Studies on the Substrate Binding Segments and Catalytic Action of Lanosterol Synthase. Affinity Labeling with Carbocations Derived from Mechanism-Based Analogs of 2,3-Oxidosqualene and Site-Directed Mutagenesis Probes

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Received September 13, 1996[⊗]

Abstract: Four 2,3-oxidosqualene analogs, **3**, **4**, **5**, and **6**, which are irreversible, time-dependent inhibitors of the enzyme lanosterol synthase, were found to attach covalently within the 231−236 (yeast numbering) segment (Figure 3). The attachment was determined by tryptic digestion of the inactivated enzyme, separation of the tryptic cleavage products by C_{18} reverse phase HPLC, and fragment identification by mass spectroscopy or Edman degradation. W232 and H234 are the targets of the chemical inactivation by cations derived from analogs **3**−**6**. 2,3-Oxidosqualene analogs **7**, **8**, and **9** inactivated the enzyme with covalent attachment to the 486−512 segment (Figure 3), which is in a domain that is predicted to be an amphipathic α-helix. Site-directed mutagenesis of various amino acid residues (76 total) in lanosterol synthase which are conserved in five different species has revealed that residues D456, H146, and H234 are essential for catalytic activity. These and other data permit the formulation of a hypothetical working model of some aspects of the activation and binding of 2,3-oxidosqualene by lanosterol synthase. The model is depicted in Figure 4. In that model D456 and protonated H146 initiate cyclization, and the domains containing 231−236 and 486−512 make contact with the reacting substrate.

Introduction

The conversion of (*S*)-2,3-oxidosqualene (**1**) to lanosterol (**2**) is remarkable not only as one of the most complex of all enzyme catalyzed reactions but also for several other reasons as well.[1] It is one of the most powerful one-step constructions known in biochemistry or synthetic chemistry. Although the reaction involves a conformationally flexible substrate and very reactive carbocation intermediates, it is efficiently channeled through a sequence of cyclization and 1,2-group rearrangements of high energy carbocations to lanosterol with complete structural and stereochemical control (Figure 1). Other enzymes transform the same substrate, 2,3-oxidosqualene, to a wide variety of naturally occurring triterpenoids with similar structural specificity, indicating not only the versatility of the substrate but also that a precise molecular interaction mechanism must exist for very fine control of the conformation of this hydrocarbon-like substrate by the catalytic protein. The understanding of the nature of the lanosterol synthase mediated transformation at the atomic level requires knowledge of the active site and the specific hydrophobic and polar interactions that lead to stereo and chemical control at each stage of the multistep process. Although X-ray crystallographic analysis of heavy atom labeled crystalline lanosterol synthase in the presence of the inhibitor 2,3-iminosqualene[2] could provide much helpful information along these lines, crystallization of the enzyme is at present a formidable challenge rather than a reality. Despite this fact, a growing body of knowledge has been developed regarding the molecular details of the cyclization process in lanosterol biosynthesis largely through (1) the study of the action of lanosterol synthase on structural analogs of 2,3-oxidosqualene, (2) gene cloning and protein sequence information on lanosterol synthase from different species, and (3) site-directed mutagenesis-enzyme activity studies to determine which amino acid subunits are critical.

The information now available regarding the biosynthesis of lanosterol from 2,3-oxidosqualene can be summarized as follows: (1) the intermediate protosterol cation has the structure shown in Figure 1 with 17β-side chain which ensures stereochemical control at C−20 of lanosterol;[3] (2) the ring B boat geometry for the intermediate protosterol[4] ring system has been confirmed;[3] (3) the methyl group at C−10 of 2,3-oxidosqualene is crucial to the correct folding of the substrate[3c] and the correct facial selectivity of addition to the 10,11-double bond;[3c] (4) the C−17 side chain in the intermediate protosterol cation is in a tight binding pocket which encloses the six terminal carbons,[3b] (5) the tetracyclization of 2,3-oxidosqualene to form the protosterol cation is not concerted but involves discrete metastable carbocations at least at the stage of C ring formation;[5] (6) the six-membered C ring is formed by ring expansion of a five-membered cyclopentylcarbinyl cation precursor;[5] (7) the initiation of cyclization, which occurs only if the substrate is properly folded and enclosed in the binding cavity,[5] probably is the result of protonation of the oxirane oxygen by a conserved and especially acidic aspartic acid unit (D456, yeast numbering);[6] (8) the closure of the A ring is concerted with oxirane

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.

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

(2) Corey, E. J.; Ortiz de Montellano, P. R.; Lin, K.; Dean, P. D. G. *J. Am. Chem. Soc.* **1967**, *89*, 2797.

(3) (a) Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1991**, *113*, 4025. (b) Corey, E. J.; Virgil, S. C.; Sarshar, S. *J. Am. Chem. Soc.* **1991**, *113*, 8171. (c) Corey, E. J.; Virgil, S. C.; Liu, D. R.; Sarshar, S. *J. Am. Chem. Soc.* **1992**, *114*, 1524. (d) Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1990**, *112*, 6429.

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S0002-7863(96)03228-3 CCC: $14.00

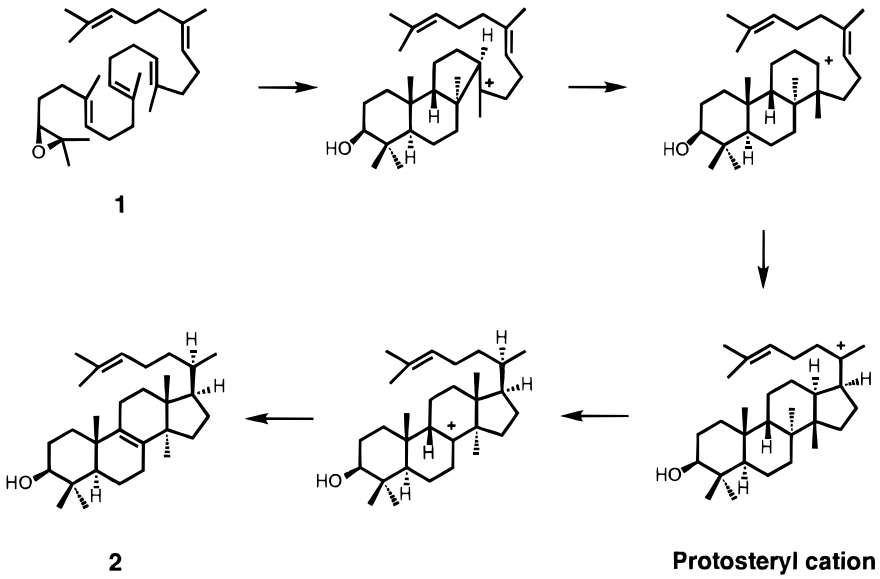

Figure 1.

