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Production of Bicyclic and Tricyclic Triterpenes by Mutated Squalene-Hopene Cyclase

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Abstract. Mutagenesis of Tyr420 to Ala in the catalytic cavity of squalene-hopene cyclase of *Alicyclobacillus acidocaldarius* resulted in an altered product spectrum. Besides hopene and diplopterol, this mutant produced significant amounts of the bicyclic α- and γ-polypodatetraenes and minor amounts of the tricyclic 13α(H)-malabaricatriene. © 1999 Published by Elsevier Science Ltd. All rights reserved.

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About 100 different carbon skeletons of mono- to pentacyclic triterpenes are formed by squalene and (3*S*)-2,3-oxidosqualene cyclases. All known triterpene cyclases present sequence similarities and contain an amino-acid repeat at corresponding sequence positions.[1,2] A well investigated cyclase is the membrane bound bacterial squalene-hopene cyclase (SHC), that catalyzes the conversion of squalene **1** into hop-22(29)-ene (diploptene) **2** and hopan-22-ol (diplopterol) (Fig.1).[3,4]

As a first step to understanding the vast diversity in cyclization products, the X-ray structure of squalene-hopene cyclase (SHC) of *Alicyclobacillus acidocaldarius* was elucidated at a 2.0 Å resolution, and a reaction mechanism proposed.[5,6] The peptide chain of SHC is organized in two α-helix bundles. From the center of a large hydrophobic patch permitting interactions with the membrane, a non-polar channel allows the access to the catalytic cavity located between the two α-helix bundles. The active site is mainly lined by 10 conserved aromatic residues (Fig. 2), which are expected to stabilize intermediate carbocations and to simultaneously determine the folding of the substrate before and during the cyclization. Asp376, together with two other Asp residues, is located at the opposite side of the channel entrance and is believed to initiate the cyclization cascade by protonation of the terminal squalene double bond.

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The deprotonation site is presumably represented by a cluster of polar amino acid associated with a water molecule and is close to the channel entrance. Surprisingly, SHC is not completely product specific. Byproducts are formed besides the main cyclization products, hop-22(29)-ene **2** and hopan-22-ol: neohopene and tetracyclic compounds, including 17-isodammara-20(21),24-diene, each representing about 0.5 to 1.5 % of the total hopene amount produced.[7]

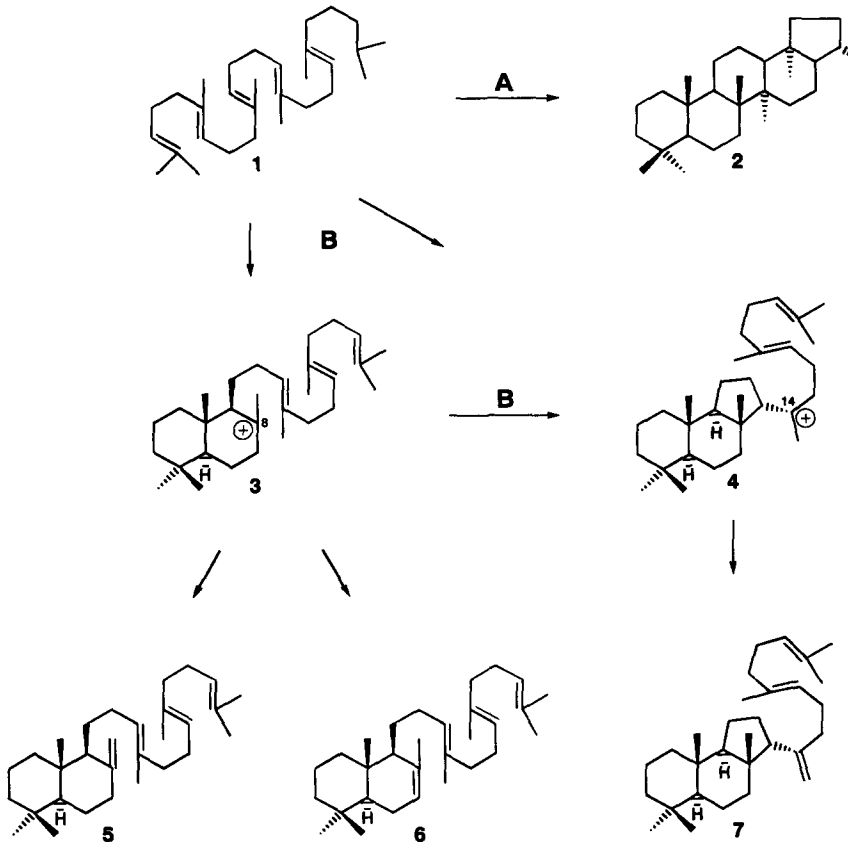

Figure 1 : Squalene **1** cyclization into hop-22(29)-ene **2** by the wild type and mutated cyclases **(A)**. Formation of polypodatetraenes **5** and **6** and malabaricatriene **7** by Tyr420Ala mutated cyclase **(B)**.

