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FULL PAPER

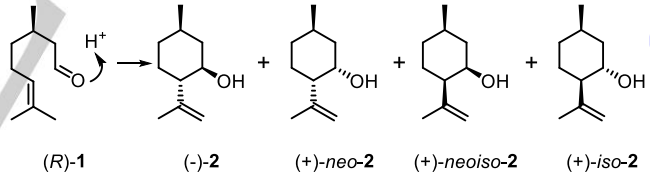
Enabling New Selectivities in the Cyclization of Citronellal by Squalene Hopene Cyclase Variants

Silke A. Bastian,[a] Stephan C. Hammer,[a] Nico Kreß,[a] Bettina M. Nestl,[a] and Bernhard Hauer*[a]

Abstract: The squalene hopene cyclase from *Alicyclobacillus acidocaldarius* (*Aac*SHC) is a highly efficient enzyme catalyst for stereoselective Brønsted acid catalysis. We engineered *Aac*SHC to catalyze the selective Prins cyclization of citronellal. Four active site variants catalyzed the diastereoselective cyclization of (*S*)-citronellal to stereoisomers (-)-*iso*-isopulegol, (+)-isopulegol and (-)-*neo*-isopulegol, respectively. The replacement of active site residues resulted in two triple variants that catalyzed the transformation of (*R*)-citronellal to give the isomers (+)-*neo*-isopulegol and (-)-isopulegol with up to >99% *de*, respectively. The newly designed library of functionally diverse active site geometries exhibits high selective control during citronellal cyclization, leading exclusively to a single diastereomer of the desired isopulegol. While the cyclization of citronellal with chemical catalysts was observed to produce the isopulegol isomer with the lowest energy, the reaction with *Aac*SHC variants proceeded with higher product selectivity. The results of this study show that variants of *Aac*SHC are excellent catalysts for the highly selective formation of isopulegol stereoisomers.

Introduction

The Prins reaction is a synthetically very useful reaction since it enables the formation of a C-C bond with the construction of two vicinal stereocenters. One of the most studied intramolecular version of this reaction is the cyclization of citronellal affording isopulegol. The acid-catalyzed cyclization of (*R*)-citronellal (**1**) to (-)-isopulegol (**2**) is an important step in the synthesis of (-)-menthol, extensively used in pharmaceuticals, agrochemicals, cosmetics and natural compounds with biological activities.[1] The reaction is catalyzed by the protonation of the carbonyl group of citronellal and proceeds via an intramolecular rearrangement followed by a deprotonation leading to the formation of isopulegol (Scheme 1). The presence of three stereogenic centers causes the formation of four stereoisomers of isopulegol, each of which occurs as a pair of enantiomers: isopulegol, *neo*-isopulegol, *iso*-isopulegol and *neoiso*-isopulegol. The selectivity of organic reactions for which multiple products can be formed is directed by the energies of different transition state structures that lead to the energy minimum for each product. In the Prins reaction of **1** to **2**, several reactive conformations could in principle occur that differ in their relative energies. These are attributable to the steric hindrance of the substituent during substrate folding. Conformations with the lowest repulsive energies possess the lowest relative energies and are therefore energetically most favored. In an achiral surrounding, the stereoselectivities or relative abundance of isomers can be estimated using the Boltzmann distribution. The ratio of isopulegol formation at ambient temperature is 0.77 (**2**), 0.15 (*neo*-**2**), 0.07 (*iso*-**2**) and 0.01 (*neoiso*-**2**).[2,3] The formed carbocation intermediates have the same structure as the four possible isopulegol stereoisomers and the deprotonation of each carbocation species leads to a corresponding isopulegol isomer.



Scheme 1. Schematic representation of the cyclization of (*R*)-citronellal (*R)*-**1** to isopulegol isomers (-)-isopulegol (-)-**2**, (+)-*neo*-isopulegol (+)-*neo*-**2**, (+)-*neoiso*-isopulegol (+)-*neoiso*-**2** and (+)-*iso*-isopulegol (+)-*iso*-**2.**

