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1-Methylidenesqualene and 25-Methylidenesqualene as Active-Site Probes for Bacterial Squalene:Hopene Cyclase

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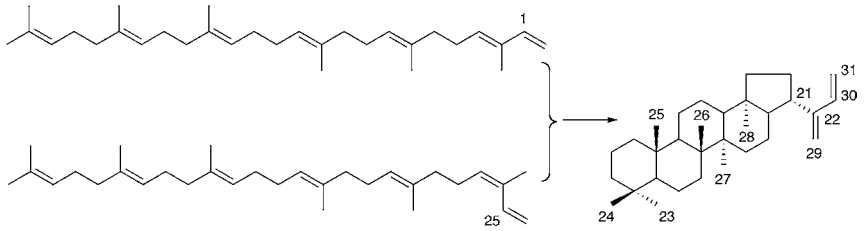
ABSTRACT



1-Methylidenesqualene and 25-methylidenesqualene were converted to 30-methylidenehop-22(29)-ene by squalene:hopene cyclase from *Alicyclobacillus acidocaldarius.* It was remarkable that both analogues generated the same product. The hopanyl intermediate cation, stabilized by the methylidene residue, enabled a rotation of the isobutenyl group at C-21 prior to the final proton elimination. In contrast, in the formation of hop-22(29)-ene, the final proton abstraction takes place regiospecifically from the *Z*-methyl group, which was verified by cyclization of (1,1,1,24,24,24-$^2H_6$)squalene into (23,23,23,30,30,30-$^2H_6$)hop-22(29)-ene.

Squalene:hopene cyclase (SHC) (E.C. 5.4.99.7) catalyzes the remarkable cyclization of squalene (**1**) into hop-22(29)-ene (**3**) in a regio- and stereospecific manner. The proton-initiated sequential cyclization of squalene, folded in an *all-chair* conformation, first generates a pentacyclic hopanyl C-22 cation (**2**), and the subsequent proton abstraction from the terminal methyl group leads to the formation of the pentacyclic ring system of hop-22(29)-ene (**3**) (Scheme 1A).[1] SHC from a thermoacidophilic bacteria *Alicyclobacillus acidocaldarius* has been the best characterized squalene cyclizing enzyme so far.[2,3] Recent crystallographic and structure-based mutagenesis studies, in combination with utilization of various active site probes, have revealed intimate three-dimensional structural details of the enzyme-catalyzed processes.

The broad substrate specificity of the bacterial SHC is remarkable. The enzyme accepts a wide variety of nonphysiological squalene analogues (C_{25}−C_{35}) and efficiently performs sequential ring-forming reactions to produce a series of unnatural cyclic isoprenoids,[4] including the recently reported unnatural C_{35} hexacyclic polyprenoid with a 6.6.6.6.6.5-fused ring system.[5] It has been described that 29-methylidene-2,3-oxidosqualene (29-MOS) (**4**), a mechanism-

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(1) For recent reviews, see: (a) Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189−2206. (b) Wendt, K. U.; Schulz, G. E.; Corey, E. J.; Liu, D. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 2812−2833. (c) Hoshino, T.; Sato, T. *Chem. Commun.* **2002**, 291−301. (d) Ourisson, G.; Rohmer, M.; Poralla, K. *Annu. Rev. Microbiol.* **1987**, *41*, 301−333. (e) Abe, I.; Zheng, Y. F.; Prestwich, G. D. *J. Enzymol. Inh.* **1998**, *13*, 385−398.

(2) For the crystal structure of *A. acidocaldarius* SHC, see: (a) Wendt, K. U.; Poralla, K.; Schulz, G. E. *Science* **1997**, *277*, 1811−1815. (b) Wendt, K. U.; Lenhart, A.; Schulz, G. E. *J. Mol. Biol.* **1999**, *286*, 175−187.

(3) For purification and cloning of *A. acidocaldarius* SHC, see: (a) Seckler, B.; Poralla, K. *Biochim. Biophys. Acta* **1986**, *881*, 356−363. (b) Neumann, S.; Simon, H. *Biol. Chem. Hoppe-Seyler* **1986**, *367*, 723−729. (c) Ochs, D.; Tappe, C. H.; Gärtner, P.; Kellner, R.; Poralla, K. *Eur. J. Biochem.* **1990**, *194*, 75−80. (d) Ochs, D.; Kaletta, C.; Entian, K.-D.; Beck-Sickinger, A.; Poralla, K. *J. Bacteriol.* **1992**, *174*, 298−302.

