Title

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Journal

Perkin 1, 17(43)

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Publication Date

2019-11-06

DOI

10.1039/c9ob01814j

Tailoring chemoenzymatic oxidation via *in situ* peracids

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Abstract

Epoxidation chemistry often suffers from the challenging handling of peracids and thus requires *in situ* preparation. Here, we describe a two-phase enzymatic system that allows the effective generation of peracids and directly translate their activity to the epoxidation of olefins. We demonstrate the approach by application to lipid and olefin epoxidation as well as sulfide oxidation. These methods offer useful applications to synthetic modifications and scalable green processes.

First reported in 1909, epoxides are commonly installed by the Prilezhaev (Prileschajew) reaction[1] where a terminal oxygen of a peracid is transferred to an olefin.[2] Through a characteristic 'butterfly' mechanism, this process reliably delivers an epoxide with retention of the stereochemistry contained within its parent alkene.[3] For most laboratory purposes, commercially-available mCPBA[4] offers a viable peracid. However, the complexities associated with its scale often complicate its industrial use, and catalytic processes such as those developed by Jacobsen or Sharpless have played an important role in advancing access to epoxides at process scales.[5] With epoxides appearing in commodity chemicals and pharmaceuticals,[6] recent attention has turned to exploring enzymatic and chemoenzymatic methods to install these groups.[7] Here we advance the use of enzymatic epoxidation methods by exploring the scope of two-phase lipase catalysis and the use of co-catalysts to expand available oxidation methodologies.

In 1990, a team at Novo Nordisk described the use of lipases as tools to prepare peracids *in situ* and subsequently epoxidize olefins (Scheme 1). Here, conventional lipase hydrolysis with H_2O_2 is intercepted,[8] and the resulting perhydrolase promiscuity was used to prepare a series of long chain per-fatty acids (Step 1, Scheme 1). To date, this method has gained utility through the optimization and commercialization of Novozyme 435 (an immobilized form of the lipase B from *Candida antarctica*, CALB).[9]

Thus far, the best chemoenzymatic epoxidation methods have been obtained with CALB, [7d,8,9] implemented in quite harsh conditions for an enzymatic reaction, such as in medium containing high H_2O_2 concentrations and toluene.[10] Because the reaction takes place in the aqueous phase, these conditions are required to displace the reaction equilibrium towards perhydrolysis instead of hydrolysis.[7d,10c,11] While the catalytic mechanism of this reaction has been carefully evaluated,[11b,12] the potential for enzyme and reaction engineering still remains an important component in developing this approach.[7d,10a–c,11a,b] Indeed, even though CALB has proved to be more stable and active in organic solvents than most lipases, [9c] its stability in the presence of high H_2O_2 concentrations could still be improved.[10ac] To increase the efficiency of the reaction, an alternative optimization strategy can also consist of increasing the ratio of perhydrolysis to hydrolysis rate. This can be accomplished by using enzymes with higher selectivity for H_2O_2 over H_2O as the nucleophilic acceptor.[11,12a]

In aqueous medium, lipases/acyltransferases such as CpLIP2 from *Candida parapsilosis*,[13] and MsAcT from *Mycobacterium smegmatis*[14] display preferences for nucleophiles other than H_2O and have been shown to efficiently catalyse perhydrolysis over hydrolysis,[14,15] suggesting that a correlation could exist between acyltransfer and perhydrolysis properties. [11b,12a] In comparison, in media with high thermodynamic activity of H_2O (a_W), lipases such as CALB favour H_2O as a nucleophile acceptor.[13b] This difference makes lipases/ acyltransferases more promising enzymes to implement for epoxidation in aqueous environments without control of the a_W, such as in simple biphasic aqueous/lipid medium appropriate for perhydrolysis reactions.