Chemical approaches including the cyclization of **1** over homogeneous and heterogeneous catalysts were investigated producing **2** as the predominant isomer followed by *neo*-**2**, *iso*-**2** and *neoiso*-**2**. Isopulegol (**2**) having the substituents of the cyclohexane ring in an equatorial position is about 15 kJ mol^{-1} more stable than *neoiso*-**2.**[2] High isopulegol (**2**) yields were obtained with most catalysts, but the ratio of isopulegol diastereomers differed strongly. By using heterogeneous catalysts including hydrous zirconia, zeolites, mesoporous silica catalysts and other solid acids fractions of isopulegol (**2**) of about 70 - 75% were obtained representing the thermodynamic equilibrium.[2,4–7] Higher **2** ratios > 90 % have been reported with homogenous catalysts such as $ZnBr_2$[8] and $Sc(OTf)_3$[9]. With novel Lewis acids like Sn-Beta[10] and Zr-Beta zeolites[11] the

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diastereoselectivity to **2** was further increased to 93 %. In addition to the desired cyclization of **1** to **2**, side product formation such as dimerizations of citronellal (**1**) and isopulegol (**2**) and the defunctionalization of **2** was observed. In recent years, the field of supramolecular catalysis has also progressed towards the Prins cyclization of citronellal (**1**) and related substrates.[12–14] Furthermore, the enantioselective Prins cyclization of achiral citronellal analogues was enabled using enantiopure supramolecular scaffolds in up to 69 % *ee*. These synthetic hosts contain well-defined cavities for stabilization of high-energy intermediates and transition states. The proposed mechanism of catalysis is similar to that employed by terpene cyclase enzymes, which control reaction outcomes by selectively stabilizing cationic intermediates and transition structures through multiple interactions with aromatic residues lining the active site.[15–18]

Squalene hopene cyclases (SHCs) have attracted interest due to their ability to synthesize bioactive polycyclic compounds that are challenging to access through conventional chemical synthesis.[19] Various non-natural substrates have been selectively converted to important polycyclic molecules.[15,20–22] A conserved aspartic acid acts as Brønsted acid for the protonation of linear (poly)isoprene compounds. The active site shape plays a tremendously important role during catalysis, as it displays the ideal chiral hydrophobic environment that is necessary to productively bind the substrate into an active site-defined arrangement while simultaneously stabilizing the evolving cationic key intermediates.[23,24] In earlier work, Siedenburg *et al.* reported the investigation of twelve SHCs to catalyze the cyclization of citronellal **1** via the protonation of the carbonyl functionality.[25,26] They discovered the SHC1 from the bacterium *Zymomonas mobilis* (*Zmo*SHC1) to form isopulegol (**2**) with **1** as substrate. Furthermore, two variants (F486C and W555Y) were identified as important for increased (**2**) formation. However, the selectivities for these biotransformations were low resulting in the formation of a mixture of isopulegol isomers.

We and others disclosed the conversion of non-natural monoterpenoid substrates in water by engineering the active site of the SHC from *Alicyclobacillus acidocaldarius* (*Aac*SHC) to create novel hydrophobic active site templates for chiral Brønsted acid catalysis.[3,27,28] We targeted residues located within 15 Å of the natural squalene binding site for site-directed mutagenesis (PDB code: 1UMP[29]). We obtained an engineered variant of *Aac*SHC (I261A) that produced exclusively (-)-*iso*-**2** from (*S*)-**1** with moderate conversion. The excellent diastereoselectivity of > 99 % *de* highlighted the exclusive conformational control by binding the substrate in one specific reactive conformation, forcing the methyl group into the unfavored axial orientation. As the results obtained from the engineering of *Aac*SHC indicated that generating active site mutations is efficient in converting the (*S*)-enantiomer of citronellal (**1**) into (-)-*iso*-**2**, we further engineered the active site of *Aac*SHC. We thus aimed to obtain variants to control reactive citronellal conformers to yield different isopulegol isomeric products.

Results and Discussion

The binding pocket of *Aac*SHC is able to control the reactivity of high-energy intermediates through a series of non-covalent interactions and substrate preorganization in order to selectively form molecules that would be difficult to access in bulk solution. Taking advantage of the evolvability of *Aac*SHC, we started to examine how many modifications within the active site of *Aac*SHC are needed to bind and activate (**1**) in different reactive conformations leading to different isopulegol isomers. 19 active site residues (L36, W169, I261, S307, W312, F365, Q366, A419, Y420, V448, W489, G490, Y495, G600, F601, F605, L607, Y609 and Y612) were chosen as the target amino acid residues for mutagenesis (see Figure 1). In order to retain the hydrophobicity of the active site, we introduced small to large hydrophobic amino acids (A, G, F, L, V and W) with varying side chains. Our previous work revealed that I261 and Y420 are crucial for the selective formation of (-)-*iso*-**2** from (*S*)-**1**, with residue I261 being located 14 Å from the proton source D376.

