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Enzymatic cyclization of 26- and 27-methylidenesqualene to novel unnatural C_{31} polyprenoids by squalene:hopene cyclase ☆

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Abstract—Squalene:hopene cyclase (SHC) from *Alicyclobacillus acidocaldarius* accepted 26-methylidenesqualene (26-MS) and 27-methylidenesqualene (27-MS) as a substrate and converted to novel pentacyclic C_{31} polyprenoids; a dammarene derivative with a 6.6.6.5+6 ring system and 26-methylidene-hop-22(29)-ene, respectively. The broad substrate specificity of the enzyme provided important information on the structure and function of SHC. Interestingly, 27-MS was also found to be a potent inhibitor of the bacterial SHC (IC_{50}=5 μM), while 26-MS just showed poor enzyme inhibition (IC_{50}=ca. 100 μM).

Squalene:hopene cyclase (SHC) (EC 5.4.99.7) catalyzes the sequential ring-forming reaction of squalene (**1a**) to produce a pentacyclic triterpene, hop-22(29)-ene (**2a**) (Scheme 1A). The enzyme binds the substrate folded in all-*chair* conformation and mediate the carbon–carbon bond-forming reaction in a regio- and stereospecific manner.[1] SHC from a thermoacidophilic bacteria *Alicyclobacillus acidocaldarius* has been the best characterized squalene-cyclizing enzyme; structure-based mutagenesis studies, in combination with employment of active site targeted probes, have revealed intimate structural details of the enzyme catalyzed processes.[2,3] Notably, the enzyme accepts a variety of nonphysiological substrate analogs (C_{25}–C_{35}) to produce a series of unnatural cyclic polyprenoids,[4] including the recently reported unnatural C_{35} hexacyclic compound with a 6.6.6.6.6.5-fused ring system.[4a]

In order to test the effect of an additional methylidene group on the polyene cyclization reaction, we have previously reported synthesis and enzymatic conversion of 1-methylidenesqualene (1-MS) (**1b**), and 25-methylidenesqualene (25-MS) (**1c**) by recombinant *A. acidocaldarius* SHC.[5] It was demonstrated that both 1-MS and 25-MS were efficiently cyclized to 30-methylidene-hop-22(29)-ene, which provided important information on the final proton abstraction for the termination of the enzyme reaction. In this paper, we describe enzymatic conversion of newly synthesized 26-methylidenesqualene (26-MS) (**1d**) and 27-methylidenesqualene (27-MS) (**1e**). It has been reported that *A. acidocaldarius* SHC cyclized 29-methylidene-2,3-oxidosqualene (29-MOS) (**1f**) to an unnatural dammarene derivative with a 6.6.6.5+6 ring system.[4b] Interestingly, 29-MOS also functioned as a time-dependent irreversible inhibitor of the bacterial SHC as in the case of vertebrate oxidosqualene cyclase.[4b,6] A partially-cyclized methylidene-extended allylic cation has been postulated to be trapped by an active site nucleophile resulting in covalent bond formation and concomitant irreversible inactivation of the enzyme.

The convergent synthesis of 26-MS[7] and 27-MS[8] started from SeO_2 oxidation of squalene to give a mixture of allylic alcohols. A mixture of squalene-26-ol and squalene-27-ol, readily separable from 1- and 25-alcohol, was then converted to a mixture of enals. After separation of 26-al and 27-al by reverse phase HPLC, Wittig condensation with methyltriphenylphosphorane finally afforded the substrates.[5,9] When incubated with purified recombinant *A. acidocaldarius* SHC, both 26-MS and 27-MS yielded a single cyclization product; 1.0 mg of **3** (from 10 mg of 26-MS) and 0.3 mg of **2e** (from 10 mg of 27-MS) were isolated from the incubation mixtures, respectively.[10]

Keywords: Squalene cyclase; Triterpene synthase; Unnatural natural products.
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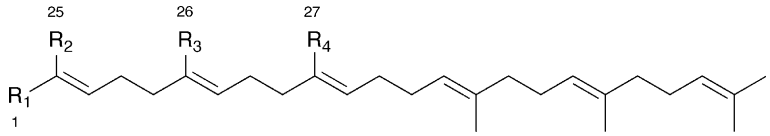
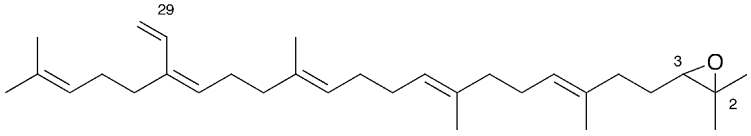