Depicted in Fig. 1, the wild-type (wt) CpLIP2 is a versatile lipase/acyltransferase, with particular efficiency for unsaturated fatty acid monoesters but also able to accept saturated acyl donors and tri-, di- and mono-glycerides as substrates.[16] Moreover, it catalyses acyltransfer to various nucleophiles, including H_2O and alcohols,[13] but also amines[17] and H_2O_2.[15] Regarding perhydrolysis, it was shown that up to 78% of olefin to epoxide group conversion could be obtained with CpLIP2 in a mixture containing weight ratios of 26% oil, 71% H_2O and 4% H_2O_2. This compared favourably with CALB that delivered a 72% yield conversion using less oil and more H_2O_2 (weight ratios of 16% oil, 54% H_2O and 29% H_2O_2).[15] Unfortunately, with CpLIP2, the significant increase of acidity induced by the concomitant hydrolysis was detrimental.[15] Therefore, among the numerous mutants of CpLIP2 available,[18] CpLIP2 Y179F (Fig. 1c) was selected for the *in situ* epoxidation experiments presented here due to its potential to catalyse perhydrolysis over hydrolysis more efficiently than the wt enzyme. Indeed, in addition to displaying boosted acyltransfer ability compared to the wt,[18a,c] the mutation Y179F was shown to enhance enzyme stability by increasing its resistance to alcohol and consequently the a_W limit for total loss of activity. [18a]

We began our studies by exploring the epoxidation of triolein (**1a**). As shown in Scheme 2, we were able to test reaction conditions using NMR analyses as a screening tool and identified methods that delivered epoxyoleic acid (**1b**) from **1a**. The first step was to identify the pH and temperature at which this reaction should take place. Considering previous studies on the stability of CpLIP2,[19] we concluded that running this reaction at pH 6.5 and at room temperature would be ideal. Next, we screened the optimal concentration of H_2O_2. We

determined that 1.5 M H_2O_2 (4.6% v:v) was the maximum concentration that could be used without inhibiting CpLIP2 Y179F activity. It allowed the production of **1b** from **1a** after 24 h, albeit as one component of a complex mixture containing mono-, di- and tri-epoxyacylglycerides.[15] While this process likely arose through the formation of an intermediate peracid, its rapid auto-oxidation prevented us from identifying this intermediate even when conducting detailed time course studies. Given the complex product mixture obtained from **1a**, we turned our attention to palmitoleic acid (**1c**), which auto-epoxidized to **1d** in 31% yield.[20]

To increase the conversion to epoxide, we explored the possibility of adding an extra ester or acid as a co-catalyst, which would serve as an additional substrate for the perhydrolysis reaction and thus allow increased production of peracids and subsequent epoxide formation. We began by screening the choice of the ester or acid component and determined that among ethyl acetate, acetic acid, methyl acetate, triacetin, methyl laurate and methyl hexanoate, with the latter providing optimal turnover. Interestingly, within the acetic acid esters, ethyl acetate was found to inhibit the enzyme. We then screened for reaction time and reaction stoichiometry and obtained optimal reaction conditions with 100 mM of olefin, 1.5 M H_2O_2, 500 mM of methyl hexanoate, 100 mM phosphate buffer at pH 6.5 and CpLIP2 Y179F at 0.4 mg mL^{-1} (0.008 mM). These conditions afforded **1d** in 71% yield from **1c** after 24 h following purification by a simple aqueous workup and column chromatography.

With this in hand, we began to evaluate the scope of the method on a series of alkene substrates. We first focused our attention to terpenes, as their alkene moieties can be easily functionalized to produce an array of building blocks for use in chemical synthesis. We selected phytol (**2a**) and methyl geranate (**3a**) as models to explore the potential of oxidizing non-fatty acid olefins and substrates with multiple olefins (Fig. 2). In the case of **2a**, addition of an ester co-catalyst was mandatory and in these two examples, methyl hexanoate was found to provide the optimal turnover. Testing this method on different terpenes of varying size, it showed to work well for converting acyclic terpenes **2a** and **3a** to their corresponding epoxides **2b**[21] and **3b**.[22] Trace amounts (<5%) of esterification of **2b** was also observed. CpLIP2 Y179F's ability to catalyse perhydrolysis over hydrolysis is exemplified in the formation of **3b**, in which the epoxide was formed while retaining its methyl ester moiety.

