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Expanding the "Terpenome": Applications of Unspecific Peroxygenases (UPOs) in Oxidations of Unnatural Terpenoids

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An unnatural tricyclic oxaterpenoid, obtained by treatment of the sesquiterpene synthase presilphiperfolan-8β-ol synthase (BcBOT2) with an unnatural farnesyl pyrophosphate ether derivative, itself obtained by chemical synthesis, was converted in oxidation studies as part of a broad screening program by selected unspecific peroxygenases (UPOs). Product analysis revealed that *Agrocybe aegerita* UPO, its mutant PaDa-I, and two commercial ones, UPO_{54} and UPO_{49}, provided new oxidation products with sufficient efficiency for subsequent upscaling that allowed product isolation and structure elucidation. As such new terpene-based oxiranes and hemiacetals were formed by UPO-mediated epoxidations and CH-activation. The structure elucidation was further supported by comparison with products generated by chemical oxidation.

1. Introduction

The realm of terpenoids, known as the "terpenome", comprises the largest number of natural products with over 80,000 representatives[1–5] contributing to a very broad spectrum of applications, be it in the fragrance, aroma,[6–8] and the pharmaceutical industry.[9] A rising trend is to develop new approaches and methods to further expand and diversify the "terpenome". Biotechnological approaches are increasingly used for the industrial production of commercially important terpenes, whereby the late stage modification of terpene scaffolds by oxidative enzymes has recently received much attention. Here, cytochrome P450 monooxygenases (P450s) have played a key role so far,[10–13] but also lipoxygenases[14] and fungal unspecific peroxygenases (UPOs)[15–18] contribute in this field. However, practically, the class of P450s shows several drawbacks such as (i) instability in organic solvents,[19] (ii) lower catalytic performance (10,000 turnover numbers versus $>$ 900,000 for UPOs),[20,21] (iii) cofactor dependency, and (iv) the "oxygen dilemma".[22,23] The latter mainly arises from the need for stoichiometric amounts of reducing agents to the redox mediators of P450s. However, often the undesired reduction of molecular oxygen generates H_2O_2, followed by unproductive side reactions with regard to a specific product formation. The formation of reactive oxygen species can then lower the enzyme's stability.[22,23] In comparison to the mechanisms on which P450 oxidation relies, UPOs follow a mechanism commonly labelled as "peroxide shunt". The highly reactive Fe(IV)=O intermediate is also found in catalysis with UPOs, but the activation of Fe(III) to Fe(IV) is achieved by H_2O_2, as the reduced form of O_2.[24,25] To circumvent the limitations of P450s, we utilize UPOs for the late stage modification of terpenoids as challenging substrates.

In this work, we present a diversification approach towards unnatural terpenoids that combines the chemical synthesis of unnatural farnesylpyrophosphate derivatives with a cationic cyclization sequence promoted by sesquiterpene synthases (STSs) and the late stage oxidation of new terpenoids collected using a set of UPOs. In recent studies, it was found that terpene synthases, and especially sesquiterpene synthases, exhibit an unusually broad substrate promiscuity yielding new terpenoid backbones.[26] In this context, we found that the presilphiperfolan-8β-ol synthase (BcBOT2) that was first isolated from the fungus *Botrytis cinerea* is the most remarkable sesquiterpene synthase.[27] Naturally, BcBOT2 catalyzes the cyclization of farnesyl pyrophosphate **3**, biosynthetically obtained from dimethylallyl pyrophosphate **1** (DMAPP) and isopentenyl pyrophosphate **2** (IPP), yielding presilphiperfolan-8β-ol **4** as the major product, among many other minor

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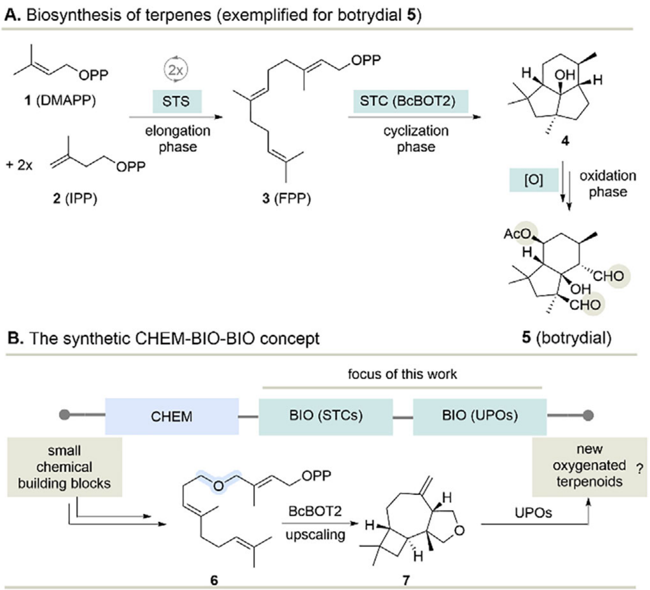


