Cyclization of (3*S*)29-Methylidene-2,3-oxidosqualene by Bacterial Squalene:Hopene Cyclase: Irreversible Enzyme Inactivation and Isolation of an Unnatural Dammarenoid

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The cyclizations of squalene and oxidosqualene are remarkable carbon−carbon bond-forming reactions occurring in triterpene biosynthesis.[1] Bacterial squalene:hopene cyclase (SHC) (E.C. 5.4.99.7) catalyzes the cyclization of squalene (**1**) folded in an all *pre-chair* conformation to hop-22(29)-ene (**2**) and hopan-22-ol (**3**) (Scheme 1A).[2] Several bacterial SHCs have been purified,[3] cloned, and expressed.[4] The SHCs are membrane-associated 70−75 kDa proteins and show 17%−27% identity to the eukaryotic oxidosqualene cyclases (OSCs). Both SHC and OSC contain several repeats of a highly-conserved, repeated motif rich in aromatic amino acids (the QW motif).[5] Very recently, crystallization of the SHC from a thermoacidophilic bacteria *Alicyclobacillus acidocaldarius* was reported.[6] The *A. acidocaldarius* SHC consists of 631 amino acids (71 524 Da) and shows its catalytic optimum at 60 °C and pH 6.0.[3c,4a]

We report herein that *A. acidocaldarius* SHC both cyclizes and is specifically labeled by [^{3}H](3*S*)29-methylidene-2,3-oxidosqualene (29-MOS) (**4**), a mechanism-based irreversible inhibitor of vertebrate OSC (lanosterol synthase).[7] Vertebrate OSCs were specifically labeled with [^{3}H](3*S*)29-MOS, and an Asp residue (D-456 in rat OSC) in the highly-conserved DCTAEA motif was linked to a partially-cyclized inhibitor.[8] This implicated the aspartate carboxylate in stabilization of the C-20 cationic center of the protosterol intermediate cation during the lanosterol-forming reaction. Indeed, site-directed mutagenesis experiments have revealed that the D-456 of the DCTAEA motif was essential for catalytic function.[9] As for *A. acidocaldarius* SHC, both D-376 and D-377 of the corresponding DDTAVV motif were shown to be crucial for the enzyme activity.[10] Comparison of the affinity labeling studies between bacterial SHC and eukaryotic OSC should provide complementary information on the two active sites.

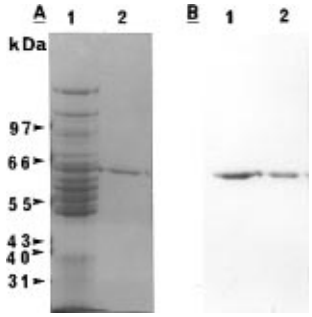

Figure 1. Affinity labeling of *A. acidocaldarius* SHC expressed in *E. coli*: (A) SDS-PAGE (7.5%) gel stained with Coomassie blue; (B) Corresponding fluorogram. Lane 1: E. *coli* cell-free extract. Lane 2: purified enzyme. Each labeling reaction was carried out at a 1 μM concentration of [^{3}H](3*S*)29-MOS (1.8 Ci/mmol) at 60 °C for 1 h.

Recombinant *A. acidocaldarius* SHC expressed in *Escherichia coli*[11] was employed for the chemical affinity labeling experiments. Both crude and purified enzymes were specifically labeled with [^{3}H](3*S*)29-MOS (1.9 Ci/mmol) after incubation at 60 °C for 1 h (Figure 1). Inhibition kinetics with purified recombinant SHC revealed that the inhibition was noncompetitive and time-dependent ($IC_{50} = 1.2$ μM, $K_I = 2.1$ μM, $k_{inact} = 0.06$ min^{-1}). A partition ratio of 610 was calculated for 29-MOS by measuring the decrease in SHC activity at increasing 29-MOS concentrations.[7a] The labeling efficiency was 10-fold lower[12] and covalent modification occurred much more slowly than that for vertebrate OSC ($IC_{50} = 0.5$ μM, $K_I = 4.4$ μM, $k_{inact} = 221$ min^{-1}, with a partition ratio of 3.8 for pig liver OSC).[7a]

Interestingly, a polycyclic C_{31} dammarene derivative (**6**) was isolated from the incubation mixture as the major cyclization product of (3*S*)29-MOS.[13] The ^{1}H NMR spectrum[14] showed the presence of five methyl singlets (δ 0.95, 0.91, 0.85, 0.81, 0.75), one vinylic methyl group (δ 1.71), three vinylic protons (δ 5.52, bs, 1H; 4.68, s, 2H), and a proton geminal to