1a $R_1 = R_2 = R_3 = R_4 = -CH_3$
1b $R_1 = -CH{=}CH_2$, $R_2 = R_3 = R_4 = -CH_3$
1c $R_2 = -CH{=}CH_2$, $R_1 = R_3 = R_4 = -CH_3$
1d $R_3 = -CH{=}CH_2$, $R_1 = R_2 = R_4 = -CH_3$ (26-MS)
1e $R_4 = -CH{=}CH_2$, $R_1 = R_2 = R_3 = -CH_3$ (27-MS)



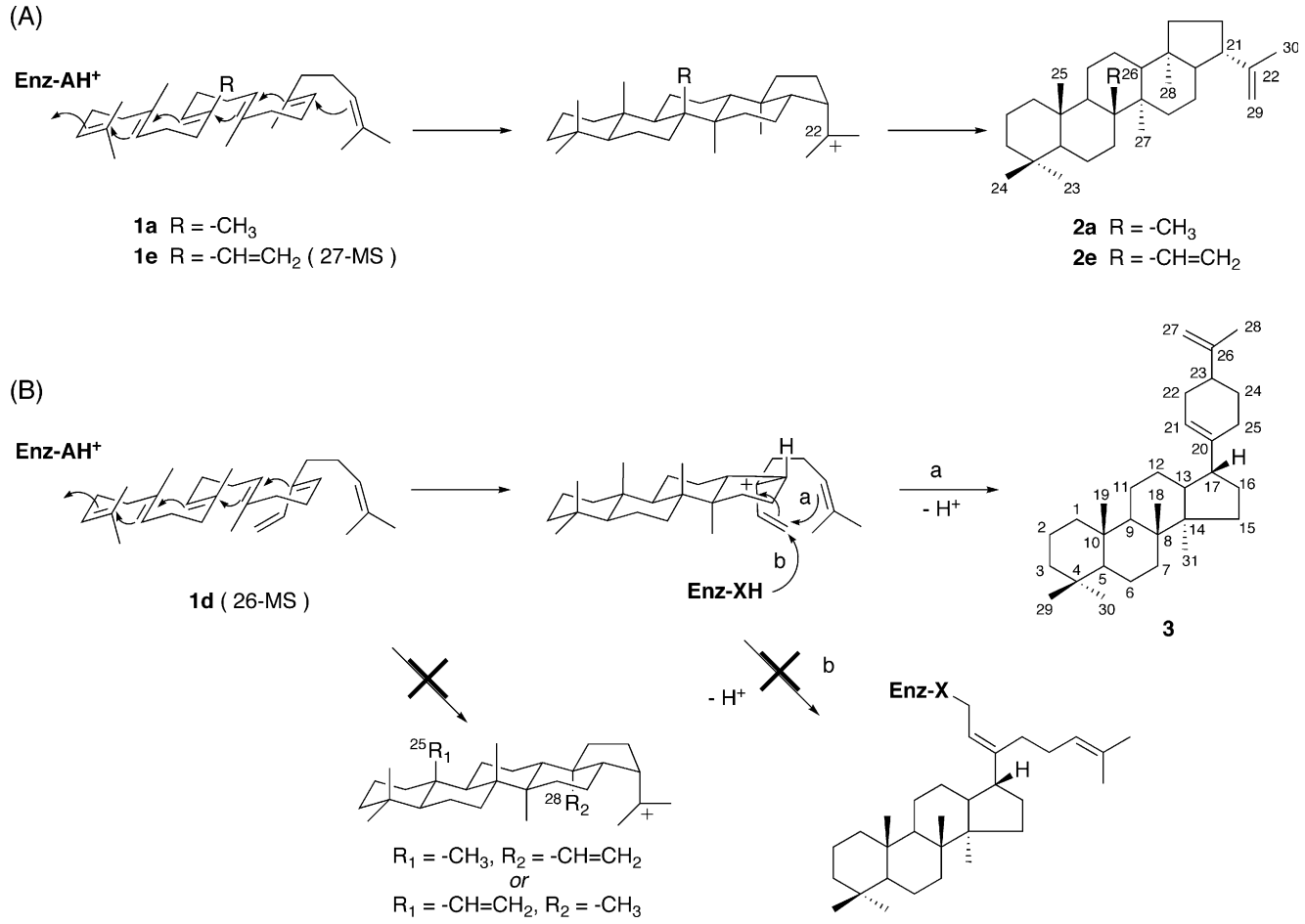
1f (29-MOS)