Scheme 1. A. Routes of terpene biosynthesis exemplified for botrydial (5), and **B.** CHEM-BIO-BIO synthesis concept reported here, detailed for the BcBOT2 product **7,** derived from FPP derivative **6** (STS = sesquiterpene synthase, and UPO = unspecific peroxygenase).

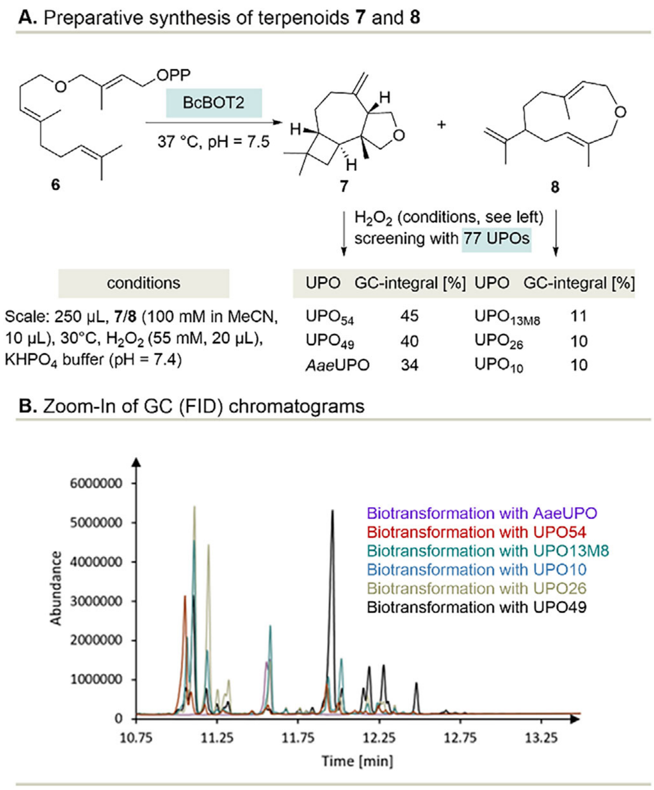
by-products (Scheme 1A).[27,28] Commonly, the cyclization phase is accompanied by an oxidation phase leading to oxygenated terpenoids. In the case of presilphiperfolan-8β-ol (**4**), the highly active phytotoxin botrydial (**5**) is the final product of the biosynthesis.[27] These functionalized terpenes often exhibit better sensory and olfactory profiles than their hydrocarbon analogs, and are therefore valorized into products relevant to the fragrance and flavor industry.[7,8]

Among the large list of unnatural farnesyl pyrophosphate derivatives accepted by BcBOT2,[29–33] the FPP ether **6** is remarkable as it yields terpenoid **7** with a tetrahydrofuran ring as the main product (Scheme 1B).[34] Overall, we conceptually combine chemical and enzymatic steps so that the overall process can be categorized as a CHEM-BIO-BIO approach. This is a nomenclature that we have introduced to classify syntheses, especially chemoenzymatic syntheses, involving one chemocatalytic and two enzymatic steps, respectively (Scheme 1B).[35] In short, the present work deals with the synthesis of oxasesquiterpene **7** from the linear precursor **6** and the broad evaluation of whether UPOs are able to use tetrahydrofuran **7** as a substrate (Scheme 1B).

