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# Size selectivity in lipase catalysed tetrol acylation

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ABSTRACT

Size selectivity of *Candida antarctica* lipase B (CAL-B) was examined in the acylation of pentaerythritol with oleic acid. Biolubricant mixtures consisting of mono-, di-, tri-, and tetraoleates were expected in variable excess. Enzymatic tetraoleate formation was suppressed under solvent conditions; however, this size selectivity was lost without solvent and tetra-acylated pentaerythritol accumulated in up to 93%. The lipase caused size selectivity persisted over a broad temperature range from 35 to 95 °C. A Fischer–Speier esterification showed that substrate bulkiness was only a minor contributor to observed size selectivity. All in all, switch on/off size selectivity using CAL-B allowed to vary pentaerythritol biolubricant compositions in an unprecedented manner.



## 1. Introduction

Lipase catalysed acylation of symmetric pentaerythritol with fatty acids potentially yields mono-, di-, and triacylated esters, and eventually the less obvious tetraester. Native lipases catalyse mono-, di-, and triglycerolester hydrolysis and by inverse hydrolysis its formation; a fourth acylation would lead to an unusual large fat molecule. Such tetraester formation is possible with achiral pentaerythritol, which is a tetrol and acylated once more than the triol glycerol. The lipase mediated synthesis of pentaerythritol tetraesters is therefore a good platform to test lipase controlled size selectivity. Pentaerythritol tetraester hydrolysis was examined before with rat pancreatic lipase and no hydrolysis occurred [1]. Every lipase is different and in this work, *Candida antarctica* lipase B is used for its potential in size selective catalysis. The entry channel with native CAL-B is rather narrow, 10 Å × 4 Å wide and 12 Å deep [2]. In view of this small entry to the catalytic site it is well possible that larger pentaerythritol tetraolates cannot be synthesised. This assumption is in line with observations that short chain fatty acids are esterified faster than longer ones [3]. The hydrolysis rate of common triglycerides was even further reduced by engineering a more restricted entry channel which enhanced size selectivity [4].

Size selectivity is a phenomenon with consequences for stereo and regio selectivity. For example the asymmetrisation of cyclic meso-1,2-dicarboxylates using porcine liver esterase showed exceptional size selectivity effects [5]. The size of the meso ring influenced R/S meso-trick hydrolysis selectivity as it was reversed from S to R with increasing ring size. Another example is lactone hydrolysis by CAL-B where increasing lactone ring sizes reversed R and S selectivity in ring opening polymerisation [6]. Also other polycondensations showed size selectivity using lipases [7]. Size selectivity is also reported for proteases. Serine prolyl oligopeptidase hydrolysed peptide chains after proline in terminal protein sections with up 30 amino acids [8].

Lipase catalysed esterifications in organic solvents is widely described [9]. Hydrophobic organic solvents with a log *P* around 2 support well water free (0.01% $H_2O$ content) lipase catalysis [10,11]. Solvent-free lipase catalysis is another possibility and an attractive option for industrial application. CAL-B showed before size selectivity with trimethylolpropane, which is in this work further explored [12].

To examine size selectivity effects in pentaerythritol oleate synthesis a fast and reliable analytical method was required. Gas chromatography (GC) and high pressure liquid chromatography (HPLC) are not well suited as oleic acid mixtures contain also other fatty acids (>10%). This multiplies product peaks with each additional acylation on a tetrol. Any chromatographic method is therefore of limited use. Pentaerythritol tetraesters are widely described but it is not excluded that in many instances they

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were less pure than thought due to the absence of a simple analytical method to assay its esterification degree using bio and chemical catalysis. Recently, an alternative convenient $^1$H NMR method was developed and proven successful in the analysis of trimethylolpropan oleate biolubricant mixtures [12]. A 400 MHz NMR spectrometer in combination with THF-$d_8$ separated the three possible methylene singlets H-C(2) of the three acylation degrees and allowed product distribution analyses. Such chemical shift separation was not observed with other deuterated solvents such as chloroform ($CDCl_3$) [13] or methanol ($CD_3OD$). It was hypothesised that the four possible pentaerythritol oleates can equally be identified using this novel and convenient $^1$H NMR method.

Pentaerythritol esters are high performance lubricants in aircraft engines [14] and various industrial machinery, but are also used as consistency wax in cosmetics [15]. It is also used, for example, as lubricant in new green refrigeration systems because it dissolves carbon dioxide that is nowadays used instead of halogen alkanes [16].

In the following pentaerythritol is acylated with oleic acid by CAL-B catalysis (Fig. 1). Size selectivity and product distribution will be influenced by various process parameters, such as presence and absence of solvent, stoichiometry, and temperature. A Fischer–Speier esterification shall distinguish substrate bulkiness from size selectivity by *C. antarctica* lipase B.

## 2. Experimental

### 2.1. Material

*C. antarctica* Lipase B, Novozym® 435 (10,000 PLU/g) was obtained from Novozymes A/S [Bagsværd, Denmark]. 3 Å Molecular sieve from Merck KGaA, [Darmstadt, Germany] was activated before use. Oleic acid (90%) and pentaerythritol were purchased at Sigma-Aldrich Chemie [Taufkirchen, Germany]. Tert-butanol (99%) was from Fluka Analytical [Buchs, Switzerland], and THF-$d_8$ from Armar AG [Döttingen, Switzerland].