cleavage;[6] (9) site-directed mutagenesis of individual tryptophan units in yeast lanosterol synthase, W/F and in some instances also W/A and W/G, showed that W143, 194, 198, 207, 218, 232, 583, 587, and 632 (which are conserved in all five known species) are not individually essential to enzyme function,[7] even though a special role for tryptophan had previously been proposed;[8] (10) the rearrangements of hydrogen and methyl on the pathway between the protosterol cation and lanosterol are thermodynamically driven with intrinsic low activation energies and require minimum assistance from the enzyme until the deprotonation step which fixes the 8,9-location of the nuclear double bond.[3b]

In this paper we describe the results of studies aimed at defining the amino acid side chains which might constitute the points of contact with the substrate not only as a part of the initial binding pocket which holds it in place for initiation of cyclization but also as crucial control elements during the later stages of ring formation and rearrangement of hydrogen or methyl. These studies are based on two experimental approaches: (1) affinity labeling of lanosterol synthase by mechanism based analogs of oxidosqualene which cause time-dependent irreversible inactivation of the enzyme and (2) site-directed mutagenesis studies of selected conserved amino acid units. The latter technique was utilized previously for the detection of the proton donor which initiates the cyclization process.[6]

It has been pointed out previously that the spatial envelope which represents the contact areas between the enzyme and the substrate probably changes as the conversion of 2,3-oxidosqualene to lanosterol progresses, one of the most interesting aspects of this unique biochemical process.[9] Initially that envelope is optimal for the binding of 2,3-oxidosqualene with tight conformational control (i.e., proper folding) to effect activation of the oxirane function and guide the cyclization to the first cationic intermediate, possibly the tricyclic 6,6,5-fused cation.[5,6] At that point there may be a modest conformational change to ensure complete control of the ensuing expansion and D-ring closure. Finally, it is possible that another conformational change occurs as the 1,2-rearrangements of hydrogen and methyl proceed which correctly positions the nucleophile that serves to terminate the reaction by proton abstraction. This dynamic behavior of the enzyme during reaction implies that the motifs of the enzyme which contact the substrate (inner shell subunits) may change as the reaction progresses. Because of the charged nature and high energy of the cationic reaction intermediates, it is also possible that long-range electrostatic (solvation type) interactions involving polar groups not in direct contact with the substrate (secondary shell subunits) may play a significant role in controlling the fidelity of the overall reaction. Clearly affinity labeling studies with carefully designed analogs of the various cationic intermediates which occur along the reaction pathway can be expected to provide useful and possibly unique information on the nature of enzymic control in the biosynthesis of lanosterol from 2,3-oxidosqualene.

Results and Discussion

Affinity labeling experiments were carried out with various synthetic analogs of 2,3-oxidosqualene which were found to be irreversible, time-dependent inactivators of lanosterol synthase. These inhibitors were synthesized with a tritium label as described later in this paper and incubated with the enzyme to effect complete inactivation. The inactive enzyme was isolated by precipitation with ammonium sulfate, and the resulting insoluble fraction was dissolved and digested with trypsin. The resulting mixture of peptide fragments was separated by reversed phase HPLC and all peaks were radioassayed. In each case only one tryptic fragment was radiolabeled except for the case of substrate **3** which gave rise to two radiolabeled fragments. Each radioactive peak was collected and submitted for analysis either by Edman sequencing or mass spectrometry or both.[10]

Affinity Labeling of Fragment WWVHTR (231−236 Yeast Numbering). 20-Oxa-2,3-oxidosqualene (**3**) has previously been used in our investigations to provide valuable information regarding the stereochemistry of the protosterol cation and also the intermediacy of a tricyclic cyclopentylcarbinyl cation. During these studies time-dependent irreversible inactivation of the enzyme was noted. Consequently, tritiated

