

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

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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.

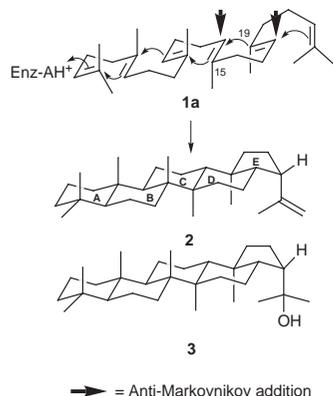
The cyclization of squalene **1** into pentacyclic triterpenes, hop-22(29)-ene **2** and hopan-22-ol **3**, is an outstanding reaction from the point of view of both stereo- and regio-chemical specificity, with the formation of five new carbon-carbon bonds and nine new chiral centers.¹ Oxidosqualene 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

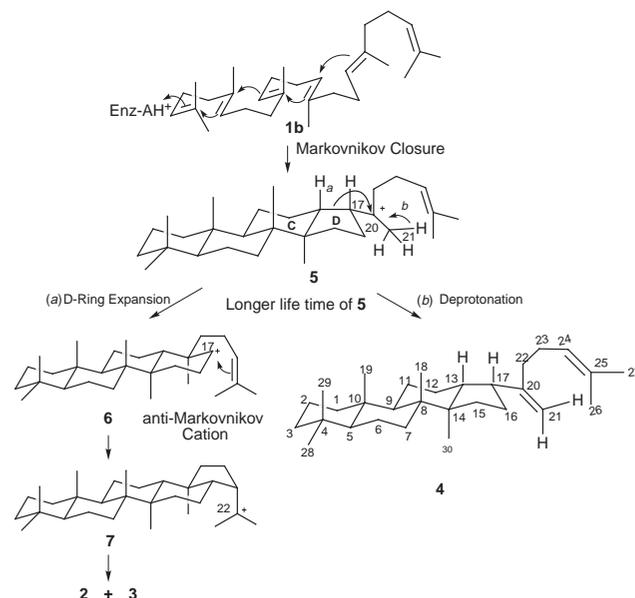
W489F). The wild-type has a catalytic optimum at 60 °C and pH 6.0.^{3,5} A large scale incubation of **1** (150 mg) for 16 h with a cell-free extract from a 6 l culture of W169F afforded **2**, **3** and **4** (an oil) (100.5, 10 and 6 mg, respectively) after the separation with a SiO₂ column (hexane-EtOAc). 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 analyses⁶ 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**.

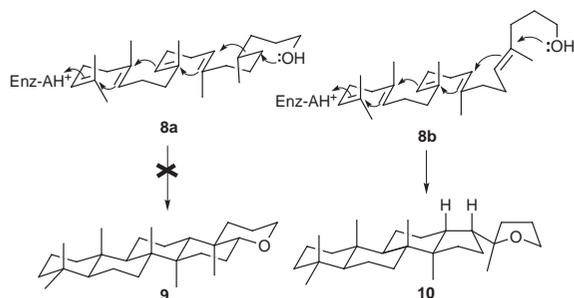
A similar ring expansion has been proposed for the C-ring formation in lanosterol biosynthesis, based on the trapping of



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** (C₂₇-OH), prepared *via* treatment of H₂IO₆ with 2,3-oxidosqualene followed by reduction with LiAlH₄, 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-dihydrosqualene⁸ and 29-methylidene-2,3-oxidosqualene⁹ 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

- I. Abe, M. Rohmer and G. D. Prestwich, *Chem. Rev.*, 1993, **93**, 2189.
- G. Ourisson, M. Rohmer and K. Poralla, *Annu. Rev. Microbiol.*, 1987, **41**, 301.
- C. Feil, R. Sussmuth, G. Jung and K. Poralla, *Eur. J. Biochem.*, 1996, **242**, 51.
- K. U. Wendt, K. Poralla and G. E. Schulz, *Science*, 1997, **277**, 1811.
- T. Sato, Y. Kanai and T. Hoshino, *Biosci. Biotechnol. Biochem.*, 1998, **62**, 407.
- 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**.
- 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.
- I. Abe and M. Rohmer, *J. Chem. Soc., Perkin Trans. 1*, 1994, 783.
- 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.
- 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.
- The mutations gave a lowering of the optimal temperature, but no change with pH. Reactions with 5 μ g 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_m s: 16.7, 277, 280 and 92 μ M; and V_{max} s: 0.09, 0.078, 0.045 and 0.017 nmol min⁻¹ μ g⁻¹, respectively, for the wild-type, W169F, W169H and W489F.

Communication 8/06948D