Methodology for the Preparation of Pure Recombinant *S. cerevisiae* Lanosterol Synthase Using a Baculovirus Expression System. Evidence That Oxirane Cleavage and A-Ring Formation Are Concerted in the Biosynthesis of Lanosterol from 2,3-Oxidosqualene

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Abstract: Lanosterol synthase [(*S*)-2,3-epoxysqualene mutase (cyclizing, lanosterol forming), EC 5.4.99.7], the enzyme from *Saccharomyces cerevisiae* which catalyzes the complex cyclization/rearrangement step in sterol biosynthesis, was overexpressed in baculovirus-infected cells and purified to homogeneity in three steps. Using pure enzyme the kinetics of cyclization were determined using Michaelis−Menten analysis for 2,3-oxidosqualene (**1**) and two analogs in which the C−6 methyl was replaced by H (**3**) or Cl (**4**). The measured V_{max}/K_M ratios for **1**, **3**, and **4** were found to be 138, 9.4, and 21.9, respectively, a clear indication that oxirane cleavage and cyclization to form the A-ring are concerted, since the nucleophilicity of the proximate double bond influences the rate of oxirane cleavage. No catalytic metal ions could be detected in purified lanosterol synthase by atomic absorption analysis. Site-directed mutagenesis studies of each of the six strongly conserved aspartic acid residues (D → N mutation) and each of the nine conserved glutamic acid residues (E → Q) revealed that only one, D456, is essential for catalytic function of the enzyme. The essential D456 residue is a likely candidate for electrophilic (specifically protic) activation of the oxirane function.

Introduction

The most remarkable step in the biosynthesis of steroids (and one of the most complex in biochemistry) is the conversion of oxidosqualene (**1**) to lanosterol (**2**) catalyzed by lanosterol synthase [*(S)*-2,3-epoxysqualene mutase (cyclizing, lanosterol forming), EC 5.4.99.7], which controls precisely the formation of four rings and six new stereocenters as shown in Scheme 1.[1] Although there have been numerous studies of the mechanism of this complicated process over the past four decades, much remains to be learned about the enzyme and how it mediates the individual cyclization and rearrangement steps. Although it has been often assumed that the cyclization to form the protosteryl cation is concerted, no compelling evidence for this proposal had been found. In addition, most of the work over the past three decades has been done with crude enzyme preparations. The lipophilic and insoluble nature of this membrane-associated enzyme necessitates the use of detergents and glycerol to obtain solutions and greatly complicates purification. Nonetheless, the application of recombinant DNA technology has led recently to the cloning of genes and determination of amino acid sequences for lanosterol synthase from various organisms.[2]

The most recent mechanistic studies of lanosterol biosynthesis, which have been conducted with purified enzyme from recombinant sources, have revealed that the cyclization involves discrete carbocations during C-ring formation and specifically that a five-membered C-ring cationic structure is first formed and then enlarged by expansion to a six-membered structure as shown in Scheme 2. Another aspect of the cyclization process is the subject of this paper, namely, the mode of activation of the epoxide function and formation of the A-ring. In addition, we describe the overproduction of the yeast (*S. cerevisiae*) enzyme in a baculovirus system and an effective method for the purification of this protein.

Results and Discussion

Overexpression of *S. cerevisiae* Lanosterol Synthase in *E. coli* and *S. cerevisiae*. In order to obtain large quantities of lanosterol synthase for detailed study, extensive attempts were made to overexpress the protein in different systems. Overexpression of the enzyme in *E. coli* was unsuccessful because active protein could not be obtained, although several different overexpression vectors and strains were used.[3] The exact reasons for this lack of success are unknown but could result from protein insolubility, improper folding, or lack of proper post-translational modification of the protein to form active enzyme.[4]

As anticipated, overexpression of the *S. cerevisiae* lanosterol synthase in *S. cerevisiae*, its native organism, provided active

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(1) For a recent review, see Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189.

(2) For recent work on the gene cloning and protein sequences for lanosterol synthase form various organisms, see: (a) Kelly, R.; Miller, S. M.; Lai, M. H.; Kirsch, D. R. *Gene* **1990**, *87*, 177 and Buntel, C. J.; Griffin, J. H. *J. Am. Chem. Soc.* **1992**, *114*, 9711 (*Candida albicans*). (b) Corey, E. J.; Matsuda, S. P. T.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11628 (*Arabidopsis thaliana*). (c) Corey, E. J.; Matsuda, S. P. T.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2211. Shi, Z.; Buntel, C. J.; Griffin, J. H.; *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7370 (*Saccharomyces cerevisiae*). (d) Kasuno, M.; Shibuya, M.; Sankawa, U.; Ebizuka, Y. *Biol. Pharm. Bull. Jpn.* **1995**, *18*, 195. Abe, I.; Prestwich, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9274 (*Rattus norvegicus*). (e) Baker, C. H.; Matsuda, S. P. T.; Liu, D. R.; Corey, E. J. *Biochem. Biophys. Res. Commun.* **1995**, *213*, 154 (*Homo sapiens*). (f) Corey, E. J.; Matsuda, S. P. T.; Baker, C. H.; Ting, A.; Cheng, H. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 327 (*Schizosaccharomyces pombe*).

(3) Vectors used: pKK223-3, pLM1, pKen2, pET-15b. Strains used: JM101, XA90, BL21-DE(3), BL21-DE(3)pLysS.

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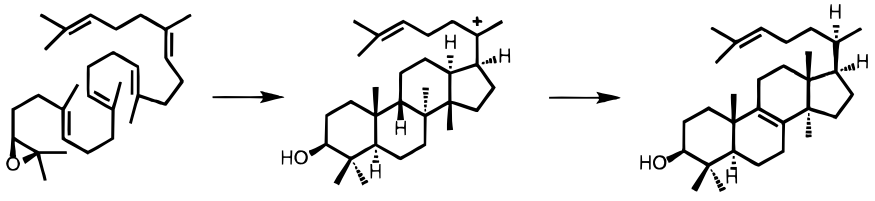
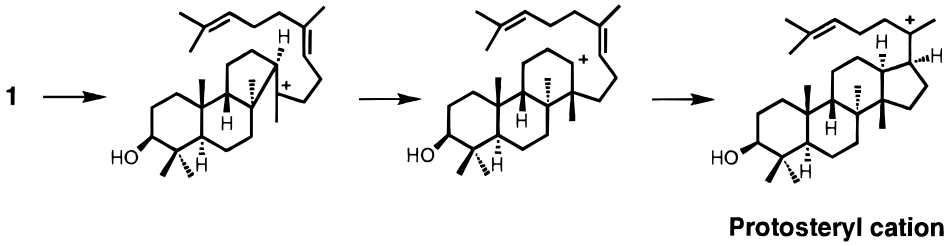
Scheme 1. Lanosterol Synthase Catalyzed Cyclization of 2,3-Oxidosqualene to Lanosterol



Scheme 2. Six-Membered C Ring Formation via Ring Expansion



protein. Several promoter systems were tried (*GAL1*, *ADH*, and *PGK*), but the best expression level was 8-fold higher than wild type.[2c] This level of overexpression proved unsatisfactory.[2c] The yeast cells (90 g) were lysed by French press and fractionated by ultracentrifugation. The enzymatically active microsomes were solubilized in a solution containing 1% Triton X-100 and 10 mM dithiothreitol (DTT), subjected to DEAE Sepharose column chromatography and a subsequent hydroxyapatite (HAT) column chromatography. At this stage, the purity was approximately 10%. Further purification by FPLC with a Mono Q column gave 50 μg of protein of only 90% purity.

Overexpression of *S. cerevisiae* Lanosterol Synthase in Baculovirus-Infected Insect Cells and Purification and Characterization. Because the *S. cerevisiae*-expressed protein was difficult to purify and insufficient for extensive studies, we turned to the baculovirus system. Baculovirus yields are relatively insensitive to protein toxicity, because polyhedrin-promoted expression peaks very late in infection when the virus is already killing the cells, and eukaryotic post-translational modifications generally occur correctly in the eukaryotic *Sf 9* cells. *Sf 9* cells were infected with an *ERG7*-recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) constructed with the PharMingen Co. transfection kit**.** The time course for expression of the *S. cerevisiae* lanosterol synthase in infected *Sf 9* cells was studied both on tissue culture plates and in spinner flasks. Maximum expression of cyclase activity was seen 3 days post-infection when cell viability (as determined by Trypan blue staining) was >80%. At 4 days, cell viability had dropped to 50%. All activity was found in the cell pellet, rather than in the media.

Using this recombinant protein source, lanosterol synthase was purified to greater than 99% purity after only three steps: (1) ammonium sulfate precipitation, (2) DEAE Sepharose ion exchange column chromatography, and (3) hydroxyapatite column chromatography (Figure 1). Starting from 20 g of cells, 2.5 mg of pure protein was obtained (Table 1). The purified protein showed a single band on SDS-polyacrylamide gel electrophoresis with a molecular weight of 83 kDa as predicted by the complementary DNA sequence.

Glycerol (20%), Triton X-100 (0.2%), and DTT (3 mM) must be added to the buffer (sodium phosphate, pH 7.0) to maintain

(4) *E. coli* lacks the post-translational modification machinery present in eukaryotes, and might be unable to produce active lanosterol synthase for this reason.

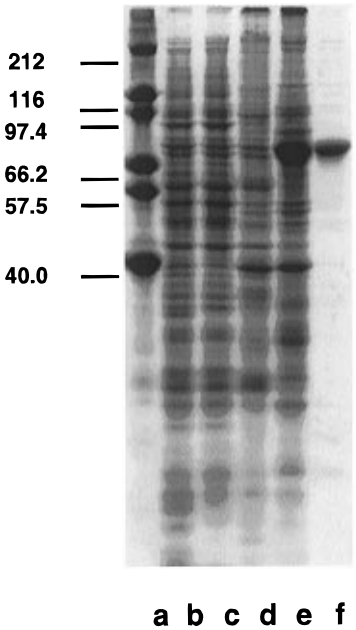


Figure 1. Protein gel of fractions from different stages of the purification. Lanes from left to right are as follows: (a) protein standards, (b) cell homogenate, (c) supernatant after first centrifugation, (d) redissolved pellet from ammonium sulfate precipitation, (e) protein solution after DEAE ion exchange chromatography, and (f) pure protein after HAT chromatography.

Table 1. Lanosterol Synthase Purification

fraction	vol. (mL)	protein conc. (mg/mL)	total amount (mg)	sp. act. (pmol/μg/h)	purif. *n*-fold
homogenate	63	332	20,900	1.26	
supernatant	63	287	18,070	1.15	1
$(NH_4)_2SO_4$	50	97.8	4,850	8.16	7
DEAE	32	0.31	9.61	182	159
HAT	40	0.050	2.0	2448	2129

enzyme activity. FPLC chromatography was unnecessary, and large amounts of protein could readily be obtained by conventional column chromatography. Protein solutions were desalted by dialysis prior to chromatography since ultrafiltration significantly reduced enzyme activity. Washing the HAT column with sodium chloride prior to elution removes all neutral and positively charged proteins and is essential to purify the protein.

The protein was digested with trypsin, and the resulting fragments were separated by reversed phase HPLC with a Vydac

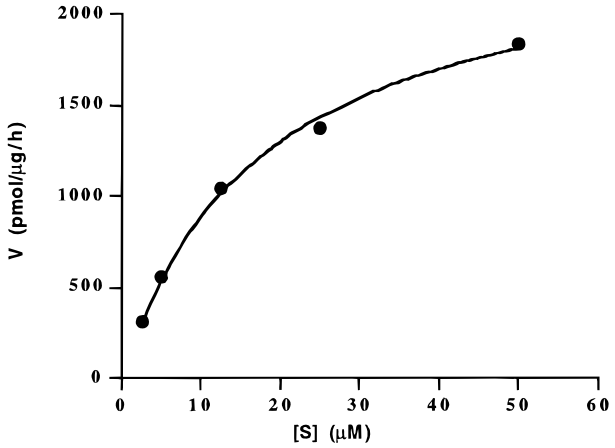


Figure 2. Michaelis−Menten plot of lanosterol synthase with 2,3-oxidosqualene.

narrowbore column (C_{18}, 300 Å, 2.1 mm × 150 mm). Two of the resulting oligopeptides were subjected to Edman degradation on a Hewlett Packard G1005 protein sequencer. The resulting sequences NTVCGVDLYYPHSTTLNIANSLVVF and LFEGIDVLLNLQNIGSFEYG are identical to the sequences predicted from the DNA sequence of *S. cerevisiae* lanosterol synthase. In 150 mM sodium phosphate buffer (pH 6.3) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT, the pure enzyme has K_M of 18 μM and V_{max} of 2448 pmol/μg/h for 2,3-oxidosqualene (Figure 2). The maximum activity of the overexpressed enzyme occurs at pH 6.3, which is the same as for the wild type yeast enzyme.