Interestingly, 26-MS was cyclized to a novel C_{31} dammarene derivative with a 6.6.6.5+6 ring system (**3**) as in the case of the previously reported enzymatic conversion of 29-MOS.[11] The ^{1}H NMR spectrum showed the presence of five methyl singlets (δ 0.93, 0.89, 0.85, 0.83, 0.80), one vinylic methyl group (δ 1.73), and three vinylic protons (δ 5.52, m, 1H; 4.70, s, 2H). A structure with the 6.6.6.5+6 ring system was uniquely consistent with both biogenetic reasoning and NMR spectroscopic data; heteronuclear (HMQC and HMBC) correlation spectroscopy. Furthermore, the stereochemistry of C-17 was confirmed by NOEs observed between Me-19/Me-18, Me-31/H-21, but absent between Me-31/H-17. No evidence was obtained for the formation of 25- or 28-methylidene-extended hopene (Scheme 1B), or similar products, in the reaction mixture. The cyclization of 26-MS was thus directional, that is, the enzyme did not accept the modification of *pro*-25β-methyl group of hopene. Moreover, the formation of the dammarene derivative suggested that the presence of the methylidene residue arrested the cyclization reaction at the tetracyclic 17-*epi*-dammarene C-20 cation with the 17α-side chain, and that final ring closure yielded compound **3** (Scheme 1B, route a).

On the other hand, 27-MS was converted to a novel unnatural C_{31} polyprenoid, 26-methylidene-hop-22(29)-ene (**2e**).[12] The ^{1}H NMR spectrum revealed the presence of five methyl singlets (δ 1.00, 0.83, 0.78, 0.75, 0.71), one vinylic methyl group (δ 1.73), and five vinylic protons (δ 6.25, dd, 1H, $J = 18.0$, 11.2 Hz; 5.23, dd, 1H, $J = 11.2$, 2.0 Hz; 4.90, dd, 1H, $J = 18.0$, 2.0 Hz; 4.77, s, 2H), indicating the structure of 26-methylidene-extended hopene, which was also supported by heteronuclear (HMQC and HMBC) correlation spectroscopy. Further, the α-axial orientation of the isopropenyl group at C-21 was confirmed by NOEs observed between Me-27/Me-28, Me-28/H-29, and Me-28/Me-30. Since formation of 27-methylidene-extended hopene was not detected, the cyclization reaction was suggested to be also directional; the enzyme tolerated the modification of *pro*-26β-methyl group of hopene, but did not accept *pro*-27α-methylidene group (Scheme 1A). Apparently, stereoelectronic effects operating at the *pro*-27α-face of the conformationally-folded substrate interrupted the cyclization reaction. On the basis of the crystal structure of *A. acidocaldarius* SHC, the *pro*-27α methyl group appears to be located at in close proximity of the active-site Phe365.[2]

Finally, 27-MS was found to be also a potent inhibitor of recombinant *A. acidocaldarius* SHC (IC_{50}=5 μM), while 26-MS just showed poor enzyme inhibition (IC_{50}=ca. 100 μM) as in the case of the previously reported 1-MS and 25-MS.[13] The Lineweaver–Burk plot analysis revealed that 27-MS acts as a noncompetitive inhibitor of SHC with $K_I = 3.2$ μM. However, the enzyme inhibition was reversible and did not shows time dependency, excluding the possibility that the partially cyclized methylidene-extended intermediate cation was trapped by an active site nucleophile to afford covalent modification of the enzyme (Scheme 1B, route b). This contrasts with 29-MOS (**1f**) that showed a time-dependent, mechanism-based irreversible inhibition of the enzyme (IC_{50}=1.2 μM, $K_I = 2.1$ μM, $k_{inact} = 0.06\ min^{-1}$).[4f] In the case of 27-MS, the absence of the oxyrane moiety resulted in small changes in the folding conformation of the tetracyclic intermediate cation, which significantly reduced the stereoelectronic interactions with the active-site nucleophile.

Supplementary material complete set of spectroscopic data (^{1}H and ^{13}C NMR, HMQC, HMBC, and NOE) of 26-methylidenesqualene (**1d**), 27-methylidenesqualene



Scheme 1. Proposed mechanism for the conversion of (A) squalene (**1a**) to hop-22(29)-ene (**2a**), and 27-methylidenesqualene (**1e**) to 26-methylidene-hop-22(29)-ene (**2e**); (B) 26-methylidenesqualene (**1d**) to a C_{31} dammarene derivative with a 6.6.6.5+6 ring system (**3**) by recombinant *A. acidocaldarius* SHC.

(**1e**), 26-methylidene-hop-22(29)-ene (**2e**), and a C_{31} dammarene derivative with a 6.6.6.5+6 ring system (**3**) (10 pages).