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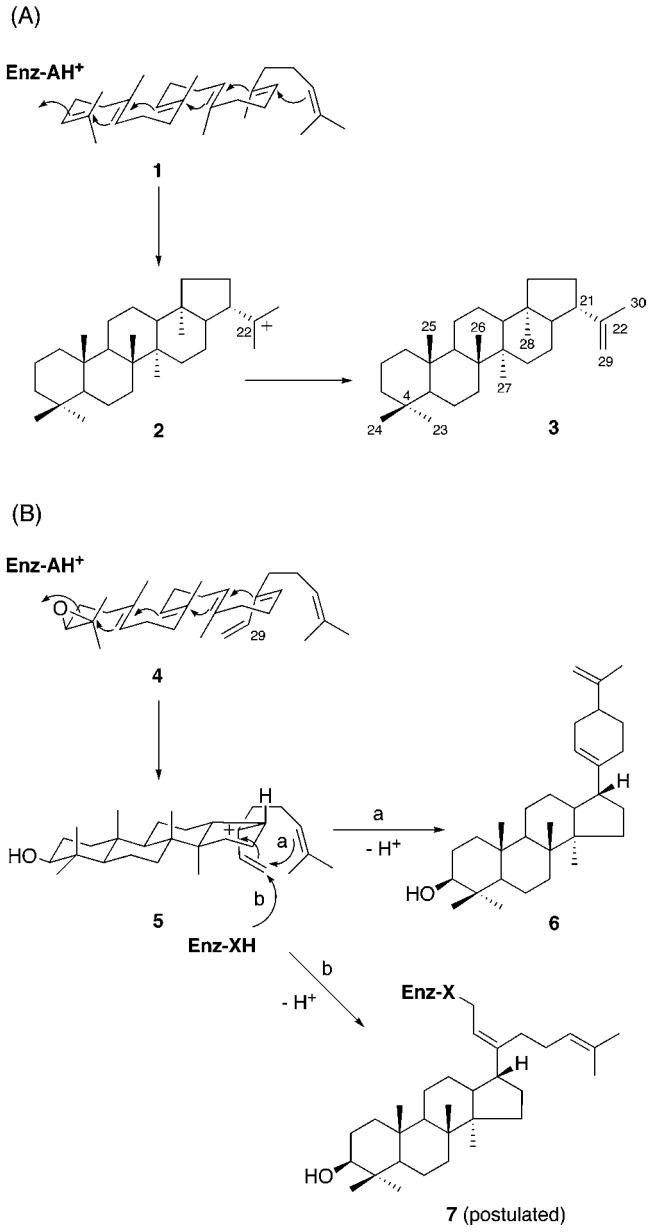
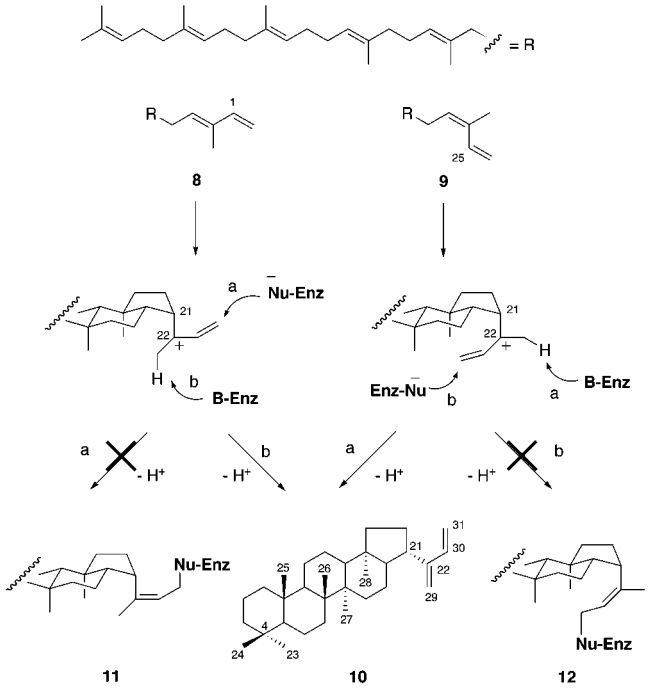
Scheme 1. (A) Proposed Mechanism for the Conversion of Squalene (**1**) to Hop-22(29)-ene (**3**) and (B) Cyclization and Enzyme Inactivation of *A. acidocaldarius* SHC by (3*S*)-29-MOS (**4**)