Epoxide Activation and A-Ring Cyclization Are Concerted. Granted that the closure of the A ring in lanosterol synthesis is a cationic process, an important question is whether epoxide opening occurs prior to or concurrently with cyclization by C−C bond formation. In order to investigate this question, a number of mechanistically relevant analogs of 2,3-oxidosqualene were synthesized and studied with regard to the catalytic action of lanosterol synthase. We discuss first the investigation of two analogs in which the 6-methyl group of 2,3-oxidosqualene (which becomes the A/B angular methyl of lanosterol) is replaced by hydrogen or chlorine, i.e., 6-desmethyl-2,3-oxidosqualene (**3**) and 6-chloro-6-desmethyl-2,3-oxidosqualene (**4**). These compounds and their 3-tritiated radioactive counterparts (^{3}H-**3** and ^{3}H-**4**) were synthesized as outlined in the section on synthesis which appears below. The 6,7-*Z* isomer of **3** and the 6,7-*E* isomer of **4** were also available from synthesis. As expected, these double bond isomers with unnatural olefinic geometry were inert to the action of lanosterol synthase. They served neither as substrates for the enzyme nor inhibitors of the cyclization of 2,3-oxidosqualene to lanosterol. In contrast 6-desmethyl-2,3-oxidosqualene (**3**) was converted by lanosterol synthase to a product of the same chromatographic mobility as lanosterol (R_f 0.5 on silica gel plates with 1:1 ether−hexane) and which was shown by ^{1}H NMR comparison with lanosterol[5] to be 10-desmethyllanosterol. It was also observed that the rate of cyclization of **3** was less than that of 2,3-oxidosqualene and that **3** caused a marked time-dependent inactivation of lanosterol synthase. Careful Michaelis−Menten kinetic studies with ^{3}H-**3** following the reaction with pure lanosterol synthase from zero to 2.5% conversion revealed that the K_M value for **3** is about 2.5 times higher than for **1** and that V_{max} is about 6 times less.[6] Similar studies and kinetic analysis with 6-desmethyl-6-chloro-2,3-oxidosqualene (**4**) and ^{3}H-**4** revealed cyclization to a lanosterol analog. For **4**, K_M was found to be 2.5 times higher than for **1** (and essentially the same as for **3**) and V_{max} was found to be about 2.5 times less than for **1**.

Comparison of the V_{max}/K_M values for **1**, **3** and **4** is instructive: for **1**, $V_{max}/K_M = 138$; for **3**, $V_{max}/K_M = 9.44$; and for **4**, $V_{max}/K_M = 21.9$. These results show that the rate at which lanosterol synthase effects cyclization of the substrate correlates with the nucleophilicity of the 6,7-double bond and strongly indicates that the 6,7-double bond participates in the closure of ring A and in the oxirane ring cleavage, i.e., oxirane cleavage and A ring formation are concerted. Put in another way, an intermediate tertiary cation (C−2 carbocation) formed by electrophilically-assisted heterolysis of the oxirane ring is unlikely to be a discrete intermediate in the conversion of 2,3-oxidosqualene to lanosterol. If the formation of a localized C−2 carbocation were involved in the rate-limiting step of the cyclization, essentially identical V_{max}/K_M values would be expected for the enzymic cyclization of **1**, **3**, and **4**. The observed order of V_{max}/K_M values, **1** (138) > **4** (21.9) > **3** (9.14), clearly correlates with the carbocation stabilizing ability of the C−6 group, CH_3 > Cl > H.

The proposal that cleavage of the electrophilically activated oxirane function of **1** and A-ring closure are concerted implies that the tight control by lanosterol synthase on the conformation of 2,3-oxidosqualene[7] enforces a suitable prefolded, three-dimensional geometry on **1**, including proximity of C−2 and C−7 which are joined during A-ring formation. The concerted A-ring closure/oxirane cleavage model has received strong support from the study of the spiro epoxide **5** in which the isopropyl subunit of 2,3-oxidosqualene is replaced by cyclopropyl. It is well-known that oxaspiropentanes such as **5** undergo extremely facile acid-catalyzed conversion to cyclobutanones, doubtless by pinacolic rearrangement of an intermediate stabilized cyclopropylcarbinyl cation.[8] Consequently, it seemed very possible that the spiro epoxide **5** would be converted by lanosterol synthase to a monocyclic cyclobutanone without involvement of any of the olefinic units and simply as a consequence of electrophilic activation of the oxirane by the enzyme. In fact, analog **5** was resistant to change by lanosterol synthase at pH 7 under conditions for the enzymic conversion of **1** to lanosterol. This rather striking result seems inexplicable in terms of a two-stage mechanism involving (1) electrophilically induced oxirane cleavage and (2) subsequent closure of the A ring. On the other hand, it does seem reconcilable with the concerted oxirane cleavage−A-ring closure pathway since that process would require a less potent activating electrophile and a substrate folding which brings C−2 and C−7 into proximity. The geometry of the oxaspiropentane subunit of **5** is such that one of the cyclopropyl methylenes would be involved in strong steric repulsion with the methylene group attached to C−7 in the appropriate conformation for the concerted oxirane cleavage−A-ring closure pathway.

Epoxides **6**, **7**, and **8** were also tested as substrate analogs of 2,3-oxidosqualene with lanosterol synthase and were found neither to be substrates nor time-dependent inhibitors, again consistent with the idea of a concerted A-ring closure process with only modest electrophilic activation provided by the enzyme. Substrates **6** and **7** clearly would require powerful

(5) Emmons, G. T.; Wilson, W. K.; Schroepfer, G. *J. Magn. Reson. Chem.* **1989**, 1012.

(6) Measurements of kinetics were carried out under the same standard conditions for **1**, **3**, and **4** (with respect to medium, concentrations, temperature *etc*.) to allow comparison. Obviously, K_M values are not absolute since detergent, which can associate with the substrate, is present.

(7) (a) Corey, E. J.; Virgil, S. C.; Cheng, H.; Baker, C. H.; Matsuda, S. P. T.; Singh, V.; Sarshar, S. *J. Am. Chem. Soc.* **1995**, *117*, 11819. (b) Corey, E. J.; Virgil, S. C.; Liu, D. R.; Sarshar, S. *J. Am. Chem. Soc.* **1992**, *114*, 1524.

(8) Trost, B. M. *Acc. Chem. Res.* **1974**, *7*, 85.

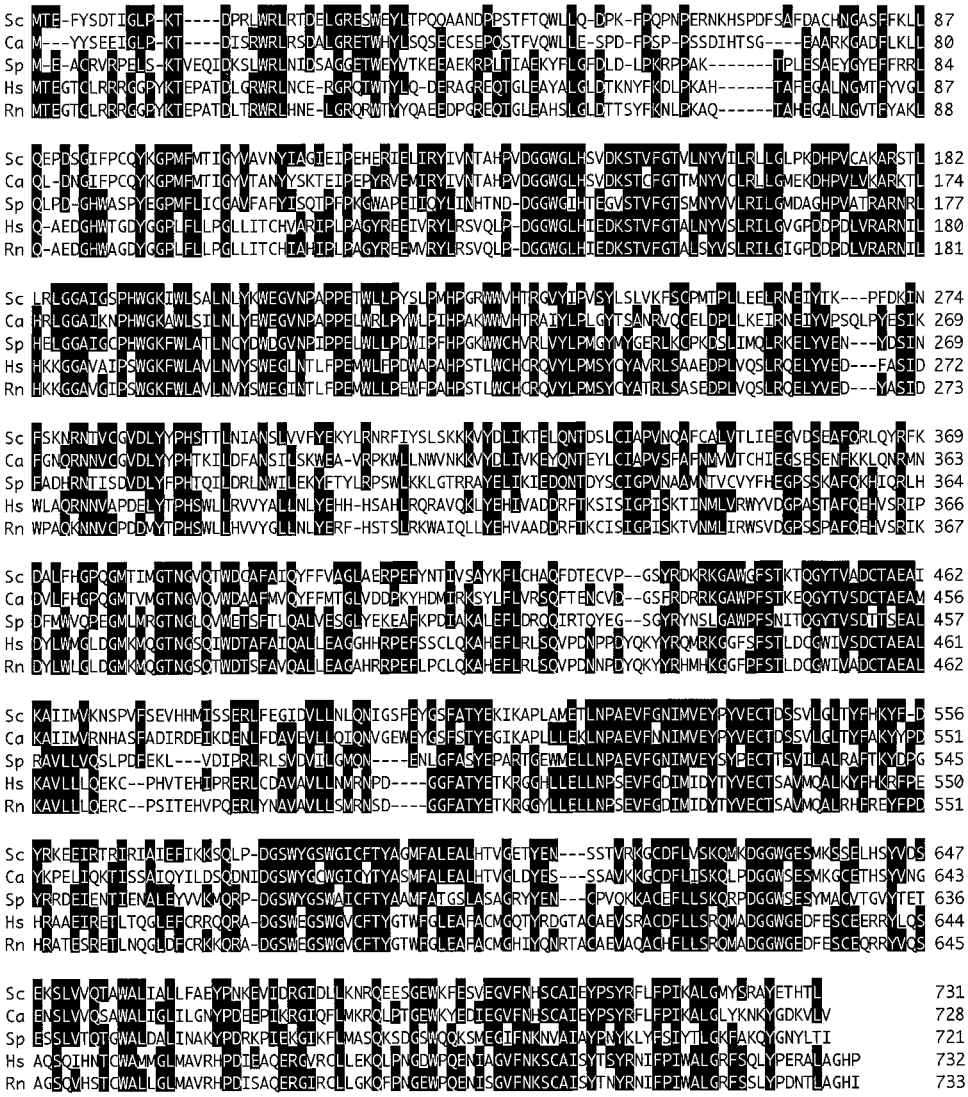


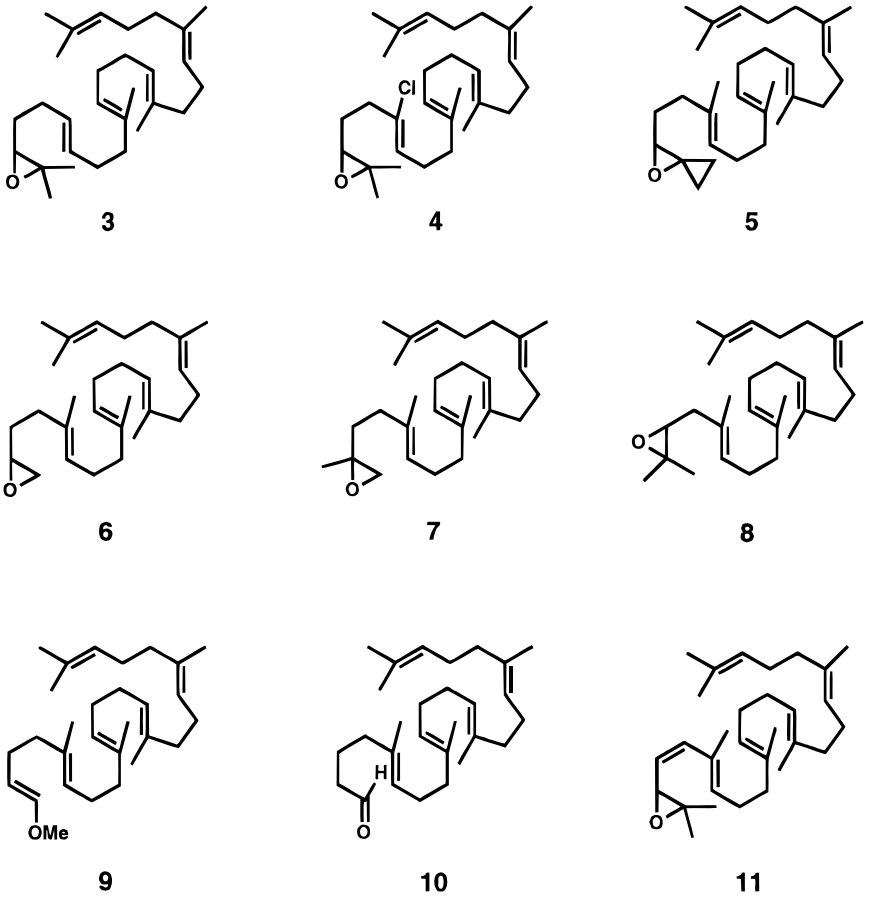
Figure 3. Alignment of lanosterol synthase amino acid sequences from *Saccharomyces cerevisiae* (Sc), *Candida albicans* (Ca), *Schizosaccharomyces pombe* (Sp), *Homo sapiens* (Hs), and *Rattus norvegieus* (Rn). Residues conserved in at least three sequences are shaded.

electrophilic activation because of the lack of charge stabilizing methyl groups at the oxirane C−terminus. Epoxide **8** (a *nor* oxidosqualene) not only has a spatially different location of the oxirane oxygen but also poor stereoelectronic geometry for concerted oxirane cleavage and ring closure. Interesting, but of less mechanistic relevance, is the complete resistance of vinyl ether **9** and aldehyde **10** to lanosterol synthase induced cyclization.

