Biosci. Biotechnol. Biochem., **63** (11), 2038–2041, 1999

Preliminary Communication

New Cyclization Mechanism for Squalene: a Ring-expansion Step for the Five-membered C-ring Intermediate in Hopene Biosynthesis

Tsutomu HOSHINO,* Masanori KOUDA, Takamasa ABE, and Shumi OHASHI

Department of Applied Biological Chemistry, Faculty of Agriculture, and Graduate School of Science and Technology, Niigata University, Ikarashi, Niigata 950-2181, Japan

Received June 23, 1999; Accepted August 9, 1999

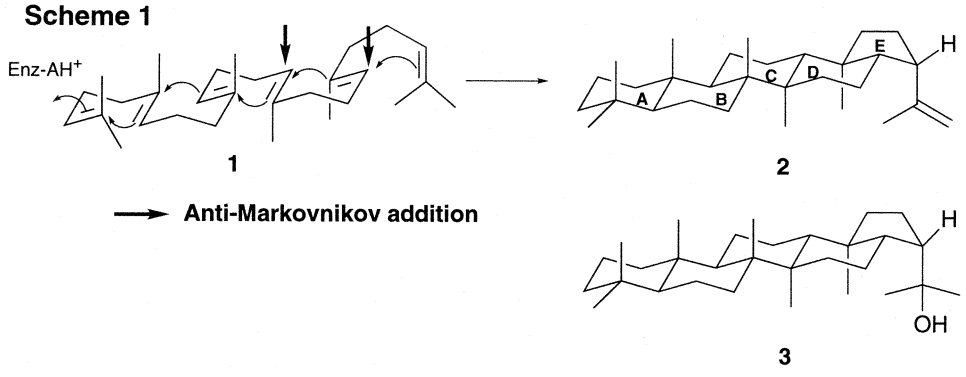
Three triterpenes having the 6/6/5-fused tri- and 6/6/6/5-fused tetracyclic skeletons were isolated from an incubation mixture of the mutated F601A enzyme, these products being in accordance with a Markovnikov closure. Successful trapping of the tricyclic cationic intermediate by using the squalene analog having a highly nucleophilic hydroxyl group leads us to propose that the ring expansion process of the 5-membered C-ring is involved in the squalene cyclization cascade.

Key words: squalene; hopene; triterpene cyclase; 2,3-oxidosqualene

The biocyclization of squalene **1** into the pentacyclic triterpene, hop-22(29)-ene **2**, which is mediated by squalene-hopene cyclase (SHC), is one of the most sophisticated biochemical reactions (Scheme 1).[1] Hopan-22-ol **3** is a minor product. (3*S*)-2,3-Oxidosqualene also undergoes polyolefin cyclization by eukaryotic cyclases (OSC) in a similar manner to **1**.[1] Recent studies on the substrate analogs and the SHC enzymes altered by site-directed mutations have given new insight into the polycyclization mechanism.[1–4] It has been believed that acyclic molecule **1** is folded into an all *pre*-chair conformation inside the enzyme cavity and that the C- and D-rings are formed by anti-Markovnikov closure (Scheme 1).[1] However, the involvement of a ring expansion process has recently been disclosed with respect to the D-ring formation.[4,5] Such a ring expansion reaction also occurs in the C-ring formation by lanosterol synthase.[6,7] X-ray analyses of *Alicyclobacillus acidocaldarius* SHC have been reported and the active sites were suggested,[8,9] but the hypotheses presented still remain inconclusive because of insufficient point mutation experiments. To date, a few mutation experiments targeted at the active sites have been reported and have revealed that the aromatic residues of Trp 169, 312 and 489[4,10,11] together with acidic Asp residues 376 and 377[12] were crucial to the catalysis. In a series of point mutation experiments targeted for aromatic amino acids,[11] we have suggested that Trp169 may reside close to the C-18 methyl of the Markovnikov-type intermediate (the dammarene skeleton).[4] Very recently, Merkofer *et al.* have reported the GC pattern of the altered products by mutated F601A SHC,[13] but the structures of the altered products have not been determined, other than that of the 17-*epi*-dammarene. We have independently investigated the function of Phe601 with the protocol of site-directed mutagenesis.[13] We report here the structures of the two unidentified compounds produced by mutant F601A, which are composed of a 6/6/5-fused tricyclic skeleton, and propose the new cyclization mechanism that ring expansion of the 5-membered C-ring intermediate occurs during the C-ring construction of **2**, based on a trapping experiment of the tricyclic carbocation intermediate by using a substrate analog with a highly nucleophilic hydroxyl group.

