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Functional Analysis of the DXDDTA Motif in Squalene-Hopene Cyclase by Site-directed Mutagenesis Experiments: Initiation Site of the Polycyclization Reaction and Stabilization Site of the Carbocation Intermediate of the Initially Cyclized A-Ring

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In order to clarify the function of the DXDDTA motif in squalene-hopene cyclase and to identify the acidic amino acid residues crucial for the catalysis, site-directed mutagenesis experiments were carried out. The following results were found: (1) residues D374 and D376 work for the initiation of polyolefin cyclization which arises from the proton attack on the terminal double bond; (2) residue D377 stabilizes C-10 carbocation of the initially cyclized A-ring intermediate, leading to subsequent B-ring closure, which was further verified by isolating the partially cyclized monocyclic product; (3) residues D313 and D447 outside the DXDDTA motif were identified as new active sites; (4)the H451 residue is likely to work in the protonated form to enhance the acidity of the carboxyl groups of D374 and/or D376.

Key words: squalene; hopene; DXDDTA motif; *Alicyclobacillus acidocaldarius*; oxidosqualene

The conversion of squalene **1** into pentacyclic hopene **2** and hopanol **3** is one of the most complicated biochemical reactions (Scheme 1).[1] This reaction is catalyzed by squalene-hopene cyclase (SHC)[EC 5.4.99-] from prokaryotic bacterium species such as *Alicyclobacillus acidocaldarius*,[2] *Zymomonas mobilis*,[3] and *Bradyrhizobium japonicum*.[4] Polycyclization proceeds with precise enzymatic control to form five new rings and nine new chiral centers. This reaction is analogous to that by oxidosqualene-lanosterol cyclases (OSLCs) [EC 5.4.99.7] from eukaryoates which catalyze the reaction of (3*S*)-2,3-oxidosqualene **4** to lanosterol **5** (Scheme 2). SHCs also convert unnatural substrate **4** to 3β-hydroxyhopene **6** and 3β-hydroxyhopanol **7** (Scheme 2).[1] Recent progress in the molecular biology of the two cyclases has disclosed a similarity in the primary structures.[2–14] A comparison of SHCs and OSLCs has shown a 20–26% identity in amino acid sequences, and both of them contain specific amino acid sequence repeats, the so-called QW motif.[15] The investigations of substrate analogs have given new insight into the polycyclization mechanism.[1,16–18] It has been believed that the cyclization of **1** proceeds by adopting all *pre*chair conformation **1b**. Very recently, it has been suggested that substrate **1** is folded in conformation **1a**, and not **1b**, (Scheme 1) to give a true intermediate of the 5-membered D-ring (17-*epi*dammarene cation, Markovnikov closure), which then undergoes a ring-expansion step resulting in the formation of the 6-membered D-ring (anti-Markovnikov adduct).[19] Site-directed mutagenesis,[19–25] affinity labeling,[25–27] and X-ray analyses[28,29] have also spurred on the identification and functional analyses of the active components. The cyclization of **1** and **4** into **2** and **5** is initiated by a proton attack on the terminal double bond and the oxirane ring, respectively, and polycyclization progressively proceeds through the generation of a series of carbocation intermediates. The acidic residues of aspartic and/or glutamic acids are likely to work as proton donors for the initiation of cyclization. The carboxylate anion is also likely to stabilize the discrete carbocation intermediates during the polycyclization process, that is, to work as negative point charges, as proposed by Johnson *et al.*[30] Shi *et al.* have indicated that aromatic residues may work to stabilize the transient carbocation, because aromatic amino acids are unusually abundant in cloned OSLCs.[9] Later, Dougherty proposed concept of the cation-π interaction for the polyolefin cyclization reaction; that is, the transient cations are stabilized through π-electrons of the aromatic rings of Phe, Tyr and Trp.[31] Poralla has suggested that Trp residues of the QW motifs would correspond to the negative point charges and would work as active sites.[15] More recently, an X-ray analysis of the SHC from *A. acidocaldarius*, has been reported, revealing that the QW motifs were located in the surface zone of the protein, and thus cannot interact with the substrate.[28] We have recently reported site-directed mutations targeted for all the conserved Trp residues, verifying that all the QW motifs were not active sites, whereas Trp169, Trp312 and Trp489 located outside the QW motifs were active components.[19–21]

Abe and Prestwich have previously proposed that the Asp residue in the VXDCTA motif of rat OSLC would stabilize the C20-protosteryl cation on the basis of an affinity labeling experiment using the suicide substrate,

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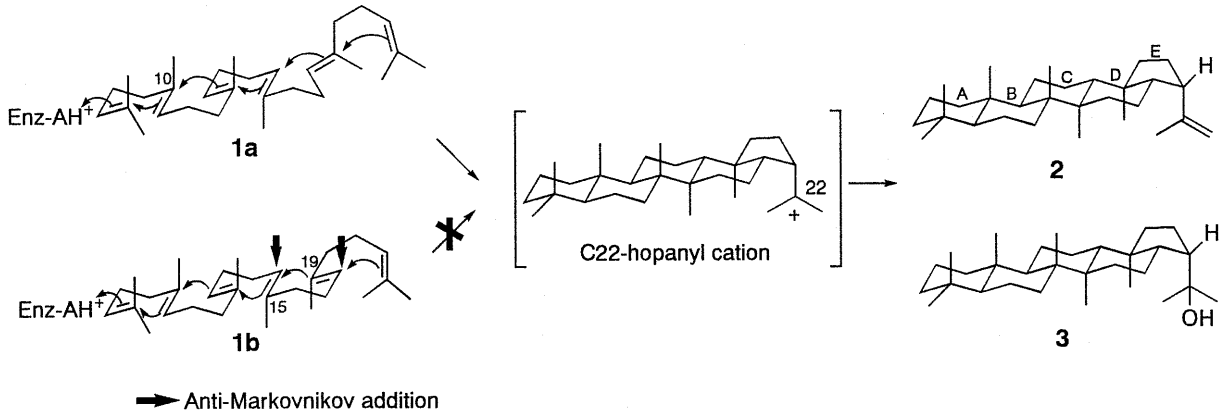
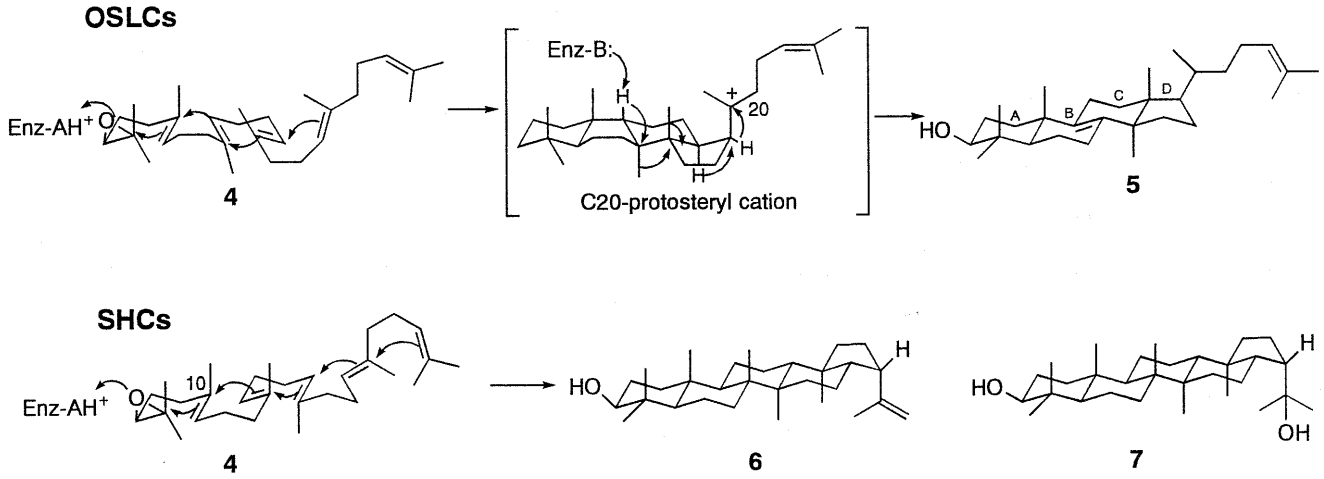
Abbreviations: SHC, squalene-hopene cyclase; OSLC, oxidosqualene-lanosterol cyclase; GC, gas chromatography; TLC, thin-layer chromatography; CD, circular dichroism



