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Enzymatic epoxidation of β-caryophyllene using free or immobilized lipases or mycelia from the Amazon region

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ABSTRACT

The chemo-enzymatic epoxidation of the terpene β-caryophyllene is reported herein. This compound can form two products, the mono-epoxide **2** and the di-epoxide **3**. Different experimental conditions, varying the source of the lipases (including mycelia from the Amazon region), the oxidizing agents (H_2O_2 aq. (AHP) or urea-hydrogen peroxide (UHP)) and the substituted acyl donors on the alkyl chain (bromide and alkyl), along with the influence of organic medium, were evaluated. Depending on the experimental conditions the formation of a single product could be obtained. CAL-B was the most efficient catalyst (conv. >99%). When using the commercial lipases product **2** was obtained in conversions of 16–27%, and using the native lipases **2** was obtained in conversions of 20–23%. With the use of mycelia UEA_06 and UEA_53 the conversions were 16 and 21%, respectively. When the 2-bromo alkylated and 2-ethylhexanoic acids were used as acyl donors only the mono-epoxide **2** was obtained in conversions of 14–54% (24 h). AHP was found to be a better oxidizing agent than UHP, a shorter time and lower amount being required to obtain **2** or **3** as the sole product in good conversions (60 up to >99%). The organic solvents were also selective. When using *n*-hexane the preferred formation of **2** was observed with >99% conversion, and when ethyl acetate or toluene were used the conversion to **3** was also >99% (in 8 and 24 h, respectively).

1. Introduction

The oxyfunctionalization of cyclic olefins is of industrial importance due to the possibility of transforming cheap and readily available substrates into valuable intermediates for fine chemical synthesis. Terpenes, which are widely distributed in nature, are of particular importance due to the fact that their oxidation products – terpenic aldehydes, alcohols and esters – are used as starting products for fragrances, flavors and therapeutic agents [1]. Epoxides can undergo various chemical reactions with a variety of nucleophiles and are readily converted into diols, amino-alcohols and ethers. An important recent application is the use of chiral epoxides as intermediates for the production of chiral pharmaceuticals [2].

Epoxides can be readily prepared from alkenes, through a number of methods including the use of *m*-chloroperoxybenzoic acid (MCPBA), transition metal catalysts and even cyclic seleninate esters [3]. Several methods for the oxidation of monoterpenes in the presence of various metals as catalysts or MCPBA have been reported. Nevertheless, most of these studies use bromide ions as promoters and/or acetic acid media resulting in complex mixtures of oxygenated derivatives with very low selectivity. In addition, the commercial production of MCPBA is under threat owing to its hazardous nature [4]. An alternative method for alkene epoxidation mediated by an enzyme (especially lipases), in the presence of a small amount of free fatty acids, was firstly described by Björkling in the early 1990s [5].

Lipases are the most widely used class of enzymes in biotechnology and they show varied stability in the presence of organic solvents, under extreme pH conditions and in ionic liquids (ILs). Apart from hydrolysis, lipases can catalyze esterification and transesterification reactions as well as the formation of peroxy-acids in low-water media [6,7].

The use of immobilized enzymes, particularly lipase originating from *Candida antarctica* B (CAL-B), in the chemo-enzymatic epoxidation of olefins, has been reported [8–12]. This lipase has been highlighted due to its excellent efficiency in catalyzing the perhydrolysis of octanoic acid. Skouridou et al. used this lipase to catalyze the formation of peroxy octanoic acid from

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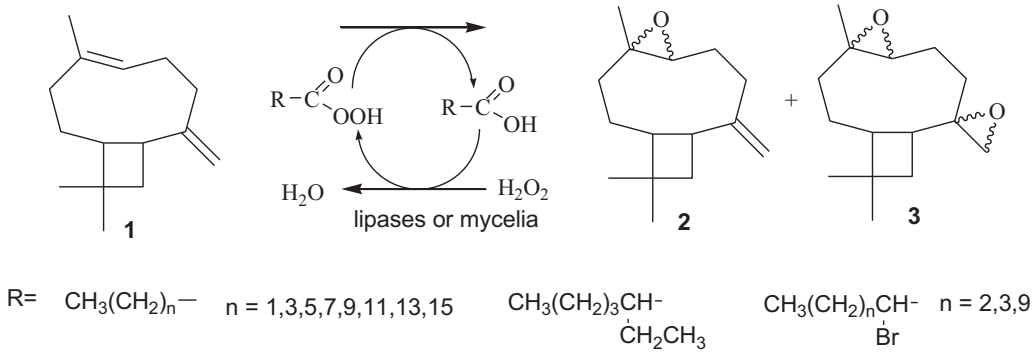

Scheme 1. The chemo-enzymatic epoxidation of β-caryophyllene (**1**) by lipases.

the corresponding carboxylic acid and aqueous hydrogen peroxide (AHP) in toluene. They varied the amount of AHP over five cycles of reaction. After five cycles the lipase activity decreased by 60–90%, depending on the amount of hydrogen peroxide used [11]. In addition to the use of aqueous hydrogen peroxide, a complex with urea, called urea hydrogen peroxide (UHP), has produced good results in the epoxidation of a range of alkenes [12].