### 2.2. General procedure for lipase catalysis in tert-butanol

0.27 g (2 mmol) pentaerythritol and 0.58 g (2 mmol) oleic acid were added (1:1 stoichiometry) to a 25 mL vial. Also other oleic acid quantities were used: 1.15 g (4 mmol), 1.70 g (6 mmol), and 2.26 g (8 mmol). 0.14 g Novozym® 435 (CAL-B) (50% weight of pentaerythritol) was employed with all substrate ratios. 0.8 g molecular sieve 3 Å was added to adsorb condensing water; and finally, 2 mL tert-butanol was filled in. The sealed vial was fixed vertically on a rotating cylinder (8 rpm) in a hybridisation oven and processed at 65 °C for up to 15 days. For reaction control an aliquot of reaction mixture was taken, tert-butanol evaporated, and a $^1$H NMR/THF-$d_8$ recorded to determine conversion, yield and product (**1**–**4**) distribution.

### 2.3. Solvent screening for optimal lipase catalysis

A 1:4 (pentaerythritol/oleic acid) substrate mixture was screened using various organic solvents (2 mL) according to the general method. After processing the immobilised lipase and molecular sieves were filtered off and the solvent evaporated with a Rotavap at 25 mbar or on a high vacuum stand. An aliquot of the raw reaction mixture was analysed by $^1$H NMR.

### 2.4. Solvent-free lipase catalysis

The same preparation and process conditions, without solvent, according to the general procedure.

### 2.5. Sulphuric acid catalysis

A series of 25 mL vials were filled with substrates as in the general procedure but without solvent. 5% concentrated sulphuric acid (96%) was added (based on pentaerythritol weight). The vials were sealed and fixed on a rotating (8 rpm) cylinder in a 100 °C preheated hybridisation oven. At defined times vials were removed and aliquots of reaction mixture were analysed by $^1$H NMR/THF-$d_8$.

### 2.6. Conversion, yield and product distribution by $^1$H NMR

A single $^1$H NMR experiment provided complete reaction progress information. The solvent of any reaction mixture was evaporated and aliquots thereof dissolved in 0.5 mL THF-$d_8$ and a $^1$H NMR recorded on a 400 MHz NMR spectrometer (Bruker Avance). This NMR-solvent separated the chemical shifts for all four possible products **1**–**4** and also oleic acid conversion was assayed at the same time. The pentaerythritol-oleates **1**–**4** distribution was determined by integrating H-C(2) singlets found at: 4.05 ppm (**1**), 4.06 (**2**) 4.08 ppm (**3**), and 4.11 ppm (**4**). Their integrams were divided by related acylation degrees (1, 2, 3, 4) and the results were normalized to 100%. The oleic acid conversion was quantified by the H-C(2′) triplet that shifted from 2.19 to 2.29 ppm upon esterification.

### 2.7. Analytical and preparative chromatography

High pressure liquid chromatography (HPLC) was realised with an Agilent PN 993967-906 and Eclipse XDB-C8 column, 4.6 × 150 mm, 5 μm; and a Charged Aerosol Detector, CAD-Ultra ESA Dionex. Fractions from selected peaks were collected and eluted a second time. Isolated compounds were then analysed by $^1$H NMR for purity. Also preparative product isolation using silica gel normal pressure chromatography with EtOAc–heptane (1:2) was realised [12]. The separation quality was controlled by thin layer chromatography (TLC) and two separation runs were required.

### 2.8. Analytical data

#### 2.8.1. Pentaerythritol monooleate (*1*)

$_\delta$H (400 MHz, THF-$d_8$): 5.33 (m, 2H, *HC*=*CH*, H-9′, H-10′); 4.05 (s, 2H, OC$H_2$, $H_{b1}$-2); 3.61 (t, 3H, O*H*, H-2); 3.51 (d, 6H, OC$H_2$, $H_a$-2); 2.27 (t, 2H, COC$H_2$, H-2′); 2.04 (m, 4H, C$H_2$. H-8′, H-11′); 1.57 (t, 2H, C$H_2$, H-3′); 1.32–1.29 (bm, 20H,C$H_2$, H-4′-7′, H-12′-17′), 0.89 (t, 3H, C$H_3$, H-18′).

$_\delta$C (100 MHz, THF-$d_8$): 173.23 (*C*=O, C-1′); 130.38 (*C*=*C*, C-9′, C-10′); 63.34 (C(O)OC$H_2$, C-2); 61.82 (HOC$H_2$, C-2); 45.15 (*C*(C$H_2$)$_4$, C-1); 34.49 (C(O)C$H_2$, C-2′); 32.75, 30.60, 30.57, 30.37, 30.16, 30.13, 30.09, 29.99, 29.96, C$H_2$, C-4′-7′, C-12′-16′; 27.9 (C$H_2$, C-8′,C-11′); 23.44 (C$H_2$, C-3′,C-17′); 14.32 (C$H_3$, C-18′).

