Enzymic products of the 2,3-oxidosqualene analog having an ethyl residue at 10-position. First trapping of the trimethylcyclohexanone ring by lanosterol synthase

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Abstract—Incubation of squalene analog, (3RS)-(6E,10E,14E,18E)-10-ethyl-2,6,19,23-tetramethyl-2,3-epoxytetracosane-6,10,14,18,22-pentaene with 2,3-oxidosqualene-lanosterol cyclase from pig liver gave four products, consisting of two mono-, one tri- and one tetracyclic lanosterol homolog, suggesting that the steric bulk size at C-10 had greater influence on the polycyclization reaction, compared to that at C-15. The formation of the trimethylcyclohexanone ring by lanosterol synthases has never been reported before. © 2001 Elsevier Science Ltd. All rights reserved.

2,3-Oxidosqualene 1 is cyclized into a variety of triterpene skeletons or lanosterol 2 by eukaryotic cyclases. The polycyclization mechanism is intriguing from the point of view that multiple C–C bond formation occurs under fine stereo- and regio-specificity. It has been believed that 1 is folded in a chair/boat/chair conformation in the enzyme cavity of lanosterol synthase. Numerous studies on substrate analogs by lanosterol synthase have appeared in order to gain insight into the polycyclization mechanism and into the substrate recognition. Among many investigations, special attention has been paid by vanTamlene, Corey and Kyler as to how the cyclization pathway is affected by the substitution of the methyl group at C-10 or at C-15. The replacement of the methyl group at C-15 with hydrogen and that of the methyl at C-10 with a vinyl group led to the normal cyclization products. However, the analog lacking methyl groups at both C-10 and C-15 afforded an unusual product having the 6,6,5-fused A/B/C tricyclic ring system, which is further linked with a four-membered ring, where the B-ring has a chair structure. This result suggests that the cyclization had proceeded in a pre-organized chair/boat/chair conformation, which is in contrast to the usual folding of 1 into the chair/boat/chair conformation by lanosterol synthase. Thus, it has been inferred that the methyl residue at the 10-position is essential for the correct folding of this substrate. They also proposed that the expansion process from a five- to a six-membered ring is involved in the C-ring formation of 2 (Scheme 1), based upon the trapping of the enzymic products having a 6,6,5-fused A(chair)/B(boat)/C-ring system from the truncated C20-analog of 1. The formation of intermediate 3 has the five-membered C-ring

Scheme 1.

Keywords: lanosterol; 2,3-oxidosqualene; squalene; lanosterol synthase; triterpene cyclase; trimethylcyclohexanone.

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An SiO₂ column chromatography by eluting with hexane:EtOAc (100:3) afforded 1 mg of 9 in a pure state. Next, an HPLC (hexane:2-propanol = 100:2) was used to obtain 8 (2 mg) in a pure form. Products 11 and 13 were indistinguishable on SiO₂ TLC, but the separation was done with argentation SiO₂ column (6% AgNO₃, hexane:EtOAc = 100:4, isolation yields: 1 mg each). The total conversion from (±)-6 was ca. 19% from the isolation yields.

Structures of all the products (Fig. 1) were unequivocally determined by detailed analyses of the NMR spectra (DEPT, COSY 45, HOHAHA, NOESY, HMQC and HMBC). Product 8 had four olefinic protons at δH 5.43 (3H, m) and 5.37 (1H, t, J = 6.8 Hz) along with exomethylene protons [δH 5.02 (1H, s) and 4.84 (1H, s)], which were correlated to δC 108.6 (t) in the HMQC spectrum. Four allyl methyl residue and one ethyl residue were also found at δH 1.80 (3H, s), 1.74 (3H, s), 1.73 (3H, s), 1.68 (3H, s), 2.22 (2H, q, J = 7.6 Hz, CH₂CH₃) and 1.14 (3H, t, J = 7.6 Hz, CH₃CH₂), suggesting a monocyclic compound for 8. The ethyl group was shown to be attached to C-9 of 8 by the HMBC correlations from the ethyl protons. The apparent NOE of H-1ax with H-3 showed the stereochemistry (Scheme 2). Product 9 also had four double bonds as well as 8, proving to be the monocyclic skeleton for 9. However, no alcoholic carbon signal was detected, but in turn a carbonyl carbon signal (δC 211.0 ppm; 1730 cm⁻¹ in CHCl₃) appeared. Detailed analyses of 2D NMR spectra of 9 revealed the presence of a 2,3,4-trimethylcyclohexane ring (Scheme 3). The fragment ion of m/z 139 further supported the presence of the trimethylcyclohexane ring in 9. As for 11, the proton signals of two olefins, three allyl methyl and exomethylene groups were found in the ¹H NMR, suggesting a tricyclic system for 11. Detailed analyses of HMBC and NOESY spectra (Scheme 2) gave the complete structure for 11. The apparent NOE of H-9 with Me-26 proved β-orientation for H-9, i.e. a boat form.

