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Green Chemistry

TUTORIAL REVIEW

The use of lipases as biocatalysts for the epoxidation of fatty acids and phenolic compounds

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Lipases are versatile enzymes that can be used for various kinds of biocatalyzed reactions. Owing to their selectivity and their mild reaction conditions, they can be often considered as more interesting than classical chemical catalysts. Besides their application in oil and fat processes, these enzymes have proved to be very attractive for other lipase-catalyzed reactions. This review discusses the latest results where lipases are used for the epoxidation of lipid substrates (namely fatty acids and their derivatives) and phenolic compounds. This chemo-enzymatic process involves a two step synthesis where the biocatalyst acts as a perhydrolase to produce peracids, which then act as catalysts to epoxidize double bonds. Various factors govern the efficiency of the reaction in terms of kinetics, yields and enzyme stability. These parameters are evaluated and discussed herein.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol. These enzymes are widely distributed among higher animals, microorganisms and plants, where they are involved in the metabolism of lipids. They can be obtained by extraction from animal or plant tissue or cultivation of microorganisms. To date, hundreds of lipases have been described and a few have been characterized in terms of tridimensional structures by X-ray crystallography. These latter showed that lipases are members of the "α/β-hydrolase fold" family, as they have a structure composed of a core of parallel β strands surrounded by α helices.[1,2] The active nucleophilic serine residue rests at a hairpin turn between a β strand and

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Chahinez Aouf

Chahinez Aouf received her Ph.D. in 2007 with Prof. M. Santelli from the University of Marseille for her work on the reductive silylation of dienes to produce optically active building blocks. Then, she joined J. Muzart's group as a postdoctoral fellow at Reims-Champagne Ardennes University and worked on the oxidative depolymerisation of starch and cellulose. In 2009, she moved to Montpellier at INRA in the Hélène Fulcrand group. Her research interests relate to the functionalization of natural phenolics for the production of new bio-based polymers. This research topic includes extraction, analysis, along with the chemical or enzymatic functionalization of natural polyphenols.

Erwann Durand

Erwann Durand has a M.Sc. in organic chemistry & biochemistry obtained from the Sciences University of Marseille. In 2010, he started a Ph.D. at UMR IATE of CIRAD Montpellier, France, on the following topic: Deep eutectic solvents, new media for lipase catalyzed reactions? Through his research he has published several journal papers to assess and develop the potential of this new "green" reaction medium for lipase-catalyzed synthesis. His expertise in organic and analytical chemistry, but also in biocatalysis and enzymology, helped him to design, synthesize and evaluate different strategies to solve problems at the interface of chemistry, physics and biology.

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an α helix in a highly conserved pentapeptide sequence Gly-X-Ser-X-Gly, forming a characteristic β-turn–α motif named the "nucleophilic elbow".[3] The active site of lipases is formed by a catalytic triad composed of Ser–Asp/Glu–His residues, and the presence of an amphiphilic α helix peptidic loop covering the active site of the enzyme in solution, just like a lid or flap, whose reorientation is triggered by interfacial activation.[4,5] However the phenomenon of interfacial activation was questioned lately with the study of *C. antarctica* B lipase, suggesting a more complex behavior.[6] The catalytic mechanism of lipases involves nucleophilic attack of the carbonyl group of the substrate by the hydroxyl residue of serine, resulting in the formation of an acyl-enzyme intermediate stabilized by an oxyanion hole. Deacylation proceeds thanks to the attack of the acyl enzyme by another nucleophile (*i.e.* H_2O) inducing the product release and the regeneration of the catalytic site.[7] Most lipases can act in a wide range of pH and temperature and possess broad substrate specificity (*i.e.* aliphatic, alicyclic, bicyclic and aromatic esters, thioesters, and activated amines), and can catalyze a variety of chemo-, regio- and enantioselective biotransformations (Fig. 1).

The catalytic polyvalence (catalytic promiscuity) of lipases, coupled with their good stability and their ability to catalyze synthesis reactions have generated much research work and industrial applications of these enzymes. Indeed, lipases are known to accept a wide diversity of substrates and can be used in many forms (liquid or solid as native enzymatic powders or immobilized biocatalysts). Lipase immobilization, which can be obtained by various strategies, has many advantages in terms of biocatalyst activity, thermostability, enzyme recovery and reuse.[8–10] Consequently, lipases are widely used in various applications in the food, chemical, cosmetic, detergent, leather, textile, perfumery, paper, biodiesel, and pharmaceutical industries. Industrial lipase applications have been reviewed extensively[11–23] (Table 1).

I. Factors governing lipase activities in synthesis reactions

The use of lipases in various synthetic bioprocesses requires a good knowledge and understanding of the many parameters governing their synthesis activity. We will discuss herein the main ones that have to be considered in lipase-catalyzed epoxidation reactions that would be described in this article.

I.1 Selection of the reaction medium

Among the different factors governing lipase activity and efficiency, the nature of the medium is obviously crucial. Reactions can be carried out in solvent free systems. Typically, in such systems, one of the substrates is used in large excess so it plays both the role of substrate and solvating medium. Such systems have the advantage of avoiding organic solvents and can be considered as environmentally friendly. However they also have some disadvantages such as difficulties in obtaining good solubilization of all the substrates, slow reaction kinetics, and a large excess of one substrate that can be problematic for further purification of final products. Accordingly, reactions in solvents are generally favored. In that context, the choice of solvent is of paramount importance. Indeed the chosen medium must allow good solubility of the different involved substrates and, in parallel, guarantee good lipase activity. Additionally, other criteria such as solvent handling (product separation and purification), solvent toxicity and biodegradability must also be considered.

Organic solvents. So far, a large majority of reactions involving lipase-catalysis have been operated in organic solvents. These solvents, pure or as mixtures, are attractive due to their ability to allow the solubilization of many substrates with different polarities. They are generally inert toward the reaction and can influence the activity, selectivity and specificity of

Jérôme Lecomte

Jérôme Lecomte has a Ph.D. in molecular chemistry (National Graduate School of Chemistry/ Montpellier II, Montpellier II University, France). He started his career in the 90s by studying biomass conversion into valuable compounds (furans, sugars, biodiesel) through heterogeneous catalysis. He joined CIRAD in 2001 and worked on the processing of cottonseed proteins into seed coatings with controlled release properties of biocides. Finally, he joined the team of Dr Pierre Villeneuve in 2006 and focused its work on the chemical and/or bioprocessing of lipids and phenolics for food (restructured lipids) and non-food applications (polymer materials, bioactive compounds).

Maria-Cruz Figueroa-Espinoza

Maria Figueroa has a Ph.D. in food science (Montpellier II University, France). She started her career by studying enzymatic polysaccharide solubilisation from rye to improve rye bread quality. She joined Montpellier SupAgro as an associate professor in food biochemistry in 2000, and CIRAD as an associate researcher in 2001. She focused its work on the chemical and/or enzymatic modification of phenolic compounds in order to obtain multi-functional compounds for food and non-food applications.

