Cyclization

Deletion of the Gly600 Residue of *Alicyclobacillus acidocaldarius* Squalene Cyclase Alters the Substrate Specificity into that of the Eukaryotic-Type Cyclase Specific to (3*S*)-2,3-Oxidosqualene**

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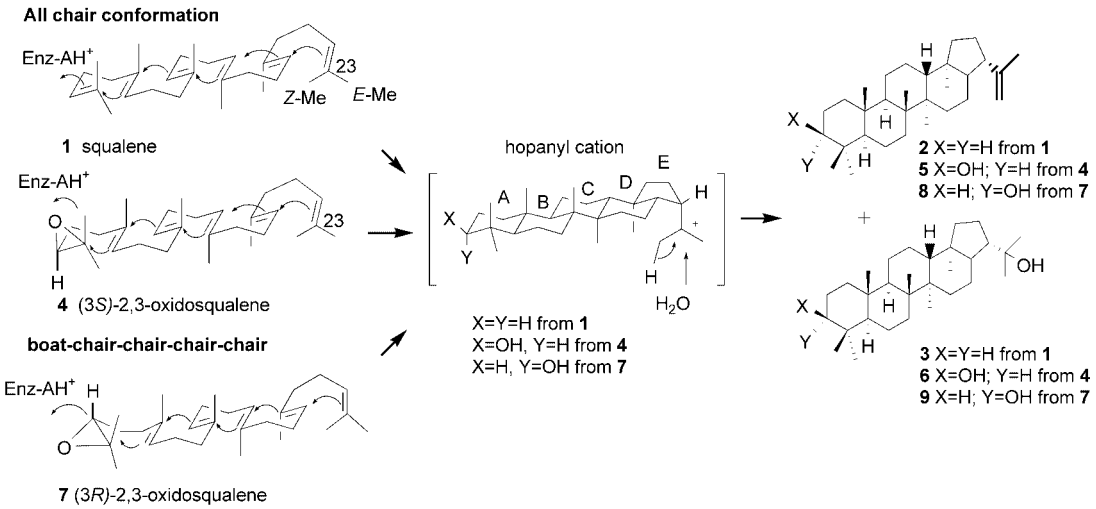
The acyclic molecule squalene (**1**) is cyclized into the pentacyclic triterpenes, hop-22(29)-ene (**2**) and hopan-22-ol (**3**; ca 5:1) by squalene–hopene cyclase (SHC) from prokaryotic species (Scheme 1).[1] The polycyclization reaction proceeds with regio- and stereochemical specificity under precise enzyme control to form the 6/6/6/6/5-fused pentacyclic ring system and nine new stereocenters. The polycyclization mechanism is analogous to that of eukaryotic oxidosqualene cyclases (OSCs).[1] SHC folds **1** into the all pre-chair conformation (a product-like conformation) inside the enzyme cavity, which leads to the final hopanyl cation through sequential ring-forming reactions. The proton elimination occurs exclusively from (23*Z*)-Me, but not from the *E*-Me, to form **2**.[1a,2a] A nucleophilic attack by a water molecule on the cation affords **3**.

Recent three-dimensional X-ray crystallographic analyses[3] and site-directed mutagenesis experiments[1a,b] have provided deeper insights into the polycyclization mechanism. Studies with the substrate analogues have also given important information on substrate recognition.[1,2] The reaction cavity is lined with aromatic amino acid residues.[3] Substrate specificity of bacterial SHCs is remarkably broad. Compounds (3*S*)-**4** and (3*R*)-oxidosqualenes **7** undergo the polycyclization reactions to form 3β- and 3α-hydroxyhopenes (**5** and **8**; ca. 1:1) and hopanediols (**6**:**9**; ca. 1:1), respectively, by a similar cyclization mechanism as **1**. Formation of the A-ring of **5** (scheme 1) proceeds by the chair conformation, while that of **8** by the boat structure.[2a]