Scheme 1. Cyclization Mechanism for Squalene **1** into Hopene **2** and Hopanol **3** by SHC.
The production ratio of **2** and **3** was *ca.* 5:1.



Scheme 2. Proposed Cyclization Mechanism for (3*S*)-2,3-Oxidosqualene **4** into Lanosterol **5** by OSLC and for **4** into 3β-Hyroxyhopene **6** and 3β-Hyroxyhopanol **7** by SHC.
The production ratio of **6** and **7** is *ca.* 5:1.

29-methylidene-2,3-oxidosqualene.[26,27] Poralla's group has presumed that the DXDDTA motif of SHCs would stabilize the C22-hopanyl cation, based on the homologous alignment between the two cyclases of SHC and OSLC.[22] The protosteryl and hopanyl carbocations are formed at the final stage of the polycyclization. Corey *et al.* have recently assigned the protonation function, which triggers the cyclization reaction, for the VXDCTA motif from *Saccharomyces cerevisiae* OSLC, this idea being inferred from point mutation experiments on the highly conserved acidic amino acid residues among all the known OSLCs.[24] Corey's hypothesis is in contrast with that proposed by Abe, Prestwich and Poralla's group; the former is the *initiation* of cyclization, whereas the latter is the *stabilization* effect of the final carbocation intermediate.

On the basis of an X-ray crystallographic analysis of SHC, Wendt *et al.* assumed that the DXDDTA motif would be responsible for the proton attack to initiate polycyclization and that the acidity would be enhanced by the basic function of His451.[28] However, the experimental evidence was not sufficient to propose the exact function of the DXDDTA motif in the SHC. Until now, only two mutations of Asp376 and 377 in the DXDDTA motif have been reported.[22] The roles of Asp374 and Thr378 in the motif are still unknown, and site-directed mutagenesis experiments are urgently required to justify the hypothesis suggested by Wendt *et al.*[28] To confirm whether their suggestion is correct and to examine whether other acidic amino acid(s) are responsible for the catalysis, we carried out site-directed mutagenesis experiments on all the acidic residues of Asp and Glu, which are highly conserved among known SHCs. Point mutation experiments would give valuable and complementary information about functions of this motif in conjunction with the X-ray analysis.

In this paper, we describe experimental evidence for the role of the DXDDTA motif. The proton-donor for the initiation of cyclization actually originates from Asp374 and 376, while the Asp377 residue stabilizes the C-10 carbocation intermediate. The role of Asp377 is unequivocally demonstrated by isolating the monocyclic skeletal compound from an incubation mixture of **1** with D377N or D377C, which has not previously been

reported as an SHC enzymic product. The Thr378 residue will be shown not have any effect on the cyclase activity, while the His451 residue will be revealed to play a significant role at the protonation stage. The two residues of Asp 313 and 447 are found as new active sites in our point mutation experiments.

Materials and Methods

Instruments. ^{1}H- and ^{13}C-NMR spectra were measured with a Bruker DPX 400 instrument, MS spectra with a JEOL SX 100 mass spectrometer, and CD spectra with a JASCO J-725 instrument. GC analyses were carried out by a Shimadzu GC-8A instrument with a DB-1 capillary column (0.53 mm × 30 m).

Regular methods. Detailed experimental protocols have been reported in the previous papers[19-21] for the overexpression systems with the pET vector in *Escherichia coli* BL21(DE3), DNA sequence analyses and enzyme purification methods.

Site-directed mutagenesis. All site-directed mutagenesis experiments were performed as described in the previous papers.[19-21] The following primers were used:

