Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

rsc.li/green-chem

View Article Online
DOI: 10.1039/C7GC01127J

Development of a Lipase-Mediated Epoxidation Process for Monoterpenes in Choline Chloride-Based Deep Eutectic Solvents

Sumanth Ranganathan[a], *Sandra Zeitlhofer*[b,c] *and Prof. Volker Sieber*[a,b,d*]

a- Technical University of Munich, Chair of Chemistry of Biogenic Resources, Schulgasse 16, Straubing 94315, Germany.

b- Fraunhofer Institute of Interfacial Engineering and Biotechnology (IGB), Bio-, Electro- and Chemo Catalysis (BioCat) Branch, Schulgasse 11a, Straubing 94315, Germany

c- University of Applied Sciences Upper Austria, School of Engineering/Environmental Sciences, Stelzhamerstrasse 23, A-4600 Wels, Austria

d- Technical University of Munich, Catalysis Research Center (CRC), Ernst-Otto-Fischer Straße 1, Garching 85748, Germany

* - Corresponding author

Email: sieber@tum.de

Tel.: +49-9421-187-300

View Article Online
DOI: 10.1039/C7GC01127J

ABSTRACT

Chemical syntheses in contemporary process industries today are predominantly conducted using organic solvents, which are potentially hazardous to humans and the environment alike. Green chemistry was developed as a means to overcome this hazard and it also holds enormous potential for designing clean, safe and sustainable processes. The present work incorporates the concepts of green chemistry in its design of a lipase-mediated epoxidation process for monoterpenes, the process uses alternative reaction media, namely deep eutectic solvents (DESs), which have not been reported for such an application before. Choline chloride (ChCl), in combination with a variety of hydrogen bond donors (HBD) at certain molar ratios, was screened and tested for this purpose. The process was optimized through the design of experiments (DoE) using the Taguchi method for four controllable parameters (temperature, enzyme amount, peroxide amount and type of substrate) and one uncontrollable parameter (DES reaction media) in a crossed-array design. Two distinct DESs, namely glycerol:choline chloride (GlCh) and sorbitol:choline chloride (SoCh), were found to be the best systems and they resulted in a complete conversion of the substrates within 8 h. Impurities (esters) were found to form in both the DESs, which was a concern; as such, we developed a novel minimal DES system that incorporated a co-substrate into the DES so that this issue could be overcome. The minimal DES consisted of urea·H_2O_2 (U·H_2O_2) and ChCl and exhibited better results than both the GlCh and SoCh systems; complete conversions were achieved within 2 h for 3-carene and within 3h for both limonene and α-pinene. Product isolation with a simple water/ethyl acetate based procedure gave isolated yields of 87.2 ± 2.4 %, 77.0 ± 5.0 % and 84.6 ± 3.7 % for 3-carene, limonene and α-pinene respectively.

Keywords: deep eutectic solvents, design of experiments, green chemistry, lipase, monoterpene epoxide

View Article Online
DOI: 10.1039/C7GC01127J

1 Introduction

The utilization of renewable resources is one of the twelve principles of green chemistry[1-3] and in addition to the improved sustainability afforded by using renewables, the molecules obtained offer novel functionalities. Recent examples of this include the synthesis of new bio-based materials such as polycarbonates from terpenes[4] or furanic polyesters from sugars[5]. Mere utilization of renewable feedstock, however, is not necessarily a more sustainable practice than using non renewable ones as it is still important that also other rules of green chemistry are followed; for example, green chemistry requires toxic and harmful chemicals to be used only sparingly, if at all, in chemical processes[1-3]. To achieve this, solvent-free synthesis[6-8] or "green" reaction media such as supercritical (SC) fluids[9, 10] or ionic liquids (IL)[11-13] can be used. However, these systems often are impractical for chemical synthesis[7, 9, 13]. An alternative approach is the use of deep eutectic solvents (DESs)[14-16]. A variety of chemical reactions that use DESs as reaction solvents have already been reported with subject areas ranging from electrochemistry[17, 18] and organic syntheses[19-21] to enzymatic reactions[22-25].

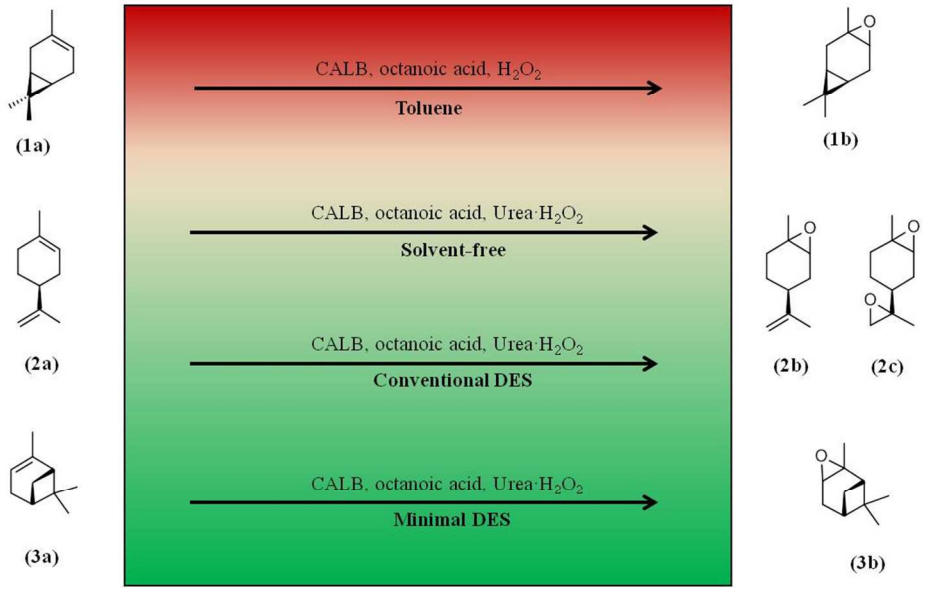
A good source of renewable feedstock is the secondary plant metabolite called terpenes that are accumulated in large quantities as by-products in the pulp, paper and fruit industries[26, 27]. Terpenes are excellent precursors for the flavor, fragrance and fine chemical industries in either a functionalized or a non-functionalized form. A specific functionalization, namely epoxidation, is instrumental in making terpene epoxides useful precursors for the production of diols, alcohols, ketones and as of late, monomers for polymers[4, 28-32]. Epoxides can be produced by different chemical means, including enzymatic apporaches[33-41]. Björkling *et al.* pioneered work on enzyme-mediated epoxidations in 1992[41] when they used a lipase (*Candida antartica* lipase B (CALB)) in the presence of an organic solvent, carboxylic acid and aqueous hydrogen peroxide (H_2O_2) to form peroxycarboxylic acid, which was able to epoxidize alkenes through the Prilezahev reaction[39]. It is important to note that the enzyme does not catalyze the epoxidation itself but provides efficient in situ formation of the oxidizing species, i.e. the peroxycarboxylic acid.

Previously, we had used this technique[41] to develop and optimize a lipase mediated epoxidation process for monoterpenes[42]. Although this process adhered to some principles of green chemistry, in that it utilized renewable reactants and enzymes as catalysts; we also used toluene as the reaction medium; which means the process cannot be considered as "green".