(6) Corey, E. J.; Cheng, H.; Baker, C. H.; Matsuda, S. P. T.; Li, D.; Song, X. *J. Am. Chem. Soc.* **1997**, *119*, 1277.
(7) Corey, E. J.; Matsuda, S. P. T.; Baker, C. H.; Ting, A. Y.; Cheng, H. *Biochem. Biophys. Res. Comm.* **1996**, *219*, 327.
(8) (a) Buntel, C. J.; Griffin, J. H. *J. Am. Chem. Soc.* **1992**, *114*, 9711. (b) Shi, Z.; Buntel, C. J.; Griffin, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7370. (c) Poralla, K.; Hewelt, A.; Prestwich, G. D.; Abe, I.; Reipen, I.; Sprenger, G. *Trends Biochem. Sci.* **1994**, *19*, 157.
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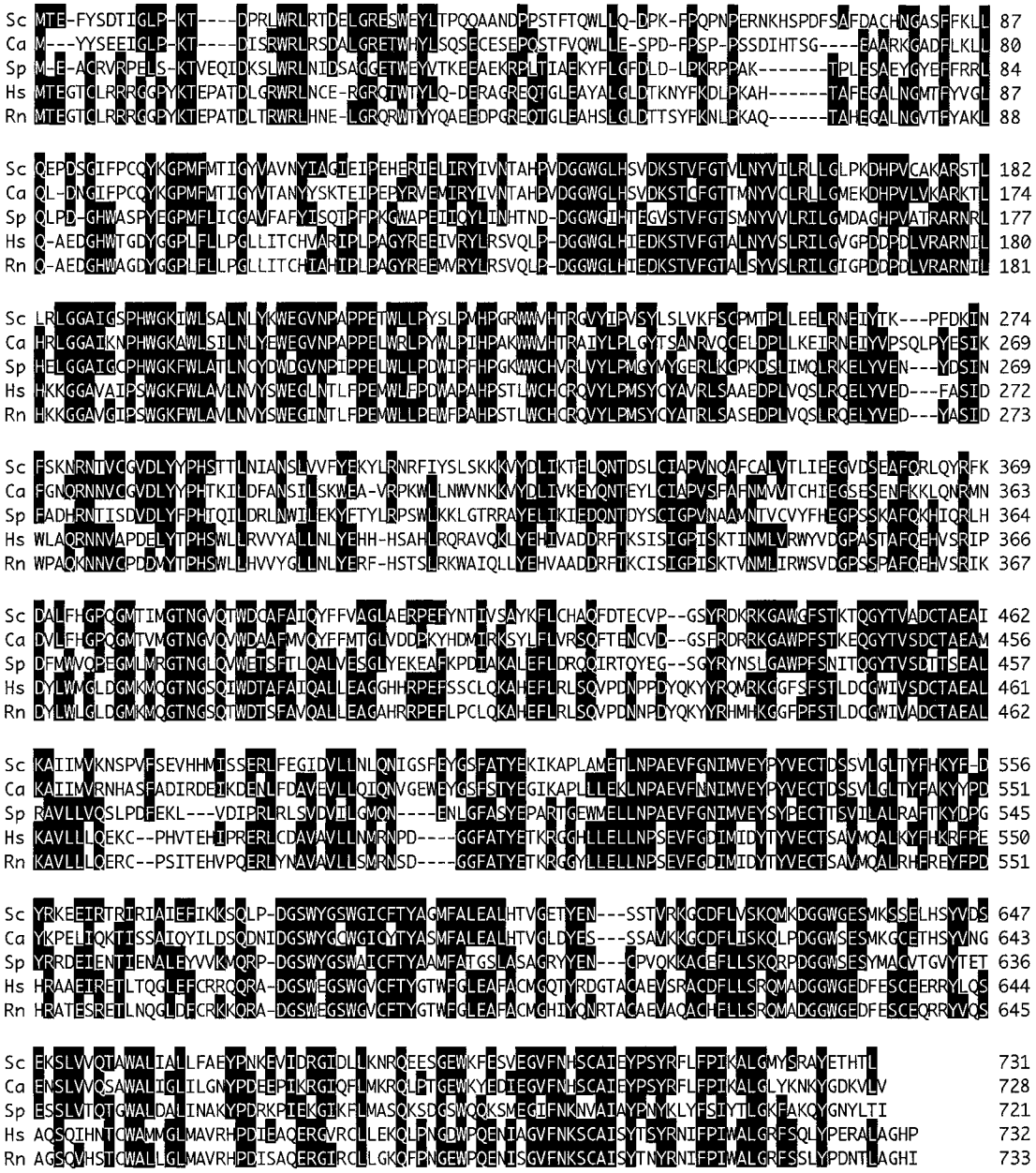


Figure 2. Alignment of lanosterol synthase amino acid sequences from *Saccharomyces cerevisiae* (Sc), *Candida albicans* (Ca), *Schizosaccharomyces pombe* (Sp), *Homo sapiens* (Hs), and *Rattus norvegicus* (Rn). Residues conserved in at least three sequences are shaded.

3 was prepared and used to label the protein. Tryptic digestion and fragment analysis by Edman sequencing and mass spectroscopy demonstrated that the protein had been labeled within the fragment 231−236 by the addition of 69 mass units at W232, a highly conserved amino acid in a highly conserved section of the enzyme (see Figure 2). The group attached to W232 corresponds to a prenyl residue (C_5H_9) which obviously can originate from the corresponding cation whose mode of formation is indicated in Figure 3.

The truncated conjugated diene **4** (Figure 3) was synthesized as a simpler analog of (20*E*)-20,21-dihydro-2,3-oxidosqualene which earlier had been found to be converted to a highly stabilized protosterol cation analog that did not undergo rearrangement of hydrogen and methyl.[3b] It was anticipated that **4** could be an irreversible inactivator of lanosterol synthase, as it indeed proved to be. Inactivation of the enzyme by incubation with tritium-labeled **4**, tryptic cleavage of the labeled enzyme and fragment analysis using mass spectrometry showed that H234 of fragment 231−236 had been labeled by the cation shown in Figure 3.

Similar experiments using the even simpler 2,3-oxidosqualene analogs **5** and **6** showed that they too irreversibly inactivated lanosterol synthase by carbocationic alkylation of H234 within the fragment 231−236 (Figure 3).

Furthermore, site-directed mutagenesis experiments showed that yeast mutants H234A, H234R, and H234K are nonviable, indicating the importance of H234. However, H234 can be replaced by phenylalanine without compromising enzyme function as proven by the viability of the H234F mutant yeast strain. Thus, it appears from the affinity labeling and site-directed mutagenesis studies that H234 may possibly be in

(10) Tryptic fragments were collected after separation by C_{18} or C_4 reverse phase HPLC and counted for radioactivity. Any radioactive peak was rechromatographed and submitted for sequencing by automated Edman degradation on an Applied Biosystems 494A, 477A (Foster City CA) or Hewlett Packard G1005A (Palo Alto CA) instrument. Tryptic peptide sequences were also determined by microcapillary HPLC/electrospray ionization/tandem mass spectrometry on a Finnigan TSQ7000 triple quadrupole mass spectrometer (San Jose, CA) as described by Hunt *et al*. (1992). These analyses were performed by Dr. William S. Lane and associates in the Harvard Protein Microchemical Analysis Laboratory. We are grateful to them for expert assistance,