Incubation of **1** (100 mg) with a cell-free homogenate of mutant F601A, that had been prepared from a 4-L culture, at 60°C and pH 6.0 for 16 h, afforded three partially cyclized products **8**, **9** and **15** in yields of 3.5, 2.2 and 20.0 mg, respectively, together with normal products **2** (39.8 mg), **3** (4.2 mg) and recovered starting material **1** (13 mg). Purification was carried out by SiO_2

Scheme 1



1 **2** **3**

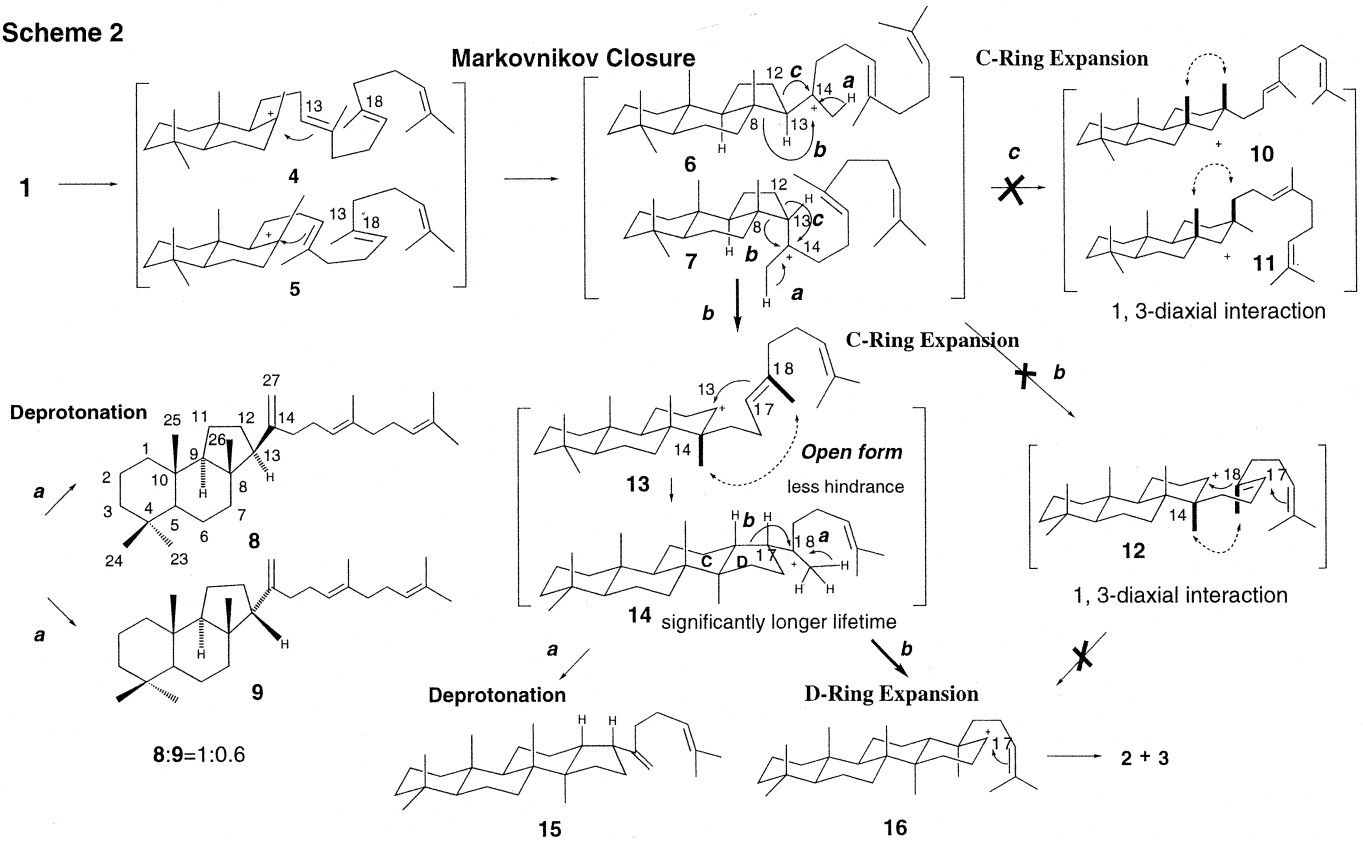
* To whom correspondence should be addressed. Fax: +81-25-262-6854; E-mail: hoshitsu@agr.niigata-u.ac.jp