This paper focuses on the development of a more sustainable or "greener" monoterpene epoxidation process that adheres to the principles of green chemistry. To the best of our knowledge, this is the first account that uses DES as solvent for lipase mediated epoxidation of monoterpenes. To begin with, we tested an enzyme-mediated process both under solvent-free conditions and then in DESs as the reaction

View Article Online
DOI: 10.1039/C7GC01127J

medium. After comparing these two approaches, we developed the process and optimized it for the DES system. The process development stages consisted of two screenings, an optimization by design of experiments (DoE) - Taguchi method, purification stage and a final scale-up phase. We also wanted to examine how using a DES as the reaction solvent affected the outcome (*i.e.*, yield of the epoxide) of the process as well as how other reaction parameters (*i.e.*, substrate type, enzyme amount, temperature of reaction and hydrogen peroxide) affected the outcome. We were subsequently able to develop a novel DES mixture that could act as both the solvent and the co-substrate source in fast and efficient epoxidations (Scheme 1)



Scheme 1: Development of the lipase-mediated epoxidation process for various reactants (1a-3a) and their corresponding epoxides (1b-3b), starting with the "ungreen" process utilizing toluene and moving on to greener processes utilizing deep eutectic solvent (DESs) and solvent free conditions.

2 Results and Discussion

2.1 Solvent free epoxidation system

The initial test of the solvent-free synthesis was performed using only terpene (3-carene (**1a**), limonene (**2a**) and α-pinene (**3a**)), octanoic acid, a peroxide source (aqueous (aq.) or urea (U) ·H_2O_2) and CALB, as specified in section 4.2.1.1. There were two distinct phases: a top organic phase that contained both the monoterpene and octanoic acid and a bottom phase containing H_2O_2. A single point measurement at the

View Article Online
DOI: 10.1039/C7GC01127J

end of 16 h revealed that 0.1 mmol 3-carene (**1a**) was totally converted to its corresponding epoxide (result not shown). Based on this result, we scaled up the process using a greater amount of the reactants at 45 °C and 60 °C. The results are shown in Figure 1.

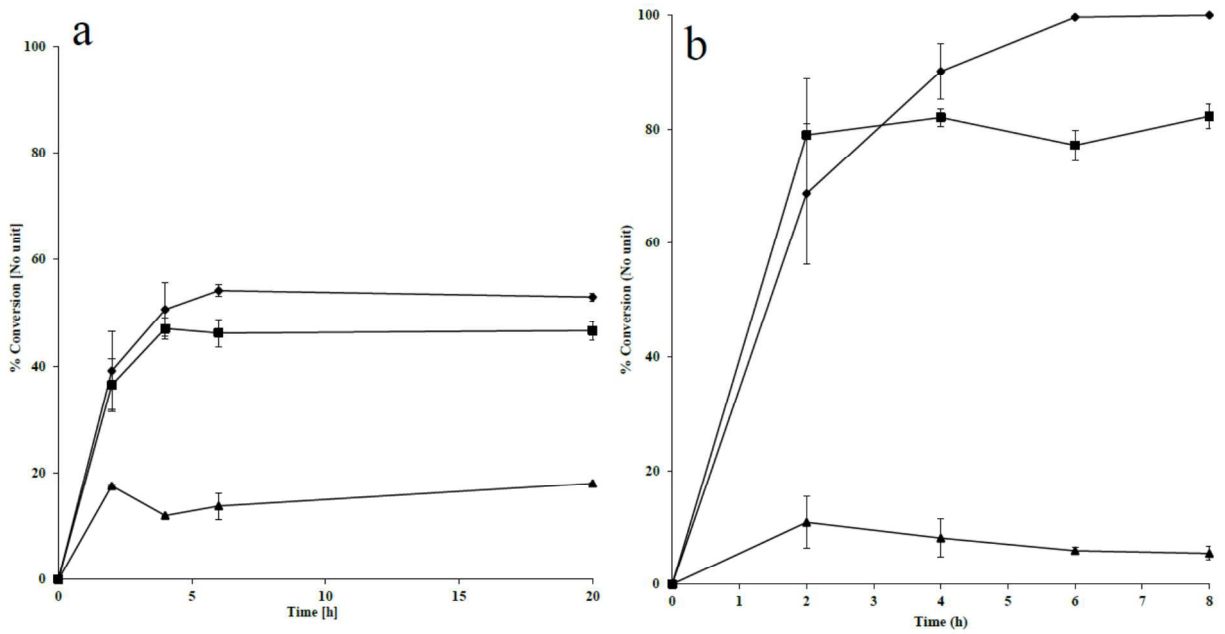


Figure 1: Conversion profile of 3-carene (diamond), limonene (square) and α-pinene (triangle) at 45 °C (a) and 60 °C (b). (Reaction conditions: 10 mmol monoterpene, 12.5 mmol aq.H_2O_2, 2.5 mmol octanoic acid, 100 mg CALB (1670 PLU), 500 rpm)

At 45°C (Figure 1 (a)), conversions of 53.0 ± 0.8 %, 46.6 ± 1.8 % and 13.8 ±0.1 % were achieved after 20 h for **1a**, **2a** and **3a** respectively. A second reaction was carried out using identical conditions, but the reaction temperature was increased to 60 °C. At this temperature (Figure **1** (b)), a conversion of 100 % was seen for **1a**, whereas **2a** and **3a** yielded 82.8 ± 2.2 % and 5.5 ± 1.2 %, respectively. When the reaction time was extended to 20 h, no increase in the conversion of **2a** could be achieved**.** Only when adding fresh enzyme after 6 h an increase in conversion was observed (data not shown), implying that inactivation of the enzyme, possibly due to the high amount of octanoic acid in the reaction medium was limiting.

More interestingly, the results of this experiment are different from those obtained in our previous work, which had implied that the best results for the lipase epoxidation of monoterpenes in toluene were to be achieved at a reaction temperature of 45 °C[42]. Our present results also indicate that the sequence in which the three substrates were oxidized, at both temperatures, was different from that reported by Bakhvalov *et al.* in 2008[43]. The present work suggests that the oxidation follows the order **1a** > **2a** > **3a** rather than **1a** >

View Article Online
DOI: 10.1039/C7GC01127J

3a > **2a**. However, the published findings are for solvent-based oxidation reactions, which have better heat and mass transfer conditions than solvent-free reactions. Additionally, each substrate behaves differently as a solvent, which could have caused the variation in the oxidation pattern. Furthermore, the results in both of the previously published studies[42, 43] were obtained using optimized conditions as opposed to the single variable change technique used in the present study.

As mentioned earlier, two distinct phases were observed when aq. H_2O_2 was used. Suspecting that the water content may have interfered with the reaction, we conducted a third test using three different temperatures and U·H_2O_2. On mixing all the reactants together, a single solid phase was obtained. After 20 h, a single point measurement was made and conversion of the monoterpenes was calculated using gas chromatography–mass spectrometry (GC–MS), as shown in Figure 2.