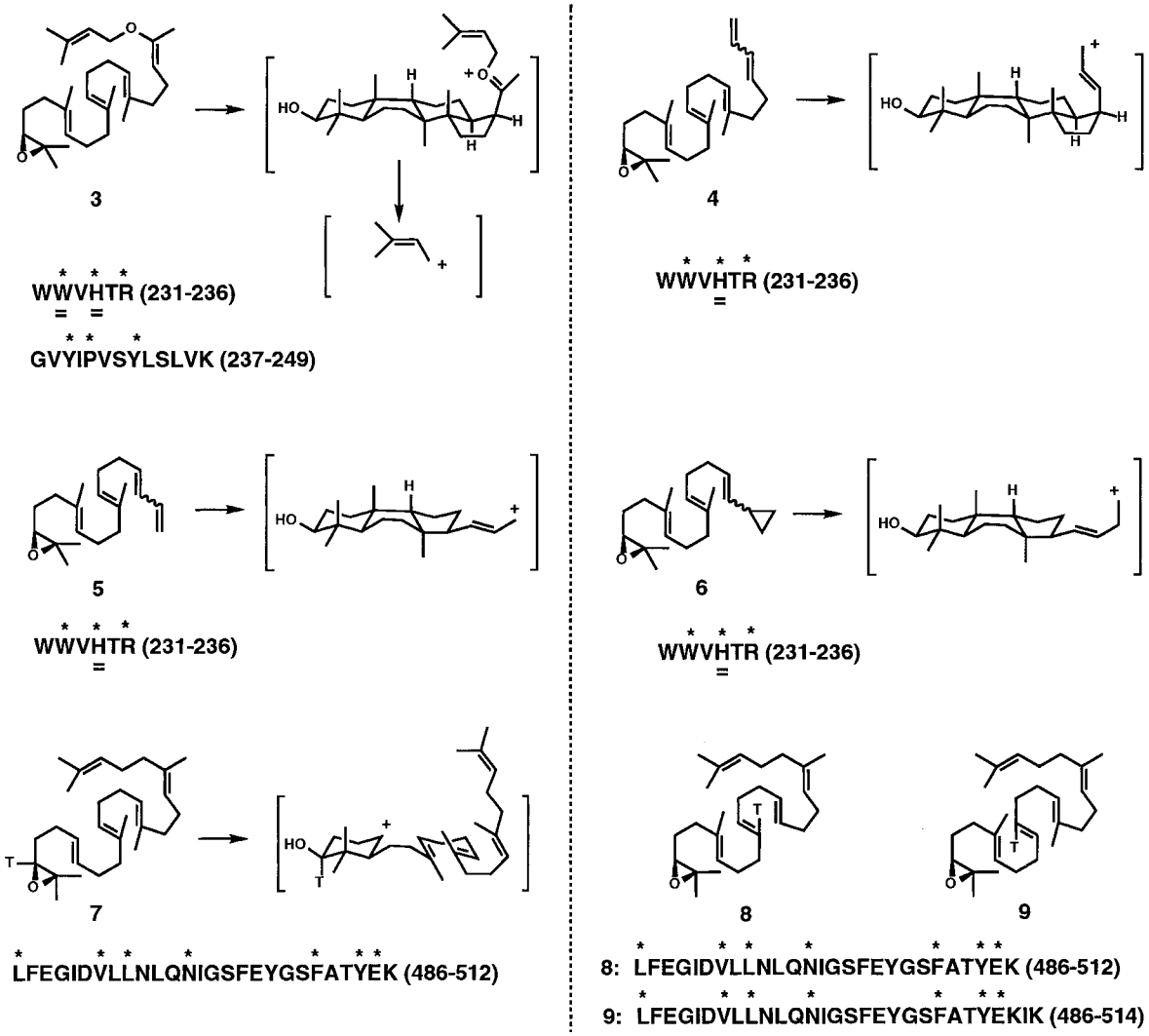


Figure 3. Carbocations derived from mechanism based analogs of 2,3-oxidosqualene**.** Sequences shown for each substrate correspond to the tryptic fragment of yeast lanosterol synthase to which labeled substrate is attached. Asterisks indicate conserved amino acid residues (from Figure 2) and double underlining indicates the amino acid residue to which the labeled substrate attaches covalently after incubation with yeast lanosterol synthase.

contact with the substrate as a π-aromatic group and may be positioned in the neighborhood of D-ring/C-17 side chain of the protosterol cation intermediate. There it may serve either as part of the binding pocket or as a polarizable π-aromatic group which provides some stabilization of an intermediate cation as a component of the secondary shell. Such stabilization may serve as the enzymic equivalent of solvation in nonenzymic carbocationic reactions of small molecules in solution. Solvation energies of such carbocations are clearly large since reaction rates increase enormously in going from alkane or cycloalkane solvents to more polar media such as CH_2Cl_2, HCOOH, or $(CF_3)_2CHOH$.

The same possibilities exist for W232 of the native enzyme which was labeled by the use of substrate analog **3** or for the W232F mutant enzyme which is also catalytically effective. Although the precise nature of the interactions involving W232 and H234 cannot now be defined, it seems likely that they play a significant role in controlling the cyclization process. Because of the complex series of molecular events involved in the cyclization process and the possible plasticity of the enzyme contact area as cyclization proceeds, it is even possible that W232 and H234 interact with the substrate at different times. For example H234 may be interacting more strongly as the B and C rings are being formed and W232 may interact more significantly as the D ring develops. In this regard it is of considerable interest that the lanosterol synthase of rat is so rapidly inactivated by 20-oxa-2,3-oxidosqualene that there is no measurable conversion to cyclization products, in contrast to observations with the yeast enzyme.[3a,5] Since the rat enzyme sequence includes C233 in place of V233 of the yeast enzyme, it is possible that C233 is labeled at the very nucleophilic SH group even more rapidly than W232 for rat (and human) lanosterol synthase.

As indicated in Figure 3, 20-oxo-2,3-oxidosqualene (**3**) also labels lanosterol synthase somewhere in the tryptic fragment 237−249, just next to the fragment 231−236 containing W232. Although some of the side chains of the conserved residue 237−244 within the 237−249 tryptic fragment may also interact with the substrate during cyclization, the catalytic importance of such interactions may be minor since the mutants of three conserved residues, Y239F, P241G, and Y244G, are viable, indicating catalytically active enzyme.

