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Synthesis of Heterocyclic Terpenoids by Promiscuous Squalene–Hopene Cyclases

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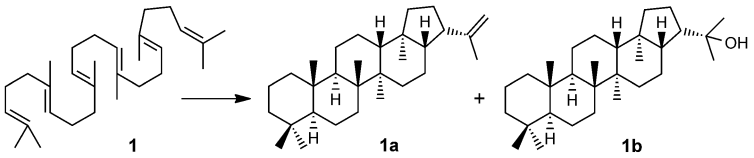
Terpenoids are central molecules of life, with functions ranging from signaling and defense to communication. Moreover, the natural terpenoid products obtained upon cyclization of a polyisoprene backbone have important applications as chiral building blocks in chemical syntheses, as fragrances, and as antimicrobial, anticancer (Taxol), and anti-malaria (Artemisinin) agents.[1,2] Therefore, the development of stereospecific synthetic routes towards these cyclic molecules is of increasing interest. Herein we highlight the potential of triterpene cyclases as general Brønsted acid catalysts for the generation of di- and triheterocycles starting from promiscuous substrates that contain a functionalized, shortened polyisoprene backbone. It was found that alcohols, ketones, and carboxylic acids could serve as terminal nucleophiles in the cyclization cascade, thereby yielding cyclic ethers, enol ethers, and lactones. This demonstrates the high versatility as well as flexibility of the catalyst for cyclization reactions.

The squalene–hopene cyclase (SHC) from *Alicyclobacillus acidocaldarius* (*Aac*SHC) has served as a model triterpene cyclase for detailed mechanistic studies since the crystal structure was solved in 1997.[3] Further characterization of this enzyme yielded unexpected results regarding its substrate spectrum. It was shown that, besides the natural substrate squalene (**1**), other linear terpenoids were also converted into cyclic products. In 1986, Neumann et al. reported cyclization of the C_{16} alcohol homofarnesol (**2**) to the heterocyclic flavor compound ambroxan (**2 a**), as well as cyclization of a homofarnesyl ether and the C_{11} derivative homogeraniol.[4] This was the first example of substrate promiscuity with terpenoids of less than 20 C atoms by a triterpene cyclase. It is worth noting that in nature substrates with shorter chain lengths (C_{10}–C_{20}) require pyrophosphate activation (class I cyclization mechanism).[1,5] In contrast, cyclization reactions catalyzed by triterpene cyclases occur by the class II mechanism, in which no activation of the substrate is required. More recently the cyclization of the C_{15} and C_{25} alcohols farnesol and geranylgeraniol, as well as of two C_{20} and C_{25} terpenes, to di-, tri-, and tetracyclic products was described.[6] The feasibility of using triterpene cyclase enzymes for novel chemistry has also recently been demonstrated by using these catalysts for Friedel–Craft alkylation, thereby integrating aromatic systems into the cyclic scaffolds of the products.[7] Further, the formation of the menthol precursor isopulegol from citronellal catalyzed by the SHC *Zmo*SHC1 from *Zymomonas mobilis* has been shown.[8] Inspired by the desire to explore the substrate promiscuity of triterpene cyclases for catalysis beyond the natural C_{30} polyisoprene backbone, we investigated whether these enzymes would form di- and triheterocycles from substrates with different carbon chain lengths and terminal nucleophiles. Stereospecific access to complex cyclic compounds difficult to produce by traditional chemical methods was established.

Terpenoids constitute one of the most diversified groups of natural products, with over 60000 different structures known to date.[1,9] This large diversity originates from the cyclization of linear terpenoid precursors catalyzed by terpenoid cyclases.[5] The cyclization reaction is initiated by carbocation formation by either pyrophosphate release (class I mechanism) or protonation of the terminal isoprene unit (class II mechanism).[1] SHCs are triterpene cyclases that catalyze cyclization of the linear triterpenoid squalene (**1**) to hopene (**1 a**) and hopanol (**1 b**) by the class II mechanism (Scheme 1). This cyclization reaction is one of the most complex reactions known in biochemistry. Hopene and hopanol are prokaryotic steroid analogues and have important functions as membrane constituents.[10]



Scheme 1. SHC-catalyzed conversion by of the natural substrate squalene (**1**) into the pentacyclic products hopene (**1 a**) and hopanol (**1 b**).