#### 2.8.2. Pentaerythritol dioleate (*2*)

$_\delta$H (400 MHz, THF-$d_8$): 5.32 (m, 4H, *HC*=*CH*, H-9′, H-10′); 4.06 (s, 2H, OC$H_2$, $H_{b2}$-2); 3.53 (m, 4H, OC$H_2$, $H_a$-2); 2.28 (t, 4H, COC$H_2$, H-2′); 2.03 (m, 8H, C$H_2$, H-8′, H-11′); 1.57 (t, 4H, C$H_2$, H-3′); 1.33–1.30 (bm, 40H, C$H_2$, H-4′-7′, H-12′-17′); 0.89 (t, 6H, C$H_3$, H-18′).

$_\delta$C (100 MHz, THF-$d_8$): 173.23 (*C*=O, C-1′); 130.38 (*C*=*C*, C-9′, C-10′); 63.34 (C(O)OC$H_2$, C-2); 61.82 (HOC$H_2$, C-2); 45.15 (*C*(C$H_2$)$_4$, C-1); 34.49 (C(O)C$H_2$, C-2′); 32.75, 30.6, 30.57, 30.37, 30.16, 30.13, 30.09, 29.99, 29.96 (C$H_2$, C-4′-7′, C-12′-16′); 27.9 (C$H_2$, C-8′,C-11′); 23.44 (C$H_2$, C-3′,C-17′); 14.32 (C$H_3$, C-18′).

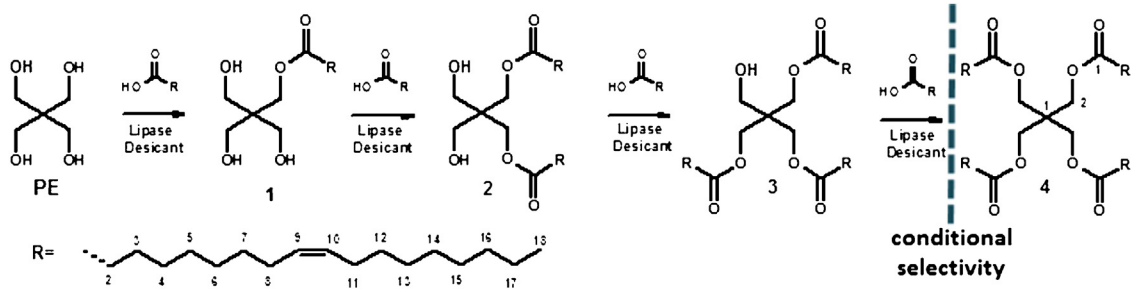


**Fig. 1.** Lipase mediated acylation of pentaerythritol (PE) with oleic acid. Using tert-butanol as solvent intermediate **3** accumulated and **4** was hardly formed. Size selectivity was lost under solvent-free conditions and tetraoleate **4** formed in excess.

*2.8.3. Pentaerythritol trioleate (**3**)*

$_{\delta}$H (400 MHz, THF-$d_8$): 5.33 (t, 6H, *HC*=*CH*, H-9′, H-10′); 4.08 (s, 6H, O*CH*$_2$, H$_{b3}$-2); 3.53 (m, 2H, O*CH*$_2$, H$_a$-2); 2.28 (t, 2H, CO*CH*$_2$, H-2′); 2.04 (s, 12H, *CH*$_2$. H-8′, H-11′); 1.57 (m, 6H, *CH*$_2$, H-3′); 1.32–1.29 (bm, 60H, *CH*$_2$, H-4′-7′, H-12′-17′); 0.89 (t, 6H, *CH*$_3$, H-18′).

$_{\delta}$C (100 MHz, THF-$d_8$): 172.98 (*C*=O, C-1′); 130.38 (*C*=*C*, C-9′, C-10′); 62.97 (C(O)O*CH*$_2$, C-2); 61.01 (HO*CH*$_2$, C-2); 44.2 (*C*(CH$_2$)$_4$, C-1); 34.41 (C(O)*CH*$_2$, C-2′); 32.75, 30.6, 30.58, 30.55, 30.16, 30.13, 30.09, 29.99, 29.96 (*CH*$_2$, C-4′-7′, C-12′-16′); 27.91 (*CH*$_2$, C-8′,C-11′); 23.44 (*CH*$_2$, C-3′, C-17′); 14.32 (*CH*$_3$, C-18′).

*2.8.4. Pentaerythritol tetraoleate (**4**)*

$_{\delta}$H (400 MHz, THF-$d_8$): 5.33 (m, 8H, *HC*=*CH*, H-9′, H-10′); 4.11 (s, 8H, O*CH*$_2$, H$_{b4}$-2); 2.29 (t, 8H, CO*CH*$_2$, H-2′); 2.04 (m, 16H, *CH*$_2$. H-8′,