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7. 26-Methylidenesqualene (**1d**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.37 (dd, 1H, $J = 17.6$, 10.8 Hz), 5.50 (t, 1H, $J = 7.4$ Hz), 5.14 (br m, 5H), 5.01 (d, 1H, $J = 16.8$ Hz), 4.92 (d, 1H, $J = 10.8$ Hz), 1.95–2.12 (m, 20H), 1.74 (s, 3H), 1.68 (s, 6H), 1.60 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 141.6, 138.8, 135.6, 135.0, 134.1, 133.0, 131.2 (×2), 124.6, 124.4, 124.3, 124.2, 123.9, 110.4, 39.7 (×3), 39.0, 28.6, 28.2, 27.9, 26.8 (×2), 26.7, 25.7, 17.7 (×2), 16.0 (×2), 16.0, 11.7. HRMS (EI): found for [$C_{31}H_{50}$] 422.3931; calcd 422.3913.
8. 27-Methylidenesqualene (**1e**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.36 (dd, 1H, $J = 17.2$, 10.8 Hz), 5.48 (t, 1H, $J = 7.4$ Hz), 5.12 (br m, 5H), 5.07(d, 1H, $J = 18.0$ Hz), 4.91 (d, 1H, $J = 10.8$ Hz), 1.95–2.26 (m, 20H), 1.73 (s, 3H), 1.68 (s, 6 H), 1.60 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 141.6, 135.2, 134.9, 134.6, 133.9, 132.9, 131.3 (×2), 124.7, 124.4 (×2), 124.3, 124.3, 110.3, 39.7, 39.7 (×2), 39.3, 28.2 (×2), 27.4, 26.9, 26.8, 26.6, 25.7 (×2), 17.7 (×2), 16.1, 16.0, 11.7. HRMS (EI): found for [$C_{31}H_{50}$] 422.3881; calcd 422.3913.
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10. The reaction mixture contained 26-MS or 27-MS (10 mg) and purified recombinant *A. acidocaldarius* SHC (30 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 as described in the previous paper.[5] The incubations were stopped by freezing and lyophilization, followed by extraction with 150 mL of hexane (×3). The combined extracts were evaporated to dryness, separated on SiO_2 TLC (developed twice first 5 cm in $CHCl_3$ then 16 cm in hexane), and finally purified by reverse phase HPLC (C8 column) to give 1.0 mg of **3** (from 26-MS) or 0.3 mg of **2e** (from 27-MS), respectively.
11. Compound **3**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.55 (m, 1H, H-21), 4.70 (s, 2H, H-27), 2.53 (m, 1H, H-23), 1.73 (s, 3H, Me-28), 0.93 (s, 3H, Me-18), 0.89 (s, 3H, Me-31), 0.85 (s, 3H, Me-29), 0.83 (s, 3H, Me-19), 0.80 (s, 3H, Me-30). ^{13}C NMR (100 MHz, $CDCl_3$): δ 147.1 (C-26), 139.5 (C-20), 120.2 (C-21), 108.2 (C-27), 57.0 (C-5), 51.0 (C-9), 49.6 (C-14), 46.2 (C-17), 45.0 (C-13), 42.2 (C-3), 42.0 (C-8), 41.5 (C-23), 40.6 (C-1), 37.5 (C-10), 35.0 (C-7), 33.4 (C-4, C-29), 33.0 (C-15), 32.0 (C-25), 31.2 (C-22), 28.3 (C-24), 27.0 (C-16), 25.3 (C-12), 21.9 (C-11), 21.5 (C-30), 20.7 (C-28), 18.7 (C-6), 18.6 (C-2), 17.0 (C-31), 16.2 (C-19), 15.9 (C-18). HRMS (EI): found for [$C_{31}H_{50}$] 422.3930; calcd 422.3913.
12. 26-Methylidene-hop-22(29)-ene (**2e**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.25 (dd, 1H, $J = 18.0$, 11.2 Hz, H-26), 5.23 (dd, 1H, $J = 11.2$, 2.0 Hz, H-31, *cis*), 4.90 (dd, 1H, $J = 18.0$, 2.0 Hz, H-31, *trans*), 4.77 (s, 2H, H-29), 2.64 (dt, 1H, $J = 7.4$, 7.4 Hz, H-21), 1.73 (s, 3H, Me-30), 1.00 (s, 3H, Me-27), 0.83 (s, 3H, Me-23), 0.78 (s, 3H, Me-25), 0.75 (s, 3H, Me-24), 0.71 (s, 3H, Me-28). ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1 (C-22), 141.2 (C-26), 114.0 (C-31), 109.9 (C-29), 56.7 (C-5), 54.7 (C-17), 51.3 (C-9), 50.3 (C-13), 48.5 (C-8), 46.3 (C-21), 45.0 (C-18), 42.1 (C-19), 42.0 (C-3), 41.8 (C-14), 40.0 (C-1), 38.2 (C-10), 38.0 (C-7), 33.6 (C-15), 33.5 (C-4, C-23), 27.2 (C-20), 25.0 (C-30), 24.0 (C-12), 21.6 (C-24, C-16), 19.7 (C-11), 18.7 (C-2, C-6), 16.7 (C-27), 16.3 (C-28), 15.1 (C-25). HRMS (EI): found for [$C_{31}H_{50}$] 422.3889; calcd 422.3913.
13. The enzyme inhibition tests were carried out as described before.[4b]