The use of H_2O_2 (AHP or UHP) as an olefin oxidant often requires an efficient catalytic process in order to achieve high conversions and selectivity, because of the poorer reactivity of H_2O_2 compared to other classic epoxidizing reagents, such as organic peracids, peresters or persulfates [9].

The simplicity of the process and efficiency of the reaction temperature and pressure allow the oxidation of double bonds of compounds *in situ*. There is no need to isolate the peracid and the reuse of the biocatalyst has shown significant advantages for all process [8].

Silva et al. studied the chemo-enzymatic epoxidation of citronellol catalyzed by CAL-B. The results showed that the organic medium seemed to be one of the most important parameters in this reaction [13].

Besides affecting the enzyme activity [14], the solvent is important because it defines a major part of the environmental performance of the processes in the chemical industry and also impacts on cost, safety and health issues [15]. Laane et al. described that the epoxidation activity is low in relatively polar solvents, quite variable in solvents intermediate and high in apolar solvents [14].

The solvent effect was also described by Svedendahl et al. In this work, the direct epoxidation of but-2-enal and 3-phenylprop-2-enal in aqueous and organic solution catalyzed by CAL-B was realized, by activating both hydrogen peroxide and the α,β-unsaturated aldehyde, bringing them to a suitable position for reaction. Substrate binding and binding of the reaction intermediates were investigated both by molecular dynamics (MD) simulations and experimental kinetics. This reaction was fasted in aqueous solution [16].

The natural sesquiterpene β-caryophyllene (BCP) is an interesting substrate due to its chiral centers, double bonds and availability, since it is found in many essential oils of spices (*e.g.* cinnamon, origanum, black pepper, basil, and cloves) and in some food/medicinal plants [17–19]. In addition, BCP is often used in cosmetics, is an FDA-approved food additive and is a component of *Cannabis sativa* extract (Sativex), an approved drug in European countries and in Canada [20]. BCP presents well-established anti-inflammatory [17] and antioxidant effects [21,22] in addition to its antispasmodic [23], antiviral [24], and local anesthetic effects [25]. According to Bhatia et al., β-caryophyllene alcohol is the fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. Its use worldwide is in the region of <0.1 metric tons per annum [26]. Park et al. investigated whether β-caryophyllene oxide exerts its anti-cancer effects through the modulation of the PI3K/AKT/mTOR/S6K1 and MAPK signaling. They observed that CPO can significantly potentiate the apoptotic effects of various pharmacological PI3K/AKT inhibitors when employed in combination in tumor cells [27].

Herein, the study of some experimental parameters which affect the chemo-enzymatic epoxidation of β-caryophyllene (**1**) to form the mono- (**2**) and/or the di-epoxide (**3**) is reported. The influence of using different biocatalysts (free lipases and mycelium-bound lipases), the amount and type of oxidizing agent (UHP or AHP), the acyl donor, and the use of different organic solvents were evaluated in an attempt to optimize the process (Scheme 1).

