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Protein Engineering of *Saccharomyces cerevisiae* Oxidosqualene-Lanosterol Cyclase into Parkeol Synthase

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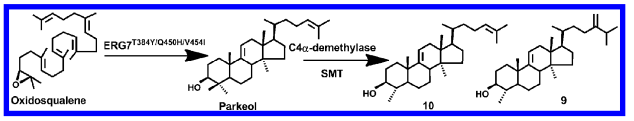
ABSTRACT



A *Saccharomyces cerevisiae* oxidosqualene-lanosterol cyclase mutant, ERG7$^{T384Y/Q450H/V454I}$, produced parkeol but not lanosterol as the sole end product. Parkeol undergoes downstream metabolism to generate compounds 9 and 10. In vitro incubation of parkeol produced a product profile similar to that of the in vivo experiment. In summary, parkeol undergoes a metabolic pathway similar to that of cycloartenol in yeast but distinct from that of lanosterol in yeast, suggesting that two different metabolic pathways of postoxidosqualene cyclization may exist in *S. cerevisiae*.

Natural evolution utilizes random mutagenesis coupled with selection pressure to generate new biocatalysts with structure or activity diversity. Protein redesign, based on sequence analysis and structure alignments as well as application of site-directed mutagenesis techniques, has complementarily facilitated construction of tailored enzymes with broadened substrate selectivity and/or product specificity.[1] Herein we describe the protein redesign of Saccharomyces cerevisiae oxidosqualene-lanosterol cyclase into parkeol synthase with 100% product specificity accuracy.

Oxidosqualene cyclases (OSCs) catalyze the conversion of a common substrate, acyclic (3*S*)-2,3-oxidosqualene (**OS**), into diverse tetracyclic or pentacyclic sterols and triterpenes, including lanosterol, cycloartenol, parkeol, β-amyrin and lupeol, etc.[2] Product diversity is mainly attributed to prefolded substrate conformation and by interactions between carbocationic intermediate and functional groups of catalytic amino acid residues of the enzyme during catalysis. Specifically, lanosterol, cycloartenol, and parkeol formations proceed via a chair−boat−chair prefolded substrate conformation and a protosteryl cation intermediate; whereas dammaradienol, β-amyrin and lupeol are biosynthesized via a chair−chair−chair conformation substrate and a dammarenyl, baccharenyl, and lupenyl cation, respectively. Another characteristic of OSC-catalyzed reactions is the accurate or multifunctional nature of their product formation profiles.[3a,b] The oxidosqualene-lanosterol cyclases (ERG7), oxidosqualene-cycloartenol synthases (CASs), β-amyrin synthases (BASs), lupeol synthases (LUP1s), marneral synthase (MRN1), and cucurbitadienol synthases make a single product or only minor byproducts (< 1% of total), whereas baruol synthase from Arabidopsis thaliana is able to make a remarkable number of products.[3b−d]

To date, several genes for accurate OSCs, including ERG7, CAS, and cucurbitadienol synthase, all of which catalyze the cyclization of **OS** into the protosteryl cation intermediate, have been cloned and sequenced.[4]

