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Metabolic Engineering to Produce Sesquiterpenes in Yeast

Beth E. Jackson,[†] Elizabeth A. Hart-Wells,[‡,1] and Seiichi P. T. Matsuda*[,†,‡]

Department of Biochemistry and Cell Biology and Department of Chemistry, Rice University, Houston, Texas 77005

matsuda@rice.edu

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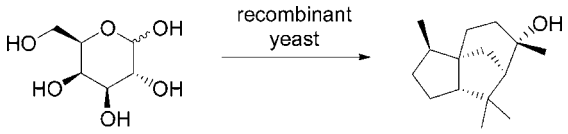
ABSTRACT



Presented here is a metabolically engineered yeast strain that produces sesquiterpenes. Epi-cedrol synthase expressed in a native yeast strain converted endogenous farnesyl pyrophosphate to 90 μg/L epi-cedrol. This system was genetically modified to increase foreign terpene yields to 370 μg/L. The best yields were obtained by overexpressing a truncated 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in a *upc2-1* mating type a background. This system allows sufficient production to characterize novel sesquiterpene synthase genes.

The sesquiterpenes and sesquiterpenoids comprise a large class of natural products with diverse biological activities, including communication and defense against insects and disease.[2] Interesting sesquiterpenes include the aphid alarm pheromone farnesene,[3] the antiinflammatory bisabolol,[4] the perfumery agent farnesol,[5] and amorphadiene, a precursor to the antimalarial drug artemisinin.[6] Because many of these compounds are only accessible in limited amounts from natural sources, sesquiterpenoids have been the targets of numerous synthetic approaches. The challenges presented by these stereochemically complex polycycles have inspired many synthetic advances and the development of new reagents. In fact, the retrosynthetic method itself was initially developed for the synthesis of the sesquiterpene hydrocarbon longifolene.[7]

Sesquiterpenes are produced by terpene synthase-mediated cyclization of the 15-carbon precursor farnesyl pyrophosphate (FPP).[8] In principle, sesquiterpenes could be produced artificially by incubating synthetic FPP in vitro with heterologously expressed sesquiterpene synthases. This origin suggests a universal biosynthetic approach to produce sesquiterpenes using terpene synthases to cyclize FPP. However, the expense of FPP and typically poor in vitro conversion make this approach impractical.

Engineered microbial systems producing sesquiterpenes would facilitate practical production of the natural products by providing an inexpensive and environmentally benign[9] alternative to synthetic routes or extraction from natural sources. *Escherichia coli* strains have been developed that biosynthesize sesquiterpenes from FPP in vivo.[10] The best yields (60 μg/L) obtained to date were obtained by increasing the expression levels of trichodiene synthase rather than optimizing precursor production.[10a]

We present here a metabolically engineered yeast system with improved sesquiterpene yields. The yeast *Saccharomyces cerevisiae* is an attractive alternative expression host. *S. cerevisiae* normally produces FPP as an intermediate in sterol biosynthesis,[11] and there has been significant progress in

[†] Department of Biochemistry and Cell Biology.
[‡] Department of Chemistry.

(1) Current address: U.S. House of Representatives, 2332 Rayburn House Office Building, Washington, DC 20515.