2. Experimental data

2.1. Materials

The terpene (β-caryophyllene) (80%) was purchased from Sigma–Aldrich. The solvents acetonitrile (99.5%), chloroform (99.8%), ethanol (99.5%), cyclohexane (99%) and *t*-butanol (99%) as well as octanoic (99.5%), dodecanoic (98%) and hexadecanoic (C16) (98%) acids were acquired from Vetec. Dichloromethane (99.5%), methanol (99.5%), and *n*-hexane (98.5%) were obtained from Synth, *t*-butyl methyl ether (MTBE) (99.9%) was obtained from Tedia, ethyl ether (98%) was purchased from Chemis, ethylacetate (99.8%), *N*,*N*-dimethylformamide (DMF) (99%) and dimethylsulfoxide (DMSO) (99%) were obtained from Grupo Química and acetone was obtained from Carlo Herba. Decanoic (98%) and tetradecanoic acids (98%) were purchased from Fluka and 2-bromopentanoic (99%), 2-bromohexanoic (97%), 2-bromohexadecanoic (97%) and 2-ethylhexanoic (99%) acids were obtained from Sigma–Aldrich. Urea-hydrogen peroxide-UHP (percentage given as 30% in H_2O_2) was supplied by Sigma–Aldrich and aqueous hydrogen peroxide 30% H_2O_2 (percentage given as wt% H_2O_2 in water) was purchased from Vetec. Lipases originating from *Candida antarctica* B (CAL-B, Novozym 435, 10,000 PLU/g immobilized on a polyacrylate resin), *Rhizomucor miehei* (Lipozyme RM IM, 5-6 BAUN/g) and *Mucor miehei* (Lipozyme IM, 5-6 BAUN/g) were donated by Novozymes. Lipases originating from *Burkholderia cepacia* (PS-C Amano I, 1638 U/g; PS Amano SD, 30,000 U/g; PS Amano – 30,000 U/g; PS Amano IM, 500 U/g; PS-C Amano II, 1000 U/g), *Rhizopus oryzae* (F-AP15, 150 u/mg), *Candida rugosa* (AY Amano 30, 30,000 u/g), *Pseudomonas fluorescens* (AK, 25,000 U/g), *Aspergillus niger* (A Amano 12, 120,000 u/g) and *Mucor javanicus* (M Amano 10, 10,000 u/g) were donated by Amano Pharmaceuticals Co. The mycelium-bound lipases isolated from Amazonian plants (named

mycelia UEA_01, UEA_06, UEA_07, UEA_23, UEA_27, UEA_28, UEA_41, and UEA_53 UEA_115) were isolated, characterized and kindly provided by Dr. Sandra Patricia Zanotto of the State University of Amazonas (*Universidade do Estado do Amazonas*; UEA) (Manaus-AM, Brasil) [28]. Three native lipases originating from *A. niger* (LAN 18.2 U/mL), *Rhizopus oligosporus* (LRO 14.9 U/mL) and *Mucor hiemalis* (lipase 32, 26.4 U/mL) were isolated from soil micro-organisms in the region of Bueno Brandão (MG, Brazil), characterized and kindly donated by Dr. Patricia O. Carvalho (USF-Bragança Paulista, SP) [29,30].

2.2. General procedure for the chemo-enzymatic epoxidation of β-caryophyllene

In a typical chemo-enzymatic epoxidation reaction of β-caryophyllene, 0.6 mL (2.5 mmol) of the substrate, 1.2 mL of aqueous hydrogen peroxide (30%), 0.16 mL (1.0 mmol) of octanoic acid and 50 mg of lipase or 100 mg of mycelium, were added to 10 mL of dichloromethane (or other organic solvent when specified). The reaction was stirred at 150 rpm, at 25 °C, for 24 h. Reaction samples were periodically collected and analyzed by gas chromatography (GC) (Agilent Technology 7820 A) with flame ionization detection. The separation was performed using a Shimadzu CBP-5-M25-025 m column, with a column temperature program of 80–250 °C (10 °C/min). The injector and detector temperatures were set at 280 °C and 290 °C, respectively. The flow rate of the carrier hydrogen gas was of 7 mL/min, resulting in an analysis time of 15 min (**1** R_t = 6.5 min, **2** R_t = 8.5 min, **3** R_t = 11 min). To obtain the pure products, the reaction mixture was washed with saturated Na_2CO_3 and purified by column chromatography using a mixture of *n*-hexane:ethyl acetate (7:3, v/v) as the eluent. The products were fully characterized by ^{1}H- and ^{13}C NMR, and the spectroscopic data compared with those reported in literature [31].

Mono-epoxide **2**: (41%, 225 mg); m.p.:62–64 °C (Lit. [31] 62–63 °C) ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm) 4.97 (1H, s), 4.85 (1H), 2.91(1H, q, *J* 10.4 Hz,), 2.58 (1H, d, *J* 9,2 Hz), 2.36 (1H, m), 2.15 (1H, q, *J* 4,4 Hz), 2.11 (2H, m) 1.80–1.55 (5H, m), 1.34–1.29 (5H, m) 0.95 (6H, m). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 62.0, 59.0, 58.0, 56.0,49.0, 47.0, 39.0, 35.0, 33.0, 31.0, 30.0, 27.0, 25.0, 22.0, 16.0.