2. Results and Discussion

2.1. Evaluation of the UPO Screening Against Terpenoid 7/8

Preliminary studies on the substrate scope of UPOs have recently been reported, revealing a variety of oxyfunctionalization modes. E.g., *Agrocybe aegerita* UPO (*Aae*UPO) has been shown to oxidize organic substrates by forming epoxides from alkenes and benzyl or allyl alcohols from the corresponding unsaturated hydrocarbons.[36–39] In general, two types of UPOs



Scheme 2. A. Preparative synthesis of terpenoid **7** along with by-product **8,** and results of primary screening, starting with a panel of 77 UPOs. The GC integrals refer to the relative integrals of the main product compared to the starting materials **7** (threshold $\geq$ 10%). **B.** Zoom-in of the overlay (GC, and FID) of high-performing UPOs resulting from the high-throughput evaluation.

are known, which are categorized according to their length. While long monomeric UPOs have an average size of 44 kDa, short UPOs are characterized by size between 29 and 32 kDa.[21] The differences in size and organization of the heme channels are also reflected in variations of substrate scopes. Short UPOs can accept bulky substrates, while long UPOs reveal a substrate scope towards smaller molecules. The substrate promiscuity of UPOs would make them the perfect complement for the oxyfunctionalization of terpenoids.

In order to carry out the principal studies depicted in Scheme 1B, it was necessary to prepare sufficient amounts of terpenoid **7** as well as get access to a set of diverse short and long UPOs. The chemoenzymatic synthesis of the terpenoid **7,** along with the macrocyclic ether **8** as a minor component (**7**:**8** = 9:1; a total of 221 mg) was achieved on a larger scale using 1.5 g of derivative **6** and 220 mg of purified BcBOT2 (Scheme 2A, 22 $\times$ 100 mL batches, each containing 67 mg of **6**, and 10 mg of BcBOT2). For the oxidation of terpenoids **7** and **8**, we chose a commercial panel of 77 UPOs, heterologously produced in *Komagataella phaffii* (former called as *Pichia pastoris*) from *Aminoverse* B.V. This panel consists of 62 wild-type UPOs and 15 UPO enzyme variants.

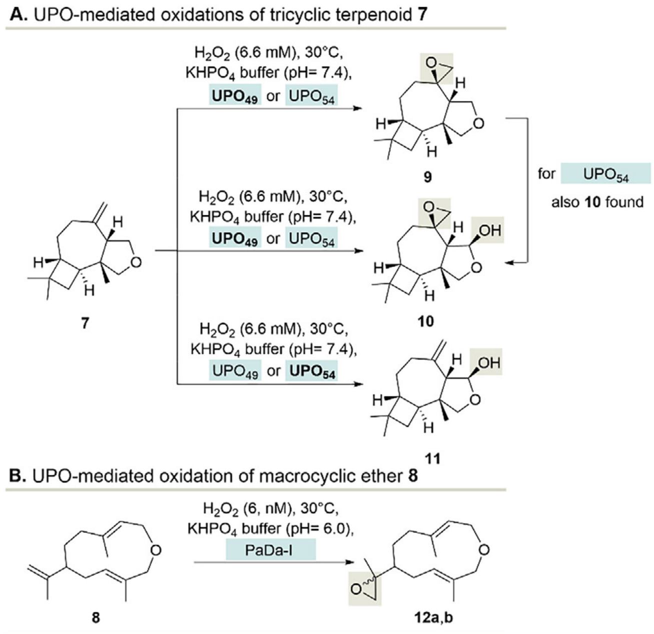
To our delight, 34 UPOs were able to transform the terpenoid mixture and within the scope of this study, we unraveled overall three different oxyfunctionalization patterns. These were indicated by their retention indices (R_I of **9** = 1756, R_I of **10** = 1918, R_I of **11** = 1750, **and** R_I of **12** = 1842) and *m/z* values collected from GC-MS analysis (Scheme 2B, Table S1 and Figures S1–S32). As a threshold, we used a value of 1% relative integral of the main product compared to the tricyclic ether **7**. A primary series of analytical scale transformations revealed several promising enzyme candidates, namely UPO_{54}, UPO_{49}, and *Aae*UPO[40,41] with up to 45% GC integral of the main product (UPO_{54}, Scheme 2A). The *Aae*UPO mutant PaDa-I, heterologously produced in *Komagataella phaffii*,[42] displays a similar reaction pattern to its wild type *Aae*UPO.[36–39] Hence, PaDa-I was investigated under optimized conditions[42] resulting in the same oxidation product with comparable GC integral (superposition of two compounds with 23% and 20%, Scheme 2A). Since PaDa-I could be recombinantly produced in-house,[43] all further reactions were performed with PaDa-I instead of the wild type *Aae*UPO.

