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Steric Bulk at Position 454 in *Saccharomyces cerevisiae* Lanosterol Synthase Influences B-Ring Formation but Not Deprotonation

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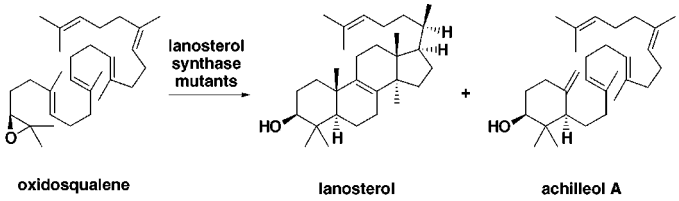
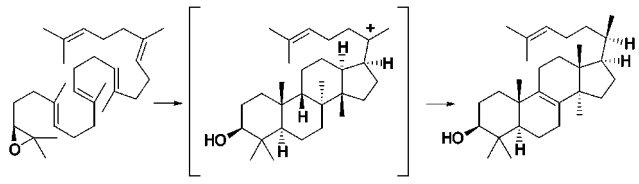
ABSTRACT



Lanosterol synthase converts oxidosqualene to the tetracyclic sterol precursor lanosterol. The mutation experiments described here show that an active-site valine residue in lanosterol synthase contributes to cyclization control through steric effects. Mutating to smaller alanine or glycine residues allows formation of the monocyclic achilleol A, whereas the leucine and isoleucine mutants make exclusively lanosterol. The phenylalanine mutant is inactive.

Animals and fungi form the tetracyclic sterol ring system from oxidosqualene in a single reaction catalyzed by lanosterol synthase (Scheme 1).[1] Oxidosqualene is protonated and cyclized to the chair-boat-chair protosteryl cation, and a series of hydride and methyl shifts terminated by the specific abstraction of the C9 proton produces lanosterol.[2] The lack of a lanosterol synthase crystal structure has hindered efforts to determine how the enzyme directs formation of the energetically unfavorable B-ring boat. Nonenzymatic biomimetic cyclization studies have established the structural and stereoelectronic requirements for cation−olefin polycyclization,[3] and a biomimetic cyclization to construct the A/B-trans-9,10-syn system has recently been achieved.[4] In nonenzymatic systems, cyclization to the desired product is achieved by optimizing intramolecular interactions between the olefins, the electrophilic carbon, and the leaving group. Enzymatic cyclizations are controlled by intramolecular forces imposed by the active site. It has been suggested that precisely localized point charges could control cyclization.[5] Aromatic residues that could contribute electron density have been mutated in the related and structurally characterized squalene hopene cyclase,[6] and some of the resultant mutants form different products.[7] Recently, purely

Scheme 1 Reaction Catalyzed by Lanosterol Synthase



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steric effects have been shown to be important in controlling deprotonation in the *Arabidopsis thaliana* cycloartenol synthase (which also cyclizes to the protosteryl cation but abstracts a different proton). The cycloartenol synthase Ile481Val mutant converts oxidosqualene to a mixture of cycloartenol, lanosterol, and parkeol (54:25:21).[8] Ile481 is strictly conserved in the known examples of cycloartenol synthase,[9] and valine is conserved at the corresponding position in lanosterol synthase enzymes.[1a] These residues correspond to active-site residue Asp374 in *Alicyclobacillus acidocaldarius* squalene-hopene cyclase, and wild-type cycloartenol synthase probably differs from the Ile481Val mutant by active-site alterations rather than changes in global architecture. We describe here a series of experiments in which *Saccharomyces cerevisiae*[10] lanosterol synthase Val454 (which corresponds to *A. thaliana* cycloartenol synthase Ile481) was mutated to the hydrophobic residues phenylalanine, leucine, isoleucine, alanine, and glycine to investigate the role of sterics in catalysis.

pSM61.21 (native *S. cerevisiae* lanosterol synthase in the integrative galactose-inducible yeast expression vector pRS305GAL)[11] was subjected to single-stranded mutagenesis.[12] The resultant lanosterol synthase Val454 derivatives were used to transform the yeast lanosterol synthase mutant SMY8.[11] The strain expressing Val454Phe remained sterol-dependent, but all other mutants produced colonies on sterol-free media (1% yeast extract, 2% peptone, 2% galactose, 13 mg/L heme) that were indistinguishable from a control expressing native lanosterol synthase in the same vector. These genetic complementation experiments suggest that when under galactose control Val454Leu, Val454Ile, Val454Ala, and Val454Gly produce enough lanosterol to meet the cell's sterol requirements. If these mutants generate byproducts, they are not sufficiently detrimental or abundant to significantly inhibit yeast growth.