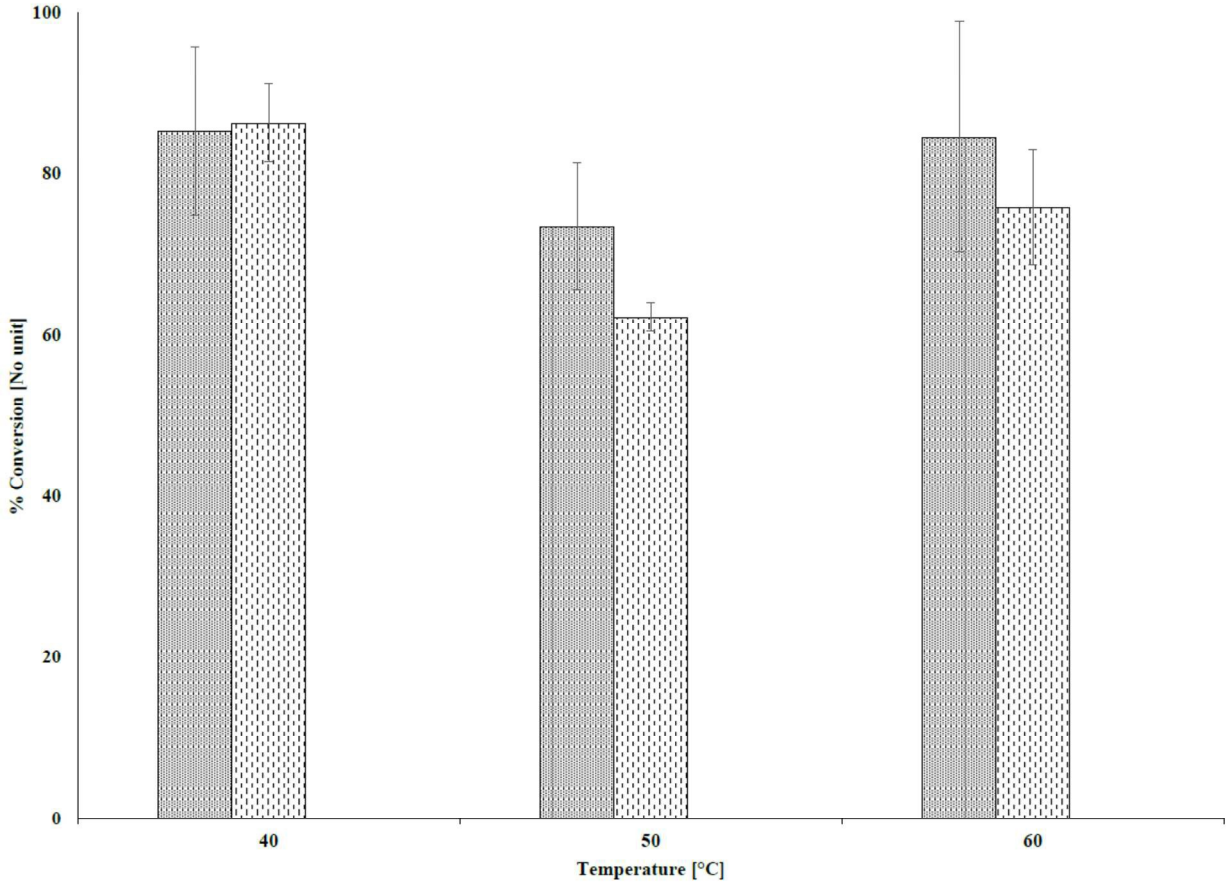


Figure 2: Conversion of 3-carene (dotted) and limonene (vertical dashes) obtained using U·H_2O_2 under solvent-free conditions at 40, 50 and 60 °C after 20 h. (The reaction conditions were 2 mmol monoterpene, 2.5 mmol U·H_2O_2, 0.5 mmol octanoic acid, 100 mg CALB (1670 PLU), 500 rpm)

View Article Online
DOI: 10.1039/C7GC01127J

Conversions of 85.3 ± 10.4 %, 73.4 ± 7.9 % and 84.5 ± 14.4 % were obtained for **1a** and 86.2 ± 4.8 %, 62.2 ± 1.8 % and 75.8 ± 7.2 % for were obtained for **2a** at 40, 50 and 60 °C, respectively after 20 h. There was no conversion of **3a** at any of the three tested temperatures. On a closer observation of Figure 2, it can be seen that the results of the epoxidations at 40 and 60 °C were identical for **1a,** but not for **2a**. However, an interesting phenomenon can be seen at 50 °C, for which the conversion was the lowest. This may have occurred due to the difference in the solubility of the octanoic acid in each of the substrates (**1a-3a**).

Both the systems, *i.e.* aq. H_2O_2 and U·H_2O_2, exhibited incomplete conversions of the starting materials (**1a-3a**) with the exception of the reaction at 60 °C using aq. H_2O_2 for **1a**. If a process has to be developed so that maximum conversion is obtained for all three substrates, optimization using, for example, the DoE approach can be carried out. However, each substrate would act as its own solvent and any optimization will be useful only for that particular substrate. Additionally, both the cases (*i.e.* aq. H_2O_2 and U·H_2O_2) had issues in terms of both handling and reproducibility (as evidenced by the high error percentage of the tests); therefore, we shifted our focus from a solvent free system towards utilizing green reaction media, namely DESs.

2.2 Conventional DES, first screening round

Because of the drawbacks experienced on using the solvent-free system (section 2.1), DESs were chosen as a "green" alternative to carry out the lipase mediated epoxidation reactions. To determine the best DES candidates, a two-step screening approach was used: an initial screening step to evaluate the fluidity of the selected DES reactants and a second step was performed to evaluate their epoxidation of **1a**. The mixtures that were assumed to be suitable for the epoxidation process were chosen from the list published by Russ and Koenig[15]. The DES mixtures were prepared in ratios described in Table 2 according to the procedure described in section 4.3. All the mixtures in Table 2 had been previously described by Russ and Koenig[15] apart from the 4-hydroxy phenyl acetic acid (HPA) and ChCl mixture (Table 2, #3). This particular chemical (HPA) was chosen because phenylacetic acid was described in the same work as having a melting point of 25 °C in the same molecular ratio. Because both the chemicals are similar in structure but for the presence of an additional OH group, this mixture was tested to see if a new DES mixture could be formed in a similar temperature range.

From previous experience it has already been established that 45 - 60 °C is the ideal temperature for performing lipase based epoxidation reactions [41, 42, 44]. Hence, only those DESs that were liquids at 60 °C were selected for the second round of screening. The DES mixtures from Table 2 were heated to 100 °C

View Article Online
DOI: 10.1039/C7GC01127J

and cooled to 60 °C before the samples were visually examined for fluidity; the results are given in Table **2**. HPA, L-(+)-tartaric acid, L-glutamic acid and D-glucose in combination with ChCl did not yield a liquid at 60 °C and malonic acid with ChCl (1:1) only yielded a very viscous liquid that could not be stirred. As a result, these mixtures were not used for the epoxidation reaction.

Literature[15] suggested that the selected mixtures are supposed to yield liquids at temperatures much lower than those tested in this work. Meng *et al.* suggested that the presence of moisture can interfere with the hydrogen bonding between DES components (urea and ChCl), which would lead to increased melting temperatures[45]. Working on the assumption that this phenomenon could be extended to other DES mixtures, the individual DES components (that were not liquid) were dried under vacuum and tested again; no changes in their behaviors were observed. Since the DES mixtures mentioned above (HPA, tartaric acid, glutamic acid and D-glucose in combination with ChCl) did not form liquids and this step was a mere screening round, they were omitted from the second round of screenings and were not investigated further.

2.3 Conventional DES, second screening round

The eight successful liquid DES mixtures from the previous screening round were screened for epoxidation activity, as described in section 4.4. The conversions after 24 h of reaction time are given in Table 2. It can be inferred that the sugar and sugar alcohol systems were the ones that performed best. For the carboxylic acid systems (Table 2, #1 & 2), no additional peroxy acid generator, *i.e.* octanoic acid, was added. These two reactions yielded minimal conversion, which may have been due to the polar nature of both these acids, as the polarity of a carboxylic acid increases with a decrease in its aliphatic chain. However, the melting temperature required to produce a DES mixture also increases[15]; as a result, these mixtures were not tested. In the case of urea: ChCl, it could be inferred that the combined effect of urea as the HBD and the additional $U{\cdot}H_2O_2$ could have led to the inactivation of the lipase after a certain amount of time. Because urea at a concentration of 6 M is known to be a denaturant of enzymes[46], we assumed that this could be a reason for the reduced conversion. In order to test this, the lipases were washed three times with water to remove the residual urea and then with ethanol to remove any terpene or terpenoid impurity present. The reactions were then repeated and the conversion was either less than the previous occasion or there was no conversion observed at all.

Considering the alcohol HBDs in combination with ChCl, we found the glycerol system (81.1 %) yielding a better conversion than ethylene glycol (57.5 %). A suitable explanation for this behavior could be obtained from the work of Rengstil *et al*[47] that described the fluidity of a DES system to be directly

View Article Online
DOI: 10.1039/C7GC01127J

proportional to the number of hydrogen bonds on offer from a HBD. As the conversion obtained for the ethylene glycol DES mixture was below 80 %, it was not used for the optimization round. The fructose based DES yielded a dark brown mixture and was not as stable as the sugar alcohols, hence it was also not included in the optimization step. Lastly, because sorbitol is a cheaper resource than xylitol, it was preferred for use in the optimization phase. As a result, only glycerol (GlCh) and sorbitol (SoCh) DES mixtures were considered further.