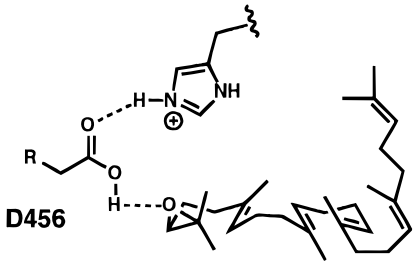
Finally, *Z*-4,5-dehydro-2,3-oxidosqualene (**11**) was synthesized (*vide infra*) and tested as a substrate for lanosterol synthase. This epoxide clearly bound very well to the enzyme since it is a strong competitive inhibitor (IC_{50} 8.3 μM) for the cyclization of 2,3-oxidosqualene (K_M 18 μM). However, epoxide **11** was totally resistant to either cyclization or epoxide cleavage by lanosterol synthase. Given the fact that epoxide **11** is very sensitive to protic activation (for example, it is very unstable on silica gel), as might be expected for an allylic epoxide, the ineffectiveness of lanosterol synthase as a catalyst for oxirane cleavage in **11** is impressive. There are two factors which may give rise to this inertness of **11**. First, the electrophilic activation by the enzyme may be weak and may be effective only for concerted nucleophilic attack by the 6,7-double bond. Second, the three-dimensional arrangement required for concerted oxirane cleavage−A-ring cyclization of **11** is somewhat different than that for **1** and involves significantly greater steric repulsions of the C−1, C−1′, and C−6 methyl groups and poorer orbital overlap between C−2 and C−7. The experimental results with substrate **11** appear to favor the concerted over the nonconcerted ring A closure pathway.

D456 Is The Prime Candidate As Electrophilic Activator of 2,3-Oxidosqualene in Lanosterol Biosynthesis. It is generally accepted that the cyclization of 2,3-oxidosqualene to lanosterol proceeds by way of cationic intermediates[1] and that the process is initiated by electrophilic activation of the oxirane function. One possibility for such electrophilic activation is Lewis acid catalysis by a cationic metal center bound to the enzyme. In order to test for the type of metal ion activation samples of purified enzyme in purified buffer solution (which had been treated to remove transition metals and zinc) was passed through a hydroxyapatite column (which itself removes these metal ions), collected, and analyzed by atomic absorption (carried out by Drs. Robert Shapiro and Bert L. Vallee of the Harvard Medical School). These analyses were negative for Mn, Cu, and Fe cations and essentially negative for Zn^{2+} (since the amount found was only 0.13 Zn^{2+} per molecule of enzyme). As a result of these findings we believe that oxirane activation is unlikely to be caused by a Lewis acidic metal ion and is probably effected by an acidic group (i.e., protic acid) on the enzyme. This conclusion is also consistent with the observations that metal ion chelators such as EDTA and 1,10-phenanthroline

Scheme 3. Oxidosqualene Analogs



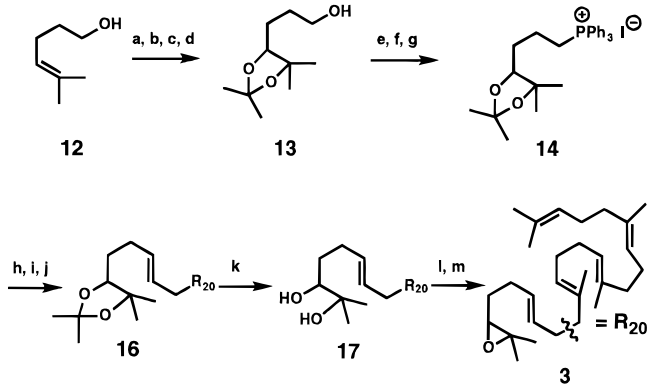
Scheme 4. Possible Role of D456 in Catalysis by Lanosterol Synthase



do not inhibit lanosterol synthase and that H_2S and trisnorsqualenethiol are also without inhibitory effect.

Information regarding the most likely acidic amino acid unit for protic activation of the oxirane function of lanosterol synthase was obtained from site-directed mutagenesis experiments involving the aspartic acid (D) and glutamic acid (E) residues of the enzyme which are conserved in five different species, identified in Figure 3. The species, *Saccharomyces cerevisiae*,[2c] *Candida albicans*,[2a] *Schizosaccharomyces pombe*,[2f] *Homo sapiens*,[2e] and *Rattus norvegicus*,[2d] arose over a large evolutionary time scale (*ca.* 880 million years). Each of the six conserved Asp residues, D140, D286, D370, D456, D580, and D629, was mutated to asparagine, and each of the nine conserved Glu residues, E216, E264, E460, E483, E487, E511, E520, E526, and E634, was mutated to glutamine. Site-directed mutagenesis experiments were carried out using the *ERG7* gene by the method of Kunkel.[9] The steps used were as follows: (1) annealing of the appropriate oligonucleotide to the uracil containing single-stranded DNA template, (2) primer extension using T4 DNA polymerase and nick sealing using T4 DNA ligase, (3) transformation into *E. coli* DH5α and selection of

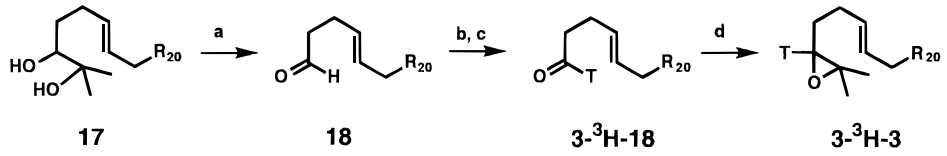
Scheme 5. Synthesis of **3**[a]



[a] (a) BnBr, NaH, 95%. (b) NMO, OsO_4, acetone/H_2O, 83%. (c) 2,2-dimethoxypropane, *p*-toluenesulfonic acid, 97%. (d) H_2, 10% Pd/C, 98%. (e) MsCl, Et_3N, CH_2Cl_2; (f) NaI, acetone, 91% over two steps. (g) PPh_3, CH_3CN, 99%. (h) *n*-BuLi, THF, −78 °C, then $R_{20}CH_2CHO$. (i) *s*-BuLi, THF, −78 °C. (j) 2-phenyl-1-propanol, −78 °C to 23 °C, 55% overall. (k) TsOH, MeOH, 60%. (l) MsCl, Pyr, CH_2Cl_2. (m) K_2CO_3, MeOH, 80% overall.

mutants using restriction fragment analysis, (4) transformation into the mutant yeast strain SMY8 (*MAT***a** *erg7::HIS3 hem1::TRP1 ura3*−52 trp1-Δ63 leu2−3,112 his3-D200 ade2 Gal^+),[2f] and (5) testing for ergosterol production by the complementation method. SMY8 transformants were selected after growth in a synthetic complete medium lacking leucine and supplemented with ergosterol. The transformant with the desired mutant was then restreaked on a YPG plate without supplementary ergosterol. Of the nine conserved glutamates none is essential since all of the individual E → Q mutants were viable in an ergosterol-free medium. Of the six conserved aspartates *only one*, D456,

Scheme 6. Synthesis of ^{3}H-Labeled **3**[a]



17 **18** **3-^{3}H-18** **3-^{3}H-3**

[a] (a) $NaIO_4$, THF/H_2O, 79%. (b) $NaBT_4$, MeOH, 90%. (c) Dess−Martin reagent, CH_2Cl_2, $NaHCO_3$, 81%. (d) Isopropyldiphenylsulfonium tetrafluoroborate, LDA, DME, −78 °C, 99%.

is essential since only the D456N mutant was not viable in the absence of ergosterol.

Incorporation of the mutagenic oligonucleotide was confirmed by restriction fragment analysis since the oligonucleotides were designed to introduce a new restriction site within the gene. To further confirm incorporation of the desired mutations DNA sequencing of the mutated *erg*7 gene was performed on the following mutants: D140N, D370N, D456N, D580N, E460Q, and E634Q. DNA sequence analysis showed both that the desired mutations had been incorporated and that no other mutations had been introduced during the mutagenesis procedure.

The essentiality of D456, its occurrence in a highly conserved region, and the dispensability of all 14 of the other conserved aspartates and glutamates all point to D456 as a likely candidate for protic activation of 2,3-oxidosqualene to initiate cyclization. A matter of concern in this regard is the question of whether D456 is sufficiently acidic to catalyze fast oxirane cleavage, since experiments by Ms. Donnette Daley in this laboratory have revealed that 2,3-oxidosqualene (and similar oxiranes) are stable in glacial acetic acid at 23 °C for many hours and that stronger acids such as trichloroacetic acid are required for catalyzed oxirane cleavage. However, considerable enhancement in the acidity of D456 by the presence of neighboring positive charge[10] is clearly a reasonable possibility. For instance, a proximate protonated histidine hydrogen bonded to the D456 carboxyl *endo* lone pair as shown in Scheme 4 should enhance the acidity of that carboxyl by several orders of magnitude.[11]

Synthesis of 2,3-Oxidosqualene Analogs. 5-Methyl-4-hexen-1-ol (**12**), prepared in 86% yield by reaction of γ-butyrolactol with isopropylidenetriphenylphosphorane in THF, was converted to acetonide **13** as shown in Scheme 5. Conversion of the alcohol **13** to the corresponding iodide and subsequent treatment with triphenylphosphine yielded the triphenylphosphonium salt **14**. Coupling of **14** with the aldehyde **15** using the modified Schlosser−Wittig reaction conditions gave 6-*E*-**16** and 6-*Z*-**16** in 1:1 ratio (reaction not optimized). The mixture of *Z* and *E* isomers of **16** was treated with TsOH in MeOH to remove the protecting group and give diols **17** and 6-*Z*-**17**, which were separated by preparative TLC using 3:1 hexane−EtOAc. The 6-*E* olefin **17** was converted to 6-desmethyl-2,3-oxidosqualene (**3**) via the monomesylate in the standard way. 6-*Z*-**17** was converted 6-*Z*-**3** by the same method. Wittig reaction of aldehyde **15** with the ylide from **14** under salt-free conditions (THF, $KN(TMS)_2$ as base) produced 6-*Z*-**16** cleanly which was further transformed into pure 6-*Z*-**3**. The stereochemistry of **3** at the 6,7-olefinic linkage was clearly shown by the observation of a 15 Hz coupling between the protons at C(6) and C(7) as contrasted with $J = ca.$ 6 Hz for the corresponding protons of 6-*Z*-**3**. These assignments are consistent with the findings that **3** is a substrate for lanosterol synthase, whereas 6-*Z*-**3** is not.

^{3}H-labeled 6-desmethyl-2,3-oxidosqualene was prepared as shown in Scheme 6. A mixture of *Z* and *E* isomers of **17** was cleaved with $NaIO_4$ to give the corresponding aldehydes **18** and 6-*Z*-**18**. The aldehydes were first reduced with $NaBT_4$ to yield ^{3}H-labeled alcohols, which were converted to ^{3}H-labeled aldehydes upon treatment with Dess−Martin reagent. When ^{3}H-labeled aldehydes **18** and 6-*Z*-**18** were treated with the ylide generated from diphenylisopropylsulfonium tetrafluoroborate, ^{3}H-labeled **3** was obtained together with ^{3}H-labeled 6-*Z*-**3**. The validity of the kinetic parameters measured using the mixture of ^{3}H-labeled **3** and 6-*Z*-**3** was confirmed by control experiments which showed that addition of 6-*Z*-**3** to a concentration five times higher than that of **3** did not change the measured kinetics, as expected since 6-*Z*-**3** is not a substrate.

The chloro analog **4** was synthesized by *N*-chlorosuccinimide chlorination[12] as shown in Scheme 7. The resulting mixture of 6-*Z* and 6-*E* isomers of **19** as well as nonchlorinated 6-*Z*-**16** was deprotected to the corresponding diol mixture and separated first by chromatography on silica gel containing 10% $AgNO_3$ to give the *Z* and *E* isomers of diol **20** and then by preparative silica gel plate chromatography to give pure **20** and the *Z* isomer. Conversion of 6-*Z* olefin **20** to **4** and of 6-*E*-**20** to 6-*E*-**4** were carried out in the standard way via the mono mesylate. ^{3}H-labeled **4** was synthesized by a process analogous to that used for the synthesis of ^{3}H-**3** as outlined in Scheme 6.

The stereochemical assignment to **4** and 6-*Z*-**4** is supported by the following data: (1) the observed and calculated[13] δ values for the olefinic proton at C(7) in **4** are 5.48 and 5.49; (2) the observed and calculated[13] δ values for the olefinic proton at C(7) in 6-*Z*-**4** are 5.60 and 5.62; and (3) epoxide **4** is a substrate for lanosterol synthase, whereas 6-*Z*-**4** is not.