The first SHC mutants with an altered product specificity were recently described.[8,9] The mutants Phe601Ala and Trp169Phe lead to an enhanced formation of one of the side-products, 17-isodammara-20(21),24-diene.[8,9] The mutant Tyr420Ala produced two new hydrocarbons which were not produced in detectable amounts by the wild-type enzyme and which represented respectively 23 and 10 % of the total hopene amount.[9]

These two hitherto uncharacterized bicyclic compounds have now been isolated[10] and identified as α-polypodatetraene **5** (with the shortest retention time) and γ-polypodatetraene **6** by ^{1}H- and ^{13}C-NMR and GC-MS and comparison of the data with those from the literature.[11,12] Polypodatetraenes were formerly isolated from several ferns.[11,12] An additional very minor fern hydrocarbon, (17*E*)-13α(H)-malabarica-14(27),17,21-triene **7**, was isolated in low amounts and identified by GC-MS and ^{1}H-NMR and comparison of the spectra with literature data,[13] as well as by GC coelution (60 m DB1 capillary column) with a thoroughly identified reference sample isolated from the sediments of Lake Cadagno.[14]

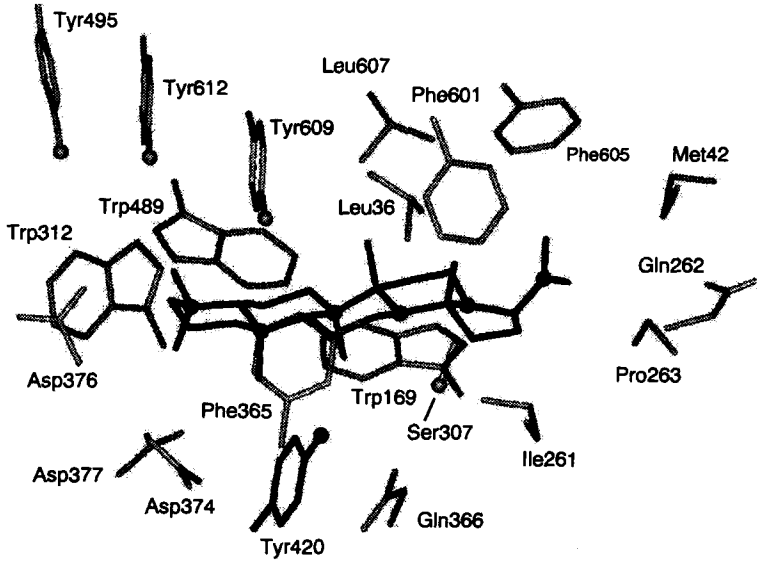

Figure 2. Essential residues of active site of hopene-squalene cyclase with a modelled hop-22(29)-ene molecule.

The positions of the intermediate carbocations are indicated as spheres on the hop-22(29)-ene skeleton. Only the side chain of the amino acids are represented. Tyr420 is drawn in black. Oxygen atoms of hydroxyl groups are also shown as spheres.

According to the proposed mechanism for the SHC catalyzed reaction, it is rather consistent to find cyclization products with less than four rings for the mutant Tyr420Ala, as compared with the wild type and the Phe601Ala and Trp169Phe mutants. Indeed, Tyr420 is much closer to the protonation site than Phe601 and Trp169. How does the replacement of this Tyr by Ala induce the synthesis by the enzyme of bi- and tricyclic triterpenes while simultaneously maintaining the production of relatively high amounts of hopene? This answer is not obvious. A possible explanation for the properties of mutant Tyr420Ala cyclase would be a less precise fit of the folded squalene into the catalytic site because of the replacement of the flat and rigid Tyr aromatic ring by the small nearly spherical Ala methyl group. As a result, alternative

routes may be followed for the cyclization of squalene. The replacement of Tyr420Ala still allows a quasi-normal conformation of the substrate in the active site of the enzyme. It does not affect in a too drastic manner the cyclization, and the regular process can be followed yielding hop-22(29)-ene **2** that is still observed as the major product. This replacement also introduces significant perturbations in the enzyme/substrate complex that allow in addition erroneous cyclizations. On the one hand, the less precise fit of the substrate might induce less tight positioning of the squalene double bonds towards the C8 or C14 cations than that required for a normal cyclization. Such an inaccurate positioning of the substrate double-bonds towards cationic sites was proposed to induce the minor mistakes observed in the case of the wild type enzyme.[7] On the other hand, the free electron pair of the Tyr420 hydroxy group might help to compensate the electron deficiency of the C8 or C14 carbocations **3** and **4** (Fig. 1). In the mutant, such a stabilization is lost, facilitating the elimination of the more acidic protons in β-position. Both processes might result in a premature termination of the cyclization into bi- and tricyclic triterpenes, and non-specific proton elimination by basic sites, normally not involved in the catalysis, would yield the observed olefins. The respective contributions of aromatic amino acid side chains with high electron densities to the folding of the substrate and/or to the stabilization of cationic intermediates have still to be addressed by additional experiments.

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