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RESEARCH ARTICLE

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# Lipoxygenase-catalysed co-oxidation for sustained production of oxyfunctionalized terpenoids

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## Abstract

Lipoxygenases (LOX) catalyse allylic oxidations and epoxidations of a co-substrate in the presence of an unsaturated fatty acid containing a 1,4-pentadiene moiety. One- and two-step enzyme assays were established to verify the role or involvement of LOX in such co-oxidations. It was shown that LOX is only involved in the formation of reactive hydroperoxides, but not in the oxidation of a co-substrate, assuming a mechanism involving free peroxy radicals for the latter. Ten mono- and sesquiterpenes were used as co-substrates and the resulting products were analysed by mass spectrometry. A semi-preparative approach was developed using (+)-valencene as an example, and the resulting products were isolated by preparative GC and their structures elucidated by NMR spectroscopy.

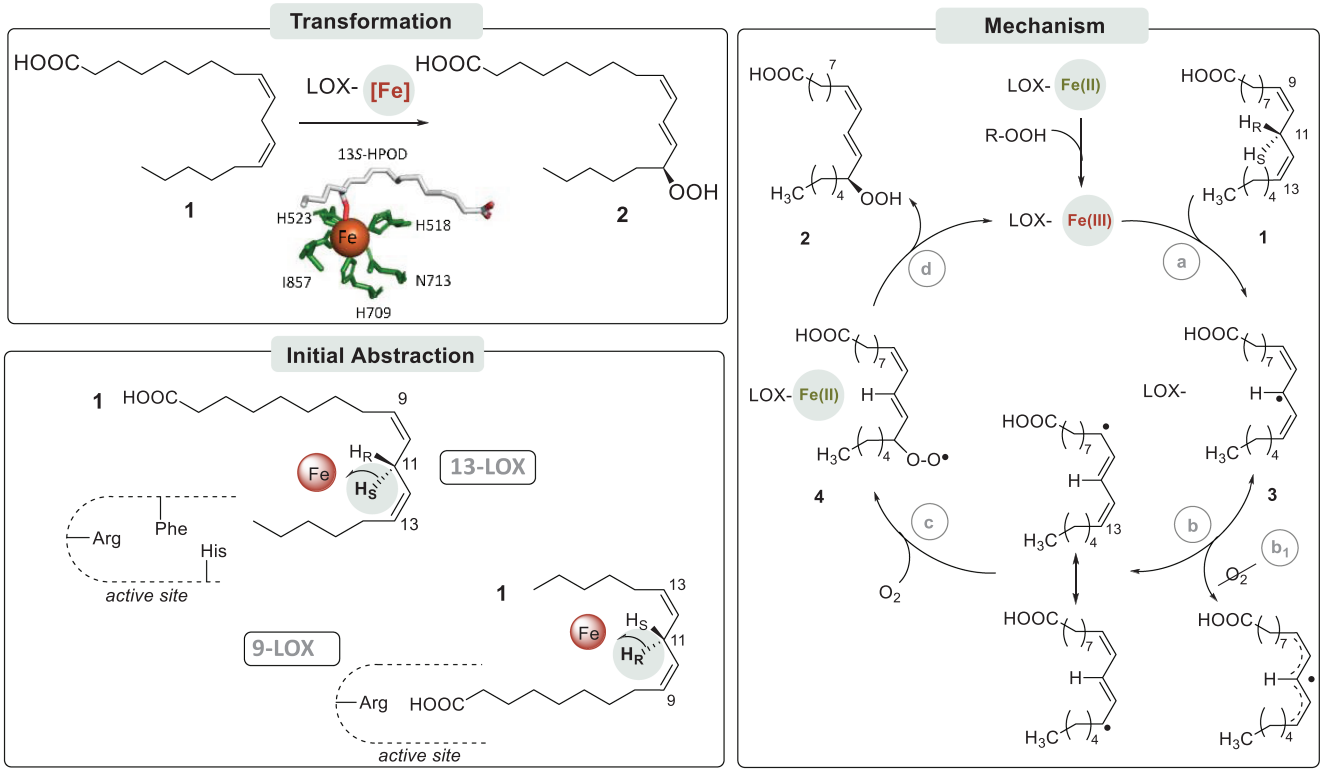
**KEYWORDS**
lipoxygenase, nootkatone, oxidation, terpenes, valencene

## 1 | INTRODUCTION

The biotechnological functionalization of terpenoids, for example, using enzymatic catalysis, enables the expansion of available terpenes under environmentally friendly and mild conditions. Thus, the structural diversity of this class of natural products can be considerably expanded and terpenoids with oxyfunctionalized moieties are particularly attractive as these are often characterized by remarkable sensory profiles.[1,2] By applying biocatalysis, terpenes without fragrance-relevant properties can be upgraded to products relevant for the fragrance and flavour industry. Potentially new olfactory properties are opened up by targeted enzymatic oxidations. An interesting but little-studied group of enzymes are lipoxygenases, which should be suitable for allyl oxidations and epoxidations in the presence of an unsaturated fatty acid.[3]