2.4 Conventional DES- optimization using the Taguchi method

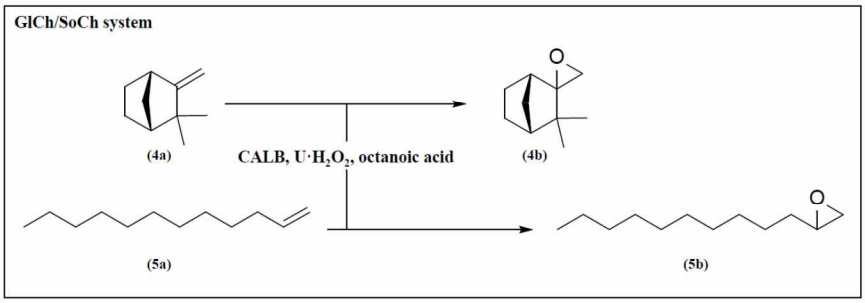
The lipase-mediated epoxidations were optimized for the GlCh and SoCh systems using the Taguchi crossed array method. A detailed explanation of the choice of parameters for this optimization, the theory behind the Taguchi method and the signal to noise ratio can be obtained from literature[48-53]. All reactions were performed in the order as described in the Supplementary section (once for each of the systems, *i.e.* GlCh and SoCh) in triplicate. Minitab (version 17) software was used to analyze the results. The response variable used was the conversion of the monoterpenes (**1a**–**3a**) to their corresponding epoxides (**1b**–**3b**). The results of the optimizations are given in detail in the Supplementary information.

The optimized set of parameters for maximum conversion of 1 mmol of terpene was similar for both DES Systems used GlCh (5 mmol ChCl, 10 mmol Glycerol) and SoCh (5 mmol ChCl, 5 mmol Sorbitol): 4 mmol U·H_2O_2, 100 mg lipase at 40° C to 50 °C. Independent of the DES used, the conversion is more efficient for **1a** and **2a**. Decreasing the amount of enzyme to 75 mg only slightly decreased the amount of conversion. SoCh was found to work slightly better at lower temperatures (40 °C). Interestingly, a strong dependence on the amount of U·H_2O_2 was found for the conversion amounts of the two DES systems.

2.5 Evaluation of the substrate range in the GlCh and SoCh systems

After the optimal conditions for the processes, i.e. the GlCh and SoCh systems, were identified, two additional substrates, camphene (**4a**) and 1-dodecene (**5a**), were tested to verify the range of the DESs. **5a** was tested because it is a monoterpene and **4a** was tested to verify if the process could be extended to the terminal double bond of linear olefins as well (Scheme 2).

View Article Online
DOI: 10.1039/C7GC01127J



Scheme 2: Lipase-mediated epoxidation of camphene (4a) and 1-dodecence (5a) to their corresponding epoxides (4b and 5b) using the optimized set of parameters for the GlCh and SoCh systems.

All reactions were performed with the optimized set of conditions described in section 2.4. The results for the new substrates in addition to those tested in the GlCh system are shown below in Figure 3.

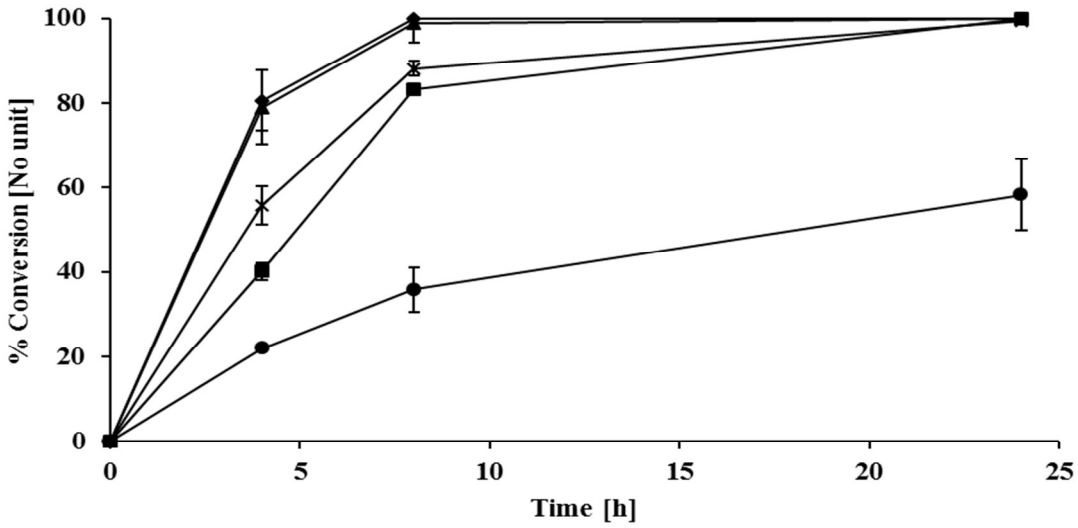


Figure 3: Conversions obtained for 1-dodecene (circle), α-pinene (square), camphene (x), limonene (triangle) and 3-carene (diamond) over time using the optimized GlCh system.

After 8 h, **1a** and **2a** were almost fully converted to their corresponding epoxides (**1b** and **2b**), as predicted by the DoE. **3a** and **4a** were approximately 83-88% converted and only 35% of **5a** was converted to 1-dodecene epoxide (**5b**) after 8 h with 69% being converted after 24 h. Although increased reaction times may ultimately improve the conversion, we did not test for this.

Similar tests were also performed for the SoCh system and the results are shown in Figure 4; similar findings were obtained. The conversions of **1a** and **2a** were 100% and that of **3a** was approximately 63 -

View Article Online
DOI: 10.1039/C7GC01127J

70% after 8 h. However, **4a** had a slightly lower conversion of 75 - 83% in the SoCh system than in the GlCh system. Sampling proved to be more difficult for the SoCh system than for the GlCh system. This was because of the separation of phases (in the reaction vessel), which took longer for the SoCh system compared to the GlCh system. **5a** had a conversion of 55 - 65 %, which was surprising given that GlCh system had a conversion of 25 - 35% for this substrate. We assume that the viscosity of the SoCh system played a major role in this difference. A possible explanation is that sorbitol and ChCl may have formed a dynamic DES system, wherein U·H_2O_2 and octanoic acid might have been dissolved better (than the GlCh system) leading to a faster peroxycarboxylic acid formation, resulting in a faster epoxidation process.

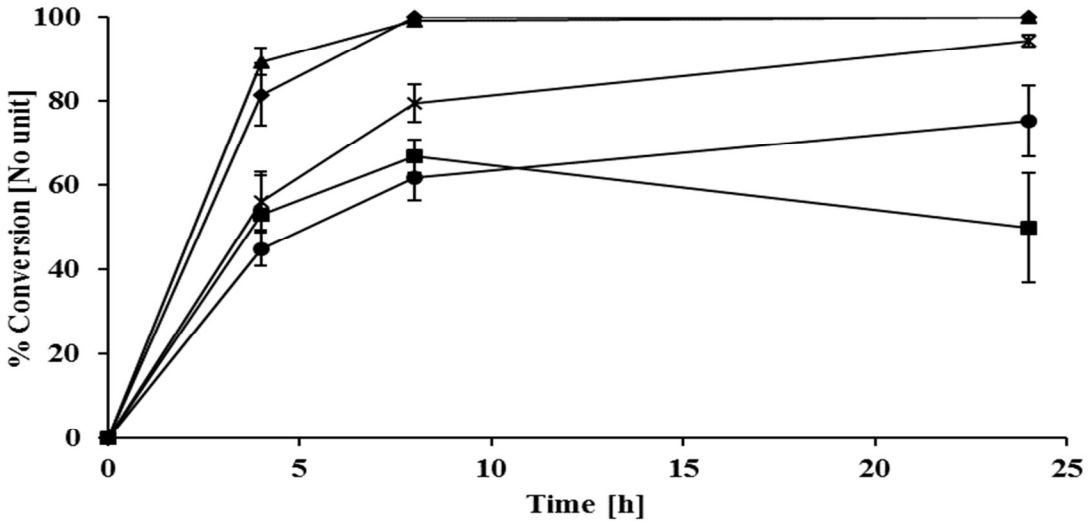


Figure 4: Conversion obtained for 1-dodecene (circle), α-pinene (square), camphene (x), limonene (triangle) and 3-carene (diamond) over time using the optimized SoCh system.

