Lanosterol Biosynthesis: The Critical Role of the Methyl-29 Group of 2,3-Oxidosqualene for the Correct Folding of this Substrate and for the Construction of the Five-Membered D Ring

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Abstract: Lanosterol synthase catalyzes the polycyclization reaction of (3S)-2,3-oxidosqualene (1) into tetracyclic lanosterol 2 by folding 1 in a chair-boat-chair-chair conformation. 27-Nor- and 29-noroxidosqualenes (7 and 8, respectively) were incubated with this enzyme to investigate the role of the methyl groups on 1 for the polycyclization cascade. Compound 7 afforded two enzymatic products, namely, 30-norlanosterol (12) and 26-normalabaranorlanosterol (13; 12/13 9:1), which were produced through the normal chair-boat-chair-chair conformation and an atypical chair-chair-boat conformation, respectively. Compound 8 gave two products 14 and 15 (14/15 4:5), which were generated by the normal and the unusual polycyclization pathways through a chair-boat-chair-boat conformation, respectively. It is remarkable that the twist-boat structure for the B-ring formation was changed to an energetically favored chair structure for the generation of 15. Surprisingly, 14 and 15 consisted of a novel 6,6,6,6-fused tetracyclic ring system, thus differing from the 6,6,6,5-fused lanosterol skeleton. Together with previous results, we conclude that the methyl-29 group is critical to the correct folding of 1, with lesser contributions from the other branched methyl groups, such as methyl-26, -27, and -28. Furthermore, we demonstrate that the methyl-29 group has a crucial role in the formation of the five-membered D ring of the lanosterol scaffold.

Keywords: alkenes · cyclization · enzyme catalysis · polycycles · terpenoids

Introduction

Oxidosqualene cyclases convert (3S)-2,3-oxidosqualene (1) into nearly 120 different cyclic triterpene skeletons.[1] These enzymes impose either the chair or boat conformation on acyclic compound 1, and the ensuing proton attack on the epoxide ring triggers the ring-forming reactions (C–C bond formation) with the correct regio- and stereochemical specificities, thus usually leading to the production of tetra- and pentacyclic scaffolds. Triterpene cyclases generate many chiral centers during the polylefin cyclization cascade, and the mechanism for the cyclization/rearrangement reactions has attracted considerable attention.[1] The mechanism for the cyclization pathway was first proposed over a half-century ago by Eschenmoser et al. in 1955,[2] who with Cornforth[3] proposed that the chair-boat-chair conformation for lanosterol biosynthesis affords protosterol cation 4 with a 17α-oriented side chain (Scheme 1b).

Virgil[4] proposed the 17β-oriented side chain 3 as the true protosteryl cation intermediate based on the analysis of the stereochemistry of the enzymatic products of 20-oxa-2,3-oxidosqualene[4] and (20E)-20,21-dehydro-2,3-oxidosqualene[5] by hog-liver lanosterol synthase. Furthermore, through the subsequent backbone-rearrangement process, 3 can be converted into 2 with a 20R configuration through a least-motion pathway that involves only a small rotation (~< 60°) about the C-17–C-20 axis, but a large rotation (120°) is required prior to proton migration from C-17 to C-20 to generate the R configuration, as observed in 4.[4] Thus, a chair-boat-chair conformation (Scheme 1a) that leads to the protosterol cation 3 is now accepted as an intermediate in lanosterol biosynthesis.[6, 7]

Numerous studies on lanosterol synthase-mediated reactions of substrate analogues of 1 have been reported.[8-11] Corey et al. and van Tamelen et al. reported the enzymatic products of 26-noroxidosqualane,[9] 28-noroxidosqualane (5),[10] and 27,28-bisnoroxidosqualenes (6; Figure 1).[11] Analogues of 26-noroxidosqualane and 5 were converted into 19-nor- and 18-norlanosterols 10,[9, 10] respectively, thus indicating that the normal chair-boat-chair conformation was imposed by lanosterol synthase. The tetracyclic product 11, which consists of the ring system of both a 6,6,5-fused tricycle and an isolated four-membered ring, was created from 6, thus indicating that 6 was folded in an abnormal chair-boat-chair conformation.[11] Interestingly, the conformation of the B ring was converted from a twist boat into a chair structure. Accordingly, Corey et al. proposed that the

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methyl-27 group at C10 of 1 was critical for the correct folding in the polycyclization and rearrangement reaction.\cite{11} Clayton et al.\cite{12} and Corey et al.\cite{13} examined the cyclization reaction of 1-nor and 25-nor analogues and reported that the 1-nor analogue underwent the polycyclization reaction to yield 29-norlanosterol, whereas the 25-nor analogue underwent no reaction. However, no report has appeared concerning the enzymatic reactions of 7 and 8, in which the methyl groups present in 1 at C-10 and C-19, respectively, are absent.