Lipoxygenases (LOX) are iron-containing non-heme dioxygenases belonging to the class of oxidoreductases.[3-5] They generally catalyse the dioxygenation of 1*Z*,4*Z*-pentadiene units found in polyunsaturated fatty acids such as linoleic acid (**1**) (Scheme 1),[6-9] resulting in the formation of chiral *E*,*Z*-configured hydroperoxides (HPOD, **2**).[10,11] The HPODs can initiate the biosynthesis of lipid mediators or serve as precursors for a number of secondary metabolites that possess antimicrobial and antifungal properties.[11-14] While plant LOX accept linoleic acid (**1**) or linolenic acid as substrates, animal LOX use arachidonic acid.[6,13,15] Lipoxygenases often exist in the form of different isozymes that differ in terms of optimal pH, substrate and product specificity, regioselectivity and co-oxidation properties.[3,6,16-18,20] In industrial processes, LOXs are used in the production of flavours or lipid-based chemicals, in the modification of lipids from raw materials, and in the bleaching of paint components due to their particular environmental friendliness.[19]

The crystal structure of the isoenzyme LOX-1 from soybean (*Glycine max*) served as a reference structure for all animal and plant LOXs.[11,21] They all have in common that they consist of two domains: A 25-30 kDa N-terminal β-barrel structure and a larger (55-60 kDa) α-helical domain at the C-terminus, where the active site




**SCHEME 1** LOX-mediated conversion of linoleic acid (**1**), the initial abstraction of an enantiotopic hydrogen and the underlying catalytic mechanism (explanation see text)

is also located. In addition, atomic absorption and Mößbauer spectroscopy led to the identification of an iron atom, a cofactor with a pseudo-octahedral coordination sphere (Scheme 1).[11,12,19] Substrate recognition is attributed to highly conserved leucine and isoleucine residues in the active site.[11] LOXes also have a tubular opening that is thought to act as an oxygen channel and control access to the active site through conserved amino acid side chains. This so-called "COFFA site" is thought to be responsible for stereocontrol in LOX-mediated oxidations and consists of the amino acid alanine in (*S*)-specific LOX, whereas glycine is found at this position in (*R*)-specific LOX.[22-24] Regioselectivity has been explained on the basis of the substrate orientation hypothesis.[25] Depending on whether it is a 13-LOX or a 9-LOX, the abstraction of the enantiotopic HS or HR hydrogen atom occurs depending on the substrate orientation in the active pocket.

Hydrogen abstraction occurs homolytically via the Fe(III) form of LOX, yielding highly reactive and mesomerically stabilized C radicals (Scheme 1, steps a and b).[5-7,11,14,20] Under aerobic conditions, the radical-enzyme complex reacts with molecular oxygen to form peroxy radical **4** (step c).[5,20] In contrast, if anaerobic conditions prevail, this peroxy radical is released from the active pocket (step b1)[5,6,20] and its reduction eventually leads to the formation of HPOD (**2**; step d). Simultaneously, the lipoxygenase is converted back to the active Fe(III) form.[3,5,20]

The bleaching properties of LOX were early combined with the idea of using its co-oxidative abilities in the oxidation of a second substrate.[18,26] The co-oxidation is attributed to the interaction with peroxy radicals formed by LOX, so a mechanism involving free peroxy radicals was suspected.[17,18,26] In addition to β-carotene (**5**) and further radical scavengers, **6-8** were also investigated as substrates for co-oxidation (Figure 1).[17,18,26,27] Moreover, mono- and sesquiterpenes **9-12** were included in the substrate portfolio, and in these cases oxidations of allylic positions were observed. In the case of (+)-valencene (**12**), the commercially valuable oxidation products nootkatone (**13**) and nootkatol (**14**) are formed. Typically, lipoxygenases from *G. max* or the basidiomycete *Pleurotus sapidus* were used in these studies.[28-34] Indeed, LOX-catalysed co-oxidation can be considered as a sustainable and natural alternative to the chemical production of oxyfunctionalized terpenoids.

The present work aims to extend the chemical potential of LOX for selected mono- and sesquiterpenes. Another aspect are preliminary mechanistic studies on the role of LOX in the co-oxidation of secondary substrates.

## 2 | EXPERIMENTAL

### 2.1 | Reaction rate and conversion rate of HPOD formation

To increase the yield of HPOD, analytical enzyme assays were performed at different pH values and temperatures. To determine the reaction rate, the conversion was followed photometrically at 234 nm over a period of 40 minutes (pH) or 20 minutes (temperature). Measurements were carried out at 30-second intervals using