In vitro incubations with synthetic substrate generated triterpene products for chromatographic and spectroscopic analysis. Recombinant yeast was resuspended in 100 mM potassium phosphate at pH 6.2 and lysed in a French Press. Racemic oxidosqualene[13] (1 mg/mL) and Triton X-100 (10 mg/mL) were added to the resultant homogenates, which were incubated at room temperature overnight. Triterpenes were recovered from the reactions essentially as described previously.[14] Reactions were typically performed to provide ~50 mg of total triterpene alcohol products.

The activities of the mutant enzymes were initially analyzed by TLC (1:1 hexane:ether). This qualitative method is useful for assaying total activity of an oxidosqualene cyclase because diverse triterpene alcohols including lanosterol, cycloartenol, parkeol, lupeol, and β-amyrin have sufficient structural similarity near the polar alcohol that they are inseparable by TLC. The Val454Leu, Val454Ile, Val454Ala, and Val454Gly mutants cyclized oxidosqualene to material that comigrated with lanosterol. The Val454Ala and Val454Gly mutants also produced a minor product less polar than lanosterol. The Val454Phe mutant did not produce detectable product (<1% the activity of wild-type, if any).

The products of the leucine and isoleucine mutants were characterized further after acetylation with acetic anhydride and pyridine. GC analysis of each reaction showed a single signal that coeluted with lanosteryl acetate; no parkeyl acetate or cycloartenyl acetate was observed (<0.5%, if any); 250 MHz ^{1}H NMR analysis confirmed that each mutant produced lanosterol. The acetylated triterpene produced by the isoleucine mutant showed methyl and acetate protons within 0.03 ppm of literature lanosteryl acetate signals:[15] δ 0.688 (C18), 0.872 (C30), 0.884 (C28), 0.884 (C29), 0.912 (C21), 1.004 (C19), and 4.49 ppm (C3). The acetylated product of the leucine mutant showed the same lanosteryl acetate protons: δ 0.687 (C18), 0.871 (C30), 0.881 (C29), 0.881 (C28), 0.911 (C21), 1.003 (C19), and 4.50 ppm (C3).

The Val454Ala mutant was expressed and assayed similarly. GC analysis of an acetylated aliquot showed two compounds in a 91:9 ratio. The crude triterpene alcohols were purified by silica gel chromatography (5% ether in hexane) followed by HPLC (YMC-Pack-SIL, 15% *tert*-butyl methyl ether:85% hexane). The 250 MHz ^{1}H NMR spectrum of the major compound matched that of literature lanosterol values,[15] and that of the minor compound was consistent with literature values for achilleol A.[16]

A control TLC assay suggested that this 91:9 quantitation was inaccurate, because achilleol A (but not lanosterol) was present in the homogenate before addition of substrate. The yeast lanosterol synthase mutant SMY8 has a functional biosynthetic pathway to oxidosqualene, which is converted to lanosterol and achilleol A in vivo when the Val454Ala mutant lanosterol synthase is expressed. Lanosterol is apparently metabolized rapidly in vivo, but achilleol A is not. Accumulated achilleol A produced in vivo would bias the analysis to overestimate its production by the enzyme.

The Val454Ala and Val454Gly mutants were consequently expressed in yeast strain LHY3,[8] a lanosterol synthase mutant

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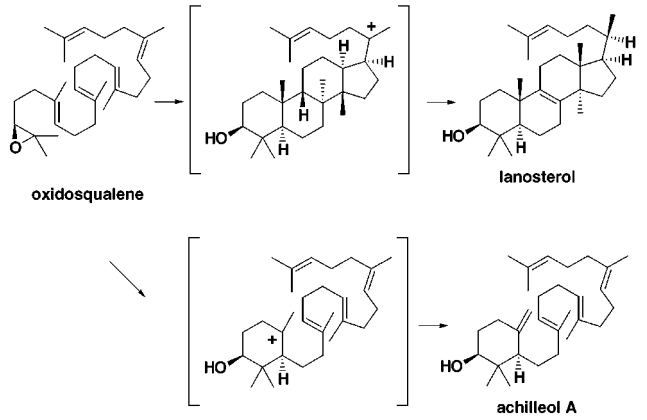
that also has a squalene synthase deletion and therefore does not accumulate oxidosqualene or achilleol A in vivo. GC analysis of the acetylated products showed compounds with relative retention times corresponding to those of lanosteryl acetate and achilleyl A acetate in both the alanine (95:5) and glycine (83:17) mutants (Scheme 2). The ^{1}H NMR

