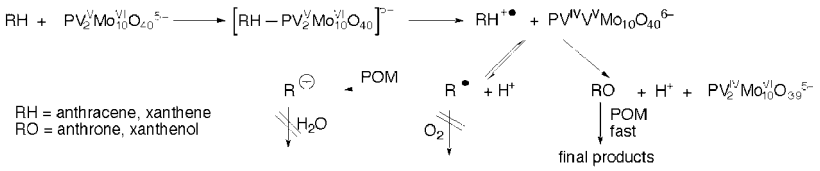

Scheme 1. Activation and oxygenation of aromatic hydrocarbons by $H_5PV_2Mo_{10}O_{40}$.

through a M-vK type mechanism is possible in a homogeneous liquid phase using POMs as catalysts (Scheme 1).

The reaction is initiated by the relatively fast formation of a donor–acceptor complex and then a radical cation. Subsequent formation of a free radical and oxygenation is discounted from the isotope incorporation experiments and anaerobic reactions. Homolytic cleavage of the C–H bond (not shown) does not occur because compounds that easily react in this way (for example, cyclohexene) did not react in this system. Nucleophilic attack of water was also discounted. The rate-determining step appears to be the oxygen transfer from the POM[15] to the activated hydrocarbon as indicated by the inverse deuterium isotope effect. Consequent reaction steps are fast, since no intermediates such as anthrone are observed, and benzylic alcohols react much faster.

Experimental Section

Reactions were carried out in 15-mL glass pressure tubes. Typically, appropriate amounts of hydrocarbon substrate (all highest purity commercial products) and $H_5PV_2Mo_{10}O_{40}\cdot 34\,H_2O$[9] were dissolved in CH_3CN. The gases $^{18}O_2$ (96.1%) or $^{16}O_2$ were added by four consecutive pump/thaw cycles on a Schlenk line. Conversions were measured by gas-liquid chromatography (HP 6890) on a 5% phenylmethylsilicone (30 m, 0.32 mm ID, 0.25 μm coating) column. Isotope incorporation was quantified by GC-MS (HP 5973 (same column)). Reactions under anaerobic conditions in CD_3CN, similarly carried out in high-pressure NMR tubes, were quantified by ^{1}H NMR spectroscopy. Labeled $H_5PV_2Mo_{10}O_{40}$ was prepared by drying the POM at 120 °C for 24 h and then adding 50 equivalents of $H_2{}^{17}O$ (10.2%) or $H_2{}^{18}O$ (94.3%) in dry CH_3CN and mixing for 18 h. The drying/exchange cycle was repeated three times. ^{18}O enrichment (see Figure 2, middle) is estimated as about 50% at the terminal oxygen atoms and to be greater than 90% (by deconvolution by assuming a 40 cm^{-1} shift, and from the equal intensities of the peaks) at the edge and corner-shared oxygen atoms. Total enrichment for potentially transferable oxygen atoms is therefore about 75%. Anhydrous reactions were carried out using $((C_4H_9)_4N)_5PV_2Mo_{10}O_{40}$ in dried solvents. The isotope effect in the oxidation of $[H_{10}]$- and $[D_{10}]$anthracene in a competitive reaction was measured by mixing anthracene (10 mM), $[D_{10}]$anthracene (10 mM), $H_5PV_2Mo_{10}O_{40}\cdot 34\,H_2O$ (1 mM) at 60 °C, in O_2 (1 atm). The isotope ratio was computed by comparing the ratio of $[H_{10}]$- and $[D_{10}]$anthracene remaining under pseudo-first-order conditions at up to 35% conversion. Samples for IR spectra (Nicolet Protégé 460) were prepared by placing a few drops of the POM-containing solution on a NaCl plate and evaporating off the solvent. ESR spectra were measured in 1-mm tubes at ambient temperature. UV/Vis kinetic measurements were performed on a diode-array instrument (HP 5842) equipped with a magnetic stirrer and a temperature bath (60 ± 0.1 °C). ^{17}O NMR spectra (Bruker DMX 400) were measured at 54.244 MHz and referenced to $H_2{}^{17}O$.