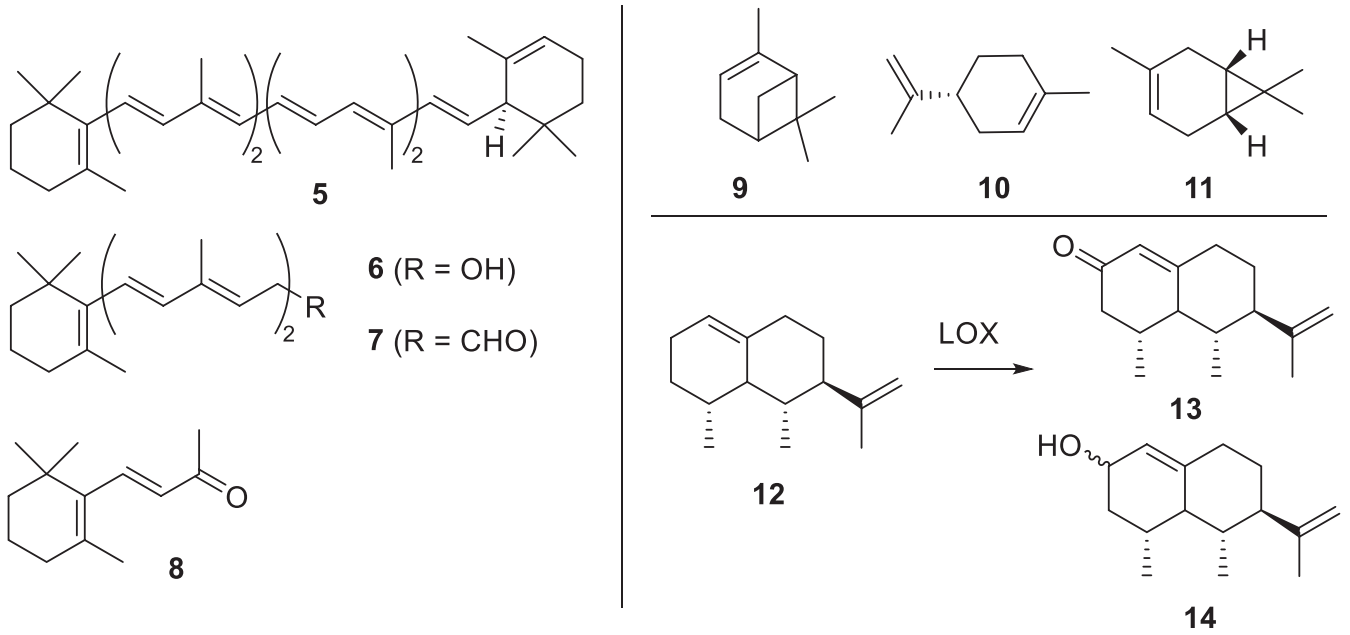



FIGURE 1 Substrates used in LOX-mediated co-oxidations with linoleic acid (**1**) as primary substrate: β-Carotene (**3**), retinol (**6**), retinal (**7**), β-ionone (**8**), α-pinene, (**9**), (+)-limonene (**10**), (+)-car-3-ene (**11**), (+)-valencene (**12**), nootkatone (**13**) and nootkatol (**14**)[28-34]

the SPECTRAmax Plus384 and Multiskan™ GO microplate reader. Temperature optimizations were performed in a range of 20-45°C. The pH optimizations were performed in a range of pH 4.0-10.0 (Appendix S1). For each analytical enzyme assay, 85.5 μmol/L of linoleic acid and 2000 U LOX from *Glycine max* (commercially available, supplier: Carl Roth, LOX ≥100000 U/mg, Unit: 1 μmol/min) were mixed. In addition, a negative control containing only linoleic acid was used. A 0.1-M Tris–HCl buffer with a pH of 9.1 was used for temperature optimization. The pH optimizations were performed at 22°C.

## 2.2 | General procedure for a one-step analytical enzyme assay

The total volume of the analytical enzyme assays was 1.0 mL. A 0.1 mol/L HEPES buffer with a pH of 6.5 served as the reaction solution. 800 μmol/L LA and 400 μmol/L cosubstrate were added to the buffer. Acetone (4% [v/v]) served as solubilizer. Biotransformation was started by the addition of 200 U LOX. Incubation was performed at 50°C for 48 hours. Subsequently, the enzyme assays were made alkaline (pH 9) with 1 mol/L NaOH and extracted twice with *n*-hexane (200 μL, 100 μL) for 1 minute. The organic phases were combined and washed with 300 μL of a saturated NaCl solution. The organic phase was then used directly for GC–MS analysis.

## 2.3 | General procedure for a two-step analytical enzyme assay

In a two-step enzyme assay, formation of HPOD occurred first, followed by co-oxidation with the co-substrate. For this purpose, 0.1-mol/L Tris–HCl buffer (pH 9.0) was provided and 800 μmol/L LA and 200 U LOX were added. Acetone (4% [v/v]) served as solubilizer. Incubation was performed at 40°C for 1 hour. Then, formic acid (1% [v/v], pH 3.0) was added and the HPOD was transferred to the organic phase by extracting twice with MtBE. The organic phase was washed with a saturated NaCl solution, dried with $MgSO_4$, filtered and the solvent was removed using a nitrogen stream. Then, the formed HPODs were dissolved in a 0.1-mol/L HEPES buffer (pH 6.5) and 400 μmol/L of the cosubstrate was added. The enzyme assay was incubated at 50°C for 48 hours and performed in analogy to the one-step enzyme assay.

