Inactivation of Two Triterpene Cyclases by 18(*E*)-(3*S*)-29-Methylidene-2,3-oxidosqualene

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The cyclizations of squalene by bacterial squalene:hopene cyclase (SHC) (E. C. 5.4.99.7) and of oxidosqualene (OS) by eukaryotic oxidosqualene-lanosterol cyclase (OSLC) (E. C. 5.4.99.7) offer impressive examples of enzyme-templated carbon−carbon bond formation during the biogenesis of sterols and other triterpenes.[1] SHC folds squalene in a chair-chair-chair conformation and then converts squalene to hop-22(29)-ene and hopan-22-ol.[2] In contrast, OSLC binds OS in a chair-boat-chair conformation and mediates the cyclization to lanosterol through a series of conformationally rigid, partially cyclized carbocationic intermediates. Several bacterial SHCs and eukaryotic OSLCs have been purified,[3] cloned and expressed.[4] SHCs and OSLCs are membrane-associated 70−85 kDa proteins and show 17% to 27% sequence identity. Rat liver OSLC (83 kDa) has optimal activity at 37 °C and pH 6.0−8.0 range,[3b,4b] while the 72-kDa SHC from a thermoacidophilic bacteria *Alicyclobacillus acidocaldarius* shows its catalytic optimum at 60 °C and pH 6.0.[3a,4a] Both SHC and OSLC contain multiple repeats of a highly conserved motif rich in aromatic amino acids (the QW motif).[5] The recently reported three-dimensional X-ray structure of the *A. acidocaldarius* SHC revealed a homodimeric structure with two α-helical domains creating a hydrophobic detergent-binding site (proposed as the catalytically active site) located in a large central cavity.[6] Since there is as yet no three-dimensional structure of OSLC, comparison of the chemical affinity labeling studies of bacterial SHC and eukaryotic OSLC provides an alternative route for obtaining comparative information on their catalytically active sites.

We report herein that (18*E*)−(3*S*)-29-methylidene-2,3-oxidosqualene [(18*E*)-29-MOS)] (**1**) is cyclized to bicyclic and pentacyclic products by SHC (Schemes 1 and 2). In addition, we show that [^{3}H]-**1** acts as a mechanism-based irreversible inhibitor that results in specific covalent modification of both *A. acidocaldarius* SHC and rat OSLC. Recently, we demonstrated that *A. acidocaldarius* SHC and rat OSLC were specifically labeled with three inhibitors: [^{3}H](18*Z*)−(3*S*)29-MOS (**2**)[7] (the Δ^{18} stereoisomer of **1**) the nonterpenoid photoactivatable inhibitor [^{3}H]Ro48−8071,[8] and [^{3}H]18-thia-oxidosqualene.[9] The initial discovery of **2** as the mechanism-based irreversible inhibitor of both cyclases[7,10] suggested that its Δ^{18} stereoisomer **1** might also irreversibly inactivate both cyclases. During the course of our work, a report by Cattel and co-workers validated this assumption; *racemic* (18*E*)-29-MOS was found to be an irreversible inhibitor of crude pig (IC_{50} = 4.0 μM) and yeast (IC_{50} = 5.0 μM) OSLC.[11] In this work with unlabeled racemic **2**, however, the covalent