E45Q, 5′-pd[GAGGACGTA*CTG*CGCTTCCATGGTGACGTTGCTCAG]-3′ (*Nco* I)
E197Q, 5′-pd[GCGCGCGTGCCC*CAG*CTGTACGAGACC]-3′ (*Pvu* II)
D374E, 5′-pd[CGTGTACTACCCG*GAG*GTGGACGACACTGCAGTCGTGGTG]-3′ (*Pst* I)
D374I, 5′-pd[CGTGTACTACCCG*ATC*GTGGACGACACTGCAGTCGTGGTG]-3′ (*Pst* I)
D374V, 5′-pd[CTACCCG*GTC*GTGGACGACACTGCAGTCGTG]-3′ (*Pst* I)
D374N, 5′-pd[CGTGTACTACCCG*AAC*GTGGACGACACTGCAGTCGTGGTG]-3′ (*Pst* I)
D376N, 5′-pd[CGGACGTG*AAC*GACACTGCAGTCGTGG]-3′ (*Pst* I)
D377C, 5′-pd[GGACGTGGAC*TGC*ACTGCAGTCGTGGTG]-3′ (*Pst* I)
D377N, 5′-pd[GGACGTGGAC*AAC*ACTGCAGTCGTGGTGTG]-3′ (*Pst* I)
D374V/D376N, 5′-pd[CTACCCG*GTC*GTG*AAC*GACACTGCAGTCGTG]-3′ (*Pst* I)
D374V/D377C, 5′-pd[CGTGTACTACCCG*GTC*GTGGAC*TGC*ACTGCAGTCGTGGTG]-3′ (*Pst* I)
D376N/D377C, 5′-pd[CCGGACGTG*AACTGC*ACTGCAGTCGTGGTG]-3′ (*Pst* I)
T378A, 5′-pd[CGTGGACGAC*GCT*GCAGTCGTGGTGTGG]-3′ (*Pst* I)
D421N, 5′-pd[GCTCGTGTTGTCCAC*GTT*GTAGGCGCCC]-3′ (*Sal* I)
D442N, 5′-pd[GACGGCGG*ATT*GGTCACCTCGCCGAAGTC]-3′ (*Eco*O65 I)
D447N, 5′-pd[CACTCGAGCACATGGGCGGTGAC*GTT*CTCTGACGGC]-3′ (*Pma*C I)
H451F, 5′-pd[GCCGAAACACTCCAGCAC*GAA*GGCGGTGACGTCC]-3′ (*Xho* I)
H451R, 5′-pd[GAAACACTCTAGCAC*GCG*GGCGGTGAC]-3′ (*Xho* I)
D530N, 5′-pd[CGCCCCAGCCAC*GTT*TGGGTTCTGATGC]-3′ (*Pfl*M I)
E535Q, 5′-pd[CGGCAGTC*CTG*GCCCCAGCCACCGTCTGGGTTCTGATGC]-3′ (*Pfl*M I)

The bold letters designate the altered bases, and the target mutations are shown by the italic letters. The underlined letters show the silent mutations for easy screening of the desired mutants by a restriction fragment analysis. The created or deleted restriction sites are shown in parentheses. The mutants of D313N and D350N have already been constructed as described in the previous paper.[20]

Thermal stability and kinetic analysis. A mixed solution, which was composed of 0.5 mM squalene, 0.2% Triton X-100 and 5 μg of the homogeneously purified enzyme in a sodium citrate buffer (60 mM, pH 6.0), was prepared in a final volume of 5 ml for the enzyme reactions. Incubation was carried out for 60 min at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, or 70°C) to examine the thermal stability of the cyclases. To determine the kinetic parameters, the incubation was conducted for 60 min at 30°C or 60°C. To terminate the reaction, 15% methanolic KOH (6 ml) was added. The lipophilic enzymatic products (**2** and **3**) and starting material **1**, which remained unchanged, were extracted four times with hexane (5 ml) from the incubation mixtures, and the amount of **2** thus produced was evaluated by GC analyses with a DB-1 capillary column (30 m in length). Kinetic parameters K_m and k_{cat} were determined according to the Lineweaver-Burk equation.

Isolation and spectroscopic data for 8 and 9 (Achilleol A). The *E. coli* transformants, in which the mutated SHCs of D377C and D377N were expressed, were cultured at 30°C for 20 h in a Luria-Bertani medium (6 l) containing 50 mg/l of ampicillin. The cells were collected by centrifugation and washed twice with a Tris-HCl buffer (pH 8.0, 50 mM). To the collected pellets, 300 ml of the Tris buffer supplemented with 1% Triton X-100 (w/v) was added. Cell-free homogenates were prepared with an ultrasonicator at 4°C and then centrifuged again to remove the cell debris. The pH value of the supernatant was adjusted to 6.0 by adding 0.5 M citric acid. Squalene **1** (50 mg) was incubated with the homogenates at 60°C for 16 h, and the enzymatic reaction was terminated by adding 15% methanolic KOH (450 ml). The products were extracted three times with hexane (750 ml) from the incubation mixtures, and remaining **1** was detected in a small amount by TLC. **8** had a lower R_f value than **2**, but higher than **1**. SiO_2 column chromatography, eluting with hexane, gave the following isolation yields for D377C: **2** (3.8 mg) and **8** (42.2 mg); and for D377N: **2** (15.4 mg) and **8** (30.9 mg). The retention time of compound **8** by the GC was identical to that of **1**.

Selected NMR data (in C_6D_6) for the monocyclic ring of **8** as δ_H values: H-1 (1.98, m; 2.03, m), H-2 (1.51, 2H, m), H-3 (1.18 m; 1.45 m), H-5 (1.67, m), H-7 (1.39 m; 1.54 m), H-8 (1.71 m; 1.90 m), H-23 (0.89, 3H, s), H-24 (0.81, 3H, s), H-25 (4.52 bs, 4.73 bs); and other protons of the isoprenoid side chain: ~5.1 (4H, m, olefinic protons), ~2.0 (12H, m, $6\times CH_2$), 1.57 (3H, s), 1.58 (9H, s, $3\times$Me) and 1.66 (3H, s). δ_C values around the monocyclic skeleton: C-1 (32.51), C-2 (23.73), C-3 (36.33), C-4 (34.87), C-5 (53.58), C-6 (149.39), C-7 (24.76), C-8 (38.22), C-23 (28.43), C-24 (26.25), C-25 (108.80), and other carbons of the side chain; 135.7 (s), 135.08 (s), 134.89 (s), 131.25 (s), 124.40 (d), 124.32 (d), 124.26 (d), 124.01 (d), 39.76 (t), 39.72 (t), 28.29 (t), 28.25 (t), 26.75 (t), 26.66 (d), 25.69 (q), 17.68 (q), 16.12 (q), 16.06 (q), 15.99 (q). EI-HRMS m/z (M^+): calcd. for $C_{30}H_{50}$, 410.3913; found, 410.3883.

Compound **4** (75 mg) was incubated under nearly the same conditions as those used for **1** with cell-free extracts of mutants D377C and D377N and purified by SiO_2 column chromatography, giving *ca.* 25 mg and *ca.* 20 mg of **9**, respectively, as isolated yields. Selected NMR data (in $CDCl_3$) for **9** (Achilleol A)[32] as δ_H values: H-1 (2.17, 2H, dd, J=10.0, 4.4 Hz), H-2 (1.79 m, 1.50 m), H-3 (3.64, 1H, dd, J=10.2, 4.4 Hz), H-5 (1.58, 1H, t), 23-Me (0.954, 3H, s), 24-Me (0.866, 3H, s), 25-CH_2 (4.77, 1H, s; 4.56, 1H, s); and as δ_C values: C-1(29.30), C-2 (31.49), C-3 (73.95), C-4 (38.99), C-5 (51.10), C-6 (147.49), C-25 (110.54). The relative stereochemistry was confirmed by the NOESY spectrum; strong correlation was observed between H-3 and Me-23 and between H-3 and H-5. The coupling constant of H-3 established that the hydroxyl group was in β-orientation. Detailed NMR and MS data are shown in the literature.[32)]

