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Hydrolysis of Fatty Acid Esters by *Candida Antarctica* Lipase B (Novozym 435) Dissolved in Anhydrous Triethylamine

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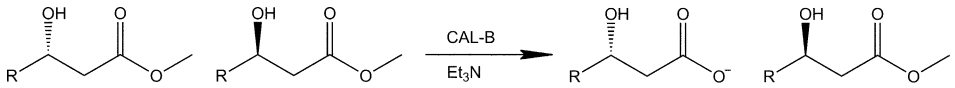
Enzyme-catalyzed stereoselective esterification of OH groups in non-aqueous media has been widely used for kinetic racemate resolution. However, rapid hydrolysis of the ester group was observed as a competing reaction if we attempted to resolve 3-hydroxy octanoic acid methyl ester with Novozym 435 in a non-aqueous medium with a catalytic amount of the base triethylamine. This hydrolysis away from the stereogenic carbon atom showed stereoselectivity, thus the preparative value of this reaction was inspected closer and it could be extended to higher homologues. 3-Hydroxy decanoic acid methyl ester, 3-hydroxy dodecanoic acid methyl ester and 3-hydroxy tetradecanoic acid methyl ester were also hydrolyzed by Novozym 435 with enhanced reaction rates and moderate selectivities if dissolved in triethylamine. Ethyl mandelate and ethyl lactate were also converted under these conditions. The same phenomenon could also be observed with a 2% DABCO solution in hexane, whereas secondary amines, for example piperidine, did not influence the reaction.

Introduction

Candida antarctica lipase B (CAL-B) is best known for the resolution of alcohols, notably secondary alcohols, by enantioselective esterification in organic solvents.[1–4] Additionally, desymmetrization reactions of prochiral diesters or cyclic anhydrides have been reported with high yield and excellent optical purity.[5–8] The enantioselective hydrolysis or esterification of racemic carboxylic acid monoesters however, showed only moderate enantioselectivity. For example, in the hydrolysis of arylaliphatic carboxylic acid ethyl esters in sodium phosphate buffer, enantiomeric ratios (*E*) from 3.5 to 9 were reported,[9] and the resolution of (*R*,*S*)-Ibuprofen by esterification with dodecanol had an *E* of 5.3.[10] CAL-B (Novozym 435) and vinyl acetate were used for the kinetic resolution of 3-hydroxy fatty acid methyl esters.[11] In our search to enhance the moderate enantioselectivity ($E = 27$), we tried to improve the results by adding bases. Lipases were reported to resolve secondary alcohols with enhanced reaction rates and higher selectivities if organic bases, particularly triethylamine **1** (Et_3N), were added to the solvent.[12] These additives have been used in an empirical manner, and their beneficial influence depended on the specific substrate/solvent/lipase combination. Though, other lipases were mostly applied under these conditions, some examples of desymmetrization reactions with CAL-B were reported with high yield and excellent enantiomeric ratios.[13,14] Reaction rates and enantioselectivities of lipase catalyzed resolutions were greatly improved in some cases by adding **1**, even to propylene carbonate or an ionic liquid solution.[15–17]

These reports have encouraged us to investigate the influence of bases on the enzymatic kinetic resolution of 3-hydroxy fatty acid methyl esters. Herein we report on our studies regarding the influence of tertiary amines i.e. **1** on the enzymatic resolution of 3-hydroxy fatty acid methyl esters, which led to an (*S*)-selective ester cleavage with approximately 80% conversion within less than ten hours.



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

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Results and Discussion

As our earlier investigations had indicated the limitations of the Novozym 435 mediated resolution of 3-hydroxy fatty acid methyl esters, we wanted to explore the reported beneficial influence of basic media on this reaction.[12] The acylation of 3-hydroxy octanoic acid methyl ester **2** with vinyl acetate was performed as described in Ref. [11] but in a 1% solution of **1** in hexane.

This did lead to a dramatic increase in the rate of conversion of the starting material by a factor of five; however, the stereoselectivity was reduced. Similar results were obtained if we increased the amount of base to a co-solvent level (10%). The reaction rate went up once more, but the stereospecificity was largely lost (Figure 1). Whereas the conversion was accelerated by a factor of five, the enantiomeric ratio dropped from $E=27$ in the absence of the additive to $E=11$ and $E=6$, respectively.[18]

We observed that immediately after the addition of the base, the concentration of the (*S*)-form of the hydroxyl acid ester started to decrease without the concomitant appearance of the acylated product. After 25% saponification had been achieved, the slower (*R*)-selective acylation could be observed. We removed the acylating agent and studied the enzymatic reaction of the ester directly in **1** as the solvent. Within five hours, 50% hydrolysis of the ester could be traced by GC and the carboxylic acid could be detected as a product in the case of **2**. Similar results were obtained for 3-hydroxy decanoic acid methyl ester **3** and 3-hydroxy dodecanoic acid methyl ester **4**, though no free acid could be detected by GC. As the hydrolysis of the esters in completely non-aqueous media is not possible, water must either be present in the solvent or in the enzyme catalyst. GC analysis of the Et_3N used shows no trace of water. Therefore, we think that the water that is inherent in the immobilized enzyme takes part in the reaction.