Herein, we describe the enzymatic products of 7 and 8 and discuss how the branched methyl groups present in 1 influence the conformation during the polycyclization cascade. We report that the methyl-29 group at C-19 is more important to the correct folding of 1, especially with regard to the boat structure of the B ring relative to the methyl-27 group at C-10. We also describe the unprecedented cyclization pathway that generates a novel skeleton that consists of a 6,6,6,6-fused tetracyclic ring system, which differs from the 6,6,5,5-fused lanosterol skeleton.

**Results**

**Syntheses of noroxidosqualenes 5, 7, and 8:** The synthesis of 7 was performed according to Scheme 2. (4E,8E)-5,9,13-Tri-methyltetradeca-4,8,12-trienal (19), prepared from squalene by treating with MCPBA and periodic acid, was subjected to the Wittig–Horner reaction with ethyl diethylphosphonoacetate to yield (2E,6E,10E)-ethyl-7,11,15-trimethylhexadeca-2,6,10,14-tetraenoate (20; 87%). Ethyl ester 20 was converted into the corresponding alcohol 21 by reduction with diisobutylaluminium hydride (DIBAL-H; 93%), which was then transformed into bromide 22 by using PBr$_3$. Commercially available geraniol 23 was treated with NBS to give bromohydrin 24 (53%), followed by bromination with PBr$_3$ to afford

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**Scheme 1. Folding conformation of (3S)-2,3-oxidosqualene 1 for lanosterol biosynthesis.**

**Figure 1. Structures of substrate analogues 5–9 and the enzymatic products 10–16.** The fundamental core of 13 is the same as that of malabaricatriene triterpene, but 13 lacks the Me-26 group of the malabaricatriene skeleton that is situated at C-8. The black numbering of the carbon atoms is used for the assignments of the NMR spectroscopic data and is arbitrary. The red numbering for 10, 12, and 13 is based on the numbering of the parent skeletons (lanosterol and malabaricatriene with C$_{30}$).
Bromide 25 was treated with sodium benzenesulphinate to yield the phenylsulfone derivative 26, which was converted into epoxide 27 (61%). The coupling reaction of 27 and 22 was carried out in THF containing HMPTA by adding nBuLi, thus yielding the phenylsulfone derivative 28 of oxidosqualene (13%). The removal of the phenylsulfonyl group was carried out with the super hydride reagent to obtain the desired 7 (76%).

The syntheses of noranalogues 5 and 8 were performed by using essentially identical methods to that for 7 with the same reagents. Compounds 21 and 18 were used for the preparation of 5 and 8, respectively (the experimental details are described in the Supporting Information). The ethyl-substituted analogue 9 was prepared according to the method described in the Supporting Information.

The structures of products 12–15 from noroxidosqualenes 7 and 8 and the cyclization pathways: Products 12 and 13 were generated by incubating (±)-7 with hog-liver lanoster synthase and were purified by column chromatography and HPLC. Compound 12 was separated as a pure compound, and 13 was acetylated before purification. The $^1$H and $^{13}$C NMR (600 and 150 MHz, respectively) spectra of 12 measured in CDCl$_3$ revealed one olefinic proton ($\delta_H=5.09$ ppm, $\tau=6.9$ Hz), two vinyl methyl groups ($\delta_H=1.68$ and 1.60 ppm, each 3H, s), and one tetrasubstituted double bond (C-8: $\delta_C=128.0$ ppm, s; C-9: $\delta_C=135.9$ ppm, s). Heteronuclear multiple-bond correlations (HMBC) were observed for Me-19/C-9, H-14/C-8, and H-14/C-9. No NOE interactions for H-9/Me-25/H-8 demonstrated that the structure of 13 is as shown in Figure 2, thus allowing 7 to adopt a chair-chair-boat conformation (Scheme 3a). The cationic intermediate 29 thus formed could be quenched by the deprotonation of H-15 to give the E geometry, which was confirmed by the clear NOE interaction of Me-26/H-16 (see the Supporting Information). This conformation is consistent with that proposed for the polycyclization reaction of 27,28-bis-noroxidosqualene 6. However, it should be noted that the ratio of fully cyclized 12/partially cyclized 13 was determined to be 9:1 by GC analysis, thus suggesting that the influence of the small hydrogen substitution at C-10 on the polycyclization cascade is not significant.