From trisnorsqualene aldehyde, compounds **5**−**10** were prepared as shown in Scheme 8. Spiro epoxide **5** was prepared by reaction of trisnorsqualene aldehyde with the ylide generated from cyclopropyldiphenylsulfonium tetrafluoroborate.[14] 1,1′-Bisnorsqualene epoxide (**6**) was prepared by coupling trisnorsqualene aldehyde with trimethylsulfonium methylide. Treatment of trisnorsqualene aldehyde with MeLi gave the corresponding alcohol which by oxidation with PCC generated the methyl ketone **21**. Upon treatment with the ylide generated from trimethylsulfonium methylide, **21** was converted to **7**. After oxidation of trisnorsqualene aldehyde with $NaClO_2$ to the corresponding acid, and methylation with diazomethane, the resulting methyl ester was treated with LDA and NCS to give the corresponding α-chloromethyl ester **22**. When **22** was treated with MeLi, epoxide **8** was generated. The vinyl ether **9** was prepared by coupling trisnorsqualene aldehyde with methoxymethylene triphenylphosphorane. Upon acidic hydrolysis, **9** was converted to aldehyde **10**.

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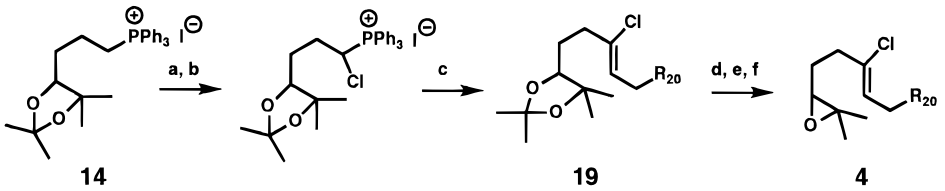
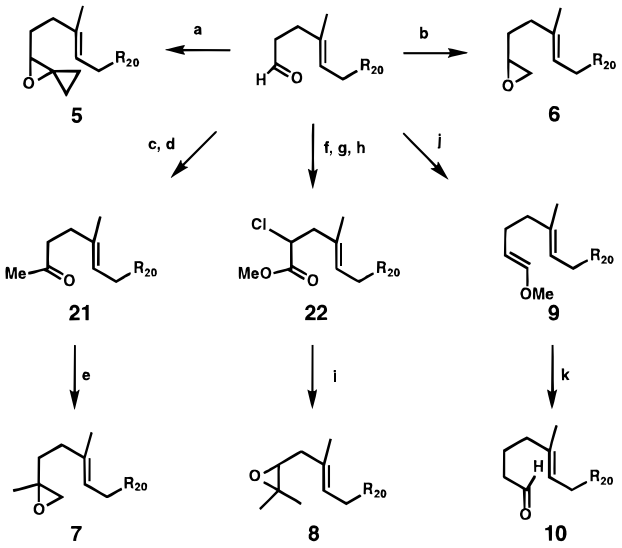
(10) Westheimer, F. H. *Tetrahedron* **1995**, *51*, 3.
(11) (a) Gandour, R. D. *Bioorganic Chem.* **1981**, *10*, 169. (b) This proposal contrasts with the position taken by Abe and Prestwich (Abe, I.; Prestwich, G. D. *Lipids* **1995**, *30*, 231) that two adjacent asp residues in rat lanosterol synthase, corresponding to D456 and C457 in the yeast enzyme, "may act as point charge to stabilize incipient cationic charge". See, also: Abe, I.; Prestwich, G. D. *J. Biol. Chem.* **1994**, *269*, 802.
(12) Corey, E. J.; Shulman, J. I.; Yamamoto, H. *Tetrahedron Lett.* **1970**, 447.
(13) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*, 4th ed.; John Wiley and Sons: New York, 1981, p 228.
(14) Trost, B. M.; Bogdanowicz, M. J. *J. Am. Chem. Soc.* **1973**, *95*, 5311.

Scheme 7. Synthesis of **4**[a]



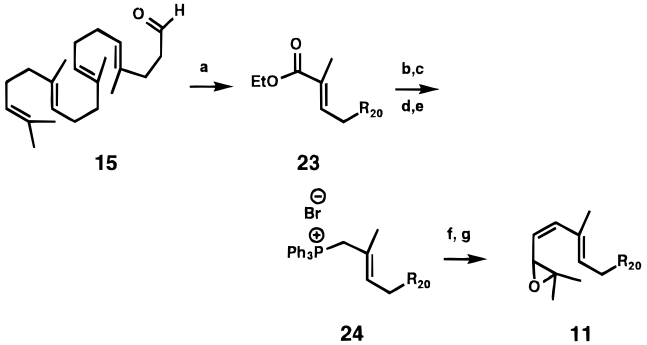
[a] (a) *n*-BuLi, THF, −78 °C. (b) NCS, THF, −78 °C. (c) *s*-BuLi, THF, −78 °C, then **15**, 36% overall. (d) TsOH, MeOH, 28%. (e) MsCl, Pyr, CH_2Cl_2. (f) K_2CO_3, MeOH, 74% overall.

Scheme 8. Synthesis of **5**−**10**[a]



[a] (a) Cyclopropyldiphenylsulfonium tetrafluoroborate, KOH, DMSO, 23 °C, 62%. (b) Trimethylsulfonium iodide, *n*-BuLi, 20%. (c) MeLi, THF, −78 °C, 21%. (d) PCC on alumina, hexane, 23 °C, 84%. (e) Trimethylsulfonium iodide, *n*-BuLi, THF, 3%. (f) $NaClO_2$, NaH_2PO_4, H_2O, *t*-BuOH, 2-methyl-2-butene. (g) CH_2N_2, Et_2O, 0 °C, 8% overall. (h) LDA, THF, −78 °C; then NCS, −78 °C, 43%. (i) MeLi, THF, 39%. (j) Methoxymethyltriphenylphosphonium chloride, LDA, THF, 0 °C, 34%. (k) HCl, H_2O, THF, 23 °C, 4%.

Scheme 9. Synthesis of **11**[a]



[a] (a) Triethylphosphonopropionate, NaH, THF, −78 °C, 77%. (b) DIBAL, CH_2Cl_2, −78 °C, 90%. (c) MsCl, Et_3N, CH_2Cl_2, −40 °C. (d) LiBr, THF; (e) Ph_3P, CH_3CN, 97% overall. (f) *t*-BuOK, THF, −78 °C. (g) Me_2C(-O-)-CHCHO, −78 °C to 20 °C, 66%.

To prepare 4,5-dehydro-2,3-oxidosqualene (**11**), triethyl phosphonopropionate was condensed with **15** using NaH as a base as shown in Scheme 9. A mixture of *E* and *Z* (ratio 4:1) of α,*β*-unsaturated esters **23** was obtained. After the *Z* isomer of **23** was removed by flash chromatography, the *trans* isomer **23** was treated with DIBAL to give the corresponding alcohol, which was converted to the triphenylphosphonium salt **24** by reaction with MsCl and subsequent reaction with LiBr and PPh_3. Coupling of **24** with the epoxide of *β*,*β*-dimethylacrolein using *t*-BuOK as a base produced 4,5-(*Z*)-dehydro-2,3-oxidosqualene (**11**).

Conclusions

In summary, new data and insights have been presented on the initiation step in sterol biosynthesis from 2,3-oxidosqualene. Specifically, the possibility has been advanced that electrophilic oxirane cleavage and cyclization to form the A ring are concerted[15] and that oxirane activation involves a particularly acidic, highly conserved aspartic acid residue, D456. An X-ray crystal structure of lanosterol synthase would be of great value in connection with these hypothesis. However, given the hydrophobic character, water insolubility, and instability of the enzyme, the prospects for such a determination in the foreseeable future seem questionable.

Experimental Section

Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on an AM-500 (500 MHz), AM-400 (400 MHz), or AM-300 (300 MHz) spectrometers using chloroform-*d* as a solvent. Chemical shifts are reported as *δ*, parts per million downfield shift from tetramethylsilane (*δ* 0.0) using the residual solvent signal as an internal standard; *δ* 7.24 (^{1}H), *δ* 77.0 triplet (^{13}C). All coupling constants are in units of Hertz. Infrared spectra (IR) were recorded on a Nicolet 5 ZDX FTIR spectrometer with an internal polystyrene sample as a reference. Mass spectral analyses were performed with JEOL model AX-505 or SX-102 spectrometers. Reactions were monitored by thin layer chromatography (TLC) using Merck 60 F_{254} precoated silica gel plates (0.25 mm thickness). After ultraviolet illumination at 254 nm, the plates were visualized by immersion in the indicated solution and warming on a hot plate. Baker silica gel and reagent grade solvents were used for flash chromatography. Preparative thin layer chromatography was performed using Merck 60 F_{254} precoated silica gel plates (0.50 mm thickness, 20 × 20 cm). $AgNO_3$ impregnated silica gel plates were prepared by the addition of 5% (w/w silica gel) $AgNO_3$ to the water used in typical chromatotron plate preparation. All substrate analogs were purified chromatographically to homogeneity by TLC analysis and purity was confirmed as >97% by ^{1}H NMR analysis. All solvents are reagent grade unless otherwise stated, and anhydrous solvents were dried immediately prior to use.

Spodoptera frugiperda (*Sf 9)* insect cells, BaculoGold Transfection Kit, and pVL1393 were purchased from PharMingen. IPL-41 powdered media and fetal bovine serum were purchased from GIBCO. Grace's liquid insect media was obtained from Invitrogen or PharMingen, Pluronic F-68 from JRH Biosciences, restriction enzymes and T4 DNA polymerase from New England Biolabs or GIBCO, and T4 DNA ligase from GIBCO. QIAEX gel extraction kit was purchased from QIAGEN. CsCl was purchased from Boehringer Mannheim Biochemicals. DEAE Sepharose ion exchange resin, dialysis membrane tubing, and Triton X-100 were purchased from Sigma. Hydroxyapatite and BioRad protein content assay kit were purchased from BioRad. Standard protein molecular weight markers were purchased from Promega. Protogel for SDS-PAGE was purchased from Kimberly Research. Aquasol from Du Pont was used for liquid scintillation counting.

(15) Kinetic studies on the trichloroacetic acid-catalyzed cyclization of 2,3-oxido-2,6-dimethylhept-6-ene and related substrates in $CHCl_3$ carried out by Ms. Donnette Daley in these laboratories have demonstrated conclusively that such chemical cyclizations also involve concerted oxirane cleavage and cyclization (manuscript in preparation for this Journal). For early studies on such cyclizations, see: Goldsmith, D. J. *J. Am. Chem. Soc.* **1962**, *84*, 3913.

Construction of Recombinant Baculovirus Transfer Vector pVL1393-*ERG7*. A 2.3 kb DNA fragment encoding lanosterol synthase from *S. cerevisiae* was excised from pSM61.5 by digestion with EcoRV and NotI, isolated by agarose gel electrophoresis, and purified using the QIAEX gel extraction protocol (QIAGEN). The fragment was then ligated into the baculovirus transfer vector pVL1393, which had previously been digested with SmaI and NotI and treated with calf intestinal phosphatase. The ligation reaction mixture was used to transform competent *E. coli* DH5α. Restriction analysis of the resulting clones confirmed construction of the transfer vector with correct orientation. One clone was grown up on a large-scale, and the recombinant plasmid (pVL1393-*ERG7*) was purified by CsCl gradient. The transfer vector and PharMingen's linearized BaculoGold viral DNA were used to transfect *Sf 9* cells using PharMingen's calcium phosphate transfection procedure.[16] After the transfected cells were incubated for 4 days at 27 °C, the media containing the recombinant virus (AcMNPV-*ERG7*) was collected. A portion of the recombinant viral stock was used to infect a fresh plate of *Sf 9* cells in order to amplify the virus.

Expression of Lanosterol Synthase in *Sf 9* Cells Using AcMNPV-*ERG7*. A 10 L bioreactor of *Sf 9* cells growing in serum-free Sf900 II media with 0.05% Pluronic F-68 was infected with AcMNPV-*ERG7* at a multiplicity of infection of *ca.* 1. At 48 h post infection, the cells were harvested yielding a wet cell paste (120 g), which was frozen at −78 °C.

Protein Purification. *Sf 9* cells (20.9 g) infected with the *ERG7*-recombinant baculovirus were suspended in 100 mM sodium phosphate buffer (pH 7.0, 63 mL). The resulting suspension was sonicated using four 30 s blasts (power lever 4) at 4 °C. The cell lysate was centrifuged (16 000 × *g*, 30 min), and to the resulting supernatant was added saturated ammonium sulfate solution (30 mL). This suspension was stirred at 4 °C for 1 h, after which the precipitated proteins were pelleted by centrifugation (16 000 × *g*, 30 min). The pellet was resuspended in 20 mM sodium phosphate buffer (pH 7.0, 50 mL) containing 20% glycerol, 1% Triton X-100, and 3 mM DTT. This solution was dialyzed against 20 mM sodium phosphate buffer (pH 7.0, 1 L) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT at 4 °C overnight and then applied to a DEAE Sepharose CL-6B column (50 mL bed volume) at 4 °C preequilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT. The column was eluted with a linear gradient from 20 mM sodium phosphate (pH 7.0) to 200 mM sodium phosphate (pH 7.0) (250 mL × 250 mL) both containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT. Fractions of 15 mL each were collected. Enzyme activity was eluted off the column between 85 and 100 mM sodium phosphate concentration.

