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Altered Product Pattern of a Squalene-Hopene Cyclase by Mutagenesis of Active Site Residues

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Abstract. Amino acid residues lining the catalytic cavity of squalene-hopene cyclase of *Alicyclobacillus acidocaldarius* have been mutated. Alterations of His451 to Ala and Trp489 to Ala resulted in reduced enzymatic activity, while the product patterns were identical to that of the wild-type. Mutation of Phe601 to Ala led to the enhanced formation of a tetracyclic triterpene, 17-isodammara-20(21),24-diene **4**, and of Tyr420 to Ala to a significant alteration of the product pattern.

Keywords: Squalene cyclization, hopene, triterpene synthase

The enzymatic formation of cyclic triterpenes from squalene or (3*S*)-2,3-oxidosqualene is a highly complex and specific cyclization process leading to about 100 different carbon skeletons. Recently the X-ray structure of the first triterpene cyclase, namely the squalene-hopene cyclase from *Alicyclobacillus acidocaldarius,* has been elucidated, and a reaction mechanism proposed.[1,2] This three-dimensional structure hopefully will form the basis for the understanding of the variability and specificity of triterpene cyclases.

The catalytic site of this membrane-bound enzyme is located in a cavity which is accessible through a non-polar channel connecting the cavity with the non-polar part of the cytoplasmic membrane where squalene **1** is dissolved. The active site comprises a cluster of aspartate residues among which Asp376 is presumably the proton donor starting the cyclization cascade (**Fig. 1**).[1-4] The cavity is lined mainly by aromatic residues which may expected to stabilize intermediate carbocations.[2] Glu45 and Glu93 abstract the proton from the final carbocation via a water molecule polarized by a hydrogen bonding network.[1] His451 is expected to be involved in the initial protonation of the terminal double bond of squalene by enhancing the acidity of the catalytic Asp376.[1,2] Tyr420 may play a structural role in the folding process of the hopene skeleton in B- or C-ring

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formation. Trp489 has been proposed to stabilize the C-10 cation during A-ring formation, whereas Phe601 is in a position to stabilize the C-18 cation.[2]

Recently we observed in wild-type hopene cyclase some degree of unspecifity in the cyclization reaction. Along with hop-22(29)-ene **5** and diplopterol (= hopan-22-ol) **6,** neohopene, tetracyclic dammara-dienes and eupha-7,24-diene were detected in a yield of 0.5 - 1.5 % of the hopene-peak.[5]

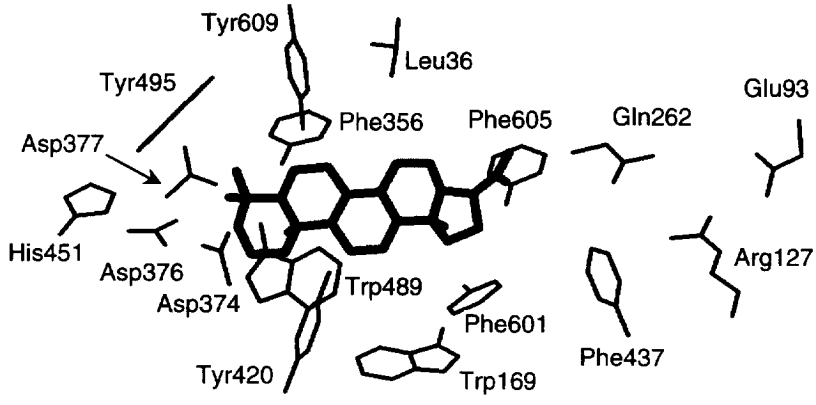


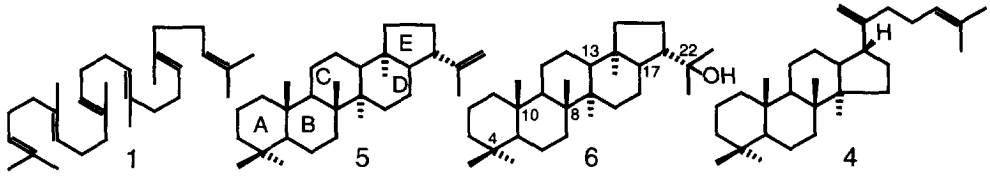
Figure 1. Essential residues of the active site cavity of squalene-hopene cyclase. The model of hopene (grey) is positioned according to Reference 2.

The availability of an X-ray structure provides the opportunity to alter the active site of the cyclase by site-directed mutagenesis, and consequently to modify the product pattern. These experiments should also help to verify current ideas about the cyclization mechanism.

