On the cyclization mechanism of squalene: a ring expansion process of the five-membered D-ring intermediate

Tsutomu Sato,[a] Takamasa Abe[b] and Tsutomu Hoshino*[ab]

[a] *Graduate School of Science and Technology, and*
[b] *Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Ikarashi, Niigata 950-2181, Japan. E-mail: hoshitsu @agr.niigata-u.ac.jp*

Received (in Cambridge, UK) 7th September 1998, Accepted 26th October 1998

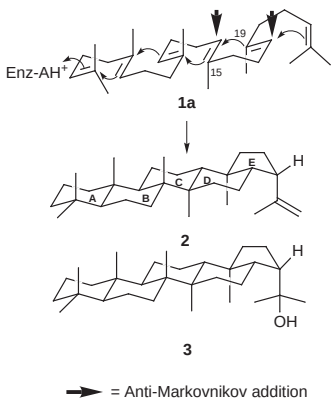
Site-directed mutagenesis experiments with W169F, W169H and W489F for the squalene-hopene cyclase, and the formation of 10 possessing the five-membered D-ring and a tetrahydrofuran moiety as the enzyme product of the analogue 8 with a hydroxy group, strongly suggest that a ring expansion reaction from the five- to the six-membered ring is responsible for the D-ring formation of hopene.

The cyclization of squalene **1** into pentacyclic triterpenes, hop-22(29)-ene **2** and hopan-22-ol **3**, is an outstanding reaction from the point of view of both stereo- and regio-chemical specificity, with the formation of five new carbon–carbon bonds and nine new chiral centers.[1] Oxidosqualene also undergoes the polyolefin cyclization analogous to squalene.[1] Recent progress on the two cyclases of squalene and oxidosqualene has spurred mechanistic studies of the polycyclization reactions. Squalene-hopene cyclase (SHC) is believed to fold the linear molecule **1** into an all pre-chair conformation **1a** inside the enzyme cavity, leading to **2** and **3** through the generation of a series of carbocation intermediates (Scheme 1).[2] Scheme 1 also shows that the C- and D-rings are formed by anti-Markovnikov closures. Site-directed mutagenesis experiments of SHC revealed that both D-376 and D-377 were crucial for the catalysis.[3] Recently, an X-ray analysis of *Alicyclobacillus acidocaldarius* SHC has been reported.[4] We have independently succeeded in an overexpression of the SHC and reported the first identification of the tryptophan residues 169 and 489 as components of the active sites; substitution of these tryptophans with valine and leucine by point mutations resulted in complete loss of the enzyme activity.[5] Here, we report that mutants of W169F, W169H and W489F produce the normal cyclization products **2** and **3** together with an abnormal tetracyclic product **4** consisting of a 6/6/6/5-fused ring system, the formation of **4** being in agreement with the Markovnikov rule. This finding leads us to propose that a ring expansion reaction is involved in the D-ring formation of **2** and **3**.