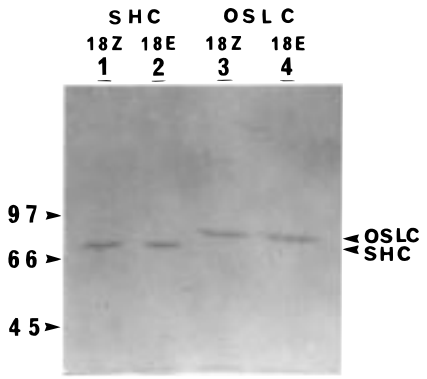
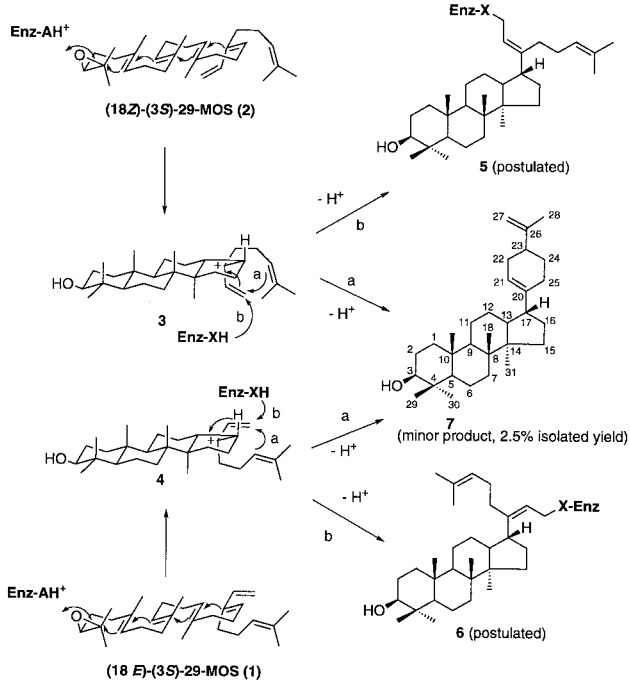


Figure 1. Fluorgram showing affinity labeling of partially purified *A. acidocaldarius* SHC and rat OSLC: (**A**) **Lane 1**, 18*Z*-isomer [^{3}H]-**2** with SHC; **lane 2**, 18*E*-isomer [^{3}H]-**1** with SHC; **lane 3**, 18*Z*-isomer [^{3}H]-**2** with OSLC; **lane 4**, 18*E*-isomer [^{3}H]-**1** with SHC. Each labeling reaction was carried out with 1 μM of either [^{3}H]-**1** and [^{3}H]-**2** (specific activities for both were 9.38 Ci/mmol) at 60 °C for 1 h for SHC and 37 °C for 1 h for rat OSLC.

Scheme 1. Proposed Mechanism for Cyclization and SHC Inactivation by 18(*E*)-(3*S*)29-MOS (1) and 18(*Z*)-(3*S*)29-MOS (2)



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(4) For cloning and functional expression of *A. acidocaldarius* SHC, see: (a) Ochs, D.; Kaletta, C.; Entian, K.-D.; Beck-Sickinger, A.; Poralla, K. *J. Bacteriol.* **1992**, *174*, 298−302. For cloning and functional expression of OSLC from rat liver, see: (b) Abe, I.; Prestwich, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9274−9278.

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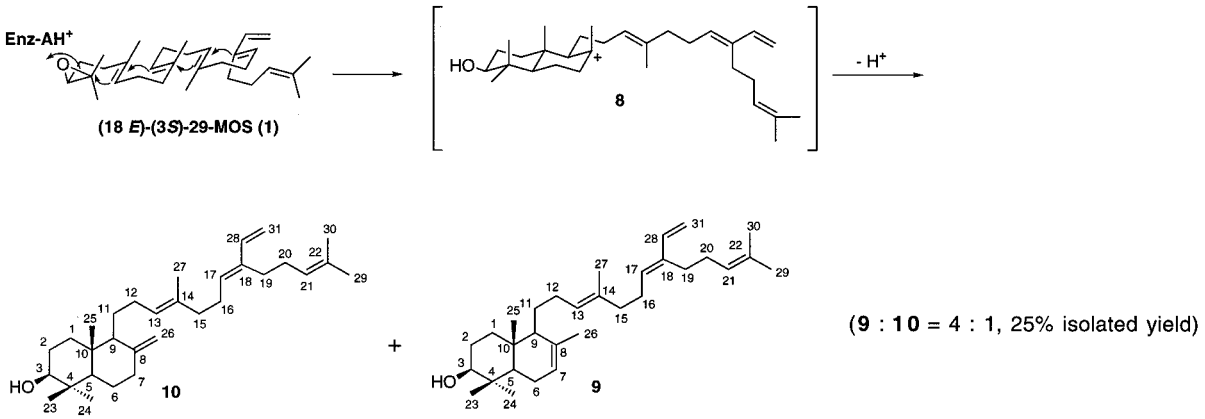
Scheme 2. Structures of Major Bicyclic Compounds Isolated from Cyclization of 1 by SHC.



protein modification and the cyclization products were not examined.