Products 14 and 15 were generated by incubating (±)-8 with hog-liver lanoster synthase and their acetates were purified with reversed-phase HPLC. $^1$H NMR (see the Supporting Information) and DEPT analysis revealed that both products had one olefinic proton ($\delta_H=5.10$ ppm, $\tau=6.9$ Hz).
be noted that the Me-19 and Me-29 groups must be placed in a trans arrangement because both 14 and 15 had no NOE interaction between the Me-19 and Me-29 groups, as seen in lanosterol.

The stereochemistry of 14 and 15 was carefully inferred on the basis of detailed analysis of the NOESY spectra. Some of the angular methyl proton signals overlapped or were contiguously located. The NMR spectra of the acetate salts of 14 and 15 were measured in different solvents such as CDCl₃, CD₂Cl₂, and (CD₃)₂CO to separate these signals (see the Supporting Information). The NOE interactions of authentic lanosterol acetate were also collected in the different solvents (see the Supporting Information) and used as a reference to determine the stereochemistry of 14 and 15. In the NOESY spectra of the 14-acetate (see the Supporting Information), definitive cross peaks of Me-29/H-5a and Me-19/Me-20β confirmed the configurations of the Me-29α and Me-19β groups. Furthermore, a strong NOE interaction between Me-29 and H-18 was observed for 14, but no NOE interaction for Me-19/H-18 indicated that both Me-29α and H-18ax were arranged in an axial disposition in the chair structure. A clear NOE interaction for Me-19β/H-21 was observed, which is indicative of Me-19β and H-21β (Figure 2b). Thus, the following stereochemistry was assigned for 14: 3S, 5R, 10S, 13R, 14R, 18R. On the other hand, clear NOE interactions for Me-19/H-5a and Me-20β/Me-29 were found for the 15-acetate, but no NOE interaction for Me-29β/ H-5a (see the Supporting Information), unambiguously indicated the stereochemistry of Me-29β and Me-19α. Definitive NOE interactions for Me-29β/H-21and Me-19α/H-18 indicated that H-21 and H-18 were oriented in axial and equatorial dispositions, respectively (Figure 2c). The chair structure of the D ring was further supported by strong NOE interactions for Me-19/H-17ax, Me-19/H-15ax, and H-15ax/H-17ax. Thus, the stereochemistry of 15 was determined as follows: 3S, 5R, 10S, 13S, 14S, 18R. It is noticeable that the configurations at C-13 and C-14 of 14 are the same as those of 2, but those of 15 are opposite to those of 2. Molecular modeling of the acetate salts of 14 and 15 supported the configurations at C-13 and C-14 (see the Supporting Information). A comparison of the proton chemical shifts of the angular Me groups of the acetate salts of 14 and 15 with those of lanosterol acetate (see the Supporting Information) further supported the configurations. Thus, the structures of 14 and 15 are depicted as shown in Figure 1.

It is possible that 29-noroxidosqualene (8) adopts a chair-boat-chair-chair conformation (path a in Scheme 3b) to afford cation intermediate 30, which could then undergo 1,2-hydride and methyl shifts in an antiperiplanar fashion followed by the deprotonation of H-9 to yield 14. A chair-boat-chair-chair conformation could be adopted for the formation of 15 (path b in Scheme 3b) to give the 6,6,5-fused tricyclic cation 31 with H-13αt. The C-13–C-14 bond axis could rotate to afford cation 32 with an α-oriented Me group at C-14, and the subsequent ring expansion could give the 6,6,6-fused tricyclic cation 33, followed by further cyclization to produce the 6,6,6,6-fused tetracycle 34. The subse-

Figure 2. Key data of COSY, HOHAHA, NOE, and HMBC spectra for proposing the structures of products a) 13, b) 14, and c) 15, which were measured in CDCl₃, CD₂Cl₂, and (CD₃)₂CO to separate these signals (see the Supporting Information).
quent backbone rearrangements and the proton elimination of H-9 could result in the production of 15.

Other folding conformations of 8 were envisaged and the corresponding cyclization products were evaluated to validate the proposed structures of 14 and 15 (see the Supporting Information, pages S50–S53). To acquire the configuration of Me-29α for 15, which is identical to that of 2, the formation for B-ring construction must be a boat form. Three other possible folding conformations that lead to products 56–58 are assumed, but the NOE interactions of the 15-acetate are inconsistent with the proposed structures of 56–58 (see the Supporting Information, page S50).