Circular dichroism of the wild-type and the mutated SHCs. SHCs have been usually purified and assayed in a sodium citrate buffer (pH 6.0) containing 0.2% Triton X-100 (buffer A). The Triton X-100 detergent has strong absorption in the UV region, because of the involvement of its phenyl residue. Therefore, for CD measurements, we employed Brij 35 ($C_{12}E_{23}$) as an alternative detergent, instead of Triton X-100. When dissolved in buffer B [10 mM sodium phosphate (pH 6.0) containing 0.6% Brij 35], wild-type SHC was found to have equivalent activity (115%) to that in buffer A. The cell-free extract was prepared from cells grown in 200 ml of the medium, and was applied to a DEAE-Toyopearl 650 M column (Tohso) which had been previously equilibrated with buffer B. The wild-type and mutated SHCs were homogeneously purified through two steps of DEAE column chromatography: the enriched fraction of the SHCs, which had been prepared by the first chromatography with a linear gradient of a 10–50 mM sodium citrate buffer (pH 6.0) containing 0.6% Brij 35, was applied again to the column by eluting with a linear gradient of 0–0.3 M NaCl in buffer B to obtain the pure SHCs. The purity was checked by SDS-PAGE. The CD spectrum of the purified enzyme (0.1 mg/ml) in buffer B was measured at a wavelength between 200 and 250 nm across a 1-mm light path at 20°C.

Results and Discussion

Characterization of all the conserved acidic amino acid residues

At present, seven SHCs[2–5)] and five OSLCs[6–14)] have been cloned and sequenced. Figure 1 depicts the amino acid alignment of two SHCs from *A. acidocaldarius* (Gram-positive) and *Zymomonas mobilis* (Gram-negative), and of OSLC from *Homo sapiens*, as typical examples. In the SHC of *A. acidocaldarius*, nine Asp and three Glu residues were found (Fig. 1) as the conserved

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                              QW6                 45                                   QW5c
A. a.    1  MAEQLVEAPAYARTLDRAVEYLLSCQKDEGYWWGPLLSNVTMEAEYVLLCHILDR-VDRDRMEKIRRYLLHEQREDGTWALYPGGPPDLDTTIEAYVALKYIGMSRDEEPMQKALRFIQSQ 120
Z. m.    9  AFHHSPLSDDVEPIIQKATRALLEKQQQDGHWVFELEADATIPAEYILLKHYLGEPEDLEIEAKIGRYLRRIQGEHGGWSLFYGGDLDLSATVKAYFALKMIGDSPDAPHMLRARNEILAR 129
H. s.   63  YFKDLPKAHTAFEGALNGMTFYVGLQAEDGHWTGDYGGPLFLLPGLLITCHVARIPLPAGYREEIVRYLRSVQLPDGGWGLHIEDKSTVFGTALNYVSLRILGVGPDDPDLVRARNILHKK 183
                                                      169                        197
A. a.  121  GGIESSRVFTRMWLALVGEYPWEKVPMVPPEIMFLGKRMPLNIYEFGSWARATVVALSIVMSRQP-------VFPLPERARVPELYETDVPPRRR-GAKGGGGW-IFDALDRALHGYQKLS 232
Z. m.  130  GGAMRANVFTRIQLALFGAMSWEHVPQMPVELMLMPEWFPVHINKMAYWARTVLVPLLVLQALKP-------VARNRRGILVDELFVPDVLP-TLQESGDPIWRRFFSALDKVLHKVEPYW 242
H. s.  184  GGAVAIPSWGKFWLAVLNVYSWEGLNTLFPEMWLFPDWAPAHPSTLWCHCRQVYLPMSYCYAVRLSAAEDPLVQSLRQELYVEDFASIDWLAQRNNVAPDELYTPHSWLLRVVYALLNLYE 304
                       QW5b                                                   312, 313                          QW5a
A. a.  233  VHPFRRAAEIRALDWLLERQAGDGSWGGIQPPWFYALIALKILDMTQHPA---FI-KGWEGLELYGVELDYGGWMF--QASISPVWDTGLAVLALRAAG---LPADHDRLVKAGEWLLDRQ 344
Z. m.  243  PKNMRAKAIHSCVHFVTERLNGEDGLGAIYPAIANSVMMYDALGYPENHPERAIARRAVEKLMVLDGTEDQGDKEVYCQPCLSPIWDTALVAHAMLEVG---GDEAEKSAISALSWLKPQQ 360
H. s.  305  HHHSAHLRQRAVQKLYEHIVADDRFTKSISIGPISKTINMLVRWYVDGPASTAFQ-EHVSRIPDYLWMGLDGMKM--QGTNGSQIWDTAFAIQALLEAGGHHRPEFSSCLQKAHEFLRLSQ 422
              350          365     374-378                           QW4      420, 421                     442  447 451
A. a.  345  ITVP-GDWAVKRPNLKPGGFAFQFDNVYYPDVDDTAVVVWALN-TLRLPD--ERRR-RDAMTKGFRWIVGMQSSNGGWGAYDVDN-TSDLPNHIPFCDFGEVT-DPPSEDVTAHVLECFGS 458
Z. m.  361  ILDVKGDWAWRRPDLRPGGWAFQYRNDYYPDVDDTAVVTMAMDRAAKLSD--LHDDFEESKARAMEWTIGMQSDNGGWGAFDANNS-YTYLNNIPFADHGALL-DPPTVDVSARCVSMMAQ 477
H. s.  423  VPDNPPDYQKYYRQMRKGGFSFSTLDCGWIVSDCTAEALKAVLLLQEKCPHVTEHIPRERLCDAVAVLLNMRNPDGGFATYETKRGGHLLELLNPSEVFGDIMIDYTYVECTSAVMQALKY 543
                        QW3       489                                        QW2  530  535
A. a.  459  FG------YDDAWKVIRRA-VEYLKREQKPDGSWFGRWGVNYLYGTGAVVSALKAVGIDTRE----PYIQKALDWVEQHQNPDGGWGEDCRSYEDPAYAGK---GASTPSQTAWALMALIA 565
Z. m.  478  AG-------ISITDPKMKAAVDYLLKEQEEDGSWFGRWGVNYIYGTWSALCALNVAALPHDH----LAVQKAVAWLKTIQNEDGGWGENCDSYA-LDYSG-YEPMDSTASQTAWALLGLMA 585
H. s.  544  FHKRFPEHRAAEIRETLTQGLEFCRRQQRADGSWEGSWGVCFTYGTWFGLEAFACMGQTYRDGTACAEVSRACDFLLSRQMADGGWGEDFESCEERRYLQS---AQSQIHNTCWAMMGLMA 661
                     QW1         601
A. a.  566  GGRAESEAARRGVQYLVETQRPDGGWDEPYYTGTGFPGDFYLGYTMYRHVFPTLALGRYKQAIERR                                                     631
Z. m.  586  VGEANSEAVTKGINWLAQNQDEEGLWKEDYYSGGGFPRVFYLRYHGYSKYFPLWALARYRNLKKANQPIVHYGM                                             659
H. s.  662  VRHPDIEAQERGVRCLLEKQLPNGDWPQENI-AGVFNKSCAISYTSYRNIFPIWALGRFSQLYPERALAGHP                                               732
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Fig. 1. Amino Acid Alignment of *Alicyclobacillus acidocaldarius* SHC (A.a.), *Zymomonas mobilis* SHC (Z.m.), *Homo sapiens* OSLC (H.s.). Target amino acid residues for the site-directed mutagenesis are shaded. The solid line shows the DXDDTA or VXDCTA motif. Dotted lines indicate the QW motifs. The active site residues of W169, W312, F365, Y420, W489, and F601, which were identified in the previous studies,[19–21,23,33,34)] are in bold type.

