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Directed Evolution To Generate Cycloartenol Synthase Mutants that Produce Lanosterol

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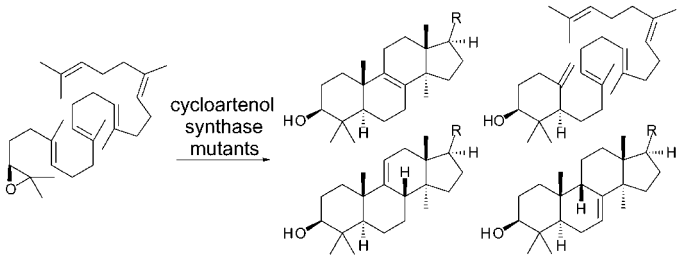
ABSTRACT



Cycloartenol synthase converts oxidosqualene to cycloartenol, a pentacyclic isomer of the animal and fungal sterol precursor lanosterol. We used directed evolution to find cycloartenol synthase residues that affect cyclopropyl ring formation, selecting randomly generated cycloartenol synthase mutants for their ability to genetically complement a yeast strain lacking lanosterol synthase. To increase the likelihood of finding novel mutations, the little-studied *Dictyostelium discoideum* cycloartenol synthase was used for the mutagenesis. Several catalytically important residues were identified.

Oxidosqualene cyclases convert oxidosqualene (Scheme 1, **1**) through carbocationic intermediates to nearly 100 different cyclic triterpene alcohols.[1] These enzymes generate triterpenoid diversity by controlling which proton is abstracted from a carbocationic intermediate. For example, both lanosterol synthase and cycloartenol synthase cyclize oxidosqualene to the protosteryl cation (**2**) and then promote rearrangement to the lanosteryl 8-cation (**3**). Lanosterol synthase abstracts the proton originating at C-9 to form lanosterol (Scheme 1, **4**), either directly from C-9 or after a hydride shift from C-9 to C-8. Cycloartenol (**5**) biosynthesis requires a hydride shift from C-9 to C-8, followed by cyclopropyl ring formation and deprotonation from C-19 (Scheme 1).

We previously identified two *Arabidopsis thaliana* cycloartenol synthase[2] (*At*CAS1) residues that contribute to the catalytic difference between cycloartenol synthase and lanosterol synthase. A selection for randomly generated *At*CAS1 mutants that genetically complement a yeast lanosterol synthase deletion strain uncovered an Ile481Val mutant that converts oxidosqualene to a mixture of cycloartenol, lanosterol, and parkeol (**6**) (55:24:21).[3] The strict differential conservation at this position (Ile is conserved in cycloartenol synthases and Val is conserved in lanosterol synthases, Figure 1) shows that the residue at this position contributes to the catalytic difference between native cycloartenol synthase and lanosterol synthase. At four other positions (*At*CAS1 Tyr410, Gly488, Phe717, and Met731) cycloartenol synthases strictly conserved one residue and the known lanosterol synthases conserved another. We generated *At*CAS1 derivatives with these residues mutated to their lanosterol synthase counter-

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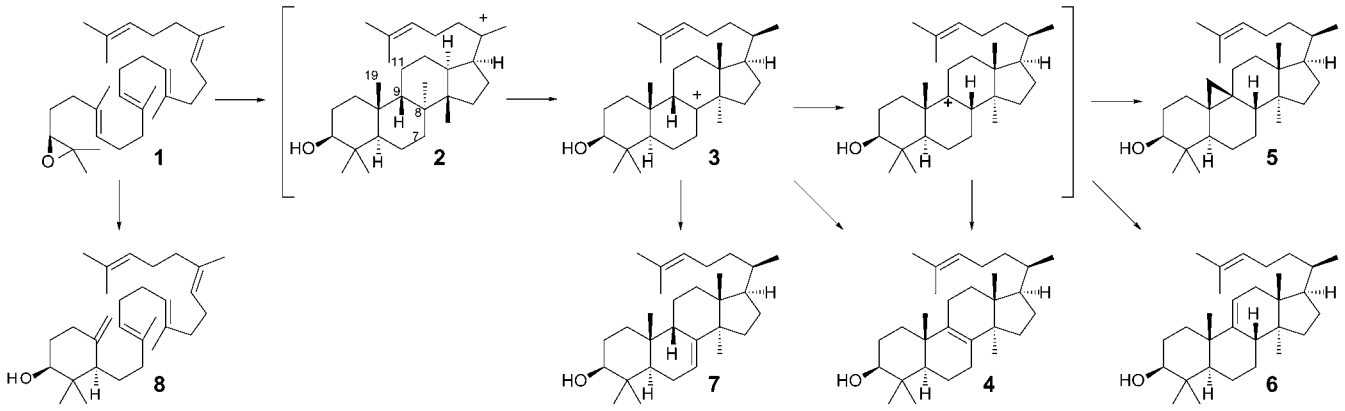
Scheme 1. Cyclization and Rearrangement of Oxidosqualene (**1**) to Cyclic Triterpene Alcohols; Lanosterol (**4**) Accessible in Principle by Either Deprotonating the C8 or C9 Cation or a Bridged Structure