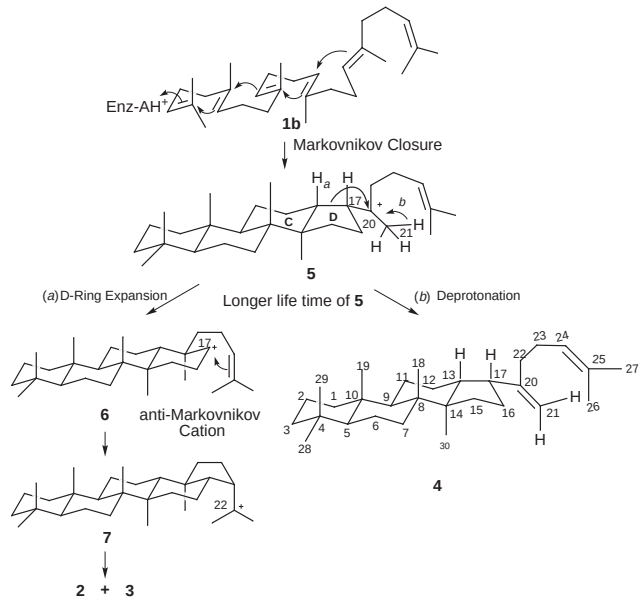
Cell-free homogenates of the mutants prepared by site-directed mutagenesis were incubated with **1** at optimal temperatures (45 °C for W169F and W169H, and 53 °C for W489F). The wild-type has a catalytic optimum at 60 °C and pH 6.0.[3,5] A large scale incubation of **1** (150 mg) for 16 h with a cell-free extract from a 6 l culture of W169F afforded **2**, **3** and **4** (an oil) (100.5, 10 and 6 mg, respectively) after the separation with a SiO_2 column (hexane–EtOAc). The incubation of other mutants conducted with the same quantities of **1** and cell-free extracts as for W169F gave the following isolated yields: for mutant W169H: **2** (73.5 mg), **3** (6.3 mg) and **4** (33.0 mg), and for mutant W489F: **2** (36.8 mg), **3** (3.3 mg) and **4** (9.8 mg). Detailed 2D NMR analyses[6] revealed that **4** had a dammarene skeleton with an exomethylene group, but the 17-side chain had an α-orientation (17-*epi*-dammarene). No other product was detected in the reaction mixtures except for recovered **1**.

Concomitant production of **4** together with the two normal products **2** and **3** indicates that a common intermediate **5** is being produced during the polyolefin cyclization process (Scheme 2). Formation of the dammarene cation **5** having a 6/6/6/5-fused ring system is thermodynamically favored by Markovnikov control. Proton elimination from the methyl group would give **4** [path (*b*)], while the ring expansion process from a five- to six-membered D-ring would give the hopanyl C22-cation **7** *via* a disfavored anti-Markovnikov C-17 cation **6** [path (*a*)], the latter being formed after the ring expansion of **5** has been processed. The ring expansion competes with the deprotonation. Steric factors also favor the formation of **5**; given that the cyclization reaction proceeds by adopting a pre-chair conformation **1a** for the D-ring construction, greater repulsion would occur due to the 1, 3-diaxial arrangement between the two methyls at the 15- and 19-positions (squalene numbering), thus resulting in the less hindered conformation **1b**.

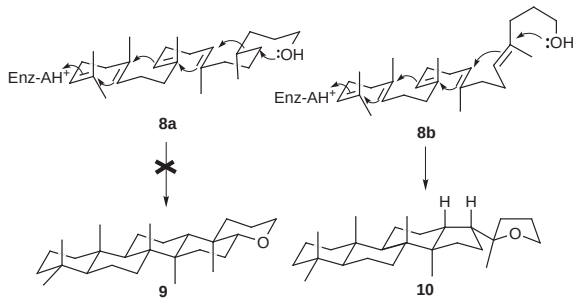
A similar ring expansion has been proposed for the C-ring formation in lanosterol biosynthesis, based on the trapping of



Scheme 1



Scheme 2



Scheme 3

the five-membered C-ring intermediate (a 6/6/5-fused ring) from incubation experiments with substrate analogues.[7] Incubation of the squalene analogue **8** (C_{27}-OH), prepared *via* treatment of H_5IO_6 with 2,3-oxidosqualene followed by reduction with $LiAlH_4$, with the wild-type SHC afforded **10**[6] almost quantitatively (Scheme 3). Compound **9** and other products were not detected. Formation of **10** strongly supported the suggestion that the cyclization reaction proceeded *via* the prefolded **8b** (like **1b**), but not through **8a** (like **1a**), and also gave unequivocal evidence for the involvement of a discrete metastable C-20 carbocation intermediate like **5** prior to the ring expansion and further cyclization; the hydroxy group would have attacked the tertiary C-20 cation thus produced due to its highly nucleophilic nature, resulting in the formation of a tetrahydrofuran ring in **10**. A dammarene cation similar to **5** was postulated for the cyclization mechanism of 2,3-dihydrosqualene[8] and 29-methylidene-2,3-oxidosqualene[9] by SHC.