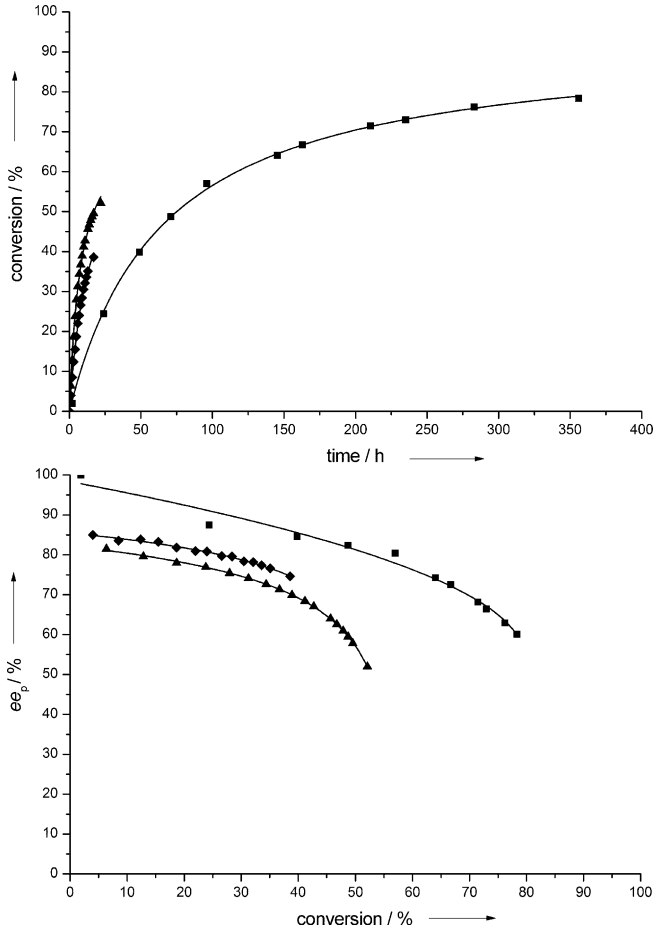

Figure 1. Enzymatic kinetic resolution of 3-hydroxy octanoic acid methyl ester by Novozym 435 and vinyl acetate in hexane containing different amounts of Et_3N. Plots of a) conversion versus time and b) enantiomeric excess versus conversion; ■ = reaction without Et_3N, ◆ = reaction with catalytic amount Et_3N, ▲ = Et_3N as a co-solvent.

However, after work-up on a semi-preparative scale of 3-hydroxy dodecanoic acid methyl ester **4**, the respective acid and traces of its oligomers could be unambiguously confirmed by mass spectrometry. Measurement of the optical rotation of the remaining ester gave a value of $[\alpha]_D=-12^\circ$ ($c=2$; CH_3Cl), which is consistent with an $ee=77\%$ of the (*R*)-form determined by GC analysis.[18,19]

In the following experiments, we used Novozym 435 in **1** as the solvent and resolved the 3-hydroxy fatty acid methyl esters **2**–**5** without adding any further solvent or acylating agent.

All reactions showed very similar rates. Once again there is, within experimental error, one plot for all data of 3-hydroxy octanoic acid methyl ester **2**, 3-hydroxy decanoic acid methyl ester **3** and 3-hydroxy dodecanoic acid methyl ester **4** up to a 70% conversion, and likewise the plot of conversion versus ee_p is represented by a joint curve. Only the 3-hydroxy tetradecanoic acid methyl ester **5** showed separated plots, which indicates accelerated reaction rates but a lower *ee* (Figure 2).

The enantioselectivity, however, is poor to moderate; calculation of the enantiomeric ratio from the regression curves of the plot ee_p versus conversion gave $E=10.3$ and for **5** $E=2.5$.[21] However, except for the latter, these values are a substantial improvement over the ester cleavage in phosphate buffer. If the hydrolysis was conducted as described by Bornscheuer,[9] we found *E* values below 2 for all compounds investigated (**2**: $E=1.7$; **4**: $E=1.8$; **5**: $E=1.6$). If the hydrolysis with Novozym 435 was conducted in hexane without **1**, no significant conversion was observed over a period of three days. Analysis after 53 h showed only in the case of **2** that 20% conversion had taken place. In contrast to the amine solvent, the (*R*)-form was hydrolyzed resulting in 5% *ee* of the (*S*) form.