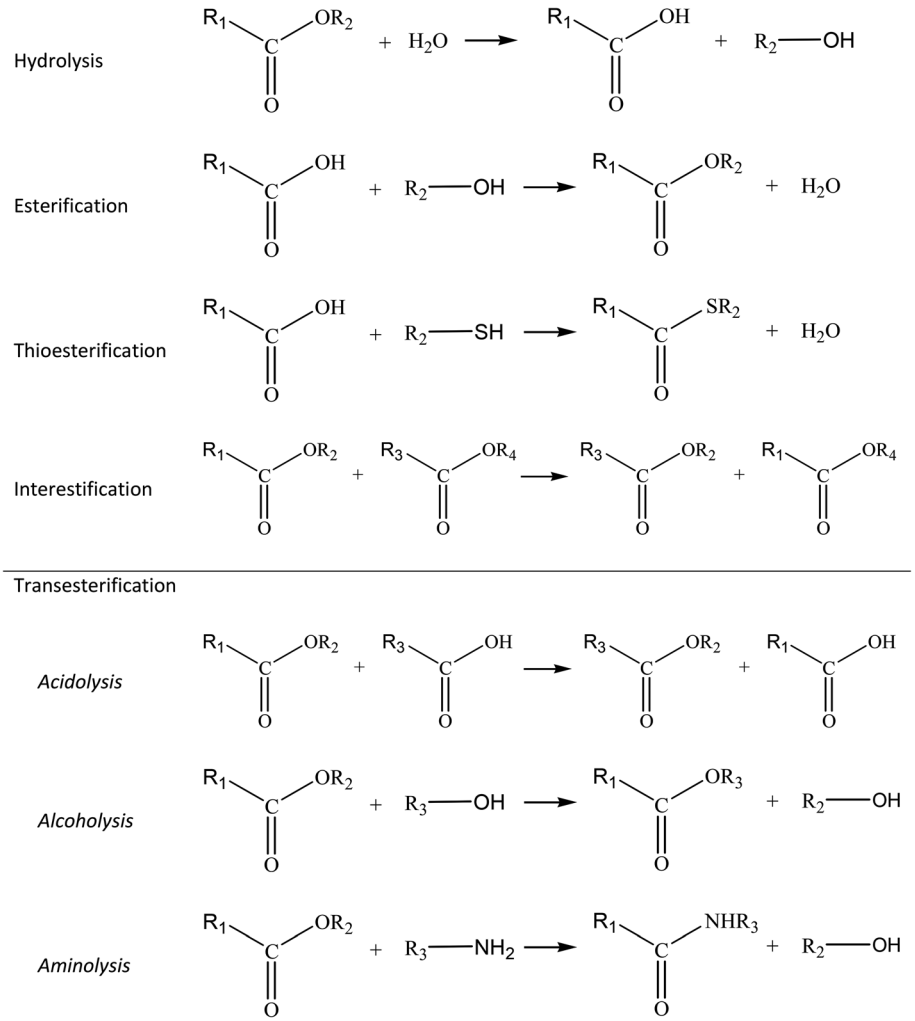

Fig. 1 Main examples of reactions catalysed by lipases.

Eric Dubreucq

Eric Dubreucq graduated in biological engineering and food sciences. Prof. Eric Dubreucq has specialized in lipid biotechnology since his recruitment to Montpellier SupAgro in 1991. He jointly leads with Dr Pierre Villeneuve the microbiological and enzymatic biotechnology of lipids and agropolymers group of UMR IATE. His activities cover the selection, the improvement and application of new enzymes. During the last few years he has developed activities in biocatalysis applied to the obtention of functionalized synthons from lipids and polyphenols. He also works on the biotechnology of natural rubber. He has co-authored 57 international papers and is a co-inventor in 10 patents.

Hélène Fulcrand

Hélène Fulcrand has a Ph.D. degree in analytical and organic chemistry. Over the last 20 years Hélène Fulcrand has devoted her research to the study of the reactivity of phenolic compounds in order to better understand the relationship between the phenolic composition of wine and its organoleptic quality. The knowledge and experience she gained in flavonoids chemistry have led her to open a new topic of research on the valorisation of polyphenols in the field of materials science. This research activity aligns with the emerging field of sustainable chemistry aimed at fighting some of the most important challenges of the 21st century.

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Table 1 Examples of the applications of lipases on the industrial scale

Field of industry	Application
Food	Hydrolysis of milk fat to obtain flavoring material in cost- or calorie-reduced foods.
	Cheese ripening and flavor enhancement.
	Oil and fat restructuring to produce *e.g.* cocoa butter equivalents, human milk fat substitutes, low calorie fats, polyunsaturated fatty acid and oleic oil-enriched oils, *etc.*
	As bread improvers to strengthen dough stability and increase bread volume, texture, color and shelf-life.
	As noodle softeners.
	Synthesis of emulsifiers and flavoring agents.
Chemical	Hydrolysis of oils and fats to obtain fatty acids, di- and monoacylglycerols, and reagents for lipid analysis.
	Synthesis of esters to obtain chiral intermediates.
	Synthesis of esters and emulsifiers.
	Transesterification of natural oils, *e.g.* for the production of some high value polyunsaturated fatty acids.
	Formulation of detergents for laundry and household uses to remove fatty stains and lipids.
Pharmaceutical	Racemic mixtures resolution.
	Synthesis of chiral synthesis intermediates, digestive aids, specialty lipids, sugar based surfactants, *etc.*
Cosmetics and perfumery	Synthesis of fragrances.
Tannery	Degreasing of leather.

the chosen biocatalyst. Moreover, they can be easily eliminated once the reaction is completed. However, their high inflammability and their toxicity can be a concern. Solvent polarity is considered to be the key parameter to anticipate lipase activity, with hydrophobic solvents being generally preferred for lipase-catalyzed reactions.[24] The negative influence of solvent polarity on lipase activity and stability seems indeed to be due to competition of solvent and enzymatic protein for water. Such a competition governs the protein hydration state which is crucial for enzyme activity.[25] Unfortunately, very hydrophobic solvents are not always adapted for lipase-catalyzed reactions involving polar substrates such as sugars, amino acids or phenolic compounds,[15] these molecules being poorly soluble in these solvents. Therefore, solvents with intermediate polarities are often used and examples have been given on the use of acetonitrile,[26,27] *tert*-butanol,[28] 2-methyl-2-butanol[29–31] or acetone.[32] However, due to the difficulties in selecting an appropriate solvent that has to preserve lipase activity, allow good solubility of all the substrates and exhibit limited toxicity, some alternatives have been studied recently.

Pierre Villeneuve

Pierre Villeneuve obtained a Ph.D. in organic chemistry from the University of Montpellier in 1995. After a two years post doctorate at USDA (Wyndmoor, PA , USA) he joined Danone R&D. Then in 2002, he went back to academic research at CIRAD (Montpellier). He jointly leads with Prof. Eric Dubreucq the microbiological and enzymatic biotechnology of lipids and agro-polymers group of UMR IATE. His research activities deal with lipid modification by biocatalysis, lipid oxidation and antioxidant and lipid chemistry. He has co-authored 112 international papers, 3 book chapters and is a co-inventor in 3 patents.