2.2. Semi-Preparative Scale Oxidation of Terpenoid 7/8 with Selected UPOs

The amount of oxygenated terpenoid products collected during the small-scale biotransformations was not sufficient to elucidate their structures. Before applying this reaction on a semi-preparative scale, the appropriate amount of the co-substrate H_2O_2, as well as a uniform and comparable addition of the UPO catalyst were investigated by monitoring their peroxidase activity.[44] Since H_2O_2 is a bottleneck for the stability and activity of UPOs,[45] the optimal supply is of relevance to eliminate undesired side reactions (e.g., overoxidation) and to achieve the highest yields as stated by Meyer et al. with a dosing rate of 0.47 µmol · min^{-1} H_2O_2 for immobilized PaDa-I[46] in a packed-bed reactor.[37] In general, enzymes are less process stable in the cell-free extract than when being immobilized.[46] Because of that, we applied a lower manual dosing of 0.275 µmol H_2O_2 every 30 min (corresponds to 0.009 µmol · min^{-1} H_2O_2) throughout 3 h, but a higher concentration of H_2O_2 (1.65 eq. or 6.6 mM) compared to conditions employed for screening (1.1 eq or 4.4 mM). The peroxidase activity was evaluated using the oxidation of ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] as a model reaction (see Supporting Information).[47]

To isolate and characterize the oxidation products, the following UPOs were employed for semi-preparative biotransformations: V_{batch} = 10 mL, terpenoid **7** (0.04 mmol), UPO_{54} (338 U), UPO_{49} (87U), and PaDa-I (5800 U) (Scheme 3, for more details see Supporting Information). Here, the tricyclic terpenoid **7** proved to be very suitable for investigating the catalytic scope of UPOs, despite the fact that only one oxidizable functional group, the exocyclic olefinic double bond, is present. The two enzymes UPO_{49} and UPO_{54} showed a similar oxidation profile, however, differences were found for the product ratios of **9–11**. UPO_{49} mainly furnishes products **9** and **10** with only traces of **11** present. In contrast, UPO_{54} mainly forms lactol **11** as the major product, with **9** and **10** remaining as minor oxidation products in small quantities.

Here, hydroxylation of the methylene group next to the ether oxygen atom occurs which provided lactol **11**. This reac-



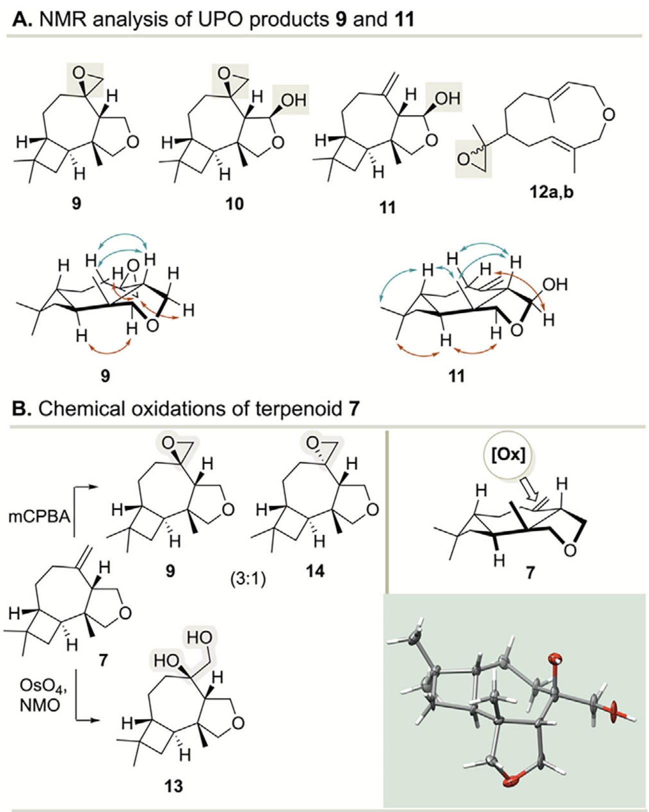
Scheme 3. Semi-preparative transformations, and conditions of **A.** terpenoid **7** using UPOs identified in the screening process, **B.** terpenoid **8** using PaDa-I, and major products **9–11** generated by the respective UPOs.

tivity resembles the oxyfunctionalization of non-activated CH bonds, which is commonly associated with P450 enzymes[10,11] and UPOs,[48] as well as the classically radical chemistry of tetrahydrofurans.[49] Here, it is noteworthy that an overoxidation to the lactone was not observed.[15] Since lactols commonly reach equilibrium via the ring-opened hydroxyaldehyde (see **15** in Scheme 5), it can be assumed that the configuration of the hemiacetal moiety in **10** and **11** resembles the thermodynamically favored diastereomer.