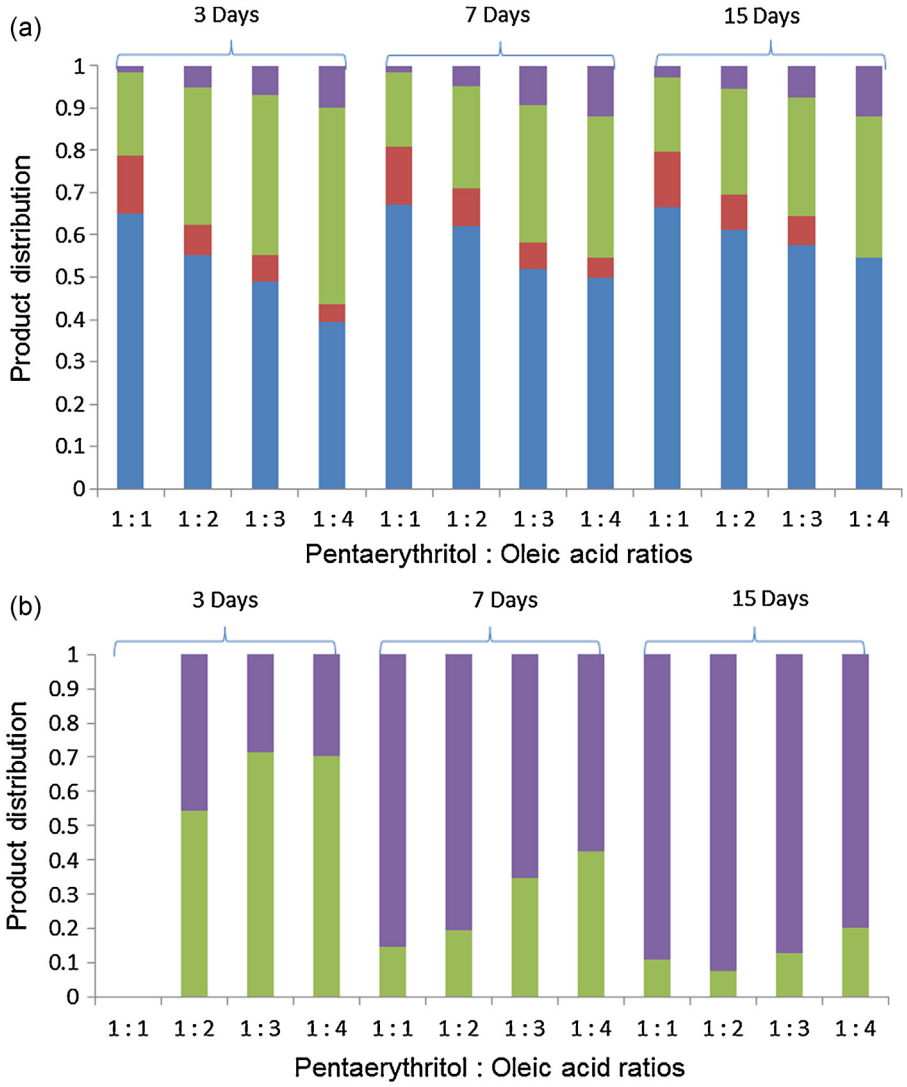


**Fig. 2.** (A) Lipase mediated catalysis of pentaerythritol with various oleic acid ratios in tert-butanol. Monooleate **1** (blue) in high proportion, dioleate **2** (red) transformed swiftly into trioleate **3** (green), while tetraoleate **4** (violet) formation was limited due to size selectivity. (B) Size selectivity (A) was lost with solvent-free conditions. A binary product mixture of tri- and tetraoleates **3**–**4** resulted and tetraoleate **4** (violet) accumulated over time up to 93% (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

**Table 1**
Oleic acid conversion in enzymatic acylation of pentaerythritol using CAL-B to screen for a suitable organic solvent.

| Solvent | Oleic acid conversion [%] |
|---|---|
| Pentane | 41.7 |
| Toluene | 40.8 |
| tert-Butanol | 39.5 |
| Dioxane | 33.8 |
| Acetone | 30.9 |
| Hexane | 21.9 |
| THF | 21.0 |
| Cyclohexane | 17.9 |
| Diphenylether | 15.9 |

H-11′); 1.59 (m, 8H, C$H_2$, H-3′); 1.32-1.29 (bm, 80H, C$H_2$, H-4′-7′, H-12′-17′); 0.89 (t, 12H, C$H_3$, H-18′).

$_{\delta}$C (100 MHz, THF-$d_8$): 172.98 (*C*=O, C-1′); 130.38 (*C*=*C*, C-9′-10′); 62.97 (C(O)O*C*$H_2$, C-2); 44.2 (*C*($CH_2$)$_4$, C-1); 34.41 (C(O)*C*$H_2$, C-2′); 32.75, 30.6, 30.58, 30.55, 30.16, 30.13, 30.09, 29.99, 29.96 (*C*$H_2$, C-4′-7′, C-12′-16′); 27.91 (*C*$H_2$, C-8′, C-11′); 23.44 (*C*$H_2$, C-3′,C-17′); 14.32 (*C*$H_3$, C-18′).