Water. Water is a natural solvent of enzymes, including lipases. Although monophasic aqueous reaction media with high a_w promote hydrolytic reactions, a number of studies have shown that biphasic aqueous–hydrophobic media can allow a displacement of the reaction equilibrium toward ester synthesis with high yield, which can be of great interest for lipase-catalyzed synthetic reactions when at least one of the reactants is water soluble and another one is hydrophobic.[32–37] This can avoid the need to control a_w and allow easier solubilization of polar and apolar co-substrates and substrate recovery.

Ionic liquids and deep eutectic solvents. Ionic liquids were the first potentially good alternative to organic solvents for biotransformations,[38–43] due to their non-volatility, their thermal stability and their high solvation properties. These new media are salts that are liquid at ambient temperature. They are constituted by an organic cation associated with an anion. Generally, cations are dissymmetric and large quaternary ammoniums or phosphoniums (tetraalkylammoniums, tetraalkylphosphoniums, alkylpyridiniums, triazoliums, pyrrolidiniums or imidazoliums) with various substitutions, while anions can be inorganic (F^-, Cl^-, BF_4^-, PF_6^-, ClO_4^-, $CuCl_2^-$, $SnCl_3^-$ *etc.*) or organic ($CH_3CO_2^-$, $CF_3CO_2^-$, $CF_3SO_3^-$, R_3BOH^- *etc.*). Many cation–anion combinations can be obtained ($>10^6$) offering the possibility to create a huge variety of ionic liquids with adjusted physico-chemical properties and can solubilize various compounds of different polarities. Accordingly, ionic liquids have been extensively studied for lipase reactions.[44] Many examples are now documented for the use of ionic liquids in lipase-catalyzed synthesis of sugar esters[45,46] or lipophilization of phenolic substrates.[47–49] However, ionic liquids can have some drawbacks. Recently, their toxicity has been questioned,[50–53] their production cost can be prohibitive for using them on an industrial scale, and finally, their high viscosity can make difficult the recovery of reaction products and immobilized enzymes.[54–56] More recently, some publications revealed that Deep Eutectic Solvents (DES) could be a

promising alternative to ionic liquids as efficient "green" media in lipase-catalyzed reactions.[57–60] These solvents, which share many characteristics with ionic liquids (non-volatile, thermally stable up to nearly 200 °C, non-flammable, *etc.*), have many other advantages: they are relatively inexpensive, environmentally benign and have a very low toxicity.[61] Moreover, unlike ionic liquids, these solvents do not require a preliminary purification step. Indeed, they result from the association of a cationic salt (ammonium or more recently phosphonium) with a hydrogen-bond donor (HBD). This results in a deep eutectic solvent with a room temperature melting point. The strong interaction between the HBD and the anion, provided by the salt, leads to a considerable reduction in the melting point of the mixture. In addition, the strong association between the components radically decreases their reactivity, making them inert in most cases. The term "eutectic point" is used to characterize the lower melting point of the mixture, which is often much lower than that of the pure constituents. Thus, the mixture can be used at a temperature that permits biocatalytic reactions. In that context, some research studies have reported highly encouraging results for enzymatic synthesis in DES alone or as a co-solvent.[62,63] Recently, our group has made significant progress in the application of these solvents in the lipase modification of polar substrates. Indeed, it has been demonstrated that DES based on choline chloride in a binary mixture with water could be effectively used for the alcoholysis of phenolic esters with 1-octanol, using immobilized *C. antarctica* lipase B as a biocatalyst.[64] In this study, we have shown that the lipase-catalyzed reactions of dissolved substrates in DES are extremely difficult to perform without the addition of water. The best results were obtained in DES based on choline chloride (ChCl) associated with urea (U) as a hydrogen-bond donor after adding water. However, further research is required to increase the potential of these green solvents. In particular, it would be very useful to determine the respective role of each component (ChCl, urea, water and substrates) in lipase activity and stability.

Supercritical fluids. Supercritical fluids appear to be promising for enzymatic reactions. In particular, supercritical carbon dioxide ($ScCO_2$) has been extensively studied and was first described as a potential medium for biotransformations by Hammond *et al.* in the mid-eighties.[65] The main advantage of supercritical fluids for biocatalysis is their flexibility in their solvent properties. Indeed, depending on pressure or temperature changes, their physical properties (viscosity and solvating properties) can be adjusted and have an impact on the enzyme selectivity.[66–68] Supercritical carbon dioxide has a dipole moment close to the one of pentane or hexane and therefore allows the solubilization of many low molecular weight apolar compounds. Additionally, supercritical fluids are considered as environmentally friendly and their handling and elimination at the complexion of the reactions are rather easy. Therefore, many research studies have been carried out where lipases are used in such fluids.[69,70] For example, Romero *et al.* showed that initial reaction rates for the synthesis of isoamyl acetate with *C. rugosa* or *Rhizomucor miehei* lipase were higher in $ScCO_2$ than in the tested organic solvents.[71] These higher rates were due to an improvement of the diffusivity of substrates in the supercritical fluid. The addition of a co-solvent has also been suggested to improve reaction kinetics and yields. For example, acetone or ethanol was used to increase medium polarity and ease reactions involving very polar substrates. The acetone–$ScCO_2$ co-solvent system was successfully used for the grafting of palmitic acid to glucose by *C. antarctica* B lipase.[72] Recently, co-solvent systems involving ionic liquids have been investigated showing an improvement of enzyme activities.[73] However, some other results suggest that the addition of a co-solvent has no significant advantage compared to the supercritical fluid alone in terms of yields and kinetics.[74] Finally, one important parameter that has to be considered when using supercritical fluids is the influence of pressurization and depressurization of the gas. Indeed, a depressurization that would be too fast could lead to the removal of water at the enzymatic protein surface causing conformational changes which can affect its catalytic activity.[74,75]

I.2 Other factors

Lipases have different catalytic properties and optimal pH. For example, extracellular lipases from *Cinnamomea antrodia* and *Penicillium aurantiogriseum* have an optimum pH = 8,[76,77] close to the one of *Rhizopus oryzae* (pH = 8.5)[78] whereas other lipases such as the ones from fungi have neutral or weakly acid optimal pH.[79] However, most lipases can be active at a wide range of pH. Similarly, lipases show in general a good thermostability that depends on the nature of the enzyme itself or its conditioning as free or immobilized forms.[10] Typically, an immobilized lipase exhibits a better temperature range than the native form. The activity of a lipase can be improved by the use of various additives. For instance, the addition of salts into the organic medium has been proposed in order to maintain constant the water content and improve the lipase activity.[80] Other authors have shown that the addition of crown ethers could favor lipase activity in organic solvents by modifying the conformation of the active site and the hydrogen bond network.[81–83] The use of tensioactive molecules can also impact the efficiency of the biocatalyst by improving its solubility in the medium.[84] Some physical parameters can also be used to improve the lipase efficiency such as the use of ultrasounds as described by Lee *et al.*[85] for the lipase-catalyzed alcoholysis of glucose with vinyl laurate in ionic liquids. Similarly, the positive effect of ultrasound was also observed in a solvent free system for the synthesis of mono- and diacylglycerols with *C. antarctica* B lipase[86] or with various immobilized lipases during triacylglycerol hydrolysis.[87] A special case to be mentioned is that of lipases/acyltransferases, which are able to catalyze acyltransfer reactions preferentially to hydrolysis even in media with very high a_w (>0.8) in the presence of various nucleophiles in aqueous solution.[88,89]