parts to identify those that control product structure. Only the Tyr410Thr mutant had an altered product profile, forming lanosterol, 9β-lanosta-7,24-dien-3β-ol (Scheme 1, **7**), and parkeol (65:33:2).[4] This mutation is cooperative with the Ile481Val mutation; the Ile481Val Tyr410Thr double mutant biosynthesizes lanosterol more efficiently (75:24:0.6),[4] but deprotonation from C7 and C11 also occurs.

Because all positions with strict differential conservation had been examined, we revisited directed evolution to identify other catalytically relevant positions. To increase the chance of obtaining mutations different from the Ile481Val found in the initial selection of*At*CAS1 mutants, we used the *Dictyostelium discoideum* ortholog (*Dd*CAS1). Randomly mutated libraries[5] of *Dd*CAS1 were transformed into the yeast lanosterol synthase deletion mutant SMY8.[6] Yeast transformants, marked by uracil prototrophy, were induced and selected on plates containing galactose and lacking ergosterol.[7] The mutant enzymes that acquired lanosterol biosynthetic ability were expected to allow SMY8 to grow in the absence of ergosterol.

Nonsaponifiable lipid was obtained from strains that grew without exogenous sterol. Yeast metabolizes lanosterol rapidly to ergosterol, so the presence of ergosterol (evidenced by GC−MS) in each positive strain confirmed its ability to biosynthesize lanosterol. SMY8 was retransformed with each mutant construct, and the transformants were rescreened by the same complementation assay to ensure that the plasmids provided the sterol independence. Plasmids recovered from positive strains were sequenced, and each had a single mutation.

Eighteen strongly complementing plasmids were isolated from 10,000 transformants. Each had one of four muta-