Scheme 2. Proposed Mechanisms for Cyclization and Enzyme Inactivation by 1-Methylidenesqualene (**8**) and 25-Methylidenesqualene (**9**)



based irreversible inhibitor of vertebrate oxidosqualene cyclase,[6] was accepted as a substrate and cyclized to an unnatural dammarene derivative with a 6.6.6.5+6 ring system (**6**) by *A. acidocaldarius* SHC (Scheme 1B).[4f] Interestingly, 29-MOS also functioned as an effective time-dependent irreversible inactivator of the enzyme (IC_{50} = 1.2 μM, K_I = 2.1 μM, k_{inact} = 0.06 min^{-1}).[4f] A partially cyclized methylidene-extended allylic cation (**5**) has been postulated to be trapped by an active site nucleophile resulting in covalent bond formation and concomitant irreversible inactivation of the enzyme (**7**) (Scheme 1B). To further understand the enzyme reaction mechanism, here we report newly synthesized active site probes, 1-methylidenesqualene (1-MS) (**8**)[7] and 25-methylidenesqualene (25-MS) (**9**)[8] (Scheme 2).

The convergent synthesis of the methylidene-extended substrate analogues involved (i) SeO_2 oxidation of squalene to give a mixture of allylic alcohols (squalen-1-ol and squalen-25-ol were readily separated from a mixture of 26- and 27-alcohol), (ii) oxidation of the allylic alcohol to the

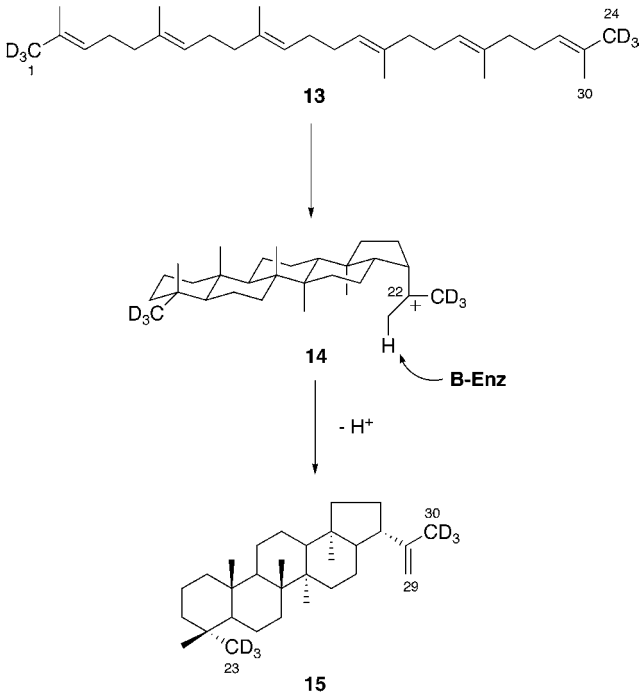
(4) For enzymatic conversion of squalene analogues (C_{25}−C_{31}) by bacterial squalene cyclase, see: (a) Rohmer, M.; Anding, C.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 541−547. (b) Bouvier, P.; Berger, Y.; Rohmer, M.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 549−556. (c) Rohmer, M.; Bouvier, P.; Ourisson, G. *Eur. J. Biochem.* **1980**, *112*, 557−560. (d) Renoux, J.-M.; Rohmer, M. *Eur. J. Biochem.* **1986**, *155*, 125−132. (e) Abe, I.; Rohmer, M. *J. Chem. Soc., Perkin Trans. 1* **1994**, 783−791. (f) Abe, I.; Dang, T.; Zheng, Y. F.; Madden, B. A.; Feil, C.; Poralla, K.; Prestwich, G. D. *J. Am. Chem. Soc.* **1997**, *119*, 11333−11334. (g) Robustell, B.; Abe, I.; Prestwich, G. D. *Tetrahedron Lett.* **1998**, *39*, 957−960. (h) Robustell, B.; Abe, I.; Prestwich, G. D. *Tetrahedron Lett.* **1998**, *39*, 9385−9388. (i) Zheng, Y. F.; Abe, I.; Prestwich, G. D. *J. Org. Chem.* **1998**, *63*, 4872−4873. (j) Sato, T.; Abe, T.; Hoshino, T. *Chem. Commun.* **1998**, 2617−2618. (k) Hoshino, T.; Kondo, T. *Chem. Commun.* **1999**, 731−732. (l) Hoshino, T.; Ohashi, S. *Org. Lett.* **2002**, *4*, 2553−2556.