Scheme 2 *S. cerevisiae* Lanosterol Synthase Val454Ala and Val454Gly Mutants Produce Significant Amounts of Achilleol A



spectra of the alanine and glycine mutant products showed signals consistent with those of lanosteryl and achilleyl A acetate literature values. The proton NMR spectrum of the alanine mutant acetate mixture showed lanosteryl acetate methyl and acetate protons within 0.02 ppm of literature values as follows: δ 0.688 (C18), 1.004 (C19), 0.911 (C21), 0.882 (C28), 0.882 (C29), 0.872 (C30), and 4.51 ppm (C3). Achilleyl A acetate signals were present within 0.02 ppm of literature values[16] as follows: methyl singlets at δ 0.793, 0.948, 1.603, and 1.685 ppm; side chain allylic methylene protons at δ 2.015; exo-methylene olefinic singlets at δ 4.628 and 4.886 ppm; and C3 at δ 4.680. Proton NMR spectra of the glycine mutant acetates supported the identification of achilleyl A acetate with signals within 0.03 ppm of literature values as follows: methyl protons at δ 0.793, 0.948, 1.603, and 1.683 ppm; side chain allylic methylene protons at δ 2.015; exo-methylene protons at δ 4.626 and 4.882 ppm; and C3 at δ 4.666. Lanosteryl acetate signals were present in the glycine mutant acetate mixture within 0.02 ppm of literature values: δ 0.687 (C18), 1.004 (C19), 0.911 (C21), 0.881 (C28), 0.881 (C29), 0.871 (C30), and 4.50 ppm (C3). An additional incubation with the alanine mutant provided crude triterpene alcohols, which were purified by silica gel chromatography (5% ether in hexane) followed by HPLC (YMC-Pack-SIL, 15% *tert*-butyl methyl ether:85% hexane). The 250 MHz ^{1}H NMR spectra of the purified compounds were then measured and found to be consistent with literature values (see Supporting Information).

A second potential complication with the quantitation is that if yeast lanosterol demethylase were active under the in vitro assay conditions, lanosterol would be consumed, exaggerating the relative amount of achilleol A. To determine whether this was a problem, a control assay was performed under conditions that minimize lanosterol demethylation. This oxidative reaction requires stoichiometric amounts of the cofactor NADPH. Washed microsomes (a partially purified enzyme preparation that should have very little NADPH) of LHY3 expressing lanosterol synthase Val454Gly were assayed to determine the product profile without lanosterol metabolism. This assay gave essentially the same results as crude homogenate (84:16), which therefore has negligible lanosterol metabolism and is a suitable enzyme source for product quantitation.

The product profiles of the lanosterol synthase Val454 mutants are summarized in Table 1. The inactivity of the

Table 1. Product Composition of *S. cerevisiae* Lanosterol Synthase Val454 Mutants

454 residue	lanosterol, %	achilleol A, %
phenylalanine	0	0
leucine	100	0
isoleucine	100	0
valine (wild-type)	100	0
alanine	95	5
glycine	83	17

Val454Phe mutant can be attributed to occluding the active site with excessive bulk or prohibiting productive cyclization by inappropriately positioned π-electron density. Alternatively, the added steric bulk might disrupt protein folding. Lanosterol synthase remains accurate when valine is mutated to leucine or isoleucine. In contrast, cycloartenol synthase produces a mixture of cycloartenol, lanosterol, and parkeol when the corresponding conserved isoleucine residue is mutated to valine.[8] Even the more dramatic Val454Ala and Val454Gly mutants do not alter deprotonation from the protosteryl cation. Instead, these mutants acquire achilleol A biosynthetic ability. Decreasing amino acid side chain steric bulk from isopropyl to methyl or hydrogen allows monocycle formation, suggesting that steric bulk contributed by the valine side chain may participate in prefolding the oxidosqualene Δ^{10} olefin to facilitate B-ring formation. It is noteworthy that like deprotonation to form the tetracyclic ring system, deprotonation to form the monocycle is accurate; no other monocyclic compounds were observed.

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Supporting Information Available: A 250 MHz ^{1}H NMR spectrum of achilleol A produced by the Val454Ala mutant and a GC trace of the acetylated Val454Ala products. This material is available free of charge via the Internet at http://pubs.acs.org.

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