In contrast to bacterial SHCs, eukaryotic OSCs selectively recognize **4** as the substrate, and never accept **1** and **7**.[1] Figure 1 shows a comparison of the amino acid alignment of some SHCs and OSCs. Gly600 is highly conserved among SHCs, but absent in OSCs.[1a,b,4] To investigate the role of the G600 from *Alicyclobacillus acidocaldarius*, we constructed the Gly600-deletion mutant (ΔG600 SHC) and then incubated with **1** or a racemic mixture of **4** and **7**. Interestingly, **1** and **7** were completely inert to the mutated cyclase (< 0.2 % detection limit), while **4** underwent the cyclization to mono- and tricyclic skeletons which have a 3β-hydroxy group. It is of note that the prokaryotic squalene cyclase could be altered into eukaryotic-type one, the substrate of which is limited to **4**.

The ΔG600 SHC was constructed by using the polymerase chain reaction (PCR), in which oligonucleotide primers were designed in inverted tail-to-tail directions to amplify the cloning vector together with the target sequence.[5] One mg each of **1** or the racemic mixture of **4** and **7** was incubated at 40–70 °C for 16 h with 1 mL of the cell-free homogenates (ca. 200 μg of the pure mutated SHC). The reaction was quenched by adding 15 % KOH/MeOH and the lipophilic products were extracted with hexane. The GC analysis showed that no cyclization products were present in the incubation mixture from **1** (< 0.2 % detection limit). On the other hand, six major products were detected in that of the racemate (**4** and **7**; total yield, 32 %). The optimum catalytic



Scheme 1. Polycyclization pathway of squalene (**1**), (3*S*)-**4**, and (3*R*)-oxidosqualene (**7**) into the hopane skeleton.

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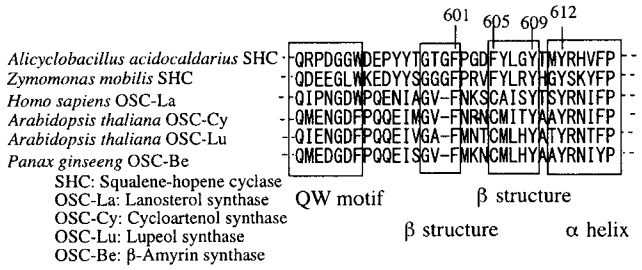


Figure 1. Amino acid alignment of some triterpene cyclases. The G598–Y605 alignment of *A. acidocakdarius* SHC corresponds to the G694–C700 sequence of human OSC, and these sequences constitute β-sheet domains.

temperature was 45 °C.[6] The distribution ratios [%] of products **10**–**15** (Figure 2) was determined to be 11.3:2.9:2.9:3.4:8.6:2.9 and there was 68.0 % recovered substrate, by the GC analysis. Products **10** and **13**–**15** were isolated in a pure state by the combination of normal and reverse-phase HPLCs, but separation of **11** and **12** was unsuccessful; column chromatography on SiO_2 impregnated with 5 % $AgNO_3$ gave no separation. A mixture of acetates of **11** and **12**, prepared with Ac_2O/py, (Ac = acetyl, py = pyridine) was subjected to a careful column chromatography on SiO_2 (5 % $AgNO_3$) which lead to the successful separation of **11** and **12** in part, but a large portion was still mixed.