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(11) Recombinant *A. acidocaldarius* SHC was expressed in *E. coli* and purified as described.[4a] The enzyme converted squalene into a 17:1 mixture of hop-22(29)-ene and hopan-22-ol and showed the apparent $K_M = 1.6$ μM and $k_{cat} = 2.4$ min^{-1} in the presence of 0.1% Triton X-100.
(12) The labeling efficiency was monitored by a DEAE batch method. Thus, the enzyme (1 μM) was incubated with [^{3}H](3*S*)29-MOS (1 μM, 1.9 Ci/mmol) at 60 °C for 1 h (total volume 300 μL). The assay mixture was diluted with 300 μL of buffer A, and 50 μL of DEAE-Sephacel (that had been preequilibrated with the same buffer) was added. After incubation for 1 h at room temperature, the mixture was centrifuged, the supernatant was removed, and DEAE resin was washed five times with 1 mL of the buffer A. The DEAE resin was resuspended in 1 mL buffer, and bound radioactivity was assessed by LSC.
(13) The reaction mixture contained (3*S*)29-MOS (20 mg), and SHC (120 mg) in 200 mL of 50 mM Na citrate (pH 6.0) and 0.1% Triton X-100 was incubated at 60 °C for 16 h. Separately, [^{3}H](3*S*)29-MOS (1.5×10^6 dpm) was incubated with SHC (0.6 mg, 1 mLl) under the same conditions. The incubations were stopped by freezing and lyophilization, followed by extraction with 300 mL of CH_2Cl_2 (×2). The combined extracts from both incubations were evaporated to dryness, separated on SiO_2 column (CH_2Cl_2, $R_f = 0.23$), and finally purified by TLC on $AgNO_3$-impregnated SiO_2 (10% EtOAc/hexane, $R_f = 0.09$) to give 0.55 mg of **6**.

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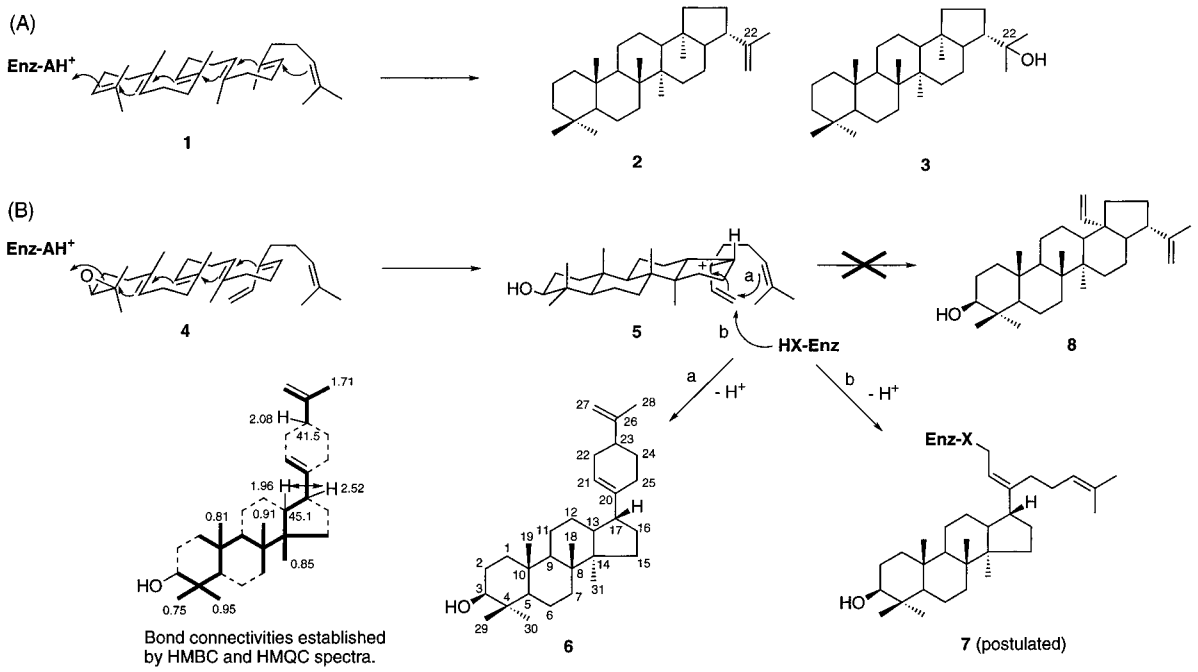
Scheme 1. (A) Proposed Mechanism for the Conversion of Squalene (**1**) to Hopene and Hopanol and (B) Cyclization and SHC Inactivation by (3*S*)29-MOS (**4**)



3β-hydroxyl group (δ 3.17, dd, J = 5, 12 Hz). A structure with the 6.6.6.5+6 ring system was uniquely consistent with both biogenetic reasoning and spectroscopic data (H−H COSY, HMQC, HMBC, and MS);[14] this structure was confirmed by comparison of NMR data of **6** with that of (20*S*)-dammarenediol and (4*S*)-limonene. No evidence was found for the methylidene-extended 3β-hydroxyhopene (**8**), or similar products, in the reaction mixture.

