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Enzymatic Formation of an Unnatural Hexacyclic C_{35} Polyprenoid by Bacterial Squalene Cyclase

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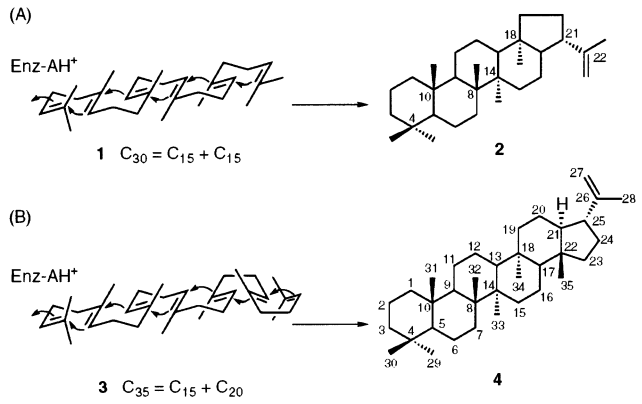
The formation of pentacyclic triterpene by squalene:hopene cyclase (SHC) (E.C. 5.4.99.7) offers an impressive example of enzyme-templated sequential polyene cyclization reactions. SHC binds squalene (**1**) in all-*chair* conformation, and mediates formation of new carbon−carbon bonds in regio- and stereospecific manner to produce the pentacyclic ring system of hop-22(29)-ene (**2**) (Scheme 1A).[1] Recent crystallographic and structure-based mutagenesis studies together with affinity labeling experiments on the SHC from a thermoacidophilic bacteria *Alicyclobacillus acidocaldarius* revealed structural details of the active site of the enzyme located in a large central cavity.[1,2] The *A. acidocaldarius* SHC is a 72 kDa membrane-bound enzyme with its catalytic optimum at 60 °C and pH 6.0.[3]

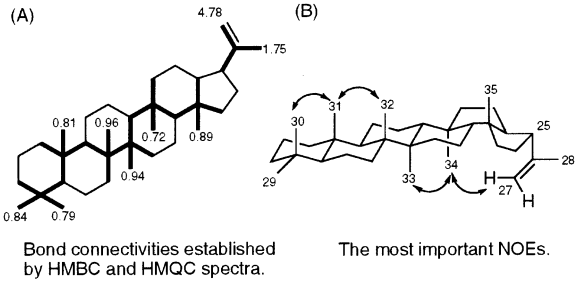
Interestingly, the bacterial SHC, catalyzing a stereochemically and mechanistically simpler process than eukaryotic oxidosqulane cyclases, shows broad substrate specificity; the enzyme accepts a wide variety of nonphysiological squalene analogues (C_{25}−C_{31}) and efficiently performs sequential ring-forming reactions to produce a series of unnatural cyclic triterpenoids.[4] On the basis of the crystal structure of the enzyme, the hydrophobic active site cavity (ca. 1200 Å^3) lined with aromatic amino acid residues appears to have enough space to accept larger substrate analogues (C_{35}) with an additional isoprene unit. By utilizing such properties of the enzyme, we conducted several enzymatic conversion experiments of synthetic substrate analogues to generate unnatural polycyclic polyprenoids. Herein we describe the enzymatic formation of a novel hexacyclic polyprenoid (**4**) from a C_{35} analogue (**3**) in which a farnesyl C_{15} unit is connected in a head-to-head fashion to a geranylgeranyl C_{20} unit by the *A. acidocaldarius* SHC (Scheme 1B). This is the first demonstration of the remarkable ability of the squalene (C_{30}) cyclizing enzyme to perform construction of the unnatural hexacyclic skeleton.

The convergent synthesis of the C_{35} analogue (**3**)[5] involved the coupling of farnesyl phenylsulfone with geranylgeranyl bromide, followed by dephenylsulfonation by $LiHBEt_3$ in the presence of catalytic amount of $PdCl_2$[1,3-bis(diphenylphosphino)propane] as described before.[4g,h] When the substrate was incubated with purified recombinant *A. acidocaldarius* SHC,[6] hexacyclic C_{35} polyprenoid (**4**) ($[\alpha]^{25}_D = -22.0°$) was isolated from the incubation mixture as a single product (10% yield from 10 mg of **3**).[7] The ^{1}H NMR spectrum revealed the presence of six methyl singlets (δ 0.84, 0.79, 0.96, 0.81, 0.94, and 0.72) almost identical with those of the C-4α, C-4β, C-8β, C-10β, C-14α, and C-18α methyl group of hop-22(29)-ene (**2**).[8] Further, there were an additional methyl singlet (δ 0.89), one vinylic methyl (δ 1.75), and two vinylic protons (δ 4.78, s, 2H). A structure with the 6/6/6/6/6/5-fused hexacyclic ring system was uniquely consistent with both biogenetic reasoning and NMR

