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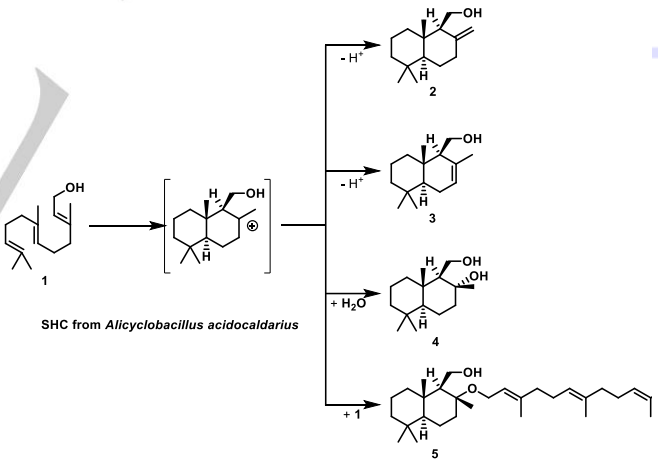
Enzymatic Addition of Alcohols to Terpenes by Squalene Hopene Cyclase Variants

Lisa C. Kühnel, Bettina M. Nestl and Bernhard Hauer*[a]

Abstract: Squalene hopene cyclases (SHC) catalyze the polycyclization of squalene into a mixture of hopene and hopanol. Recently, amino acid residues lining the catalytic cavity of the SHC from *Alicyclobacillus acidocaldarius* were replaced by small and large hydrophobic amino acids. The alteration of leucine 607 to phenylalanine resulted in increased enzymatic activity towards the formation of an intermolecular farnesyl-farnesyl ether product from farnesol. Furthermore, the addition of small chain alcohols acting as nucleophiles led to the formation of non-natural ether-linked terpenoids and thus, to a significant alteration of the product pattern compared to wildtype. It is proposed that the mutation of Leu at position 607 may facilitate premature quenching of the intermediate by small alcohol nucleophiles. This mutagenesis-based study opens the field for further intermolecular bond forming reactions and generation of non-natural products.

The squalene-hopene cyclase from *Alicyclobacillus acidocaldarius* (*Aac*SHC) is a triterpene cyclase that catalyzes in nature the cyclization of the linear triterpene squalene to hopene and hopanol. This complex polycyclization is one of the most demanding biochemical reactions forming five new C-C bonds and nine stereocenters.[1] The reaction is initiated by binding the substrate in a specific product-like conformation and triggering of cation formation by protonation of a double bond. The highly acidic Asp376 of *Aac*SHC is the electrophilic residue that initiates the cyclization by protonating the terminal isopropylidene of squalene. The enzyme provides a highly conserved, hydrophobic active site containing electron-rich amino acids that facilitate the production of carbocations as well as the shielding of carbocations from solvent.[2] *Aac*SHC cyclase exhibits a remarkably broad substrate tolerance forming new C-C and C-X bonds. Functionalized acyclic terpene derivatives can be used as substrates by SHCs for the synthesis of numerous non-natural carbopolycyclic and heteropolycyclic products in a stereoselective manner. One commercial interesting example is the enzymatic synthesis of the high valuable flavor compound ambroxan starting from the simple alcohol precursor homofarnesol in the grams-scale.[3] Various nucleophiles attacking the carbocationic intermediates in the reaction enabled the stereoselective syntheses of a multitude of polycyclic products. Starting from small sesquiterpenes like farnesol (C_{15}) up to C_{35} non-natural polyprenoids were successfully converted.[4–9] SHCs can be also utilized for carbon-carbon, carbon-oxygen and carbon-nitrogen bond formation reactions using functionalities such as alcohols, aromatic systems, carboxylic acids, ketones or amides.[10–12] Previous work demonstrated that SHCs can perform Friedel–Crafts alkylations with aromatics as well as hydroamidations revealing the potential of these enzymes for chiral Brønsted acid catalysis.[13,14]