## 3. Results and discussion

### 3.1. Lipase catalysed tetrol acylation in tert-butanol

Size selectivity induced pentaerythritol trioleate **3** accumulation was observed and at any time no tetraoleate **4** was formed in the first 24 hours (Fig. 2A). A solvent screening was realised to ensure optimal substrate solubility, pentane, toluene and tert-butanol were well suited for this biotransformation (Table 1) [17]. Tert-butanol was chosen for all experiments as the most convenient and greenest solvent of the available options. The biotransformation of oleic acid (1:1, n:n) with pentaerythritol was quantitative (98.5%) at 65 °C/three days. The same lipase quantity was also used with increasing oleic acid ratios (Fig. 2A) and conversions decreased slowly to 71%, which is close to expected 75% using a 1:4 ratio. If

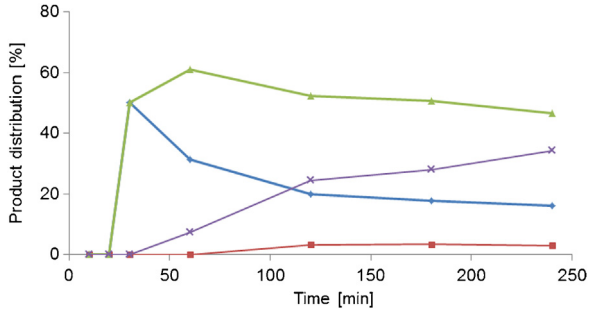


**Fig. 3.** Fischer–Speier esterification of pentaerythritol with oleic acid (1:1, n/n). Monooleate **1** (green) was formed rapidly, dioleate **2** (red) was readily transformed into trioleate **3** (blue) and the tetraoleate **4** (violet) fraction increased constantly. The trioleate's **3** bulkiness retarded tetraoleate **4** formation to some degree (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

size selectivity limits conversions trioleate **3** is not transformed into tetraoleate **4**. With prolonged process time, up to 15 days, the oleic acid conversions hardly improved (Fig. 2A). As a consequence, product mixtures were obtained in all transformations with any substrate ratios. This is a usual occurrence with multiple reactions on the same molecule and was observed before with similar trimethylolpropane-oleate biolubricants [18]. Product mixtures after multiple reactions on the same molecule are also known in chemical catalyses of star and wheel like structures [19].

The $^1$H NMR of obtained product mixtures showed that mono acylated pentaerythritol **1** was rapidly generated with all substrate ratios followed by dioleate **2** formation. The dioleate **2** was swiftly transformed into trioleate **3**, which accumulated, and tetraoleate **4** formation was largely suppressed. Nevertheless in some cases minor quantities of **4** were recovered (1–12%) (Fig. 2B). The fourth acylation was sluggish due to diffusion-like kinetics as observed before with trimethylolpropane which is structurally close to pentaerythtitol [12]. It was therefore not excluded that bulkiness of **3**

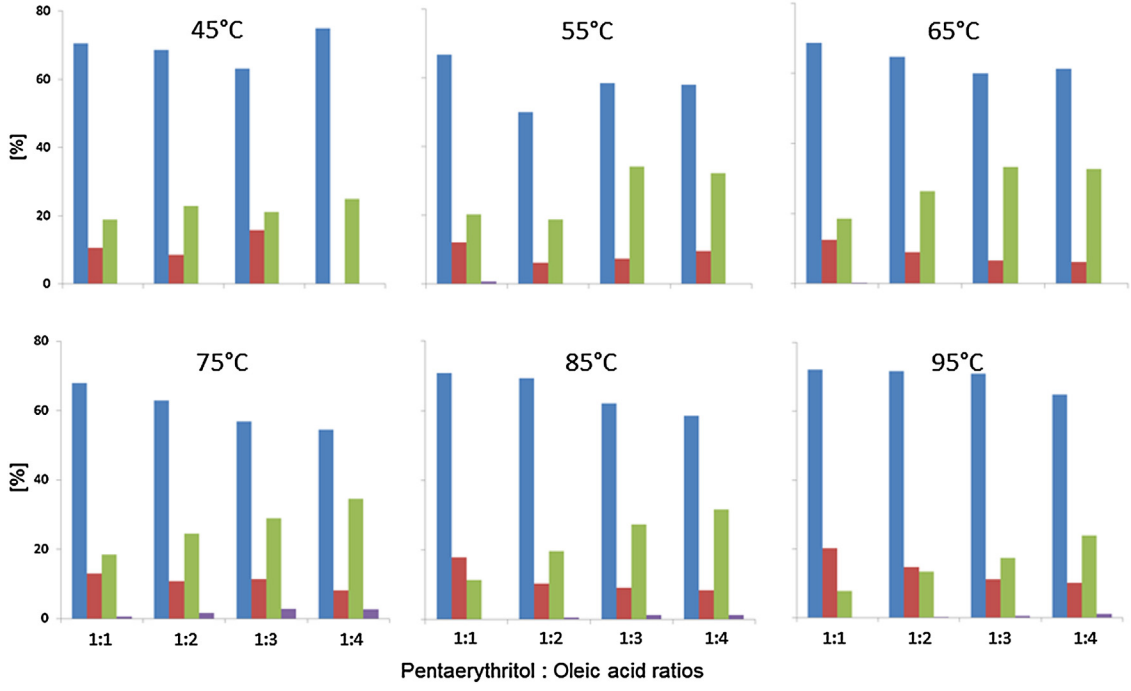


**Fig. 4.** Temperature influence on size selectivity of CAL-B using tert-butanol/24 h. *X*-axes pentaerythritol/oleic acid ratios (1:1 to 1:4). Monooleate **1** (blue), dioleate **2** (red), trioleate **3** (green). At all temperatures tetraoleate **4** (violet) formation was largely suppressed due to size selectivity (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