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Structure−reactivity−mechanism studies of *Sc*ERG7 have indicated that several amino acid residues, including Trp232, His234, Phe445, Tyr510, and Tyr707 are critical for product specificity, and mutations at these positions can result in the isolation of parkeol.[5] In parallel, a double site-directed mutant of OSC7 from Lotus japonicas (*Lj*OSC7), in which the Asn478 and Val482 (which correspond to Gln450 and Val454 of *Sc*ERG7, respectively) were changed to His and Ile of the CAS at the corresponding positions, resulting in the production of parkeol and cycloartenol.[6] Furthermore, random and directed evolution approaches to explore critical residues, many of which alter the product specificity of *Arabidopsis thaliana* CAS (*At*CAS) from cycloartenol production to lanosterol formation, established the importance of Tyr410 and Ile481 in the accurate cycloartenol biosynthesis.[5g,7] It was also further established that the mutation at His477 position generates an enzyme that makes parkeol (73%) as its major product. Interestingly, although the AtCAS$^{H477N/I481V}$ double mutations can cooperate to convert *At*CAS into an accurate *Sc*ERG7, the simultaneous mutational effect at Tyr410, His477, and Ile481 are not synergistic.[3c,7] On the other hand, a parkeol synthase from Oryza sativa was recently reported by Ito et al.[8] Nevertheless, the corresponding positions of Thr384 and Gln450 of *Sc*ERG7 were replaced with Tyr and Asp, respectively. To further substantiate the functional role of the *Sc*ERG7 Thr384, Gln450, and Val454 positions in affecting ERG7-catalyzed cyclization/rearrangement cascades toward lanosterol, cycloartenol, or parkeol production, we generated single, double, and triple mutations which bear Tyr, His, and Ile at positions 384, 450, and 454, respectively, and established the product profiles.

The ERG7^{T384Y}, ERG7^{Q450H}, and ERG7^{V454I} single mutations, ERG7$^{T384Y/Q450H}$, ERG7$^{Q450H/V454I}$, ERG7$^{T384Y/V454I}$ double mutations, and ERG7$^{T384Y/Q450H/V454I}$ triple mutations were generated using the QuikChange site-directed mutagenesis kit and transformed into the *S. cerevisiae* TKW14 strain, as previously described.[5c,e] The genetic selection results showed that all mutations supported ergosterol-independent growth of TKW14, except for the ERG7^{Q450H} single- and ERG7$^{T384Y/Q450H/V454I}$ triple-mutations. Subsequently, the nonsaponifiable lipids (NSL) from each mutant were extracted and applied to a GC−MS spectroscopy for product ratio determination.

Table 1. Product Profiles of *S. cerevisiae* ERG7$^{T384Y/Q450H/V454I}$ Single, Double and Triple Mutants

AA substitution	**2**	**3**	**4**	**5**	**6**	**7**	**8**
T384Y	63	31.5	3.5	–	–	1	1
Q450H	–	–	–	100	–	–	–
V454I	90	–	–	–	10	–	–
T384Y+Q450H	1	98	1	–	–	–	–
Q450H+V454I	75	–	–	–	16	–	9
T384Y+V454I	10	84	6	–	–	–	–
T384Y+Q450H+V454I	–	100	–	–	–	–	–

Table 1 shows the product profile produced from individual or collective mutations of the ERG7$^{T384X/Q450X/V454X}$. The three single-point mutants showed that the accurate enzymatic function of ERG7 was interrupted when residues with different characteristics were substituted into the respective active-site residues. The ERG7^{T384Y} single mutant produced lanosterol (**2**), parkeol (**3**), 9β-lanosta-7,24-dien-3β-ol (**4**), protosta-13(17),24-dien-3β-ol (**7**) and protosta-16,24-dien-3β-ol (**8**) in the ratio of 63:31.5:3.5:1:1 without compounds achilleol A (**5**) and (13αH)-isomalabarica-14(26),17*E*,21-trien-3β-ol (**6**). Specifically, the nonviable ERG7^{Q450H} single mutant produced minor amount of **5** as the sole product. Alternatively, the ERG7^{V454I} mutant produced poor amounts of **2** and **6** in a relative ratio of 90:10. Previously, Joubert et al. reported that the ERG7^{V454I} mutant produced 100% of **2**, yet no **6** was identified.[7e] The discrepancy of experimental results between the two studies may be attributed to the poor amount of the produced products and the lack of authentic sample for validation at that time, since compound **6** was first fully characterized after Joubert's publication.[7e] The ERG7$^{T384Y/Q450H}$ double mutant produced **2**, **3**, and **4** in the ratio of 1:98:1, whereas the ERG7$^{T384Y/V454I}$ yielded the same product profile in the relative ratio of 10:84:6. This result indicates that the double-position mutations at position 384 and either at position 450 or 454 have synergistic effects that favor the formation of parkeol. Finally, the ERG7$^{T384Y/Q450H/V454I}$ triple mutant further eliminated the biosynthesis of **2** and **4** and generated parkeol (**3**) as the sole product. Steady-state kinetic analysis revealed that the ERG7$^{T384Y/Q450H/V454I}$ mutant exhibited a 18-fold decrease in the V_{max}/K_M value for **OS** as compared to the wild-type enzyme.[7g] Therefore, the simultaneous substitutions on these three amino-acid residues effectively presented a dominant tendency for the generation of parkeol (Scheme 1).