Hoshino and coworkers observed in wildtype *Aac*SHC some degree of unspecificity in the cyclization of the truncated squalene substrate farnesol (**1**).[5] A conversion of 64% was observed for **1** giving four products (**2** – **5**) that were formed from one common bicyclic intermediate (Scheme 1). Along with deprotonated alkene products albicanol (**2**) and drimenol (**3**) and the corresponding alcohol drimane-8, 11-diol (**4**) as major product, the nucleophilic attack of the hydroxyl group of a second farnesol molecule to the bicyclic skeleton forming the ether linkage product **5** was detected



Scheme 1. Cyclization reaction of farnesol (**1**) and resulting products (**2-5**). Major product **4** (45%) is followed by minor products **2** (3%), **3** (7%) and **5** (9%).[5]

This was the first reported example where the carbocation generated on the farnesol molecule was quenched by reacting with an available substrate molecule to produce an ether. On the basis of the crystal structure of the enzyme, the hydrophobic active site cavity lined with aromatic amino acid residues appears to have enough space to accept two molecules of farnesol. This result prompted us to evaluate the manipulation of the enzyme reaction by substrate analogues that would lead to chemically and structurally novel, non-natural ether products.

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We report herein the enzymatic cyclization of farnesol in which the terpenoid unit is connected with geraniol or small alcohol nucleophiles using *Aac*SHC variants. In contrast to the cyclization of farnesol, the incubation of geraniol with purified recombinant *Aac*SHC wildtype did afford very small amounts of its cyclization product. This may reflect the fact that the active site of the enzyme does not favour a reactive binding of the geraniol substrate.[5,12,15] Our previous studies suggested that the alteration of the active site pocket of *Aac*SHC facilitated the conversion of truncated terpene-like substrates without disturbing the important catalytic machinery.[12,16]

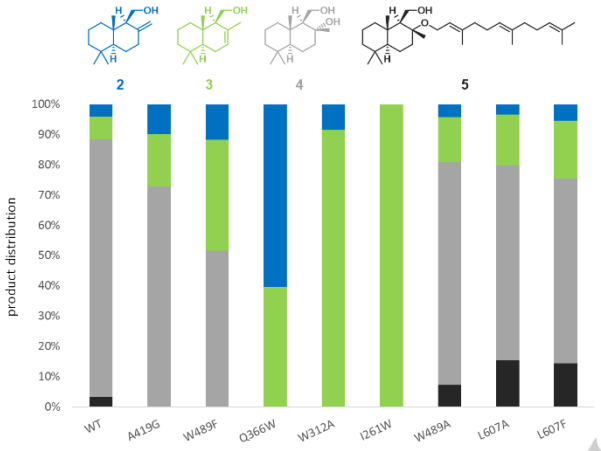
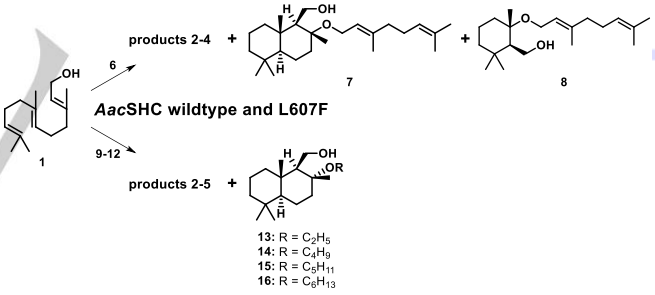


Figure 1. Library screening of *Aac*SHC wildtype and variants for the cyclization of farnesol (**1**) towards products **2-5**. Biotransformations were performed with purified protein in triplicates.

Mutant design was approached by site-directed mutagenesis of active site amino acid residues. The introduction of small hydrophobic and aromatic residues that preserve the hydrophobic nature of the active site was explored to fine-tune and re-size the active site of the *Aac*SHC. The analysis of the single mutant library identified variants that were highly active towards the truncated squalene substrate farnesol (conversion of **1** ≥50 %, see also Supporting Information Figures S1 and S4). Selected variants are shown in Figure 1. The analysis of the *Aac*SHC mutant library allowed us to identify regions in the active site responsible for favouring either deprotonation or addition products (Figure 2). Replacement of active site residues resulted in small but important alterations in the precise positioning and folding of the substrate within the active site. Further, these results suggested that small changes in the active site geometries are sufficient to alter the selectivity of the reaction. The synthesis of **4** as major product suggests that cation hydroxylation during the annulation phase generally occurs by attack of water inside the active site cavity. Remarkably, most variants altered the product selectivity in favour of the deprotonation products **2** and **3.** Three variants demonstrated significant increases in the formation of bicyclic products **2** and **3**, influencing the cation deprotonation. An excellent product selectivity (>99 %) towards **3** was observed by variant I261W (Figure 1). The observation that most variants showed an increased formation of cyclization products **2** and **3** can be explained by the re-sizing of the active site that prevented the reactive carbocation intermediate from reacting with water or other nucleophiles.