When epoxide **9** was subjected to the established conditions, UPO_{54} led to the doubly oxidized terpenoid **10** as the main product (Figure S31). Both sites, the alkene, and the tetrahydrofuran, were oxidized with identical stereochemistry as determined for **9** and **11**. The substrate range of the terpenome was further extended by the chemoselective oxidation of the 1,1-disubstituted exocyclic double bond of macrocyclic ether **8** induced by PaDa-I (*Aae*UPO, respectively). It yielded a diastereomeric mixture (≈1.7:1 based on NMR integrals of epoxide protons; see Supporting Information) of oxiranes **12a,b**.

2.3. Key Elements of Structure Elucidation and Mechanistic Considerations

Besides high-resolution mass spectrometry, structure analysis relied on different NMR methods (Scheme 4). Comparing the NMR data of the oxidized biotransformation products **9–12** with those of the starting materials **7** and **8**, both the ^{1}H- and the ^{13}C-NMR data revealed the locations where the oxidations must have taken place. This applies in particular to the signals belonging to the olefinic double bond as well as the methylene group next to the furan oxygen atom. For the epoxides **9**, **10**, and



Scheme 4. Details on the structure elucidation of **912**: **A**. selected NOESY correlations (green = above the ring, and brown = under the ring), and **B**. chemical oxidative derivatizations of **7** and the crystal structure[50] of **13**.

12, expected chemical shifts of $\delta(^1H)$ between 2.7 and 2.2 ppm, and $\delta(^{13}C)$ between 60 and 49 ppm were found. On the other hand, the lactols hemiacetals showed typical chemical shifts of $\delta(^1H)$ between 5.5 and 5.2 ppm, and $\delta(^{13}C)$ between 104 and 101 ppm. NOESY correlations indicated, but did not unequivocally prove, that the epoxidation must have occurred from the upper side. To facilitate structure elucidation, the chemical oxidation with m-CPBA was conducted which afforded two epimeric epoxides **9** and **14** in a 3:1 ratio with preference for re-side attack. Here, the conformation of the tricyclic substrate **7** must have controlled the facial selectivity of the oxidation. With the synthetic compound in hand, it was possible now to collect analytical data for the minor diastereomer. Indeed, we detected diastereoisomer **14** in the crude product collected with both enzymes. For the main biocatalyst UPO_{49} a ratio of 5:1 was found as judged by GC-MS analysis. We extended the question of facial selectivity to the Os(VIII)-mediated dihydroxylation, which yielded a crystalline product that allowed the open stereochemical problem to be solved unambiguously by X-ray crystallography.

The analysis of the dihydroxylation product **13** showed that the tertiary alcohol is located at the same position of the seven-membered ring as the methyl group, and the former single allylic proton. This strengthens the interpretation of the NOESY data collected for the epoxides from UPO-biotransformation and complements the oxidative transformations previously performed with **7**.[29] The stereochemical orientation of the newly introduced hydroxy group in lactols **10** and **11** was likely determined by the equilibrium of lactol groups with the ring opened aldehyde **15** (Scheme 5).