## 2.4 | General procedure for a two-step semi-preparative approach

The total volume of the semipreparative preparation was 60 mL. First, a 0.1-mol/L HEPES buffer with a pH of 9.0 was provided and 12-mmol/L linoleic acid was added, resulting in an ACN content of 8% [v/v], which served as a solubilizer. The addition of 2000 U LOX initiated the biotransformation (30 minute, 40°C, 75 rpm) and formation of HPOD. Finally, the pH was lowered to pH 6.5 and the mixture was diluted by adding buffer solution. The ACN content was increased to 15% [v/v]. Then, 1.2 mmol/L cosubstrate was added before the mixture was incubated for another 72 hours.

The pH was then brought to alkaline with 1 mol/L NaOH and excess hydroperoxides were removed by addition of a saturated $Na_2S_2O_3$ solution. The mixture was then extracted with *n*-pentane, the organic phases were combined, washed with a saturated $NaHCO_3$ solution and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure (850 mbar, 40°C).

## 2.5 | Analysis

Analysis of successful co-oxidations was performed by comparing the original chromatograms with those obtained after LOX-HPOD treatment. An additional negative control also provided information on the sensitivity of the co-substrates to auto-oxidation. All co-substrates used were analysed chromatographically, and the retention indices as well as the purities and possible by-products were determined by mass spectrometry.

## 3 | RESULTS AND DISCUSSION

The optimizations for HPOD production and co-oxidation were performed separately. First, the optimal conditions for HPOD formation were verified. Linoleic acid (LA, **1**) served as substrate, while (+)-valencene (**12**) was used for the subsequent co-oxidation. Co-oxidation was then assessed by GC-based on the percentage area of nootkatone (**13**) formed.

Evaluation of enzymatic HPOD formation was performed photometrically at 234 nm and an extinction coefficient of 25 000 mol/L/cm.[35,36] To optimize reaction rate and turnover rate, HPOD formation was followed temporally at different temperatures and pH values (Appendix S1). By plotting the absorbance as a function of time, increasing curves were obtained from which the linear range and subsequently the reaction rate could be determined. Here, a high reaction rate is consistent with increased LOX activity. Regarding the temperature optimization, two maxima could be determined, one generated by the isoenzyme LOX-1 (20°C) and the other by the remaining isoenzymes (35-40°C).[37] Here, conversion rates of more than 95.2% ± 2.9% could be achieved (Appendix S1). With respect to pH optimization, a pronounced maximum above pH 8 could be determined, which can also be attributed to LOX-1 activity.[6,16,27,35,38] The conversion rate could be determined to more than 95.6% ± 4.2% (Appendix S1). An influence of ionic strength on HPOD formation cannot be demonstrated.

Investigations on the involvement of LOX in co-oxidation were carried out by developing one- and two-step enzyme assays (Figure 2A). Here, it was shown that LOX is not directly involved in co-oxidation, but only in the enzymatic generation of HPOD. In contrast, peroxidation-induced co-oxidation yielded an identical product spectrum as chemical oxidation with hydrogen peroxide. Therefore, the results testify to HPOD-induced co-oxidation in the form of a free peroxy radical mechanism, which was already assumed by WALDMANN and AZIZ.[17,26] Another peculiarity of a two-step enzyme assay was revealed when different incubation times for HPOD generation were considered (Figure 2B). A decrease in HPOD concentration was observed with increasing incubation time. The reason is the multifunctionality of LOX, which is why HPOD lyase properties of LOX degrade the hydroperoxides to secondary metabolites and are no longer available for subsequent co-oxidation.[8]

Since it has been shown that a one-step assay leads to higher co-oxidation yields, a variety of optimizations were carried out using the co-substrate (+)-valencene (**12**). Studies on the auto-oxidation of the substrate LA and the co-substrate showed that the latter is subject to significant autooxidation, which was validated using negative controls. The substrate LA is not subject to autooxidation. In addition, several optimizations were carried out. A substrate to cosubstrate molar ratio of 2:1 and an enzyme activity of 200 U LOX were found to contribute to the increase in product yield. Moreover, a 0.1-mol/L HEPES buffer with 4% (v/v) acetone as solubilizer and incubation at 50°C over 48 hours were found to be the optimized conditions for a one-step assay. Thus, the yield of nootkatone formed could be increased from 2.1% ± 0.7% to 39.2% ± 0.1%.