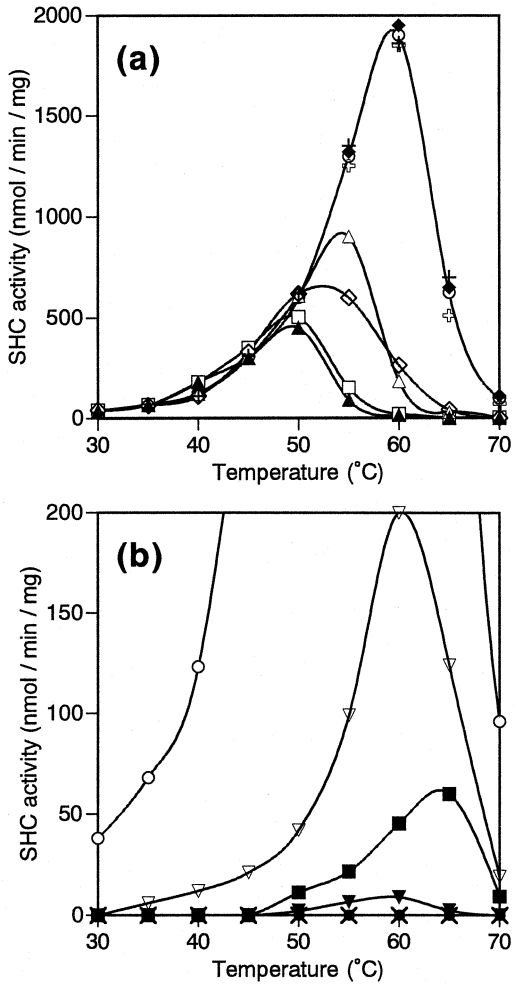


Fig. 2. Effect of Incubation Temperature on the Enzyme Activities of the Wild-type and Mutant SHCs targeted for All the Conserved Acidic Amino Acid Residues.

(a) represents the following enzymes: ○; Wild-type, ✣; E45Q, ◆; E197Q, □; D350N, △; D421N, ◇; D442N, +; D530N, ▲; E535Q. (b) shows the following enzymes: ○; Wild-type, ▽; D313N, ×; D374N, ●; D376N, ▼; D377N, ■; D447N.

acidic amino acids. Among these twelve residues, three residues of D374, D376 and D377 were situated in the DXDDTA motif and the two residues, D350 and D530, were in the QW motif. The remaining seven residues were E45, E197, D313, D421, D442, D447 and E535. As for acidic amino acids, two functions could be assumed for squalene cyclization: one is protonation toward the π-electron of the terminal double of **1** to initiate the cyclization, and the another one is stabilization of the cationic intermediates during cyclization through the carboxylate anion, this function having been previously proposed by Johnson *et al.*[30] To identify the functions of Asp and Glu inside and outside the DXDDTA motif, all of which are conserved among the known SHCs, *in vitro* mutagenesis experiments were carried out. The Asp and Glu residues were replaced by those of Asn and Gln, respectively, to remove the functions of both protonation and the carboxylate anion.

Figures 2a and 2b show the relationship between the incubation temperature and cyclase activity for the wild-type and altered SHCs. The results are classified into four categories: (1) E45Q, E197Q and D530N had the same activities and the optimal temperature (60°C) as those of the wild-type; (2) the four residues, D350, D421, D442 and E535, had more or less equivalent activities to each other, but showed lower specific activities and optimal temperatures than those of the wild-type at 50°C, 55°C, 53°C, and 50°C, respectively, for mutants D350N, D421N, D442N and E535Q; (3) mutants D313N, D377N and D447N had significantly lower activity at the overall incubation temperatures examined, while the optimal temperatures remained unchanged or higher than those of the wild-type at 60°C, 60°C and 65°C for the mutants D313N, D377N and D447N, respectively; (4) the cyclase activities of mutants D374N and D376N were completely quenched, despite the incubation temperature being varied.

Each incubation was conducted at pH 6.0 and 30°C and for 60 min to determine the kinetic data of k_{cat} and

Table 1. Kinetic Data and Optical Temperatures for the Wild-type and Mutant SHCs Targeted for All the Conserved Asp and Glu Residues (D→N and E→Q)

The kinetic values of K_m and k_{cat} were determined from Lineweaver-Burk plots. The enzyme activities were assayed by estimating the amount of **2** produced by incubating at 30°C for 60 min under which conditions the purified proteins were not denatured.

SHC	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m	Relative activity (%)	Optimal temperature for 60 min (°C)
Wild-type	1.67×10	6.43	3.85×10^{-1}	100.0	60
E45Q	1.69×10	6.22	3.68×10^{-1}	95.6	60
E197Q	1.64×10	6.43	3.92×10^{-1}	101.8	60
D313N*	—	—	—	0	60
D350N	1.69×10	6.35	3.76×10^{-1}	97.6	50
D374N*	—	—	—	0	—
D376N*	—	—	—	0	—
D377N*	—	—	—	0	60
D421N	1.67×10	6.58	3.94×10^{-1}	102.3	55
D442N	1.67×10	6.35	3.80×10^{-1}	98.8	53
D447N*	—	—	—	0	65
D530N	1.67×10	6.43	3.85×10^{-1}	100.0	60
E535Q	1.69×10	6.35	3.76×10^{-1}	97.6	50

* No activity was detected at the lower temperature of 30°C. Thus, the enzyme activities for these mutants were evaluated at the higher temperature of 60°C (see Table 2).

