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Importance of *Saccharomyces cerevisiae* Oxidosqualene-Lanosterol Cyclase Tyrosine 707 Residue for Chair-Boat Bicyclic Ring Formation and Deprotonation Reactions

Tung-Kung Wu,* Tsai-Ting Wang, Cheng-Hsiang Chang, Yuan-Ting Liu, and Wen-Shiang Shie

Department of Biological Science and Technology, National Chiao Tung University, 300, Hsin-Chu, Taiwan (Republic of China)

tkwmll@mail.nctu.edu.tw

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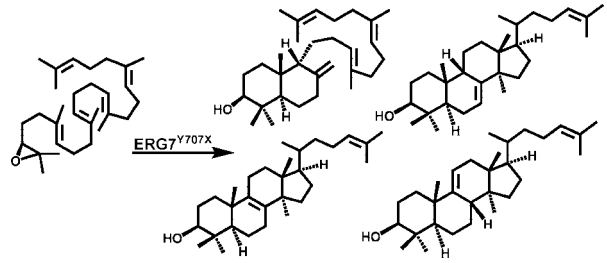
ABSTRACT



A contact mapping strategy was applied to identify putative amino acid residues that influence the oxidosqualene-lanosterol B-ring cyclization reaction. A bicyclic intermediate with two altered deprotonation products, in conjunction with lanosterol, were isolated from the ERG7^{Y707X} mutants, indicating that the Tyr707 residue may play a functional role in stabilizing the chair-boat bicyclic C-8 cation and the lanosteryl C-8/C-9 cation intermediates.

Enzyme active sites provide highly optimized microenvironments for enzyme activity. Changes at the active site center can have large effects on enzyme activity and provide a promising way to access new reactive functionalities.[1] However, in order to redesign proteins to acquire a new or higher specificity and activity, it is a prerequisite to identify important plasticity residues in the active site cavity with the capability of producing diverse product profiles. In parallel, the characterization of mutated amino acids that successfully alter product specificity forms the basis of understanding the contribution that specific amino acids make to catalysis. We report herein the elucidation of plasticity residues within *Saccharomyces cerevisiae* oxidosqualene-lanosterol cyclase (ERG7), which represents the first step in undertsanding the enzyme's structure−function relationships and opens up the possibility of generating new reactivities.

The oxidosqualene-lanosterol cyclase-templated cyclization/rearrangement of (3*S*)-2,3-oxidosqualene (**OS**, **1**) to lanosterol (**LA**, **2**) proceeds through a series of discrete, conformationally rigid, partially cyclized carbocation intermediates.[2−5] The reaction for **LA** formation is characterized by a precise chair-boat (C−B) B-ring conformation, 6-6-

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6-5 tetracyclic protosteryl cation formation, hydride and methyl group migration to the lanosteryl C-8/C-9 cation, and highly specific deprotonation. The formation of pentacyclic β-amyrin, catalyzed by β-amyrin synthase, proceeds similarly with the exception of chair-chair (C−C) bicyclic conformation, and the cationic cyclization of the tetracyclic dammarenyl cation, which is followed by annulation of a fifth ring.

Among various triterpene biosyntheses, the concerted or nonconcerted A/B ring cyclization mechanism and the C-B versus C-C B-ring conformation have been of specific interest to most researchers.[3,6] In the oxidosqualene-lanosterol cyclase-catalyzed cyclization, although it was proposed that expoxide opening was concerted with A-ring formation or even B-ring formation, truncated monocyclic cyclization products have been isolated from various *S. cerevisiae* ERG7 mutants.[4a,5a−d,6a] Furthermore, although B-ring boats are well-represented in triterpenoids with three or more rings, biosynthesis of bicyclic triterpene alcohol has only been achieved with C-C *trans*-decalin derivatives.[7] No known bicyclic triterpene alcohols derived from a C-B bicyclic cation have been reported. The energetically unfavorable boat conformation in oxidosqualene-lanosterol cyclase and oxidosqualene-cycloartenol synthase is probably achieved by enzyme residues through a steric and/or an electronic effect.[8] Recently, we have reported on the isolation of enzymatic products derived from the carbocationic intermediates of monocyclic, tricyclic, tetracyclic, truncated-rearranged, and altered deprotonated products produced by single amino acid replacement of Tyr99, Trp232, His2434, Phe445, Tyr510, or Phe699 in *S. cerevisiae* ERG7.[5] Previously, it was predicted that the human oxidosqualene-lanosterol cyclase's (human OSC) Tyr98 residue (corresponding to Tyr99 in *S. cerevisiae* oxidosqualene-lanosterol cyclase, ERG7) was spatially positioned to enforce the energetically unfavorable B-ring boat conformation of **OS** by pushing the methyl group at C-8 below the molecular plane.[8] However, our site-saturated mutagenesis experiments established that the probable function of ERG7 Tyr99 is to achieve both the C-B tricyclic Markovnikov C-14 (lanosterol numbering) cation stabilization and the regio- or stereochemical control of the protons at the C-15 position for subsequent deprotonation, but not B-ring formation.