Scheme 1



Scheme 2



spectroscopic data: homonuclear (^{1}H−^{1}H COSY) and heteronuclear (^{1}H−^{13}C, HMQC, and HMBC) correlation spectroscopy (Scheme 2A). In addition, NOE experiments (NOESY and NOE difference spectroscopy) allowed us to elucidate the ring junctions and to evaluate the stereochemistry of ring substituents. Thus, occurrence of NOEs between Me-30/Me-31, Me-31/Me-32, Me-33/Me-34, Me34/H-27, whereas no NOE were observed between Me-34/Me-35, indicated the α-axial orientation of Me-33, Me-34, and the isopropenyl group at C-25, as well as the β-axial orientation of Me-35 (Scheme 2B).

The cyclization of the C_{35} analogue (C_{15} unit + C_{20} unit) was directional; it was initiated by the C_{15} unit (Scheme 3B), and not by a proton attack on the terminal double bond of the C_{20} unit (Scheme 3C). This suggests α-orientation of the *pro*-C14 methyl group is crucial to the correct folding and binding of the substrate prior to the cyclization, as in the case of the formation of hop-22(29)-ene from C_{30} squalene (C_{15} unit × 2) (Scheme 3A). The alternative β-orientation of the *pro*-C14 methyl group may interact repulsively with the *pro*-C10 methyl or a nearest neighbor from the substrate binding pocket of the enzyme. Further, the substrate should be folded in *chair*−*chair*−*chair*−*chair*−*boat*−*boat* conformation of the C_{35} analogue (**3**) (Scheme 4D) to achieve the above-mentioned stereochemistry of the C-18, C-22, and C-25 of

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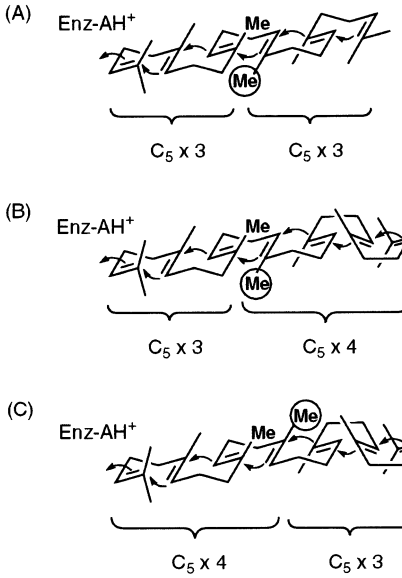
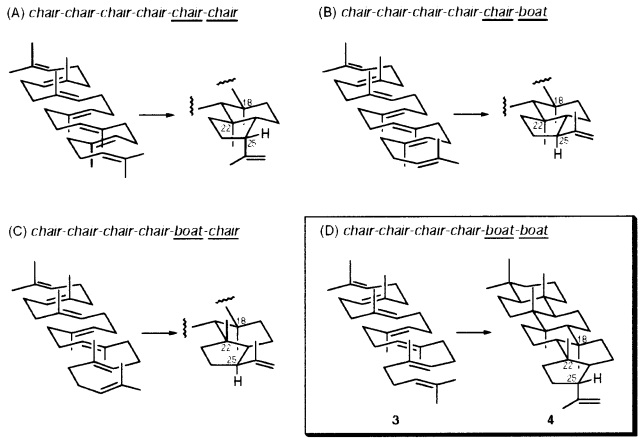
Scheme 3



Scheme 4



the cyclization product (**4**); that is the α-axial orientation of Me-34 and the isopropenyl group at C-25, as well as the *β*-axial orientation of Me-35. Other folding conformations of the substrate (Scheme 4A−C) lead to formation of products whose stereochemistry is not consistent with the results of the NOE experiments.