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Oxidosqualene Cyclase Residues that Promote Formation of Cycloartenol, Lanosterol, and Parkeol**

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Eukaryotes make sterols from three different cyclic intermediates.[1] Lanosterol (**7**) is the initial carbocyclic sterol precursor in animals[2] and fungi.[3] Cycloartenol (**5**) and parkeol (**8**) are isomeric sterol biosynthetic intermediates in plants[4, 5] and sea cucumbers,[6] respectively. Lanosterol, cycloartenol, and parkeol synthases cyclize (*S*)-2,3-oxidosqualene ((*S*)-**1**) [7] to the intermediate protosteryl cation **2**[8] and form different products by promoting cationic cyclization

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and rearrangement reactions terminated by multiple deprotonation options (Scheme 1). A conserved active-site valine in lanosterol synthase participates in tetracycle formation,[9] and a conserved isoleucine at the corresponding position in cycloartenol synthase influences both cyclization and deprotonation.[10, 11] In search of other catalytic residues, we mutated conserved lanosterol-synthase residues where cycloartenol synthase maintains a different residue, and thereby generated an enzyme that makes parkeol as its major product.

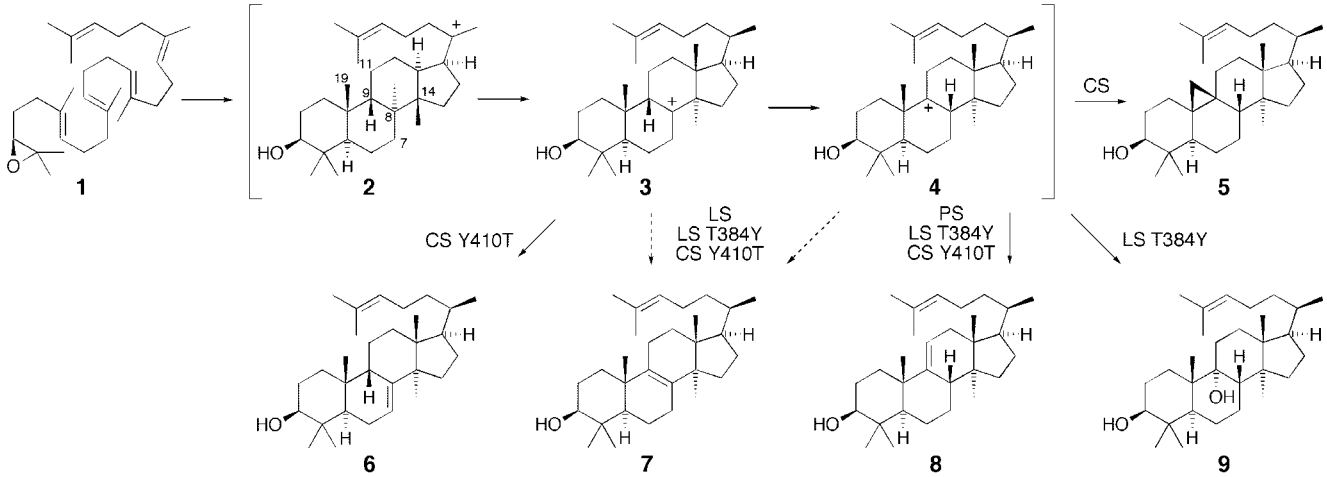
The Thr384 residue is conserved in lanosterol synthase. All known cycloartenol synthases have Tyr at this position, which corresponds[12] to an active-site residue in the squalene–hopene cyclase[13] crystal structure.[14, 15] The conservation pattern and the predicted active-site location are consistent with a role in the catalytic differences between these enzymes. We mutated Thr384 in *Saccharomyces cerevisiae* lanosterol synthase into Tyr, and expressed the mutant gene with the vector pRS305GAL in the lanosterol-synthase/squalene-synthase double-mutant yeast strain LHY3.[10] Incubation with exogenous racemic 2,3-oxidosqualene (**1**) as previously described[9] produced a triterpene alcohol fraction (which comigrates with lanosterol during thin layer chromatography) and a more polar product. The triterpene alcohol fraction exhibited ^{1}H NMR spectroscopic signals characteristic of a lanosterol (**7**) and parkeol (**8**) mixture, and GC-MS after acetylation confirmed these structures. The more polar product was shown to be lanost-24-ene-3β,9α-diol (**9**; Table 1, Scheme 1) by NMR spectroscopy (^{1}H, ^{13}C, DEPT, COSY-DEC, and HSQC); this is a known synthetic compound[16] that has not been isolated from nature or from an enzymatic reaction. Parkeol (**8**) results from C-11 deprotonation, and the diol **9** is formed by quenching an intermediate C-9 carbocation with water, a process that is precedented in the biosyntheses of diplopterol,[17] dammarenediol,[18] and lupanediol.[19] The 9α stereochemistry is consistent with water attack concerted with hydride migration from C-9 to C-8.