The most active fractions were combined, dialyzed against 7.5 mM sodium phosphate buffer (pH 7.0, 1 L) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT at 4 °C overnight, and then applied to a hydroxyapatite (HAT) column (25 mL bed volume) preequilibrated with 7.5 mM sodium phosphate buffer (pH 7.0) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT at 4 °C. The column was washed with seven column volumes of 1 M sodium chloride solution, followed by one column volume of 7.5 mM sodium phosphate buffer (pH 7.0) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT. The enzyme was eluted with a linear gradient from 7.5 mM sodium phosphate (pH 7.0) to 200 mM sodium phosphate (pH 7) (180 mL × 180 mL) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT. Fractions of 10 mL each were collected. Enzyme activity was eluted between 110 and 150 mM sodium phosphate concentration. Aliquots from fractions containing the most cyclase activity were analyzed by SDS-PAGE for purity.[17]

Enzyme Activity Assay. To 47.5 μL of the enzyme solution was added 2.5 μL solution containing racemic 2,3-oxidosqualene (20 mg/mL) and Triton X-100 (200 mg/mL) to yield a final concentration of 1 mg/mL substrate and 1% Triton X-100. The resulting solution was incubated at 18 °C for 8 to 12 h. The reaction mixture (5 mL) was spotted on a TLC plate, and water was removed by placing the TLC plate *in vacuo* for 15 min. The TLC plate was then developed in diethyl ether to 2 cm and then fully developed in hexane−diethyl ether (1:1). The TLC plate was visualized by staining with *p*-anisaldehyde. R_f values for 2,3-oxidosqualene and lanosterol were 0.91 and 0.54, respectively.

Quantitative assays were performed in duplex with the following modifications. The substrate, 2,3-oxidosqualene, was radiolabeled with tritium (100 mCi/mmol). The assays for homogenate, supernatant, and ammonium sulfate precipitated fractions were terminated at 30 min, and the assays for DEAE and HAT fractions were terminated at 2.5 h. After chromatography, the areas corresponding to oxidosqualene and lanosterol were added to scintillation cocktail and counted.

To measure the Michaelis−Menten constants and reaction rates of 2,3-oxidosqualene, 6-desmethyloxidosqualene, and 6-chlorooxidosqualene, the following procedures were used. Substrate or modified substrate at different concentrations in 20% Triton X-100 in water was added to lanosterol synthase in 100 mM sodium phosphate buffer (pH 7.0) containing 20% glycerol, 0.2% Triton X-100, and 3 mM DTT. After vortexing, the reaction mixture was incubated at room temperature. Aliquots were withdrawn for analysis at different times before the reaction reached 5% conversion. K_m and V_{max} were obtained by fitting the initial rates vs. substrate concentrations to the Michaelis−Menten equation. Those parameters can also be obtained from the double reciprocal plot of initial rates vs. substrate concentrations.

Protein Content Determination. Protein content of homogenate, supernatant, and ammonium sulfate precipitated fractions were determined based on the pellet weight. The protein amount in DEAE column chromatography fractions were determined by BioRad protein content determination methods. The protein concentration in HAT column chromatography fractions were determined by comparing with known amounts of protein standards on SDS-PAGE.

Expression Fold Determination. Wild type *S. cerevisiae* was hydrated by soaking with water and lysed by suspending in five times weight of 100 mM sodium phosphate buffer (pH 7.0) and vortexing against one volume of glass beads. The homogenate was then incubated with tritium labeled substrate for 3.5 h and assayed as described above. The specific activity of the homogenate solutions from wild type yeast (2.18 pmol/min/mg) and *Sf 9* cells (21.0 pmol/min/mg) were used to calculate the overexpression fold, which is 10-fold.

Mutagenesis. Mutants were generated according to the method of Kunkel.[9] Uracil-containing single-stranded DNA was prepared using helper phage M13KO7 from *E. coli* RZ1032 transformed with the vector pSM61.21, a *LEU2*-based pRS305[18] derivative containing the *S. cerevisiae* lanosterol synthase gene driven by the GAL promoter. Oligonucleotides (approximately 40 bases long) containing the desired mutation as well as silent mutations which created or deleted a restriction site were used as primers. Oligonucleotides were synthesized by an automated DNA synthesizer. Mutant clones were screened by restriction fragment analysis. Some of the mutants, D140N, D370N, D456N, D580N, E460Q, and E634Q, were sequenced by the dideoxynucleotide method using the USB Sequenase Kit (version 2.0) to further confirm incorporation of the mutagenic oligonucleotide.[19] *E. coli* strain DH5α was used for all DNA manipulations. *E. coli* were transformed, selected, and propagated according to published procedures.[19] The plasmid pSM61.21 was linearized with BstEII and transformed into *S. cerevisiae* strain SMY8 (*MAT***a** *erg7::HIS3 hem1::TRP1 ura3*−52 trp1-Δ63 leu2−3,112 his3-D200 ade2 Gal^+)[2f] in order to integrate into the *LEU2* locus in the genome by homologous recombination. SMY8 transformants were selected at 30 °C on synthetic complete medium lacking leucine, supplemented with hemin chloride (13 mg/L), ergosterol (20mg/L), and Tween 80 (0.5%). Complementation analysis was performed on 1% yeast extract, 2% peptone, 2% galactose, and 2% agar, supplemented with heme chloride (13 mg/L), and Tween 80 (0.5%).[20]

5-Methyl-4-hexen-1-ol (12). To a stirred solution of γ-butyrolactone (11.0 g, 128 mmol) in CH_2Cl_2 (50 mL) at −78 °C was added gradually 1 M diisobutylaluminum hydride in CH_2Cl_2 (150 mL). After the

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(20) *Guide to Yeast Genetics and Molecular Biology*; Guthrie, C., Fink, G. R., Ed.; Academic: New York, 1991.

reaction mixture was stirred at −78 °C for 1 h, it was quenched with MeOH (10 mL) and saturated Rochelle's salt (10 mL). The mixture was warmed to 23 °C and stirred for 2 h. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined extracts were dried ($MgSO_4$) and concentrated *in vacuo* to yield the desired product as a colorless oil. The crude product was used directly for the next reaction without purification.

To a suspension of isopropyltriphenylphosphonium iodide (20.6 g, 47.7 mmol) in THF (50 mL) at −78 °C was added *t*-BuOK (5.9 g, 52.7 mmol) in THF (40 mL). The mixture was stirred at 0 °C for 30 min, γ-butyrolactol (4.0 g, 45.5 mmol) in THF (20 mL) was added dropwise, and the resulting mixture was stirred overnight. The reaction mixture was treated with water and extracted with ether (3 × 50 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by flash column chromatography using 3:7 ether−pentane (4.50 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.11 (m, 1 H), 3.61 (t, J = 6.6, 2 H), 2.03 (m, 2 H), 1.67 (s, 3 H), 1.59 (s, 3 H), 1.56 (m, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 132.0, 123.8, 62.5, 32.6, 25.6, 24.3, 17.5; IR (neat) 3334 (br), 2965, 2928, 2877, 2871, 2863, 1450, 1436, 1059, 1041 cm^{-1}; HRMS (CI) 114.1043 (114.1045 calcd for $C_7H_{14}O{\cdot}NH_4^+$).

Triol Monoacetonide 13. To a solution of **12** (1.55 g, 13.6 mmol) in THF (30 mL) at 0 °C was added sodium hydride (359 mg, 15.0 mmol), followed by benzyl bromide (1.78 mL, 15.0 mmol, dropwise). The resulting solution was stirred at 23 °C overnight, filtered, and concentrated *in vacuo*. The crude product was purified by flash column chromatography using 5% ethyl acetate in hexane to give the benzyl ether of **12** (2.6 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42−7.26 (m, 5 H), 5.13 (m, 1 H), 4.51 (s, 2 H), 3.49 (t, 2 H), 2.09 (m, 2 H), 1.71 (s, 3 H), 1.69 (m, 2 H), 1.64 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.8, 132.2, 129.0, 128.7, 128.3, 127.6, 127.4, 123.9, 72.8, 69.9, 33.5, 25.7, 24.5, 17.6; IR (neat) 2965, 2927, 2855, 1454, 1363, 1102, 1076 cm^{-1}; HRMS (CI): 222.1867 (222.1858 calcd for $C_{14}H_{20}O$ + NH_4^+)

N-Methylmorpholine oxide (630 mg, 5.4 mmol), the benzyl ether of **12** (1 g, 4.9 mmol), and OsO_4 (8 mg) were dissolved in 5:2 acetone−water solution with stirring, and the resulting solution was stirred at 23 °C overnight, after which it was quenched with sodium hydrogen sulfite (50 mg). After another 20 min of stirring, the solution was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. The crude diol was purified by flash column chromatography using first 20% ethyl acetate in hexane then ethyl acetate (970 mg, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36−7.25 (m, 5 H), 4.51 (s, 2 H), 3.51 (t, J = 5.9, 2 H), 3.35 (m, 1 H), 3.32 (m, 1 H), 2.63 (s, 1 H), 1.86−1.33 (m, 4 H), 1.17 (s, 3 H), 1.12 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.0, 128.3, 127.7, 127.6, 78.1, 72.9, 70.3, 28.8, 26.9, 26.3, 23.2; IR (neat) 3423 (br), 2970, 2933, 2862, 1364, 1090, 1077 cm^{-1}; HRMS (CI):256.1909 (256.1913 calcd for $C_{14}H_{22}O_3$ + NH_4).

To a solution of the above prepared benzyl ether diol (640 mg, 2.69 mmol) in 2,2-dimethoxypropane (30 mL) was added *p*-toluenesulfonic acid (20 mg). The resulting solution was stirred for 30 min, saturated sodium bicarbonate solution (20 mL) was added, and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (3 × 25 mL). The combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated *in vacuo* to give the acetonide (730 mg, 97%). ^{1}H NMR ($CDCl_3$) δ 7.37−7.25 (m, 5 H), 4.51 (s, 2 H), 3.68 (m, 1 H), 3.57−3.48 (m, 2 H; m), 1.82 (m, 1 H), 1.70 (m, 1 H), 1.56 (m, 2 H), 1.31 (s, 3 H), 1.27 (s, 3H), 1.24 (s, 3 H), 1.09 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.5, 128.3, 127.6, 127.5, 106.4, 83.1, 80.1, 72.8, 69.9, 28.5, 27.1, 26.8, 26.0, 22.8; IR (neat) 2981, 2935, 2860, 1370, 1217, 1197, 1115, 1008 cm^{-1}; HRMS (CI) 279.1965 (279.1960 calcd for $C_{17}H_{27}O_3$).

A mixture of the above prepared benzyl ether acetonide (650 mg, 2.34 mmol) and 10% palladium−carbon (0.5 g) in ethanol (30 mL) was hydrogenated at room temperature under atmospheric pressure. After the reaction was complete, the mixture was filtered, and the filtrate was concentrated *in vacuo* to afford alcohol acetonide **13** (430 mg, 98%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.71−3.68 (m, 3 H), 2.20 (s, 1 H), 1.74 (m, 2 H), 1.57 (m, 2 H), 1.43 (s, 3 H), 1.34 (s, 3 H), 1.25 (s, 3 H), 1.10 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 106.7, 83.5, 80.5, 62.7, 30.6, 28.5, 26.9, 26.3, 25.9, 22.8; IR (neat) 3417 (br), 2982, 2937, 2870, 1371, 1236, 1217, 1200, 1117, 1063, 1034, 1008 cm^{-1}; HRMS (CI) 189.1483 (189.1491 calcd for $C_{10}H_{21}O_3$).

Triphenylphosphonium Salt 14. The alcohol acetonide **13** (300 mg, 1.60 mmol) and triethylamine (640 mg, 6.32 mmol) were dissolved in dichloromethane (40 mL) at 0 °C, and methanesufonyl chloride (724 mg, 6.32 mmol) was added with stirring. The reaction mixture was stirred at 0 °C for 1 h, allowed to warm to room temperature, and poured into a solution of 5% aqueous sodium bicarbonate solution. The methanesulfonate ester was isolated by extraction with ether and removal of solvent. The mesylate was then heated at reflux with sodium iodide in acetone under argon for 3 h. The mixture was cooled to room temperature, and the solvent was removed *in vacuo*. After workup, the crude product was purified by flash chromatography to generate the corresponding iodide (430 mg, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67 (m, 1 H), 3.31−3.18 (m, 2 H), 2.09 (m, 1 H), 1.89 (m, 1 H), 1.56 (m, 2 H), 1.32 (s, 3 H), 1.27 (s, 3 H), 1.25 (s, 3 H), 1.10 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 106.7, 82.3, 80.1, 30.9, 30.2, 28.5, 26.8, 25.9, 22.8, 6.6; IR (neat) 2981, 2936, 2866, 1377, 1370, 1271, 1236, 1217, 1168, 1002 cm^{-1}; HRMS (CI): 316.0767 (316.0774 calcd for $C_{10}H_{19}O_2I$ + NH_4).