Interestingly, the optimum pH for co-oxidation was found to be 6.5. The reason for this could be the isoenzyme LOX-3, whose pH optimum coincides with that of co-oxidation. Isoenzyme LOX-3 is also characterized by strong co-oxidizing properties under aerobic

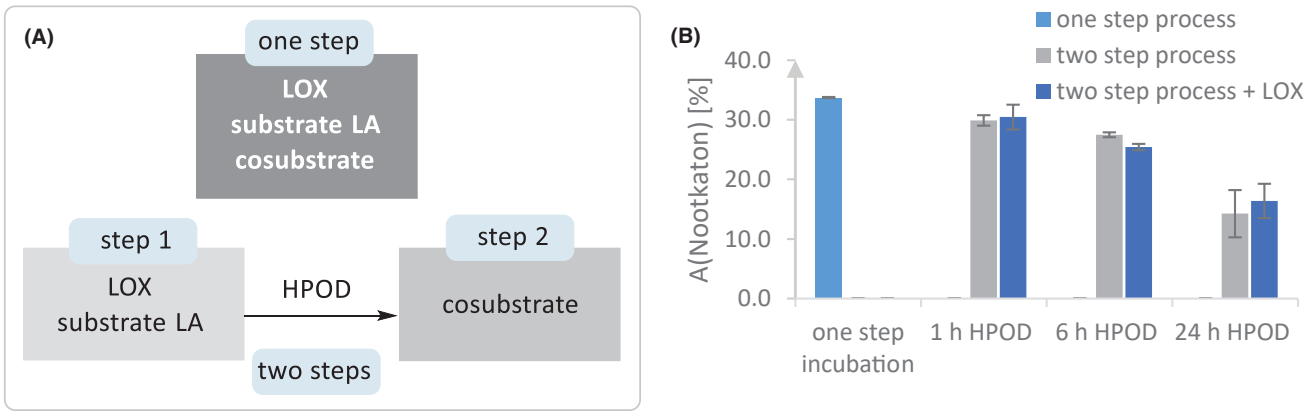



FIGURE 2 A, Schematic representation of the one-step and two-step analytical assay. The two-step assay is divided into HPOD formation (step 1) and co-oxidation of a co-substrate (step 2). In the one-step assay, the HPOD formation and co-oxidation occur simultaneously in one batch. B, Comparison of a one-step with a two-step assay. The two-step assay was run once with LOX and once without LOX addition. Three different incubation times for HPOD formation were also chosen and compared

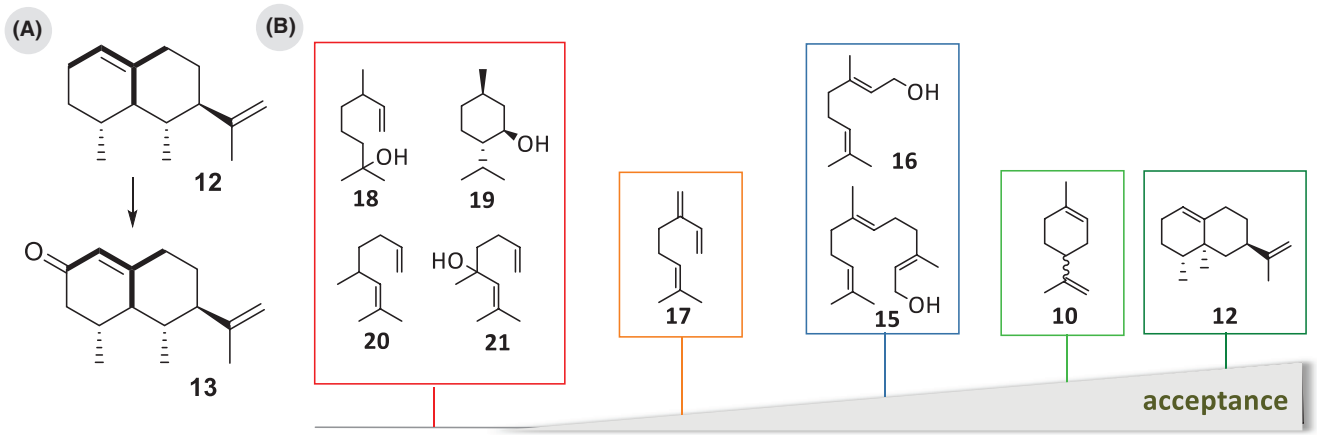



FIGURE 3 A, Structures of (+)-valencene (**12**) and nootkatone (**13**); B, Mono- and sesquiterpenes used in this study and acceptance by the LOX-HPOD co-oxidation system

conditions.[16,18,39] In this context, it is hypothesized that the ability of isoenzyme LOX-3 is due to the release of fatty acid radicals that form reactive peroxy radicals with molecular oxygen outside the active site.[5] These peroxy radicals could subsequently act as initiators for the peroxidation of the cosubstrate.

A total of 10 mono- and sesquiterpenes were selected for co-oxidation with the HPOD formed by LOX catalysis. The latter were (+)-valencene (**12**) and *E*,*E*-farnesol (**15**). Geraniol (**16**), myrcene (**17**), dihydromyrcenol (**18**), L-menthol (**19**) and the two enantiomers of limonene (**10**) served as monoterpenes. In addition, the by-products anomadiene (**20**) and isolinalool (**21**), which deviate from the isoprene rule and are formed during the production of dihydromyrcenol and linalool, were included in the studies.