Besides the factors that have been described above governing lipase activity some other parameters also have an influence on reaction rates and final yields. One can cite the nature

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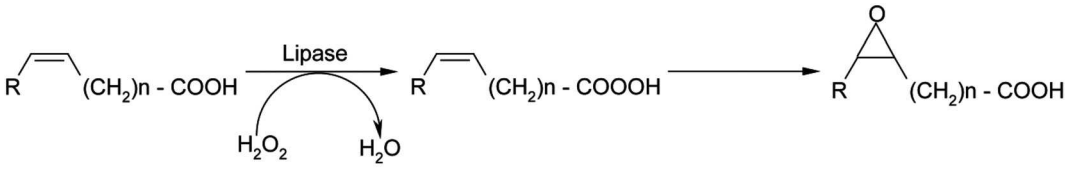

Fig. 2 Chemo-enzymatic epoxidation of unsaturated fatty acids involving a lipase-catalyzed perhydrolysis step.

and concentrations of substrates, the enzyme load, the enzyme conditioning (immobilization, *etc.*), the water activity of the system, and the nature of the biocatalytic process (batch, fluidized beds, and columns). These parameters will be discussed in the subsequent section concerning lipase-catalyzed epoxidation reactions.

II. Lipases in epoxidation of fatty acids

The worldwide demand for replacing petroleum-derived raw materials with renewable ones is quite significant.[90] Besides polysaccharides and sugars, plant oils are the most important renewable raw materials of the chemical industry.[91] Annual production of oils and fats is expected to reach 185 million tons in 2016, the main plant sources being palm, soya, canola (or rapeseed) and sunflower oils. The latter give access to various fatty acids which greatly differ in terms of carbon chain length, number and position of C═C double bonds on the aliphatic chain and the presence of side functional groups. Accordingly, many oleochemicals are obtainable from fatty acids, which can be obtained by chemical, enzymatic or microbial processes.[91] Among them, epoxidized fatty acids or triacylglycerols are of great interest to the oleochemistry industry. Among the major applications of epoxidized vegetable oils and fatty acids is their use as plasticizers for polyvinyl chloride (PVC) and other plastic materials.[92] Epoxy fatty acids can also be used as PVC-stabilisers owing to their ability to slow down degradation by scavenging the free HCl released during PVC decomposition when exposed to heat and light.[93] In addition, epoxidized derivatives of fatty acids can be used as diluents for paints, as intermediates for polyurethane-polyol production, as corrosion protecting agents and as additives to lubricating oils.[94]

Epoxidized fatty acids can be obtained from natural sources. For instance, vernolic acid (12*S*,13*R*-epoxy-9-*cis*-octadecenoic acid) can be found in the seed oils from several Asteraceae genera, including *Stokesia*, *Vernonia* and *Crepis*[95,96] or certain Euphorbiaceae species such as *Euphorbia lagascae* and *Bernardia pulchella*.[97] In the seed oils of these plants, vernolic acid can compose 50% to 90% (w/w) of the total fatty acids. However, industrial extraction and production of such epoxidized fatty acids from natural sources appears rather unrealistic. Therefore, to date, epoxidized oils and fatty acids are currently produced by chemical epoxidation of unsaturated plant oils. Although there are several methods available to epoxidize the double bonds of unsaturated fatty acids, the only method applied on the industrial scale is the Prileshajev epoxidation reaction. Indeed, this reaction is used on the industrial scale to produce more than 200 000 t per year of epoxidized soybean oil. In this reaction, short chain peroxy acids are generated from the corresponding acid and hydrogen peroxide in the presence of a strong mineral acid. Then, these peroxy acids react with unsaturated fatty acid C═C double bonds to obtain epoxidized fatty acids (Fig. 2). Peroxy acids are prepared either in a separate step or *in situ*. Due to the potential danger of handling peroxy acids, the *in situ* method is generally preferred for large-scale epoxidation of unsaturated triglycerides.[98] However, this chemical method for epoxidation has some disadvantages. First, side reactions do occur *via* oxirane ring opening, leading to diols, hydroxyesters, estolides and other dimers, which are believed to be catalyzed by the presence of a strong mineral acid.[99] As a result, the selectivity of this process never exceeds 80%.[98] Moreover, the presence of a strong acid in an oxidative environment can cause equipment corrosion. Finally, this acid must be recycled or neutralized before discharge into the environment. Therefore, using enzymatic processes to epoxidize oils and fats or fatty acids appeared as an alternative and promising solution for more selective epoxidation reactions. In that context, among the various potential enzymes that are known to catalyse epoxidation reactions (cytochrome P450 monooxygenases, diiron-center oxygenases, lipoxygenases, peroxygenases), lipases were the most widely investigated. Indeed, lipolytic enzymes have been shown to produce peroxy fatty acids from hydrogen peroxide and fatty acids by perhydrolysis reaction. These peroxy acids subsequently epoxidize unsaturated fatty acids *via* a non-catalyzed reaction.

II.1 Lipase-catalyzed perhydrolysis

Among the different lipases used in the lab scale or in industry the most widely used is most probably the one from *C. antarctica*. This yeast is known to produce two types of lipases (A and B), B being the most widely studied and used. The latter contains 317 amino acids and has a molecular weight of 33 kDa, and shows no significant interfacial activation phenomenon.[100,101] In 1990, an immobilized form of lipase B (Novozyme 435) was shown to catalyze the conversion of saturated fatty acids with 4 to 22 carbon atoms into peroxy fatty acids in the presence of hydrogen peroxide.[102] The same group showed that the lipase-mediated synthesis of peroxy acids from carboxylic acids and hydrogen peroxide can be used to perform *in situ* epoxidation of alkenes.[103] In such an enzymatic

epoxidation involving *C. antarctica* B lipase, the reaction system consists of an aqueous phase containing hydrogen peroxide, an organic phase containing the lipid substrate and the immobilized enzyme as solid phase.[97,104] At the complexion of the reaction, the immobilized lipase is removed by filtration after reaction and generally reused.

In such a perhydrolysis reaction, hydrogen peroxide acts as the nucleophile instead of water in the deacylation step of the serine hydrolase. However, the perhydrolase activity of lipases and esterases is generally much lower than their esterase activity and some of them, such as subtilisin, do not exhibit perhydrolase activity.[105] In contrast, some hydrolases display better perhydrolysis activity than hydrolysis and are therefore described as perhydrolases.[106–109] Perhydrolysis by lipases presumably occurs with a carboxylic acid first reacting with the active site serine group to form an acyl-enzyme intermediate, which in turn reacts with hydrogen peroxide to form a peroxy acid. However, the Ser-His-Asp catalytic triad of serine hydrolases is not the only determinant for perhydrolase activity because some of these enzymes do not exhibit any perhydrolysis activity.[105] Accordingly, some authors proposed an alternative mechanism where the catalytic serine stabilizes the carboxylic acid substrate with a hydrogen bond instead of forming an acyl-enzyme intermediate.[110] An explanation proposed to elucidate the difference in activities between hydrolases and perhydrolases concerns the electronegative microenvironment of the active site. The more hydrophobic environment present in perhydrolases compared to other hydrolases would protect the peroxy acid against hydrolysis.[111] Besides, the presence in the structure of the enzyme of amino acids particularly sensitive to oxidation by H_2O_2 and by peroxy acids can also explain the difference in enzymatic activities.[112] The alignment of the amino acid sequences of six hydrolases and six perhydrolases was studied by Bernhardt *et al.*[105] in order to observe which residues appeared in perhydrolases but not in esterases. They showed that the substitution of a single amino acid was sufficient to shift the hydrolase activity of an aryl esterase from *Pseudomonas fluorescens* to make perhydrolysis the preferred reaction in aqueous solution. A molecular basis for the increase in perhydrolase activity is the presence of a hydrogen bond formed between a carbonyl oxygen atom of the enzyme and the peroxide nucleophile. This peroxide hydroxy–carbonyl hydrogen bond stabilizes the hydrogen peroxide attack on the putative acyl-enzyme intermediate, hence facilitating the perhydrolysis reaction.[105]