Since the mutants of W169V and W489L were completely inactive,[5] it appears that the tight binding to **1** comes from the aromatic ring residue, not from the hydrophobic aliphatic residues of SHC. To date, cation–π interactions induced by aromatic moieties, resulting in the carbocation stabilization, have been proposed for the catalysis and/or acceleration of the polycyclization reaction.[10] Kinetic values for the mutants were compared with that of the wild-type.[11] For the mutant W169F, K_m increased 17-fold, but V_{max} remained unchanged. On the other hand, for the mutant W489F, K_m increased 5.5-fold, but V_{max} was only 14% of the wild type. These kinetic results imply that the W169 would bind to **1** rather than stabilizing the carbocation, while W489 may exhibit both binding and cation stabilization, and also suggest that the higher electron density of the π-electrons, the greater affinity to **1**. The looser binding of the phenylalanine or histidine residues to **1**, near the D-ring, in the mutant SHCs would lead to the longer lifetime of **5**, as inferred from the thermodynamic and steric preferences. Compared to W169F, W169H significantly increased the amount of **4** 5.5-fold; the histidine residue may abstract a proton from the 21-methyl [path (*b*)], indicating that the position of W169 in the cavity may possibly be close to the 21-methyl of **5**, but further evidence is required to confirm this.

Notes and references

1 I. Abe, M. Rohmer and G. D. Prestwich, *Chem. Rev*., 1993, **93**, 2189.
2 G. Ourisson, M. Rohmer and K. Poralla, *Annu. Rev. Microbiol*., 1987, **41**, 301.
3 C. Feil, R. Sussmuth, G. Jung and K. Poralla, *Eur. J. Biochem*., 1996, **242**, 51.
4 K. U. Wendt, K. Poralla and G. E. Schulz, *Science*, 1997, **277**, 1811.
5 T. Sato, Y. Kanai and T. Hoshino, *Biosci. Biotechnol. Biochem*., 1998, **62**, 407.
6 All the HRMS (EI) and NMR (H-H COSY 45, HOHAHA, NOESY, DEPT, HMQC and HMBC) spectra were consistent with the proposed structures of **4** and **10**.
7 E. J. Corey and H. Cheng, *Tetrahedron Lett*., 1996, **37**, 2709; E. J. Corey, S. C. Virgil, H. Cheng, C. H. Baker, S. P. T. Matsuda, V. Singh and S. Sarshar, *J. Am. Chem. Soc*., 1995, **117**, 11 819; T. Hoshino and Y. Sakai, *Chem. Commun*., 1998, 1591.
8 I. Abe and M. Rohmer, *J. Chem. Soc., Perkin Trans. 1*, 1994, 783.
9 I. Abe, T. Dang, Y. F. Zheng, B. A. Madden, C. Fei, K. Poralla and G. D. Prestwich, *J. Am. Chem. Soc*., 1997, **119**, 11 333.
10 K. Poralla, *Bioorg. Med. Chem. Lett*., 1994, **4**, 285; I. Abe and G. D. Prestwich, *Proc. Natl. Acad. Sci. U.S.A*., 1995, **92**, 9274; D. A. Dougherty, *Science*, 1996, **271**, 163.
11 The mutations gave a lowering of the optimal temperature, but no change with pH. Reactions with 5 μg of purified SHC were conducted at 30 °C and pH 6.0 for 1 h; thermal denaturation of the SHCs was not found. The kinetic values of K_m and V_{max} were determined from Lineweaver–Burk plots as follows: K_ms: 16.7, 277, 280 and 92 μM; and V_{max}s: 0.09, 0.078, 0.045 and 0.017 nmol min^{-1} μg^{-1}, respectively, for the wild-type, W169F, W169H and W489F.

Communication 8/06948D