Table 2. Specific Activities and Kinetic Parameters of Mutant SHCs, measured at 60°C for 60 min
The enzyme activities were assayed by estimating the amount of **2** formed by the incubation of **1**.

SHC	Specific activity (nmol/min/mg)	Relative activity (%)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m	Relative activity (%)	Activity for substrate **4*****
Wild-type	1.91×10^3	100.0	1.67×10	2.89×10^2	1.73×10	100.0	+++
D313N	2.00×10^2	10.5	1.61×10	2.87×10	1.78	10.3	+++
D374N*	0	0	—	—	—	0	+
D376N*	0	0	—	—	—	0	++
D377N*,**	5.91	0.3	—	—	—	0	++
D447N	4.53×10	2.4	1.21×10	9.30	7.69×10^{-1}	4.5	+++

* The activity was so low that the kinetic values could not be determined.
** This mutant produced **8** together with **2**. The specific activity was determined by estimating **8** to be 12.0 nmol/min/mg.
*** The conversion ratios are roughly shown, which were determined by estimating the amount of **6** produced from **4** (0.5 mg) with 2 ml of the cell-free extract of each mutant (incubated at 60°C for 16 h). The symbols (+++, ++, and +) represent conversion ratios of 100%, 50–90%, and 20–49%, respectively. With the same enzyme amount, wild-type SHC completely converted **4** into **6** with a short incubation time of 60 min.

K_m, and the results, which were estimated from Lineweaver-Burk plots, are summarized in Table 1. Under the incubation conditions, no thermal denaturation was apparent for any of the mutants. The seven mutants, E45Q, E197Q, D350N, D421N, D442N, D530N and E535Q, which belong to categories (1) and (2) just described, had the same kinetic values of K_ms and k_{cat}s as those of the wild-type, verifying that the seven acidic residues were not active sites. The lower specific activities (Fig. 2a) would have been due to gradual denaturation of the protein during the progress of the cyclization reaction,[21] because high energy is released during this reaction.[28] Thus, the four residues, D350, D421, D442 and E535, would be responsible for reinforcing the enzyme structure (Fig. 2a), but not as catalytic sites. The K_m and k_{cat} values for mutants D313N, D377N and D447N, which are classified into the type (3), were not determined, because the activities at 30°C were too low to measure the cyclase activity (Fig. 2b, Table 1). At the elevated temperature of 60°C, the mutants had sufficiently higher activities to obtain the kinetic parameters (Fig. 2b and Table 2). The k_{cat} values for mutants D313N and D447N were very low at 9.9% and 3.2% of the wild-type values, respectively, whereas there was little changes in the K_m values, demonstrating these Asp residues to be components of the catalytic sites. The crucial role of D313 and D447 for the catalysis has never been reported. The kinetic data for mutants D374N, D376N and D377N, all of which were aligned in the DXDDTA motif, could not be measured due to the excessive loss of cyclase activity in the overall range of temperatures examined, verifying that the three residues, D374, D376 and D377, in the DXDDTA motif were critical to the cyclase activity (Tables 1 and 2).

CD spectra were measured to examine whether the protein structure was altered by the mutations, the six mutants being selected. As examples of those mutants which underwent remarkable loss of cyclase activity, the following five were selected: D374N, D376N and D377N, which are involved in the DXDDTA motif, and two other mutants of D313N and D447N, which are located outside the motif. The D421N mutant was selected as one that was labile to heat denaturation. The CD spectra of these mutants were superimposable to that of the wild-type (Fig. 3), indicating that no significant change in the enzyme structure had taken place by mutation.

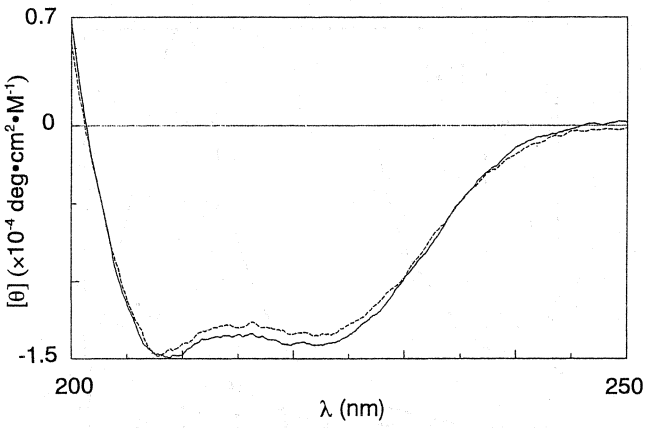


Fig. 3. CD Spectra for the Wild-type and Mutated D374N SHCs.
The solid line and dotted line show the wild-type SHC and mutant D374N, respectively. The CD spectra of D313N, D376N, D377N, D421N and D447N were almost identical to that of the wild-type.

Further mutations of the DXDDTA motif and H451

Further point mutation experiments were carried out in order to understand the DXDDTA motif of SHCs in detail, and to compare with the VXDCTA motif of OSLCs (Table 3). The D374 residue was replaced by Glu, Ile, or Val. The Val residue, corresponding to the D374 residue of SHC, is conserved in the VXDCTA motif of all known OSLCs. The mutations of D374 into Ile and Val resulted in the complete loss of enzyme activity, while mutant D374E still retained the activity, although it was only a little. This result indicates that the position at 374 must be acidic. The alteration of D377 into Cys, which is highly conserved in known OSLCs, resulted in remarkedly decreased activity. The point mutation experiments targeted for D374 and D377 revealed that this position must be an acidic amino acid for **1** to show catalytic activity; thus the doubly mutated SHCs for the DXDDTA motif had no activity (Table 3).

The Thr residue of the DXDDTA motif in SHCs is also conserved in the VXDCTA motif of OSLCs. We presumed that the hydroxyl group of the T378 residue may contribute to the catalytic function; therefore, the

Table 3. Specific Activities and Optimal Temperatures of the Wild-type and Mutant SHCs Targeted SHCs Targeted for the Asp Residues in the DXDDTA Motif

Other mutants are shown here, along with the doubly mutated SHCs, which are not included in Tables 1 and 2. The assay methods were the same as these described in Table 2.

SHC	Amino acid sequence	Specific activity (nmol/min/mg)	Relative activity (%)	Optimal temperature for 60 min (°C)	Activity for substrate **4****
Wild-type	DVDDTA	1.91×10^3	100.0	60	+++
D374E	EVDDTA	1.61	0.1	60	++
D374I	IVDDTA	0	0	—	+
D374V	VVDDTA	0	0	—	++
D377C*	DVDCTA	3.82	0.2	60	++
D374V/D376N	VVNDTA	0	0	—	−
D374V/D377C	VVDCTA	0	0	—	+
D376N/D377C	DVNCTA	0	0	—	++

* This mutant produced **8** together with **2**. The specific activity for the formation of **8** was 42.2 nmol/min/mg.
** The conversion ratio of **4** is roughly represented by +++ 100%, ++ 50–90%, + 20–49%, − 0%.