The capacity of a lipase to transform a fatty acid into its corresponding peracid was then used to carry out *in situ* epoxidation of alkenes.[102,103] When unsaturated fatty acids or their alkyl esters were treated with hydrogen peroxide in the presence of *C. antarctica B* lipase, their epoxidized derivatives were produced in a two steps reaction[113] (Fig. 2). First, the unsaturated fatty acids are converted into the corresponding unsaturated peroxy acid owing to the perhydrolysis activity of the lipase, and then the resulting unsaturated peroxy or carboxylic acids are epoxidized *via* an uncatalyzed Prileshajev reaction that is often referred to as "self-epoxidation reaction" in spite of the fact that it proceeds predominantly *via* an intermolecular process.[113]

This chemo-enzymatic reaction can also be applied to oils and fats for the production of epoxidized plant oils.[98,104,114] The reaction is then carried out directly onto unsaturated triacylglycerols that are treated with H_2O_2 in the presence of a suitable lipase. Peroxy fatty acids are formed that epoxidize the C=C double bonds. The resulting mixture contains epoxidized triglycerides, a small amount of epoxidized free fatty acids and some epoxidized mono- and diglycerides. However the elimination of these side products at the end of the reaction can be tedious and, therefore, a way to prevent their formation has been found by adding free fatty acids to the starting material.[114] Under these conditions, perhydrolysis occurs but all the hydroxyl groups of partial glycerides (mono- and diacylglycerols) are then reesterified by the excess of free fatty acids. The resulting final material only contains epoxidized oil (triacylglycerols) and epoxidized free fatty acids that can be easily removed by alkaline washing. With this strategy, various plant oils (rapeseed, sunflower, soybean and linseed oil) were epoxidized with conversions and selectivities above 90%.[98]

II.2 Factors governing the efficiency of lipase-catalyzed epoxidation

Lipase-catalyzed epoxidation reactions have been carried out with *C. antarctica* B on different substrates such as free fatty acids and their corresponding alkyl esters, triacylglycerols and various olefins.[113–115] Several parameters govern the efficiency of these reactions. Hydrogen peroxide concentration is the most critical parameter influencing the reaction rate and the degree of epoxidation. Orellana-Coca *et al.*[116] showed that an excess of hydrogen peroxide compared to the amount of double bonds is necessary in order to yield a total conversion within a short time period. However, a large excess of hydrogen peroxide results in the accumulation of peroxy acids in the final product. These unreacted peroxy acids can be a potential problem for reasons of safety and contamination of the final product. Moreover, a high hydrogen peroxide concentration in the reaction medium negatively affects enzyme activity.[116–119] Indeed, in the presence of 6–12 M hydrogen peroxide, the enzyme is rather stable at 20 °C whereas at 60 °C the enzyme loses activity rapidly. The rate of deactivation increases with increasing H_2O_2 concentration.[119] Accordingly, a step by step addition of hydrogen peroxide can be advantageous to reduce the deactivation of the biocatalyst.[102,103,116] Some potential alternatives to hydrogen peroxide as an oxidant were also investigated. For example, Ankudey *et al.*[120] proposed the use of urea hydrogen peroxide and suggest that this oxidant would limit the deactivation of the lipase in comparison with what was observed with hydrogen peroxide. However, these observations were not confirmed by Törnvall *et al.*[119] who did not observe any significant differences between those two oxidants in terms of enzyme deactivation effect.

Temperature is also a major parameter influencing reaction rate and conversion. An increase of temperature has a positive

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effect on the reaction rate but it was observed that temperatures above 50 °C would result in hydrogen peroxide decomposition and possible enzyme inactivation.[116] The effect of the nature of the medium is also important. A majority of the studies involves the use of an organic solvent, mainly toluene, to favour the solubilisation of the lipid substrate and therefore ease the contact between the substrate and the biocatalyst. Additionally, in systems where toluene is used, the stability of the enzyme seems to be improved. For example, Warwel and Rüsch gen. Klaas observed that *C. antarctica* B lipase was very stable, with 75% of residual activity after 15 reaction cycles.[113] Similarly, Hagström *et al.* suggested that the use of toluene would reduce the direct exposure of the enzyme to hydrogen peroxide.[121]

Orellana-coca *et al.*[116] performed the epoxidation of linoleic acid in toluene and observed a quantitative conversion of double bonds to give the corresponding diepoxide when operational temperature was set between 40 °C and 60 °C. Comparable results were obtained later on using oleic acid or methyl oleate as the substrate with a 90% conversion into epoxystearic acid after 6 h at 50 °C.[122] The use of toluene can also be advantageous when carrying out the epoxidation on triacylglycerols. On plant oils such as sunflower, soybean or linseed, Rüsch gen. Klaas and Warwel were able to obtain yields above 90% after 16 hours.[98] Some others, using a rather high hydrogen peroxide concentration (60%), were able to epoxidize soybean oil in a very good conversion (95%) at 50 °C using toluene.[123] Other organic solvents such as *t*-butanol[124] or dichloromethane[125] were also proposed with satisfactory results.

More recently, the use of hydrophobic and hydrophilic ionic liquids was proposed in order to improve reaction yields in lipase-catalyzed epoxidation of methyl oleate.[126] The authors observed that hydrophilic ionic liquids were advantageous, resulting in the best yields and reaction kinetics. For example, of the nine different tested lipases, *Aspergillus niger* lipase in hydrophilic BMI.BF4 yielded the epoxidized compound in 89% in the first reaction hour, whereas hydrophobic BMI.PF6 yielded the same product in 67%.