The synthesis of unlabeled and tritium-labeled **1** was accomplished following minor modifications of the published synthetic routes to **2**.[10,11] Briefly, the (18*E*) and (18*Z*) isomers were separated as the (3*S*)−(2,3)-epoxysqualene-29-aldehydes; processing of the two isomers by methylidenation afforded the desired compounds **1** and **2** with no isomerization. The tritium-labeled materials were obtained by reduction of the 29-aldehydes with sodium borotritide (75 Ci/mmol), reoxidation, and olefination. The 18*E*-geometry of **1** was confirmed by ^{1}H NMR, which revealed a double doublet at δ 6.25 (J = 17.4, 11.0 Hz) for one vinyl proton attached to C-29. The analogous proton of the vinyl ABC system of the 18*Z* isomer **2** appeared at δ 6.68 (J = 17.5, 11.0 Hz).[10,12] On the basis of ^{1}H NMR data, there was no detectable amount of the 18*Z* isomer **2** present in the samples of **1** employed for enzymatic studies.

Incubation of [^{3}H]-**1** (9.38 Ci/mmol) with either partially purified recombinant *A. acidocaldarius* SHC[4a] (60 °C for 1 h) or rat OSLC[3b] (37 °C for 1 h) resulted in specific covalent modification of each cyclase, as detected by fluorography of SDS−PAGE separated proteins (Figure 1). Inhibition kinetics revealed that the inhibition was noncompetitive and time-dependent for both SHC (IC_{50} = 4.5 μM, K_I = 7.4 μM, k_{inact} = 0.015 min^{-1}) and rat OSLC (IC_{50} = 7.2 μM, K_I = 21 μM, k_{inact} = 0.048 min^{-1}). Interestingly, the same polycyclic C_{31} dammarene derivative **7** previously obtained[7] from cyclization of **2** by SHC was also isolated from the incubation mixture as a minor cyclization (2.5% isolated yield) product of *E*-isomer (**1**) (Scheme 1). Two bicyclic products **9** and **10** (ratio = 4:1, isolated yield 25.0%) were isolated as the major products (Scheme 2) of the SHC cyclization of **1** and were fully characterized by spectroscopic means.[13,14] It seems that both Δ^{18} stereoisomers **1** and **2** could interrupt the SHC cyclization at the respective tetracyclic 17-epi-dammarene C-20 cations **3** or **4**. These two cations could subsequently be further cyclized to generate the proposed species **7**, or could be trapped by an active-site nucleophile to give a covalently modified enzyme. It is worth noting that the different orientation of the 29-methylidene group in cations **3** and **4** could result in trapping by different nucleophilic residues in the active site of SHC.

The bicyclic compounds **9** and **10** most likely arise from the corresponding bicyclic cation **8**, followed by proton elimination from the C-7 or the 26-methyl group. The elimination appears to be a spontaneous, nonenzymatic process based on the ratio of trisubstituted to disubstituted products isolated. Although the modification of SHC at this stage by cation **8** cannot be ruled out, similar bicyclic products were not observed from the cyclization reaction of **1** with rat OSLC. Since (18*Z*)-2,3-oxidosqualene (e.g., compound **1** without the 29-methylene) was cyclized to a mixture of two 6,6,5-fused tricyclic products with trans/syn/trans A/B/C ring junctions by OSLC,[15] **1** could be cyclized to a 6,6,5-fused tricyclic cation in the labeling reaction. This cation would then be trapped by an active site nucleophile causing the covalent modification of OSLC. It is most likely that the labeling site of rat OSLC by **1** would be different from that of **2**, which has been shown to be covalently linked to the D-456 residue of the DCTAEA motif.[4b] Active site mapping of both SHC and OSLC modified by the Δ^{18} stereoisomers [^{3}H]-**1** and [^{3}H]-**2** will hopefully clarify these issues, and the results will be reported in due course.