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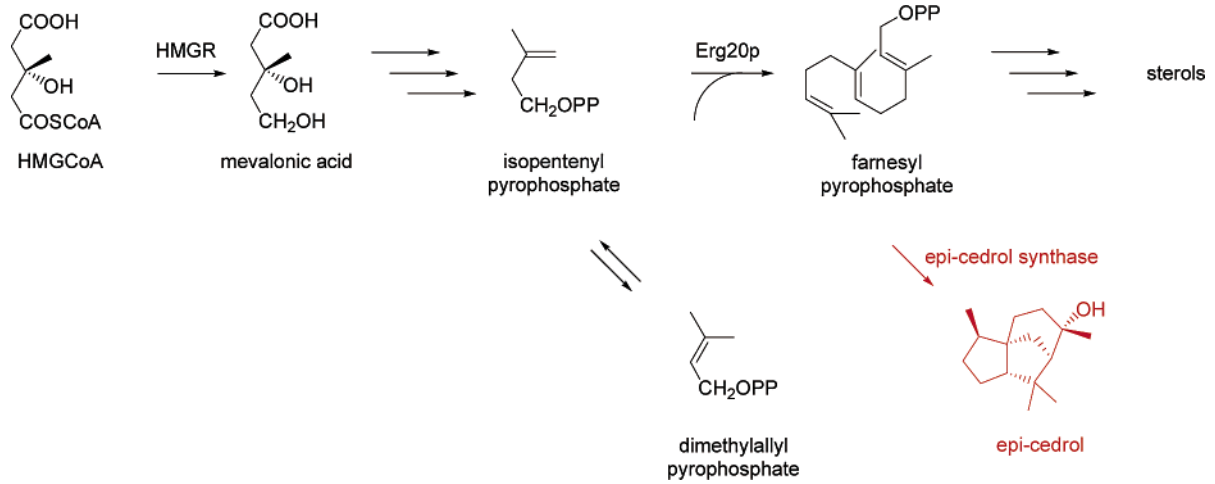
Scheme 1. Native Yeast Sterol Biosynthetic Pathway and Engineered Route to Epi-cedrol[a]



[a] In the native yeast sterol biosynthetic pathway (black), HMGCoA is converted to farnesyl pyrophosphate by an enzymatic sequence that includes hydroxymethylglutaryl CoA reductase (HMGR) and farnesyl pyrophosphate synthase (Erg20p). Expressing epi-cedrol synthase allows the production of the foreign sesquiterpene epi-cedrol (red).

elucidating how sterol homeostasis is controlled.[12] The yeast sterol biosynthetic pathway (Scheme 1) has now been deduced, and the relevant genes are known, which greatly facilitates efforts to increase FPP production. We first investigated whether FPP in the wild-type yeast sterol biosynthetic pathway is accessible for diversion to foreign sesquiterpenes. Of the many available sesquiterpene synthases that might be used in a metabolic engineering effort, we chose the *Artemisia annua* epi-cedrol synthase because it generates a relatively nonvolatile product.[13] Most sesquiterpene synthases convert FPP to cyclic carbocationic intermediates and then deprotonate to form $C_{15}H_{24}$ hydrocarbons. Epi-cedrol synthase catalyzes similar cationic chemistry but terminates cyclization by adding water to an intermediate carbocation to form epi-cedrol, a $C_{15}H_{26}O$ sesquiterpene alcohol. The greater mass and polarity of epi-cedrol relative to sesquiterpene hydrocarbons minimize losses due to volatility, ensuring accurate and consistent yields.

The epi-cedrol synthase was subcloned into the galactose-inducible, high-copy yeast expression vector pRS426GAL[14] and used to transform the native mating type α yeast strain JBY574[15] using the lithium acetate method.[16] This transformant was cultured in synthetic complete medium lacking uracil[16] and used to inoculate 5 mL of synthetic complete medium lacking uracil, containing the inducing sugar galactose.[17] The cell pellet was saponified to recover the epi-cedrol, but insignificant yields were obtained. As an alternative means of isolating epi-cedrol, we included Diaion HP-20 (Supelco) resin to adsorb hydrophobic components. After the culture reached saturation, the resin was readily removed from the culture by filtration through a polyethylene frit. The resin was washed with deionized water, and the organic components were eluted with ethanol. The solvent was removed, and the residue was partitioned between pentane and water. The organic fraction was shown by GC to contain 90 μg of epi-cedrol/L of culture.[18] Epi-cedrol from this and other yeast strains was identified by GC/MS. Yields were determined by GC/FID and are shown in Table 1. To determine whether any epi-cedrol had been derivatized to an ester, the organic eluent was hydrolyzed by saponification, and the nonsaponifiable lipids were examined by GC. Saponification did not increase epi-cedrol yields, establishing that ester derivatives are not a significant product sink. The mechanism by which sesquiterpenes escape the cell and bind to resin is unknown, but resin adsorption without saponification is an efficient means of isolating epi-cedrol biosynthesized in yeast.