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for the B ring and that of H-13 with H-27 also verified
β-arrangement for the isoprenoid side chain. The fission
ion m/z 261 (20%) of EIMS further supported
the tricyclic ring. 

Detailed analyses of HMBC and NOESY data estab-
lshed the lanosterol skeleton having ethyl group at
Me-19 and C-9 (Scheme 2) verified the double bond position. Detailed analyses of HMBC and NOESY data estab-
lshed the lanosterol skeleton having ethyl group at
C-14. EIMS of 13 showed m/z 440 (M+, 8%), 411
(M−-Et, 100%) and 393 (M−-Me−H₂O, 35%). The
fission patterns are quite similar to those of authentic
lanosterol [m/z 426 (M+, 60%), 411 (M−-Me, 100%),
393 (M′-Me−H₂O, 32%)], further demonstrating a
lanosterol homolog for 13. The stereochemistry at C-20
has remained uncertain, but should have the same
R-configuration as 2, based on the reaction mechanism
and on the fact that the chemical shift difference for the
Me-21 was minimal between 2 and 13; δH of 2 and 13
were 1.15 and 1.14 (d, J = 6.3 Hz), and δC for C-20 of
2 and 13 were 36.68 and 36.67, respectively, in C₆D₆
solution.

The product distribution of monocyclic (8 and 9), tri-
cyclic 11 and fully cyclized 13 was in a ratio of 3:1:1.

From 5, tri-cyclic 10 and completely cyclized 12 were
produced in a ratio of 1:2.6, but no monocyclic com-
pound was accumulated. The cavity size or the binding
site to accommodate the Me group at C-10 of 1 is
possibly more accurate than that at C-15, leading to
higher production of monocyclic products from 6. A
looser binding of the ethyl group with the cyclase, due
to the slightly bulky size at C-10, would have prevented
the completion of the polycyclization reaction, thus
resulting in the accumulation of two carbocation inter-
mediates 15 and 16. Deprotonation from Me-28 of 15
(path b) could give 11, while an enlargement of the
five-membered C-ring of 15 into the six-membered ring
(path a), followed by a further cyclization and back-
bone rearrangement, could give 13 according to Scheme
1. Proton elimination from 16 (path c) could give 8.

Intermediates 15 and 16 could be formed by the cycliza-
tion of (3S)-6, because 8, 11 and 13 have a β-oriented
OH group.

The Tetrahymena pyriformis cyclase catalyses the con-
version reaction from squalene into pentacyclic tetra-
hymanol. This cyclase also accepts both enantiomers of
(3R)- and (3S)-1 to give α- and β-hydroxytetra-
hymanol, respectively, along with the production of the
triterpene having a trimethylcyclohexanone moiety, the
monocyclic ketone being produced only from (3R)-1,
but not from (3S)-1. However, the stereochemistry of
the trimethylcyclohexanone ring has remained unresolved. The chemical shifts of the cyclohexanone ring of 9 were compared with those of 19 having the 2R,3S,4R-stereochemistry, which is available from the chemical synthesis from (±)-1 by using the Lewis acid SnCl4. A careful comparison of the NOESY spectrum of 9 with that of 19 showed that a strong NOE between Me-25 and H-2 was observed for 9, whereas there was no NOE between them for 19 (Scheme 3). Detailed NOE analyses allowed us to propose the structure 9 (Fig. 1 and Scheme 3). Compound 19 is produced from (3S)-1 via chair-formed 18, which is then subjected to the 1,2-rearrangement reactions of the hydride and the methyl in an antiparallel concerted manner. On the other hand, it is likely that the formation of 9 proceeds from (3R)-6 via twist boat-formed 17 and that the rearrangement reactions proceed as shown in Scheme 3, taking into consideration the stereochemistry (2S,3R,4R) of the trimethylcyclohexanone ring of 9. The deprotonation from the OH group, formed after the epoxide ring-opening, could give ketone 9. Formation of 9 led to a surprising and important question, because lanosterol synthase is believed to be active only to (3S)-1, but inert to (3R)-1. One possible explanation of this paradox may be that the larger steric bulk size of the ethyl group may have induced incorrect placement of the (3R)-epoxide at the catalytic site intrinsic to the (3S)-epoxide, where (3R)-6 had been constrained to fold with a boat form in the enzyme cavity. Despite the occurrence of looser binding around the A/B-ring formation site, the cyclase had still a binding ability to (3S)-6 which enabled it to form the chair structure, leading to the production of 8 and to further cyclization to form 11 and 13; the yield (31%) of 8, 11 and 13 from (3S)-6 was higher than that (7.7%) of 9 from (3R)-6.