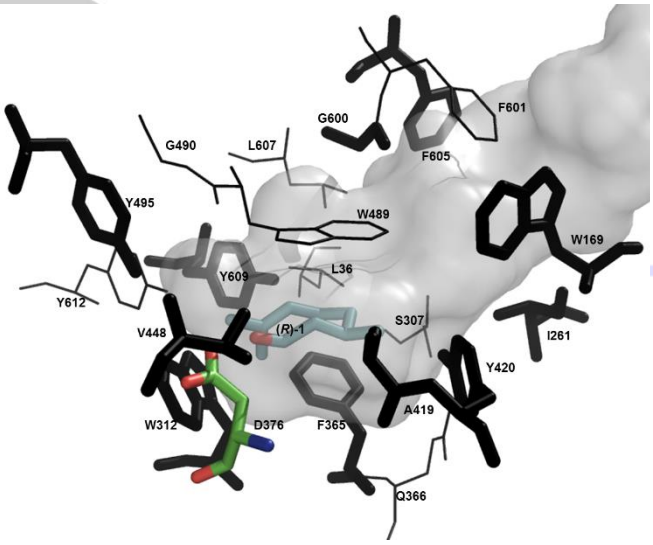
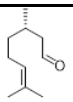
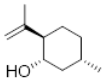
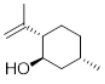
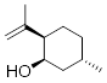
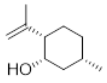
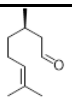
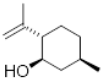
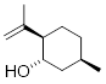
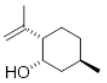
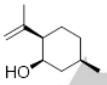


Figure 1. Mutagenesis of the active site of *Aac*SHC for the selective formation of isopulegol isomers from citronellal (**1**). Representation of the residues lining the active site of *Aac*SHC. The catalytic aspartic acid (D376) is shown in green sticks, the residues targeted by mutagenesis are shown in black sticks with diastereoselective variants in bold. The average distance between the carboxyl group of the catalytic D376 and the hydrophobic residues of active variants (A419, Y609, F365, Y420, G600, W169, I261) is 6.7 to 13.7 Å. The distance between D376 and W312 is 4.3 Å.

Table 1. Product formations [%] in the conversion of citronellal enantiomers (*S*)- and (*R*)-**1** with *Aac*SHC wildtype and variants after 40 h.

 (*S*)-**1**	 (+)-**2**	 (-)-*iso*-**2**	 (-)-*neo*-**2**	 (-)-*neoiso*-**2**
wildtype		1.15 ± 0.01		
I261A[3]		7.70 ± 0.22		
W169A[3]		7.90 ± 0.23		
Y609A	2.65 ± 0.19			
W312G			0.47 ± 0.07	
 (*R*)-**1**	 (-)-**2**	 (+)-*iso*-**2**	 (+)-*neo*-**2**	 +)-*neoiso*-**2**
wildtype	0.33 ± 0.14		0.11 ± 0.09	
Y609G	2.59 ± 0.38	2.58 ± 0.11	3.36 ± 0.10	
F365A/ Y420W/ G600F			4.64 ± 0.18	
A419G/ Y420C/ G600A[a]	9.09 ± 0.03		1.26 ± 0.03	
A419G/ Y420C/ G600A[b]	13.25 ± 0.42		1.55 ± 0.14	

[a] Reaction performed at 30°C using 2 mM (*R*)-citronellal, 0.2 % (*v/v*) Triton X-100 and 3-5 mg/ml purified protein. [b] Reaction performed at optimized reaction conditions at 20°C with 2 mM (*R*)-citronellal, 0.1 % (*v/v*) Triton X-100 and 2 mg/ml purified protein.