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Supporting Information Available: Experimental details (enzyme assay, inhibition kinetics, time dependency of inactivation; comparative ^{1}H NMR data for compound **7**, as well as 500 MHz H−H COSY, HMQC, HMBC, and ^{13}C NMR spectra of **9** and **10**) (15 pages).

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(12) Unlabeled and tritium-labeled **1** were prepared from the corresponding (18 *E*)−(3*S*)-2,3-oxidosqualen-29-al as described.[10,11]

(13) The reaction mixture containing (18*E*)−(3*S*)29-MOS (8 mg) and SHC (120 mg) in 200 mL of 50 mM Na-citrate, pH 6.0, 0.1% Triton X-100, was incubated at 60 °C for 16 h. Separately, [^{3}H](18*E*)−(3*S*)29-MOS (1.5×10^6 dpm) was incubated with SHC (0.6 mg, 1 mL) under the same conditions. The reactions were stopped by freezing and lyophilization, followed by extraction with two 300 mL-portions of CH_2Cl_2. The combined extracts from each incubation were evaporated to dryness and then separated on silica gel to give a mixture of **9** and **10** (4: 1 ratio, 2.0 mg) as one spot on TLC (CH_2Cl_2, R_f = 0.34) and **7** (CH_2Cl_2, R_f = 0.26). The partially purified **7** was purified to homogeneity by $AgNO_3$-impregnated TLC (10% EtOAc/hexane, R_f = 0.09) to give 0.2 mg.

(14) The structure of **7** (predicted on mechanistic grounds and from TLC R_f value) was confirmed by comparison to an authentic sample using ^{1}H NMR. The structures of **9** and **10** (ratio = 4: 1) was deduced from detailed spectroscopic studies, and the NMR assignment was performed according to H−H COSY, HMQC and HMBC (J = 6 Hz) experiments. **9**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.19 (dd, J = 17.5, 10.5 Hz, 1H, H-28), 5.38 (t, J = 7.0 Hz, 1H, H-17), 5.33 (brs, 1H, H-7), 5.10 (m, 2H, H-13 and H-21), 5.07 (d, J = 17.5 Hz, H-31a); 4.86 (d, J = 11 Hz, H-31b); 3.18 (m, 1H, H-3), 2.34 (m, 1H, H-9), 2.17 (m, 2H, H-16), 2.16 (m, 2H, H-19), 2.10 (m, 2H, H-12), 2.01 (m, 2H, H-15), 1.98 (m, 2H, H-20), 1.90 (m, 2H, H-6), 1.80 (m, 2H, H-1), 1.65 (s, 3H, Me-26), 1.63 (s, 3H, Me-30), 1.55 (s, 3H, Me-29), 1.54 (s, 3H, Me-27), 1.51 (m, 2H, H-2), 1.37 (m, 2H, H-11), 1.15 (m, 1H, H-5), 0.90 (s, 3H, Me-24), 0.79 (s, 2H, Me-25), 0.68 (s, 3H, Me-23); **10**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.19 (dd, J = 17.5, 10.5 Hz, 1H, H-28), 5.38 (t, J = 7.0 Hz, 1H, H-17), 5.10 (m, 2H, H-13 and H-21), 5.07 (d, J = 17.5 Hz, H-31a); 4.86 (d, J = 11 Hz, H-31b), 4.78 (s, 1H, H-26a), 4.49 (s, 1H, H-26b), 3.18 (m, 1H, H-3), 2.34 (m, 1H, H-9), 2.17 (m, 2H, H-16), 2.16 (m, 2H, H-19), 2.10 (m, 2H, H-12), 2.01 (m, 2H, H-15), 1.98 (m, 2H, H-20), 1.80 (m, 2H, H-1), 1.63 (s, 3H, Me-30), 1.55 (s, 3H, Me-29), 1.54 (s, 3H, Me-27), 1.51 (m, 2H, H-2), 1.37 (m, 2H, H-11), 0.93 (s, 3H, Me-24), 0.80 (s, 2H, Me-25), 0.70 (s, 3H, Me-23); HRMS for both **9** and **10** (EI, 80 eV) $C_{31}H_{50}O$: calcd 438.3862, found 438.3859.

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