We then turned to screen a series of alkenes using the same reaction conditions. Here, our goal was to understand the scope of this reaction in context to a low enzyme and co-catalyst loading (Fig. 2). We began with the epoxidation of geraniol (**4a**). While its corresponding epoxide **4b**[23a] was formed in low yield, the reaction was inhibited by the formation of the unreactive hexanoate ester of **4a**. Once the alcohol was *tert*-butyldimethylsilyl (TBS) protected to **5a**, the reaction proceeded smoothly to deliver epoxide **5b**.[23b] Similar to the epoxidation with mCPBA,[22–23] this reaction regioselectively epoxidizes the more nucleophilic alkene, which can be seen in **3b** and **5b**. Oxidation of linalool (**6a**) generated epoxide **6b** *in situ*, which underwent an intramolecular reaction by attack of the tertiary hydroxyl-group yielding furan **6c** and pyran **6d**.[24] Protection of this hydroxyl-group, as a TBS ether, provided a facile conversion of **7a** to the corresponding epoxide **7b**. Trace amounts (<5%) of TBS deprotection and conversion to **6c** and **6d** was also observed in epoxidation of **7a**.

The method was also capable of epoxidizing cyclic terpenes. In the first example, α-pinene (**8a**), its product pinene oxide **8b**[25] was observed to be unstable and undergo isomerization followed by hydration, common among terpene epoxides, to yield sobrerol **8c**.[26] In a second example, epoxide **9b** was obtained from (1*S*)-(+)-3-carene (**9a**),[27] demonstrating that depending on the terpene structure and its ability to rearrange, oxidation to a stable terpene epoxide is viable under these chemoenzymatic conditions.

While many of the olefins shown in Fig. 2 contain varying functional groups that were not affected by the defined reaction, some of the alcohol-containing substrates underwent rearrangements or further reactions as suggested by the epoxidation of **6a** to **6c/6d** and the rearrangement of **8b** to **8c**. Other alcohols such as 3-cyclohexene-1-methanol (**10a**), cyclohex-2-en-1-ol (**12a**) and prenol (**14a**) were not tolerated, often returning unreacted starting materials instead of the desired epoxides **10b**, **12b** and **14b**. Here, protection of **10b**, **12b** and **14b** as TBS ethers **11a**, **13a** and **15a**, respectively, enabled the conversion to epoxides **11b**,[28] **13b**,[29] and **15b**.[30]

Next, we tested the method to epoxidize olefins within conjugated aromatic systems (Fig. 2). The first example, *cis*-stilbene (**16a**), underwent epoxidation to **16b**.[31] Indene (**17a**) underwent epoxidation followed by ring opening of its respective epoxide to afford the enzyme-directed cis-diol **17b**.[32] The third aromatic example, benzopyran **18a** was successfully epoxidized to **18b**.[33] While these yields were not optimized for each substrate, the fact that very reactive epoxides such as **16b** or **18b** were obtained demonstrated the mild nature of these conditions.

Glycals were also found to be applicable in this chemoenzymatic method. These substrates could be activated by the lipase reaction to yield their corresponding epoxides but underwent spontaneous opening to form diols due to the reaction being conducted under aqueous conditions.[34] This olefin activation was observed by the conversion of glycals **19a-22a** to **19b**,[35] **20b**,[36] **21b**,[37] and **22b**,[38] respectively (Fig. 2). Based on the glycals tested, per-benzylated **22a** was less reactive than the per-acetylated **19a-20a** or silyl-protected **21a**. Interestingly, the epoxidation to **21b** was possible in the presence of hydroxyl-groups. The stereochemical outcome was confirmed to deliver glucosides **19b** and **21b** from glucals **19a** and **21a**, as well as galactosides **20b** and **22b** from galactals **20a** and **22a**. While H_2O intercepted each of the glycal epoxides, one can envision the development of protocols to apply these epoxides for glycal assembly.[39]

Next, we tested if the method was applicable in the epoxidation of more complex molecules. Using the same conditions as the previous examples, mycophenolic acid (**23a**), β-caryophyllene (**24a**), cholesteryl chloride (**25a**), pregnenolone (**26a**) and dehydroepiandrosterone (**27a**) were effectively epoxidized to their corresponding products **23b, 24b**,[40] **25b**,[41] **26b**,[42] and **27b**[43] without unwanted side reactivity (Fig. 2). The reaction with **24a** returned only product **24b** along with 5–10% of an unidentified by-product. Just like with mCPBA, substrate control leads to the α-epoxides of **25b**, **26b**, and **27b** as the major product.