contributed to observed size selectivity. This led to the question if the observed size selectivity was purely caused by *C. antarctica* lipase B or if it was enhanced by substrate bulkiness. Native lipases hydrolyse and synthesise mono-, di- and triglycerides but not pseudo tetraglycerides as the investigated tetraoleate **4**. The catalytic active site with CAL-B is located at the end of a narrow access port, which is a 10 Å × 4 Å wide and 12 Å deep channel [2]. Consequently, there is limited space to transform bulky trioleate **3** into tetraoleate **4**.

### 3.2. Solvent-free lipase catalysis

Solvent-free conditions caused size selectivity loss yielding mainly tetraoleate **4** and no monooleate **1** and dioleate **2**. Over prolonged reaction time, the tetraoleate **4** fraction increased up to 93% and the rest was trioleate **3** (Fig. 2B). The poor solubility of pentaerythritol in oleic acid was the origin of the narrow product distributions in solvent-free conditions (Fig. 2B). The absence of mono and diacylated products **1** and **2** was explained by poor pentraerythritol dissolution. The first acylation (**PE** → **1**) was therefore slow. The second (**1** → **2**) and third (**2** → **3**) acylations were, by contrast, fast as the intermediates **1** and **2** were dissolved and became further acylated. The trioleate **3** accumulated due to CAL-B's size exclusion effect. A further reaction (**3** → **4**) to tetraoleate **4** was observed and size selectivity was lost most likely as a result of different kinetics. Whether the size limiting channel in CAL-B was altered by solvent-free conditions is not determined and further work is needed. All in all, solvent-free conditions led to tetraoleate **4** excess with only one of three other esters. This was not observed under solvent conditions.

### 3.3. Fischer–Speier esterification

Acid catalysed esterification was conducted to estimate the size selectivity effect caused by substrate bulkiness, in particular of intermediate **3**. A catalysing proton is very small in comparison to steric *C. antartica* lipase B and any substrate bulkiness effect should become apparent with acid catalysis. The solvent-free Fischer–Speier esterification was realised at 100 °C with 5% sulphuric acid [20]. Resulting product distribution was much broader than in solvent-free CAL-B catalysis. With sulphuric acid all four possible esters **1**–**4** were formed (Figs. 2B and 3). Initially, trioleate **3** accumulated with acid catalysis as under solvent conditions using CAL-B. But the accumulation of **3** was temporary, and then its concentration dropped. The initially observed trioleate **3** excess was explained by the expected substrate bulkiness effect determining the reaction rate for the fourth esterification (**3** → **4**). All in all, acid catalysis was non-selective in comparison to lipase catalysis (Fig. 3). In CAL-B catalysis no tetraoleate **4** was formed after a processing times that was 6 times longer (Fig. 2A; see 1:1 ratio). In final conclusion, substrate bulkiness was a minor cause in observed size selectivity with CAL-B.

### 3.4. Temperature variation and selectivity

Catalyst selectivity improves in general by lowering process temperature [21]. This is based on the theory about the activated complex described by the Eyring equation $\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$ that allows to separate enthalpic from entropic influence. The entropy $\Delta S^{\#}$ contribution in the transition state becomes less important with lower temperature and consequently selectivity should improve [22]. In the process examined here, four consecutive reaction steps occurred (**PE** → **1** → **2** → **3** → **4**). The well observable size selectivity concerned the **3** → **4** transformation. Starting with the lowest possible reaction temperature, 35 °C/24 h, oleic acid converted sluggishly (Fig. 4). With raising temperature, conversions