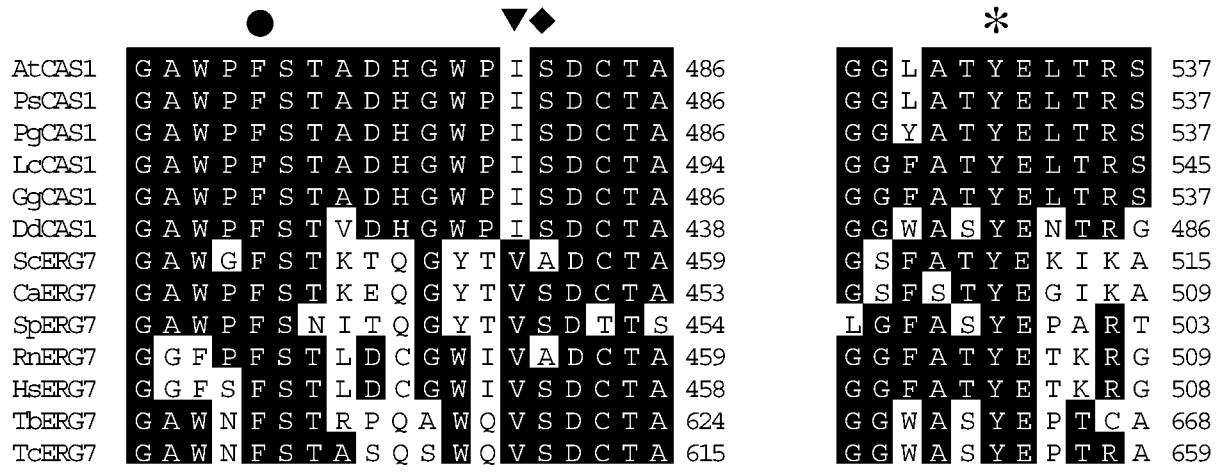


Figure 1. Conservation pattern of the mutated residues. Ile481 (▼) is differentially conserved as Ile in cycloartenol synthases (CAS1) and Val in lanosterol synthases (ERG7). Phe472 (●) and Tyr532 (*) are conserved in the known cycloartenol synthases and lanosterol synthases, and Ser482 (◆) is nearly conserved. Cycloartenol synthases are from *A. thaliana* (At),[2] *Pisum sativum* (Ps),[12a] *Panax ginseng* (Pg),[12b] *Luffa cylindrica* (Lc),[12c] *Glycyrrhiza glabra* (Gg),[12d] and *D. discoideum* (Dd).[12e] Lanosterol synthases are from *S. cerevisiae* (Sc),[13c,d] *Candida albicans* (Ca),[13a,b] *Schizosaccharomyces pombe* (Sp),[6] *Rattus norvegicus* (Rn),[13e,f] *Homo sapiens* (Hs),[13g,h] *Trypanosoma brucei* (Tb),[13i] and *Trypanosoma cruzi* (Tc).[13j]

tions: *Dd*CAS1 Tyr363Cys, Phe424Ser, Ser434Pro, and Tyr481His. Phe424Ser arose only once, but the others were observed repeatedly. Because all previous analyses of cycloartenol synthase mutants had been done in *At*CAS1, we maintained consistency by generating the four corresponding *At*CAS1 mutants using site-specific mutagenesis: Tyr410Cys, Phe472Ser, Ser482Pro, and Tyr532His. The *At*CAS1 Tyr410Cys and Tyr532His mutants genetically complemented the sterol auxotrophy in SMY8, allowing growth comparable to the wild-type lanosterol synthase cDNA expressed identically in SMY8 (colonies formed after 2 days). However, *At*CAS1 Phe472Ser complemented less efficiently (colonies formed in 7 days), and yeast that were dependent on *At*CAS1 Ser482Pro for sterol did not generate colonies after 2 weeks. At all four mutated positions, *At*CAS1 and *Dd*CAS1 conserve the same residue, and *At*CAS1 could acquire single nucleotide mutations parallel to those observed in *Dd*CAS1. The strongly complementing *At*CAS1 Tyr410Cys and Tyr532His mutants did not appear in an earlier screen for spontaneously occurring mutations.[3a] This probably reflects the higher mutagenesis rate in the current study rather than differences between the two genes.

The *At*CAS1 Tyr410Cys mutant converts oxidosqualene to lanosterol, 9β-lanosta-7,24-dien-3β-ol, and achilleol A (**8**) (75:24:1).[8] This product composition is readily rationalized in light of previous experiments. Tyr410 is conserved in cycloartenol synthases and corresponds to a Thr that is conserved in animal and fungal lanosterol synthases. This position was shown previously to be catalytically important in both cycloartenol synthase[2,12] and lanosterol synthase.[6,13] The *At*CAS1 Tyr410Thr mutant does not produce cycloartenol but instead lanosterol, 9β-lanosta-7,24-dien-3β-ol, and parkeol (65:33:2).[4] Mutating the corresponding Thr384 residue in yeast lanosterol synthase to the Tyr conserved in cycloartenol synthase compromised selectivity. Although lanosterol remained the major product, parkeol and lanost-24-ene-3β,9α-diol appeared as byproducts.[9] Neither cycloartenol synthase nor lanosterol synthase has been structurally characterized, but the structure of a related enzyme known as squalene-hopene cyclase (SHC)[10] has been solved to 2.0 Å resolution, and Tyr410 corresponds to the active-site SHC Ser307 residue.[11] The *At*CAS1 Tyr410Cys mutant makes significant amounts of lanosterol because the substituted Cys sterically and electronically resembles the Thr that is important in animal and fungal lanosterol synthases.