In addition to olefins, this method is capable of performing sulfide oxidations to their respective sulfoxides *via in situ* peracid formation (Fig. 3). In the presence of both an alkene and a sulfide, sulfide oxidation appears to take preference over olefin epoxidation to retain aromaticity in the conversion of thiophene **28a** to sulfone **28b**,[44] though further studies are needed to assess the reaction's regioselectivity with additional substrates. While the sulfoxide product of benzothiophene was not observed as a major product, this is consistent with its known high reaction rate from sulfoxide to sulfone.[44b] On the other hand, sulfides **29a-32a**[45] all showed conversion to their corresponding sulfoxides **29b-32b** as a major product, where sterics play a key role in the induction of stereochemistry for **30b-32b**. While many known sulfide oxidations tend to over-oxidize to the sulfone, this reaction's product selectivity is useful for when only the sulfoxide is desired.[46]

Our next series of studies explored the use of lactones as co-catalysts to further optimize the reaction. As shown in Scheme 3, *in situ* formation of the corresponding ω-hydroxy peracid (Step 1) would be capable of conducting the epoxidation (Step 2), generating an ω-hydroxy acid that could regenerate the lactone (Step 3), a feature that was not possible by esters such as methyl hexanoate (Scheme 1). Here, we could envision a process wherein the recycling of the ω-hydroxy acid to lactone serves as a means to reduce the co-catalyst requirement.

A series of lactones were tested in the epoxidation of β-caryophyllene (**24a**) to **24b** in which the previously optimized conditions were applied, replacing the 500 mM of methyl hexanoate with 300 mM of lactone to account for higher reactivity when the lactone is regenerated. While only five- and six- membered ring lactones will be in equilibrium with their respective hydroxy acids in aqueous medium due to their higher stability, additional lactones were also tested for their efficacy as a co-catalyst.[47] NMR analyses (Fig. 4) indicated that β-butyrolactone and δ-valerolactone present optimal turnover of **24a** to its respective epoxide **24b**, showing the efficacy of using lactones in the place of esters or acids as co-catalysts. As expected, other lactones, such as caprolactone, underwent significant hydrolysis (Step 3, Scheme 3) during the oxidation process, therein identifying a structure-function relationship that could enable tuning the reactivity.

In our final study, we briefly explored the effect of mutagenesis as a means to modify the enzymatic activity, as suggested by CpLIP2 Y179F (Fig. 1c) and then compared these to CALB's activity. Interestingly, we found that the CpLIP2 mutants and CALB have different selectivities for the co-catalyst used in the reaction. Using CpLIP2 Y179F with **24a**, no starting material could be seen with methyl hexanoate after 24 h as compared to β-butyrolactone that returned traces of **24a**. This was confirmed by a higher recovery yield to **24b** (67% *versus* 41%, respectively). Similar results were observed with mutants CpLIP2 Y179F_S369A and CpLIP2 S369A, both known to improve acyltransferase activity.[18b] Conversely, with CALB, while some starting material remained after 24 h, the β-butyrolactone appeared to be a better co-catalyst due to less byproduct formation, suggesting that the co-catalysts behave differently depending on the lipase. In addition, the wt and some other mutants of CpLIP2 were tested in the same conditions. Preliminary results suggest that mutations can change the selectivity toward the co-ester and the subsequent final epoxidation yield that can be obtained. Therefore, the co-catalyst should be adapted to each enzyme or enzyme mutation to achieve optimal results for the epoxidation.

Conclusions

Here we report an oxidation method that operates at low enzymatic loading (0.4 mg mL^{-1}, $< 10^{-4}$ equivalents) in media containing low concentrations of H_2O_2 ($< 5\%$ v:v) and 5 equivalents of a co-catalytic ester (Scheme 1) or 3 equivalents of lactone (Scheme 3). Our studies have shown that this reaction can be used to epoxidize olefins (Fig. 2) and glycals (Fig. 2), as well as oxidize sulfides (Fig. 3). This method offers several advantages compared to commonly used peracid conditions. The first arises from the mild nature of the reaction, as both the epoxidation (Fig. 2) and oxidation (Fig. 3) were conducted at pH 6.5 using phosphate buffer. As shown in Fig. 2, we were able to prepare and isolate epoxides from substrates that are well known to undergo rearrangements (**9b**,[27c] Fig. 2) and hydrolytic opening (**16b**[32] and **18b**,[33] Fig. 2) under peracid conditions. Second, the reaction is conducted without organic solvent, a greener and cost-effective advantage, with the substrate floating as a wax or solid on top of the reaction medium. While organic solvents are needed for chromatography, use of them during extraction was only to expedite screening efforts. Comparable yields were obtained by removal of the aqueous phase, washing with H_2O and air or vacuum drying prior to further purification.