One major drawback of both the systems was the formation of caprylate esters of both glycerol and sorbitol, which we detected using GC-MS (Supplementary Information). To produce pure epoxides (**1b-5b**) and avoid the formation of esters, we decided to shift the search toward DES mixtures that did not contain any alcohol groups. This led to the development of the "minimal" DES system which consisted of ChCl: U·H_2O_2 that was to be used as both the peroxide source and the solvent.

2.6 Minimal DES results

We already demonstrated that the urea: ChCl (Table 2, # 9) system was liquid at the desired temperature, *i.e.* 60 °C and yielded a conversion of 67 %. We therefore elected to use this system, albeit with a small

View Article Online
DOI: 10.1039/C7GC01127J

modification: U·H_2O_2 was used instead of urea for a novel DES to be formed. Doing so meant that additional amounts of U·H_2O_2 did not need to be added, as the compound already contains urea for DES formation and the H_2O_2 needed for epoxidation. This method was used along with the same reaction conditions outlined in section 4.6 and the epoxidation was successful within 2 h for **1a** and **2a**, whereas it took 3 h for **3a**; this is shown both in Figure 5 and also and in Table 5 of the Supplementary Information. It can be seen that after 2 h, **1a** was completely converted to **1b**, **2a** was 99 ± 1 % and **3a** was converted to 92 ± 6%. After 3 h, all the samples were completely converted to their epoxides.

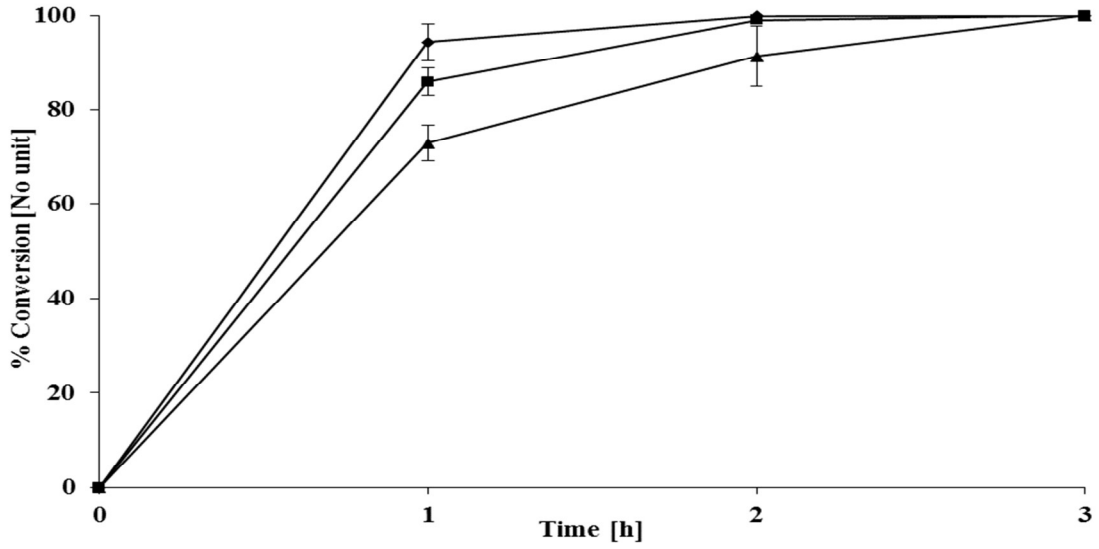


Figure 5: Conversion profile of 3-carene (diamond), limonene (square) and α-pinene (triangle) using the ChCl:U·H_2O_2 DES mixture

This surprisingly good result could have been due to the urea and the ChCl forming a proper DES, with the remaining H_2O_2 being dissolved in the DES. This resulted in the educts and the peroxy acid generator having better solubility, which led to faster reaction kinetics. This makes this reaction medium, which was the simplest of all of the ones tested, the most effective one as well. In fact, it performed even better than the toluene system that we had previously developed[42]. It should be noted that this process itself was not optimized using the Taguchi method, but the results of the previous optimizations were used here. The epoxides produced were then purified according to the procedure described in section 4.8.

To analyze the purity of epoxide **1b**, we carried out GC-MS and nuclear magnetic resonance (NMR) analyses. As described above, in the samples from the GlCh and SoCh systems, esters formed between octanoic acid and the alcohol groups of the DES was detected as impurity peaks in the GC-MS. No such peak was present when the synthesis was performed in U·H_2O_2 (Supplementary Information). In theory the cholinium species could also lead to side product formation as it also contains an alcohol group. Since

View Article Online
DOI: 10.1039/C7GC01127J

the GC-MS exhibited no impurities (Supplementary Information, Figure 6), we performed an additional NMR analysis of purified **1b** and compared the spectra of the samples from the two different systems. The chemical shift presents the spectrum for **1b** from the GlCh system (cyan) and ChCl:U·H_2O_2 system (red) (Figure 6). The NMR shifts of **1b** match to the ones reported in literature[42, 44]. It can be seen, however, that peaks that correspond to octanoic acid esters at around 0.9, 1.3 and 2.3 ppm, are present in **1b** from the GlCh system, while these peaks are absent in **1b** from the ChCl:U·H_2O_2 system. Apparently, lipase based esterification of choline with CALB is less efficient, possibly due to the positive charge of the molecule, leading to decreased side product formation. We can therefore conclude that the ChCl:U·H_2O_2 system is much more efficient in producing epoxides in a purer form than the GlCh or SoCh systems.

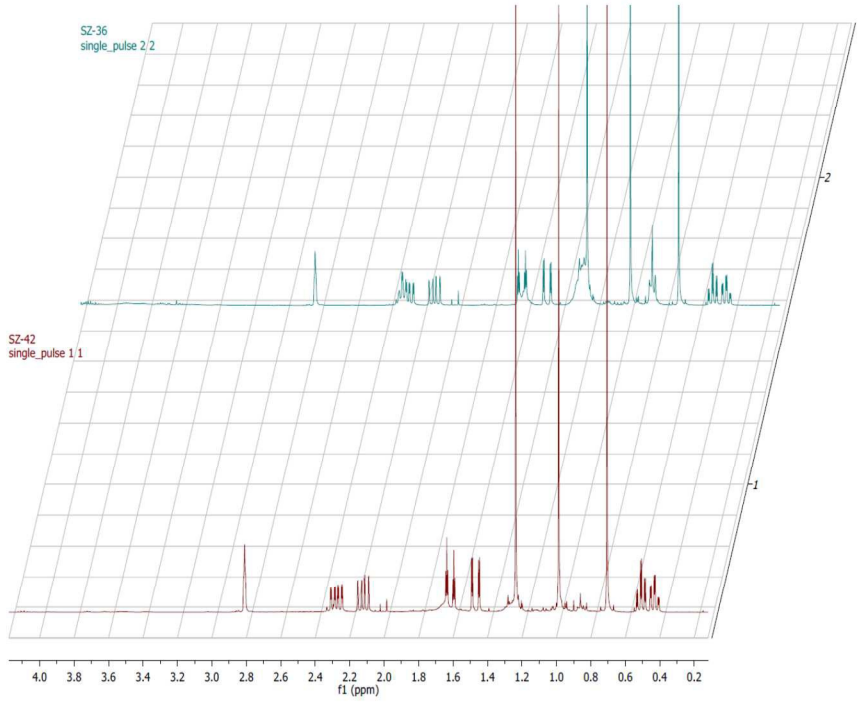


Figure 6: NMR spectrum of 3-carene epoxide produced by GlCh (top) and ChCl:U·H_2O_2 (bottom) systems.