Di-epoxide **3**: (38%, 225 mg); m.p.: 73–75 °C (Lit. [31] 72–73 °C) ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.08, (1H, q, *J* 4.8 Hz,), 2.99 (1H, d, *J* 2.4 Hz), 2.89 (1H, q, *J* 4,8 Hz), 2.67 (2H, t, *J* 3,6 Hz), 2.57 (1H, d, *J* 5,2 Hz), 2.34 (1H, m), 2.22–2.06, (5H, m), 2.01 (1H, s), 1.81–1.41 (5H, m), 1.29 (3H, s), 0.94 (3H, s). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 63.0, 60.0, 50.0, 49.0, 40.0, 39.0, 34.0, 30.1, 29.8, 29.7, 27.2, 17.0.

3. Results and discussion

Some experimental parameters which may influence the chemo-enzymatic epoxidation of β-caryophyllene to form mono-**2** and/or di-epoxide **3** will be discussed below.

3.1. Screening of lipases and mycelia

In a first approach, thirteen commercially available lipases and three native lipases were used to obtain epoxides derived from β-caryophyllene using $H_2O_{2\ aq}$ (30%) as an oxidant agent in 24 h of reaction (Table 1). Using the commercial lipases PS-C Amano I, F-AP15, PS-C Amano SD, PS Amano, AY Amano 30, PS Amano IM, M Amano 10, AK Amano 20, PS-C Amano II, A Amano 12, Lipozyme RM IM, and Lipozyme IM (Table 1, entries 2–13) the mono-epoxide **2** was obtained in moderate conversions which ranged from 16 to 27%. Similar results were obtained with the use of non-commercial lipases (Table 1, entries 14,15), such as LAN (20%) and LRO (23%). It should be noted that these data are similar to those obtained with commercial lipases and di-epoxide **3** was not detected.

Table 1
Evaluation of different lipases in the chemo-enzymatic epoxidation of β-caryophyllene.[a]

Entry	Lipases	Activity	Conversion (%)[b]	
			2	3
1	CAL-B	10,000 PLU/g	0	>99
2	PS-C Amano I	1638 U/g	17	0
3	F-AP15	150 u/mg	16	0
4	PS-C Amano SD	23,000 U/g	16	0
5	PS Amano	30,000 U/g	16	0
6	AY Amano 30	30,000 u/g	23	0
7	PS Amano IM	500 U/g	17	0
8	M Amano 10	10,000 u/g	23	0
9	AK Amano 20	25,000 U/g	25	0
10	PS-C Amano II	1000 U/g	25	0
11	A Amano 12	120,000 u/g	27	0
12	Lipozyme RM IM	5–6 BAUN/g	24	0
13	Lipozyme IM	5–6 BAUN/g	26	0
14	LRO	14.9 U/mL	23	0
15	LAN	18.2 U/mL	20	0

[a] Reaction conditions: β-caryophyllene (2.5 mmol), 30% H_2O_2 (5.0 mmol), octanoic acid (1.0 mmol), lipase (50 mg), dichloromethane (10 mL), 150 rpm, r.t. (~25 °C).
[b] Determined by GC.

However, when CAL-B (Table 1, entry 1) was used as the biocatalyst, di-epoxide **3** was obtained as a single product with >99% conversion in 24 h of reaction.

The epoxidation of β-caryophyllene was then carried out using nine mycelium-bound lipases from the Amazon region. The results are presented in Table 2. In the reactions catalyzed by mycelia, the mono-epoxide **2** was obtained as a single product. After 24 h of reaction, the conversion degrees were 2–21%. In the case of the mycelia UEA_06 and UEA_53, the conversions were 16 and 21%, respectively. These results were also similar to those obtained with some commercial lipases, such as PS Amano (16%) and AY Amano 30 (23%). As observed in Table 2, the conversion degrees increased over time. For example, after 168 h of reaction, the highest conversions were achieved using the mycelium UEA_06 (64%), followed by UEA_23 (55%), UEA_01 (49%) and UEA_41 (48%).

These data show that both of the native lipases (LAN and LRO) and the mycelia have great potential for use in epoxidation reactions. This is the first report which uses these native lipases and mycelia in epoxidation reactions in the preparation of β-caryophyllene epoxides. Also, these results are of interest considering the huge diversity of microorganisms which have not yet been explored.