Affinity Labeling of Fragment 486−512. In the preceding paper (*J. Am. Chem. Soc*. **1997**, *119*, xxxx)[6] the conversion of 6-desmethyl-2,3-oxidosqualene (**7**) to 19-norlanosterol was described. Kinetic studies with **7** provided strong evidence that the cleavage of the oxirane ring and formation of the A ring are concerted. During the course of this research it was observed that **7** is a time-dependent, irreversible inactivator of lanosterol synthase. Consequently, tritiated **7** was synthesized

and used to inactivate the enzyme. After tryptic digestion and chromatographic separation, a single labeled fragment was isolated which mass spectrometric analysis showed to be 486−512 (Figure 3). This same fragment was labeled by the substrate 10,15-desdimethyl-2,3-oxidosqualene (**8**).[3c] The 486−512 segment of lanosterol synthase is predicted to be α-helical by statistical analysis.[11] Assuming the α-helical geometry for 486−512 in the enzyme it seems reasonable to consider that amino acids L486, I490, L494, and possibly I499, which could interdigitate snugly with and control the folding of the substrate during cyclization, may be contact units of the binding pocket. The helical 486−512 segment is just upstream (toward the carboxyl terminus) of the highly conserved 454−466 segment (also predicted to be helical)[11] which contains D456, the likely initiator of cyclization.[6] The region from S471 to S475 is predicted to be a loop which interconnects the two helical domains 454−470 and 476−501.[11]

Site-Directed Mutagenesis Experiments. A total of 76 *S. cerevisiae* lanosterol synthase mutants were constructed in which a single highly conserved amino acid was replaced by another amino acid, usually that which is most closely related structurally. Site-directed mutagenesis experiments were carried out using the *ERG7* gene by the method of Kunkel.[12] The steps used were as follows: (1) synthesis of the appropriate oligonucleotide and annealing to the single strand, uracil containing DNA template, (2) primer extension using T4 DNA polymerase to form the complimentary strand, (3) use of T4 DNA ligase to seal the nick, (4) transformation of the resulting double stranded DNA into *E. coli* DH5α and selection of mutants using restriction fragment analysis, (5) insertion into the mutant yeast strain SMY8 (*MAT***a** *erg7::HIS3 hem1::TRP1 ura3*−52 trp1-Δ63 leu2−3,112 his3-D200 ade2 Gal$^+$),[7] and (6) 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 vector was then tested for viability by restreaking on a YPG plate without supplementary ergosterol. Yeast cells with an *Erg7* mutation leading to catalytically incompetent lanosterol synthase do not grow in a medium lacking ergosterol. As a double check on the mutagenesis methodology, 18 of the mutants (E460Q, E460A, E460K, E634Q, C540A, C590A, D140N, D370N, D456N, D580N, M532Q, M532A, M532N, M532S, M532H, P214G, P225G, and W232G) were subjected to sequencing analysis. In every case the expected sequence was verified experimentally.

Table 1 provides a summary of the mutation data currently available. The following residues appear to be essential for viability of the yeast mutants in an ergosterol-free medium and for enzyme function: D456, H146, H234, and M532. The underlined entries in Table 1 correspond to nonviable mutants. At present it is not possible to assign a role to M532. As mentioned earlier, D456 possibly is the proton donor in oxirane activation[6] and H234 may be within a region which contacts the substrate and may play a role in cation stabilization. H234 can be replaced by Phe but not by Ala, Lys, or Arg without loss of activity, consistent with this possibility. H146 in the protonated form may intensify the proton donor ability of D456 through hydrogen bonding.[6] Consistent with this hypothesis is the loss of enzyme function in the mutants H146A but not H146K or H146R.

Table 1. Site-Directed Mutagenesis of the Highly Conserved Residues

C/A	540, 590, 619
D/N[6]	140, 286, 370, **456**, 580, 629
D/E[6]	456
E/Q[6]	216, 264, 460, 483, 487, 511, 520, 526, 539, 634
E/A	460
E/K	460
H/A	**146**, 227, **234**, 291
H/F	234
H/K	146, **234**
H/R	146, **234**
I/A	531, 705, 716
L/A	145, 163, 166, 199, 202, 622, 719
M/A	379, **532**
P/G	214, 215, 221, 225, 228, 241
R/Q	236, 436
W/F[7]	143, 194, 198, 207, 218, 232, 583, 587, 632
W/G	218, 232
W/A	143, 232, 583, 632
Y/F	160, 239, 288, 510, 535, 537, 593, 707
Y/G	244
Y/A	707

Inactive mutants: D456N, H146A, H234A, M532A, H234K, H234R

Residues found to be essential for catalytic activity: D456, H146, H234, M532

It is interesting that none of the cysteine SH groups of the conserved C540, C590, and C619 residues is critical to activity. Their function may be secondary, e.g., to help maintain three-dimensional structure through hydrogen bonding. The hydrophobic residues ile, leu, and pro which are conserved are of interest as possible members of the substrate binding site(s), since they are well suited to precise van der Waals contacts with the folded substrate (leading to control of conformation) and compatible with highly reactive carbocation intermediates. It is clear from the data in Table 1 that, if these are residues which contact the substrate, none of them is so critical as to cause malfunctioning of lanosterol synthase in one-at-a-time mutations to alanine. The same is true of the various conserved trp residues (W → F mutation), as discussed previously.[7] The W units in the two conserved DGGW sequences (140−143 and 629−632) and the conserved DGSW sequence (580−583) can be replaced by phe indicating that these four-subunit sequences may have more to do with connecting rigid elements of the three-dimensional structure (e.g., as hinges between domains) than with catalysis or substrate binding.

Deletions at N- and C-Termini of ERG7. The yeast lanosterol synthase gene has also been modified to allow the construction of mutants which produce a truncated enzyme lacking either 19, 31, 52, 65, or 75 amino acid units at the N-terminus or 21 residues at the C-terminus. Only one of these mutants was viable in an ergosterol-free medium, that lacking only the 19 units of the N-terminus.

Site-directed mutagenesis primers were designed to introduce a *Sal*I restriction site followed by a new ATG start site at positions 19, 31, 52, 65, and 75, and these oligonucleotides were used to mutagenize the *ERG7* gene in the shuttle vector, p61.21, using the method of Kunkel.[12] With the new start site in place the original start site could be removed by cutting the gene with the restriction enzyme, *Sal*I, and then religating the vector

(11) (a) See: Rost, B.; Schneider, R.; Sander, C. *Trends Biochem.* **1993**, *18*, 120. We thank Dr. Schneider for carrying out the computer analysis. (b) The fact that analogs of 2,3-oxidosqualene lacking methyl groups at carbons 6, 10, or 15 are capable of causing irreversible inactivation and covalent labeling of lanosterol synthase, even though they can be converted to tetracyclic products, indicates that for each of these analogs there is a certain probability (or frequency) that once initiated the cyclization can go astray because a cationic intermediate attacks the enzyme as a consequence of less than perfect control.