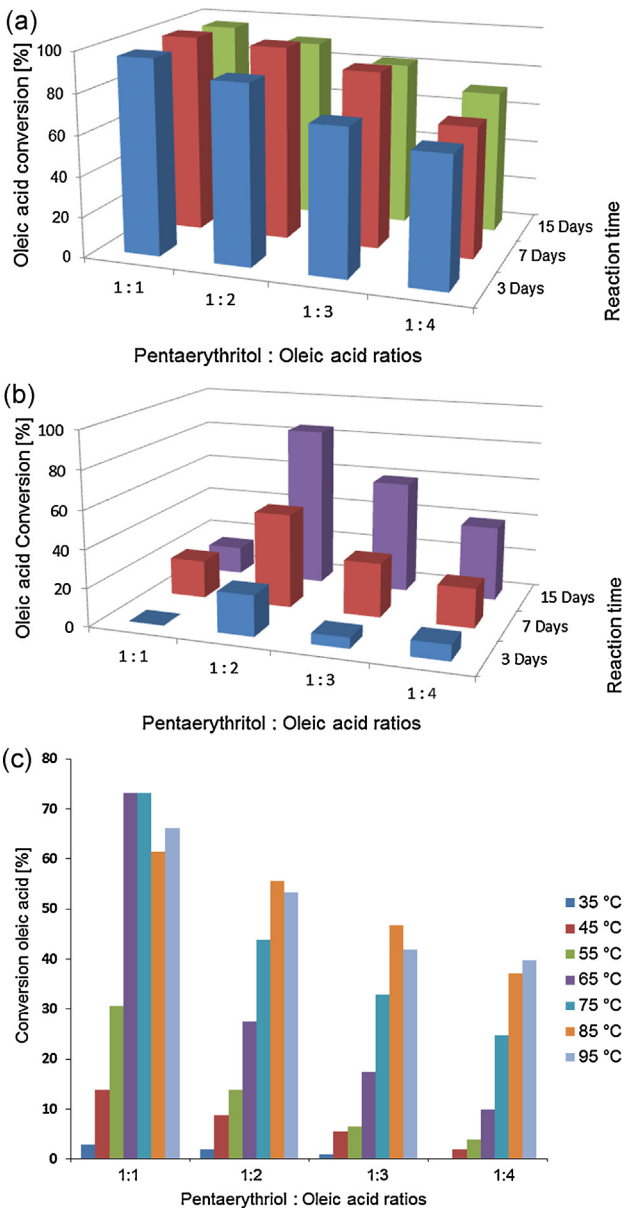


**Fig. 5.** (A) Oleic acid conversions under solvent conditions. (B) Solvent-free transformation resulted in low oleic acid conversions. (C) Temperature dependence of oleic acid conversions with tert-butanol/24 h.

improved from 2.9% to 73% and declined to 66% above the optimal temperature with 95 °C/24 h. The size selectivity persisted up to 65 °C as no tetraoleate **4** was detected (Fig. 4). However, when the temperature rose above 75 °C a minor tetraester **4** formation was observed. The trioleate **3** excess was reduced at higher temperatures, but this was also the case with lower temperatures <65 °C. The cause of this non-linear temperature influence was not established. Data recording from experiments realized at lower temperature was less reliable as conversions were low and signals week what explains the absence of the dioleate **2** at 45 °C/1:4 ratio (Fig. 4). Finally, the temperature variation experiments showed that there was persisting size selectivity with CAL-B from low to high reaction temperatures.

### 3.5. Comparison of persistence and absence in size selectivity

The size selectivity of CAL-B depended on the use or absence of tert-butanol. This solvent dissolved the substrates at the same time

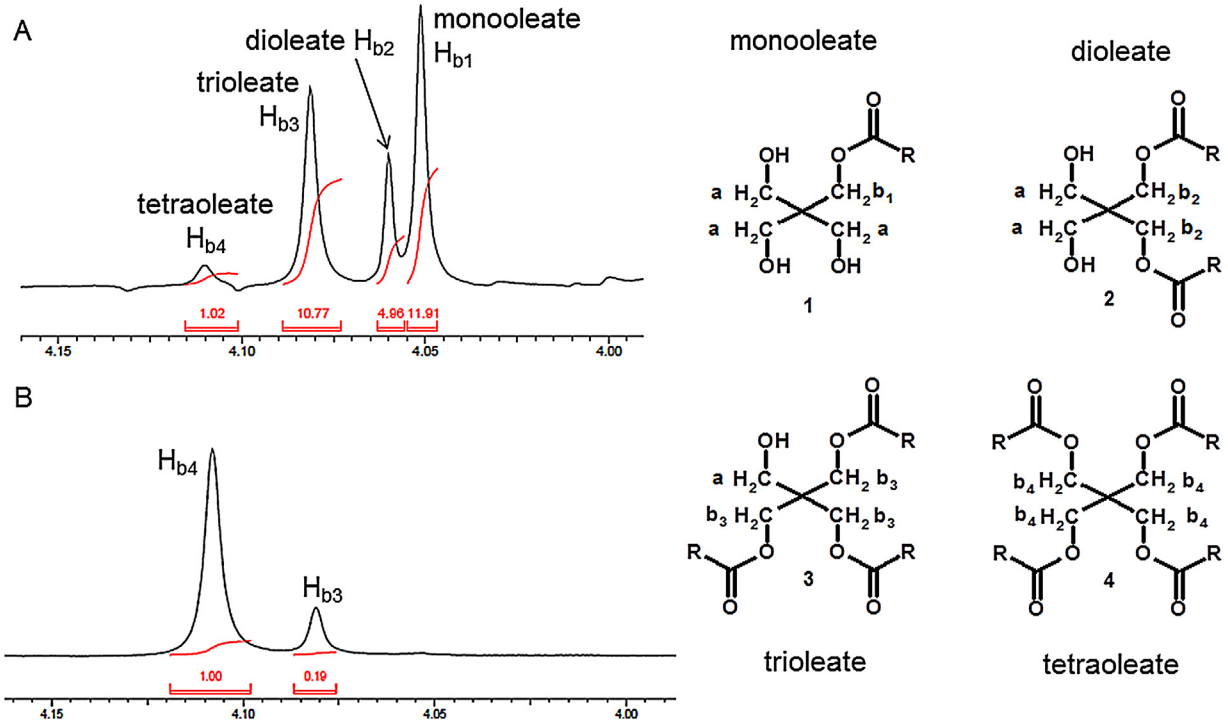


**Fig. 6.** $^1$H NMR (400 MHz, THF-$d_8$) of $H_x$-C(2) product singlets (x = a, b1, b2, b3 and b4) in biolubricant mixtures. (A) All four possible products **1**–**4** (right). *Conditions*: CAL-B in tert-butanol/3d/65 °C with 1:1 PE/OA ratio. (B) Two product mixture of **3** and **4** (20:80). *Conditions*: Solvent-free CAL-B catalysis with 1:4 PE/OA ratio after 15 days/65 °C. Right: proton labeling for $H_x$-C(2) assignment in $^1$H NMR spectra A and B for **1**–**4**. $H_a$ protons appeared all at $\delta_{H_a} = 3.53$ ppm and are not visible in A and B.