Abbreviations: SHC, squalene-hopene cyclase; OSC, oxidosqualene cyclase

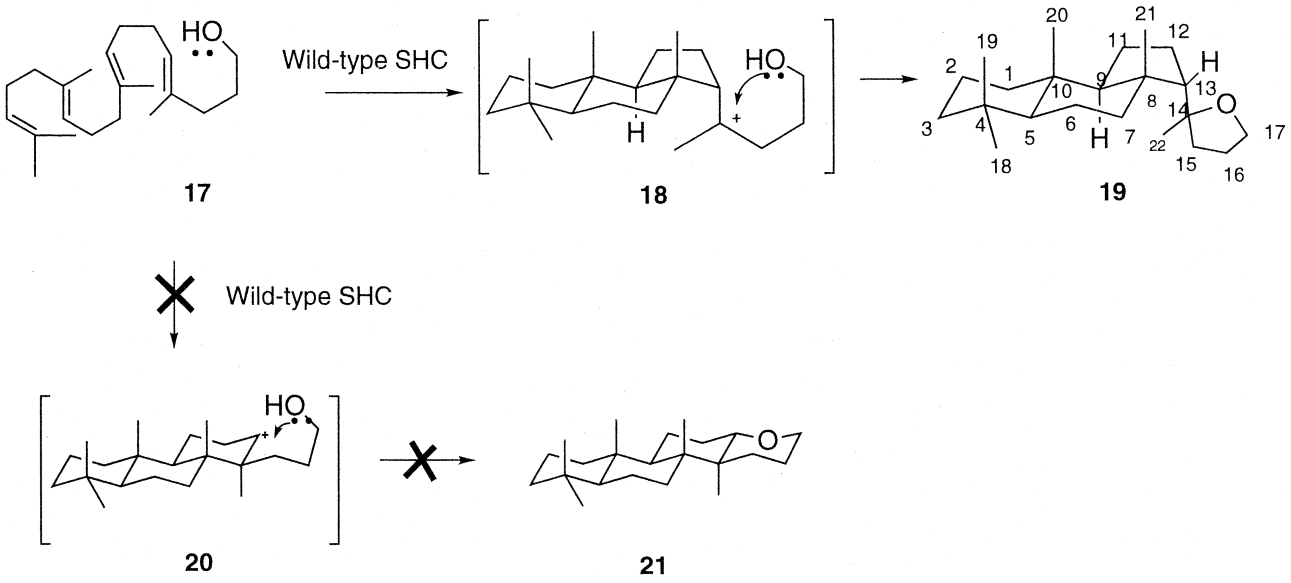
column chromatography, eluting with hexane, and separation of **8** and **9** was accomplished by C_{18} reversed phase HPLC (THF:H_2O=6:4). The structures of these metabolites were determined by a detailed analyses of 2D NMR data, including ^1H–^1H COSY 45, HOHAHA, NOESY, HMQC and HMBC, to be the tricyclic molecules for **8** and **9**,[14] and the tetracyclic molecule for **15**.[4] The 6/6/5-fused tricyclic compounds have never previously been reported as SHC enzymatic products. No premature products having mono- and/or bicyclic skeletons could be detected. The accumulated amounts of **8** and **9** were less than that of **15**; the ratio of (**8**+**9**) to **15** being 1 to 3.5, which was further confirmed by a GC analysis. However, tricyclic **8** and **9**, which were significantly enhanced by this mutation, should be taken into account in the polycyclization mechanism. Concomitant accumulation of tricyclic **8** and **9** together with tetracyclic 17-*epi*-dammarene **15** strongly suggests that the Phe601 residue is well-enough aligned within the enzyme cavity to participate in the construction of the 6/6-fused C/D ring system. Tricyclic **8** and **9** indicate the existence of carbocation intermediates **6** and **7**, which could be formed in accordance with a Markovnikov closure after the B-ring formation has been completed. Cationic intermediates **6** and **7** could be subjected to the deprotonation reaction from the methyl at C-14 to give **8** and **9** (path *a*), but they could also, by an alternative path (*b*), undergo further cyclization to give **15** through the ring expansion process of 5-membered C-ring intermediate **6** or **7** into **13** (Scheme 2). If cyclization proceeds *via* **12** to directly give cation **16**, greater steric repulsion (1,3-diaxial interaction between each methyl at C-14 and C-18 of **12**) would occur; thus, less-hindered open conformation **13** could be arranged to give **15** *via* **14**.[4] The ring expansion process (path *b*) of tricyclic **6** or **7** competes with the deprotonation reaction (path *a*). An alternative path (*c*) for the C-ring expansion step, which does not lead to the formation of natural **2**, could be presumed, but greater steric repulsion due to 1,3-diaxial interaction would also occur between each methyl at C-8 and C-13 in **10**, or between the methyl at C-8 and the isoprenoid chain at C-13 in **11**. Thus, the path (*c*) is unlikely. Phe601 may be arranged at the appropriate position surrounding C-13 and C-14 of tricyclic **6** and **7** or between C-13 and C-18 of tetracyclic **13**. Computational studies have revealed that equilibrium between the tertiary cation (Markovnikov closure, **6** or **7**) and the nearly isoenergetic secondary cation (anti-Markovnikov closure, **13**) can be readily shifted by selective positioning of a nucleophilic group,[15] supporting the feasibility of such a ring-expansion process. The aromatic quadrupole of Phe601 would correspond to the nucleophile. The formation of **15** further suggests that this residue may also be responsible for the ring expansion reaction from **14** to **16**, if it is localized at a favorable position around C-17 and C-18 in **14**. Wendt *et al.* have suggested, on the basis of an X-ray analysis, that Phe601 might be situated only at the C-18 carbocation of **14**,[9] but the isolation of **8**, **9** and **15** in this experiment strongly suggests that this aromatic amino acid also stabilizes the C-14 cation in tricyclic intermediates **6** and **7**, in addition to the 18-cation in **14**.