In contrast, the chair-folding in the B-ring formation results in a β-oriented Me-29 group. All of the possible folding conformations are listed in the Supporting Information (page S51). Examples 1–4, in which no bond rotation and no ring expansion occur, would lead to 59–62, but these structures do not agree with the NOE data of 15-acetate. The reaction schemes of example 5 (see the Supporting Information, page S51) and path b in Scheme 3 are accompanied by a C-13–C-14 bond rotation on Markovnikov cation intermediates prior to the ring expansion (5–6-membered ring), then further cyclization and backbone rearrangements lead to product 15 (63 is identical to 15) that fully coincides with the observed NOE data. Thus, the bond rotation prior to the ring expansion is required to fulfill the stereochemistry of 15. A higher production of 15 than 14 (15/14 5:4) indicates that the Me-29 group is more crucial for the correct folding of 1 for the biosynthesis of 2 relative to Me-27 (12/13 9:1).

In our experiment, the incubation of 5 (see Figure S3 in the Supporting Information for the synthetic details) gave 10 as the sole enzymatic product (see Figure S10 and the NMR spectroscopic data in the Supporting Information, pages S54–55), which was in good agreement with the results reported by van Tamelen et al.[10]

Time-dependent inactivation by noroxidosqualenes 5, 7, and 8:

Compounds 12–15 were produced in very small amounts. We investigated the possibility that time-dependent inactivation occurred during the incubations of 7 and 8. Kinetic analysis revealed the following parameters: IC₅₀ = 180 μM, kₘₐₓ = 0.0037 min⁻¹, Kᵢ = 0.013 μM for 7; IC₅₀ = 76 μM, kₘₐₓ = 0.0068 min⁻¹, Kᵢ = 0.015 μM for 8 (see Figure S11 in the Supporting Information). It was reported that 6 displayed time-dependent irreversible inhibition.[11] Time-dependent inhibition was also observed for 5 in our experiment (kₘₐₓ = 0.0065 min⁻¹, Kᵢ = 0.020 μM), although this finding has not been previously reported.[10]

Cyclization pathway of the bulkier ethyl-substituted oxidosqualene 9:

Previously,[15–17] we reported the results of incubation experiments of analogues of 1 bearing an ethyl substituent at C-10 and/or C-15 with hog-liver cyclase (see Figure S12 in the Supporting Information). Analogue 64 substi-
tuted with an ethyl group at both C-10 and C-15 afforded only the monocyclic products 67 and 69. Analogue 65 with an ethyl substituent at C-10 gave monocycles 68 and 70, tricycle 71, and tetracycle 73 (68/70/71/73 1:1:1:1). Analogue 66 with an ethyl group at C-15 yielded tricycle 72 and tetracycle 74 (72/74 1:2.6). The polycyclization reaction of 64 afforded the monocyclic compounds 68 and 70 as the main products, but the reaction of 66 proceeded to the tri- and tetracyclic stages 72 and 74, respectively. An ethyl group is slightly bulkier than a methyl group, thus leading us to conclude that the binding site that accommodates the methyl group of C-10 of 1 is more discriminating than the binding site for the methyl group of C-15.

Synthesized (±)-9 (6 mg) was incubated with partially purified hog-liver cyclase to examine the effect of the bulkier substituent at C-19 on the polycyclization pathway. The enzymatic product was purified by column chromatography on SiO2 gel and normal-phase HPLC (hexane/THF 100:0.5) to yield pure 16 (1.8 mg) as the sole enzymatic product. The 1H and 13C NMR spectra (in CDCl3) indicated the following functional groups: one olefinic proton (δH = 5.11 ppm, t, J = 6.8 Hz), two vinylcyclic methyl protons (Me-26: δH = 1.682 ppm, s, 3H; Me-27: δH = 1.605 ppm, s, 3H), and one tetrasubstituted double bond (δC = 134.4 ppm, s; δC = 134.3 ppm, s), thus indicating a lanosterol skeleton for 16. The detailed analyses of the 2D NMR spectra were in good agreement with homolanoester structure, in which ethyl group is substituted at C-20 (see Figure S13 in the Supporting Information for the NMR spectroscopic analyses). The 20R stereochemistry is credible in light of the cyclization pathway (Scheme 3c). High production of 16 indicates that the binding site that accepts C-19 is somewhat more loosely packed than the binding sites for C-10 or C-15 and can accept the bulky ethyl group, albeit with the somewhat lower affinity of 9 for this cyclase relative to that observed for 1 (Km = 231 μM, Vmax = 0.47 μM min-1 for 9; Km = 75 μM, Vmax = 0.33 μM min-1 for 1). Kinetic analysis showed that 9 did not undergo time-dependent inhibition. The polycyclization reaction of the bulkier ethyl-substituted 65 and 66 halted at the premature stage to afford mono- and tricyclic products (see Figure S12 in the Supporting Information). In the reaction of 9, full polycyclization occurred without stopping at the intermediate stage. In contrast, the methyl-deficient 26-noroxidosqualene and 27-noroxidosqualene 7 (substituted with a less-bulky hydrogen atom) gave 19-norlanosterol and 30-norlanosterol as the main products (ca. 90%; see Figure 1), respectively, which resulted from the complete polycyclization reaction that proceeded through the normal folding conformation. Therefore, these results strongly indicate that the binding site involved in the early steps of the polycyclization is tightly packed and more discriminating, whereas the binding site for the later step is less compact and can accept larger substituents.

