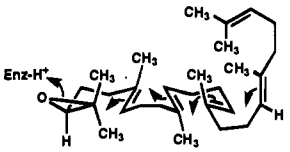
New Insights Regarding the Cyclization Pathway for Sterol Biosynthesis from (*S*)-2,3-Oxidosqualene

E. J. Corey,* Scott C. Virgil, Hengmiao Cheng, C. Hunter Baker, Seiichi P. T. Matsuda, Vinod Singh, and Sepehr Sarshar

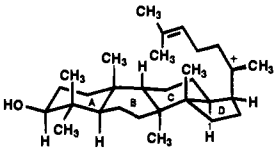
Department of Chemistry, Harvard University Cambridge, Massachusetts 02138

Received June 19, 1995

After a quiet period of over two decades in the study of the mechanistic details of the cyclization step in sterol biosynthesis, there has recently been a renaissance in this field due to the availability of a powerful arsenal of new chemical, biochemical, and biological tools.[1-3] It is clear that the enzyme lanosterol synthase, which catalyzes the conversion of (*S*)-2,3-oxidosqualene (**1**) to lanosterol (via a protosterol intermediate, **2**),[2,4] must exert very fine control over the conformation of the flexible, hydrocarbon-like substrate in order to channel the reaction through a sequence of four cyclizations and four 1,2-group migrations with complete structural and stereochemical control. The precise molecular interactions which result in this remarkable conformational control remain to be defined, however. The idea has been widely entertained that the cyclization step is concerted (nonstop), i.e., without the intermediacy of mono-, bi-, or tricyclic carbocations, and it usually has been assumed that the conformation of the bound substrate corresponds to that which is best suited to undergo the completely concerted process **1** → **2**. We report herein new data on the enzymic cyclization which indicates that this process is probably not concerted and that it occurs in stages with discrete carbocation intermediates, but with tight conformational control.



1



2

We have previously reported[2a] that 20-oxa-2,3-oxidosqualene (**3**) is converted by the lanosterol synthase of yeast (*Saccharomyces cerevisiae*)[5] to the 17β-acetylprotosterol derivative **4**. When this reaction was scaled up and examined in detail, it was discovered that a second, isomeric tetracyclic compound (**5**) is formed as a minor product. The yields of **4** and **5** after conversion to the *tert*-butyldimethylsilyl (TBS) ethers and isolation by HPLC were 40.4 and 3.4%. The structure **5** was demonstrated by X-ray crystallographic analysis of the *p*-bromobenzoate derivative (Figure 1).[6] ^{1}H NMR analysis was completely consistent with this assignment of structure; in addition to a singlet due to the acetyl methyl at 2.07 δ, the C(17) proton (α to C=O) appeared as a triplet at 2.87 δ (J = 8.5 Hz), indicating vicinal coupling to C(16)H$_2$, but not to C(13)H. In contrast, under the same conditions (*S*)-2,3-oxidosqualene is converted cleanly to lanosterol by the yeast synthase with no sign of abnormal cyclization products comparable to **5**.

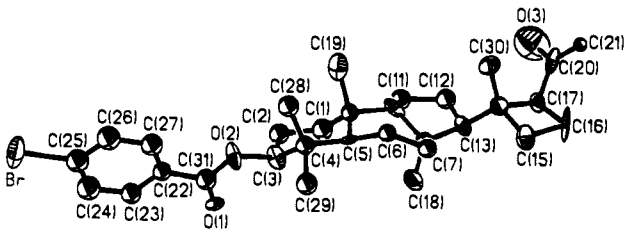
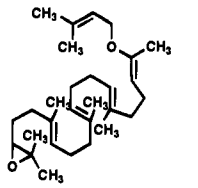
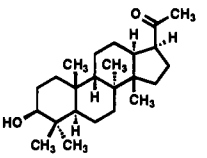


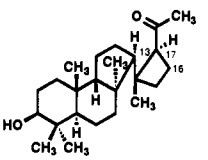
Figure 1. X-ray structure of the *p*-bromobenzoate of **5**.