The kinetic data for the wild-type and the F601A mutated SHCs in the formation of **2**, which were deter-

Scheme 2



Scheme 3



mined from Lineweaver-Burk plots at 30°C, were as follows: K_ms, 16.7 and 1215 μM; and V_{max}s, 0.097 and 0.29 nmol min^{-1} μg^{-1}, respectively for the wild-type and F601A. The K_m values were markedly increased by this mutation. The looser binding to the active site, which is responsible for construction of the 6/6-fused C/D ring system, may have afforded a mixture of diastereomers **8** and **9** *via* improperly folded intermediate **4** or **5**. Either **6** or **7** may be a true intermediate for the ring expansion process. We have previously succeeded in trapping intermediate **14** by using squalene analog C27-OH having a highly nucleophilic nature.[4] Substrate analog C22-OH **17** was prepared to validate the assumption of the ring expansion of the 5-membered C-ring in **6** or **7**. Compound **17** was synthesized by subjecting **1** to the epoxidation reaction in CH_2Cl_2 with *m*-chloroperbenzoic acid to yield three regio-isomers of 2,3-, 6,7- and 10,11-oxidosqualene. These were treated with H_5IO_6 to give a mixture of the three corresponding aldehydes, and one of them, the C22-aldehyde, being isolated by column chromatography (C_{18} reversed phase) with CH_3CN:H_2O (8:2) and then reduced with $LiAlH_4$ to obtain the desired **17**. Incubation of **17** with wild-type SHC afforded **19**[14] with an isolation yield of 32%, together with recovered **17** (55%), without trapping any other products (Scheme 3). The formation of **19** strongly suggests that the polycyclization reaction proceeds *via* tricyclic cation **18** (like **7**) to afford the tetrahydrofuran ring as a result of the nucleophilic attack by the hydroxyl group on the cation **18**. Compound **21**, produced *via* anti-Markovnikov intermediate **20**, was not detected in the incubation mixtures. This trapping experiment unambiguously supports the idea that the polycyclization of squalene proceeds *via* discrete meta-stable 6/6/5-fused tricyclic C-14 cation **7** prior to the ring expansion and the further cyclization and that the cyclization reaction does not proceed in such a manner, *i.e. via* anti-Markovnikov closure, as shown in Scheme 1.