Discussion

X-ray crystallographic analysis of human lanosterol synthase was reported in 2004. It revealed that the tyrosine (Tyr) residue Tyr98 is well positioned to enforce the energetically unfavorable boat folding for the B-ring formation of 3 by pushing the methyl group at C-10 of 1 below the molecular plane (Scheme 1a). A less-bulky hydrogen atom in 7 is substituted at C-10. The increased space between the Tyr residue and the hydrogen atom at C-10 may have allowed freer motion of this substrate, thus leading to the adoption of the energetically favored chair structure in the B ring and the production of 13. The altered chair folding for the B ring could further exert influence on the conformation for the subsequent C-ring formation; a chair form for the C ring in lanosterol biosynthesis could be changed into a boat structure (Scheme 3a).

Wu et al. reported that the F699N or F699M variants of S. cerevisiae lanosterol synthase produced the tricyclic compound (14E,17E)-(13αH)-malabarica-14,17,21-triene, the skeleton of which is the same as that of 13 (i.e., 26-nomalabaricatriene). This report is the first that describes the successful alteration of the conformation of the B ring from boat to chair by using mutagenesis experiments. The phenylalanine (Phe) residue Phe699 is located near the D-ring-forming residue (as seen in 29). Furthermore, it was demonstrated that by varying the size of the side chain through a single amino acid substitution at the residues of Tyr99-Tyr707-IIe705-Phe699, which are highly conserved (see Figure S14 in the Supporting Information) and are in proximity to the B-, C-, and D-ring-formation sites, the substrate conformation and polycyclization cascade are altered. Thus, the appropriate sizes of the active-site residues to allow for steric bulk are crucial to determine the substrate folding. In turn, the steric size at the methyl-substituted positions on substrate 1 also influenced the conformation and/or the polycyclization cascade (e.g., truncation of the annulation reaction), as demonstrated by the incubation experiments of 7, 8, and ethyl-substituted analogues 9 and 64-66 with the native lanosterol cyclase from hog-liver. Therefore, it is apparent that the steric-size match between the substrate and the catalytic site is critical to the correct folding of 1 and the cyclization pathway.

Analogue 8 could undergo two different cyclization pathways. Product 14 could be produced under a normal polycyclization pathway (through a chair-boat-chair conformation). However, it is difficult to discern why 8 was folded in a chair-chair-boat during the polycyclization cascade.
to afford 15 (path b in Scheme 3). Notably, the conformation of the B ring is changed from the boat to chair form. As described above, the recognition site of the Me-29 group of 1 is less compact than necessary because the bulkier ethyl-substituted 9 was also accepted as the substrate and folded in the normal conformation (Scheme 3c) to afford homolanosterol 16. Binding between the less-bulky hydrogen atom at C-19 and the loosely packed binding site may have given rise to excess space and free motion at the B-ring-formation site, thus allowing the thermodynamically favored chair structure to be formed instead of the constrained boat conformation. Furthermore, the improper arrangement of 8 inside the reaction cavity could further contribute to the misfolded boat form at the C-ring-formation site, as mentioned in the production of 13. The increase in the cleft volume at the D-ring-formation site, which could be generated by the lack of the Me-29 group, could further allow the C-13–C-14 bond rotation in the Markovnikov cation 31 prior to the ring expansion, although this rotational movement is usually unlikely when the substrate is tightly constrained by the enzyme active sites.