We previously generated ERG7 wild-type and mutant homology models, based on human OSC X-ray crystal structure, to investigate the effects of amino acid substitutions on the cyclization mechanism and product profiles.[5] To explore the plasticity residues involved in **OS** B-ring cyclization, we applied a contact mapping strategy that aims to probe amino acids within van der Waals radii distance of the C-8 carbon of **OS** or **LA**, based on the assumption that amino acids within the active site are most likely to contribute to a catalytic outcome. Mutational studies of plasticity residues may provide insight into the role of these residues in the determination of the B-ring configuration or in generating an evolved enzyme with new reactivities. Among the various putative ERG7 C-8 contact mapping residues under investigation, the Tyr707 residue is of particular interest and was therefore subjected to genetic selection and product characterization. In this study, we describe the identification of the ERG7 Tyr707 residue as a crucial component for B-ring stabilization and/or final deprotonation, as well as the characterization of the mutational effects on product specificity/diversity.

We first generated ERG7$^{\Delta Y707}$ deletion and ERG7^{Y707X} site-saturated mutations and expressed them in a yeast HEM1 ERG7 double-knockout mutant TKW14, as previously described.[5] The genetic selection results of Tyr707X mutants showed that most mutations at Tyr707 cannot abolish the activity of ERG7 cyclase with the exception of the Tyr707Arg mutation (Table 1), indicating that the Tyr707 mutations are not detrimental to the catalytic activity of ERG7. However, the loss of activity of ERG7$^{\Delta 707}$ deletion mutant suggested that the existence of Tyr707 is essential for the catalytic function of ERG7. Next, the TKW14[pERG7^{Y707X}] mutant strains were cultured and nonsaponifiable lipid (NSL) extracts were isolated for product characterization. Silica gel column chromatography coupled with thin layer chromatography (TLC) and gas chromatography−mass spectrometry (GC-MS) were used to analyze the triterpenoid products with a molecular mass of 426 Da. The ERG7^{Y707X} mutants showed one to four distinct products. Three products were identified as authentic **LA** (**2**), 9β-lanosta-7,24-dien-3β-ol (**3**), and parkeol (**4**) by comparison with authentic standards using ^{1}H and ^{13}C NMR as well as GC-MS. A fourth distinct compound, with a 1.5 min reduced retention time compared to **LA** on the GC column, was isolated and further character-

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Table 1. Product Profiles of *S. cerevisiae* TKW14 Expressing the ERG7$^{\Delta Y707}$ Deletion and ERG7^{Y707X} Site-Saturated Mutants[9]

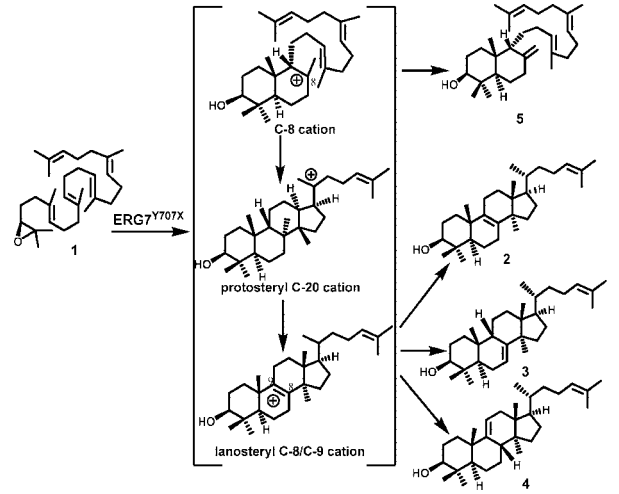
ERG7^{mut}	**5**	**2**	**3**	**4**
Y707H	83.6	4.7	6.2	5.5
Y707Q	82	4.3		13.7
Y707E	12.3	70.8	10.5	6.4
Y707D	21.8	67.3	7.6	3.3
Y707T	9.8	65.1	13.5	11.6
Y707S	21.3	42.9	20.5	15.3
Y707C	6	47.1	42.6	4.3
Y707G	28.3	48.2	16.3	7.2
Y707A	18.9	56.9	19.9	4.3
Y707L		85.3	14.7	
Y707I		87.9	12.1	
Y707F		89.2	7.7	3.1
Y707V		100		
Y707P		100		
Y707M		100		
Y707N		100		
Y707W		100		
Y707K		100		
Y707R		No new product		
Δ707				