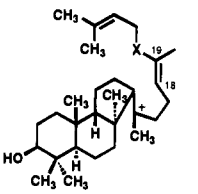
3



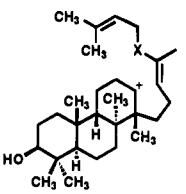
4



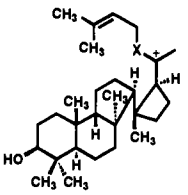
5



6 X = CH_2
8 X = O



7 X = CH_2
9 X = O



10 X = CH_2
11 X = O

The formation of **5** is not readily rationalized on the basis of a mechanism which does not involve intermediate carbocations prior to generation of the 20-oxa analog of **2**. On the other hand, if the cyclization of (*S*)-2,3-oxidosqualene proceeds via the discrete tricyclic cation **6** (the product of Markovnikov closure with respect to ring C), and if the resulting cyclopentylcarbinyl cation has a finite lifetime before ring expansion to form the tricyclic cyclohexyl carbocation **7** and further cyclization to the protosterol cation **2**, the formation of the tetracyclic product **5** from 20-oxa-2,3-oxidosqualene (**3**) is simply explained as due to an enhanced trapping rate of carbocation **8** (before rearrangement to **9**) because of the much greater nucleophilicity of the 18,19-double bond of **8** relative to **6**. That is, in cation **8**, the cyclization to the tetracyclic cation **11** is accelerated by the electron-donating 20-oxa function to the point where the normally very unfavorable closure of a four-membered ring competes with the 1,2-ring-expansive shift which converts **8** to **9**. In the case of sterol biosynthesis from 2,3-oxidosqualene, intermediate **6** undergoes ring expansion to **7** exclusively

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

(2) For a revision of the stereochemistry of the tetracyclization reaction, see: (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.

(3) For recent work on the gene-cloning and protein sequences for lanosterol synthase from various organisms, see: (a) Kelly, R.; Miller, S. M.; Lai, M. H.; Kirsch, D. R. *Gene* **1990**, *87*, 177. 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 (*Saccharomyces cerevisiae*). (d) Kasuno, M.; Shibuya, M.; Sankawa, U.; Ebizuka, Y. *Biol. Pharm. Bull. Jpn.* **1995**, *18*, 195 (*Rattus norvegieus*). (e) Baker, C. H.; Matsuda, S. P. T.; Liu, D. R.; Corey, E. J. *Biochem. Biophys. Res. Commun.* **1995**, *213*, 154 (*Homo sapiens*).

(4) Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1990**, *112*, 6429.

(5) All experiments reported herein were carried out with the lanosterol synthase of *S. cerevisiae*.

(6) The coordinates of the *p*-bromobenzoate of **5** can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, U.K.

0002-7863/95/1517-11819$09.00/0

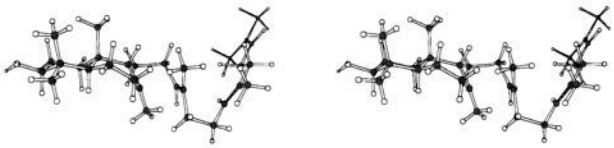


Figure 2. Stereopair model for formation of a six-membered C ring in the conversion of 2,3-oxidosqualene to lanosterol.

because the competing closure to **10** is relatively very slow. Other explanations for the diversion of the cyclization of **3** to the 6,6,5,4-tetracyclic system **5** are conceivable, but they are generally more complex and plagued by inconsistencies, and in any event, they contravene the concerted cyclization hypothesis.[7]

Granted that cations **6** and **7** are formed sequentially as discrete intermediates in the tetracyclization **1** → **2**, the following question arises. Why is **6** formed at all since, in principle, **7** could be formed directly in a stereoelectronically more favorable manner (better orbital overlap) from the bicyclic cation corresponding to the A/B ring system? In addition to the Markovnikov factor, which obviously favors formation of the tertiary cation **6** over the secondary cation **7**, there is also a large steric factor in favor of the five-membered-ring closure to cation **6**. Direct formation of a six-membered C ring entails severe nonbonded steric repulsions between the two methyl groups on the carbons being joined (C(8) and C(14), steroid numbering) and their nearest neighbors, as shown in Figure 2, which corresponds to a C(8)/C(14) distance of *ca.* 2.5 Å (early transition state). In contrast, ring closure to form the five-membered structure **6** involves relatively minor steric repulsions, as shown in Figure 3.[8] The prefolded conformations of the bicyclic cation leading to **6** and **7** differ with respect to the dihedral angle about the C(11)−C(12) bond.[9]

The evidence presented herein against a concerted mechanism for the enzymic cyclization of 2,3-oxidosqualene raises the question of whether discrete carbocations are involved in the formation of the A and B rings. For this reason, we have examined the behavior of 1,1′-bisnor-2,3-oxidosqualene (**12**) (a homologue of **1** lacking the methyl substituents on the oxirane ring) as a substrate for cyclization using purified yeast enzyme. Studies reported earlier with **12** as substrate were inconclusive,[10] since they were performed with a crude enzyme preparation (from rat liver) and afforded only the corresponding 1,2-diol, indicating preferential reaction with a liver epoxide hydrolase.

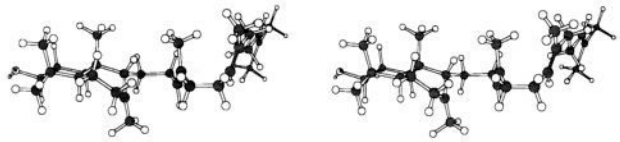
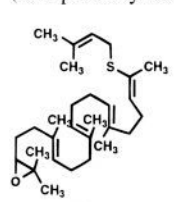


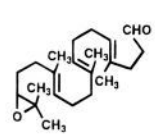
Figure 3. Stereopair model for formation of a five-membered C ring in the conversion of 2,3-oxidosqualane to lanosterol.