The above prepared iodide (210 mg, 0.70 mmol) and triphenylphosphine (600 mg, 2.29 mmol) were dissolved in acetonitrile (20 mL), and the resulting solution was heated at reflux under argon overnight. The solvent was then removed *in vacuo*. The crude product was purified by flash column chromatography using 2% MeOH in $CHCl_3$ to give **14** (390 mg, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85−7.67 (m, 15 H), 4.05(m, 1 H), 3.59 (m, 1 H), 3.73 (m, 1 H), 2.00 (m, 1 H), 1.86 (m, 2 H), 1.68 (m, 1 H), 1.35 (s, 3 H), 1.29 (s, 3 H), 1.25 (s, 3 H), 1.04 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 135.0, 133.7, 130.4, 118.5, 117.6, 106.8, 82.6, 80.6, 29.4, 29.2, 28.5, 27.1, 26.0, 23.1, 20.5; IR (neat) 2979, 2934, 2927, 2865, 2361, 2342, 2333, 1438, 1113 cm^{-1}; HRMS (FAB) 433.2298 (433.2296 calcd for $C_{28}H_{34}O_2P$).

Acetonide 16. To the triphenylphosphonium salt **14** (150 mg, 0.268 mmol) in THF (2 mL) at −78 °C was added *n*-BuLi (161 μL, 1.67 M solution in hexane), and the resulting solution was stirred at room temperature for 30 min. The reaction mixture was then cooled to −78 °C, and the aldehyde $R_{20}CH_2CHO$ (84.6 mg, 0.268 mmol) in THF (2 mL) was added. The aldehyde $R_{20}CH_2CHO$ was prepared by peracid epoxidation of squalene, cleavage to a diol mixture by aqueous acid, chromatography, diol cleavage with periodate, and vacuum distillation. After 5 min stirring at −78 °C, *s*-BuLi (227 μL, 1.3 M solution in cyclohexane) was added, and the reaction mixture was stirred at −78 °C for 3 h. 2-Phenylpropanol (40.0 mg, 0.295 mmol) was then added, and the solution was warmed to room temperature. After workup, the product was purified by flash chromatography using 4% diethyl ether in hexane (69.1 mg, 55%). The product consisted of a 1:1 mixture of *Z* and *E* isomers. R_f 0.77 (silica gel, 1:1 diethyl ether−hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.06 (s, 3 H), 1.21 (s, 3 H), 1.31 (s, 3 H), 1.40 (s, 3 H), 1.38 (m, 1 H), 1.49 (m, 1 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.95−2.19 (m, 18 H), 3.66 (m, 1 H), 5.09 (m, 4 H), 5.36 (m, vinyl *H* for cis double bond), 5.41 (m, vinyl *H* for trans double bond); IR (neat) 2980, 2929, 2854, 1376, 1217 cm^{-1}; MS (CI) *m/z* 488 (M + NH_4^+); HRMS: 470.4129 (470.4124 calcd for $C_{32}H_{54}O_2$).

Diol 17. To a solution of the acetonide **16** (58.5 mg) in THF (0.4 mL) and MeOH (3 mL) was added *p*-toluenesulfonic acid (5.00 mg), and the resulting solution was stirred at room temperature for 24 h. The solvent was then removed *in vacuo*, and the product was purified by flash chromatography using 20% diethyl ether in hexane (32.0 mg, 60%). The isomers **17** and 6-*Z*-**17** were then separated by preparative TLC using 3:1 hexane-EtOAc.

Data for 17: R_f 0.62 (silica gel, diethyl ether); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.13 (s, 3 H), 1.18 (s, 3 H), 1.38 (m, 1 H), 1.50 (m, 1 H), 1.57 (s, 12 H), 1.66 (s, 3 H), 1.95−2.21 (m, 20 H), 3.36 (dd, J = 10.5 Hz, J = 2 Hz, 1 H), 5.09 (m, 4 H), 5.43 (m, 2 H, vinyl H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.01, 16.05, 17.69, 23.21, 25.71, 26.46, 26.65, 26.76, 28.24, 29.78, 31.17, 31.44, 39.71, 39.74, 73.02, 78.09, 124.24, 124.40, 124.53, 129.65, 131.10, 131.29, 134.74, 135.17; IR (neat) 2966, 2927, 2855, 1448, 1380, 1007 cm^{-1}; MS (EI) *m/z* 430 (M^+); HRMS: 430.3821 (430.3811 calcd for $C_{29}H_{50}O_2$).

Data for 6-*Z*-17: R_f 0.67 (silica gel, diethyl ether); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.13 (s, 3 H), 1.18 (s, 3 H), 1.38 (m, 1 H), 1.50 (m, 1 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.93−2.23 (m, 20 H), 3.37 (d, J =

10.4 Hz, 1 H), 5.09 (m, 4 H), 5.37 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.00, 16.06, 17.68, 23.23, 24.44, 25.68, 25.88, 26.51, 26.68, 26.79, 28.27, 31.59, 39.62, 39.74, 73.03, 78.21, 124.27, 124.42, 124.71, 129.05, 130.54, 131.25, 134.68, 135.16; IR (neat) 2966, 2927, 2855, 1448, 1380, 1007 cm^{-1}; MS (EI) *m/z* 430 (M^+); HRMS: 430.3821 (430.3811 calcd for $C_{29}H_{50}O_2$).

6-Desmethyltrisnorsqualene Aldehyde (18). Diol **17** (19.0 mg, 0.0442 mmol) was dissolved in THF (0.27 mL), water was added to saturation (slight cloudiness) followed by $NaIO_4$ (28.4 mg, 0.133 mmol), and the resulting solution was stirred at room temperature for 1 h. After workup, the product was purified by flash chromatography using 3% diethyl ether in hexane (13.0 mg, 79%). R_f 0.8 (silica gel, 1:1 diethyl ether−hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.58 (s, 12 H), 1.66 (s, 3 H), 1.97−2.65 (m, 20 H), 5.07 (m, 4 H), 5.31 (m, vinyl *H* for cis double bond), 5.39 (m, vinyl *H* for trans double bond); IR (neat) 2961, 2927, 2856, 1729, 1446 cm^{-1}; MS (EI) *m/z* 370 (M^+); HRMS: 370.3247 (370.3236 calcd for $C_{26}H_{42}O$).

^{3}H-Labeled 6-Desmethyltrisnorsqualene Aldehyde (3-^{3}H-18). Aldehyde **18** (10.7 mg, 0.0288 mmol) was dissolved in MeOH (1 mL), and the resulting solution was cooled to 0 °C. Under stirring conditions was added [^{3}H]-$NaBH_4$ (25 mCi, 359.8 mCi/mmol) in MeOH (1 mL), and the stirring was continued for 2 h. Acetone (100 mL) was added to quench the unreacted [^{3}H]-$NaBH_4$. The solvent was removed *in vacuo*, and the product was purified by flash chromatography using 20% diethyl ether/hexane to give the ^{3}H-labeled alcohol (9.6 mg, 0.0257 mmol, 90%). A solution of the resulting alcohol in CH_2Cl_2 (1 mL) was treated with $NaHCO_3$ (23.5 mg, 0.280 mmol) and Dess−Martin reagent (18.7 mg, 0.0431 mmol). After stirring for 1 h at 23 °C, saturated $Na_2S_2O_3$ solution (1 mL), saturated $NaHCO_3$ solution (1 mL), and CH_2Cl_2 (1 mL) were added. The stirring was continued until two phases became clear. The reaction was then worked up, and the product was purified by flash chromatography using 3% diethyl ether in hexane (7.8 mg, 0.0211 mmol, 81%). This material was used to make ^{3}H-labeled 6-desmethyloxidosqualene (8.6 mg, 68 μCi/μmol, 99%) following method (b) which is described below.

6-Desmethyl-2,3-oxidosqualene (3). Diol **17** (7.0 mg, 0.0163 mmol) was dissolved in CH_2Cl_2 (80 μL) and pyridine (13.2 μL, 0.163 mmol) with stirring at 23 °C, MsCl (1.51 μL) was added, and stirring continued at room temperature for 12 h. MeOH (1 mL) and K_2CO_3 (35 mg) were then added, and after stirring for another 15 min, the solvent was removed *in vacuo*, and the product was purified by flash chromatography using 3% diether-hexane to give **3** (5.5 mg, 80%). R_f 0.86 (silica gel, 1:1 diethyl ether−hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24, (s, 3 H), 1.28 (s, 3 H), 1.51−1.66 (m, 2 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.93−2.13 (m, 18 H), 2.72 (m, 1 H), 5.09 (m, 4 H), 5.43 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.01, 16.05, 17.67, 18.79, 24.89, 25.68, 26.68, 26.79, 28.26, 28.93, 29.50, 31.22, 39.71, 39.74, 58.27, 64.05, 124.27, 124.42, 124.50, 129.00, 130.97, 131.25, 134.92, 135.15; IR (neat) 2966, 2927, 2855, 1448, 1380, 1007 cm^{-1} IR (neat) 2961, 2927, 2855, 1448, 1378 cm^{-1}; MS (EI) *m/z* 412 (M^+); HRMS: 412.3710 (412.3705 calcd for $C_{29}H_{48}O$).

6-*Z*-3. Diol 6-*Z*-**17** (2.8 mg, 0.00651 mmol) was dissolved in CH_2Cl_2 (80 μL) and pyridine (5.26 μL, 0.0651 mmol) with stirring at room temperature, MsCl (0.655 μL in 10 μL CH_2Cl_2, 0.00847 mmol) was added, and the stirring was continued at room temperature for 12 h. MeOH (0.5 mL) and K_2CO_3 (15 mg) were then added. After stirring for another 15 min, the solvent was removed *in vacuo*, and the product was purified by flash chromatography using 3% diether-hexane to give 6-*Z*-**3** (0.50 mg, 19%). R_f 0.86 (silica gel, 1:1 diethyl ether−hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (s, 3 H), 1.29 (s, 3 H), 1.51−1.66 (m, 2 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.95−2.24 (m, 18 H), 2.72 (m, 1 H), 5.09 (m, 4 H), 5.38 (m, 2 H); IR (neat) 2961, 2927, 2855, 1448, 1378 cm^{-1}; MS (EI) *m/z* 412 (M^+); HRMS: 412.3710 (412.3705 calcd for $C_{29}H_{48}O$).

^{3}H-Labeled 6-Desmethyl-2,3-oxidosqualene (3-^{3}H-3). To a solution of diphenylisopropylsulfonium boron tetrafluoride (121 mg, 0.383 mmol) in DME (2 mL) at −78 °C was added LDA solution (0.100 mL, 0.38 M solution in DME), and the resulting solution was stirred at −78 °C for 30 min. Aliquots of this solution were then added to a solution of 6-desmethyl trisnorsqualene aldehyde (3-^{3}H-**18**) (6.1 mg, 0.0165 mmol) in DME (1 mL) at −78 °C until the solution turned yellow. The reaction mixture was stirred at −78 °C for 1 h and then worked up by extraction. The product was purified by flash chromatography using 3% diethyl ether in hexane (6.9 mg, 99%). The specific activity of the product was 68 μCi/μmol; trans/cis ratio was 53:47.

Acetonide of 6-Chloro-6-desmethyl-2,3-dihydroxysqualene 19. To the triphenyl phosphonium salt **14** (208 mg, 0.371 mmol) in THF (1 mL) at −78 °C was added *n*-BuLi (222 μL, 1.67 M solution in hexane), and the resulting solution was stirred at room temperature for 30 min. The reaction mixture was cooled to −78 °C, and *N*-chlorosuccinimide (59.2 mg, 0.443 mmol) in THF (1 mL) was added. After stirring for 20 min at −78 °C, *s*-BuLi (616 μL, 1.3 M solution in cyclohexane) was added until the solution became clear, and the reaction mixture was stirred at −78 °C for 90 min. To this mixture was added the aldehyde $R_{20}CH_2CHO$ (95.0 mg, 0.300 mmol) in THF (1 mL) with stirring which was continued at −78 °C for 5 min. The reaction mixture was then warmed to room temperature, and after extractive workup, the product was purified by flash chromatography using 4% diethyl ether in hexane (54.0 mg, 36%). The product was a mixture of 6-*E* and 6-*Z* chloroolefins and 6-*E*-6-desmethyloxidosqualene (1:1:2) as determined by 400 MHz ^{1}H NMR analysis.

Conversion of Acetonide 19 to the Corresponding Diol (20). To a solution of the acetonide **19** (50.0 mg, 0.100 mmol) in THF (0.2 mL) and MeOH (2 mL) was added *p*-toluenesulfonic acid (5.00 mg, 0.0263 mmol), and the resulting solution was stirred at room temperature for 48 h. The solvent was then removed *in vacuo*, and the crude product was subjected to flash chromatography using 20% diethyl ether in hexane to give a mixture of chlorinated and nonchlorinated compounds. The nonchlorinated component was then removed by flash chromatography on silica gel containing 10% silver nitrate using diethyl ether as an eluant. The compounds eluted first were the 2,3-dihydroxy-6-*Z* and *E*-chloro olefins (1:1) (13.0 mg, 28%). The *Z* and *E* isomers were then separated by use of a preparative TLC silica gel plate using 50% diethyl ether−hexane.