and conserved CAL-B activity due to its preferable hydrophobicity log *P* = 0.8 [23], 0.35 [24,25]. This lipase catalysed mono-, di- and triglyceride formation, however, the synthesis of pseudo tetraglyceride **4** remained well restricted. The available space in the channel toward the catalytic site in CAL-B is small and caused most likely observed size selectivity. This hypothesis was tested by examining if trioleates **3** bulkiness reduces the reaction rate of the fourth acylation to a significant degree. For this purpose, sulphuric acid was used as there a small proton catalyses instead of sterically hindered CAL-B. Also the temperature was varied. Due to the robustness of immobilised CAL-B (Novozym® 435) it was possible to conduct lipase mediated esterification close to 100 °C, which allowed to compare sulphuric acid with enzyme catalysis. The catalyst comparison showed that substrates bulkiness is a negligible cause for observed trioleate **3** accumulation. Nevertheless, trioleate **3** excess was observed using sulphuric acid, but tetraoleate **4** formation set in immediately. This contrasted with CAL-B use where hardly any tetraoleate **4** was formed, and this up to 95 °C with a 6-fold longer reaction time.

Why did solvent-free CAL-B catalysis yield tetraoleate **4** in excess (93%) and monooleate **1** and dioleate **2** were absent? The data suggested diffusion-like kinetics at the beginning of the reaction (Fig. 5A–C). The rate determining step was pentaerythritol solubility in the solvent-free reaction mixture. The further acylation of intermediate **1** and **2** was fast and led to a kinetically controlled accumulation of the tetraoleate **4** fraction. All in all, CAL-B allowed to produce tri- and tetraoleates (**3** and **4**) in excess.

### 3.6. Analysis

$^1$H NMR analysis of reaction mixtures allowed to quantify substrate conversion, yield and product distribution at once. Deuterated tetrahydrofuran (THF-$d_8$) separated all four possible $H_{b(1-4)}$-C(2) singlets of the products **1**–**4** (Fig. 6). Other deuterated solvents such as $CDCl_3$ [26,27] or $CD_3OD$ did not separate these four singlets with the available 400 MHz NMR spectrometer. As aforementioned, the four individual $H_{b(1-4)}$-C(2) singlets appeared in a narrow perimeter at: $\delta_{Hb1}$ = 4.05 ppm (**1**), $\delta_{Hb2}$ = 4.06 ppm (**2**), $\delta_{Hb3}$ = 4.08 ppm (**3**) and at $\delta_{Hb4}$ = 4.11 ppm (**4**) (see labelling in Fig. 6). The proton integrams were divided by the corresponding acylation degrees and normalised to 100%. Oleic acid conversion was determined from H-C(2′) triplet that shifted from 2.19 to 2.29 ppm upon esterification.

HPLC analysis was considered too, but found less convenient than $^1$H NMR analysis because of the use of impure oleic acid (90%) that contained also other fatty acids. In HPLC multitudes of peaks were recorded. Fractions of HPLC-peaks recovered in a first separation run were eluted a second time and the recovered fractions were again analysed by $^1$H NMR. The spectra showed that even after two chromatographic separations impurities had to be expected. In some contrast, thin layer chromatography (TLC) indicated simpler mixtures and suggested easier separation. But following preparative chromatography on silica-gel showed that product purification remained challenging as experienced with HPLC. At least two separation runs were needed to isolate pure products. All in all, the convenient $^1$H NMR/THF-$d_8$ method facilitated size selectivity examinations by far.

## 4. Conclusions

The lipase mediated acylation of pentaerythritol with oleic acid showed size selectivity effects. Process parameters were varied to cause excessive generation of one of the four possible products. *C. antarctica* lipase B (CAL-B) exercised size selectivity using tert-butanol and pentaerythritol-trioleate **3** accumulated, while the corresponding tetraoleate **4** was not formed. This contrasted sharply with solvent-free conditions where tetraoleate **4** was obtained in up to 93% purity. Also the temperature influence on the lipase's size selectivity was examined. Below 65 °C, no tetraoleate was detected after 3 days, above this temperature

some minor tetraoleate formation was observed, but even with 95 °C CAL-B size selectivity persisted rather well. The obtained four biolubricants **1**–**4** are envisioned for direct use and also as intermediates for further esterification of remaining hydroxyl groups with specific substituents. All in all, lipase mediated esterification using CAL-B with and without solvent enabled size selectivity variation in the acylation of pentaerythritol with oleic acid.

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