*Aac*SHC variants with alanine or phenylalanine at position 607 displayed highest activities and selectivities towards the farnesyl-farnesyl ether product **5**. An approximate four-fold increased formation of **5** was obtained with variant L607F compared to the wildtype (Table 1). Up to 65.1% ± 5.4% of **1** was successfully converted into the enzymatic products. Inspired by these results, we further investigated the *Aac*SHC mutant library for increased formation of **5**. Alternatively, we became interested in expanding the reaction scope towards new cyclization-addition products quenching the carbocation intermediate with geraniol (**6**) and small alcohols as nucleophiles (**9**-**12**). To determine the substrate specificity of the L607F variant towards the nucleophilic addition of further terpenoids, biotransformations using farnesol (**1**) with geraniol (**6**) were performed. GC analysis of the incubation mixture of **1** and 1 % (*v/v*) **6** with *Aac*SHC wildtype and variant L607F afforded five product peaks. Table 1 shows the GC profile of the product distribution pattern. Interestingly, in addition to **2**-**4,** two novel ether products **7** and **8**, in which the geraniol unit is connected to the drimane (**7**) and cyclogeraniol (**8**) skeletons, were formed (Figure S2). An approximately 4-fold increased formation of these ethers products was obtained with variant L607F, while no farnesyl-farnesyl ether **5** was detected (Table 1). This might be explained by the fact that an excess amount of **6** was utilized in biotransformations competing with the farnesol substrate. Recent investigations of Syrén *et al.* on the cyclization of geraniol and geranyl alkyl ethers by *Aac*SHC revealed that increased alkyl substituents provided increased activities for the cyclization and selectivities towards the geranyl alkyl ether.[12]



Scheme 2. Reactions leading to novel ether-products (**7, 8, 13-16**) and other cyclization products (**2-5**) using *Aac*SHC wildtype and L607 variants

It is anticipated that aliphatic alcohols (ethanol **9**, *n*-butanol **10**, *n*-pentanol **11** and *n*-hexanol **12**) were subjected to the enzymatic reaction with *Aac*SHC wildtype and L607F in order to synthesize novel non-natural ethers (Scheme 2). Reduced conversions of **1** were observed with 1% (*v/v*) **9** and **12** as nucleophiles (conversions of 41.2% ± 10.6% and 22.5 ± 4.03%, respectively). A similar observation about inhibition of *Aac*SHC in the presence of 2% ethanol was be made by Gärtner in 1987.[17] Whereas farnesol was less converted in the presence of **9** and **12**, formation of

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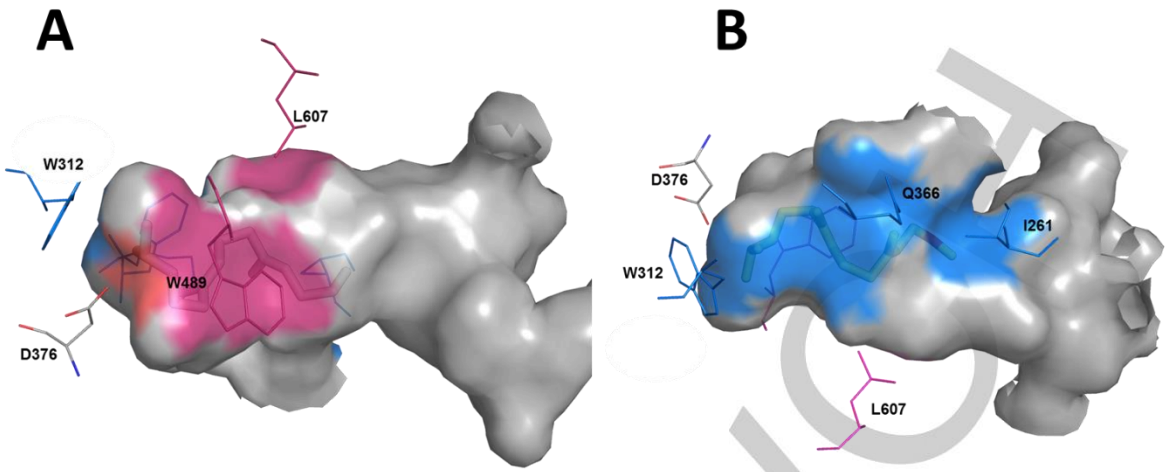