(5) Abe, I.; Tanaka, H.; Noguchi, H. *J. Am. Chem. Soc.* **2002**, *124*, 14514−14515.

(6) (a) Xiao, X.-y.; Prestwich, G. D. *J. Am. Chem. Soc.* **1991**, *13*, 9673−9674. (b) Abe, I.; Bai, M.; Xiao, X.-y.; Prestwich, G. D. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 32−38. (c) Abe, I.; Prestwich, G. D. *J. Biol. Chem.* **1994**, *269*, 802−804. (d) Abe, I.; Prestwich, G. D. *Lipids* **1995**, *30*, 231−234. (e) Madden, B. A.; Prestwich, G. D. *J. Org. Chem.* **1994**, *59*, 5488−5491. (f) Madden, B. A.; Prestwich, G. D. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 309−314. (g) Abe, I.; Prestwich, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9274−9278.

(7) 1-Methylidenesqualene (**8**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.35 (dd, 1H, J = 17.2, 10.8 Hz), 5.46 (t, 1H, J = 7.4 Hz), 5.15 (brm, 5H), 5.05 (d, 1H, J = 17.2 Hz), 4.90 (d, 1H, J = 10.8 Hz), 2.23 (dt, 2H, J = 7.5, 7.5 Hz), 2.02 (m, 18H), 1.73 (s, 3H), 1.68 (s, 3 H), 1.60 (s, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.6, 135.0, 134.9, 134.8, 134.3, 133.4, 132.8, 131.1, 124.7, 124.4 (×2), 124.3 (×2), 110.3, 39.8, 39.7 (×2), 39.3, 28.3 (×2), 26.9, 26.8, 26.7, 26.6, 25.7, 17.6, 16.0 (×2), 15.9 (×2), 11.6; HRMS (EI) found for [$C_{31}H_{50}$] 422.3889, calcd 422.3913.

Scheme 3. Cyclization of (1,1,1,24,24,24-$^2H_6$)Squalene (**13**) to (23,23,23,30,30,30-$^2H_6$)Hop-22(29)-ene (**15**)



enal, and (iii) Wittig condensation with methyltriphenylphosphorane, as described previously.[9] When incubated with purified recombinant *A. acidocaldarius* SHC, interestingly, both 1-MS and 25-MS yielded the same cyclization product (35% and 38% yield from 10 mg of 1-MS and 25-MS, respectively) that afforded identical spectra (^{1}H and ^{13}C NMR, HMQC, HMBC, and GC−MS).[10,11] The ^{1}H NMR spectrum revealed the presence of six methyl singlets (δ 0.84, 0.79, 0.81, 0.96, 0.93, and 0.74) almost identical with those of the Me-23, Me-24, Me-25, Me-26, Me-27, and Me-28 of hop-22(29)-ene (**3**). In addition, five vinylic protons (δ 6.36, dd, 1H, J = 17.6, 10.8 Hz; 5.25, d, 1H, J = 17.6 Hz; 5.10, d, 2H, J = 13.6 Hz; 5.00, d, 1H, J = 10.8 Hz) indicated the presence of a conjugated diene system, suggesting the structure of a methylidene-extended hopene. Moreover, the α-axial orientation of the isobutenyl group at C-21 was confirmed by NOEs observed between Me-25/Me-26, Me-27/Me-28, and Me-28/H-31. The structure of the unnatural novel C_{31} polyprenoid was thus determined to be 30-methylidene-hop-22(29)-ene (**10**).

It was remarkable that both 1-MS and 25-MS efficiently afforded the same cyclization product (Scheme 2). In both cases, the cyclization reactions were directional; a proton attack on the regular terminal bond first generated a pentacyclic hopanyl C-22 cation, and the subsequent proton elimination from the terminal methyl group yielded the conjugated diene system. It is likely that the stabilization of the intermediate allylic cation by the presence of the methylidene residue enabled a rotation of the isobutenyl group at C-21 around the C-21−C-22 bond prior to the final proton abstraction by the active site basic residue of the enzyme.