In conclusion, this is the first report that a triterpene having a trimethylcyclohexanone ring was produced by mammalian cyclase. It is quite interesting from the aspect of molecular evolution that the same cyclohexanone skeleton is also constructed by the squalene cyclase of a protozoa Trypanosoma pyriformis. This supports the idea that triterpenoid cyclases should have evolved from a common ancestor cyclase; that is, a variety of natural triterpene skeletons may have been created by subtle changes in the active sites. This study also gave further evidence that lanosterol is biosynthesized via the ring-expansion process of the five-membered C-ring as shown in Scheme 1. It is noteworthy that the ethyl group migrates in a similar way to the methyl of natural 1. Kyler et al. reported that the vinyl appendage (the same C2 unit as ethyl group) at C-10 had no influence on the cyclization as for Baker’s yeast cyclase, leading to the complete polycyclization to give a lanosterol homolog without any abortive cyclization products having been trapped. The specificity for squalene analogs is different between pig liver and Baker’s yeast.

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References

8. NMR data for 8, 9, 11, 13 and 19. 1H NMR (600.13 MHz) and 13C NMR (150.9 MHz) in CDCl3 ppm relative to 7.28 and 128.0 ppm of the solvent peak.

Compound 8: (analog of Achilleol A), δH 0.92 (3H, s, H-25), 1.13 (3H, s, H-24), 1.14 (3H, H, t, J = 7.6 Hz, H-31), 1.48 (1H, m, H-6ax), 1.68 (3H, s, H-30), 1.72 (1H, m, H-6eq), 1.73 and 1.74 (3H each, s, H-28 and H-29), 1.77 (1H, t, J = 6.3 Hz, H-3), 1.80 (3H, s, H-23), 1.85 (2H, bt, J = 7.7 Hz), 1.98 (1H, m, H-5ax), 2.02 (1H, m, H-8), 2.22 (2H, q, J = 7.6 Hz, H-27), 2.22 (4H, t, J = 7.7 Hz, H-15 and 19), 2.30 (8H, m, H-11, 12, 16, 20), 2.38 (1H, m, H-5eq), 2.41 (1H, m, H-8), 3.24 (1H, dd, J = 13.8, 6.6 Hz), 4.84 (1H, s, H-26), 5.02 (1H, s, H-26), 5.37 (1H, bt, J = 6.8 Hz), 5.43 (3H, m, H-10, 13, 17), δC 13.6 (C-31), 16.1 (C-28 and C-29), 16.2 (C-25), 17.7 (C-30), 23.6 (C-27), 24.7 (C-7), 25.3 (C-23), 26.3 (C-24), 27.1 and 27.2 (C-16 and C-20), 28.4 (C-12), 29.05 (C-11), 32.6 (2C, C-5 and C-6), 35.9 (C-8), 40.2 (C-15 and C-19), 40.6 (C-2), 51.8 (C-3), 76.7 (C-1), 108.6 (C-26), 124.3 (C-10), 124.9 (C-13, 17 and 21), 131.1 (C-22), 134.9 and 135.1 (C-14 and C-18), 141.7 (C-9), 148.0 (C-4). [3]13(S) (EtOH) −7.8 (c = 0.08).

