μg/mL cholesterol, and 0.2 μg/mL sparking ergosterol provided crude material from which 4-desmethyl sterols were removed by silica gel chromatography to yield 4 mg of FF-MAS. ^{1}H NMR indicated ~90% FF-MAS, 4% lanosterol, 2% 4,4-dimethyl-5α-ergosta-8,14,24(28)-trien-3β-ol, and 2% cholesterol (Figure S3, Supporting Information). Comparing ^{13}C NMR and DEPT spectra (Figure S4, Supporting Information) with literature values[2a,6a] further confirmed the structure assignment of FF-MAS.

(21) Except for the accumulation of lanosterol at low concentrations of supplemented cholesterol (Figure 1B), amounts of sterols other than FF-MAS, ergosterol, and cholesterol were consistently minimal.

(22) T-MAS was identified by comparison of its ^{1}H NMR and mass spectra with those of an authentic standard and with published data.[2a,6a]

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