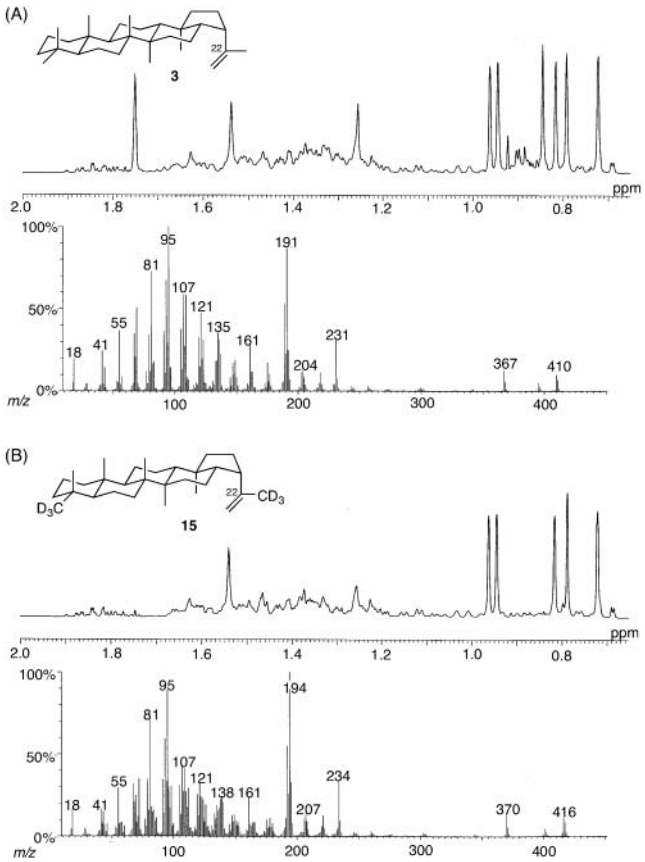


Figure 1. Comparison of ^{1}H NMR and MS spectra of (A) hop-22(29)-ene (**3**) (M = 410) and (B) (23,23,23,30,30,30-$^2H_6$)hop-22(29)-ene (**15**) (M = 416). In the ^{1}H NMR spectrum, retention of C-22 vinyl protons (δ 4.78) and a complete loss of C-30 vinylic methyl protons (δ 1.75) were observed.

(8) 25-Methylidenesqualene (**9**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.36 (dd, 1H, J = 17.6, 10.8 Hz), 5.47 (t, 1H, J = 7.4 Hz), 5.14 (brm, 5H), 5.07 (d, 1H, J = 17.6 Hz), 4.92 (d, 1H, J = 10.8 Hz), 2.23 (dt, 2H, J = 7.5, 7.5 Hz), 2.02 (m, 18H), 1.74 (s, 3H), 1.68 (s, 3H), 1.60 (s, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.61, 135.0, 134.9, 134.8, 134.3, 133.4, 132.8, 131.1, 124.7, 124.4 (×2), 124.3 (×2), 110.3, 39.8, 39.7 (×2), 39.3, 28.3 (×2), 26.9, 26.8, 26.7, 26.6, 25.7, 17.6, 16.0 (×2), 15.9 (×2), 11.6; HRMS (EI) found for [$C_{31}H_{50}$] 422.3902, calcd 422.3913.

(9) Sen, S. E.; Prestwich, G. D. *J. Med. Chem.* **1989**, *32*, 2152−2158.

(10) The recombinant *A. acidocaldarius* SHC was prepared as described in a previous publication.[5] The reaction mixture containing the methylidene-extended squalene **8** or **9** (10 mg) and purified recombinant SHC (30 mg) in 300 mL of 50 mM Na-citrate, pH 6.0, 0.1% Triton X-100, was incubated at 60 °C for 16 h. The incubations were stopped by freezing and lyophilization, followed by extraction with 150 mL of hexane (× 3). The combined extracts were evaporated to dryness and separated on SiO_2 TLC (developed twice first 5 cm in $CHCl_3$ then 16 cm in hexane, the R_f values for the substrate and the product were 0.8 and 0.6, respectively) to give 3.5 mg (from **8**) or 3.8 mg (from **9**) of compound **10**.