Data for 2,3-dihydroxy-6-chloro-6-desmethylsqualene (20). R_f 0.83 (silica gel, diethyl ether); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.15 (s, 3 H), 1.20 (s, 3 H), 1.45 (m, 1 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.76 (m, 1 H), 1.95 (m, 14 H), 2.25 (m, 2 H), 2.41 (m, 1 H), 2.52 (m, 1 H), 3.33 (d, J = 10.7 Hz, 1 H), 5.09 (m, 4 H), 5.49 (t, J = 6.8 Hz, 1 H); IR (neat) 3406 (br), 2966, 2925, 2854, 1446, 1382, 1158, 1079 cm^{-1}; MS (FAB) *m/z* 487 ($M + Na^+$); HRMS: 487.3310 (487.3319 calcd for $C_{29}H_{49}ClO_2$).

Data for 6-*E*-20. R_f 0.91 (silica gel, diethyl ether); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.16 (s, 3 H), 1.21 (s, 3 H), 1.48 (m, 1 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.79 (m, 1 H), 2.05 (m, 14 H), 2.16 (m, 2 H), 2.51 (m, 2 H), 3.33 (d, J = 10.7 Hz, 1 H), 5.09 (m, 4 H), 5.60 (t, J = 7.5 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.96, 16.00, 16.06, 17.62, 23.50, 25.57, 26.67, 26.84, 26.95, 27.34, 28.25, 28.35, 29.47, 30.80, 39.34, 39.80, 57.52, 73.09, 124.29, 124.45, 124.59, 125.55, 128.53, 133.50, 133.98, 134.97, 135.34; IR (neat) 3406 (br), 2966, 2925, 2854, 1446, 1382, 1158, 1079 cm^{-1}; MS (FAB) *m/z* 487 ($M + Na^+$); HRMS: 487.3310 (487.3319 calcd for $C_{29}H_{49}ClO_2$).

6-Chloro-6-desmethyltrisnorsqualene Aldehyde (21). A solution of a 1:1 mixture of **20** and 6-*E*-**20** (13 mg, 0.0267mmol) in THF (0.30 mL) was treated with water to slight turbidity, then $NaIO_4$ (25 mg, 0.117 mmol) was added, and the resulting solution was stirred at room temperature for 1 h. After workup, the product was purified by flash chromatography using 3% diethyl ether in hexane (6.9 mg, 61%). R_f 0.76 (silica gel, 1:1 diethyl ether−hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.57 (s, 12 H), 1.65 (s, 3 H), 1.93−2.24 (m, 16 H), 2.61−2.70 (m, 4 H), 5.09 (m, 4 H), 5.49 (t, J = 6.9 Hz, vinyl *H* for cis double bond), 5.59 (t, J = 7.6 Hz, vinyl *H* for trans double bond), 9.76, 9.79 (s, s, 1 H); IR (neat) 2964, 2918, 2852, 1728, 1438 cm^{-1}; MS (EI) *m/z* 404 (M^+); HRMS: 404.2831 (404.2846 calcd for $C_{26}H_{41}ClO$).

^{3}H-Labeled 6-Chloro-6-desmethyltrisnorsqualene Aldehyde (^{3}H-21). Aldehyde **21** (6.9 mg, 0.0171 mmol) was dissolved in MeOH (0.5 mL), and the resulting solution was cooled to 0 °C. A solution of [^{3}H]-$NaBH_4$ (25 mCi, 359.8 mCi/mmol) in MeOH (0.5 mL) was added with stirring, and the stirring was continued for 2 h. Acetone (100 mL) was added to consume the unreacted [^{3}H]-$NaBH_4$. The solvent was removed *in vacuo*, and the product was purified by flash chromatography using 20% diethyl ether−hexane to give the ^{3}H-labeled alcohol (5.5 mg, 0.0135 mmol, 79%). The alcohol was then dissolved in CH_2Cl_2 (0.5 mL) and treated with $NaHCO_3$ (11.3 mg, 0.135 mmol)

and Dess−Martin reagent (8.8 mg, 0.0203 mmol). After stirring for 1 h, saturated $Na_2S_2O_3$ solution (0.5 mL), saturated $NaHCO_3$ solution (0.5 mL), and CH_2Cl_2 (1 mL) were added. The stirring was continued until two phases were clear. The reaction was then worked up, and the product was purified by flash chromatography using 3% diethyl ether in hexane (4.3 mg, 0.0106 mmol, 78%). This material was then used to make ^{3}H-labeled 6-chloro-6-desmethyl-2,3-oxidosqualene (4.7 mg, 64 mCi/mmol, 99%) following the method (b) described below.

6-Desmethyl-6-chloro-2,3-oxidosqualene (4). Method (a). Diol **20** (3.1 mg, 0.00667 mmol) was dissolved in CH_2Cl_2 (50 μL) and pyridine (5.59 μL, 0.0691 mmol), and MsCl (0.695 μL in 220 μL CH_2Cl_2, 0.00898 mmol) was added with stirring at room temperature. After 12 h, MeOH (1 mL) and K_2CO_3 (25 mg) were added, and stirring was continued for 15 min. The solvent was removed *in vacuo*, and the product was purified by flash chromatography using 3% diether/hexane to give **4** (2.2 mg, 74%), further purified by normal phase HPLC using 0.1% isopropyl alcohol in hexane. R_f 0.5 (silica gel, 1:4 diethyl ether−hexane); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.25 (s, 3 H), 1.28, (s, 3 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.69 (m, 1 H), 1.81 (m, 1 H), 1.97 (m, 14 H), 2.25 (m, 2 H),, 2.41 (m, 1 H), 2.47 (m, 1 H), 2.69 (dd, J = 6.3 Hz, J = 5.9 Hz, 1 H), 5.10 (m, 4 H), 5.48 (t, J = 6.8 Hz, 1 H); IR (neat) 2948, 2926, 2857, 1444 cm^{-1}; MS (EI) *m/z* 446 (M^+); HRMS: 446.3303 (446.3315 calcd for $C_{29}H_{47}ClO$).

Synthesis of 6-*E*-4. 6-*E*-**20** (6.0 mg, 0.0123 mmol) was converted by method (a) to 6-*E*-**4** (3.7 mg, 67%). R_f 0.5 (silica gel, 1:4 diethyl ether−hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (s, 3 H), 1.29, (s, 3 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.72 (m, 1 H), 1.82 (m, 1 H), 1.93 (m, 14 H), 2.13 (m, 2 H), 2.48 (m, 2 H), 2.72 (dd, J = 6.9 Hz, J = 5.8 Hz, 1 H), 5.09 (m, 4 H), 5.60 (t, J = 7.5 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.94, 16.00, 16.05, 17.67, 18.79, 24.86, 25.68, 26.68, 26.79, 26.88, 27.18, 28.17, 28.27, 30.76, 39.18, 39.74, 58.53, 63.22, 124.14, 124.26, 124.41, 125.45, 128.44, 131.25, 132.60, 133.77, 134.92, 135.28; IR (neat) 2948, 2926, 2857, 1444 cm^{-1}; MS (EI) *m/z* 446 (M^+); HRMS: 446.3303 (446.3315 calcd for $C_{29}H_{47}ClO$).

Synthesis of 3H-Labeled 4. Method (b). To a solution of diphenylisopropylsulfonium boron tetrafluoride (121 mg, 0.383 mmol) in DME (2 mL) at −78 °C was added LDA solution (0.100 mL, 0.38 M in DME), and the resulting solution was stirred at −78 °C for 30 min. Aliquots of this solution were then added to a solution of 6-desmethyl-6-chlorotrinorsqualene aldehyde 3-^{3}H-**21** consisting of a 1:1 mixture of *Z*/*E* isomers (4.3 mg, 0.0104 mmol) in DME (1 mL) until the solution turned yellow. The reaction mixture was stirred at −78 °C for 1 h and then worked up. The product was purified by flash chromatography using 3% diethyl ether in hexane (4.7 mg, 99%). The exact ratio of *Z*/*E* was 55:45, and the specific activity was 64 μCi/μmol.

Biosynthetic Product from 6-Desmethyl-2,3-oxidosqualene and Lanosterol Synthase. Compound **3** (6.9 mg) was dissolved in dH_2O/Triton X-100 (8:2, 200 μL), and the resulting solution was added to a solution of lanosterol synthase (1.2 μM, 6 mL) in buffer containing sodium phosphate (200 mM, pH 6.4), glycerol (20%), Triton X-100 (0.2%), and DTT (3 mM). After incubation at 20 °C for 48 h, the unconverted starting material and the product were extracted with $CHCl_3$, and the product was purified by flash chromatography using 5% diethyl ether in hexane and then by preparative TLC using 25% diethyl ether in hexane (0.15 mg, 2.2%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.687 (s, 3 H), 0.742 (s, 3 H), 0.864 (s, 3 H), 0.899 (d, J = 6.9 Hz, 3 H), 0.998 (s, 3 H), 1.142−2.003 (m, 24 H), 1.584 (s, 3 H), 1.662 (s, 3 H), 3.218 dd, J = 11.8 Hz, J = 4.5 Hz, 1 H), 5.082 (t, J = 6.1 Hz, 1 H). Comparison with the assigned methyl peaks in the ^{1}H NMR spectrum of lanosterol[5] indicates the absence of the A/B ring angular methyl group.

Spiro Epoxide 5. Trisnorsqualene aldehyde (65.6 mg, 0.171 mmol) and cyclopropyldiphenylsulfonium tetrafluoroborate (53.7 mg, 0.171 mmol) were introduced into a 25-mL round-bottomed flask fitted with a stir bar and septum. DMSO (2 mL) was added to the flask, and the stirred solution at room temperature was treated with freshly crushed KOH (19.2 mg, 0.342 mmol). After 2 h at room temperature, the reaction was complete as indicated by silica gel TLC using triethylamine-treated plates. The reaction mixture was extracted four times with hexane. The combined organic layers were washed twice with saturated aqueous $NaHCO_3$ solution, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The final product (62% yield) was purified on a silica gel column (1.1 cm × 19 cm) pretreated with triethylamine. ^{1}H NMR ($CDCl_3$) δ 5.16−5.07 (5H; m; H's of C=C*H*-), 3.45 (1H; t, J = 5.9; H's of epoxide ring), 2.16−1.96 (18H; m; Hs of CH_2-C=CH), 1.68 (3H; s; CH_3), 1.62 (3H; s; CH_3), 1.60 (12H; s; H's of CH_3), 1.10−0.83 (4H; m; H's of cyclopropyl ring); IR (neat) 2966, 2916, 2871, 2852, 1580, 1475, 1439, 1024 cm^{-1}; HRMS 424.3684 (424.3705 calcd for $C_{30}H_{48}O$).

1,1′-Bisnorsqualeneoxide 6. A suspension of dry trimethylsulfonium iodide (106 mg, 0.26 mmol) in anhydrous THF (1 mL) was cooled to 0 °C and treated with 319 μL of 1.626 M *n*-BuLi in hexane to form the ylide over 15 min at 0 °C with stirring. This solution was added to 100 mg of trisnorsqualene aldehyde in 1 mL of THF via cannula. The reaction mixture was stirred for 30 min at 0 °C and then allowed to warm to 25 °C. After 1 h all of the starting material had been consumed. The product was separated by extractive isolation and purified on a silica gel column using 20:1 hexane−Et_2O as the eluant. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.09 (m, 5H), 2.90 (m, 1H), 2.74 (dd, J = 4.9, 4.0 Hz, 1H), 2.47 (dd, J = 2.7, 4.9 Hz, 1H), 2.06 (m, 20H), 1.68 (s, 3H), 1.60 (m, 15H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 135.20, 135.00, 134.97, 133.88, 131.32, 125.02, 124.46, 124.32, 52.17, 47.26, 39.80, 39.69, 35.87, 31.07, 28.32, 26.82, 26.72, 26.66, 25.76, 17.74, 16.11, 16.07, 16.00.

Methyl Ketone 21. A solution of azeotropically dried trisnorsqualene aldehyde (353.8 mg, 0.92 mmol) in THF (5 mL) was cooled to −78 °C and treated with excess MeMgCl in THF (1.5 mL of a 2.9 M solution). After 4 h the reaction mixture was quenched by the addition of brine, and the product was isolated by extractive workup and purified by silica gel chromatography on a flash column (2.7 cm × 24 cm) using 25% diethyl ether in hexane as the eluant to give the methyl carbinol (76.6 mg, 0.191 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.18−5.08 (m, 5H), 3.78 (t × q, J = 6.15 Hz, 1H), 2.14−1.97 (m, 20H), 1.68 (s, 3 H), 1.60 (s, 15H), 1.14 (d, J = 20.1 Hz, 3H). HRMS (EI^+) *m/z* [M^+] calculated for $C_{28}OH_{48}$ 400.3705, observed 400.3694. This carbinol (76.6 mg, 0.191 mmol) was dissolved in hexane (2 mL) and treated with pyridinium chlorochromate on alumina (1.2 g). After stirring for 24 h at 23 °C, the reaction mixture was filtered through silica gel, and the crude product was purified on a silica gel flash column using 5% diethyl ether in hexane as eluant to give pure methyl ketone **21** (64 mg, 0.161 mmol) in 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.13 (m, 5H), 2.51 (t, J = 7.26 Hz, 2H), 2.23 (t, J = 7.93, 2H), 2.13 (s, 3H), 2.05−1.97 (m, 16H), 1.67 (s, 3H), 1.59 (s, 15H). HRMS (EI^+) *m/z* [M^+] calculated for $C_{28}OH_{46}$ 398.3549, observed 398.3535.