2.7 Product purification and isolated yields

The utilization of DES has implications for product purification. The low solubility of DES in organic solvents can be exploited for a simple extraction process. Accordingly, *n*-hexane was first used as the extraction solvent as described in detail in section 4.8.10. Isolated yields close to 90 % could be obtained (Table 1). However, the utilization of *n*-hexane counteracts the green principles of the process as it is

View Article Online
DOI: 10.1039/C7GC01127J

considered a harmful organic solvent[54]. An effective replacement for the extraction solvent was to be investigated. The high water solubility of the DES constituents combined with the low water solubility of the products actually might allow a water based extraction process. Hence, a new water based purification scheme was developed. Water indeed dissolves the DES and three phases appear - the upper organic phase with the product and octanoic acid, the middle phase with lipase beads and a lower DES phase that can be discarded. The octanoic acid in the organic phase could then be deprotonated and transferred to the aqueous phase yielding pure epoxide as an upper phase. However, this led to the loss of terpene epoxide on the walls of the separating funnel, due to the work in small scale. To solve this issue, we used ethyl acetate in combination with water in order to facilitate better separation of the DES and the organic phases. On using the protocol described in 4.8.2, we were able to isolate the products with relative ease and the results obtained are shown below in Table 1. It can be seen that the water and ethyl acetate purification procedure is equal to or even slightly better than the n-hexane process.

Table 1: Comparison of isolated yields (%) of terpene epoxides (1b-3b) obtained on using the *n*-hexane and water + ethyl acetate processes.

S.No.	Product	Isolated yield (%) obtained on using n-hexane	Isolated yield (%) obtained on using water/ethyl acetate
1	**1b**	89.8 ± 5.9	87.2 ± 2.4
2	**2b** (70 %), **2c** (30 %)	74.0 ± 4.5	77.0 ± 5.0
3	**3b**	80.4 ± 7.0	84.6 ± 3.7

3 Conclusions

This work presents the epoxidation of monoterpenes under solvent-free conditions. Owing to the incomplete conversion of reactants, with the exception of **1a**, it can be inferred that individually tailored optimizations are necessary for each monoterpene. To overcome these issues, DES, which is considered a green reaction medium, was used to epoxidize monoterpenes. Two of these systems, *i.e.* GlCh and SoCh, were successful in yielding complete conversions of the starting material within 6-8 h. However, both these systems produced ester impurities. To avoid this, a novel "minimal DES" consisting of ChCl and U·H_2O_2 was developed, which achieved a total conversion of the reactants within 2-3 h. We were able to reduce the reaction time by a quarter using this new DES system. In addition to this, we developed a purification procedure using water and ethyl acetate that enables a good recovery of terpene epoxides whilst maintaining the green aspect. To summarize, we believe that this new system could inspire future works in this field not just at the laboratory scale but also at the industrial scale.

View Article Online
DOI: 10.1039/C7GC01127J

4 Materials and Methods

4.1 Materials

All the materials in this study were used as purchased without further modification or purification steps. **1b** was produced in house [42] and was used as an analytical standard. (+)-limonene (96%) was purchased from Acros Organics, Germany. Toluene (≥ 99.9%) was purchased from Merck KGaA, Germany. Gylcerol (≥ 99.5%) was purchased from Roth chemicals, Germany. D-sorbitol (min. 99%) and sodium hydroxide (min. 99%) were obtained from Applichem GmBH. 3-carene (≥ 90%), α-pinene (98%), 1-dodecene (95%), camphene (95%), choline chloride (≥ 98%), D(-) fructose (≥ 99%), potassium carbonate(≥ 99%), L-(+)-tartaric acid (≥ 99%), laevulinic acid (99% FG), Malonic acid (99%), Octanoic acid (98%), urea-hydrogen peroxide ($U{\cdot}H_2O_2$) (97%), urea (molecular biology grade), xylitol (99 %) and zinc bromide (98%) were bought from Sigma Aldrich, Germany. Ethyl acetate (LC-MS grade, min. 99.95%), aqueous hydrogen peroxide (aq. H_2O_2) (35 %) and n-hexane (> 95%) were obtained from Th.Geyer GmBH, Germany. Ethylene glycol (≥ 99.5%) was purchased from VWR chemicals, Germany. The enzyme *Candida antartica* lipase B (CALB) was procured from two suppliers - Chiral Vision (IMMCALB-T2-TXL, 15000 PLU/g) was used for optimization reactions and c-LEcta (CALB Immo plus, 16700 PLU/g) was used for all the other reactions. Both the commercial CALB preparations used in this work were immobilized covalently on to identical hydrophobic supports with a similar enzyme loading. Moreover, previous tests performed showed no characteristic difference in reactivity (results not shown).

4.2 Methods

4.2.1 Solvent free epoxidation systems

The tests for the solvent-free epoxidation systems were carried out using two different peroxide sources: aqueous (aq.) H_2O_2 and urea (U)$\cdot H_2O_2$.

4.2.1.1 Aq. H_2O_2

An initial test was carried out to determine whether a solvent-free epoxidation was even possible for monoterpenes; this was done using 2 mmol **1a**, 2.5 mmol aq. H_2O_2 (35%), 0.5 mmol octanoic acid, 100 mg (1670 PLU) CALB, 40 °C and 500 rpm for a duration of 16 h.

View Article Online
DOI: 10.1039/C7GC01127J

The scaled-up version was carried out using 10 mmol monoterpene (**1a**, **2a** and **3a**), 12.5 mmol of aq. H_2O_2 (35%), 2.5 mmol of octanoic acid, 100 mg (1670 PLU) CALB, 45 and 60 °C and 500 rpm for a duration of 20 h (45 °C) and 8 h (60 °C).

4.2.1.2 U·H_2O_2

The test was performed using 2 mmol monoterpene (**1a-3a**), 2.5 mmol U·H_2O_2, 100 mg (1670 PLU) CALB, 0.5 mmol octanoic acid, 40, 50 and 60 °C and 500 rpm for a reaction time of 20 h.

4.3 Conventional DES, first screening round

Several DES mixtures described by Russ and Koenig[15] were prepared with the assumption that they would be appropriate reaction media for the lipase-mediated epoxidation reaction. ChCl was used as the halide salt and different HBDs at certain ratios (described in detail in Table 2) were used to form the DES mixtures. For the preparation of the DES, ChCl and the corresponding HBD were carefully weighed into an empty 20 ml reaction vessel. The vessel was then heated to 100 °C for 120 minutes, after which the samples were cooled to 60 °C. The fluidity of the DES mixture was visually examined and noted. The samples that were liquid at 60 °C were then used as the reaction media in the second round of the screening process.

Table 2: List of HBDs and ChCl screened as DES for the first round of screening. ChCl:HBD are given in molar ratios. T °C refers to the melting point of the mixtures and RT refers to room temperature as described by Russ & Koenig[15]. Conversion refers to the amount of 3-carene converted to its respective epoxide during the second round of screening.