(11) 30-Methylidenehop-22(29)-ene (**10**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.36 (dd, 1H, J = 17.6, 10.8 Hz, H-30), 5.25 (d, 1H, J = 17.6 Hz, H-31 trans), 5.10 (d, 2H, J = 13.6 Hz, H-29), 5.00 (d, 1H, J = 10.8 Hz, H-31 cis), 3.10 (dt, 1H, J = 7.4, 7.4 Hz, H-21), 0.96 (s, 3H, Me-26), 0.93 (s, 3H, Me-27), 0.84 (s, 3H, Me-23), 0.81 (s, 3H, Me-25), 0.79 (s, 3H, Me-28), 0.74 (s, 3H, Me-24); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.3 (C-22), 141.6 (C-30), 114.8 (C-29), 112.7 (C-31), 56.1 (C-5), 54.6 (C-17), 50.4 (C-9), 49.5 (C-13), 45.2 (C-18), 42.1 (C-3), 42.0 (C-14), 41.9 (C-8), 40.3 (C-1), 39.7 (C-21), 37.4 (C-10), 33.6 (C-15), 33.4 (C-23), 33.3 (C-7), 28.4 (C-20), 24.0 (C-12), 21.6 (C-24), 21.4 (C-16), 20.9 (C-11), 18.7 (C-6), 16.8 (C-27), 16.7 (C-26), 16.1 (C-28), 15.8 (C-25); LRMS (EI) *m*/*z* 422, 407, 367, 231, 215, 201, 191, 173, 161, 145, 133, 119, 105, 95; HRMS (EI) found for [$C_{31}H_{50}$] 422.3885, calcd 422.3913.

In contrast, in the formation of hop-22(29)-ene, the final proton elimination from the hopanyl C-22 cation takes place regiospecifically from the *Z*-methyl (Me-30) group, but not from the *E*-methyl (Me-24) of squalene.[12] This was verified by enzymatic conversion of (1,1,1,24,24,24-$^2H_6$)squalene (**13**), which was chemically synthesized as described by Ourisson and co-workers[4b] (Scheme 3).[13] The ^{1}H NMR and MS spectra of the cyclization product (51% yield from 10 mg of **13**) indicated retention of C-29 vinyl protons (δ 4.78) and complete loss of C-30 vinylic methyl protons (δ 1.75) of the cyclization product (M = 416) (Figure 1), clearly demonstrating exclusive formation of (23,23,23,30,30,30-$^2H_6$)hop-22(29)-ene (**15**) by *A. acidocaldarius* SHC.

On the basis of the crystal structure of *A. acidocaldarius* SHC, active-site residues involved in the regiospecific proton abstraction at H-29 have not been completely identified yet.[2] Furthermore, no product with a hydroxyl group resulting from cation hydration was detected in the reaction mixture, which was confirmed by GC−MS analysis. It has been suggested that the water network around Glu45 at the bottom of the active site cavity may be the only location in which water is available for cation quenching.[2]

Finally, 1-MS and 25-MS were found to be poor enzyme inhibitors of recombinant *A. acidocaldarius* SHC (IC_{50} = ca. 100 μM).[14] Moreover, the enzyme inhibition did not show time dependency. We first expected that the methylidene-extended analogues would also act as mechanism-based irreversible inhibitors of the enzyme as in the case of 29-MOS.[4f] However, the methylidene-extended hopanyl intermediate cation was not trapped by active-site nucleophile to give covalent modification of the enzyme (Scheme 2).

In summary, this paper presents enzymatic formation of an unnatural novel C_{31} polyprenoid by *A. acidocaldarius* SHC. It was remarkable that both 1-MS and 25-MS afforded the same product, which provided important information on the final proton abstraction for the termination of the polyene cyclization reaction. Further studies of the enzyme reaction by using active-site probes in combination with structure-based mutagenesis are now in progress in our laboratories.

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Supporting Information Available: Complete set of spectroscopic data (^{1}H and ^{13}C NMR, HMQC, HMBC, NOE, and MS) of 1-methylidenesqualene (**8**), 25-methylidene-squalene (**9**), 30-methylidenehop-22(29)-ene (**10**), (1,1,1,-24,24,24-$^2H_6$)squalene (**13**), and (23,23,23,30,30,30-$^2H_6$)hop-22(29)-ene (**15**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) The regiospecific proton elimination from the hopanyl cation was briefly mentioned in a recent review article.[1c] However, experimental details have not been published in the literature.

(13) The deurterium-labeled squalene (10 mg) was incubated with recombinant *A. acidocladarius* SHC as described above, affording 5.1 mg of (23,23,23,30,30,30-$^2H_6$)hop-22(29)-ene (**15**). There was no great difference observed between the yield for the cyclization of squalene and hexadeuteriosqualene.

(14) The enzyme inhibition tests were carried out using the purified recombinant *A. acidocladarius* SHC as described previously.[4f,i]