As shown herein, we were able to prepare peracids from ester and lactone co-catalysts *in situ* and apply them in an effective manner for epoxidation and oxidation methods. These studies provide *in situ* access to peracids that have yet to be explored in a synthetic context. For many co-catalyst examples such as 3-hydroxybutaneperoxoic acid, access to this peracid and its use are conveniently provided from β-butyrolactone. Studies are now underway to develop methods to match individual substrates with their corresponding co-catalyst oxidants and engineered enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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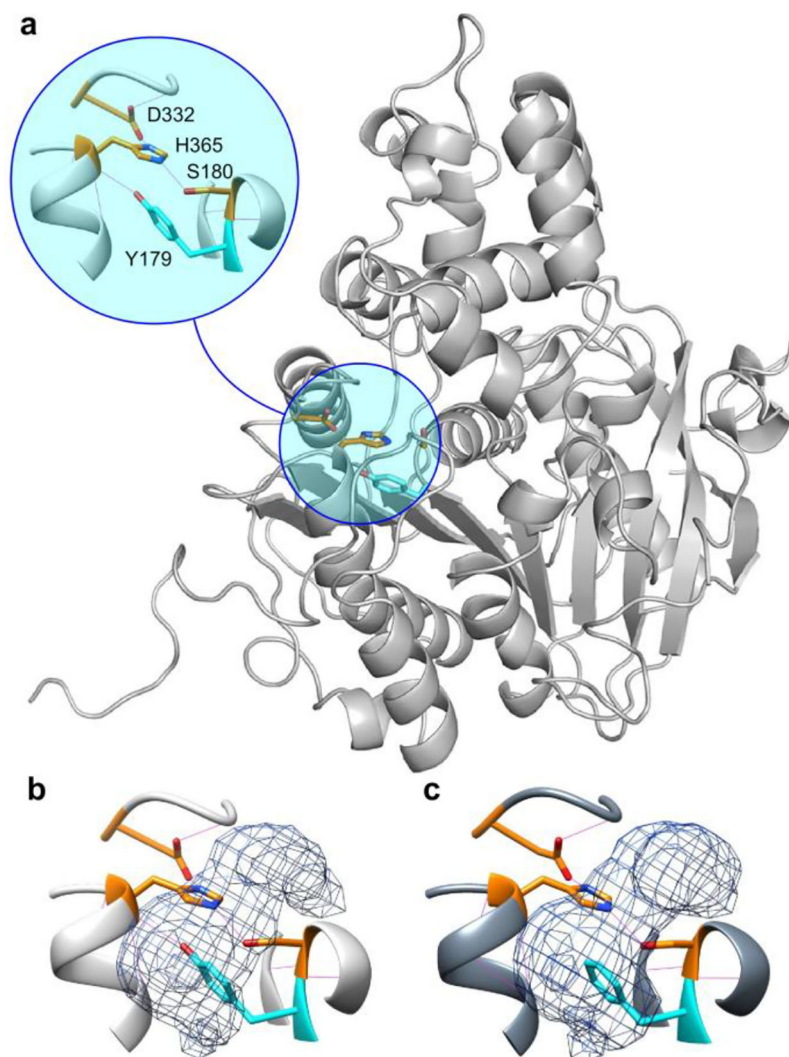
Fig. 1.
Structural features of CpLIP2. **a)** Structure of CpLIP2 with the active site identified by a circle. Expansion of the active site identifying the catalytic triad comprised of H365, S180 and D332 with Y179 playing a putative role in oxyanion formation. **b)** Close up of the active site pocket within wtCpLIP2. **c)** Close up of the active site pocket in CpLIP2 Y179F.
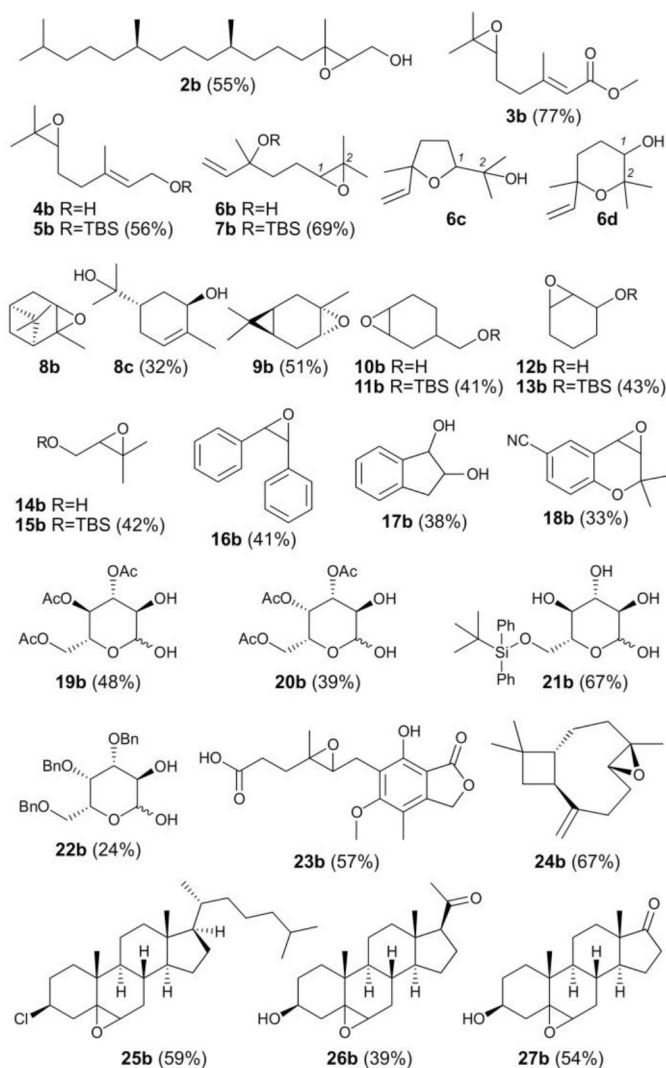
Fig. 2.