A plausible explanation for this product specificity is the hypothetical H-bonding base dyad between His234 and Tyr510 and the function of either His234 or Tyr510 as a base to influence other active site interactions during the deprotonation reaction. The homology model of ERG7

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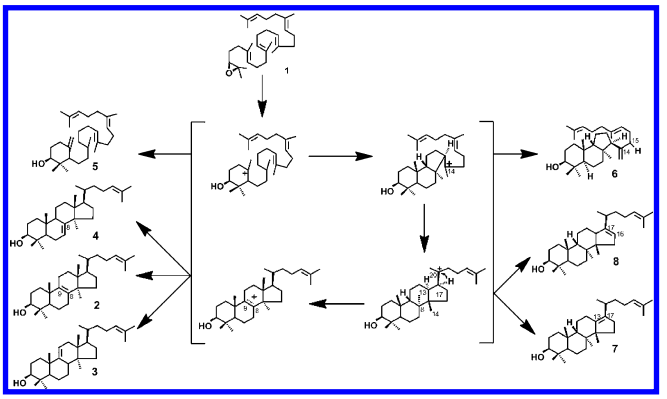
Scheme 1. Proposed Cyclization/Rearrangement Pathways of Oxidosqualene within *S. cerevisiae* ERG7$^{T384X/Q450X/V454X}$ Mutants



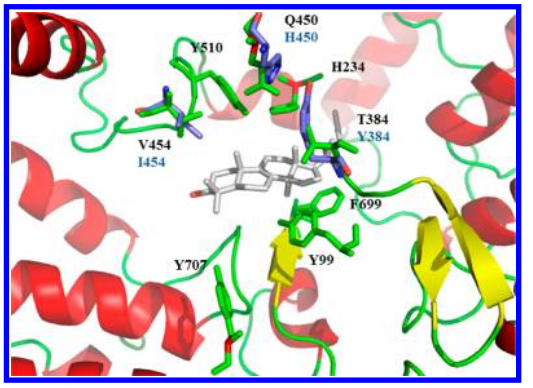


Figure 1. Local view of the superimposed homology model of S. cerevisiae ERG7$^{T384Y/Q450H/V454I}$ (blue) and ERG7^{WT} (green) complexed with protosteryl C-17 cation.

showed that Val454, Tyr510, Gln450, His234, and Thr384 are spatially proximally located to each other (Figure 1). Perhaps the substitution of Thr384 to Tyr, Gln 450 to His, and Val454 to Ile affected the His234:Tyr510 H-bonding base dyad interaction and subsequent deprotonation. Consistent with the observation is the increased production of achillolA when His was substituted for Gln450, and of parkeol when T384Y/Q450H double mutation was performed. Finally the T384Y/Q450H/V454I triple mutation may possibly tune the proper orientation for specific deprotonation reaction, thus producing parkeol as a sole product. However, exact contributions of the mutations on the product profile and proportions remain unclear and await X-ray structure determination of the mutated proteins.