The endocyclic epoxidation of β-caryophyllene with *Nemania aenea*, *Diplodia gossypina* and *Chaetomium cochliodes* has been

Table 2
Conversions to mono-epoxide (**2**) obtained in the chemo-enzymatic epoxidation of β-caryophyllene using different mycelia.[a]

Entry	Mycelium	Conversion to **2** (%)[b]			
		24 h	72 h	120 h	168 h
1	UEA_01	14	38	43	49
2	UEA_06	16	49	59	64
3	UEA_07	13	38	37	40
4	UEA_23	8	47	49	55
5	UEA_27	2	2	8	9
6	UEA_28	11	42	40	45
7	UEA_41	10	44	47	48
8	UEA_53	21	34	41	41
9	UEA_115	15	29	40	46

[a] Reaction conditions: β-caryophyllene (2.5 mmol), 30% H_2O_2 (5.0 mmol), octanoic acid (1.0 mmol), mycelia (100 mg), dichloromethane (10 mL), 150 rpm, r.t. (~25 °C).
[b] Determined by GC.

reported, but showed very low productivities because of the strong antifungal activity of β-caryophyllene and β-caryophyllene epoxide. It is unlikely that antifungal agents will be produced with fungi because of the apparent strong product inhibition [32–34].

Considering the above results and in order to evaluate some other experimental parameters which may influence the formation of mono- or di-epoxide derived from β-caryophyllene, CAL-B was selected as the biocatalyst.

3.2. Influence of acyl donors

The stability of the enzyme may also be influenced by the size of the acyl donor [35]. In the next part of this study, the influence of the acyl donor chain length on the chemo-enzymatic epoxidation of **1** using CAL-B as the biocatalyst and AHP as an oxidant agent was investigated. As previously reported, the formation of peroxy-acid is an important step in this reaction [5,9].

In this study, the following alkyl acids were used as acyl donors: acetic (C2), butyric (C4), hexanoic (C6), octanoic (C8), decanoic (C10), dodecanoic (C12), tetradecanoic (C14) and hexadecanoic (C16). In 24 h of reaction only the di-epoxide **3** was formed in conversions of >99% using the various alkyl acids (C4-C16), except when acetic acid was the acyl donor where both the mono- (**2**) and di-epoxide (**3**) were detected, in conversions of 80% and 20%, respectively. It is well described in the literature that acetic acid is not the best acyl donor in enzymatic reactions because it may be aggressive toward the enzyme, forming strong bonds at the active site [7].

Another important factor to be considered is the steric effect of the acyl donor. Bulky chains, in general, might reduce the rate of nucleophilic attack by H_2O_2 which consequently reduces the reaction rate [36]. Thus, in order to expand the studies related to the influence of acyl donors, 2-ethylhexanoic, 2-bromo-valeric, 2-bromo-hexanoic and 2-bromo-hexadecanoic acids were used to evaluate both steric and electronic effects on the epoxidation of β-caryophyllene (**1**).

With the use of 2-ethyl-hexanoic acid only the product **2** was obtained, in 14% conversion in 24 h reaction. This result reinforces the fact that the formation of peroxy-acid is an important step in the formation of the corresponding epoxide. In this case, steric hindrance was most likely the parameter which influenced the low formation of **2**. This data is also consistent with those presented by Hollmann et al., who evaluated the CAL-B activity in esterification reactions with methyl valeric acid at positions α-, β-, γ-with octan-1-ol. The enzyme activity showed a great dependence on the position of the methyl group. When α- and β-substituted acids were used, the product conversions were low (<25%), and it was postulated that the substituents in these positions hinder the proper connection of the substrate at the active site of the enzyme [37].

When 2-bromo-valeric, 2-bromo-hexanoic and 2-bromo-hexadecanoic acids were used, only mono-epoxide **2** was formed, in conversions of 47, 54 and 36%, respectively, in 24 h of reaction. These results probably reflect both the electron withdrawing effect of the halogen atom close to the carboxyl group and the steric effect of the alkyl chain. Recently, it was described that the CAL-B activity may be strongly influenced by the presence of strong acids. In general, acids with pK_a values lower than 4.8 decreased the lipase activity. These acids can protonate the aspartate or glutamate at the active site of CAL-B. If one of these residues is protonated, they cannot act effectively in the catalytic triad of CAL-B to activate Ser105 (*via* His224) for the nucleophile attack and thus the lipase activity is decreased in the presence of strong acids. This also explains the lower conversion (80%) when acetic acid (pK_a 4.75) was used as the acyl donor rather than octanoic acid (99%), in 24 h of reaction [37].

Table 3
Influence of time on the chemo-enzymatic epoxidation β-caryophyllene (**1**).

Entry	Time (h)	AHP		UHP	
		2	**3**	**2**	**3**
1	2	60	nd	16	nd
2	4	32	31	50	nd
3	6	34	58	60	nd
4	8	13	83	82	8
5	24	nd	99	14	86

Reaction conditions: β-caryophyllene (2.5 mmol), UHP or AHP (5.0 mmol), octanoic acid (1.0 mmol), CAL-B (50 mg), dichloromethane (10 mL), r.t., nd = not detected.