(12) Kunkel, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 488.

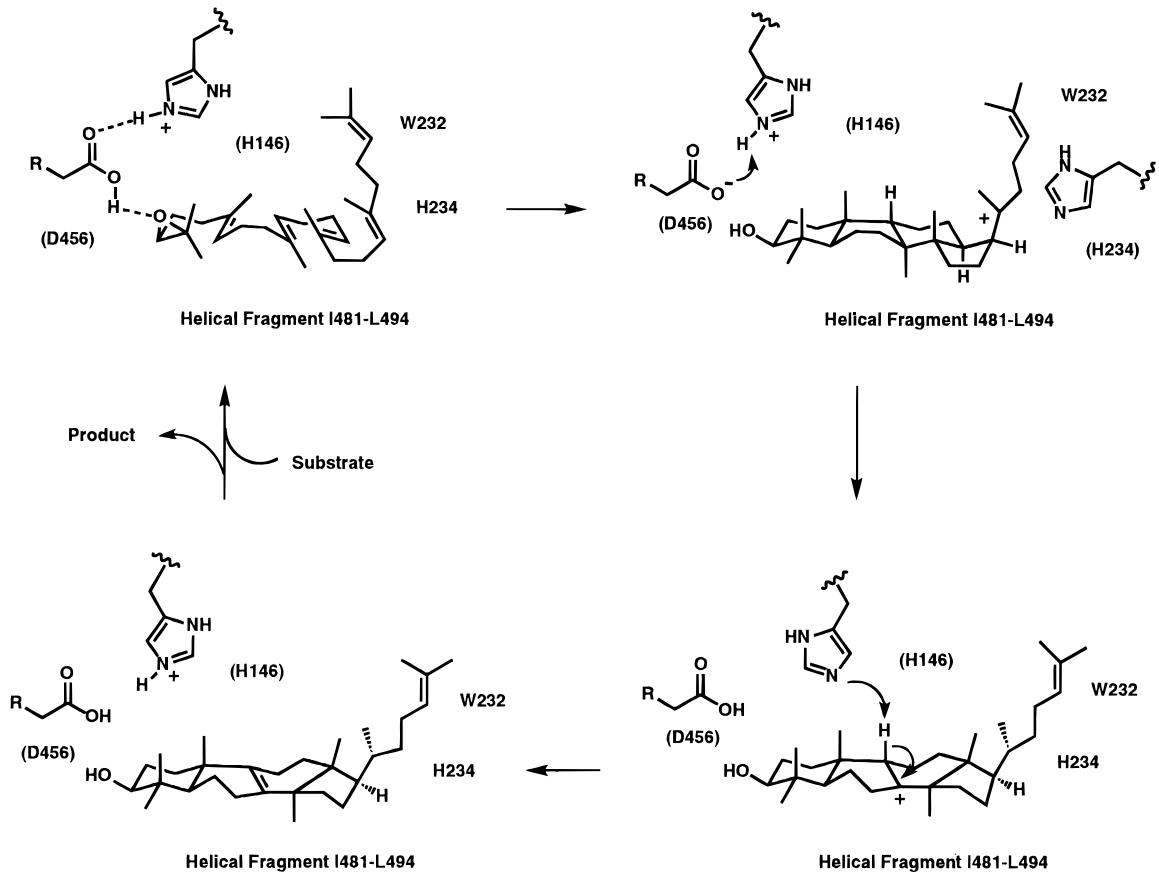


Figure 4. Hypothetical possibility for lanosterol biosynthesis.

together. Oligonucleotides were also designed to introduce a new stop site at the C-terminus 21 residues upstream of the wild type stop site. Once the DNA constructs were created, they were used to transform the *S. cerevisiae* strain SMY8 using the standard lithium acetate procedure. After plating the transformed yeast on ergosterol-free media, it was determined that deleting even 31 residues from the n-terminus and 21 residues from the c-terminus resulted in loss of cyclase activity.

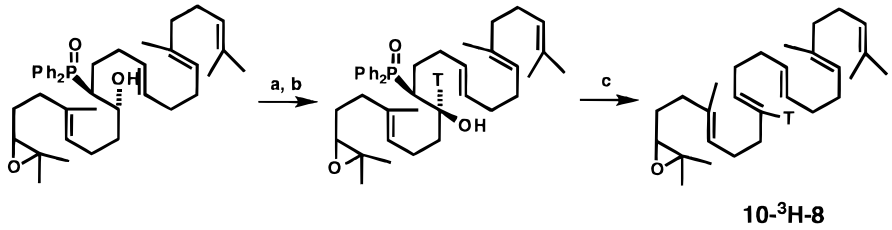
A hydrophobic loop on the outside of the protein which might be responsible for the detergent and glycerol requirement was found by digesting purified lanosterol synthase with trypsin for a short time in the absence of Triton X-100 and glycerol. The amino acid sequence of the purified peptide, which is the major product of incubation of lanosterol synthase with trypsin at 0 °C for 30−60 min, was determined by Edmann degradation to be ESWEYLTPQQAANDPPSTFTQWLLQDPK (29−56). The next step was deleting the nucleic acid sequence responsible for this amino acid sequence from the *ERG7* gene. Two different mutant genes were created using standard site-directed mutagenesis techniques. One of the mutants deleted all 28 amino acids from the peptide chain, and the other deleted a 12 residue hydrophobic section (PPSTFTQWLLQD). The SMY8-derived strains transformed with these constructs were unable to grow in the absence of ergosterol.