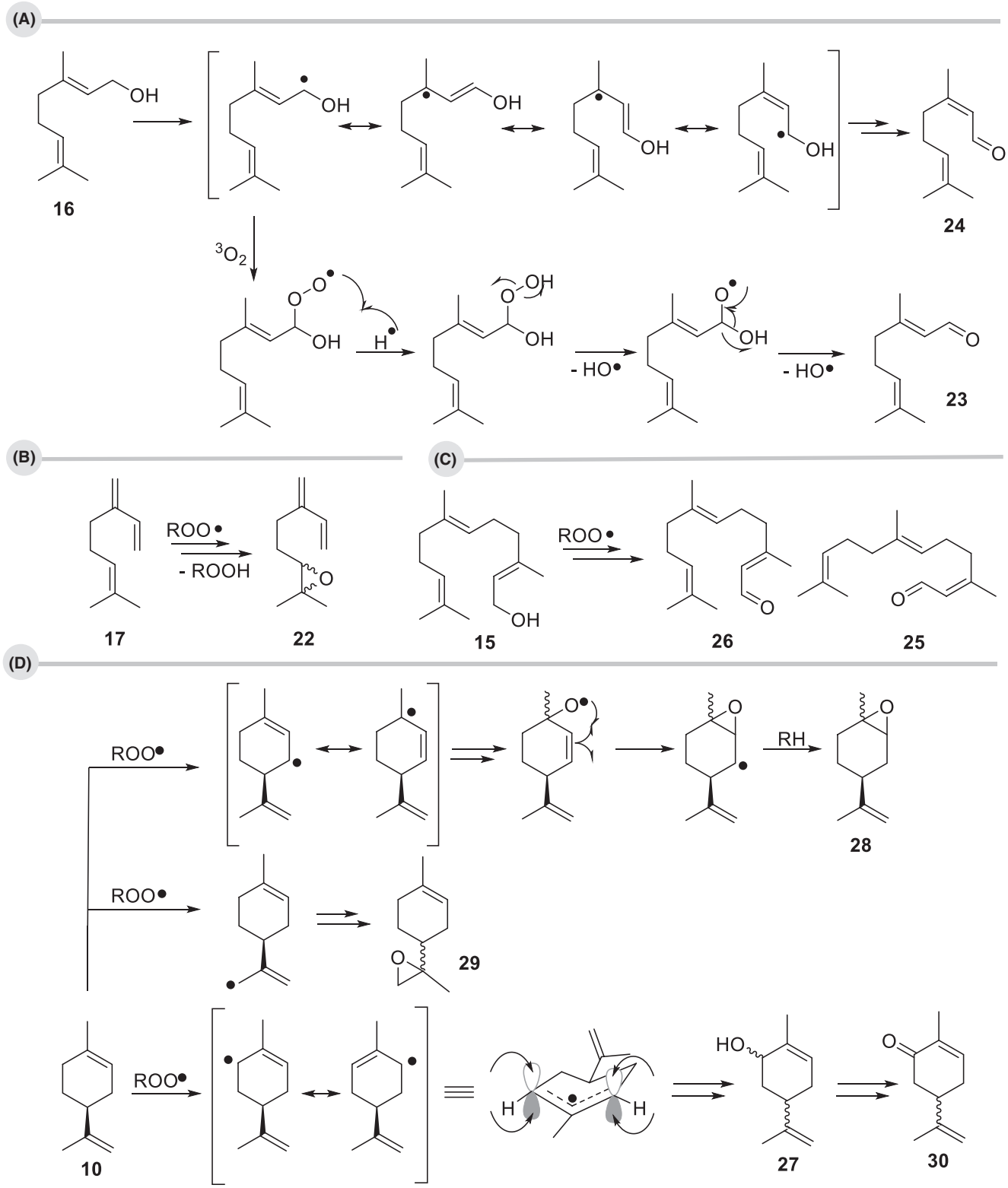
Previous studies of co-oxidations with the basidiomycete *P. sapidus* revealed oxidation of the allylic position (Figure 3A).[40] Since a free peroxy radical mechanism can be assumed for LOX from *G. max*, the regioselectivity of such an allylic oxidation is essentially based on the bond dissociation energies of the allylic CH bonds. In addition, steric accessibility is another important factor.[41] Finally, the stabilization capabilities of the formed radical are also crucial for regioselectivity.[41]

It is therefore hardly surprising that no conversion was observed for the substrate L-menthol (**19**). Likewise, isolinalool (**21**), dihydromyrcenol (**18**) and anomadiene (**20**) were not converted by the catalytic system, although these compounds contain alkenes. With the substrate myrcene (**17**), allylic oxidation was also not observed, but epoxidation to myrcene oxide (**22**) was found to occur instead (Scheme 2B). Studies by Weidmann showed that steric hindrance favours epoxidation over allylic oxidation,[41] but since there is no obvious steric hindrance present in myrcene, the lack of acceptance could be associated with the observation that acyclic compounds generally show lower reactivity with respect to allylic hydrogen abstraction (Figure 3B).[41]

Co-oxidation of geraniol (**16**) was shown to result in the formation of the oxidation products geranial (**23**) and neral (**24**) (Scheme 2A). Free fatty acid peroxy radicals formed outside the active pocket presumably act as initiators for the formation of the allyl radical, which, after rotation around the C2/C3 bond, also explains the formation of a tertiary nerolyl radical. In the reaction with (*E*,*E*)-farnesol (**15**), the products (*E*,*Z*)-farnesal (**25**) and (*E*,*E*)-farnesal (**26**) were detected after co-oxidation, and these are formed according to a mechanism analogous to that of geraniol (**16**) (Scheme 2C).