S.No.	HBD	Type	ChCl : HBD	T °C	Fluidity at 60 °C	Conversion (%)
1	Valeric acid	Carboxylic acid	1:2	RT	Yes	No conversion
2	Laevulinic acid	Carboxylic acid	1:2	RT	Yes	17.4
3	4-hydroxy phenyl acetic acid	Carboxylic acid	1:2	No data	No	NA
4	Malonic acid	Dicarboxylic acid	1:1	10	Yes*	NA

View Article Online
DOI: 10.1039/C7GC01127J

5	L (+) tartaric acid	Dicarboxylic acid	2:1	47	No	NA
6	L-Glutamic acid	Amino acid	1:2	13	No	NA
7	Glycerol	Alcohol	1:2	-40	Yes	81.1
8	Ethylene glycol	Alcohol	1:2	-20	Yes	57.5
9	Urea	Amide	1:2	12	Yes	66.9
10	D-Fructose	Sugar	1:2	5	Yes	100
11	D-Glucose	Sugar	1:2	14	No	NA
12	D-Xylitol	Sugar alcohol	1:1	RT	Yes	100
13	D-Sorbitol	Sugar alcohol	1:1	RT	Yes	100

*** Was liquid, but highly viscous; NA - not applicable**

4.4 Conventional DES, second screening round

All the DES mixtures that were liquids at 60 °C (Table 2) from the previous screening round were used as the reaction media for the lipase mediated epoxidation of **1a**. A typical lipase reaction screening experiment consisted of 1 mmol **1a**, 0.25 mmol octanoic acid, 3 mmol U·H_2O_2, 100 mg (1670 PLU) CALB and the liquefied DES mixtures from the first screening round. The reaction was carried out at 60 °C and 500 rpm in an oil/sand bath. In order to have sufficient reaction medium for the epoxidation reactions, the DES mixtures were prepared at an increased factor of five whilst maintaining the same molecular ratio (For example: 5 mmol ChCl with 10 mmol glycerol). A single point measurement was taken at the end of 24 h to determine the conversion of 1a to 1b, and the measurement is described in Table 2.

View Article Online
DOI: 10.1039/C7GC01127J

4.5 Optimization

The lipase-mediated epoxidation process was optimized using the DoE Taguchi method. The theory behind this method has already been described in detail in our previous work[42] as well as in various other studies[48-52], as such, it will not be discussed in the present study. Although this method was used in the present study, DESs were used instead of organic solvents. The parameters chosen and the levels used are given in Supplementary Information. The optimizations were performed using two L_9 orthogonal arrays (Supplementary Information) and the performance criterion used was "larger is better". Each row in an array corresponds to the combination of parameters at their respective levels. The constant parameters used in the process were: 500 rpm mixing and the source of lipase (CALB from Chiral Vision with a loading of 15000 PLU/g). The reactants were mixed in the order described in the Supplementary information. The arrays for the trials and the analysis of the results were generated using Minitab (version 17) software.

4.6 Minimal DES mixture

ChCl (7.5 mmol) and U·H_2O_2 (15 mmol) were mixed at room temperature between 45 min to 1 h with a magnetic stirrer. The resultant fluid mixture was then used as both a solvent and a peroxide source for the lipase-mediated epoxidation reaction. The following reaction conditions were used: 5 mmol monoterpene (**1a-3a**), 100 mg (1670 PLU) CALB, 1.25 mmol octanoic acid, 50 °C and 500 rpm.

4.7 Analytics and sampling

The GC-MS and NMR settings, heating profile of gas chromatography and mass spectrometry details, in addition to the retention times of the reactants and products have already been described in our previous works[42, 44]. The sampling was performed as follows (as DESs do not follow the traditional rules of solvents, this study utilized different sampling techniques to analyze the compounds):

- Up to 1 mmol of the starting material: 2 µl of organic phase (DES and enzyme free) was mixed with 198 µl *n*-hexane. 10 µl of this sample was then transferred to 990 µl ethyl acetate
- Up to 10 mmol starting material: 2 µl of organic phase was added to 998µl ethyl acetate
- Up to 100 mmol of starting material: 1 µl of organic phase was mixed with 999 µl ethyl acetate

The samples were then subjected to GC-MS measurements. For the NMR measurements, 20 µl of pure epoxide was mixed with 600 µl deuterated chloroform ($CDCl_3$) and the sample was measured with ^1H proton NMR.

View Article Online
DOI: 10.1039/C7GC01127J

4.8 Purification procedure

Terpene epoxides (1b, 2b, 2c and 3b) were produced in the minimal DES setup as mentioned in 4.6 and was purified using an adapted version of our previous work[42]. The purification procedure was developed after an initial screening step (detailed description of the development is described in the supplementary information). Two purification processes were tried for the effective recovery of the terpene epoxides (**1b-3b**) in triplicates.

4.8.1 Purification using *n*-hexane

The first method that was tried used *n*-hexane as the extraction solvent to extract the nonpolar fractions (epoxides (1a-3b) and octanoic acid). This mixture was vortexed for 30 seconds to 1 minute. This mixture was then cooled down to -20 °C for a time period of 1-2 h, which yielded three phases: a top organic phase; a middle phase containing the lipase and a bottom DES phase (Supplementary information, SI figure 3). The top organic phase was decanted and if necessary filtered - when lipase beads were found in the organic phase. The organic phase was washed 3-5 times with 5 ml saturated sodium bicarbonate solution ($NaHCO_3$) for complete removal of octanoic acid. The organic phase was then dried using anhydrous sodium sulphate (Na_2SO_4). The *n*-hexane was then removed using vacuum distillation, following which, the acid free epoxide was weighed and the isolated yield of the process was calculated.

4.8.2 Purification using water and ethyl acetate combination

Owing to the harmful nature of the *n*-hexane and in the interest of making the process greener, the following protocol was adapted. First, 10 mass equivalents of distilled water was added to the DES mixture. This mixture was then vortexed at maximum speed for 30 s to 1 min. To this mixture, 10 ml of ethyl acetate was added and vortexed for 30s to 1 min. This mixture was then transferred to a separating funnel and 20 ml of saturated sodium bicarbonate ($NaHCO_3$) solution was added to this mixture. The organic phase was retained while the aqueous phase was discarded. This was repeated till the octanoic acid was completely neutralized. The organic phase was then dried using anhydrous sodium sulphate (Na_2SO_4) as before. The excess ethyl acetate was then removed using vacuum distillation, the terpene epoxide (**1b-3b**) weighed and the isolated yield of the process calculated.

Acknowledgments

We gratefully acknowledge the financial support of “Bayerisches Staatsministerium für Wirtschaft und Medien, Energie und Technologie”. Additionally, we would like to thank Ms. Melanie Iwanow, Mr. Paul Stockmann and Dr. Luciana Vieira for the theoretical and practical inputs that were helpful in completing the paper in its present form.