Thus, considering the above results, octanoic acid was selected as the acyl donor for use in the subsequent experiments, which included the influence of the type and amount of oxidizing agent and organic solvent. The use of octanoic acid as an acyl donor has been previously described in the literature. [8,11,13]

3.3. The influence of the type and amount of oxidizing agent

The type and concentration of peroxide donor used in chemo-enzymatic epoxidation are important factors. A high concentration of peroxide donor can cause inhibition of the enzyme by changing its tertiary and/or quaternary structure. In addition, an excess of peroxides can cause changes in the substrate reactivity through direct oxidation and the peroxide, depending on its strength (expressed as the % of free oxygen), can also change the course of the enzymatic reactions through the excessive production of peroxy-acids [12,36].

Thus, the chemo-enzymatic formation of **2** and **3** using two different peroxide donors, AHP (aqueous hydrogen peroxide – 30%) and UHP (urea hydrogen peroxide – 30%), was investigated as a function of the reaction time. Aliquots were withdrawn at 2, 4, 6, 8 and 24 h and analyzed by GC. The results are presented in Table 3.

With the use of AHP or UPH, mono-epoxide **2** was obtained as a single product in conversions of 60 and 16%, respectively, in 2 h of reaction (Table 3, entry 1). Also, in the case of UPH, only mono-epoxide **2** was obtained in 4 and 6 h of reaction forming the product in conversions of 50 and 60%, respectively (Table 3, entries 2 and 3). However, after 4 and 6 h using APH as the oxidizing agent, both products **2** (32 and 31%, respectively) and **3** (31 and 58%, respectively) were formed (see also Table 3, entries 2 and 3).

In relation to the results obtained after 8 h of reaction, the conversions into mono-epoxide **2** were 13% and 82% and di-epoxide **3** were 83% and 8% for AHP and UHP, respectively (Table 3, entry 4). However, in 24 h of reaction, **3** was the main product, which was formed in conversions of 99% and 86%, respectively (Table 3, entry 5).

The above results show that the formation of **2** and **3** was time dependent. The data also revealed that, in general, the formation of mono-epoxide **2** was favored in the presence of UHP, since this reagent releases the oxidant agent in a more controlled manner [12]. On using AHP, the formation of di-epoxide **3** was favored. As recently described, the H_2O_2 concentration used determines the rate and amount of peracid formed, which, in turn, influences the efficiency of the chemo-enzymatic reaction [36].

In the next part of this study, the influence of each oxidant amount was evaluated in the epoxidation of β-caryophyllene. Fig. 1 presents the conversions into products **2** and **3** as a function of UPH (in mmol).

It was observed that with an increase in the amount of UHP from 1 to 3 mmol the conversion to mono-epoxide also increased, from 20 to 96%, respectively. For this amount of UHP the two products were detected, and from 5.0 to 16 mmol of oxidizing agent the

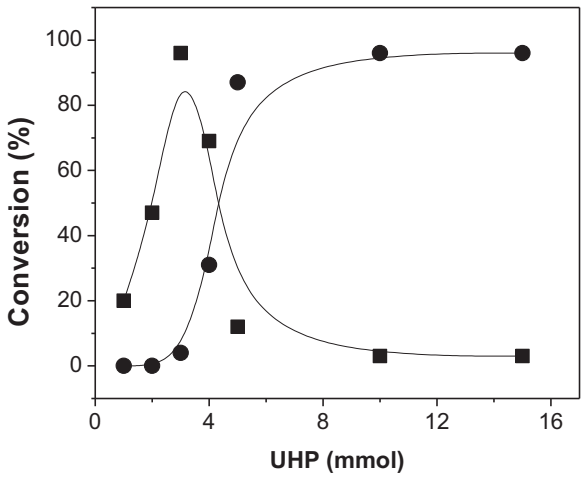

Fig. 1. Influence of the amount of UHP on the chemo-enzymatic epoxidation of β-caryophyllene to form the mono-epoxide 2 (■) and di-epoxide 3 (●). Reaction conditions: β-caryophyllene 2.5 mmol, UHP 1.0–15.0 mmol, octanoic acid 1.0 mmol, CAL-B 50 mg, dichloromethane 10 mL, r.t. 24 h.

di-epoxide **3** was obtained as the main product in conversions of 87–96%.

The results obtained using AHP as the peroxide donor are presented in Fig. 2.

As can be observed, with 2.0 mmol of AHP the mono-epoxide was obtained with a conversion degree of 93%. With the use of larger amounts of AHP, ranging from 2.5 to 15 mmol, both products were formed. The mono-epoxide **2** was obtained in 81–26% and the di-epoxide **3** in 19–74% conversions. In the case of 4.0 mmol of AHP the di-epoxide was obtained as the main product, in conversion degrees of 94 to 99%. These results were to be expected considering that AHP is considered to be a more energetic oxidant agent than UHP.