Evaluated substrates. Epoxide products **2b**-**27b** and rearranged epoxides **6c**, **6d** and **8c** prepared by oxidation of alkenes **2a**-**27a** using 1.5 M H_2O_2 and 500 mM methyl hexanoate in phosphate buffer pH 6.5 for 24 h. The reported yields were obtained after reacting each of substrates **2a**-**27a** under identical conditions. Unless stated otherwise in the text, the remaining mass was unreacted starting material or product lost/decomposed during purification. While additional enzyme and/or co-catalyst was shown to improve conversion, we report on a direct comparison of the efficiency of this reaction with a single substrate-optimized condition. Structures of the starting materials are provided in Supplemental Figure S1.
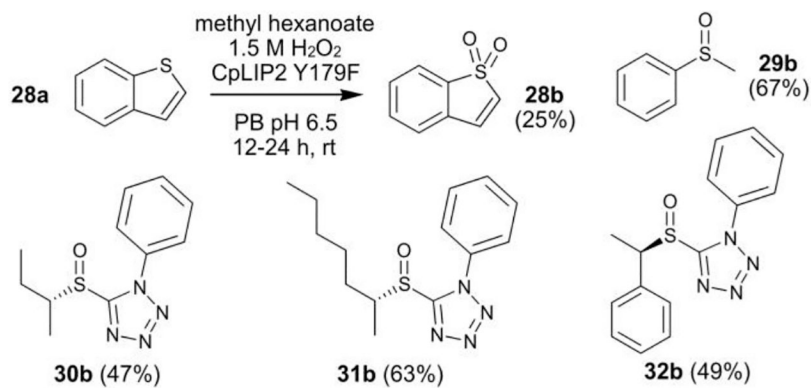
Fig. 3.
Exemplary application to sulfoxide preparation. While the oxidation of benzo[*b*]thiophene (**28a**) led to the formation of the sulfone **28b**, conditions were identified that enabled the conversion of **29a**-**32a** to their corresponding sulfoxides **29b**-**32b**. The reported yields were obtained after reacting each of substrates **28a**-**32a** under identical conditions used in Fig. 2. Unless stated otherwise in the text, the remaining mass was unreacted starting material. PB denotes phosphate buffer. Structures of the starting materials are provided in Supplemental Figure S1.