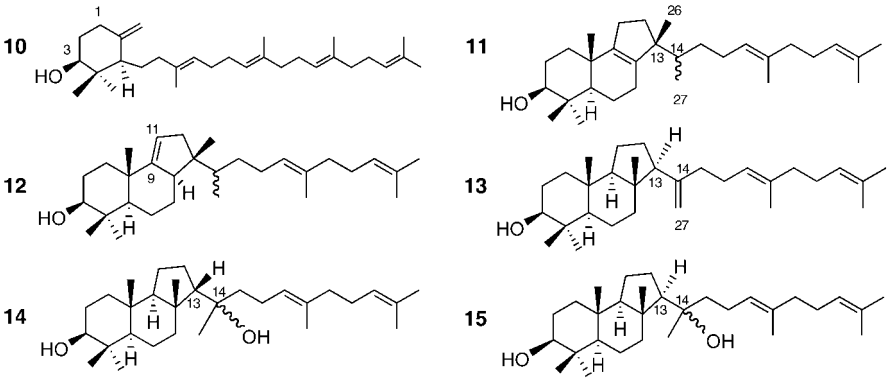
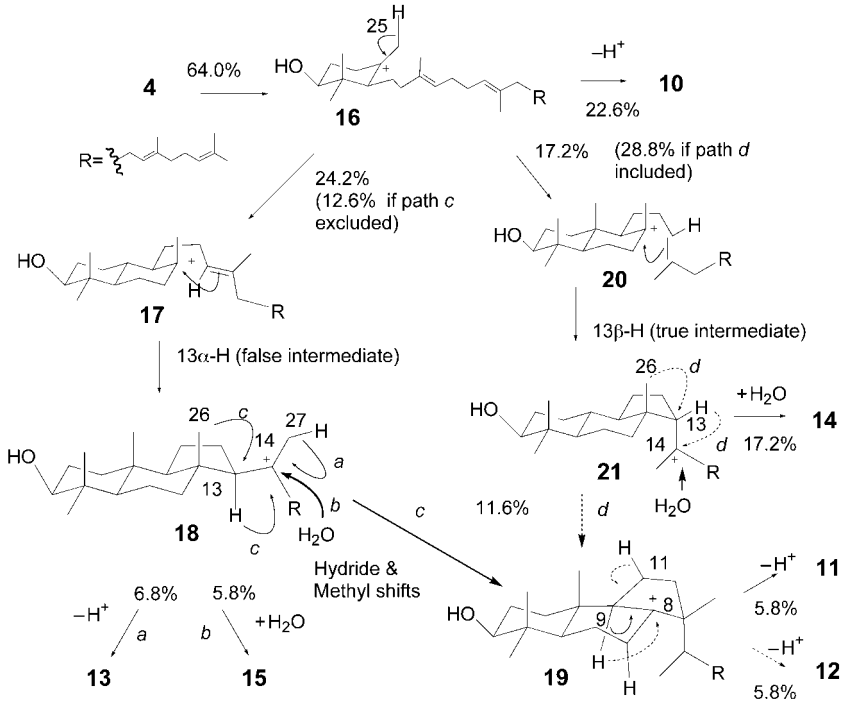


Figure 2. Cyclization products of **4** by the ΔG600 mutant.

Structures of all the isolated products as shown in Figure 2 were determined by NMR spectroscopy (^1H, ^{13}C, DEPT, COSY 45, HOHAHA, NOESY, HMQC, HMBC).[7] In particular, the H-3 signals of all the products were dd (J = 10–12 and 5–6 Hz), characteristic of axial–axial and axial–equatorial spin–spin couplings, thus all the products have a 3β-hydroxy group. Product **10** was identified as achilleol.[8a] Products **11**–**15** have three allyl methyl groups (δ_H = 1.69–1.88 ppm), which suggests the involvement of a tricyclic ring skeleton. Products **11** and **12** have a podiodatriene skeleton[8b] (podioda-8,17,21-trien-3β-ol for **11** and podioda-9(11)-17,21-trien-3β-ol for **12**). Compound **11** was recently isolated as an *Arabidopsis* OSC product, and was named thalianol.[8c] Compound **12** is a novel tricyclic triterpene. Product **13** is malabarica-14(27),17,21-trien-3β-ol. Diols **14** and **15** have a similar skeleton to **13** bearing a second hydroxy group at C-14. The NOESY spectra of **13**–**15** clearly indicated that the stereochemistry at C-13 of **13** and **15** is opposite to that of **14** (13α-H for **13** and **15**; 13β-H for **14**). Production of 3β-hyroxylated skeletons from the racemic mixture, but no formation of 3α-hydroxylated one, indicated that only **4** underwent cyclization, while the enantiomer **7** was unaffected.[2] The recovered epoxysqualene, further purified by normal-phase HPLC (hexane:2-PrOH = 100:2), showed an $[\alpha]_D^{25} = +1.17$ (c = 0.84, EtOH), further verifying that 3*S*-(−)-**4** was selectively converted.[9] Thus, the conversion of **4** by the mutant cyclase was 64 %.