In conclusion, we propose a new cyclization mechanism by which the formation of 6-membered C- and D-rings, anti-Markovnikov adducts, in hopene biosynthesis occurs as a result of two ring expansion steps of the 5-membered C- and D-rings which have heen produced in advance by Markovnikov closure, and that Phe601 would facilitate the C/D ring expansion reaction through cation-π interaction.[16]

Acknowledgment

This work was supported in part by a grant-aid to T.H. (Nos. 09660111 and 11660104) from the Ministry of Education, Science and Culture of Japan.

References

1) Abe, I., Rohmer, M., and Prestwich, G. D., Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. *Chem. Rev.*, **93**, 2189–2206 (1993).
2) Robustell, B., Abe, I., and Prestwich, G. D., Synthesis and enzymatic cyclization of (3*S*)11-fluoro-2,3-oxidosqualene. *Tetrahedron Lett.*, **39**, 957–960 (1998).
3) Hoshino, T. and Kondo, T., The cyclization mechanism of squalene in hopene biosynthesis: the terminal methyl groups are critical to the correct folding of this substrate both for the formation of the five-membered E-ring and for the initiation of the polycyclization reaction. *J. Chem. Soc. Chem. Commun.*, 731–732 (1999).
4) Sato, T., Abe, T., and Hoshino, T., On the cyclization mechanism of squalene: a ring expansion process of the five-membered D-ring intermediate. *J. Chem. Soc. Chem. Commun.*, 2617–2618 (1998).
5) Pale-Grosdemange, C., Feil, C., Rohmer, M., and Poralla, K., Occurrence of cationic intermediates and deficient control during the enzymatic cyclization of squalene to hopanoids. *Angew. Chem. Int. Ed. Engl.*, **37**, 2237–2240 (1998).
6) Corey, E. J. and Cheng, H., Conversion of a C-20 2,3-oxidosqualene analog to tricyclic structures with a five-membered C-ring by lanosterol synthase. Further evidence for a C-ring expansion step in sterol biosynthesis. *Tetrahedron Lett.*, **37**, 2709–2712 (1996).
7) Hoshino, T. and Sakai, Y., Further evidence that the polycyclization reaction by oxidosqualene-lanosterol cyclase proceeds *via* a

ring expansion of the 5-membered C-ring formed by Markovnikov closure. On the enzymic products of the oxidosqualene analogue having an ethyl residue at the 15-position. *J. Chem. Soc. Chem. Commun.*, 1591–1592 (1998).

8) Wendt, K. U., Poralla, K., and Schulz, G. E., Structure and function of a squalene cyclase. *Science*, **277**, 1811–1815 (1997).

9) Wendt, K. U., Lenhart, A., and Schulz, G. E., The structure of the membrane protein squalene-hopene cyclase at 2.0 Å resolution. *J. Mol. Biol.*, **286**, 175–187 (1999).

10) Sato, T., Kanai, Y., and Hoshino, T., Overexpression of squalene-hopene cyclase by the pET vector in *Escherichia coli* and first identification of tryptophan and aspartic acid residues in the QW motif as active sites. *Biosci. Biotechnol. Biochem.*, **62**, 407–411 (1998).

11) Sato, T. and Hoshino, T., Kinetic studies on the function of all the conserved tryptophans involved inside and outside the QW motifs of squalene-hopene cyclase: stabilizing effect of the protein structure against thermal denaturation. *Biosci. Biotechnol. Biochem.*, **63**, 1171–1180 (1999).

12) Feil, C., Sussmuth, R., Jung, G., and Poralla, K., Site-directed mutagenesis of putative active-site residues in squalene-hopene cyclase. *Eur. J. Biochem.*, **242**, 51–55 (1996).