Figure 2. Surface presentation of the *Aac*SHC catalytic domain (PDB code 1UMP, grey surface) and farnesol (**1**) as sticks presented separately. The active site aspartic acid D376 is shown as lines (red surface). (**A**) Replacement of residues W489 and L607 (shown in pink) provided increased formation of farnesyl-farnesyl ether product **5** in the course of the cyclization of **1**. (**B**) Active site of AacSHC was rotated 180° compared to their position in (**A**) around the x-axis. Also shown in blue are the residues W312, Q366 and I261 that were replaced by alanine or tryptophan in order to favour the formation of elimination products **2** and **3**.

farnesyl butyl- as well as pentyl ethers **14** and **15** was monitored using **10** and **11** (Table 1, Figures S3 and S5). Farnesol (**1**) incubated with **10** and **11** were competent substrates for the enzyme and efficiently converted to their corresponding ether products. 61.7% ± 2.3% of farnesol was converted in when incubated with *n*-butanol (10) and 40.5 ± 6.95% with *n*-pentanol, respectively. Again, improved ether formations were obtained by variant L607A compared to the wildtype.

Table 1. Product distribution [%] for the conversion of farnesol (**1**) with **1**, geraniol (**6**), *n*-butanol (**10**) and *n*-pentanol (**11**) using *Aac*SHC wildtype and variant

nucleo-phile	variant	2	3	4	5	7	8	14	15
1	WT	4	7	85	4				
	L607F	6	19	61	14				
6	WT[a]	17	17	55	0	2	9		
	L607F[a]	4	14	41	0	8	33		
10	WT[a]	14	15	66	1			4	
	L607F[a]	14	27	43	7			9	
	L607F[b]	14	19	36	3			28	
11	WT[a]	15	17	57	1				10
	L607F[a]	14	24	43	2				17

Reaction conditions: farnesol (2 mM in DMSO), 1-2.5 % (*v/v*) nucleophile, citrate buffer pH 6 (60 mM), 0.01 % CHAPS, 1 mg/mL purified catalyst, 65°C, 24 h [a] 1% (*v/v*) of geraniol (**6**), *n*-butanol (**10**) and *n*-pentanol (**11**) [b] 2.5% (*v/v*) of *n*-butanol (**10**)

A raise in the concentration of *n*-butanol from 1% (*v/v*) to 2.5% (*v/v*) led to 3-fold enhanced formation of **14**, however, enhancing the amount of **10** up to 2.5% (*v/v*) resulted in an abolished activity towards farnesol. In particular, variant L607F generated up to 28% more of **14**, while a drop in the formation of **4** was obtained. Remarkably, while the increase of **14** occurred at the expense of product **4,** we have seen little differences in the formation of deprotonation products **2** and **3** when adding the alcohols as nucleophiles. These data suggest that *n*-butanol (**10**) and *n*-pentanol (**11**) are competing with water in the active site to perform the nucleophilic attack at the final bicyclic cation. This insight aligns with observations that mutations of the active site of *Aac*SHC affect the cyclization reaction and the subsequent quenching of the carbocation intermediate (nucleophilic addition favored over deprotonation). Analogously, active site variants have a great plasticity to produce non-natural products by connecting a farnesol unit with small alcohols in an intermolecular fashion.