The TLC trace of the ERG7$^{T384Y/Q450H/V454I}$ also identified two triterpene products with lower R_f values than that of the lanosterol/parkeol position. The NSL fractions of ERG7$^{T384Y/Q450H/V454I}$ were purified by $AgNO_3$-impregnated silica gel column for structure characterization using GC–MS and NMR (^{1}H and ^{13}C NMR, DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOE) spectroscopic techniques. The first compound was identified by NMR spectroscopy as 4α,14α-dimethyl-24-methylene-5α-cholest-9(11)-en-3β-ol (**9**), a product with a parkeol nucleus but only one methyl group remaining at the C-4 carbon, and its C-24 carbon presents a germinal-substituted double bond. The second compound was characterized as 4α,14α-dimethyl-5α-cholest-9(11),24-dien-3β-ol (**10**), a product with m/z of 412 and presents a 4-demethylated $\Delta^{9(11)}$ moiety of parkeol, based on closely matched values of NMR spectra relative to the previously mentioned compound, except for the position of the methyl group substitution. Specifically, the ^{13}C NMR spectrum revealed the presence of two pairs of tertiary–quaternary substituted double bonds (δ_c 125.96, 131.55 and 116.96, 147.32 ppm). Connectivities from HMBC, COSY, and distinct NOE spectra further confirmed the structure as **10** (see the Supporting Information). These two metabolites have been reported from an extract of Neolitsea aciculata and sea cucumber, respectively.[13] However, these two compounds are being reported for the first time in parkeol metabolic pathways within yeast.

In *S. cerevisiae*, the partial key transformations from lanosterol to ergosterol take place in the following order: (1) C-14 demethylation/C-15 deprotonation; (2) two consecutive C-4 demethylations; and (3) C-24 methylation/deprotonation.[9a] However, in the present study, parkeol metabolic products homologous to those of postlanosterol pathways were not detected in our experiments. The isolation of compounds **9** and **10** indicated that the postparkeol metabolism in *S. cerevisiae* proceeds first to remove a methyl group from the C-4 position to generate **10**, and is followed by the subsequent methylation/deprotonation at the C-24 position to obtain **9**. Recently, Ito et al. expressed an *Oryza sativa* cycloartenol synthase (AK121211) gene in yeast and elucidated a metabolic pathway of cycloartenol similar to the present study but different from that of previously established by Nes et al.[8,9b] Taken together, these findings indicate that two different metabolic pathways of postoxidosqualene cyclization may exist in *S. cerevisiae* and that parkeol and cycoartenol may drive a distinct metabolic pathway from that of lanosterol (Scheme 2).

To unequivocally characterize the parkeol metabolic pathway in *S. cerevisiae* in vitro, cell-free extract of the yeast HEM1 ERG7 double-knockout strain, TKW14, was incubated with exogenous parkeol, and the product profile was determined by GC–MS. As expected, neither compound **9** nor **10** could be detected in the cell-free extract of TKW14 alone. In contrast, a mixture of compounds **9** and **10** could be clearly detected following incubation of parkeol with cell-free extract of TKW14 for 24 h (Figure 2). The results support the hypothesis that a distinct parkeol metabolic pathway, which is first demethylated to produce **10** and subsequently methylated/deprotonated at C-24 to obtain **9**, exists in *S. cerevisiae*. This is the first demonstration of the functional conversion of an oxidosqualene-lanosterol cyclase mutant into parkeol synthase with 100% product specificity accuracy.

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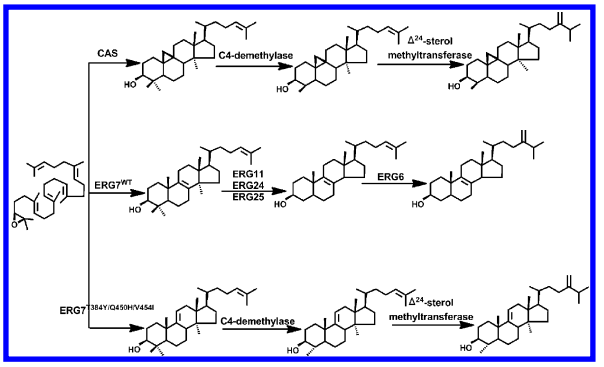
Scheme 2. Proposed Metabolic Pathway of Lanosterol, Cycloartenol[8], and Parkeol in *S. cerevisiae*