ized with NMR (^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC, ^{1}H $-^1$H COSY and NOE) and demonstrated to be (9*R*,10*S*)-polypoda-8(26),13*E*,17*E*,21-tetraen-3β-ol (**5**) based on the following data. The compound showed a distinct ^{1}H NMR chemical shift with three unseparated olefinic protons (δ 5.163, 3H) and two methylene protons (δ 4.576, δ 4.748), as well as five methyl singlets (δ 0.802, 0.952, 1.023, 1.614, 1.720) and two unseparated methyl singlets (δ 1.644). The ^{13}C NMR spectrum revealed the presence of one secondary-quaternary (δ = 109.42, 149.26 ppm) and three tertiary-quaternary substituted double bonds (δ = 124.96, 134.94; 124.45, 135.05; and 124.54, 131.34 ppm). The HSQC spectrum showed that the olefinic methylene protons at δ 4.576 and 4.748 are attached to the carbon at 149.26 ppm. The aforementioned NMR information indicated a bicyclic triterpene alcohol with three double bonds on the side chain as well as a double bond of two germinal protons. Furthermore, the presence of NOEs among Me-24/Me-25, Me-25/H-26, Me-25/H-1, Me-25/H-9, Me-23/H-3, Me-23/H-2, and Me-23/H-5, as well as the absence of NOEs among Me-23/H-9, Me-23/ Me-25, Me-24/H-3, Me-24/H-5, and Me-25/H-5, confirmed the structure to be **5**, a C-B 6-6 bicyclic product with a C-9α hydrocarbon side chain configuration and $\Delta^{8(26),13,17,21}$ double bonds. This is the first reported isolation of a bicyclic triterpene alcohol derived from a C−B bicyclic cation, although a stereoisomer with a C-9β hydrocarbon side chain configuration has previously been reported as a natural product isolated from the bark of *Cratoxylum cochinchinense*.[7b] Comparison of the ^{1}H and ^{13}C NMR spectra of these two stereoisomers further confirmed the structural assignment.

The product profiles of each mutant are summarized in Table 1. No products with *m*/*z* = 426 were observed in the nonviable mutants, ERG7^{Y707R} and ERG7$^{\Delta 707}$. However, in the viable mutants, nine of them (Y707X, X = H, Q, D, E, S, T, C, G, and A) produced other products besides **2**, including two altered deprotonation products, **3** and **4**, as well as the novel bicyclic product, **5**. In the ERG7^{Y707Q} and ERG7^{Y707H} mutants, large amounts of **5** were generated as the major product, while **2** was the major product in the other viable mutations. The ERG7^{Y707L}, ERG7^{Y707I}, and ERG7^{Y707F} mutants produced only **2**, **3**, and a slight amount of **4**. Six mutations (Y707X, X = V, P, M, N, W, K) produced only **2** in line with the wild-type ERG7. Interestingly, most nonpolar amino acid substitutions formed **2** or other altered deprotonation products but no truncated cyclization product.

The product profile of the ERG7^{Y707X} mutants suggested that **OS** is folded in a chair-boat-chair conformation and initiates ring cyclization through opening of the oxirane ring and subsequent A-ring annulation (Scheme 1). A bicyclic

Scheme 1. Proposed Cyclization/Rearrangement Mechanism Occurring in the ERG7^{Y707X} Site-Saturated Mutants



C-8 cation (lanosterol numbering) was formed without disruption at the monocyclic C-10 cation position, and it was followed by deprotonation of Me-26 to produce C-B truncated **5**. Further cyclization of the C- and D-ring proceeded to produce a tetracyclic protosteryl C-20 cation. Then skeletal rearrangement of the two hydrides and two methyl groups, H-17α → 20α, H-13α → 17α, Me-14β → 13β, Me-8α → 14α, and/or one hydride shift from H-9β to H-8β generated the lanosteryl C-8/C-9 cation, which underwent deprotonation at C-8, C-7, and C-11 to yield **2**, **3**, and **4**, respectively.

Tyr707 of ERG7 is a strictly conserved residue in both squalene cyclases (SHCs) and oxidosqualene cyclases, and corresponds to Tyr609 in SHC from *Alicyclobacillus acidocaldarius* and Tyr704 in human OSC. Tyr609 of SHC has been previously suggested to assist the function of Phe365 (corresponds to Phe445 of ERG7), to stabilize the C-8 cation via cation-π interaction, by both an electronic and steric effect. Substitution of the Tyr609 of SHC with Phe, Ala, Leu, Cys, and Ser caused a perturbation in the active site

and lowered the π-electron density around the C-8 position, thus quenching the cyclization at the bicyclic stage and resulting in truncated bicyclic product formation.[7d−g] The homology modeling of ERG7 revealed that Tyr707 is spatially proximal to both the C-10 and C-8 positions of **2**, with the hydroxyl group of the phenolic side chain pointing toward the B-ring capable of stabilizing the bicyclic cationic intermediate (Figure 1). The phenolic oxygen of Tyr707 was found at a distance of 5.6 and 5.1 Å from C-10 and C-8 of **2**, respectively. Interestingly, a distance of ~6.2 Å was observed between the phenolic oxygen of Tyr707 and the bicyclic C-8 cation intermediate.