The results of our experiments using two different triterpene cyclases, namely the squalene–hopene cyclases *Aac*SHC and *Zmo*SHC1, as biocatalysts are presented in Table 1. To assess the catalytic performance of the investigated enzymes, the natural substrate squalene (**1**) was included as control, as well as the previously investigated C_{16} substrate, homofarnesol (**2**).[11] In addition, two other substrates of similar chain length with a carboxyl and keto group in place of the alcohol functionality, namely homofarnesoic acid (**3**) and farnesylacetone (**4**), were selected, and cyclization to heterocyclic products was observed with both SHCs. The conversion of homofarnesol to the flavor compound ambroxan (**2 a**) was found to be significantly higher

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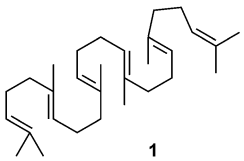
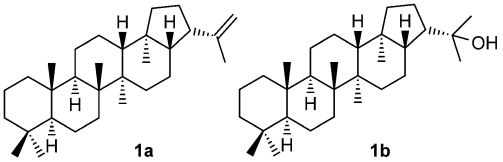
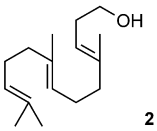
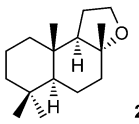
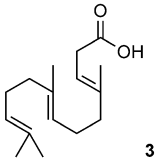
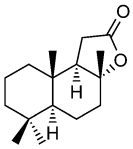
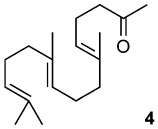
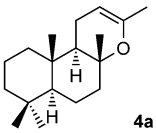
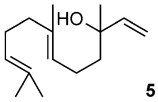
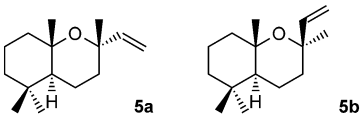
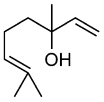
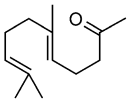
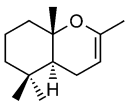
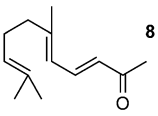
Table 1. Conversion of substrates (10 mM) after 20 h by partially purified SHCs.

Substrate	Product	Product formation[a] with *Aac*SHC (0.35 mg mL^{-1})	*Zmo*SHC1 (0.35 mg mL^{-1})	*Zmo*SHC1 (4 mg mL^{-1})
 1	 1a 1b	97.3 ± 2.9[b]	0.8 ± 0.1[b]	4.3 ± 0.4[b]
 2	 2a	4.4 ± 0.5	34.8 ± 1.4	65.6 ± 0.9
 3	 3a	2.3 ± 0.3	7.7 ± 0.2	22.9 ± 3.2
 4	 4a	0.7 ± 0.1	0.2 ± 0.0	2.5 ± 0.2
 5	 5a 5b	1.4 ± 0.8[c]	0.5 ± 0.0[c]	0.9 ± 0.1[c]
 6		n.c.	n.c.	n.c.
 7	 7a	1.1 ± 0.1	1.7 ± 0.1	23.0 ± 0.6
 8		n.c.	n.c.	n.c.

[a] Shown as percentage of the concentrations of both substrate and product calculated with internal standard calibration. [b] Hopene/hopanol: 1:6 for *Aac*SHC, 1:9 for *Zmo*SHC1. [c] (−)-caparrapioxide/(−)-8-*epi*-caparrapioxide: 1:2 for *Aac*SHC, 1:3 for *Zmo*SHC. n.c.: no conversion detected. NMR data for the cyclic products are provided in the Supporting Information.

for *Zmo*SHC1 than for *Aac*SHC, thus demonstrating that the substrate scopes of these triterpene cyclases differ.[11] Among amber odorants, ambroxan is the most valuable and has been used in the flavor and fragrance industries for a long time.[12,13] In nature, ambroxan occurs as a metabolite in the gut of the sperm whale, and it is disappearing from the world market due to excessive whaling.[12]