Scheme 2 shows the mechanisms for the formation of products **10**–**15**. (3*S*)-Oxidosqualene **4** is cyclized into monocyclic cation **16**, then deprotonation from Me-25 yields **10**. Formation of bicyclic cations **17** and **20** guides further cyclization, leading to the 6/6/5-fused tricyclic Markovnikov cations **18** and **21**, respectively. The deprotonation from Me-27 of **18** affords **13** (path *a*). Attack by water on the C-14 cation gives diol **15** (path *b*). Sequential 1,2-shift reactions of hydride and Me-26 of **18** give cation **19** (path *c*). Deprotonation at C-9 of **19** gives **11** and hydride shift of H-9 to C-8 and the proton elimination of H-11 affords **12**. The



Scheme 2. Proposed polycyclization pathways of **4** into **10**–**15**; see text for details.

alternative route via **21** (path *d*) may be possible, but path *c* would be favorable owing to the antiparallel rearrangement reaction. A water attack on cation **21** gives **14**. Previously we have demonstrated that **21** (13β-H) is a true intermediate, but **18** (13α-H) is a false one.[1a, 10a]

The lack of reaction of **1** and **7** suggests that the terminal double bond of **1** and the epoxide ring of **7** may have failed to be in a close proximity to the D374XD376D377 motif,[10b] which is responsible for the initiation of the polycyclization reaction. The specific activity of the mutant enzyme for **4** significantly decreased (ca. tenfold),[6] compared to that of the native SHC, which suggests the looser binding of the epoxide ring of **4** to the mutant enzyme than to the wild-type. The secondary structures of the β-sheet and α-helix domains[3, 11] (Figure 1) may be disorganized by the loss of the G600, resulting in inappropriate positioning of F601,[10a] F605,[10c] Y609,[10d,e] and Y612[10d,e] in the reaction cavity, which are crucial for the sequential ring-forming reactions to give the fully cyclized **2**. Residues Y609 and Y612 are important in forming 6/6-fused bicyclic cations **17** and/or **20**,[10d,e] thus monocycle **10** may have accumulated owing to the inappropriate positions of these residues. However, the Phe365, aligned prior to the G600, would still be correctly placed in the reaction cavity, which leads to the formation of **18** and **21** by the stabilization of cations **17** and **20**.[12] The incorrect placement of the F601 and F605 residues, which are essential for the formation of tetra- and pentacycles,[10, 12] would interrupt the polycyclization reaction at the tricyclic ring stage, possibly because of the decreased cation/π interaction. A high production (24.2 %) of the false intermediate **18** (13α-H)[1a, 10a] further indicated that the enzyme structure was disordered in part.

There are a few reports on the altered substrate specificity of the squalene cyclase. A triple mutant of D377C/V380E/V381A,[13] a double mutant of D376C/C435S,[14] and the single mutants[10b, 15] of D374N and D376N had no effect on **1**, but all the mutants had a cyclization activity for both enantiomers of **4** and **7**, albeit with significantly lower activity than the wild-type, which indicates that all the mutants had no stereospecificity for the racemic epoxides. The decreased acidity of the DXDD motif by the substitution with Cys or Asn would have led to no reaction for **1** and to a lowered activity for **4** and **7**. In contrast, the ΔG600 mutant selectively accepts **4**, but has no effect on **7**. Thus, the altered substrate specificity of the ΔG600 mutant is completely different from those of the previously reported mutants. The following differences are also noted: The ΔG600 mutant still had the original DXDD motif, and the location of the deleted G600 residue in the reaction cavity is distal from the initiation site of the DXDD motif.[3] The X-ray structure of the wild-type SHC, which was cocrystallized with 2-azasqualene,[3c] shows that the G600 is located in a close proximity to the D-ring formation site of **1**.

