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SYSTEMATIC INVESTIGATION OF THE KINETIC RESOLUTION OF 3-HYDROXY FATTY ACID ESTERS USING *CANDIDA ANTARCTICA* LIPASE B (CALB) AND THE INFLUENCE OF COMPETING OLIGOMERIZATION ON THE ENANTIOMERIC RATIOS

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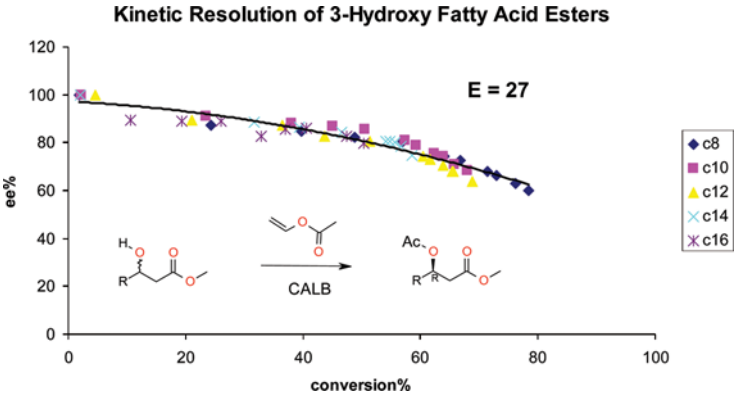
GRAPHICAL ABSTRACT



Abstract *The kinetic resolution of 3-hydroxy fatty acid esters C8:0 to C16:0 with* Candida antarctica *lipase B shows common plots of the enantiomeric excesses of the product and substrate, respectively, versus the conversion and an enantiomeric ratio E of 27 calculated from ee(p). Differences in E, either calculated from the products or the substrates, could be explained by competing oligomerization as a second substrate-consuming process. This reaction is slow compared to acylation, and the remaining enantiomer was oligomerized.*

Keywords CALB; 3-hydroxy fatty acid ester; kinetic resolution

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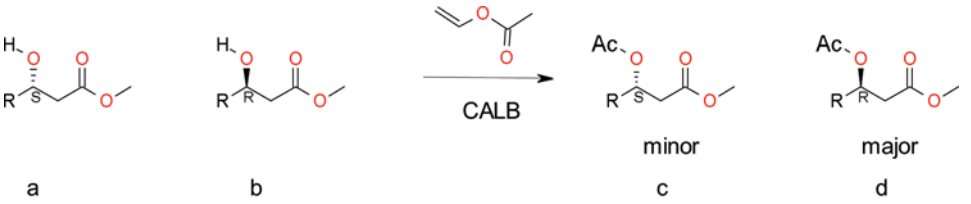
INTRODUCTION

3-Hydroxy fatty acid esters are important building blocks for a wide range of target molecules, and research for convenient and highly selective methods for their synthesis is still ongoing.[1–5] As the 3-oxo species are readily available,[6,7] one standard procedure to get the hydroxy esters is by reducing them. The gold standard is doubtlessly the Noyjori's Ru–Binap-catalyzed hydrogenation, which always leads to greater than 95% conversion and enantiomeric excess (ee), as recently reported by Brückner. Thus, depending on the catalyst, nearly quantitative yields of each desired enantiomer can be obtained.[8,9] However, one alternative to this somewhat expensive and sensitive catalyst is the enzymatic resolution of the racemic mixture from the conventional hydrogenation of the respective oxo-ester.[10,11] Though this method will produce, in principle, only 50% of the theoretical yield of the racemic starting mixture, it is effective when both separated enantiomers are needed. In our search for easily obtained, homochiral, liposome-building, amphiphilic molecules, we used *Candida antarctica* lipase B (CALB, Novozym 435) for the resolution of the homolog-even 3-hydroxy fatty acid esters C8:0 to C16:0, according to Eq. (1):



R=	C_5H_{11}	C_7H_{15}	C_9H_{19}	$C_{11}H_{23}$	$C_{13}H_{27}$
	1	2	3	4	5

Absolute configuration of the products was assumed as described.[10,11]

Surprisingly, there are few reports regarding the kinetic resolution of 3-hydroxy fatty acid esters. Bornscheuer et al. investigated the influence of diverse factors, such as solvent polarity and water content, on the transesterification of **1a**/**b**-, **2a**/**b**-, and 3-hydroxy hexanoic acid methyl ester using *Pseudomonas cepia* lipase (PCL). They obtained moderate to good enantiomeric ratios E (5–17), depending on the solvent, and they reported the kinetic resolution of these three molecules using PCL in supercritical CO_2.[12,13] The same lipase was used by Ikunaka for the resolution of 3-hydroxy tetradecanoic acid and its methyl ester to gain homochiral compounds for the synthesis of important pharmaceutical molecules.[14] Other than those mentioned, only short-chain homologs seem to be investigated (e.g., Garcia used CALB to separate the enantiomers of 4-chlorobutyrate and valeric acid ester by aminolysis of the esters).[15] To our knowledge, there has been no systematic investigation of the kinetic resolution of long-chain fatty acid esters.