The acid analogue of **2**, the C_{16} terpenoid homofarnesoic acid (**3**), was converted to sclareolide (**3a**) as single product by both *Zmo*SHC1 and *Aac*SHC. Sclareolide (also known as norambreinolide) is a tricyclic lactone that occurs as a natural product with several bioactive properties (phytotoxic, cytotoxic, and antifungal).[14] Besides being an intermediate in the chemical route to **2a** and other natural products, **3a** can be used as an additive in food, in order to enhance organoleptic properties, and it is a potential supplement for weight loss.[14]

With farnesylacetone (**4**), low but significant product formation was observed with both tested SHCs. Product characteri-

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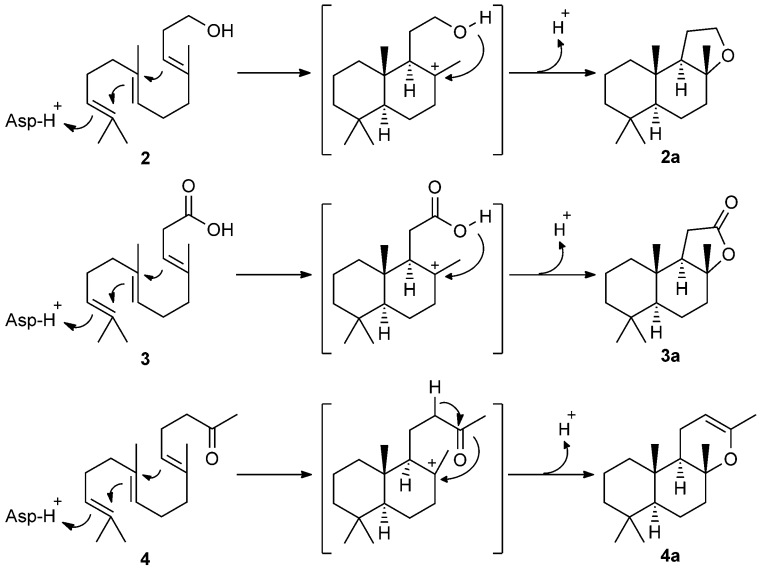
zation led to the identification of the tricyclic sclareoloxide (**4a**, a cyclic enol ether). Enol ethers, in general, represent a versatile class of compounds used in organic chemistry, as they are able to participate in olefin metathesis reactions.[15] Sclareoloxide is an intermediate in the synthesis of drimane-type sesquiterpenoids and ambroxan,[13,16] and also provides access to puuphenones, an important group of marine metabolites offering different biological activities used in antitumor and anti-malaria treatments.[17] To the best of our knowledge, this is the first example of using a triterpene cyclase for the stereospecific formation of lactones and cyclic enol ethers. The suggested cyclization mechanism is displayed in Scheme 2. We propose that after protonation of the substrates' terminal C=C double bond by the catalytic aspartate residue in the active site of the SHC, the heterocyclic products are created by intramolecular, nucleophilic attack of the alcohol, keto, or carboxyl group during the final ring closure step of the cyclization cascade (Scheme 2).



Scheme 2. Proposed reaction mechanisms for the cyclization reactions of homofarnesol (**2**) to ambroxan (**2a**), homofarnesoic acid (**3**) to sclareolide (**3a**), and farnesylacetone (**4**) to sclareoloxide (**4a**).

The next step of our substrate engineering approach was analysis of the formation of diheterocycles. The tertiary alcohol nerolidol (**5**; C_{15}, a regioisomer of farnesol) is a common precursor for the organic synthesis of many cyclic compounds.[6,18] Whereas *Aac*SHC-catalyzed cyclization of farnesol had previously been shown to furnish bicyclic products without the incorporation of oxygen,[7] **5** showed conversion to two stereoisomeric heterocyclic products: (−)-caparrapioxide (**5a**) and (−)-8-*epi*-caparrapioxide (**5b**). No other stereoisomer (e.g., (+)-caparrapi oxide) was obtained, unlike the situation for traditional chemical synthesis.[18–20] Caparrapioxides **5a** and **5b** have been found in nature in the defense secretion of termites, in sea hare, and in the extract of sun-cured Greek tobacco.[21] Because of their unusual structural features, they are interesting chemical products that could have special biological activities, as they were reported to have the fragrance of ambergris.[19,22]