Why was the six-membered D ring constructed for 14 and 15 instead of the five-membered D ring? The intermediary cation 33 cannot generate a stable tertiary cation such as 3 in the subsequent annihilation reaction, but affords only a secondary cation irrespective of the formation of five- or six-membered D rings. Therefore, a six-membered ring with less steric strain than a five-membered ring could be constructed for both products 14 and 15. The H-9β atom of intermediate 30 was eliminated for the formation of 14 (path a in Scheme 3), as seen in the biosynthesis of 2, but H-9α of intermediate 34 was abstracted for that of 15 (path b in Scheme 3). At the present time, the reason remains uncertain why the sterically different H-9 could be eliminated. One possible explanation is that the basic (polar) residue responsible for the deprotonation reaction of H-9α may be different from that for H-9β. The X-ray crystal structure of human lanosterol synthase has revealed that the phenolic Tyr503 residue that is hydrogen bonded to the histidine (His) residue His232 and situated above the molecular plane of 3 is involved in the deprotonation reaction of H-9β.[17] The H-9β atom of 30 could be eliminated in the same mechanism as that of 3 to form 14. Another polar residue that is located below the molecular plane of 34 may have abstracted H-9α to yield 15. For the production of 13 and 11, the different other polar residues would have worked for the deprotonation reaction.

Further studies, including X-ray crystallography studies of the complex between 29-nor-2,3-iminosqualene (a potent inhibitor) and lanosterol cyclase are required to validate our assumptions or to understand precisely the polycyclization reaction with intermediaries cations 29 and 30–34, respectively, as discussed in the enzymatic reaction of 6 with hog-liver cyclase.[18]

Conclusion

In conjunction with previously published results[16–13] we conclude that the Me-29 group of 1 is critical to the correct folding of the substrate in the enzyme cavity, with lesser contributions from the other branched methyl groups, such as Me-26, Me-27, and Me-28. Although lanosterol synthase folds 1 in a normal chair-boat-chair-chair conformation, the nor-substrate 8 was folded in two different conformations. The unusual chair-boat-chair-chair conformation afforded the 6,6,6,6-fused tetracyclic skeleton 15, whereas the normal chair-boat-chair-chair conformation gave the 6,6,6,6-fused tetracycle 14. Products 14 and 15 were diasteromers that have opposite stereochemistry at C-13 and C-14. This report is the first to describe the importance of the Me-29 group for the correct folding of 1 and the generation of the new triterpene scaffold by using hog-liver cyclase. Furthermore, this study highlights the reason why a five-membered D ring is constructed during lanosterol biosynthesis instead of a six-membered ring.

Experimental Section

General analytical methods: NMR spectra of the enzymic products were recorded in CDCl3, on Bruker DMX 600 and DPX 400 spectrometers, the chemical shifts are given in ppm relative to the solvent peak δH = 7.26 and δC = 77.0 ppm as the internal reference for the 1H and 13C NMR spectra, respectively. The chemical shifts are given in ppm relative to the solvent peak in CD3OD (δH = 7.28 and δC = 128.0 ppm). The chemical shifts of the solvent peaks were assigned to be δH = 2.04 and δC = 29.8 ppm in (CD3)2CO. The coupling constants J are given in Hz. GC analyses were recorded on a Shimadzu GC-8A chromatograph equipped with a flame-ionization detector (a DB-1 capillary column; 30 m × 0.25 mm × 0.25 μm; J&W Scientific Inc.). GC-MS spectra were recorded on a JEOL SX 100 or a JEOL JMS-Q1000 GC MS instrument equipped with a ZB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm; Zebron) by using the EI mode operated at 70 eV. HRMS (EI) was performed by using a direct-inlet system. HPLC was carried out with Hitachi L-1700 (pump) and L-7405 (UV detector), and the HPLC peaks were monitored at λ = 210 or 214 nm. Specific rotation values were measured with a Horiba SEPA-300 polarimeter.

Syntheses of noroxidosqualenes 5, 7 and 8: All the synthetic experiments were carried out in a N2 atmosphere. The syntheses of 5, 7, and 8 were performed by using essentially identical methods and with the same reagents. The detailed synthetic processes are described in the Supporting Information (see Figures S1–S4).

Preparation of ethyl-substituted oxidosqualene 9 (see Figure S4 in the Supporting Information): Triphenylphosphine (17 g, 64.8 mmol) and ethyl 2-bromobutyrate 48 (10 g, 51.3 mmol) were heated to reflux for 12 h in toluene to give phosphorane 49. The Wittig reaction of (4E,6E,12E)-4,9,13,17-tetramethyloctadeca-4,8,12,16-tetraenal (18) with 49 gave (2E,6E,10E,14E)-ethyl-2-ethyl-6,11,15,19-tetramethylcyclo-2,6,10,14,18-pentaeneoate (50; 50, %), 1.05 g (2.53 mmol) of which was subjected to reduction with DIBAL-H (hexane solution, 0.98 mol L−1, 5.93 mL (5.81 mmol)) to yield alcohol 51 (96%). Next, 51 was transformed into the bromohydryln derivative 52, which was then converted into the corre- sponding bromide 54. The phenylsulfone derivative 41, prepared from 3-methylbut-2-en-1-ol (39), and 54 were coupled with nBuLi to afford C63 phenylsulfone 55, followed by reduction with super hydride to yield 9.