We have observed that substitution of isoleucine at position 261 to smaller alanine or glycine residues resulted in enhanced enzyme activity most likely by inducing a reorientation of the neighboring amino acid side chains.[3] Individual mutations were introduced into the *Aac*SHC gene by site-directed mutagenesis. *Aac*SHC wildtype, variants plus deleterious variant (D376C) were produced in *Escherichia coli* and purified by ion exchange chromatography. The activity of the wildtype and designed variants for the cyclization of **1** was investigated by monitoring the formation of **2** using gas chromatography. Activities of the different variants are shown in Table 1 and Supporting Information Figures S1-S4. Several variants emerged that displayed moderate monoterpenoid cyclase activity with high selectivities > 99 % *de* and initial activities up to 8% conversion (Table 1 and Tables S1-S2). Four *Aac*SHC variants exhibited preference towards in each case (only) one isomer of **2** (Figure 2). Three of these positive variants had single point mutations to alanine (I261A, W169A, Y609A), while the tryptophan at position 312 was mutated to glycine. The modifications of amino acids located in close proximity to the reactively bound substrate within *Aac*SHC's active site cavity, might directly influence the mode of substrate binding and folding. We assume that the creation of additional space through the introduction of small hydrophobic side chains might promote a certain preorganization of the substrate molecule The most active variants I261A and W169A catalyzed the reaction to give (-)-*iso*-**2** in > 99 % *de*. Similar to the best variant I261A from our first mutant library, residue W169 is located at a neighboring position at the back end of the enzyme's active site pocket (see Figure 1). Substitutions W312G and Y609A resulted in the formation of (-)-*neo*-**2** and (+)-**2**, respectively, whereby diastereoselectivities of > 99 % *de* and up to 2-fold increased conversions compared to the wildtype were obtained. Both residues are in close proximity to the catalytic D376 and the bound substrate and thus will have an active influence on binding and folding of the substrate in the active site. The screening of the library, however, failed to yield any mutant for the formation of (-)-*neoiso*-**2**.

The screening of the *Aac*SHC mutant library with (*R*)-**1** has shown that while the reaction of the (*S*)-enantiomer is facilitated by single point mutagenesis, its impact is smaller for the (*R*)-enantiomer. Similar conversions were obtained in the cyclization of the (*R*)-enantiomer using single point variants (see Supporting Information Tables S4-S5). However, no variant was detected that demonstrated excellent product selectivity. In contrast, the best variants A419G, Y420C and Y609G provided a mixture of (-)-**2**, (+)-*neo*-**2** and (+)-*iso*-**2** (Table 1 and Table S4). This suggests that single amino acid exchanges in the active site of *Aac*SHC are not able to stabilize the reactive binding of the substrate in one specific productive conformation. To investigate how such a product specific *Aac*SHC variant could be crafted, the active site mutant library was further extended.

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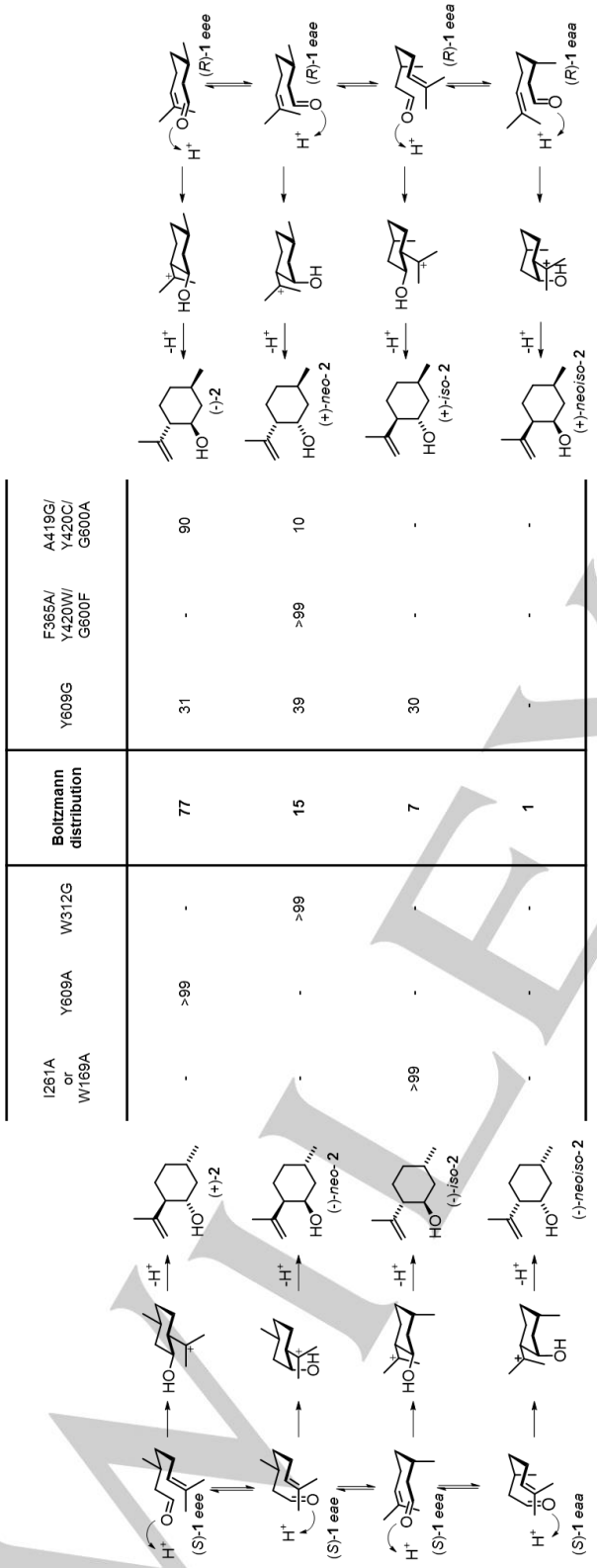