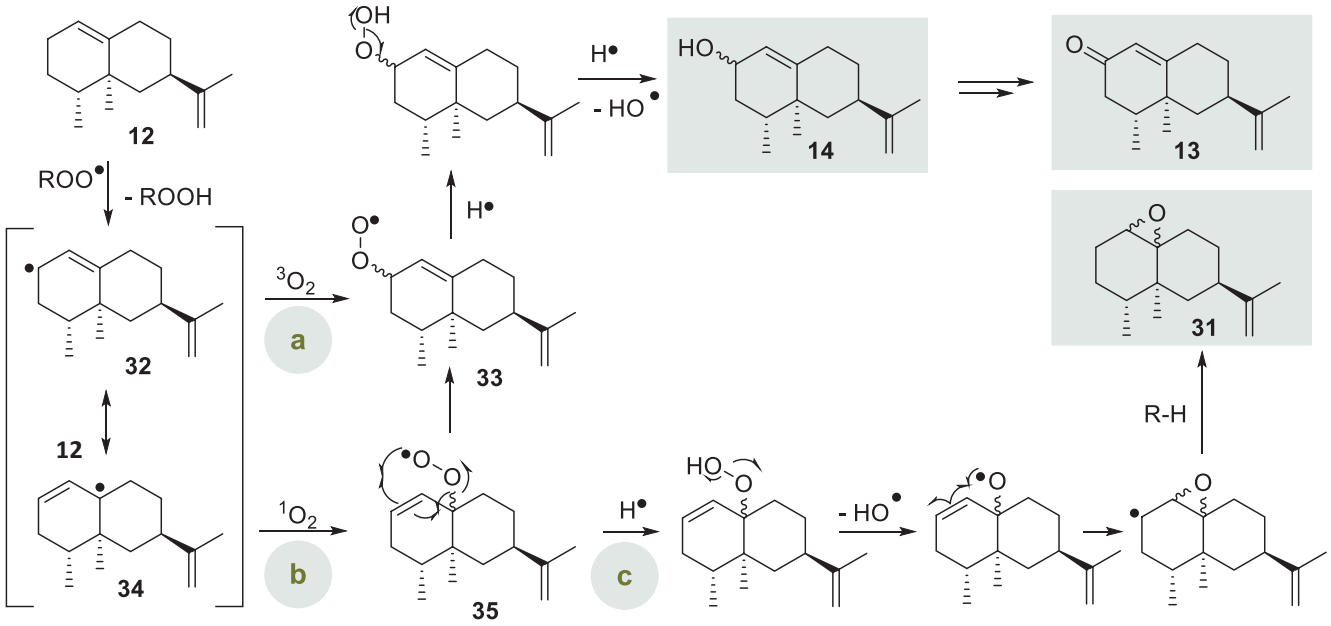
For the HPOD-induced co-oxidation of both limonene enantiomers (**10**), an identical product spectrum with five main compounds was obtained in both cases, so that none of the enantiomers is preferentially accepted (Scheme 2D). Thus, stereocontrol induced by a chiral template, like the enzyme, of the co-oxidation can be excluded and a free peroxy radical mechanism is operating. The latter is further supported by the fact that the diastereomers of the formed epoxides (**28**, **29**) and carveol (**27**) were detected.

Compared to the previous reactions, limonene (**10**) is a much more approachable substrate. This can be attributed to the fact that cyclic substrates exhibit higher reactivity towards allylic hydrogen abstractions than comparable acyclic compounds.[42] Regarding the regioselectivity, the formation of the carveol (**27**) is initially surprising, since the possible more stable tertiary radical is not formed. Here, steric hindrance by the neighbouring iso-propenyl group might prevent the formation of the more stable radical. The formation of the limonene epoxides (**28**, **29**) is unexpected as this oxidation competes with allylic oxidation. Depending on whether the reaction occurs at the *endo*- or *exo*-cyclic double bond, the products 1,2-limonene epoxide (**28**) and 8,9-limonene epoxide (**29**) are formed. Moreover, since the introduction of the oxygen function can occur via the "*re*"- or "*si*"-faces in each case and in fact both diastereomeric pairs were detected in the product spectrum. GC analysis using a chiral hydrodex-β-6TBDM stationary phase revealed that carvone (**30**) had formed as a racemate. The formation of both enantiomers of carvone (**30**) is a strong indication that all four possible stereoisomers of carveol (**27**) must have formed before.[43] Carvone is also of olfactory interest. While the sensory profile of (−)-carvone has a peppermint note, (+)-carvone (**30**) is characterized by a caraway odour.[34]