We investigated whether mating type influences foreign sesquiterpene production. *S. cerevisiae* exists in two haploid mating types, α and **a**. Both mating types synthesize and secrete pheromones that trigger cell fusion with the opposite mating type. The mating pheromone secreted by α cells (including JBY574) is an unmodified peptide, whereas **a** cells secrete a dodecapeptide mating pheromone that bears a farnesyl group derived from FPP.[19] The yeast ergosterol

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(18) GC was performed using a HP 6890 series GC equipped with a 30 m × 0.25 mm i.d., 0.1 μm df Rtx-5 capillary column, a FID detection system with a split ratio of 1:40 helium carrier gas. The injector and detector were at 250 °C, and the temperature program was 70 °C for 1 min, 15 °C/min to 250 °C.
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Table 1. Epi-cedrol Yields from Recombinant Strains[a]

yeast strain	genotype	epi-cedrol (μg/L)
JBY574	MATα	90 ± 20
JBY575	MAT**a**	180 ± 10
CJ2-A	MAT**a** *upc2-1*	110 ± 20
BEJY7	MAT**a** pGal1-trHMG1:LEU2	70 ± 20
BEJY8	MAT**a** pGal1-ERG20::LEU2	230 ± 90
BEJY8[pEH12.2]	MAT**a** pGal1-ERG20::LEU2 [trHMG1 pRS314GAL]	190 ± 30
EHY24	MAT**a** *upc2-1* pGal1-ERG20::LEU2	230 ± 70
EHY24[pEH12.2]	MAT**a** *upc2-1* pGal1-ERG20::LEU2 [trHMG1 pRS314GAL]	170 ± 50
EHY41	MAT**a** *upc2-1* pGal1-trHMG1:LEU2	370 ± 40
EHY42	MATα *upc2-1* pGal1-trHMG1:LEU2	280 ± 80

[a] All strains have *ura3-52 trp1-*$^{\Delta}$*63 leu2-3,112 his3-*$^{\Delta}$*200 ade2* alleles and are Gal+. Products were quantitated from crude mixtures by GC-FID. The reported yields ± standard deviation are from at least three replicates.

pathway was manipulated in both **a** (JBY575) and α (JBY574) mating types. Yields in JBY575 were 180 μg/L, suggesting that FPP is more accessible in the **a** strain for diversion to foreign sesquiterpene biosynthesis. The **a** strain may normally biosynthesize more FPP as starting material for mating factor prenylation or may less rigorously channel FPP to sterol biosynthesis. We consequently used the **a** strain for further investigation.

An additional genetic change known to broadly increase sterol biosynthesis is a dominant mutation of the Upc2p transcription factor,[20] which belongs to a superfamily of regulatory DNA binding proteins.[21] The *upc2-1* allele substitutes an Asp residue for the Gly at amino acid position 888.[20b] Yeast containing *upc2-1* exhibit aerobic sterol uptake in excess of 5-fold relative to wild type.[20a] More important for the present work, *upc2-1* elicited an increase in the metabolic flux of sterol biosynthesis, indicated by the substantial increase in steryl esters detected in yeast carrying this mutant allele.[20a] Steryl ester accumulation was not a result of changes in esterification or ester hydrolytic activity. Therefore, the process by which *upc2-1* induces an overall increase in the rate of sterol biosynthesis remains unknown.[22] The utility of *upc2-1* to increase the availability of the sterol intermediate FPP was examined in our system. Expressing epi-cedrol synthase in the *upc2-1* mutant CJ-2A actually decreased foreign sesquiterpene yields relative to wild-type strain. FPP is apparently less accessible to the epi-cedrol synthase in the *upc2-1* mutant. Because the molecular basis of sterol overproduction in this mutant remains unknown, this result is difficult to interpret.