Epoxide 7. The above methyl ketone **21** in THF (2 mL) was added to a cold (−78 °C) solution of dimethylsulfonium methylide. The reaction mixture was allowed to warm to room temperature and kept for 16 h. After extractive isolation the desired epoxide was purified on a silica gel flash column (1.5 cm × 28 cm) using 4% diethyl ether in hexane as the eluant to give epoxide **7** (2 mg, 0.0048 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.14−5.09 (m, 5H), 2.62 (d × d, J = 7.54, 9.69 Hz, 2H), 2.04−1.98 (m, 20H), 1.68 (s, 3H), 1.60 (m, 9H), 1.55 (s, 6H), 0.94 (t, J = 7.35 Hz, 3H). HRMS (EI^+) *m/z* [M^+] calculated for $C_{29}H_{48}O$ 412.3705, observed 412.3695.

Trisnorsqualene Methyl Ester. A solution of trisnorsqualene aldehyde (469 mg, 1.2 mmol) in *tert*-butyl alcohol (30 mL) and 2-methyl-2-butene (10 mL) was cooled to 0 °C and treated with $NaClO_2$ (1.09 g, 12 mmol) in 20% NaH_2PO_4 (10 mL) aqueous solution. After 1 h the reaction mixture was treated with a saturated aqueous solution of $Na_2S_2O_3$. After isolation by extractive workup, a solution of the resulting trisnorsqualene carboxylic acid in diethyl ether (20 mL) was cooled to 0 °C. To the stirred solution was added excess CH_2N_2 in diethyl ether. After 3 min, acetic acid (2 N) was added to consume the excess CH_2N_2. The product was isolated by extractive workup and purified on a silica gel column (2.9 cm × 23 cm) using 5% diethyl ether in hexane as the eluant to give the methyl ester. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.14−5.07 (m, 5H), 3.66 (s, 3H), 2.31−2.26 (m, 2H), 2.43−2.38 (m, 2H), 2.06−1.98 (m, 18H), 1.68 (s, 3H), 1.60 (s, 15H). HRMS (EI^+) *m/z* [M^+] calculated for $C_{28}H_{46}O_2$ 414.3498, observed 414.3496.

α-Chloro Methyl Ester 22. A solution of azeotropically dried trisnorsqualene methyl ester (39.6 mg, 0.095 mmol) in THF (1 mL) at −78 °C was added to a solution of LDA gradually. After 15 min the

resulting solution of enolate was added to a suspension of *N*-chlorosuccinimide in THF (1 mL) at −78 °C. After stirring for 30 min the reaction mixture was quenched by the addition of brine. The product was isolated by extractive workup and purified on a silica gel column (1.7 cm × 28 cm) using 2% diethyl ether in hexane as the eluant to give the α-chloro methyl ester **22** in 43% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.26 (t, J = 5.88 Hz, 1H), 5.14−5.07 (m, 4H), 4.39−4.33 (m, 1H), 3.66 (s, 3H), 2.75−2.49 (m, 2H), 2.09−1.93 (m, 16H), 1.68 (s, 3H), 1.66 (s, 3H), 1.60 (s, 12H). HRMS (EI$^+$) *m/z* [M$^+$] calculated for $C_{28}O_2H_{45}Cl$ 448.3108, observed 448.3091.

Oxide 8. A solution of the azeotropically dried α-chloro methyl ester **22** (18.3 mg, 0.041 mmol) in THF (1 mL) was cooled to −78 °C and treated with an excess of MeLi (80 mL, 1.53 M in diethyl ether). After stirring for 5 min, the reaction mixture was warmed to 0 °C. After stirring for 2 h, the reaction mixture was quenched by the addition of saturated aqueous $NaHCO_3$ solution. The product was isolated by extractive workup and purified on a silica gel flash column (1.7 cm × 28 cm) using 3% diethyl ether in hexane as the eluant to give oxide **8** (6.6 mg, 0.016 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.23−5.09 (m, 5H), 2.79 (t, J = 6.24 Hz, 1H), 2.18−1.99 (m, 18H), 1.60 (m, 9H), 1.68 (m, 6H), 1.57 (s, 3H), 1.32 (s, 3H), 1.28 (s, 3H). HRMS (EI$^+$) *m/z* [M$^+$] calculated for $C_{29}H_{48}O$ 412.3705, observed 412.3690.

Enol Ether 9. A stirred solution of methoxymethyltriphenylphosphonium chloride (86 mg, 0.25 mmol) in toluene (1 mL) at 0 °C was treated with LDA over 5 min to form the red Wittig reagent. To a solution of azeotropically dried trisnorsqualene aldehyde (89 mg, 0.23 mmol) at 0 °C was added the preformed Wittig reagent. The reaction mixture immediately turned yellow and was quenched after stirring for 1 h at 0 °C by the addition of saturated aqueous NH_4Cl solution. The product was isolated by extractive workup and chromatographed on silica gel to give a mixture of diastereomers (1:1.3) enol ether 9 (32 mg) in 34% yield. ^{1}H NMR (300 MHz, $CDCl_3$) (Mixture of diastereomers, 1:1.3 *E*/*Z*) δ 6.29 (MeO-C*H*═C, *E*, d, J = 12.5 Hz, 1H), 5.85 (MeO-C*H*═C, *Z*, d, J = 6.2 Hz, 1H), 5.14−5.10 (m, 5H), 4.71 (MeO-CH═C*H*, *E*, m, 1H), 4.31 (MeO-CH═C*H*, *Z*, m 1H), 3.57 (s, 3H), 3.49 (s, 3H), 2.07−2.00 (m, 20H), 1.68 (s, 3H), 1.60 (s, 15 H). HRMS (EI) calculated 412.3705, observed 412.3693.

Bisnorsqualene Aldehyde (10). A stirred solution of enol ether **9** (12 mg, 29 mmol) in THF (1 mL) was treated with 12% HCl (0.2 mL). After 4 h at 23 °C the reaction mixture was quenched by the addition of ice-cold saturated aqueous $NaHCO_3$ solution. The product was isolated by extractive workup and chromatographed on silica gel to give the aldehyde **10**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.76 (s, 1H), 5.14−5.09 (m, 5H), 2.39 (t, J = 7.4 Hz, 2H), 2.06−2.01 (m, 20H), 1.60 (s, 12H), 1.55 (s, 6H). HRMS (EI) calculated 398.3549, observed 398.3533.

Ester 23. To a solution of triethyl phosphonopropionate (151 mg, 0.633 mmol) in THF (2 mL) at −78 °C was added NaH (25.3 mg, 0.633 mmol), and the resulting solution was stirred for 30 min. Then the aldehyde (100 mg, 0.316 mmol) was added, and the reaction mixture was stirred for 1 h. After workup, the trans isomer was purified by flash chromatography using 1% diethyl ether in hexane (97.0 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (t, J = 7.0 Hz, 3 H), 1.58 (s, 12 H), 1.66 (s, 3 H), 1.81 (s, 3 H), 1.92−2.27 (m, 16 H), 4.15 (q, J = 7.0 Hz, 2 H), 5.10 (m, 4 H), 6.72 (t, J = 7.3 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.2, 141.9, 135.2, 134.9, 134.0, 131.2, 127.7, 125.1, 124.2, 124.2, 124.1, 60.3, 39.7, 38.3, 28.3, 28.2, 27.4, 26.8, 26.7, 25.7, 17.7, 16.0, 14.3, 12.4; IR (neat) 2964, 2926, 2856, 1711, 1444, 1269, 1121, 1079 cm^{-1}; MS (EI) *m/z* 400 (M$^+$); HRMS: 400.3333 (400.3341 calcd for $C_{27}H_{44}O_2$).

Triphenylphosphonium Salt 24. To a solution of ester **23** (130 mg, 0.325 mmol) in CH_2Cl_2 (5 mL) at −78 °C was added DIBAL (0.65 mL, 1.0 M solution in CH_2Cl_2), and the resulting solution was stirred at −78 °C for 30 min. Saturated NH_4Cl solution (5 mL) was then added. After workup, the product was essentially pure (105 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.58 (s, 12 H), 1.65 (s, 3 H), 1.66 (d, J = 1.0 Hz, 3 H), 1.93−2.14 (m, 16 H), 3.97 (s, 2 H), 5.10 (m, 4 H), 5.37 (m, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.2, 134.7, 131.3, 126.2, 124.6, 124.4, 124.2, 69.1, 39.7, 39.3, 36.1, 35.8, 28.2, 26.8, 26.3, 25.7, 17.7, 16.1, 16.0, 13.7; IR (neat) 3324 (br), 2964, 2918, 2854, 1382, 1010 cm^{-1}; MS (EI) *m/z* 358 (M$^+$); HRMS: 358.3245 (358.3236 calcd for $C_{25}H_{42}O$).

To a solution of the alcohol prepared above (89.0 mg, 0.249 mmol) in CH_2Cl_2 (2 mL) at −40 °C was added Et_3N (70.0 μL, 0.497 mmol) and MsCl (28.9 μL, 0.375 mmol). The reaction mixture was stirred at −40 °C for 30 min. Then LiBr (216 mg, 2.49 mmol) in THF (4 mL) was added, and the resulting solution was warmed to room temperature. After extractive workup, the corresponding bromide was dissolved in CH_3CN (2.5 mL), followed by addition of triphenylphosphine (326 mg, 1.25 mmol). The resulting solution was stirred at 40 °C for 12 h. Then solvent was removed *in vacuo*, and product was purified by flash chromatography eluting with first $CHCl_3$ then 4% MeOH in $CHCl_3$ to give triphenylphosphonium salt **24** (165 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 3 H), 1.45 (s, 3 H), 1.54 (s, 9 H), 1.62 (s, 3 H), 1.72−2.03 (m, 16 H), 4.53 (d, J = 14.6 Hz, 2 H), 4.91 (m, 1 H), 5.04 (m, 3 H), 5.22 (m, 1 H), 7.62-7.80 (m, 15 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.4, 136.2, 134.9, 134.8, 134.1, 134.0, 133.9, 130.2, 130.1, 124.6, 124.2, 124.1, 123.9, 122.0, 121.9, 118.8, 117.9, 39.6, 38.5, 28.1, 28.0, 26.6, 26.5, 25.5, 18.4, 17.5, 15.9, 15.8, 15.7; IR (neat) 2965, 2922, 2871, 1438, 1112 cm^{-1}; MS (FAB$^+$) *m/z* 603 (M$^+$); HRMS: 603.4100 (603.4120 calcd for $C_{43}H_{56}P^+$).

4,5-Dehydrooxidosqualene (11). To a solution of triphenylphosphonium salt **24** (39.0 mg, 0.0571 mmol) in THF (2 mL) at −78 °C was added *t*-BuOK (7.59 mg, 0.676 mmol) in THF (0.31 mL), and the reaction mixture was warmed to room temperature and stirred for 30 min. Then it was cooled to −78 °C, and to it was added the epoxyaldehyde in ether until the red color disappeared. After the resulting solution was stirred at −78 °C for 5 min and then room temperature for 1 h, it was worked up. The product was purified by flash chromatography with Et_3N treated silica gel using 3% diethyl ether in hexane (16.0 mg, 66%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.18 (s, 3 H), 1.20 (s, 3 H), 1.56 (s, 3 H), 1.57 (s, 3 H), 1.60 (s, 3 H), 1.61 (s, 3 H), 1.69 (s, 3 H), 1.98−2.21 (m, 16 H), 3.63 (d, J = 7.4 Hz, 1H), 5.23−5.30 (m, 4 H), 5.33 (dd, J = 11.8 Hz, J = 7.4 Hz, 1 H), 5.49 (t, J = 7.0 Hz, 1 H), 6.06 (d, J = 11.8 Hz, 1 H); ^{13}C NMR (100 MHz, benzene) δ 138.0, 135.3, 134.0, 135.0, 132.6, 131.1, 125.5, 125.3, 124.9, 124.8, 124.7, 61.2, 40.2, 39.7, 28.7, 27.2, 27.1, 27.0, 25.8, 24.4, 19.7, 17.7, 16.5, 16.2, 16.1; IR (neat) 2961, 2923, 2854, 1448, 1377 cm^{-1}; MS (EI) *m/z* 424 (M$^+$); HRMS: 424.3695 (424.3705 calcd for $C_{30}H_{48}O$).

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