Moreover, as in the case of the formation of hop-22(29)-ene, the cyclization reaction proceeded without rearrangement of carbon and hydrogen and was terminated by proton elimination at C-27. No product with a hydroxyl group resulting from cation hydration was detected in the reaction mixture, which was confirmed by LC−MS analysis. On the basis of the crystal structure of the enzyme, 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.[1b] The enzyme thus acted as a chaperone for the reactive carbocationic intermediates against addition of water or deprotonation by base, and terminated the reaction by specific proton elimination at C-27.

In summary, this communication presents the first demonstration of the enzymatic formation of unnatural hexacyclic C_{35} polyprenoid by squalene cyclizing enzyme. It was remarkable that the enzyme accepted the substrate with an additional isoprene unit and tightly controlled the stereochemistry of the formation of the 6/6/6/6/6/5-fused hexacyclic ring system. Manipulation of the enzyme reaction by substrate analogues (C_{35}−C_{45}) along with utilization of rationally engineered mutant enzymes would lead to further production of chemically and structurally disparate unnatural polycyclic polyprenoids, which is now in progress in our laboratories.

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Supporting Information Available: Comparative NMR data for the hexacyclic C_{35} polyprenoid **4** and hop-22(29)-ene (**2**), as well as complete set of NMR spectra of **4** (^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, HMBC, NOESY, and NOE difference spectroscopy) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 5.22−5.10 (brm, 7H), 2.09−0.97 (brm, 24H), 1.68 (s, 6H), 1.60 (brs, 12H), 1.58 (s, 3H), 1.56 (s, 3H), 1.55 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 135.8 (×3), 134.9 (×2), 131.2 (×2), 124.4 (×2), 124.3 (×4), 118.3, 39.7 (×5), 26.8 (×2), 26.6 (×5), 25.7 (×2), 17.7 (×2), 16.0 (×3), 15.6, 13.3. HRMS (FAB): found for $[C_{35}H_{59}]^+$ 479.4641; calcd. 479.4617.

(6) The SHC cDNA was PCR amplified from *A. acidocaldarius* (IFO15652T strain from the Institute for Fermentation, Osaka, Japan), and cloned into the *Nde*I/*Hind*III site of pET-22b(+) (Novagen). Recombinant enzyme was then expressed in *Escherichia coli* BL21(DE3)pLysS, and purified as described before.[3d, 4f−i]

(7) The reaction mixture contained the C_{35} polyprene (**3**) (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, 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 1.0 mg of compound **4**.

(8) ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 4.79 (s, 2H, H-27), 2.52 (m, 1H, H-25), 1.75 (s, 3H, Me-28), 0.96 (s, 3H, Me-32), 0.94 (s, 3H, Me-33), 0.89 (s, 3H, Me-35), 0.84 (s, 3H, Me-29), 0.81 (s, 3H, Me-31), 0.79 (s, 3H, Me-30), 0.72 (s, 3H, Me-34). ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 148.7 (C-26), 110.0 (C-27), 56.1 (C-5), 54.9 (C-17), 50.3 (C-9), 50.3 (C-21), 49.4 (C-13), 46.4 (C-25), 42.1 (C-3), 42.1 (C-14), 42.1 (C-18), 41.9 (C-8), 41.9 (C-19), 40.3 (C-1), 40.3 (C-22), 37.4 (C-10), 33.6 (C-15), 33.4 (C-29), 33.2 (C-4), 33.2 (C-7), 31.9 (C-20), 27.4 (C-24), 25.0 (C-28), 24.0 (C-12), 22.7 (C-23), 21.6 (C-16), 21.6 (C-30), 20.9 (C-11), 18.7 (C-2), 18.7 (C-6), 16.7 (C-32), 16.7 (C-33), 16.0 (C-34), 15.8 (C-31), 15.8 (C-35). The NMR assignments were performed according to data from ^{1}H−^{1}H COSY, HMQC, HMBC, NOESY, differential NOE experiments, and by comparison with that of hop-22(29)-ene (**2**). LRMS (FAB): *m/z* 479, 439, 409, 288, 232, 218, 191. HRMS (FAB): found for $[C_{35}H_{58} + K]^+$ 517.4188; calcd. 517.4176. $[\alpha]^{25}_D = -22.0°$ ($c = 0.10$ in $CHCl_3$).

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