The results show that the conversion into **2** and/or **3** was dependent on the amount and type of peroxide donor employed. In both cases, in reactions in which the amount of peroxide was < 2.5 mmol, mono-epoxide was the main product. With the use of between 3–4 mmol of AHP and UHP the regioselectivity decreased and a mixture of products was detected (Figs. 1 and 2). As previously noted, on using more than 5 mmol of peroxide the formation

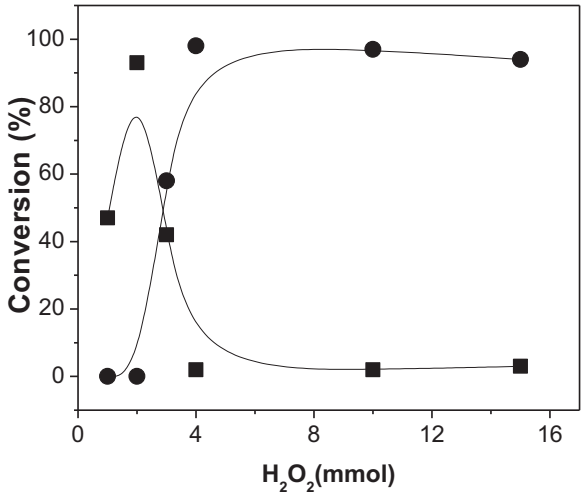

Fig. 2. Effect of the amount of H_2O_2 (30%) in the chemo-enzymatic epoxidation of β-caryophyllene to form the mono-expoxide 2 (■) and di-epoxide 3 (●). Reaction conditions: as in Fig. 1, except for AHP (1.0–15.0 mmol).

Table 4
Effect of organic solvent on the chemo-enzymatic epoxidation of β-caryophyllene.[a]

Entry	Solvent (Log *P*)[c]	Conversion (%)[b]			
		8 h		24 h	
		2	**3**	**2**	**3**
1	*n*-Hexane (3.50)	>99	0	>99	0
2	Cyclohexane (3.20)	>99	0	76	24
3	Toluene (2.50)	0	>99	0	>99
4	Chloroform (2.00)	62	38	0	>99
5	*t*-Butanol (1.45)	59	0	64	0
6	MTBE (1.43)	>99	0	93	7
7	Dichloromethane (0.93)	19	81	0	>99
8	Ethyl ether (0.85)	99	0	90	10
9	Ethyl acetate (0.68)	0	>99	0	>99
10	THF (0.49)	82	0	>99	0
11	Acetone (−0.23)	>99	0	92	8
12	Ethanol (−0.24)	98	0	>99	0
13	Acetonitrile (−0.33)	>99	0	29	71
14	Methanol (−0.76)	0	0	0	0
15	DMF (−1.00)	7	0	22	0
16	DMSO (−1.30)	0	0	0	0

[a] Reaction conditions: substrate 2.5 mmol, H_2O_2 5 mmol, octanoic acid 1 mmol, CAL-B 50 mg, solvent 10 mL, 150 rpm, 8 and 24 h, r.t. (~25 °C).
[b] Conversions were determined by GC.
[c] Ref. [14].

of the corresponding peroxy-acid was also favored and thus the formation of **3** increased (87–99%), in 24 h of reaction.

Thus, the choice of peroxide type and amount proved to be important parameters in this reaction, in which products **2** or **3** can be selectively obtained in good conversion degrees.

3.4. Effects of the organic medium

The influence of the organic medium was then evaluated. It is well established that enzyme activity is strongly affected by the choice of organic solvent [13,14,16]. Log *P* (logarithm of the partition coefficient of the solvent for the standard octanol/water two-phase system) is the most useful parameter to classify the biocatalytic reaction of a solvent [14].

Thus, in order to evaluate this effect in the epoxidation of β-caryophyllene, sixteen solvents with different polarities were selected and the conversions to epoxides **2** and **3** in 8 and 24 h are presented in Table 4.