Compound 9: δH 0.784 (3H, s, H-26), 0.903 (3H, d, J = 7 Hz, H-25), 1.069 (3H, d, J = 6.9 Hz, H-24), 1.146 (3H, t, J = 7.5, H-31), 1.38 (1H, m, H-5eq), 1.72 (m, H-5 ax), 1.45 (2H, m, H-7), 1.60 (1H, m, H-4), 1.69 (3H, s, H-30), 1.75 and 1.73 (3H each, s, H-28 and H-29), 1.80 (3H, s, H-23), 2.01 (2H, m, H-8), 2.17 (1H, m, H-6ax), 2.20 (2H, q, J = 7.5 Hz, H-27), 2.20–2.35 (6H, m, H-11, 12, 15 and 16), 2.25 (1H, m, H-6eq), 2.31 (1H, q, J = 6.9 Hz, H-2), 5.36 (1H, bt, J = 6.9 Hz, H-21), 5.40 (3H, m, H-10, 13, 17), δC 9.23 (C-24), 13.60 (C-31), 14.05 (C-25), 16.09 and 16.20 (C-28 and -29), 17.71 (C-30), 21.05 (C-26), 24.12 (C-27), 27.11 and 27.22 (C-16 and -20), 28.98 (C-12), 29.45 (C-11), 30.32 (C-8), 34.65 (C-4), 36.25 (C-7), 36.93 (C-6), 42.45 (C-3), 50.07 (C-2), 124.4 (C-10), 124.75, 124.77 and 124.92 (C-10, 13 and 17), 130.88 (C-22), 135.0 and 135.3 (C-14 and 18), 141.85 (C-9) and 211.03 (C-1). Selected δH in CDCl3 relative to the solvent peak (7.26
ppm), 0.85 (s, H-26), 1.00 (d, J = 6.7 Hz, H-25), 1.11 (d, J = 6.9 Hz, H-24), 0.98 (t, J = 7.6 Hz, H-31). [α]D 25 (EtOH) +1.25 (c = 0.08). These data are superimposable to those of the trimethylcyclohexanone ring from *T. pyriformis*.

Compound 11: H: 0.91 (3H, s, Me-25), 1.00 (3H, m, Me-31), 1.05 (3H, s, Me-26), 1.10 (3H, s, Me-24), 1.35 (1H, m, H-27), 1.41 (1H, m, H-1), 1.48 (1H, m, H-1), 1.50 (1H, H-5), 1.55–1.66 (8H, m, H-7, 6, 11 and 2), 1.68 (3H, s, Me-30), 1.69 (1H, m, H-27), 1.74 (3H, s, Me-29), 1.80 (3H, s, H-23), 1.80 (1H, m, H-9), 1.88 (1H, m, H-12), 2.07 (1H, m, H-12), 2.09 (1H, m, H-15), 2.22 (2H, t, J = 7.5 Hz, H-19), 2.23 (1H, m, H-15), 2.30 (2H, t, J = 7.5 Hz H-20), 2.32 (1H, m, H-16), 2.43 (1H, m, H-16), 2.51 (1H, dd, J = 9.3, 3.0 Hz, H-13), 3.14 (1H, dd, J = 11.2, 5.3 Hz), 4.93 (1H, s, H-28) and 5.17 (1H, t, J = 6.8, H-21), 5.45 (t, J = 6.7, H-17). [α]D 25 (EtOH) +8.9 (c = 0.09).

Compound 13: δ: 0.87 (3H, s, H-18), 0.97 (3H, s, H-30), 1.00 (3H, m, H-31), 1.08 (3H, s, H-19), 1.14 (d, J = 6.3 Hz, H-21), 1.16 (3H, s, H-29), 1.21 (2H, dd, J = 12.8, 2.0 Hz, H-5 and H-1), 1.30 (1H, m, H-22), 1.42 (1H, m, H-28), 1.53 (3H, m, H-28, 16 and 6), 1.58 (4H, m, H-2 and 15), 1.67 (3H, m, H-17 and 20), 1.72 (3H, H-1, 16 and 22), 1.75 (3H, s, H-27), 1.77 (1H, m, H-12), 1.83 (3H, s, H-26), 1.93 (1H, m, H-12), 2.04 (1H, m, H-7), 2.10 (2H, br, J = 8 Hz), 2.15 (1H, m, H-23), 2.20 (1H, m, H-7), 2.33 (1H, m, H-23), 3.16 (1H, dd, J = 10, 6 Hz, H-3), 5.42 (t, J = 7 Hz H-24). δ: 10.7 (C-31), 15.7 (C-30), 17.5 (C-18), 17.7 (C-27), 18.5 (C-19), 18.7 (C-16), 18.9 (C-21), 21.2 (C-11), 25.4 (C-23), 25.8 (C-15), 25.8 (C-26), 28.3 (C-2), 28.3 (C-29), 28.5 (C-28), 28.9 (C-6), 30.5 (C-7), 31.5 (C-12), 35.8 (C-1), 36.7 (C-20), 36.8 (C-22), 37.7 (C-10), 39.0 (C-4), 46.8 (C-13), 51.1 (C-5), 51.5 (C-17), 53.7 (C-14), 78.6 (C-3), 125.7 (C-24), 130.8 (C-25), 133.3 (C-8), 136.2 (C-9). [α]D 25 (EtOH) +11.4 (c = 0.07).

9. During the polycyclization reaction, a thermodynamically favored C-8 carbocation intermediate is produced, but no A/B-fused bicyclic product was accumulated, suggesting that the lifetime of the bicyclic cation might be significantly short.