The triterpene portions of both sterols and holothurins, the latter typically containing a $\Delta^{9(11)}$-double bond and a 14α-methyl substitute, isolated from sea cucumber have been demonstrated as being derived from parkeol.[10] Holothurin saponins exhibit potent biological activity, including hemolytic, antitumor, anti-inflammatory, and antibacterial activities.[11] Furthermore, parkeol and its derivatives, such as spheciosterol sulfates A−C and topsentiasterol sulfate E, have shown important biological activities in inhibiting NF-κB and PKC activation.[10b,c,12] Therefore, molecular cloning of the native parkeol synthase gene or genetic engineering of oxidosqualene-lanosterol cyclase into parkeol synthase creates an opportunity for utilizing parkeol as a core framework for the tailoring enzymatic modification in the development of compounds with potential bioactivity.

In summary, a *S. cerevisiae* ERG7$^{T384Y/Q450H/V454I}$ mutant with 100% of altered product specificity from lanosterol to parkeol was generated for the first time. Parkeol undergoes a metabolic pathway similar to that of cycloartenol in yeast but distinct from that of lanosterol in yeast, by first performing demethylation at the C-4 position and subsequent methylation/deprotonation at the C-24 position to generate **10** and **9**, respectively. Genetic engineering of ERG7 into parkeol synthase expands the enzymatic activity of oxidosqualene-lanosterol cyclase and opens a new avenue for obtaining parkeol as a substrate for tailored enzymatic modifications in the development of parkeol-derived compounds with potential bioactivity. Further studies should focus on tailored parkeol modification and engineering oxidosqualene-lanosterol cyclases that generate diverse new enzymatic functions.

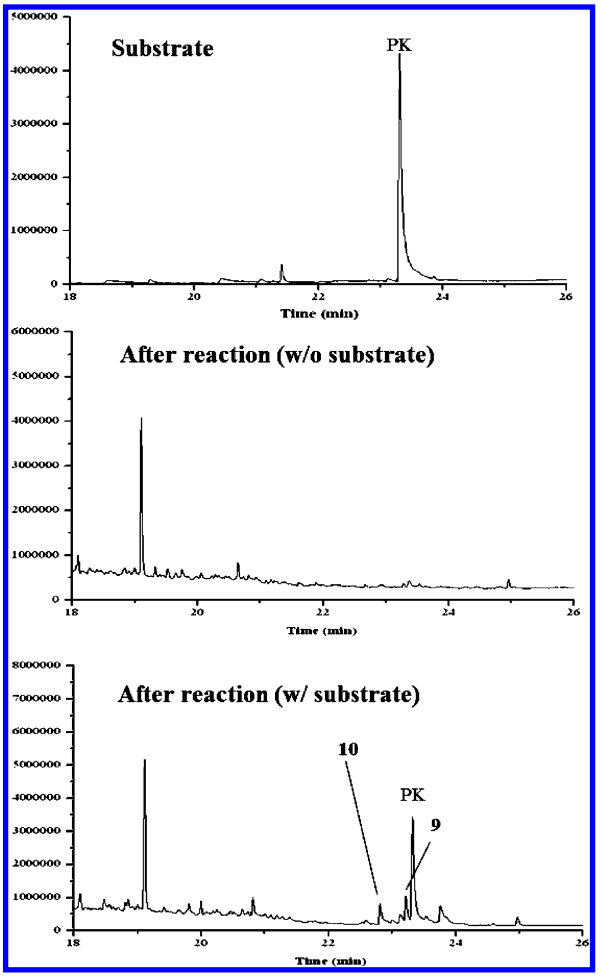


Figure 2. GC−MS profile of the in vitro enzymatic reaction products of TKW14[ERG7] strain.

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Supporting Information Available. Full experimental and characterization data, including ^{1}H and ^{13}C NMR spectra, for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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