Preparation of hog-liver cyclase (lanosterase synthase) and the incubation conditions of 2,3-iminosqualene and its analogues: Hog liver (100 g) was homogenized at 4°C in a Waring blender with triis(hydroxymethyl)amino-
methane hydrochloride (Tris-HCl) buffer (90 mM, 0.1 mM, pH 7.4) containing ethylenediaminetetraacetic acid (EDTA: 1 mM), phenylmethylsulfonyl fluoride (40 μM), and mercaptoethanol (1 mM). The reaction mixture was centrifuged for 10 min at 17000 g at 4°C. The supernatant was further centrifuged at 100000 g for 90 min. The microsomal pellets were resuspended and homogenized in the original quantity of Tris-HCl buffer containing EDTA (1 mM), mercaptoethanol (1 mM), and 0.5% Triton X-100 by using a Potter-Elvehjem homogenizer and was centrifuged at 100000 g for 90 min. The supernatant was used as the enzyme source with a typical protein content of 20–25 mg/mL.

Substrate Folding in Lanosterol Biosynthesis

Product 12: \[
{\text{C}_{38}}\text{H}_{62}\text{O}: 521.4429; \text{HRMS (EI): found: 521.4431.}
\]

Substrate Folding in Lanosterol Biosynthesis

Product 13-acetate: \[
{\text{C}_{39}}\text{H}_{64}\text{O}_{3}: 543.4441; \text{HRMS (EI): found: 543.4444.}
\]

An aerobic incubation of the synthesized (±)-6 (40 mg) afforded HPLC with hexane/2-ProOH (100:0.05) as the eluent.

Anoxic incorporation of the synthesized (±)-6 (40 mg) of Figure S2 in the Supporting Information afforded two enzymatic products 14 and 15. Lipophilic materials were extracted with hexane after saponification. The products were partially purified by column chromatography on SiO2 gel, followed by acetylation with Ac2O/pyridine. The pure acetates of enzymatic products of 14 and 15 (0.7 mg) and 14-acetate (0.4 mg) were separated by HPLC with hexane/2-ProOH (100:0.05).

Spectroscopic data for products 10 and 12–16:

Product 10: \[
{\text{C}_{34}}\text{H}_{56}: 496.4121; \text{HRMS (EI): found: 496.4120.}
\]

Product 11: \[
{\text{C}_{38}}\text{H}_{62}\text{O}: 521.4429; \text{HRMS (EI): found: 521.4431.}
\]

Product 12: \[
{\text{C}_{39}}\text{H}_{64}\text{O}_{3}: 543.4441; \text{HRMS (EI): found: 543.4444.}
\]

Product 13-acetate: \[
{\text{C}_{40}}\text{H}_{66}\text{O}_{4}: 565.4452; \text{HRMS (EI): found: 565.4453.}
\]

Product 14-acetate: \[
{\text{C}_{41}}\text{H}_{68}\text{O}_{4}: 589.4474; \text{HRMS (EI): found: 589.4474.}
\]

Product 15-acetate: \[
{\text{C}_{42}}\text{H}_{70}\text{O}_{5}: 613.4496; \text{HRMS (EI): found: 613.4497.}
\]
2.048 (s, 3H; acetyl Me), 2.07 (m; H-7), 2.08 (m; 2H; H-23), 2.01 (t; C-2), 2.04 (t; C-2), 2.40 (t; C-17), 2.65 (t; C-7), 2.75 (q; C-25), 27.87 (q; C-27), 29.28 (t; C-12), 29.37 (t; C-22), 29.92 (t; C-21), 31.01 (t; C-15), 35.55 (t; C-1), 37.18 (s; C-14), 37.24 (s; C-10), 37.77 (s; C-4), 39.54 (s; C-13), 45.24 (d; C-18), 49.96 (d; C-5), 80.93 (d; C-3), 125.1 (d; C-23), 131.2 (s; C-24), 133.9 (s; C-9), 136.1 (s; C-8), 149.2 (d; C-4), 140.8 (d; C-20), 145.6 (d; C-13), 153.4 (s; C-10), 37.77 (s; C-4), 39.54 (s; C-13), 45.24 (d; C-18), 49.96 (d; C-5), 80.93 (d; C-3), 125.1 (d; C-23), 131.2 (s; C-24), 133.9 (s; C-9), 136.1 (s; C-8), 171.0 ppm (s, acetyl CO); the following $^{13}$C NMR signals are indistinguishable from each other because they have very similar chemical shifts: C-10/C-14, C-12/C-22, C-6/C-16; MS (EI): m/z (%): 69 (100), 229 (84), 241 (48), 289 (30), 213 (37), 397 (25), 439 (36), 454 (68) [M]$^{+}$; HRMS (EI): cale for C$_{33}$H$_{53}$O$_{6}$: 454.3811; found: 454.3817; assignments of the $^{1}$H and $^{13}$C NMR data in C$_{6}$D$_{6}$ are shown in Figure S9 in the Supporting Information.