Using vinyl acetate as solvent and acylating reagent, we found significant similarity in the reaction rates of all hydroxy esters. There is, within experimental error, only one common plot for all data from **1a**/**b** to **4a**/**b**, with up to a 50% conversion rate. Only the most hydrophobic **5a**/**b** ester had a significantly slower reaction

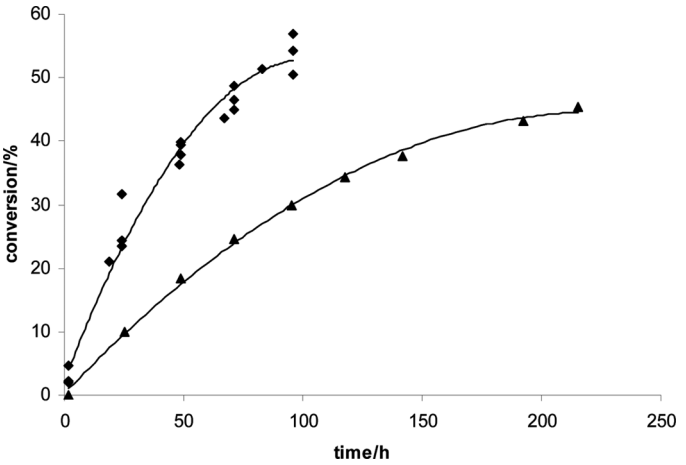


Figure 1. Resolution of 3-hydroxy fatty acid esters C8:0–C16:0 by CALB and vinyl acetate. Plots of conversion versus time for C8–C14 (◆) $y = -0.0048x^2 + 0.9903x + 2.205$; $R^2 = 0.9775$ and C16 (▲) $y = -0.0009x^2 + 0.3958x + 0.2193$; $R^2 = 0.9978$.

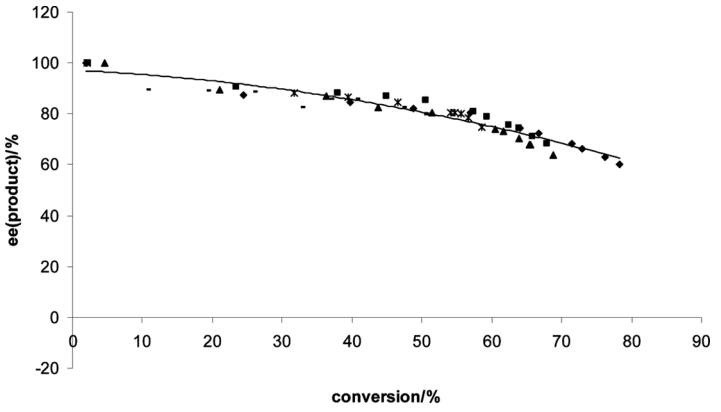
(Fig. 1). As CALB is known to hydrolyze long-chain triglycerides very slowly, we were not astonished by the slower reaction but by the homogeneity of the other data and the sudden drop from one homolog to the next.

Amazingly, when plotting conversion rate versus the ee of the remaining substrates, ee(p) or the respective products ee(s), all five compounds were represented by common curves (Figs. 2a and b).