Some other studies proposed the use of a solvent free system; however, the epoxidation of linoleic acid in such a system was not complete at 30 °C due to the formation of a solid or a highly viscous oily phase, creating mass transfer limitations.[116] Increasing the temperature up to 60 °C and using some excess of hydrogen peroxide helped in improving the rate of epoxide formation. Nevertheless, when a chemo-enzymatic reaction occurred in a solvent-free medium under conditions optimized for achieving high reaction rates and yields, the enzyme was found to suffer loss in activity, hence limiting its recycling.[122]

Concerning potential side reactions that can occur when performing lipase-catalyzed epoxidation reactions, many publications observed hydrolysis or dihydroxylation of reaction products. These side-reactions can be limited or reduced when adding free fatty acids in the reaction medium. Indeed, this addition results in the improvement of reaction kinetics and yields.[109,114,125,127]

III. Synthesis of phenolic epoxy prepolymers catalysed by lipase

Epoxidized vegetable oils and derivatives, which are biodegradable, environmentally friendly and renewable resources, have recently found industrial applications as plasticizers, additives for PVC and rubber-like materials.[128–132] This kind of application is directly related to their chemical structure which consists of flexible and aliphatic chains. However, for advanced technological applications, epoxy resins with improved physico-chemical properties such as good thermal stability, low internal stress and high mechanical strength are required. These high-performance epoxy resins are usually synthesized from aromatic derivatives. Nowadays, almost 90% of the world production of epoxy resins is based on the reaction of bisphenol A (BPA) and epichlorohydrin, yielding diglycidyl ether of bisphenol A (DGEBA).[133] However, an increasing body of research is focusing on the use of bio-based phenolic compounds in the production of epoxy prepolymers according to several synthesis routes.

III.1 Epichlorohydrin synthesis of phenolic epoxy prepolymers

According to the US Department of Health and Human Services report, epichlorohydrin was produced in 2009 by 27 manufacturers worldwide with about 1.26 million metric tons global production in 2006.[134] This large production combined with the inherent reactivity of epichlorohydrin, which is highly prone to undergo ring opening and/or nucleophilic substitution reactions with various carbon and heteroatom nucleophiles, render epichlorohydrin an excellent substrate for the synthesis of petroleum-based and more recently bio-based epoxy prepolymers.

In Japan, significant work has been performed to transform wood lignin into thermosetting epoxy resins. Indeed, acid (hydrochloric or sulphuric acid) and phenol derivatives are added to kraft lignin to cause the cleavage of lignin intermolecular bonds at the same time to generate the phenolic hydroxyl group in the molecule, followed by epoxydizing the phenolic hydroxyl group with epichlorohydrin to provide the lignin-based epoxy resin. This method was developed by Shiraishi and co-workers[135,136] who used bisphenol A as a phenol derivative. However, other hydroxyl containing compounds, including resorcinol, *p*-cresol, glycols and glycerol, can replace bisphenol A.[137–142] Another method consists of developing bio-based epoxy resins from pure phenolic compounds obtained through chemical treatment of lignin. As an example, vanillin is obtained from the lignin sulfonic acid in sulphurous acid pulp waste liquor through the oxidation decomposition process in alkali solution.[142] Fig. 3 shows the synthetic method and the chemical structure of the di-functional epoxy resin derived from vanillin. The raw material of the epoxy resin is the dihydric phenol derivative which is the reaction product

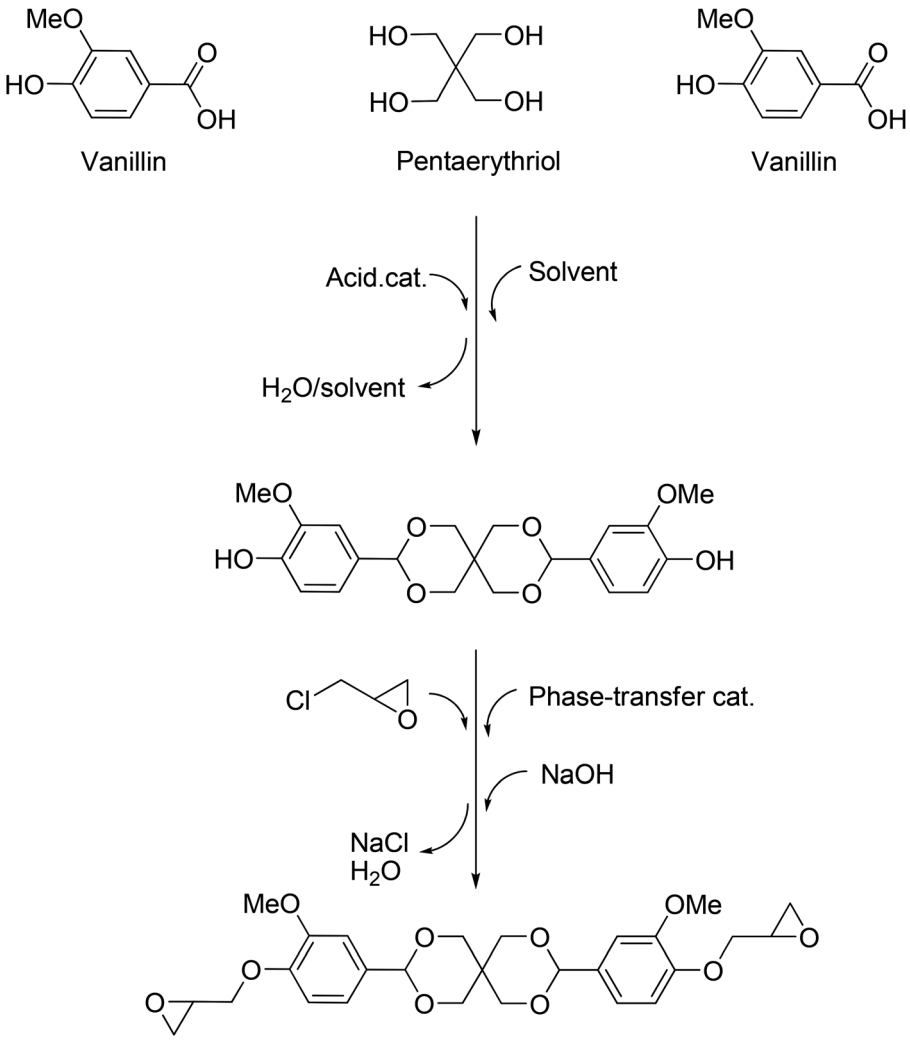

Fig. 3 The synthetic route for a difunctional epoxy resin based on vanillin.

obtained from the dehydration condensation of vanillin and pentaerythriol.[143,144]

Cardanol, a major component of cashew nut shell liquid, is a phenol derivative having a *meta* substituent of a C_{15} unsaturated hydrocarbon chain with one to three double bonds. It has drawn considerable attention from many researchers for the production of phenolic resins under different sets of conditions. Thus, cardanol-based epoxidized novolac resin was reacted with methacrylic acid in the presence of triphenylphosphine in order to produce the cardanol-based epoxidized novolac vinyl ester resin.[145] The modification of the cardanol-based novolac resins by the epoxidation reaction with epichlorohydrin enhances the performance of such resins in various fields.[146,147]

Aside from lignin and cardanol, the other terrestrial source of phenolic compounds is tannins. Tannins, which are widely present in the agricultural and forest biomass residues, constitute an important group within the phenolic compounds. They may be subdivided into hydrolysable and condensed tannins. The former are esters of gallic acid (gallo- and ellagitannins) while the latter are polymers of polyhydroxyflavan-3-ol monomers, also known as catechins.[148] In our previous work,[149] catechin was reacted with epichlorohydrin in alkaline medium to lead to the expected tetraglycidyl ether of catechin along with a benzodioxane derivative. The formation of this cyclic by-product is related to the *ortho* positions of the two phenolic hydroxyl groups carried by the B-ring of catechin. Under the same experimental conditions, the reaction of gallic acid and epichlorohydrin afforded the tetraglycidylated derivative with 72% yield[150] (Fig. 4). These epoxy prepolymers were subsequently cured with isophorone diamine to give the corresponding crosslinked networks.