The cyclization of (3*S*)29-MOS was directional, that is, it was initiated by oxirane ring opening and not by a proton attack on the terminal double bond; this has been previously observed for the cyclization of oxidosqualene by bacterial squalene cyclases.[15] It appears most likely that the presence of the methylidene residue arrested the sequential ring formation reaction at the tetracyclic 17-epi-dammarene C-20 cation **5** and that final ring closure yielded compound **6** (Scheme 1B, route a). For the labeling reaction, it is likely that the methylidene-extended 17-epi-dammarene cation (with the 17α-side chain) was trapped by an active-site nucleophile resulting in covalent bond formation and concomitant irreversible inactivation of the enzyme (Scheme 1B, route b). In comparison, rat OSC initiated cyclization of (3*S*)29-MOS from a *chair*−*boat*−*chair* conformation and the C-20 protosterol intermediate cation (with the 17β-side chain) was trapped by nucleophilic attack of the D-456 residue in the DCTAEA motif. Identification of the peptides in the *A. acidocaldarius* SHC active site modified by [^{3}H](3*S*)-29-MOS is now in progress.[16]

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Supporting Information Available: Experimental details including enzyme assay, inhibition kinetics, time dependency of inactivation, determination of partition ratio, comparative ^{13}C NMR data for compound **6**, (4*S*)-limonene, and (20*S*)-dammarenediol, and 500 MHz H−H COSY, HMQC, and HMBC spectra of **6** (7 pages). See any current masthead page for ordering and Internet access information.

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(14) ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.52 (bs, 1H, H-21), 4.68 (s, 2H, H-27), 3.17 (dd, 1H, J = 5, 12 Hz, H-3), 2.52 (m, 1H, H-17), 2.18 (m, 1H, H-25a), 2.12 (m, 1H, H-22a), 2.08 (m, 1H, H-23), 1.96 (m, 1H, H-13), 1.92 (m, 1H, H-22b), 1.87 (m, 1H, H-25b), 1.71 (s, 3H, Me-28), 0.95 (s, 3H, Me-30), 0.91 (s, 3H, Me-18), 0.85 (s, 3H, Me-31), 0.81 (s, 3H, Me-19), 0.75 (s, 3H, Me-29). ^{13}C NMR (125 MHz, $CDCl_3$): δ 149.6 (C-26), 143.5 (C-20), 120.3 (C-21), 108.3 (C-27), 79.0 (C-3), 55.9 (C-5), 50.9 (C-9), 49.6 (C-14), 46.2 (C-17), 45.1 (C-13), 41.5 (C-23), 40.8 (C-8), 39.1 (C-1), 38.9 (C-4), 37.2 (C-10), 35.1 (C-7), 33.1 (C-15), 32.1 (C-25), 31.2 (C-22), 28.3 (C-24), 28.0 (C-30), 27.4 (C-2), 27.0 (C-16), 25.2 (C-12), 22.1 (C-11), 20.7 (C-28), 18.3 (C-6), 17.0 (C-31), 16.2 (C-19), 15.9 (C-18), 15.4 (C-29). The NMR assignments were performed according to data from H−H COSY, HMQC, and HMBC (J = 6 Hz) experiments and by comparison with those of (20*S*)-dammarenediol and (4*S*)-limonene. The absolute configuration of C-23 cannot be deduced from the spectroscopic data. LRMS (EI, 80 eV): *m*/*z* 438 (M^+, 32), 247 (65), 207 (100), 189 (16), 148 (42), 121 (21). HRMS (EI, 80 eV) for $C_{31}H_{50}O$: calcd 438.3899, found 438.3884.

(15) Cell-free homogenates of hopanoid-producing microorganisms are known to initiate cyclization reactions of oxidosqualene into pentacyclic triterpenes via oxirane ring opening. See: (a) Rohmer, M.; Anding, C.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 541−547. (b) Rohmer, M.; Bouvier, P.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 557−560. (c) Bouvier, P.; Berger, Y.; Rohmer, M.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 549−556. (d) Abe, I.; Rohmer, M. *J. Chem. Soc., Perkin Trans. 1* **1994**, 783−791.

(16) Consistent with the different orientation of the side chain, preliminary data show covalent modification in the N-terminal 80 amino acids.