Site-directed mutagenesis of gene fragments was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech).[6] The mutated gene fragments were controlled by sequencing. To yield a complete cyclase gene the corresponding wild-type fragments were exchanged by the mutated ones. The cyclases were prepared from transformed *E. coli* cells by isolation of the cell membrane fraction and solubilization by Triton X-100®. This crude preparation was used for the enzymatic test. Due to the low activity of the mutated cyclases, the activity tests were performed at 50 °C for 12 h (wild-type, 1 h) as described.[3] After extraction of squalene and the cyclization products (*n*-hexane : isopropanol, 3:2) from the assay mixture, Triton X-100® was removed by separation on a small silica gel column (*n*-hexane : ethyl acetate, 1:1). The hydrocarbon mixture (squalene und cyclization products) were analysed by GC and GC/MS.[3,5]

Some properties of the mutants are described in **Table 1**. GC traces of squalene and the reaction mixture are shown in **Fig. 2**. In comparison to the wild-type, the product patterns of mutants Tyr420Ala and Phe601Ala show significant differences.

Table 1. Properties of squalene-hopene cyclase mutants	
His451Ala	reduced activity; wild-type product pattern
Tyr420Ala	reduced activity; altered product pattern
Trp489Ala	reduced activity; wild-type product pattern
Phe601Ala	reduced activity; altered product pattern



Mutant His451Ala is not fully inactivated. This result is not surprising, since the residual acidity of Asp376 may suffice for a slower protonation step. Interestingly His451 is not a conserved residue; in other hopene cyclases, Arg is at the corresponding position.

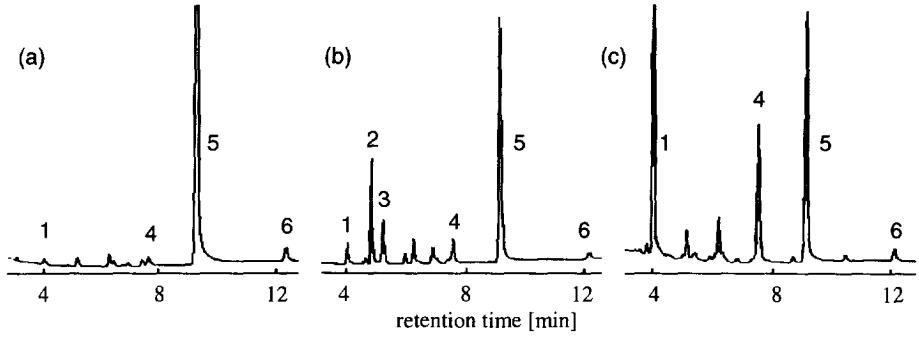


Figure 2. Gaschromatographic traces of the product pattern of wild-type squalene-hopene cyclase (a), mutant Tyr420Ala (b) and mutant Phe601Ala (c). **1**, squalene; **4**, 17-isodammara-20(21),24-diene; **5** hopene; **6**, diplopterol. Compounds **2** - **4** are discussed in the text.

Mutant Tyr420Ala has a lower activity and yields a significantly altered distribution of products (**Fig. 2b**). According to GC/MS, compounds 2 and 3 likely correspond to a mono- and a bi- or tricyclic triterpene. These structures are under investigation and may provide insight into the function of Tyr420.

Mutant Trp489Ala resulted in a significant loss of activity without any alteration of the product pattern. The stabilization of the cation at C-10 by Trp489 seems not to be essential for the product pattern, but rather for the enhancement of the reaction velocity, which is in accordance with the proposed π-cation interaction.[7]

The product significantly enhanced in mutant Phe601Ala (no. 4 in **Fig. 2c**) is identical with one of the minor products of the wild-type enzyme, tentatively characterized as 17-isodammara-20(21),24-diene **4**.[5] This was shown by comparison of the GC retention time and the mass spectra obtained by GC/MS. Phe601 has been proposed to be well positioned for the stabilization of cation C-18 during a 5-exo Markovnikov oriented D-ring closure.[2] The significant increase of product **4** in mutant Phe601Ala corroborates this role since a loss of stabilization of the 5-exo C-18 cation will facilitate premature quenching of the intermediate. Based on experiments with substrate analogues, a similar 5-exo intermediate has been proposed for the C-ring closure by oxidosqualene-lanosterol cyclase.[8]

This structure-based mutagenesis study provides for the first time light on structure-activity relationships in a triterpene cyclase and opens the field for the engineering of cyclases generating unusual triterpenes.

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