For further characterization of the catalytic potential of triterpene cyclases, the C_{10} tertiary alcohol linalool (**6**) and the C_{13} ketone geranylacetone (**7**), truncated homologues of **5** and **4**, respectively, were used for biotransformations. The C_{10} monoterpenoid **6** did not show conversion with either of the tested enzymes. In contrast, with geranylacetone as a substrate, formation of the cyclic enol ether product **7a** was observed after incubation with *Zmo*SHC1 and *Aac*SHC (Table 1). This finding is consistent with the observations made by Hoshino et al. that several linear terpenoids are accepted as substrates but not those with chain lengths of less than 15 C-atoms, such as the C_{10} alcohol geraniol.[6,23] However, we noted that the C_{13} substrate **7** was converted to a two-ring product. The cyclization product **7a** is composed of a hexahydrochromene scaffold and represents an interesting cyclic enol ether compound. Similarly to all other cyclic products reported here, this product is stereochemically defined, and in organic chemistry it is not trivial to synthesize.[24] It provides access for the production of edulans, which exhibit a rose-like aroma.[25] and it can be a valuable precursor for the production of octahydrobenzopyrane derivatives, which are compounds that exhibit pleasant odors.[22,26]

Another interesting finding is that pseudoionone (**8**), which differs from **7** by an additional C=C double bond, did not show conversion with the two investigated triterpene cyclases. This double bond imposes constraints on the substrate backbone conformation, and forces it to adopt a fold that cannot undergo cyclization (see Scheme S1 in the Supporting Information).

For *Zmo*SHC1, a clear "shift" of substrate activity was shown towards substrates of shorter carbon-chain length than that of the natural substrate squalene. Thus, compared to *Aac*SHC, a completely different biocatalytic behavior was demonstrated, even though in the active sites of *Aac*SHC and *Zmo*SHC1, 30 of the 36 amino acids (predicted to be in direct contact with the substrate) are conserved.[27] Hence, a number of amino acids seem to be crucial for the substrate binding and catalysis of the enzymes, as their exchange or deletion has been shown to have great influence on substrate specificity and biocatalytic activity.[28] Given that the overall sequence identity between *Zmo*SHC1 and *Aac*SHC is relatively low (41%), the "second-sphere" architecture, 5–10 Å away from the site of catalysis, could differ significantly, and thus determination of the crystal structure of *Zmo*SHC1 would seem to be indispensable for an

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explanation of the difference in promiscuous substrate specificity between the two triterpene cyclases.

The conversion rates shown in columns 3 and 4 of Table 1 are low for several substrates. However, additional experiments revealed that the biotransformation rates were increased with higher enzyme concentration. For example, with *Zmo*SHC1, enhanced rates were observed for the conversions of **2** to **2a** (66%), **3** to **3a** (23%), and **7** to **7a** (66%; column 5 in Table 1) under these conditions.

The potential of triterpene cyclases as general Brønsted acid catalysts was demonstrated by the generation of bi- and triheterocyclic natural compounds from promiscuous substrates containing a functionalized, shortened polyisoprene backbone. The syntheses of functional products, such as cyclic ethers, lactones, and enol ethers demonstrated that triterpene cyclases can form the basis of a toolbox for catalyzing a variety of stereospecific cyclization reactions under mild reaction conditions. *Zmo*SHC1, in particular, displays beneficial properties such as shifted substrate specificity towards shorter substrates, and has the potential to be used for the generation of flavor and fragrance compounds in addition to important natural products that are beyond chemical synthesis. The non-pyrophosphate-dependent conversion of these compounds represents an example of the use of promiscuous enzymes in novel biosynthetic routes.

Experimental Section

Complete information of the general experimental procedures, cloning, protein expression, partial purification of the membrane-bound protein fraction, determination of the protein concentration, biotransformations, sample preparation and the analytical data derived from GC, NMR and MS analyses as well as the evaluation and quantification methods are provided in the Supporting Information.

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Keywords: biocatalysis · polycyclization · promiscuity · squalene–hopene cyclases · terpenoids

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