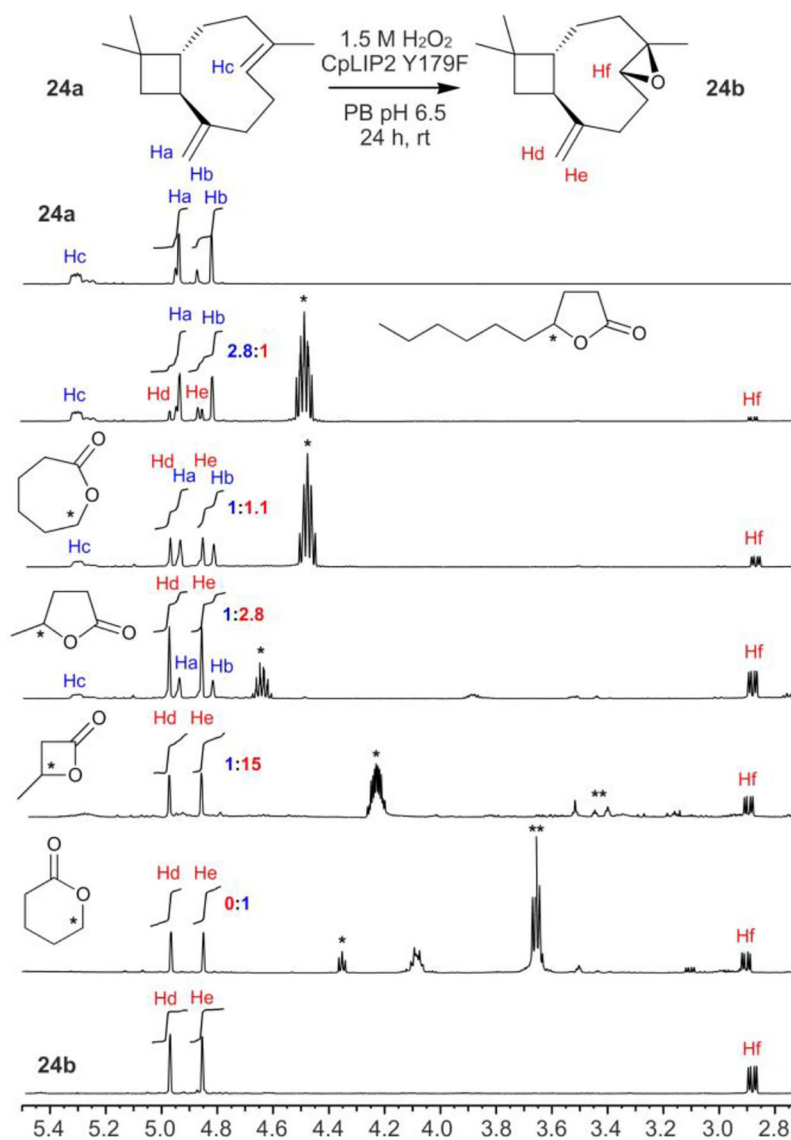
Fig. 4.
Functional role of lactones. NMR analyses from the epoxidation of β-caryophyllene (**24a**) to epoxide **24b** illustrates the relative efficacy of lactones as peracid surrogates. Protons are assigned by Ha-Hc in **24a** and Hd-Hf in **24b**. An asterisk (*) or double asterisk (**) denotes the position of peaks from the lactone or its corresponding ω-hydroxy peracid, respectively. Ratios represent yield of **24a**:**24b** based on NMR integration.
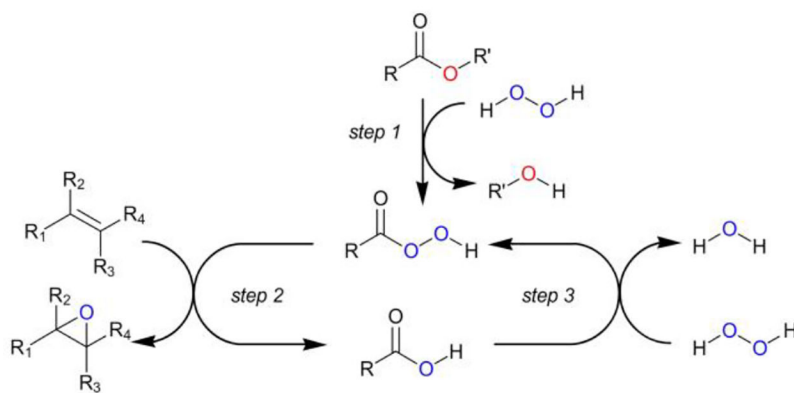
Scheme 1.
Schematic representation of enzymatic epoxidation. The lipase-catalysed exchange between H_2O_2 (oxygen atoms in blue) with an ester or acid (oxygen atoms in red) generates an alcohol or H_2O, respectively. The corresponding peracid in step 1 can, in turn, be used to epoxidize an olefin in step 2. The resulting acid can then be transformed back into the peracid with H_2O_2 in step 3, forming a continuous cycle. R represents aryl or alkyl functionality. R' can be H for acids or alkyl or aryl groups for esters.
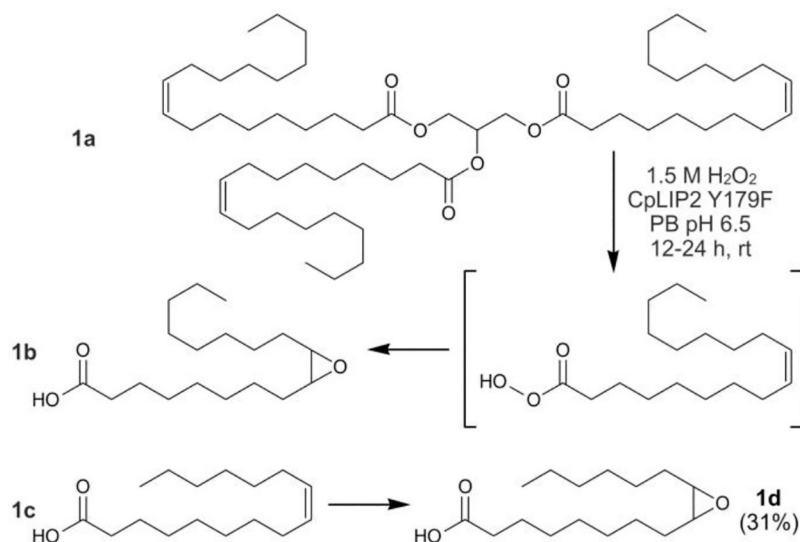
Scheme 2.