Why did the ΔG600 SHC accept only **4**? It is difficult to get a clear answer to this question at the present time, but one possible explanation is as follows. The recent study of the homology modeling of human OSC,[16] based on the crystal structure information of the wild-type SHC,[3] clearly indicates that the β-sheet domain of the OSC, consisting of the G694–C700 sequence (Figure 1), is more loosely packed compared to that of the SHC, which is composed of the G598–Y605 sequence.[16] Thus, the loss of the Gly600 makes the D-ring formation region less compact. The loosely packed domain structure of the mutant cyclase is more flexible, thus, a somewhat free motion of the substrate molecules can be allowed around the D-ring formation sites. The unconstrained motion of the substrates will influence the positions of the substrate heads; the epoxide ring of **4** could be located near to the DXDD motif, but the terminal double bond of **1** and the epoxide ring of **7** failed to have access to the motif. The proposed working hypothesis would agree with the previous reports[10e, 17] that the folding conformation and the polycyclization pathway of **1** are directed by the steric bulk size of active sites. However, other factors may be also involved in the alteration of substrate specificity. Further studies are necessary including the X-ray crystallographic analysis and the functional analyses of the related mutants, in order to gain the exact answer for the altered substrate specificity.

In conclusion, this is the first example in which the substrate specificity of prokaryotic cyclase could be successfully altered into that of the eukaryotic-type, which is quite interesting from the evolutionary aspect of squalene cyclases. This study is also indicates how eukaryotic cyclases selectively accept **4**. The rationally engineered mutagenesis of bacterial SHC will lead to further creation of novel eukaryotic-type cyclases.

Experimental Section

A plasmid of pBH, which was a pUC119 derivative having the *Bam* HI-*Hind* III fragment of *shc* gene (1623–1896 nucleotide position),[10b,d] was used as a template. A pair of primers, 5′-TTCCCAGGGGATTTCTACCTCGGC-3′ and 5′-***G****GTAC****C***GGTG-TAGTACGGCTCATCCCAGC-3′, was prepared for deleting a GGG codon of Gly600. The bold of **G** and **A** indicate the silent mutation for creating *Kpn* I site (italics).

The enzyme purification and incubation conditions were carried out according to the published methods.[10b,d]

The hexane extract from the incubation mixture was subjected to column chromatography on SiO_2 with a step-wise gradient elution of hexane→hexane/EtOAc(100:10), to give residual oxidosqualene and **10** in a pure state, but the separation of **11**–**15** failed. Isolation of **14** and **15** was by normal-phase HPLC (hexane:2-PrOH = 100:2). A reverse-phase HPLC eluting with THF/H_2O (55:45) gave **13** in pure state. As a next step, the acetate mixture of **11** and **12**, prepared with Ac_2O/py, was subjected to a careful column chromatography on SiO_2 (5 % $AgNO_3$) by eluting with hexane/EtOAc (100:0.05), leading to the separation of **11** and **12** in part.

NMR spectra were recorded in C_6D_6, the chemical shifts being relative to the solvent peak $\delta_H = 7.28$ and $\delta_C = 128.0$ ppm as the internal reference for ^1H and ^{13}C NMR spectra, respectively. The product distribution pattern was monitored by GC. GC-MS and MS spectra were obtained with electronic impact at 70 eV using a DB-1 capillary column (0.32 mm × 30 m), the oven temperature being elevated from 220 to 270 °C (3 °C min^{-1}). Specific rotation values were measured at 25 °C in EtOH. The CD spectra of the ΔG600 and the wild-type SHCs were measured in a solution of 10 mM sodium phosphate (pH 6.0) containing 0.6 % Brij 35.

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