Recently, Fourcade *et al.*[151] described the esterification reaction of some bio-based polyols such as glycerol, pentaerythriol, dipentaerythriol and sorbitol with ethyl-4-hydroxy benzoate to produce poly(4-hydroxybenzoates) as phenolic resins. In a subsequent step, the poly(4-hydroxybenzoates) are glycidized with epichlorohydrin. These renewable resource-based epoxy resins aim to be involved in structural adhesive formulations.

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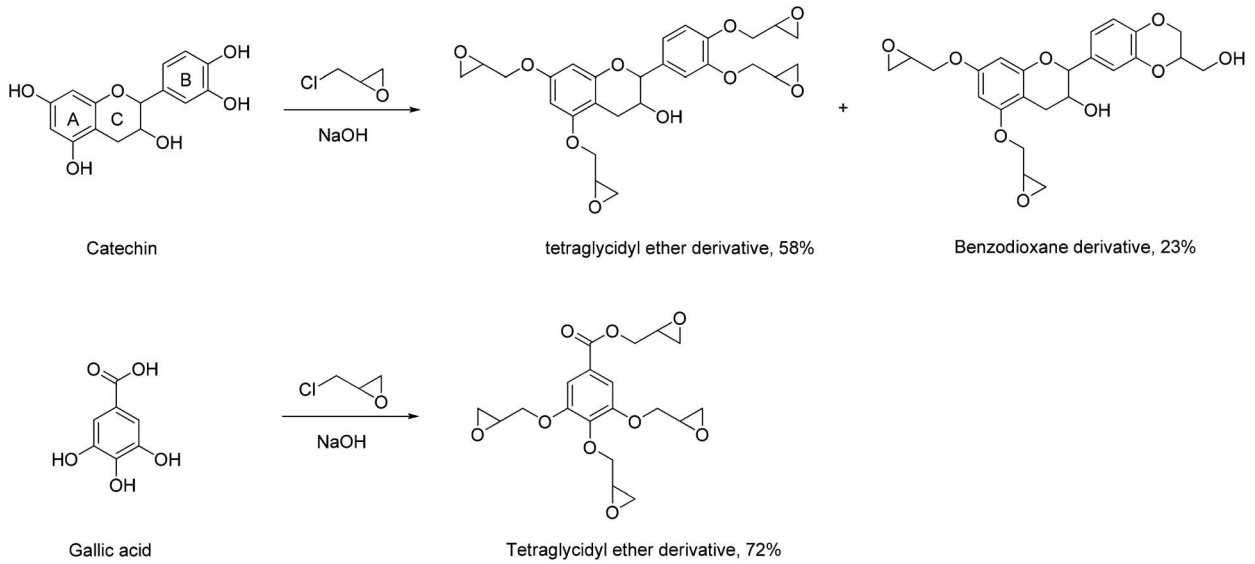

Fig. 4 Catechin and gallic acid reactions with epichlorohydrin.

III.2 Catalytic epoxidation of natural phenolic compounds

Exposure to epichlorohydrin in its liquid or vapour form can rapidly irritate eyes, skin and the respiratory tract.[134] This compound is also classified as a probable human carcinogen (group 2A) by the International Agency of Research on Cancer (IARC).[152] For this reason, more and more research studies are examining the possibility to overcome the epichlorohydrin use in the production of bio-based epoxy resins.

Thus, epoxidized cardanol was synthesized by reaction of cardanol with glacial acetic acid and hydrogen peroxide using Amberlite IR-120 as a catalyst.[153] Subsequently, a thermosetting resin system consisting of benzoxazine and epoxy deriving both from cardanol was developed. The cardanol epoxy resin contains a free phenolic function which acts as a cure accelerator for the ring opening reaction of benzoxazine besides its typical function as a hardener for epoxy resin.

Unlike cardanol, the major part of natural phenolic compounds does not have any aliphatic double bond within their structures. Thus, the synthetic route to incorporate epoxy groups into the backbone of phenolic compounds is the alkaline assisted alkylation of phenolic hydroxyls followed by the epoxidation of the resulting double bonds. Epoxides can be readily prepared from alkenes through a number of methods, including treatment with sulfonic peracids, *meta*-chloroperbenzoic acid (*m*CPBA) or dimethyldioxirane and transition metal catalysed oxidation with hydrogen peroxide or molecular oxygen.[154–157] However, the hazardous nature of these techniques precludes their use on a large scale. The alternative method to epoxidize alkenes is lipase mediated oxidation with hydrogen peroxide in the presence of a small amount of fatty acids. This process is widely applied in epoxidized oils manufacturing.[98,119,158]

This technology was recently adapted to the functionalization of gallic acid (derived from the hydrolysis of gallotannins) and vanillic acid (subunit of lignin), allowing the synthesis of bio-based epoxy prepolymers.[159] The treatment of gallic acid and vanillic acid with allyl bromide under alkaline conditions allowed total *O*-allylation of these phenolic compounds to give the tetraallylated derivative of gallic acid and the diallylated derivative of vanillic acid in 84% and 94% yields respectively (Fig. 5). In order to epoxidize these two allylated products, two methods were performed: a classical epoxidation using *meta*-chloroperbenzoic acid (*m*CPBA) at different molar ratios and the chemo-enzymatic process based on the immobilized lipase B from *C. antarctica* (Novozym 435) catalysing the formation of caprylic peracid from caprylic acid (C8) and hydrogen peroxide.

It was observed that the epoxidized product yield is highly influenced by the oxidant concentration. The treatment of allylated gallic acid with caprylic acid (molar ratio C8/C=C, 1 : 1) and hydrogen peroxide (molar ratio H_2O_2/C=C, 1.8 : 1) in the presence of Novozym 435 (20 wt% relative to the weight of the substrate) gave the tri-epoxidized derivative of gallic acid as the main product (60% yield) along with the tetra-epoxidized product with 18% yield. Comparatively, to reach the proportions of tri- and tetra-epoxidized derivatives obtained with the chemo-enzymatic oxidation, it was necessary to use 2 to 3.2 molar equivalents of *m*CPBA per allylic double bond, which represents a large excess of this compound.