Auto-epoxidation. Oxidation of triolein (**1a**) undergoes release of the corresponding peroleic acid (brackets), which is subsequently converted to epoxyoleic acid (**1b**). Similarly, palmitoleic acid (**1c**) produces its analogous peracid to yield epoxypalmitoleic acid (**1d**). PB denotes phosphate buffer. The yield of **1d** represents isolated material with the remaining mass attributed to unreacted starting material.
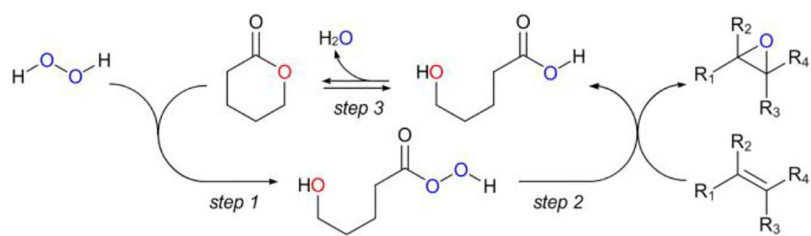
Scheme 3.
Lactone co-catalysed epoxidation. The lipase-catalysed exchange between H_2O_2 (oxygen atoms in blue) with a lactone (oxygen atoms in red) generates a ω-hydroxy peracid in step 1, which in turn can be used to epoxidize an olefin (step 2) and then cyclize back to the corresponding lactone (step 3).