When dichloromethane (log *P* = 0.93) and chloroform (log *P* = 2.00) were used both products were obtained, with conversions of 19–62% for **2** and 38–81% for di-epoxide **3**, in 8 h of reaction. With the use of *n*-hexane (log *P* = 3.50), cyclohexane (log *P* = 3.20), *t*-butanol (log *P* = 1.45), MTBE (log *P* = 1.43), ethyl ether (log *P* = 0.85), THF (log *P* = 0.49), acetone (log *P* = −0.23), ethanol (log *P* = −0.24), acetonitrile (log *P* = −0.33) and DMF (log *P* = −1.00), the mono-epoxide **2** was obtained as a single product. With the use of *t*-butanol, THF, DMF and ethanol, the conversions were of 59, 82, 98, and 7%, respectively (Table 4, entries 5, 10, 12 and 15). On using the other solvents, the conversions were all >99%. It is interesting to note that with the use of both toluene and ethyl acetate, the di-epoxide **3** was obtained as a single product with conversions of >99% (Table 4, entries 3 and 9).

Within 24 h of reaction using toluene, chloroform, dichloromethane or ethyl acetate the di-epoxide **3** was obtained in >99% conversion (Table 4, entries 3, 4, 7 and 9). In the same reaction time and using *n*-hexane, THF or ethanol only mono-epoxide **2** was formed with conversions of >99% (Table 4, entries 1, 10 and 12).

When the epoxidation reaction was carried out in the presence of cyclohexane, MTBE, diethyl ether, acetone or acetonitrile, both products were obtained but, in most cases, with the predominant

formation of mono-epoxide **2** (Table 4, entries 2, 6, 8, 11 and 13). Interestingly, with the use of acetonitrile the main product was the di-epoxide **3** with 71% conversion.

When a more polar solvent, such as DMF, was used the degrees of conversion into **2** were 7 and 22% at 8 and 24 h, respectively (Table 4, entry 15). In the presence of methanol and DMSO no product was detected (Table 3, entries 14 and 16).

These interesting results indicate that the appropriate choice of organic solvent and reaction time may promote the selective formation of only one product. However, no direct relationship with the log *P* values was detected. In other studies reported in the literature the solvent was found to affect the formation of products in enzyme-catalyzed reactions, but with no direct correlation to log *P* [38–40].

In addition to the polarity of the organic solvents, problems related to the reagents and or product solubility must be considered and obtaining an ideal combination of these parameters can be a difficult task. For example, as presented in Table 4, good results were also achieved when polar solvents such as MTBE, dichloromethane, acetone, ethyl acetate, ethanol and acetonitrile were used. Using these solvents it is expected a higher solubility of the oxidizing agent (PHA), thus a higher production of the octanoic peroxy acid and consequently an increase in the production of corresponding epoxides **2** and **3**.

However, besides the selectivity, the solvent must be as "green" as possible. The idea of "green" solvents expresses the goal to minimize the environmental impact resulting from the use of solvents in chemical production [15].

In this regard, according to Hellweg et al., dichloromethane and chloroform cannot be considered as good solvents. The use of chlorinated solvents should be avoided because, along with nitrobenzene, they have the potential for long-term and widespread exposure [41]. In this study, with the use of the above cited solvents product **3** was obtained with a conversion of >99%.

As reported, to obtain mono-epoxide **2**, hexane was the most appropriate solvent, the product being formed in a conversion of >99% in 8 h. Hexane can be considered as an environmentally favorable solvent. To produce only the di-epoxide **3** in conversion degrees of >99%, toluene can be selected, also considering the assessment of environmental parameters [15,41,42].

Finally, the results of this study demonstrate that the organic medium is an important parameter in chemo-epoxidation reactions catalyzed by lipases. Bearing in mind the goal of minimizing the environmental impact, depending on the medium, only one product (**2** or **3**) could be obtained with high selectivity and conversion degrees.

4. Conclusions

The chemo-enzymatic epoxidation of β-caryophyllene was carried out under mild conditions. CAL-B was the most effective catalyst and this reaction was dependent on the oxidant agent and acyl donor, products **2** and/or **3** being formed in excellent conversions (>99%). In relation to obtaining mono-epoxide **2** as a single product, the organic solvent was the most important parameter. Using *n*-hexane, cyclohexane, MTBE, acetone or acetonitrile, **2** was obtained in conversions of >99% in 8 h of reaction. The di-epoxide **3** was obtained in conversions of >99% using 4 mmol of AHP in 24 h of reaction. Using carboxylic acids with linear alkyl chains (C4–C16), and toluene, chloroform, dichloromethane or ethyl acetate as the organic solvent, **3** was also obtained in conversions of >99%. Thus, from these results, it was concluded that chemo-enzymatic epoxidation reactions are strongly influenced by several experimental parameters, but especially the oxidizing agent, acyl donor and organic medium. Depending on the experimental conditions, only one epoxide (**2** or **3**) was obtained, this being an advantage associated with the use of biocatalysts in these reactions.

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