**Product 16:** $\left[\varepsilon\right]_{D}^{25} = 18.62$ ($c = 0.188$ in CHCl$_{3}$); $^{1}$H NMR (400 MHz, CDCl$_{3}$); $\delta = 0.687$ (s, 3H; Me-18), 0.795 (t, $J = 7.2$ Hz; Me-31), 0.808 (3H; s, Me-29), 0.882 (s, 3H; Me-30), 0.978 (s, 3H; Me-19), 0.998 (s, 3H; Me-16), 1.16 (m; H-15), 1.20 (m; H-22), 1.22 (m; H-1), 1.36 (m; H-22), 1.37 (m; H-20), 1.38 (m; H-21), 1.50 (m; H-6), 1.53 (m; 2H; H-16, H-21), 1.56 (m; H-2), 1.58 (brd, $J = 11.6$, 2.0 Hz; H-5), 1.605 (s, 3H; Me-27), 1.63 (m; H-2), 1.64 (m; H-12), 1.67 (m; H-6), 1.682 (s, 3H; Me-26), 1.71 (m; H-17), 1.73 (m; H-11), 1.77 (m; H-12), 1.82 (m; H-23), 1.97 (brm, 2H; H-23), 2.01 (m, 2H; H-11), 2.03 (m, 2H; H-7), 2.07 (m; H-16), 3.23 (dd, $J = 11.6$, 4.4 Hz; H-3), 5.11 ppm (t, $J = 6.8$ Hz; H-24); $^{13}$C NMR (100 MHz, CDCl$_{3}$); $\delta =$ 9.15 (q; C-31), 15.42 (q; C-29), 15.67 (q; C-18), 17.62 (q; C-27), 18.26 (t; C-6), 19.15 (q; C-19), 21.05 (t; C-11), 22.53 (t; C-21), 24.35 (t; C-23), 24.43 (q; C-30), 25.74 (q; C-26), 26.49 (t; C-7), 27.85 (t; C-16), 27.93 (t; C-2), 27.96 (q; C-28), 30.64 (t; C-22), 30.69 (t; C-12), 30.79 (t; C-15), 35.60 (t; C-1), 37.01 (s; C-10), 38.89 (s; C-4), 40.86 (d; C-20), 44.46 (s; C-13), 45.95 (d; C-17), 48.97 (s; C-14), 50.38 (d; C-5), 78.98 (q; C-3), 125.3 (d; C-24), 130.8 (s; C-25), 134.3 (s; C-9), 134.4 ppm (s; C-8); the following $^{13}$C NMR signals are indistinguishable from each other because they have very similar chemical shifts: C-2/C-16, C-15/C-22; MS (EI): m/z (%): 69 (100), 81 (32), 95 (46), 123 (30), 407 (47), 425 (82), 440 (33) [M]$^{+}$; HRMS (EI): cale for C$_{35}$H$_{59}$O$_{6}$: 440.4018; found: 440.4018.

**Kinetic analyses $k_{\text{on}}$ and $K_{d}$ of time-dependent inhibition by 5, 7 and 8:** The partially purified lanostanol synthase described above (1.8 mL) was used. Noroxidosqualene 5, 7, and 8 were added to the enzyme solution, which was adjusted to a final volume of 2.0 mL with Tris-HCl buffer (0.1 M, pH 7.4): 30 µt, 60 µt, and 121 µt for 5 and 8; 60 µt, 121 µt, and 242 µm for 7. Each of the solutions was incubated at 37°C for the intervals of 0, 30, 60, 120, and 300 min. From each of the reaction tubes, an aliquot of the preincubated mixture (200 µL) was added to a tube containing (±)-1 (200 µg) and then further incubation was conducted for 8 h. To quench the reaction, KOH in MeOH (15%, 1 mL) was added and the solution was heated at 80°C for 30 min. The hexane extract was subjected to GC analysis to estimate the amount of lanosterol produced. The kinetic parameters of $k_{\text{on}}$ and $K_{d}$ were determined using Kists-Wilson plots. Ethyl-substituted 9 did not undergo any time-dependent inhibition.

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