	I261A or W169A	Y609A	W312G	Boltzmann distribution	Y609G	F365A/ Y420W/ G600F	A419G/ Y420C/ G600A
(+)-2	-	>99	-	77	31	-	90
(-)-neo-2	-	-	>99	15	39	>99	10
(+)-iso-2	>99	-	-	7	30	-	-
(-)-neoiso-2	-	-	-	1	-	-	-

Figure 2. Diastereoselective cyclization of (*S*)-**1** (left) and (*R*)-**1** (right) to **2** can proceed via four energetically most favored chair-like transition states. Dependent on the axial (a) or equatorial (e) orientation of the substituents during prefolding, these arrangements can be described with the three-letter code given on the left, whereby the letters correspond to the substituents in the following order: isopropylene, carbonyl and methyl. Comparison of the product probability according to the Boltzmann distribution with the diastereoselective *Aac*SHC that were found in the active site mutant library to possess very defined product preferences, giving specific access to (-)-*iso*-**2**, (+)- or (-)-**2** and (-)- or (+)-*neo*-**2**. Standard deviations for selectivity are <1 %.

We generated combination mutants of active site residues to examine synergistic effects of these mutations on the activity and selectivity in the cyclization of the (*R*)-enantiomer of **1**. By combining mutations at positions A419, Y420, F365 and G600, eleven double as well as triple variants were created (Table S3). Combinations of mutations of active site residues had beneficial effects on the selectivity of *Aac*SHC towards (-)-**2** generating novel diastereoselective cyclase variants. The generation of triple variants improved the selectivity of *Aac*SHC in the cyclization of (*R*)-**1**. Further analysis revealed that variant A419G/Y420C/G600A was the most active variant (10-fold increased conversion) and highly selective generating a mixture of (-)-**2** and (+)-*neo*-**2** in a 9:1 ratio (Table 1 and Figure 2). Due to the fact that we have observed in preliminary work that temperature and the concentration of detergent used had significant advantages in the cyclization of non-natural compounds, we optimized the conditions for the cyclization of (*R*)-**1** using the most active variant A419G/Y420C/G600A. We noted an 1.5-fold increase of product formation in cyclization reactions containing less detergent (0.1 % (*v/v*) Triton X-100) and decreasing reaction temperature from 30°C to 20°C. Utilizing half the concentration of purified protein under optimized reaction conditions yielded in

Notably, variant F365A/Y420W/G600F selectively protonated the (*R*)-enantiomer leading to the formation of (+)-*neo*-**2** with an excellent diastereoselectivity (>99% *de*) and 10-fold enhanced conversion.

The high levels of selectivity achieved in the SHC-catalyzed Prins reaction of **1** are the results of precise control over the substrate conformation and its interaction with catalytic functional groups within the active site. The observed product selectivity of active site variants may be attributed to the enzyme's innate hydrophobic active site cavity that stabilizes the formation and preservation of highly reactive carbocation species and shield them from quenching by water molecules. Furthermore, the directing effect of the substrate's methyl group allows the functional positioning and reactive folding of the substrate molecule within the active site for cyclization. In certain active site contours, the required positioning of this methyl group prior to catalysis might be sterically impeded by surrounding amino acid residues and thus might prevent the substrate enantiomer from being productively bound. This observation is in accordance with findings described in previous work showing that the positioning of the methyl group of a terpene substrate plays an important role for the convertibility by SHCs.[30,31] Single point mutations in the active site of *Aac*SHC are sufficient to entail a distinctive product specificity through the selective binding and activation of the substrate in differing reactive conformations. Bioinformatic analyses, in particular of sequences of terpene cyclases, have shown that active site residues of SHCs reveal an exceptionally high degree of conservation.[32] In enzyme superfamilies, small changes in the composition of amino acids of the active site can create differing activities.[33,34] As active sites provide highly optimized environments for catalysis, changes at these centers can have large effects on e.g. enzyme activity or substrate specificity.[35] It is striking that exchanges of several of these highly conserved amino acid residues led to remarkably improved variants for the cyclization of citronellal (**1**) with excellent diastereoselectivity.