Repeated experiments with **12**[10] and pure lanosterol synthase from yeast resulted in absolutely no reaction products of any kind, arguing for the possibility that the closure of ring A is not an internal S_N attack by the adjacent double bond on the electrophilically activated epoxide and that the intermediacy of a carbocation in the closure of ring A is a reasonable possibility. Interestingly, we found that (*R*)-2,3-oxidosqualene, the enantiomer of **1**, is cyclized by pure yeast lanosterol synthase to 3-*epi*-lanosterol (identical with an authentic sample) at a rate approximately 0.02 times that for **1**.[11] This result implies that a perfect fit in the A-ring region is not required for epoxide activation and that epoxide **12** can interact with the initiating electrophilic site of lanosterol synthase.

We have also obtained experimental results which suggest a link between complementary binding of lanosterol synthase to 2,3-oxidosqualene and epoxide activation. 20-Thia-2,3-oxidosqualene (**13**)[12] is *completely* inert to cyclization by yeast lanosterol synthase, in contrast to the 20-oxa analog **3**, even when large amounts of purified enzyme and very long reaction times are used. Furthermore, when exposed to mixtures of 2,3-oxidosqualene (**1**) and the 20-thia analog **13**, lanosterol synthase converts **1** to lanosterol (at a modestly diminished rate), but leaves **13** completely unchanged. Thus, it is clear that **13** is not a substrate for cyclization by lanosterol synthase, even to mono- or bicyclic products, and functions only as a moderate competitive inhibitor. It appears that, because of the larger space requirement of sulfur relative to oxygen or methylene, substrate **13** does not fit into the space available in the vicinity of the D-ring/side-chain region and, further, that this *misfit prevents epoxide activation. This remarkable result argues for a connection between goodness of fit of a correctly folded form of oxidosqualene and the initiation of cyclization*, which is tantamount to a requirement for the correct prefolding and binding of the substrate prior to cyclization. The following sequence of action by lanosterol synthase then follows: (1) enzymic binding to induce the correct folding of 2,3-oxidosqualene; (2) a conformational change, dependent on step 1, which correctly positions an electrophilic group for activation of the epoxide; and (3) cyclization to produce **2** via intermediates **6** and **7** (and possibly others).



13



14

In summary, this communication presents clear evidence against a concerted, no-intermediate pathway for the lanosterol synthase promoted cyclization of (*S*)-2,3-oxidosqualene (**1**) to protosterol cation **2** and in favor of the discrete carbocationic intermediates **6** and **7**.

Acknowledgment. This research was supported financially by the National Institutes of Health, the National Science Foundation, and NSERC of Canada.

JA9519750

(7) The fact that lanosterol synthase correctly controls the cyclization of 20-oxa-2,3-oxidosqualene (**3**) to the protosterol **4** indicates that **3** is bound by the enzyme in the same way as 2,3-oxidosqualene (**1**) itself and that the formation of coproduct **5** is due to the trapping of a normal reaction intermediate.

(8) Acid-catalyzed nonenzymic cyclization of 2,3-oxidosqualene generates (in low yield) 6/6/5-fused and not 6/6/6-fused tricyclic product; see: van Tamelen, E. E. *J. Am. Chem. Soc.* **1982**, *104*, 6480. For another study of chemical cyclization, see: Berckmoes, K.; DeClereq, P. J.; Viterbo, D. *J. Am. Chem. Soc.* **1995**, *117*, 5857.

(9) In the stereoelectronically most favorable structure for formation of **6**, the bonds about C(11)−C(12) are eclipsed, whereas in that leading to **7**, the bonds about C(11)−C(12) are staggered (*ca.* 60° change in dihedral angle).

(10) Corey, E. J.; Lin, K.; Jautelat, M. *J. Am. Chem. Soc.* **1968**, *90*, 2724.

(11) This result can be explained by assuming that *ent*-**1** and **1** are bound by the enzyme in similar conformations, except that the A-ring region of *ent*-**1** is held in a way that corresponds to the boat form of 3-*epi*-protosterol. Nonspecific cyclization of racemic 2,3-oxidosqualene has previously been reported for the methylotroph *Methylococcus capsulatus*. See: Rohmer, M.; Bouvier, P.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 557.

(12) The synthesis of **13** was carried out (V. Singh, 1988) by Horner−Emmons coupling of aldehyde **14**[2a] with $Me_2C{=}CHCH_2SOCH(CH_3)PO(OC_2H_5)_2$, separation of *E* and *Z* isomers by chromatography, and reduction of sulfoxide to sulfide. A different synthesis of **13** has recently been reported; see: Zheng, Y. F.; Dodd, D. S.; Oehlschlager, A. C.; Hartman, P. G. *Tetrahedron* **1995**, *51*, 5255.