The double bonds of the diallylated vanillic acid have been epoxidized in the same manner to give mono- and di-epoxidized products. The highest yield of epoxidation by the chemo-enzymatic method was obtained using caprylic acid (molar ratio C8/C=C, 1 : 1) and hydrogen peroxide (molar ratio H_2O_2/C=C, 2 : 1) in the presence of Novozym 435 (20 wt% relative to the weight of the substrate). Indeed, these reaction conditions led to the formation of the di-epoxidized product in 70% yield. A smaller amount (17%) of the mono-epoxidized product was also obtained (Fig. 6). However, the use of up to

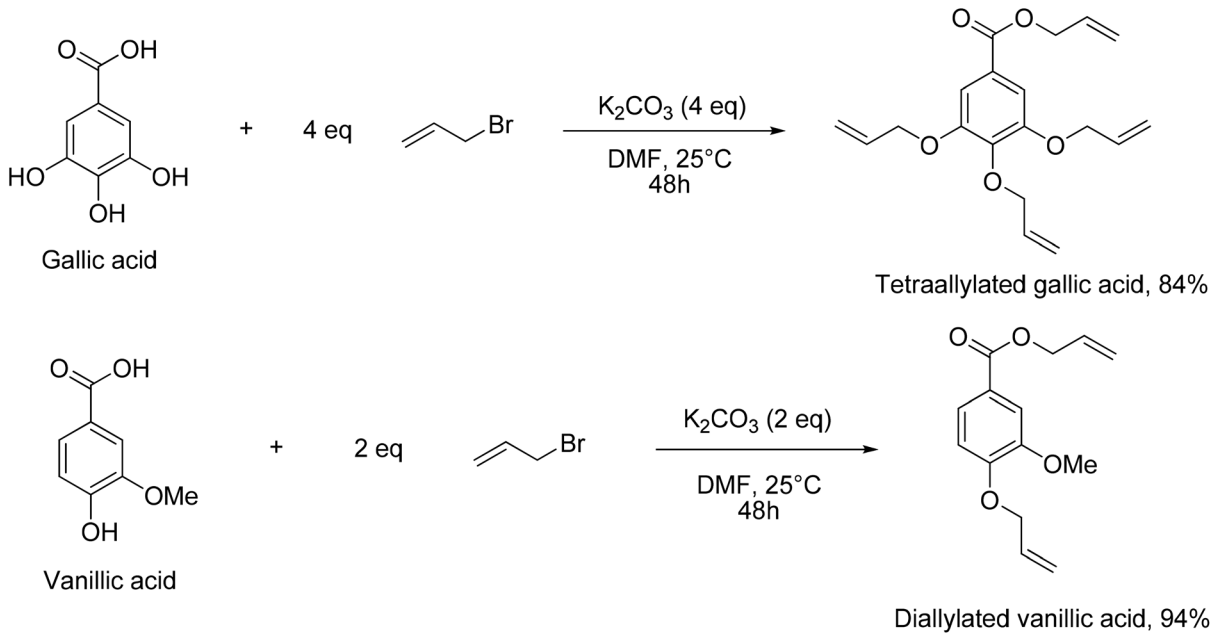

Fig. 5 Alkaline assisted allylation of gallic acid and vanillic acid.

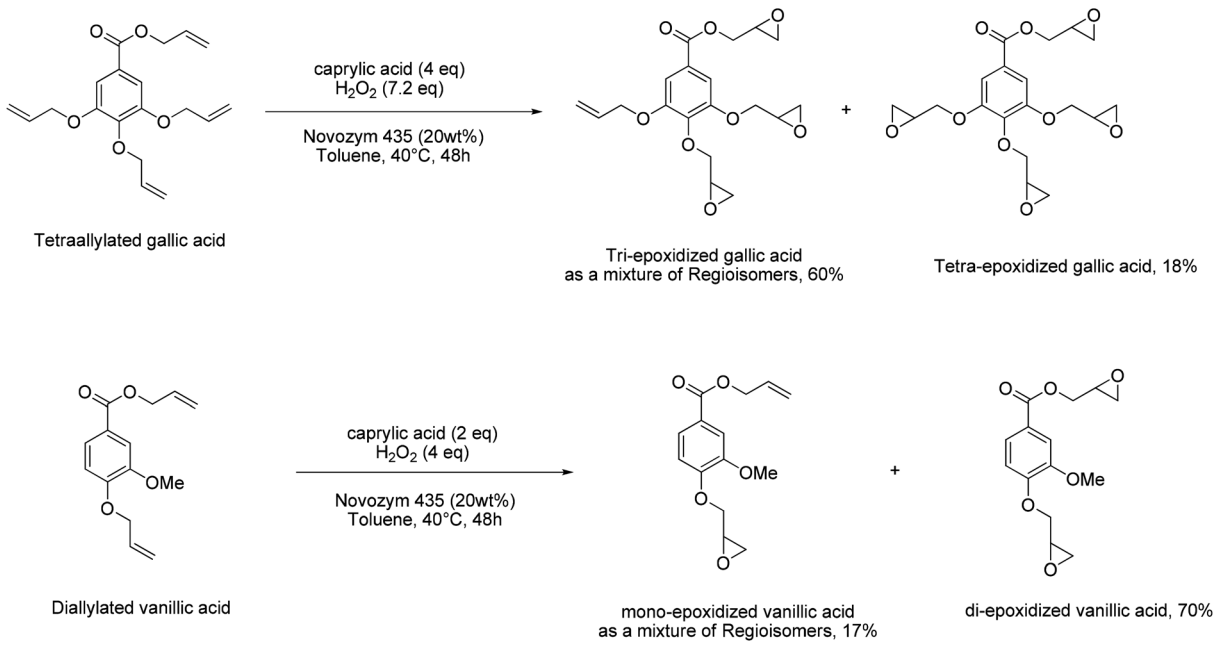

Fig. 6 Chemo-enzymatic epoxidation of allylated gallic acid and vanillic acid.

3 molar equivalents of *m*CPBA per double bond led to the formation of only 53% of the di-epoxidized product of the diallylated vanillic acid.

Hence, it was shown that the lipase-assisted generation of percaprylic acid in the presence of hydrogen peroxide is more efficient for the epoxidation of allylic double bonds of the phenolic acids such as gallic and vanillic acids than the widely used *meta*-chloroperbenzoic acid. Moreover, the hydrolysis of allylic esters by the action of Novozym 435 in aqueous medium was not observed.

IV. Conclusion

Lipases are versatile enzymes that can be used for various kinds of biocatalyzed reactions. Owing to their selectivity and

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their mild reaction conditions, they can be often considered as more interesting than classical chemical catalysts. Besides their application in oil and fat processes, these enzymes have proved to be very attractive for other lipase-catalyzed reactions. In particular, it was shown that lipases can be used in chemo-enzymatic epoxidation processes involving a two-step synthesis where the biocatalysts act as perhydrolases to produce peracids, which then act as catalysts to epoxidize double bonds. Such chemo-enzymatic epoxidation is of considerable interest because this method occurs under mild conditions and limits undesirable ring opening of the epoxide. The enzymatic process can be seen as a promising alternative to the classical Prileshajev epoxidation reaction that is carried out on the industrial scale to produce epoxidized plant oils or fatty acids. However, a lot of work is yet to be done for a potential industrial application of such lipase-catalyzed reactions. Notably, improvements in enzyme performances must be made in order to increase their resistance and stability to deactivation caused by hydrogen peroxide. Finally, the ability of lipases to participate in epoxidation reactions can also be used advantageously on unusual lipase substrates such as phenolic compounds. Indeed, we have documented herein the chemo-enzymatic epoxidation of allylated gallic and vanillic acids with lipase B from *C. antarctica* and aqueous hydrogen peroxide in the presence of caprylic acid. This recyclable and catalytic method demonstrated a good performance in the epoxidation of the allylic double bonds comparatively to the hazardous peracid *m*CPBA. These results involving a lipase are promising for the subsequent development of an alternative method for the synthesis of epoxy resins prepolymers free of bisphenol A and epichlorohydrin under mild conditions.

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