**SCHEME 2** Proposed mechanisms for the co-oxidation with the LOX-HPOD-system: A, geraniol (**16**) to geranial (**23**) and neral (**24**), B, myrcene (**17**) to myrcene epoxide (**22**), C, (*E*,*E*)-farnesol (**15**) to (*E*,*E*)-farnesal (**26**) and (*E*,*Z*)-farnesal (**25**), D, limonene enantiomers (**10**) to carveol (**27**), carvon (**30**), 1,2 limonene epoxide (**28**) and 8,9-limonene epoxide (**29**)

Next, we developed a semi-preparative two-step protocol using (+)-valencene (**12**) as substrate (40°C, pH 6.5, 72 hours; yield: 56% crude product). After product isolation by preparative GC purification, the structures of the products were identified by NMR spectroscopy and mass spectrometry. The purities of the products ranged from 63% to 92%. In addition to the well-known main




SCHEME 3 Postulated mechanism for the formation of nootkatol (**14**), nootkatone (**13**) and valencene epoxide (**31**) by a co-oxidation of (+)-valencene (**12**) with the LOX-HPOD system

product nootkatone, the two alcohols *exo*- and *endo*-nootkatol (**14**) were identified, as previously reported.[44] Nootkatone (**13**) has a pronounced grapefruit note and is therefore of great interest to the fragrance and flavour industry.[45] The isolated nootkatol alcohols (**14**) exhibit citrus and fresh notes. In addition, (+)-*cis*-valencene epoxide and (−)-valencene epoxide (**31**) were isolated, the formation of which has now been demonstrated for the first time in co-oxidation experiments with lipoxygenases.

As for the postulated mechanism of the isolated products and due to the formation of stereoisomers a free peroxy radicals must be placed in the centre of mechanistic considerations (Scheme 3). Instead of molecular oxygen as radical initiator, peroxy radicals formed by LOX catalysis could serve as initiator either by an early release of a fatty acid radical from the active pocket and reaction with molecular oxygen or by homolytic cleavage of the fatty acid HPOD.[5,18,26] These radicals initiate an allylic hydrogen abstraction in (+)-valencene (**12**). In the presence of triplet oxygen ($^3O_2$) (pathway a), a peroxy radical should form starting from the less stable secondary radical **32**, which further reacts to form hydroperoxide **33**.[46] The addition of molecular oxygen shows no facial selectivity, thus leading to the formation of the diastereomeric nootkatol alcohols (**14**) after homolytic cleavage. Another allylic hydrogen abstraction and reaction with molecular oxygen leads to the ketone nootkatone (**13**) starting from the alcohols **14**. In contrast, reaction with singlet oxygen ($^1O_2$), starting from the more stable tertiary radical **34**, leads to the peroxy radical **35**.[46,47] Subsequently, a SCHENCK rearrangement gave derivative **33** (pathway b).[48] Alternatively, the peroxy radical **35** is generated which abstracts a free hydrogen radical in a termination step (pathway c), giving the diastereomeric epoxides **31** after homolytic cleavage and release of a hydroxyl radical.

## 4 | CONCLUSION

In the present work, we describe LOX-catalysed co-oxidations with selected terpenes that were optimized using one- and two-step enzyme assays. We found that LOX is not directly involved in the co-oxidation, but only in the formation of the intermediate reactive hydroperoxides. Consequently, the co-oxidation should rely on a mechanism involving free peroxy radicals. In addition to cyclic substrates such as (+)-valencene (**12**), also linear mono- and sesquiterpenes were used as co-substrates. It was found that allylic oxidations preferentially occur at endocyclic double bonds. In the case of geraniol (**16**) and (*E*,*E*)-farnesol (**15**), oxidation of the allylic hydroxyl group present was observed. The oxidations with the co-substrates limonene (**10**) and (+)-valencene (**12**) revealed that the co-oxidation is not stereoselective. For (+)-valencene (**12**), after performing a semipreparative biotransformation, the products were isolated and the stereoisomers of nootkatol (**14**) and *cis*-valencene epoxide (**31**) were detected in addition to the fragrance-relevant nootkatone (**13**).

At this point, however, it is not clear whether oxidation, despite the lack of stereocontrol, still occurs in the presence of the protein or is triggered exclusively by the intermediate peroxide.

The LOX-HPOD system thus offers a gentle and natural alternative to chemical allylic oxidations. Importantly, access to oxyfunctionalized terpenes provides new applications for the fragrance, flavour and cosmetics industry. In the future, other commercially available terpenes or even essential oils may be investigated using this oxidation protocol expanding the practical opportunities. A more general look at this oxidation system can interrogate the evolutionary significance of this simple oxidation system, which tames triplet oxygen in a much simpler way than, for example, cytochromes.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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