View Article Online
DOI: 10.1039/C7GC01127J

References

1. R. A. Sheldon, I. W. C. E. Arends and U. Hanefeld, in *Green Chemistry and Catalysis*, Wiley-VCH Verlag GmbH & Co. KGaA, 2007, DOI: 10.1002/9783527611003.ch1, pp. 1-47.
2. R. A. Sheldon, *Green Chemistry*, 2016, **18**, 3180-3183.
3. F. Pena-Pereira, A. Kloskowski and J. Namiesnik, *Green Chemistry*, 2015, **17**, 3687-3705.
4. O. Hauenstein, M. Reiter, S. Agarwal, B. Rieger and A. Greiner, *Green Chemistry*, 2016, **18**, 760-770.
5. E. de Jong, M. A. Dam, L. Sipos and G. J. M. Gruter, in *Biobased Monomers, Polymers, and Materials*, American Chemical Society, 2012, vol. 1105, ch. 1, pp. 1-13.
6. K. Tanaka and F. Toda, *Chemical Reviews*, 2000, **100**, 1025-1074.
7. R. B. Nasir Baig and S. V. Rajender, in *An Introduction to Green Chemistry Methods*, Future Science Ltd, 2013, DOI: doi:10.4155/ebo.13.4, ch. 2, pp. 18-38.
8. H. M. Marvaniya, K. N. Modi and D. J. Sen, *International Journal of Drug Development and Research*, 2011, **3**, 42-51.
9. M. Poliakoff and P. Licence, *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, 2015, **373**.
10. E. J. Beckman, *The Journal of Supercritical Fluids*, 2004, **28**, 121-191.
11. T. Welton, *Chemical Reviews*, 1999, **99**, 2071-2084.
12. P. Wasserscheid and W. Keim, *Angewandte Chemie International Edition*, 2000, **39**, 3772-3789.
13. M. Petkovic, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, *Chemical Society Reviews*, 2011, **40**, 1383-1403.
14. A. A. Papadopoulou, E. Efstathiadou, M. Patila, A. C. Polydera and H. Stamatis, *Industrial & Engineering Chemistry Research*, 2016, **55**, 5145-5151.
15. C. Russ and B. Koenig, *Green Chemistry*, 2012, **14**, 2969-2982.
16. E. L. Smith, A. P. Abbott and K. S. Ryder, *Chem Rev*, 2014, **114**, 11060-11082.
17. C. A. Nkuku and R. J. LeSuer, *The Journal of Physical Chemistry B*, 2007, **111**, 13271-13277.
18. Q. Li, J. Jiang, G. Li, W. Zhao, X. Zhao and T. Mu, *Science China Chemistry*, 2016, **59**, 571-577.
19. D. A. Alonso, A. Baeza, R. Chinchilla, G. Guillena, I. M. Pastor and D. J. Ramón, *European Journal of Organic Chemistry*, 2016, **2016**, 612-632.
20. N. Guajardo, C. R. Müller, R. Schrebler, C. Carlesi and P. Domínguez de María, *ChemCatChem*, 2016, **8**, 1020-1027.
21. N. V. Guajardo, C. Carlesi, R. Schrebler and J. Morales, *ChemPlusChem*, 2016, DOI: 10.1002/cplu.201600594, n/a-n/a.

View Article Online
DOI: 10.1039/C7GC01127J

22. E. Gutiérrez-Arnillas, M. S. Álvarez, F. J. Deive, A. Rodríguez and M. Á. Sanromán, *Renewable Energy*, 2016, DOI: 10.1016/j.renene.2016.02.058.
23. S. Wang, X. Meng, H. Zhou, Y. Liu, F. Secundo and Y. Liu, *Catalysts*, 2016, **6**, 32.
24. S. H. Kim, S. Park, H. Yu, J. H. Kim, H. J. Kim, Y.-H. Yang, Y. H. Kim, K. J. Kim, E. Kan and S. H. Lee, *Journal of Molecular Catalysis B: Enzymatic*, 2016, **128**, 65-72.
25. D. González-Martínez, V. Gotor and V. Gotor-Fernández, *European Journal of Organic Chemistry*, 2016, **2016**, 1513-1519.
26. J. L. Bicas, A. P. Dionísio and G. M. Pastore, *Chemical Reviews*, 2009, **109**, 4518-4531.
27. W. Schwab, C. Fuchs and F.-C. Huang, *European Journal of Lipid Science and Technology*, 2013, **115**, 3-8.
28. J. L. F. Monteiro and C. O. Veloso, *Topics in Catalysis*, 2004, **27**, 169-180.
29. D. S. Seigler, in *Plant Secondary Metabolism*, Springer US, Boston, MA, 1998, DOI: 10.1007/978-1-4615-4913-0_18, pp. 312-323.
30. S. Kandi, V. Godishala, P. Rao and K. V. Ramana, *Biomedicine and Biotechnology*, 2015, **3**, 8-10.
31. R. Mewalal, D. K. Rai, D. Kainer, F. Chen, C. Külheim, G. F. Peter and G. A. Tuskan, *Trends in Biotechnology*, 2016, DOI: http://dx.doi.org/10.1016/j.tibtech.2016.08.003.
32. C. Lorenzo and A. Eugenio, *Recent Patents on Food, Nutrition & Agriculture*, 2011, **3**, 9-16.
33. G. Sienel, R. Rieth and K. T. Rowbottom, 2000, DOI: 10.1002/14356007.a09_531.
34. J. W. Brown, Q. T. Nguyen, T. Otto, N. N. Jarenwattananon, S. Glöggler and L.-S. Bouchard, *Catalysis Communications*, 2015, **59**, 50-54.
35. L. Vanoye, J. Wang, M. Pablos, C. de Bellefon and A. Favre-Reguillon, *Catalysis Science & Technology*, 2016, **6**, 4724-4732.
36. Z. Wang, in *Comprehensive Organic Name Reactions and Reagents*, John Wiley & Sons, Inc., 2010, DOI: 10.1002/9780470638859.conrr583.
37. Z. Wang, in *Comprehensive Organic Name Reactions and Reagents*, John Wiley & Sons, Inc., 2010, DOI: 10.1002/9780470638859.conrr343.
38. Z. Wang, in *Comprehensive Organic Name Reactions and Reagents*, John Wiley & Sons, Inc., 2010, DOI: 10.1002/9780470638859.conrr585.
39. Z. Wang, in *Comprehensive Organic Name Reactions and Reagents*, John Wiley & Sons, Inc., 2010, DOI: 10.1002/9780470638859.conrr513.
40. S. W. May, *Enzyme and Microbial Technology*, 1979, **1**, 15-22.
41. F. Björkling, H. Frykman, S. E. Godtfredsen and O. Kirk, *Tetrahedron*, 1992, **48**, 4587-4592.

View Article Online
DOI: 10.1039/C7GC01127J

42. S. Ranganathan, J. Tebbe, L. O. Wiemann and V. Sieber, *Process Biochemistry*, 2016, **51**, 1479-1485.
43. O. BAKHVALOV, V. FOMENKO and N. SALAKHUTDINOV, *Chemistry for Sustsainable Development*, 2008, **16**, 633-691.
44. S. Ranganathan, T. Gärtner, L. O. Wiemann and V. Sieber, *Journal of Molecular Catalysis B: Enzymatic*, 2015, **114**, 72-76.
45. X. Meng, K. Ballerat-Busserolles, P. Husson and J.-M. Andanson, *New Journal of Chemistry*, 2016, **40**, 4492-4499.
46. P. J. Rossky, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, **105**, 16825-16826.
47. D. Rengstl, V. Fischer and W. Kunz, *Physical Chemistry Chemical Physics*, 2014, **16**, 22815-22822.
48. R. N. Kacker, E. S. Lagergren and J. J. Filliben, *Journal of research of the National Institute of Standards and Technology*, 1991, **96**, 577-591.
49. J. Antony and F. J. Antony, *Work Study*, 2001, **50**, 141-149.
50. J. A. Ghani, I. A. Choudhury and H. H. Hassan, *Journal of Materials Processing Technology*, 2004, **145**, 84-92.
51. R. S. Rao, C. G. Kumar, R. S. Prakasham and P. J. Hobbs, *Biotechnology Journal*, 2008, **3**, 510-523.
52. S. P. Kondapalli, S. R. Chalamalasetti and N. R. Damera, *Application of Taguchi based Design of Experiments to Fusion Arc Weld Processes: A Review*, 2013.
53. H. Evangelaras, C. Koukouvinos and M. V. Koutras, in *Encyclopedia of Statistical Sciences*, John Wiley & Sons, Inc., 2004, DOI: 10.1002/0471667196.ess7146.
54. F. P. Byrne, S. Jin, G. Paggiola, T. H. M. Petchey, J. H. Clark, T. J. Farmer, A. J. Hunt, C. Robert McElroy and J. Sherwood, *Sustainable Chemical Processes*, 2016, **4**, 7.