In conclusion, we were able to modify the product specificity by engineered variants of *Aac*SHC that have altered specificity. We demonstrated that the residue at position 607 in the active site of *Aac*SHC is important for the intermolecular coupling reaction of farnesol with alcohol nucleophiles. We believe it is a combination of the hydrophobic environment in the mutant active site, displacement of water and L607 by phenylalanine, and the conformation of the substrate being favourable for cyclization, which led to the ether product formation. For further production of non-natural products, manipulation of the enzyme reaction by terpene analogues with different isoprene chain length and/or with aromatic ring systems is now in progress in our laboratories.

Experimental Section

Overexpression and solubilisation of *Aac*SHC wildtype and variants

AacSHC wildtype and variants were overexpressed from a pET-22b plasmid construct containing the *Aac*SHC gene under control of an IPTG-inducible T7 promotor (*lac*-operon). To overexpress the target enzymes, 400 mL TB medium containing 100 mg/ml ampicillin was inoculated with cultures of recombinant *E. coli* BL21(DE3) and shaken overnight (18 h) at 37 °C (180 rpm), whereby expression was started by auto-induction. Cells were harvested by centrifugation for 15 min at 7000 x g at 4 °C and the cell pellet was resuspended in 3x volume of lysis buffer containing DNA*se* I and 0.1 mM phenylmethylsulfonyl fluoride. Cell lysis was done by ultrasonication on ice. After centrifugation for 20 min at 7000 x g and 4°C, the supernatant was discharged, the cell debris pellet was resuspended in the same volume (*w/v*) of solubilization buffer (60 mM citrate pH 6.0, 1 % detergent CHAPS) and incubated over night while shaking at 4 °C. The suspension was heat-shocked at 50 °C for 30 min and centrifuged for 20 min at 7000 x g and 4 °C to partially purify the thermostable cyclase enzymes from residual *E. coli* proteins. Protein concentration was determinded via Bradford Assay using the BradfordUltra reagent (expedeon).

Biotransformations with *Aac*SHC wildtype and variants

Enzymatic conversions were carried out in a 500 µl scale in glass vials using 1 mg/ml partially purified *Aac*SHC variant in elution buffer (60 mM citrate pH 6.0, 1 % detergent CHAPS) and 2 mM substrate for 96 h at 60 °C while shaking with 600 rpm. Experiments were performed in triplicates. Reaction setups in buffer served as negative control (to monitor background activity). Prior to chromatographic analysis 1 mM of 1-decanol was added to the biotransformation as internal standard and the reaction mixtures were extracted twice with 500 µl methyl-*tert*-butyl ether (MTBE). The organic phases were dried over Na_2SO_4 and subsequently analyzed via GC-FID/MS.

Analytical methods

GC-FID/MS analyses were performed on an Agilent GC/FID system 7890A with inert MSD 5975C, equipped with a DB-5HT column (30 m × 0.25 mm × 0.1 µm, Agilent, Santa Clara USA) and a quadrupole mass analyzer. Separation method: 1 µl injection volume, injection temperature: 275 °C, detector temperature: 410 °C. Gradient: column temperature set at 70 °C for 2 min, then increased to 230 °C at 20 °C/min, to 240 °C at 20 °C/min, to 270 °C at 20 °C/min, to 380 °C at 20 °C/min and held for 2 min.

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Keywords: squalene hopene cyclase • active site mutagenesis • cyclization • intermolecular reaction • ether formation

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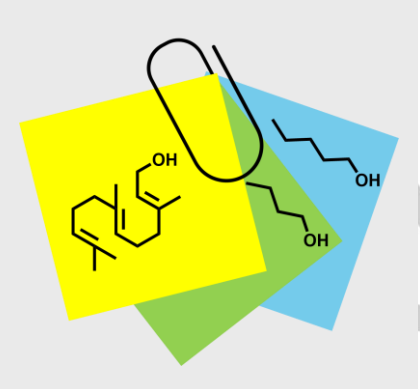
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

Connected molecules: Non-natural ether-linked terpenoids were generated by adding small chain alcohols to farnesol. A variant of the squalene hopene cyclase from *Alicyclobacillus acidocaldarius* was identified facilitating the connection of the carbocation intermediate with small alcohol nucleophiles.



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