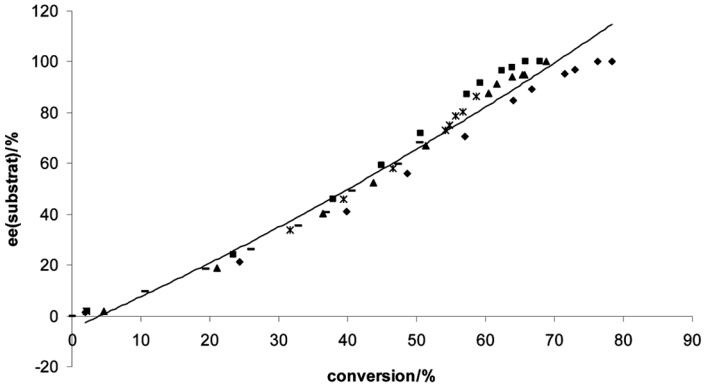
Calculation of the average enantiomeric ratio at a 30% conversion from the regression curves of the product using the equations of Chen and Shi showed $E = 27$. The calculation of E is according to the equations in Refs. 16 (see also Refs. 10 and 11). This is different from the results reported by Adam et al. for the resolution of 2-hydroxy esters, which differ significantly in ee (21% to 98%, respectively) with the enantiomeric ratios ranging from 5.8 to 55, respectively.[16] Moreover, $E = 27$ is much greater than that found by Bornscheuer et al., who used PCL for the resolution of **1a**/**b**-, **2a**/**b**-, and 3-hydroxy hexanoic acid methyl ester in his systematic investigation of the influence of solvents on this reaction.[12]

Incomprehensibly, however, E differs significantly when calculated from either the ee of the subtrate [ee(s)] or the ee of the product [ee(p)]; because of the oligomerization of the bifunctional molecules, the enantiomeric ratio amounts to only 13 when calculated from ee(s). The summation of the relative percentages for the (R) forms of the compounds **1a**/**b** and **1c**/**d** showed an excess of 4.46% for a 40% conversion and 12.3% at about an 80% conversion, respectively. This was not limited to compound **1** but was found less marked for all others as well. From Fig. 3, one can see that the common graph for the esters **1a**/**b** to **5a**/**b** has up to 6% excess of the (R) forms, and the curve for the hydroxy octanoic ester is clearly separated and significantly steeper.

Acidic racemization of the alcohol group could be discounted; a filtered charge of hydroxy ester **5**, with a defined ratio of (R)- and (S)-alcohol and acetate (**5a** = 20.02%; **5b** = 42.53%, **5c** = 1.78%, **5d** = 35.67%), was stirred for several days in the



(a)



(b)

Figure 2. Common graphs of enantiomeric excess (ee) versus conversion for the resolution of 3-hydroxy fatty acid esters C8:0–C16:0 by CALB and vinyl acetate. c8 (◆), c10 (■), c12 (▲), c14 (∗), and c16 (−). (a) $y = 0.0046x^2 + 1.161x - 3.8909$; $R^2 = 0.9711$; (b) $y = -0.004x^2 - 0.131x + 97.264$; $R^2 = 0.9205$.

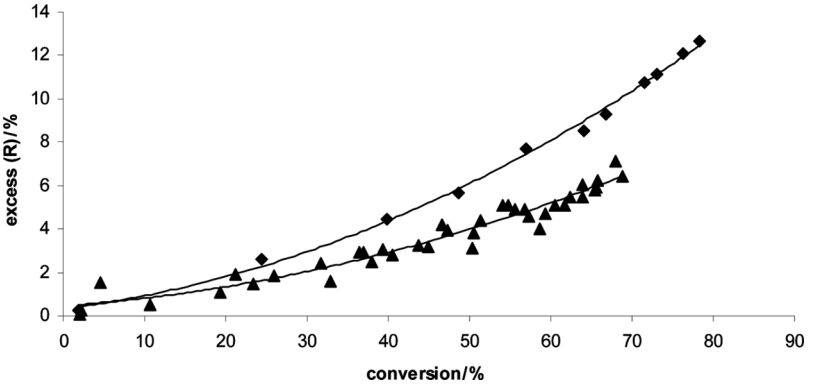


Figure 3. Plots of conversion versus excess of the (R)-form of the 3-hydroxy esters C8 (◆) $y = -0.0014x^2 + 0.0458x + 0.3237$; $R^2 = 0.9963$ and C10–C16 (▲) $y = -0.0009x^2 + 0.0264 + 0.4608$; $R^2 = 0.9484$.

respective vinyl acetate without a significant change in the ratios, which is clear evidence for the involvement of the enzyme in the process.

A closer look at the absolute amounts [gas chromatographic (GC) area counts] of the substrate isomers in the reaction solution revealed that over time, the sum of the area counts of both (R) and (S) forms decreased strongly, but the (S)-form reduction was stronger. We determined that there must be another process competing with the acylation that consumed the hydroxy esters. As no free hydroxy acid could be detected, an enzymatic hydrolysis of the substrates could be ruled out.