Conclusions

Our results demonstrate the feasibility of tailoring the central active site pocket of *Aac*SHC for the complex and highly selective Prins reaction of citronellal (**1**) to isopulegol (**2**). With only few mutations within the enzyme´s active site, amino acid positions could be identified that play a crucial role in the specific binding of the non-natural substrate and for the control of high energy intermediates during the reaction. By screening the active site library, several single and triple variants were obtained that shifted the product distribution towards a targeted isomeric selectivity. In contrast to chemical catalysts, the control of high-energy carbocations in the cyclization of **1** were selective, leading to specific isopulegol isomers. In the future, random mutagenesis and screening will be used to identify amino acid substitutions to increase the activity of *Aac*SHC variants while maintaining their high product selectivity.

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, TB medium containing 100 mg/ml ampicillin was inoculated with cultures of recombinant *E. coli* BL21(DE3) and shaken overnight (16 h) at 37 °C (180 rpm), whereby expression was started by auto-induction. Cells were harvested by centrifugation at 7000 g at 4 °C and the cell pellet was resuspended in 3x volume of lysis buffer containing *DNAse* I and 1 µM phenylmethylsulfonyl fluoride. Cell lysis was done by ultrasonication on ice After centrifugation at 7000 g for 20 min, 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 Triton X-100) 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 g to partially purify the thermostable cyclase enzymes from residual *E. coli* proteins. Subsequently, the supernatant was further purified using anion exchange chromatography.

Purification of *Aac*SHC wildtype and variants via ion-exchange chromatography

Prior to usage, the column material diethylaminoethyl–sephacel (DEAE-Sephacel) was freshly regenerated with 5 column volumes (CV) of regeneration buffer (12 mM citrate, pH 6.0, 0.2 % Triton X-100, 500 mM NaCl) and equilibrated with 5 CV wash buffer (12 mM citrate, pH 6.0, 0.2 % Triton X-100). The enzyme-containing supernatant from heat-shock purification was diluted with the four fold volume of Milli-Q water (to reduce the salt concentration within the preparation), filtered through 0.2 µm filters and loaded on the DEAE-Sephacel resin. The loaded sample was washed with 5 CV of wash buffer (12 mM citrate, pH 6.0, 0.2 % Triton X-100) and eluted with 5(-10) CV elution buffer (12 mM citrate, pH 6.0, 0.2 % Triton X-100, 200 mM NaCl). Protein concentration was determinded via Bradford Assay using the BradfordUltra reagent (expedeon).

Biotransformations with purified *Aac*SHC wildtype and variants

Enzymatic conversions were carried out in a 500 µl scale in glass vials using 3-5 mg/ml IEX-purified *Aac*SHC variant in elution buffer (12 mM citrate, pH 6.0, 0.2 % Triton X-100, 200 mM NaCl) and 2 mM substrate for 40 h at 30 °C while shaking with 600 rpm. Experiments were performed in triplicates. Reaction setups with pure buffer and with *Aac*SHC variant D376C 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 ethyl acetate. 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) and a quadrupole mass analyzer. Separation method: 1 µl injection volume, injection temperature: 250 °C, detector temperature: 320 °C. Gradient: column temperature set at 75 °C for 3 min, then increased to 120°C at 6 °C/min, then to 150 °C at 10 °C/min and to 300 °C at 30 °C/min. Total run time was 18.5 min. Citronellal (**1**) and isopulegol (**2**) isomers were separated as depicted in Figures S1 and S2. Since biotransformations were performed with enantiopure substrate, each product isomer could be clearly assigned without the application of a chiral column.

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Keywords: biocatalysis • squalene hopene cyclase • active site mutation • isopulegol • mutagenesis

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Layout 1:

FULL PAPER

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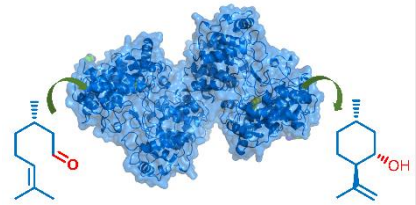

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Page No. – Page No.

Enabling New Selectivities in the Cyclization of Citronellal by Squalene Hopene Cyclase Variants