Table 4. Kinetic Data and Optimal Temperatures of the T378A Mutant Targeted for the DXDDTA Motif

H451 has been assumed to be hydrogen-bonded to D376 of DXDDTA.[28] The enzyme activities were determined from the amount of **2** produced under incubation conditions of 30°C for 60 min, under which conditions the purified proteins were not denatured.

SHC	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m	Relative activity (%)	Optimal temperature for 60 min (°C)	Activity for substrate **4****
Wild-type	1.67×10	6.43	3.85×10^{-1}	100.0	60	+++
T378A	1.65×10	6.37	3.86×10^{-1}	100.3	55	+++
H451F*	—	—	—	0	—	+
H451R	1.69×10	1.22	7.22×10^{-2}	18.8	60	+++

* No activity could be detected, so the kinetic values could not be determined.
** The conversion ratio of **4** is roughly represented in a similar way to that shown in Tables 2 and 3: +++ 100%, +: 20–49%.

T378 residue was altered into Ala. No effect was apparent on the kinetic values with only a change in the optimal temperature occurring: a slight decrease (55°C) from that of the wild-type (60°C) (Table 4), indicating that T378 was not an active site.

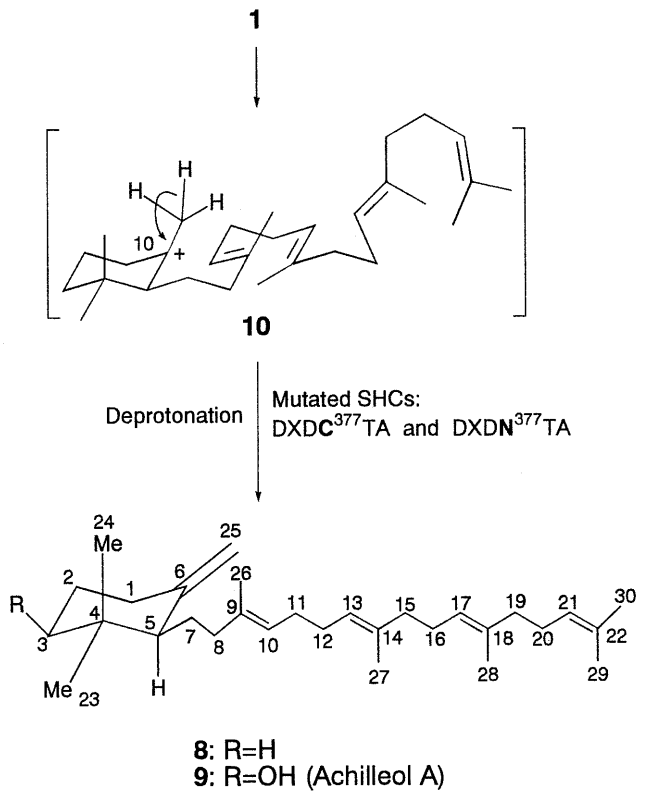
Wendt *et al.* have suggested that the D376 residue of DXDDTA participates in hydrogen-bonding with H451.[28,29] To confirm this idea, two mutants of H451R and H451F were constructed, because the side chain of the histidine residue is considered to have two functions, *i.e.*, as a base for the proton acceptor and as an aromatic ring having a π-electron; both these functions have been assumed for histidine in the oxidosqualene cyclases.[25] The mutant of H451F had no activity, but the mutant of H451R retained the activity, although it was only 19% of that of the wild-type (Table 4), suggesting that the H451 worked as the basic function. Indeed, among the seven known SHCs, five have an Arg residue and two have a His residue at the equivalent position of 451.[5] The protonated H451 residue would work to enhance the acidity of D374 and/or D376.[28]

Compound 8 from the mutants D377C and D377N

We found that mutants D377C and D377N produced a new product **8** in the incubation mixture of **1**, together with **2** and **3** (Scheme 3). The structure of **8** was determined by detailed NMR analyses. Substrate **1** has eight methyls and five olefinic protons. The ^{1}H-NMR spectrum of **8** in $CDCl_3$ showed four olefinic protons (-C*H*=) at δ_H 5.34–5.48 (m) and exomethylene protons (=CH_2) at 4.82 and 4.99 (each a singlet), the latter signals being correlated with δ_C 108.8 in the HMQC spectrum. This carbon was further confirmed to be a methylene group by DEPT 135 pulse sequence. Compound **8** had seven methyl signals as singlets, of which two resonated in a higher field at δ_H 0.81 and 0.89, while the remaining five methyls resonated in a lower field at δ_H 1.57 (s), 1.58 (s, 3 × Me) and 1.66 (s), suggesting that the two methyl groups were situated on the cyclized ring, while the other five methyl groups were at the allyl positions. These NMR data suggest that **8** was a monocyclic compound having an exomethylene group, which could be produced by the deprotonation of one of eight methyls in **1**. The relative stereochemistry of the cyclohexane moiety was established by the NOESY spectrum, a NOE cross peak between H-5 and 23-Me suggesting that the side chain of the isoprenoid chain was in an equatorial position. Detailed analyses by 2D NMR, including HMQC and HMBC, unambiguously clarified the structure of **8** (Scheme 3). Assignments of the NMR signals are shown in the Materials and Methods section.

Product **8** has not previously been reported as an SHC enzymatic product. The production ratios of **2**, **3** and **8** by D377C and D377N were *ca.* 5:1:55 and *ca.* 5:1:10, respectively, and no product other than **2** and **3** was detected in the incubation mixtures. The ratio of **8** to **2** remained unchanged, despite the incubation temperature being changed.