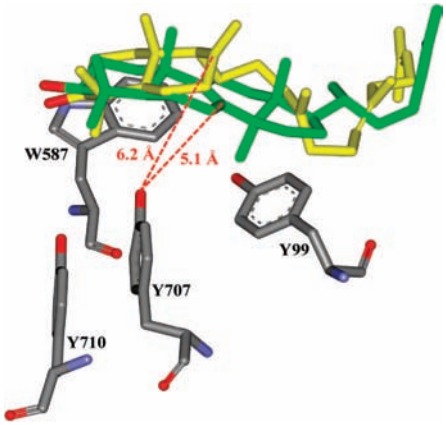


Figure 1. Homology model of wild-type ERG7 complexed with lanosterol (green) or bicyclic C-8 cation (yellow). The distance of Tyr707 to the C-8 position of lanosterol or bicyclic C-8 cation is 5.1 and 6.2 Å, respectively.

The product profile analysis of ERG7 mutants revealed that the truncated bicyclic product accumulated with polar, acidic, or smaller hydrophobic side chain substitutions. Substitutions of Tyr707 with polar or acidic side chain groups may reduce polarity and allow interaction with the bicyclic C-8 cation or other residues nearby. Consistent with the results is the observation that in human OSC, the Tyr704 is H-bonded to the backbone carbonyl group of Trp581 (corresponding to Trp587 of ERG7) through a water bridge. The reduced polarity may destroy the H-bonding of Tyr707 to the backbone carbonyl group of Trp587 or form a new interaction with other residues, thus destabilizing the bicyclic cation or disturbing the active site structure. Alternatively, substitution of Tyr707 with small hydrophobic side chains such as Gly or Ala, resulting in additional space in the cavity and complete loss of polarity, may disturb the H-bonding pattern with Trp587 and direct compound **5** formation. Interestingly, there were significant accumulations of **5** in Tyr707His and Tyr707Gln mutations, whereas no truncated or altered deprotonation product could be identified in the Tyr707Asn mutation. A possible explanation for this behavior is the similarity in H-bonding but with different orientation of His and Gln, thus deviating the electron density distribution for truncated bicyclic product formation. For the Tyr707Asn mutation, although a deleted methylene group from the side chain of Gln may enlarge the active site cavity, the generated space may be enough to insert a water molecule, thus compensating for the lost interaction and restoring the product profile. It is interesting to note that Tyr707Ile, Tyr707Leu, and Tyr707Phe mutants make altered deprotonation products. The mutations may destroy the original H-bonding but retain the ordered water with a slight shift in active site geomentry. This in turn could cause relocation of the final acceptors and result in altered deprotonation product formation. To our surprise, most nonpolar amino acid substitutions formed **2** without any truncated cyclization product. Perhaps the loss of H-bonding in these mutants did not cause an obvious interference in the active-site cavity with water retention, thus following the normal pathway to **2** formation. However, the precise molecular interactions between the enzymatic active site and the specific product profile still awaits final X-ray structure determination.

In summary, oxidosqualene cyclase mutants producing a truncated C-B bicyclic cyclization intermediate were isolated for the first time for **LA** biosynthesis. Formation of the truncated bicyclic triterpene alcohol as well as altered deprotonation products in the ERG7^{Y707X} mutants indicated that the Tyr707 residue plays a functional role in stabilizing the bicyclic C-8 cation and final lanosteryl C-8/C-9 cation intermediates. This result provides new structure−function relationship information indicating how enzymes act to control the cyclization/rearrangement mechanism. However, the Tyr707 mutations did not affect the B-ring boat conformation, indicating the importance of other residue(s) in controlling C-B versus C-C substrate conformation. Further studies should focus on characterizing the residues that influence substrate C−B versus C−C conformation and divergent evolution of new enzymatic functions.

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Supporting Information Available: Details of experimental procedures, GC-MS and NMR spectra, and and NMR assignments of (9*R*,10*S*)-polypoda-8(26),13*E*,17*E*,21-tetraen-3β-ol. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) The TKW14[pERG7^{Y707X}] strain expressed ERG7^{Y707X} as the only oxidosqualene cyclase. The in vivo product profiles represent metabolic equilibrium of lanosterol and related triterpenes under physiological conditions, which may vary from that of in vitro assay.