A Working Hypothesis for the Interactions between Substrate and Lanosterol Synthase. The studies described in this and the preceding paper (*J. Am. Chem. Soc*. **1997**, *119*, 1277)[6] implicate D456 in the initiation of the lanosterol synthase catalyzed cyclization of 2,3-oxidosqualene. In addition, the results of affinity labeling and site-directed mutagenesis experiments suggest important roles for H146, W232, H234, and the 486−498 helical segment. Taken together these findings suggest the working hypothesis which is shown in Figure 4. The preceding paper (*J. Am. Chem. Soc*. **1997**, *119*, 1277)[6] discusses not only the role of D456 in activation of the oxirane function but also the possibility of assistance of an *endo* hydrogen bond to the D456 carboxyl[13,14] from a proximate ammonium ion. Clearly, protonated H146 is an excellent candidate for the role of the neighboring ammonium ion which enhances the acidity of D456. Such a function is also consistent with the observed pH-rate profile maximum at pH 6.3 for lanosterol synthase and the fact that H146 can be replaced by K or R but not A. In addition after formation of the protosterol cation and 1,2-rearrangement steps, H146 in the unprotonated form may well be positioned to accept the proton in the final step of lanosterol biosynthesis, thereby allowing proton recycling to regenerate the reaction-initiating form of the enzyme. In the model shown in Figure 4, W232 and H234 are placed to serve as part of the binding pocket which maintains the correct three-dimensional folding of the substrate and provide some cation stabilization (possibly as neighboring polarizable π-aromatic groups) during the intermediate stages of the reaction. Finally, the amphipathic helical segment I481-L494 may also serve as part of the lipophilic binding site which controls the folding of the substrate. For example, I481, L486, I490, and L494 side chains might form a section of the lipophilic pocket which binds the substrate. Obviously, the scheme shown in Figure 4 must be considered as only a guiding hypothesis at this stage. However, it does serve to unify the data obtained from the investigations outlined herein in a mechanistically reasonable way.

Methods for the Synthesis of Analogs of 2,3-Oxidosqualene. The seven substrates used in the affinity labeling studies described herein, compounds **3**−**9**, were synthesized by either known or standard procedures. The reaction sequences and the

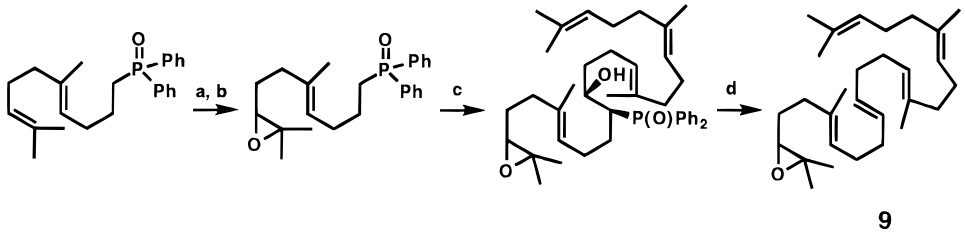
(13) Westheimer, F. H. *Tetrahedron* **1995**, 51, 3.
(14) Gandour, R. D. *Bioorg. Chem.* **1981**, *10*, 169.

Scheme 1. Synthesis of ^{3}H Labeled **8**[a]



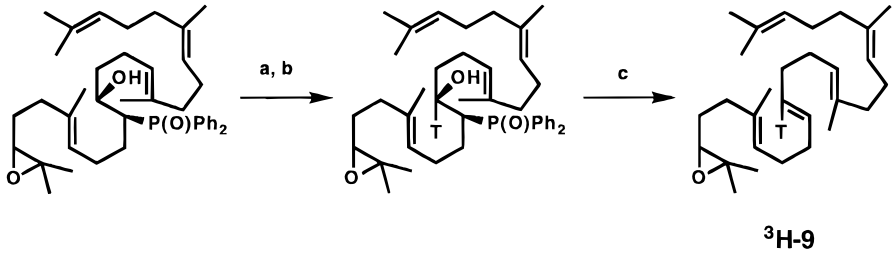
[a] (a) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −60 °C, 85%. (b) [^{3}H]-$NaBH_4$, THF, 0 °C, 99%. (c) NaH, DMF, 40 °C, 87%.

Scheme 2. Synthesis of **9**[a]



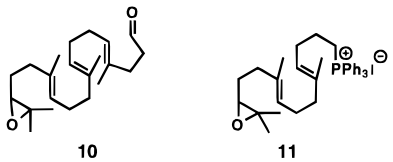
[a] (a) NBS, THF/H_2O, 36%. (b) K_2CO_3, MeOH, 96%. (c) n-BuLi, **11**, THF, −78 °C; AcOH, −78 °C to 0 °C. (d) NaH, DMF, 40 °C, 95%.

Scheme 3. Synthesis of ^{3}H-Labeled **9**[a]



[a](a) Dess−Martin reagent, $NaHCO_3$, CH_2Cl_2, 76%. (b) [^{3}H]-$NaBH_4$, THF, 0 °C. (c) NaH, DMF, 40 °C, 15% overall.

detailed experimental procedures are available as Supporting Information. Substrates **3**,[3a] **7**,[6] and ^{3}H-labeled **7**[6] were synthesized as previously described. Substrate **4** was prepared by a Wittig reaction between the ylide from allyltriphenylphosphonium chloride and the requisite C_{23} heptanor-2,3-oxidosqualene carboxaldehyde **10** in THF solution. The ^{3}H formyl labeled substrate **4** was similarly prepared, as a 1:1 mixture of *E* and *Z* forms about the newly created double bond, using R_{20}-$CH_2CH_2C^3HO$, made from Dess−Martin oxidation of $R_{20}CH_2$-CH_2CH^3HOH. Substrate **5** was synthesized similarly from ^{3}H-formyl labeled 12,13-oxidofarnesyl acetaldehyde, $R_{15}CH_2$-CH_2C^3HO, and allylidenetriphenylphosphorane. Tritiated substrate **6** was synthesized by Wittig coupling of cyclopropylcarboxaldehyde with the Wittig reagent from $\bar{\omega}$-oxidobishomofarnesyltriphenylphosphonium iodide (**11**) (tritiated at C-1 of the



bishomofarnesyl group). Substrate **8** was synthesized as shown in Scheme 1 by the β-hydroxyphosphonate modification of the Wittig olefination process.[3a] The tritiated form of substrate **9** was similarly prepared from the carbinol-tritiated form of the β-hydroxyphosphonate which was made by ^{3}H−$NaBH_4$ reduction of the corresponding β-keto phosphonate by the method described earlier[3a] and outlined in Schemes 2 and 3. The purity of each chromatographically purified substrate was shown to be >96% by ^{1}H NMR analysis.