*At*CAS1 Tyr532His does not biosynthesize cycloartenol but a mixture of 45% lanosterol, 31% parkeol, and 24% achilleol A. *At*CAS1 Tyr532His is less efficient than native *At*CAS1, generating ~5 mg/L culture (~10% the yield from native enzyme). Tyr532 is strictly conserved in both cycloartenol synthase[2,12] and lanosterol synthase[6,13] and therefore probably does not play a role specific to cyclopropyl ring formation. Instead, the formation of substantial amounts of the monocyclic achilleol A in the Tyr532His mutant suggests that Tyr532 may facilitate cyclization to the tetracyclic cation. Interestingly, Trp is conserved at the corresponding position in enzymes that initially cyclize to the dammarenyl cation, and the Tyr/Trp dichotomy may influence not only the number of rings that form but also the stereochemistry of intermediate tetracyclic cations. *At*CAS1Tyr532 corresponds to the active site SHC Tyr420 residue, and mutating SHC Tyr420 also generated partially cyclized products.[14] The *At*CAS1 Tyr532His mutation neither introduces (as does the Ile481Val mutation) nor structurally mimics (as does the Tyr410Cys mutation) a lanosterol synthase residue yet allows lanosterol biosynthesis. Deprotonation to lanosterol and parkeol rather than ring-closure to cycloartenol may be an indirect effect. Tyr532His is compatible with lanosterol biosynthesis, but the absence of this substitution in native lanosterol synthases suggests that His may compromise accurate, efficient lanosterol production.

Like Tyr532, Phe472 is strictly conserved in both cycloartenol and lanosterol synthases and consequently does not contribute to the catalytic difference between native lanosterol synthases and cycloartenol synthases. *At*CAS1

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(5) Mutant libraries were generated by propagation in the mutagenic *E. coli* strain XL1-Red (Stratagene) according to the manufacturer's instructions.

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(8) Each enzyme was assayed in vitro using synthetic oxidosqualene and protein expressed from the galactose-inducible vector pRS305GAL in the yeast strain LHY4 as described previously.[3] Catalytically efficient mutants typically provided >50 mg product from a crude yeast lysate generated from 1 L of culture. Because each polycyclic triterpene has multiple distinctive methyl signals, known triterpene alcohols were readily identified by ^{1}H NMR of a partially purified mixture. Acetate derivatives were prepared, and GC−MS analyses with comparison to authentic samples were performed to ensure that no oxidosqualene cyclase products were overlooked in the NMR. GC−FID was used for quantitation.

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Phe472 corresponds to the catalytically important active site Phe365 residue in SHC.[15] The *At*CAS1 Phe472Ser mutant has severe catalytic defects. Although in vitro reactions provided insufficient material (<5 μg/L culture, <0.01% the yield from native enzyme) to rigorously characterize the products by ^{1}H NMR,[16] its ability to complement the lanosterol synthase mutant implies that Phe472Ser promotes low-level lanosterol biosynthesis.[17] Enzymes with a Phe472Ser probably would not be efficient enough to support competitive growth in nature.

*At*CAS1 Ser482 is strongly but incompletely conserved, being present in all but the *Saccharomyces*[13c,d] and rat[13e,f] lanosterol synthases (which have Ala substitutions). *At*CAS1 Ser482 corresponds to the SHC active-site Val375 residue. In vitro reactions did not provide any evidence of oxidosqualene cyclization. This difference in behavior between the *Dd*CAS1 Ser434Pro mutant and the parallel *At*CAS1 Ser482Pro mutant highlights the importance of maintaining identical backgrounds when comparing the effects of mutations.

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(16) ^{1}H NMR is essential for reliable characterization because many triterpene alcohols have similar chromatographic and fragmentation properties (lanosterol and its Δ-7 isomer are essentially indistinguishable by GC−MS).

(17) Our experience with expressing lanosterol synthases indicates that the yeast complementation assay is considerably more sensitive than GC for identifying low levels of lanosterol biosynthesis.

These experiments show that random mutagenesis and selection can uncover a variety of mutations that allow a cycloartenol synthase to biosynthesize lanosterol and thereby genetically complement a yeast lanosterol synthase mutant. Each mutation maps to the active site. The substitutions generated by directed evolution are absent in native lanosterol synthases and are probably incompatible with efficient, accurate lanosterol biosynthesis. Tyr410Cys mimics the Thr in native lanosterol synthase, but Phe472Ser and Tyr532His replace residues that are strictly conserved (or nearly so) in both cycloartenol and lanosterol synthases. The latter two mutations are perhaps best viewed as generating enzymes that have broadened specificity and make lanosterol as one of the products. The tetracyclization and rearrangement of oxidosqualene to cation **3** appear to be more robust than the ring closure from **3** to cycloartenol, and mutations in residues with conservation patterns that seem inconsistent with a specific role in cyclopropyl ring formation can disrupt cyclopropyl ring formation while allowing tetracyclization.

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