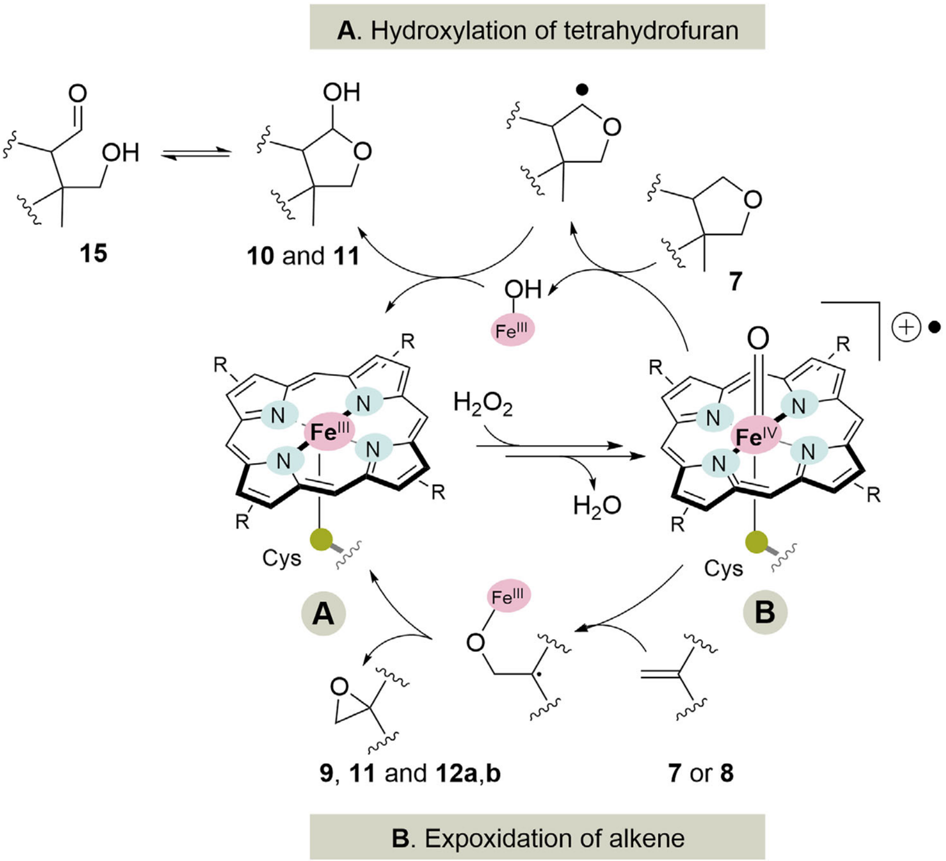
Mechanistically, the epoxidation products **9**, **12a,** and **12b** as well as the formation of the lactols **10** and **11,** can be summarized as shown in Scheme 5 case **A** (top): epoxidation, and case **B** (bottom): hydroxylation). The heme unit **A** serves as a starting point for the UPO-mediated oxidations, which after loading with H_2O_2, transforms into an iron hydroperoxide intermediate, and then furnishes the iron(IV) oxo species **B** after the elimination of water. In analogy to similar oxo species discussed for P450 enzymes, **B** can directly load the oxygen atom onto alkenes, whereby epoxides are formed (Scheme 5, lower reaction). Alternatively, it can remove an H radical from the substrate, and after recombination of the C radical thus formed (here in a neighboring position to the furan oxygen atom) with the hydroxyl group on the iron, the lactol moiety is generated. The preference for radical formation in the 2-position of tetrahydofurans by H-abstraction is well established in radical chemistry.[34]

3. Conclusion

In summary, we present the first-time successful oxyfunctionalization of the complex tricyclic terpenoid **7** and the macrocyclic ether **8** using a CHEM-BIO-BIO approach. This approach combines the chemical multistep synthesis of the non-natural FPP-derivative **3** along with the subsequent cyclization catalyzed by BcBOT2 and finally the chemo- and regioselective oxyfunctionalization catalyzed by commercial UPOs, including the well described PaDa-I mutant. Selected UPOs were found to catalyze two very different modes of oxidations, namely the epoxidation of the alkene moiety, and alternatively, the hemolytic cleavage of the C—H bond next to the tetrahydrofuran oxygen atom and trapping of OH to yield the corresponding hemiacetal. This type of radical hydroxylation is particularly noteworthy and fosters the potential of UPOs as powerful catalysts, and leads to products for potentially unique flavors and fragrances. Overall, the use of UPOs for the selective oxyfunctionalization of terpenoids paves the way to expand the "terpenome". Our further studies will focus on (i) upscaling each step at once (up to 2 L scale) which needs a systematic optimization of catalytic conditions of each step, and (ii) screening alternative UPOs to expand the product portfolio. This preliminary work can be regarded as the first step towards a chemoenzymatic synthesis campaign toward new drug-like terpenoids. For example, the lactol unit in **11** opens the possibility of a facile ring opening with the formation of two functional groups. These can further be oxidized, either chemically or enzymatically, to access more highly oxidized terpenoids, very much in the sense of the biotransformations from **4** to botrydial **5** (Scheme 1A).[27,28]

4. Experimental Section

A detailed description of the experimental procedures as well as the analytical protocols is given in the Supporting Information.



Scheme 5. Mechanistic considerations on the UPO-mediated oxidations reported here.

The authors have cited additional references within the Supporting Information.[51–54]

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Biocatalysis · CH-activation · Terpene synthases · Terpenoids · Unspecific peroxygenases

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