Sterol homeostasis has been investigated because of its role in human disease, and control mechanisms have been discovered that are directly applicable to increasing sesquiterpene yields in yeast. Hydroxymethylglutaryl CoA reductase has been shown to be a rate-limiting step in sterol biosynthesis, and overexpressing this enzyme may initially appear to be a straightforward way to dramatically increase sterol precursor biosynthesis.[23] *S. cerevisiae* encodes two isozymes of this protein, Hmg1p and Hmg2p. Although the yeast isozyme Hmg1p is regulated by transcription,[24] translation,[25] and ubiquitin-mediated proteolysis,[26] removing the noncatalytic N-terminal transmembrane-spanning domain (1656 nucleotides) from Hmg1p generates an active, cytosolic truncated protein. High-level expression of the truncated Hmg1p significantly increases accumulation of the sterol precursor squalene.[27] In an effort to exploit this higher flux through the mevalonate pathway, we generated a yeast strain in which both this truncated Hmg1p (trHmg1p) and epi-cedrol synthase were overexpressed from the GAL1 promoter. However, this strain also produced significantly less epi-cedrol than the wild-type strain; overexpressing trHmg1p makes FPP less accessible to foreign terpene synthases. To investigate the possibility of synergistic effects between the *upc2-1* mutation and trHmg1p overexpression, we combined both of these traits in the yeast strain EHY41, which expresses the trHmg1p reductase in the *upc2-1* background. Although neither trait alone was effective in promoting foreign sesquiterpene biosynthesis, the double-mutant EHY41 is our best sesquiterpene-producing yeast strain (Table 1).

Because the above experiments established that it is difficult to predict how genetic alterations impact foreign terpene biosynthesis, we reassessed mating type contributions in the presence of trHmg1p overexpression and the *upc2-1* mutation. We constructed the EHY42 yeast strain, which is otherwise identical to EHY41 but has the α mating type. As seen in wild type, the **a** strain biosynthesizes more epi-cedrol than the α strain (Table 1).

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Overexpressing FPP synthase (Erg20p) can alter sterol levels in yeast. Heterologous expression of the extrachromosomal ERG20 gene increased ERG20 activity 20% with a concomitant 28% increase in ergosterol.[28] We overexpressed FPP synthase in the wild-type **a** strain JBY575 and derivatives containing either *upc2-1*, trHmg1p, or both together. All of these strains generated similar amounts of epi-cedrol, but none produced significantly more epi-cedrol than the wild-type strain. Overexpressing FPP synthase does not increase sesquiterpene formation in the backgrounds we examined.

In our starting strain, sufficient FPP is available that 90 μg/L epi-cedrol can be biosynthesized by high-level expression of epi-cedrol synthase. Genetic optimization to increase FPP availability increased yields 4-fold. These yields are 6-fold higher than those seen in *E. coli*[10] and establish the potential of *S. cerevisiae* to generate milligram amounts of sesquiterpenes. This system should, in principle, be general to make any of the hundreds of known sesquiterpenes by expressing the appropriate sesquiterpene synthase in place of epi-cedrol synthase. Current yields are clearly insufficient for practical production. However, yields are more than adequate to support detailed NMR characterization of the product, and this system should provide the technically simplest route to characterize the products of putative sesquiterpene synthases.

It is important to note that although it was possible to increase the yield by manipulating key enzymes or regulatory proteins, the effects on sesquiterpene biosynthesis were generally less than those on sterol biosynthesis. These discrepancies could result from controls necessary to distribute flux through each branch of the isoprenoid pathway. FPP serves as a major branch point in sterol biosynthesis, and an FPP-derived signal participates as part of a feedback mechanism that decreases precursor biosynthesis.[29] Mevalonate kinase activity is decreased by FPP[30] and other isoprenoid precursors.[31] These regulatory mechanisms probably contribute to the difficulty in overproducing FPP, and further genetic optimization toward increased yields may focus on circumventing these controls.

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