Table 1. Product composition of *S. cerevisiae* lanosterol-synthase mutants.

Mutation	**7** [%]	**8** [%]	**9** [%]
none	100	0	0
Val454Ile[9]	100	0	0
Val454Leu[9]	100	0	0
Thr384Tyr	79	11	10
Thr384Tyr/Val454Ile	13	64	23
Thr384Tyr/Val454Leu	20	60	20

Mutating the lanosterol-synthase residue Val454 has also been shown to alter the product structure.[9] To determine how these two catalytically relevant positions interact, we made a lanosterol-synthase mutant with both Thr384 and Val454 converted into their cycloartenol-synthase counterparts. The Thr384Tyr/Val454Ile derivative produced primarily parkeol (**8**), with lanosterol (**7**) and 9α-diol (**9**) by-products (Table 1). The methylene added by mutating valine to isoleucine shifted production dramatically from lanosterol to parkeol, and doubled the amount of water-quenched material. This is a synergistic effect; the Val454Ile mutation alone did not change the product profile.[9] The Tyr384 derivative allowed parkeol formation, and greater bulk at position 454 amplified this effect and also increased diol formation. The larger residue may displace the substrate or basic residue to expose the C-9 carbocation to water. We examined the importance of shape at position 454 by replacing the *sec*-butyl side chain of Ile with the isobutyl side chain of Leu. The Thr384Tyr/Val454Leu mutant is also a parkeol synthase (albeit slightly less accurate than the Thr384Tyr/Val454Ile derivative; see Table 1) and generated the same two by-products.

These mutants are particularly informative when compared to complimentary experiments in which *A. thaliana* cycloartenol synthase was mutated to resemble lanosterol synthase at the residues Tyr410 and Ile481, which correspond to Thr384 and Val454, respectively.[20] The Tyr410Thr mutation abolished cycloartenol formation completely; the resultant enzyme converted 2,3-oxidosqualene (**1**) into 65% lanosterol



Scheme 1. Lanosterol synthase (LS), cycloartenol synthase (CS), and parkeol synthase (PS) cyclize 2,3-oxidosqualene (**1**) to the protosteryl cation **2** and catalyze rearrangements and deprotonations to form their respective triterpene alcohol products. It is not established whether the intermediate C-8 or C-9 cations, **3** and **4**, actually occur but these are drawn to facilitate visualization of which protons depart. Cycloartenol (**5**) and parkeol (**8**) result from C-19 and C-11 deprotonation, respectively. Lanost-24-ene-3β,9α-diol (**9**) is easily envisioned as a water-quenched C-9 cation. Losing the proton originally at C-9 (possibly after shifting to C-8) yields lanosterol (**7**). 9β-Lanosta-7,24-dien-3β-ol (9β-Δ7 lanosterol, **6**) could result from deprotonation of the C-8 cation or from deprotonation concerted with a methyl shift from C-8 to C-14. Y = Tyr, T = Thr.