Formation of **8** could be interpreted as shown in Scheme 3. The polycyclization reaction of **1** was quenched at the monocyclic intermediate **10**, a deprotonation reaction subsequently occurring from C-10 methyl



Scheme 3. Reaction Mechanism for Squalene **1** into Monocyclic Product **8** together with Hopene **2** and Hopanol **3** by Mutant SHCs of D377C and D377N.

group to form the exomethylene group. The formation of **8** strongly suggests that D377 acted to stabilize C-10 carbocation intermediate **10**. The structure of **8** is a 3-deoxyhomolog of Achilleol A which has been isolated from the plant, *Achillea odorata L.*[32] Achilleol A **9** would be produced by the cyclization of 2,3-oxidosqualene **4**, but the cyclization reaction would have halted at the monocyclic stage without completing the polycyclization process, as shown in Scheme 3. A trial was carried out to synthesize Achilleol A **9** by incubating **4** with the D377C or D377N mutants. We have succeeded in producing **9** as one of the enzymic products by using these mutated SHCs; the production ratios of **6**, **7** and **9** with D377C and D377N were *ca.* 5:1:14 and *ca.* 5:1:10, respectively. The structure was confirmed to be that of **9** by NMR analyses and by a spectroscopic comparison with data in the literature.[32] Compounds similar to **9** having a monocyclic skeleton have also been found as the enzymatic products of substrate analog, 10,15-diethyl-2,3-oxidosqualene.[16]

Insight into the product distribution and the cyclization with oxidosqualene 4

We have recently reported that mutated SHCs, targeted for W169 and W489, produced a partially cyclized compound having a tetracyclic skeleton (17-*epi*dammarene).[19] The mutations of the different active sites have afforded some of the partially cyclized products together with **2** and **3**.[19,23] This product distribution was dependent on the mutants employed; bicyclic for F365A[33] and Y420A, tri- and tetracyclic for F601A,[34] tetra- and pentacyclic products for F605A, and tri-, tetra- and pentacyclic products for I261A (unpublished results from this laboratory; these results will be published in due course). These products arose from the discrete carbocation intermediates that were generated in each reaction step of polyene cyclization. Thus, an analysis of the product distribution is very useful for identifying the active sites and for understanding their functions. The mutated SHCs for either D374 or D376 of the DXDDTA motif afforded no enzymic products and resulted only in the recovery of **1** (Tables 1 and 2). This finding strongly suggests that these two acidic residues are necessary for protonation at the terminal double bond. However, the mutation at D377 of the DXDDTA motif afforded monocyclic product **8** together with fully cyclized **2** and **3**, suggesting that the role of D377 can be identified neither as initial protonation,[28] nor as stabilization of the final C-22 hopanyl cation intermediate.[22] Thus, it can be concluded that the D377 residue has the function for stabilizing the transient C-10 carbocation intermediate (hopene numbering). To gather further evidence for the role of the DXDDTA motif, (3*S*)-oxidosqualene **4** was also incubated as another substrate. The polycyclization of **4** would not require the higher acidity than that of **1**, because the epoxide ring is more labile to acid. Both D374 and D376 are essential for the cyclization of squalene substrate **1** (Tables 1–3), but only one of either D374 or D376 was necessary for the cyclization of **4** (Tables 2 and 3); **4** was converted into **6** and **7** by the doubly mutated SHCs, D376N/D377C and D374V/D377C (Table 3), in which only one acidic residue of D374 and D376, respectively, remained unchanged in the DXDDTA motif. As shown in Table 3, the D374V/D376N mutant had no activity for the cyclization of **4**, suggesting that D377 had no acidic function; that is, it was not responsible for the initial protonation. Thus, this acid residue D377 would work as the carboxylate anion to stabilize the cation intermediate, as was observed on substrate **1**. Indeed, Achilleol A **9** was found in the incubation mixtures of **4** with the two mutants D377C and D377N, as already described. When squalene **1** was incubated, the mutated SHCs targeted for H451, D313 and D447 also did not produce any enzymic products such as mono-, bi- or tricyclic compounds even when using a large amount of the mutated SHCs, suggesting that these residues would be responsible for the initial protonation reaction together with D374 and D376.

The acidic residue is lacking in OSLCs, which corresponds to the D377 residue of SHCs. As already described, the D377 residue was found to stabilize the C-10 carbocation intermediate, leading to subsequent B-ring closure for the construction of the A/B-fused ring system. At the present time, we cannot account for the reason why the D377 residue is lacking in OSLCs. One possible answer may be that B-ring formation by OSLCs might proceed in a concerted manner and in a combination with closure of the A-ring under a pre-organized state in the active site, *i.e.*, not *via* a discrete carbocation intermediate. This idea has been suggested by Jenson *et al.*[35] On the other hand, the B-ring forma-

tion by SHCs might proceed *via* discrete transient carbocation, so that stabilization of the C-10 cation might be required for subsequent ring closure to construct the fused A/B-ring system, although further experimental evidence is required to formally propose this assumption.

In conclusion, we have gathered definitive evidence for the functions of the DXDDTA motif: initiation of the polycyclization reaction and stabilization of the carbocation generated on the A-ring. The present studies targeted all the conserved acidic amino acids, and we have identified for the first time D313 and D447 as new active sites. The D374 residue of DXDDTA was clearly identified as a catalytic site in this study, although the mutation of this position has not been reported before. On the basis of an X-ray analysis, Wendt *et al.* have suggested the role of the aspartic residue in the initiation mechanism,[28] although the experimental evidence was not sufficient because of lacking of the site-directed mutagenesis experiment. We now have strong evidence in hand for the initiation mechanism for the cyclization of squalene: (1) The D374, D376 and H451 residues work to protonate the terminal double bond in the initial reaction stage. (2) The D377 residue stabilizes the C-10 carbocation prior to subsequent ring closure for the B-ring formation. This function is unequivocally supported by the isolation of monocyclic molecule **8**. The polycyclization reaction of squalene would be initiated through intricate cooperation among the D374, D376, H451, D313 and D447 amino acid residues. There is an indication that the enhanced acidity is achieved by the presence of protonated histidine forming an Asp:His salt bridge.[24,28] The crucial roles of D313 and D447, which have not yet been mentioned in the X-ray analysis,[28,29] were clearly found by the site-directed mutagenesis experiments. The residues of D313 and D447 may serve their acidic protons to give the protonated form of H451 and/or may protonate the carbonyl goups of the D374 and D376 residues, leading to the enhanced acidity of D374 and D376, but further evidence is required to propose this assumption. A clear molecular model must be available in future for understanding the complicated mechanism achieved by the combination of several amino acids. Usage of site-directed mutagenesis not only helps to understand the fundamental issue of reaction mechanism, but also leads to the creation of the ever unknown "unnatural" natural products, as represented by the production of monocyclic triterpene **8** with the D377N mutant. Examples of "unnatural" natural products have recently been reported, which could be produced by alterations of the active sites of sesquiterpene and polyketide synthases.[36-38] The DXDDTA motif has also been found in diterpene cyclases such as copalyl pyrophosphate synthases,[39-43] abietadiene synthase[44] and the *ent*-kaurene synthase from *Phaeosphaeria* sp. L487.[45] Functional analysis of the DXDDTA motif as described in this paper will also offer valuable help for understanding the catalytic mechanism of diterpene cyclases.

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