Time dependent irreversible inhibition of lanosterol synthase was demonstrated for each of the substrate analogs **3**−**9** as follows. Each of the analogs **3**−**9** was incubated with pure recombinant lanosterol synthesis for 24 h at 23 °C, and the activity of the enzyme was compared to that of control enzyme as a function of time. Enzyme activity was measured by incubation with radiolabeled oxidosqualene, TLC separation, and radioassay of the product lanosterol. In each case (**3**−**9**) the degree of enzyme inactivation was proportional to the time of exposure to the inhibitor, and there was no appreciable inactivation of the control. In each case the use of radiolabeled analog led to the radiolabeling of lanosterol synthase. The enzyme was specifically labeled as demonstrated by the fact that for inhibitors **4**−**9** only *one* of the tryptic fragments was radioactive and for **3** only two (adjacent) fragments were radioactive (see Figure 3).

Experimental Section

Protocol for Affinity Labeling. Lanosterol synthesis was incubated at 23 °C with the ^{3}H-labeled inhibitor (specific activity 10μCi/μM), the resulting complex was precipitated by ammonium sulfate, and digested with trypsin. The resulting mixture of peptide fragments was separated by C_{18} or C_4 reverse phase HPLC, and the radioactive fraction was rechromatographed and submitted for amino acid sequencing by either Edman degradation or mass spectroscopy at the Harvard Microchemical Facility. The following detailed procedure was used.

A. Enzyme Inactivation and Precipitation of the Enzyme-Inhibitor Complex. A solution of lanosterol synthase (6 mL) (100 μg/mL 7.5 nmol) in pH 7.0 or 6.4 buffer containing 150 mM sodium phosphate, 20% glycerol, 0.2% Triton X-100, and 3 mM DTT in a 15 mL centrifuge tube was incubated with ^{3}H-labeled inhibitor (400 μM) at 23 °C for 16 h. Saturated ammonium sulfate solution (3.0 mL) was

then added. After vortexing, the solution was kept at 4 °C overnight and then centrifuged at 1000Xg for 1 h. The supernatant was removed, and the protein pellet on the side of the tube was washed to the bottom using a buffer solution of 100 mM NH_4HCO_3 and 1% 1-*O*-octyl-β-D-glucopyranoside (2 mL) and resuspended. After centrifugation at 1000Xg for 1 h, the supernatant was decanted. The precipitate was redissolved in 1 mL of 100 mM NH_4HCO_3 and 1% 1-*O*-octyl-β-D-glucopyranoside solution, transferred to an 1.5 mL microcentrifuge tube, and centrifuged at 12 000 g for 15 min. After repeating this process four times, the pellet was finally resuspended in 400 μL of 100 mM NH_4HCO_3 and 1% 1-*O*-octyl-β-D-glucopyranoside solution.

B. Reduction, Alkylation, and Trypsin Digestion. To 200 μL of the above prepared solution was added 45 mM DTT (20 μL). The resulting solution was heated at 50 °C for 15 min. After cooling to room temperature, 100 mM iodoacetamide solution (20 μL) was added, and the mixture was stored at 23 °C for 15 min. The solution was diluted with 560 μL water, and the protein was digested with 20 μg of sequencing grade trypsin from Promega at 37 °C for 24 h.

C. RP HPLC Separation and Sequencing. After the digestion was complete, the solution (250 μL per injection) was subjected to reverse phase HPLC separation using either a C_4 column (3.9 × 150 mm, Waters Delta Pak, 5 μ, 300 Å) or a C_{18} column (4.6 × 250 mm, Vydac, 5 μ, 300 Å). With the C_4 column, the following conditions were used: flow rate, 700 μL/min; chart speed, 4 min/cm; detection at 214 nm; solvent A: 0.06% trifluoroacetic acid (TFA) in water; solvent B: 0.05% TFA in acetonitrile; linear gradient: 0 min, 40% B, 5 min, 40% B, 45 min, 90% B, 15 min, 90% B, 10 min, 40% B. Fractions (700 μL) were collected, and 10% of every fraction was counted for radioactivity. With the C_{18} column, the following conditions were used: flow rate, 800 μL/min; chart speed, 4 min/cm; detected at 214 nm; solvent A: 0.06% TFA in water; solvent B: 0.05% TFA in acetonitrile; linear gradient: 0 min, 10% B, 2 min, 10% B, 60 min, 60% B, 20 min, 95% B, 15 min, 95% B, 5 min, 10% B. Fractions (800 μL) were collected, and 10% of every fraction was counted for radioactivity. The radioactive fractions from several different injections were combined, lyophilized, and dissolved in 500 μL of 1:1 H_2O:CH_3CN (for the C_4 column) or 3:1 H_2O:CH_3CN (for the C_{18} column). The labeled peptide was then repurified by either C4 or C18 reverse phase HPLC and submitted for identification/sequencing.

Mutagenesis. Mutants were generated according to the method of Kunkel.[12] 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 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, E460Q, E460A, E460K, E634Q, C540A, C590A, D140N, D370N, D456N, D580N, M532Q, M532A, M532N, M532S, M532H, P214G, P225G, and W232G, were sequenced by the dideoxynucleotide method using the USB Sequenase Kit (version 2.0) to further confirm incorporation of the mutagenic oligonucleotide. *E. coli* strain DH5α was used for all DNA manipulations. *E. coli* were transformed, selected, and propagated according to published procedures. The plasmid pSM61.21 was linearized with BstEII, 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 (20 mg/L), and Tween 80 (0.5%). Complementation analysis was performed on 1% yeast extract, 2% peptone, 2% galactose, and 2% agar, supplemented with hemin chloride (13 mg/L) and Tween 80 (0.5%).

Acknowledgment. This research was generously supported by the National Institutes of Health.

Supporting Information Available: Experimental procedures and spectral data (10 pages). See any current masthead page for ordering and Internet access information.

JA963228O