13) Merkofer, T., Pale-Grosdemange, C., Wendt, K. U., Rohmer, M., and Poralla, K., Altered product pattern of a squalene-hopene cyclase by mutagenesis of active site residues. *Tetrahedron Lett.*, **40**, 2121–2124 (1999). The structures of tricyclic products **8** and **9** have not been determined in ref. 13. We have independently reported the function of Phe601, described here, at the annual meeting of Japan Society for Biosci., Biotechnol., and Agrochem. March, 1999, Fukuoka, Japan. Abstract p. 306. The GC pattern of mutant F601A, which was constructed by us, was the same as that in the ref. 13.

14) All the HRMS(EI) and NMR spectra were consistent with the proposed structures of of **8**, **9** and **19**. Selected NMR data measured in C_6D_6 (600.13 MHz for ^{1}H- and 150.917 MHz for ^{13}C-NMR) are presented. For **8**: δ_H (ppm), H-1 (0.95, 1.52), H-2 (1.48, 1.63), H-3 (1.31, 1.52), H-5 (0.85, dd, J=12.3, 2.3 Hz), H-6 (1.29, 1.62), H-7 (1.29, 1.92), H-9 (1.30), H-11 (1.48, 1.57), H-12 (1.82, 1.95), H-13 (2.19, t, J=9.8 Hz), H-23 (1.02, s), H-24 (0.98, s), H-25 (0.963, s), H-26 (0.851, s), H-27 (5.04, s, 5.22, s); δ_C (ppm), C-1 (40.25), C-2 (19.82), C-3 (42.90), C-4 (33.16), C-5 (57.56), C-6 (19.81), C-7 (41.47), C-8 (43.99), C-9 (63.35), C-10 (37.39), C-11 (18.77), C-12 (25.81), C-13 (57.56), C-14 (149.27), C-23 (33.7), C-24 (21.5), C-25 (15.61), C-26 (15.37), C-27 (110.6). For **9**: δ_H (ppm), H-1 (1.10, 1.58), H-2 (1.58, 1.78), H-3 (1.26, 1.48), H-5 (0.94, dd, J=12.1, 2.2 Hz), H-6 (1.50, 1.65), H-7 (1.43, 1.71), H-9 (1.65), H-11 (1.52, 1.65), H-12 (2.16, 1.86), H-13 (2.33, t, J=9.6 Hz), H-23 (0.947, s), H-24 (0.972, s), H-25 (0.996, s), H-26 (1.098, s), H-27 (4.91, s, 5.17, s); δ_C (ppm), C-1 (40.79), C-2 (18.82), C-3 (42.71), C-4 (33.12), C-5 (57.31), C-6 (19.65), C-7 (37.09), C-8 (45.87), C-9 (55.95), C-10 (37.13), C-11 (21.07), C-12 (28.16), C-13 (57.04), C-14 (154.61), C-23 (33.60), C-24 (21.59), C-25 (15.78), C-26 (25.01), C-27 (109.35). Strong NOE correlation between H-13 and H-9 in **8** shows that the isoprenoid side chain of **8** was in an *exo*-orientation, while no NOE was observed between them in **9**, indicating the *endo*-orientation of the side chain. For **19** (data around the THF moiety): δ_H (ppm), H-9 (1.58), H-12 (2.01, 2.14), H-13 (1.93, dd, J=4.2, 9.4 Hz), H-15 (1.58, 1.74), H-16 (1.72, 2H, m), H-17 (3.76, 2H, m), H-21 (1.07, s), H-22 (1.31, s); δ_C (ppm), C-8 (44.38), C-9 (59.61), C-10 (37.45), C-12 (25.19), C-13 (60.12), C-14 (85.35), C-15 (38.30), C-16 (26.63), C-17 (65.98), C-21 (26.63), C-22 (24.50). No NOE between H-13 and H-9 suggests the *endo*-orientation for the THF moiety.

15) Jenson, C. and Jorgensen, W. L., Computational investigations of carbenium ion reactions relevant to sterol biosynthesis. *J. Am. Chem. Soc.*, **119**, 10846–10854 (1997).

16) Dougherty, D. A., Cation-π interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science*, **271**, 163–168 (1996).