(**7**), 33 % 9β-lanosta-7,24-dien-3β-ol (9β-Δ7-lanosterol, **6**), and 2 % parkeol (**8**; Scheme 1).[20] Adding the Ile 481 Val mutation increased lanosterol formation to 75 % and lowered 9β-Δ7-lanosterol and parkeol production to 24 % and 0.6 %, respectively.[20]

Both lanosterol-synthase and cycloartenol-synthase product specificity is markedly altered by mutating a hydroxylated residue to its counterpart in the other enzyme (exchanging threonine for tyrosine, and vice versa). Exchanging the differentially conserved aliphatic residues (valine and isoleucine) amplifies the effect. A striking difference between the two studies is that although a cycloartenol synthase with lanosterol-synthase residues produces mostly lanosterol, introducing cycloartenol-synthase residues into lanosterol synthase generates a parkeol synthase that does not form cycloartenol. Thus, cycloartenol synthase promotes a kinetically favored route to cyclopropane ring formation that can be modified to produce thermodynamically favored products, but modifying lanosterol synthase to form a cyclopropyl ring requires an additional motif that remains unidentified.

Cycloartenol (**5**), parkeol (**8**), and the 9α-diol **9** are probably derived from the C-9 cation **4**, and are formed by enzymes with Tyr at the position studied (lanosterol-synthase Thr 384 Tyr mutants, native cycloartenol synthase, and cycloartenol synthase Ile 481 Val mutants[11]). 9β-Δ7-Lanosterol (**6**) is most readily rationalized as deriving from the C-8 cation **3**, and is formed exclusively by an enzyme bearing Thr in place of Tyr 410. Lanosterol (**7**) could be derived from either cation, and is produced by enzymes with either Tyr or Thr. The residues in question may stabilize different intermediate cations or facilitate the formally *syn* hydride shift which is essential to form cycloartenol (**5**) but not lanosterol or parkeol (**7** and **8**, respectively).

As no natural parkeol synthase has been cloned, sequence information is unavailable. This enzyme may resemble the mutant enzymes developed in this study, with tyrosine and isoleucine (or possibly leucine) at positions corresponding to residues 384 and 454 in *S. cerevisiae* lanosterol synthase, respectively. Experiments to test this hypothesis by cloning and characterizing parkeol synthase are in progress.

Experimental Section

DNA manipulation, microorganisms, and culture conditions: Oligo-directed mutagenesis, transformation, and DNA amplification techniques followed established protocols.[21] Yeast transformation and protein expression were performed as described previously.[9]

Enzymatic reaction, product identification, and quantitation: Recombinant yeast was grown on a 4-L scale in inducing medium, and in vitro reactions with racemic 2,3-oxidosqualene (**1**; 100 mg) were performed as described.[9] Reactions generally approached completion (as judged by thin layer chromatography), and isolated yields were generally $\approx$80 %. Lanosterol (**7**) and parkeol (**8**) were identified by comparison of their ^{1}H NMR spectra to those of authentic standards. Although NMR spectra for lanost-24-ene-3β,9α-diol (**9**) have not been reported previously, the 24,25-dihydro analogue (lanostane-3β,9α-diol) has been characterized.[22] NMR spectroscopic signals in the ring system of **9** matched very closely those of the dihydro derivative, and the side chain provided signals corresponding to those of lanosterol (**7**).[22] Selected data for compound **9**: ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, referenced to $Si(CH_3)_4$; atom numbering as in ref. [22]): δ = 0.786 (s, H-18), 0.808 (s, H-29), 0.924 (s, H-30), 0.996 (s, H-28), 1.035 (s, H-19), 1.602 (s, H-27), 1.681 (s, H-26), 3.201 (m, H-3); ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C, referenced to $CHCl_3$ at 77.0 ppm): δ = 14.59, 15.40, 16.73, 17.62, 18.27, 18.58, 21.42, 23.74, 24.85, 25.71, 27.36, 27.95, 27.95, 28.28, 29.13, 29.52, 33.82, 35.80, 36.39, 38.80, 40.54, 42.64, 45.18, 45.69, 47.45, 50.36, 77.17, 78.64, 125.16, 130.95. Ratios of products were determined by ^{1}H NMR quantitation using well-resolved signals.

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