In a separate experiment, pure **1a**/**b** was incubated with CALB in hexane. Continuous GC/MS analysis over 8 days showed a 14% decline in the amount of the hydroxy ester, but no decomposition product was formed. A workup of the reaction by short-way distillation easily separated the remaining hydroxy ester from the residue. It showed an ee of 5% for the (S) form. An alkaline methanolysis of the residue resulted in educt **1a/b**, with an ee of 6% for the (R) alcohol. From this, one has to conclude that beside the desired acylation reaction, oligomerization of the 3-hydroxy esters also occurred. Because of the preferred acylation of the (R) form, the concentration of the (S) form rose in the solution, and as the oligomerization seems to be nonselective (E$\sim$1), its concentration decreased more rapidly than that of the (R) form. As confirmed by Fig. 3, this process should increase with decreasing concentration of the (R) form.

Lipases, especially CALB, are known to catalyze the synthesis of polyesters from methyl diesters and diols and the lactonization of ω-hydroxy fatty acid esters.[18,19] This is clearly the case for the reported experiments in which one ester acts as the acyl donor while the hydroxyl group of another molecule plays the role of the substrate to form an oligomer or polyester. This is contrary to the results reported by Adlercreutz for the reaction of lactic acid and its esters.[20] From the absence of dimer formations, they concluded that the hydroxy group would not play the role of a nucleophile, and they did not find any CALB-catalyzed esterifications of lactic acid and dodecanoic acid. As Adam et al. have shown, α-hydroxy esters undergo enzyme-catalyzed acylation, and lactic acid is probably a special exception. In most other cases, it is likely that an oligomerization side reaction will occur with other bifunctional molecules as well.

EXPERIMENTAL

Unless otherwise stated, all materials used were of analytical grade and bought from Merck, Darmstadt. CALB Batch No. LC2 00009 was provided by Novo Nordisk.

Preparation of 3-Hydroxy Fatty Acid Methyl Ester

The 3-hydroxy fatty acid methyl esters were synthesized from the respective keto esters by sodium borohydride reduction in methanol.[21] In the cases of homologs C14:0 and C16:0, tetrahydrofuran (THF)/methanol (1:1) was used as the solvent to increase solubility. After quantitative conversion, the products were purified by short-way distillation. Yields were not determined, but the purity was measured using gas chromatography.

Keto esters were prepared according to Clay et al.'s method b.[22]

Kinetic Resolution Experiments

Five μl of the enantiomeric mixture **a**/**b** and 10 μl of vinyl acetate were added to 30 mg of CALB in 1 ml of the respective solvent. GC analysis of the reaction mixture was performed at regular time intervals. The details of the GC are as follows: HP 6890 GC, Autosampler 7683, split/splitless injector, mass selective detector (MSD) 5973, Cyclodex-B (30 m, i.d. 0.25-mm, 0.25-μm film) from Agilent, He 1 ml/min. All separations were carried out isothermally. The temperature and retention times for each mixture are as follows: **1** (140 °C), [a: 9.6 min, b: 9.9 min, c: 11.8 min, d: 12.1 min]; **2** (150 °C), [a: 15.9 min, b: 16.4 min, c: 19.8 min, d: 20.2 min]; **3** (160 °C), [a: 25.7 min, b: 26.4 min, c: 31.3 min, d: 31.9 min]; **4** (160 °C), [a: 63.3 min, b: 65.3 min, c: 76.1 min, d: 77.6 min]; and **5** (160 °C), [a: 159.7 min, b: 164.4 min, c: 189.2 min, d: 192.8 min].

The conversion of compound **5** in vinyl acetate was stopped by filtering off the enzyme after 4 days (**5a** = 20.02%; **5b** = 42.53%, **5c** = 1.78%, **5d** = 35.67%). The enzyme-free solution was stirred for an additional 4 days with regular GC inspections and showed no racemization or any other change in enantiomer distribution.

OLIGOMERIZATION

For the oligomer identification, 0.5 mmol of **1a**/**b** (87 mg, 92 μl, respectively) were added to 550 mg of CALB in 3 ml of hexane. The mixture was stirred at room temperature, and 50-μl analytic samples were taken regularly, diluted with 500 μl of hexane, and measured by GC. After 8 days, the solvent was evaporated and short-way distillation was done with a Büchi BKR-51 (1.7×10^{-1} mbar/100 °C) to give a clean fraction of compound **1** (59 mg).

Two ml of 10% KOH in methanol were added to the distillation residue, and the mixture was stirred at 60 °C for 15 min to transform the nonvolatile oligomer mixture to the methyl ester. The methanol phase was extracted with 1 ml of isooctane, which was directly analyzed and showed **1** as the main compound.

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