Dissolving the esters in **1** without enzyme did not result in any conversion over a period of four days. Thus, the presence of both the enzyme and the amine was necessary to conduct the reaction.

Some references regarding the effect of **1** report that other nitrogen bases also enhance reaction rates and selectivity if added to the solvent, for example, pyridine, *N*,*N*-dimethylaminopyridine (DMAP), lutidine, imidazole, pyrrolidine, or *N*,*N*-diisopropylamine.[12] Amine **1**, however, has been shown to be advantageous over all others. The commonly accepted explanation for the enhanced catalytic effect is the basicity of the amines, which makes them trap traces of acid that should be

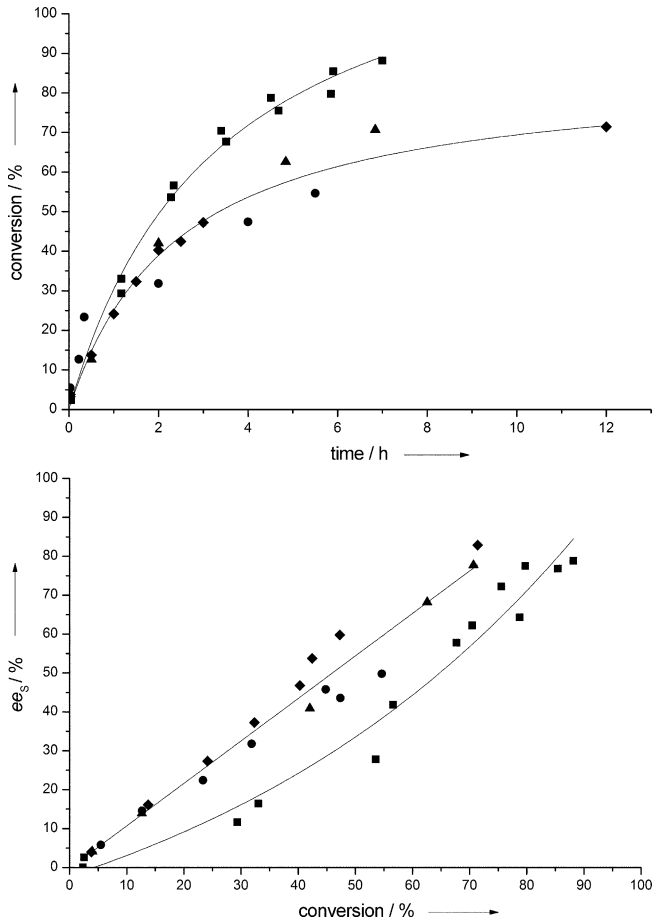

Figure 2. Hydrolysis of 3-hydroxy fatty acid esters **2**(●), **3**(▲), **4**(◆), and **5**(■) by Novozym 435 dissolved in Et_3N; a) reaction rate; b) plot enantiomeric excess versus conversion.

responsible for enzyme-deactivation. This idea is supported by the finding that inorganic basic materials, such as silver oxide, potassium hydrogen carbonate, or solid-state buffer materials, also enhance lipase catalyzed resolutions.[12,14,16] Amine **1** is the preferred base, which is mentioned by other authors in this field.

We tested other amines but unsuccessfully. In pyridine or piperidine, Novozym 435 did not catalyze the ester cleavage or any other reaction of **2** or **3** at all. It was only in diethylamine (Et_2NH) that we found 13% conversion of the ester **2** to the respective amide with 20% *ee* (*R*-form) after three days. As both secondary amines are more basic than **1** (piperidine: $pK_b = 2.88$; diethylamine: $pK_b = 2.90$; triethylamine: $pK_b = 3.25$) the reason for the activation of the enzyme in this hydrolysis cannot exclusively be explained by the basicity of the solvent. A pH effect cannot be excluded, of course, but we believe that there is a special effect for sterically hindered amines, that is, amines with tertiary nitrogen atoms such as **1** or DMAP or else with bulky alkyl groups like diisopropylamine. One of the few base-supported enzymatic ester cleavages was reported by Guo and Sih. They employed either dextromethorphan or levomethorphan in the respective reaction with aryloxypropionic esters.[21] Both enantiomeric alkaloids enhance the reaction rate and selectivity, independent of their own chirality if we trace back to the fact that both bear the same tertiary amino center.

From these references and our experiments we concluded that the acyl binding site of the enzyme may be activated by sterically hindered amines. Indeed, **1** could be replaced by a 2% hexane solution of diazo[2.2.2]bicyclooctane (DABCO) **6**, which has a similar structure around the N atom. Reaction rates, *ee* values, and, consequently, the enantiomeric ratios of the educts **2**–**5** ($E = 5$) were similar to those in **1** as shown by Figure 3.

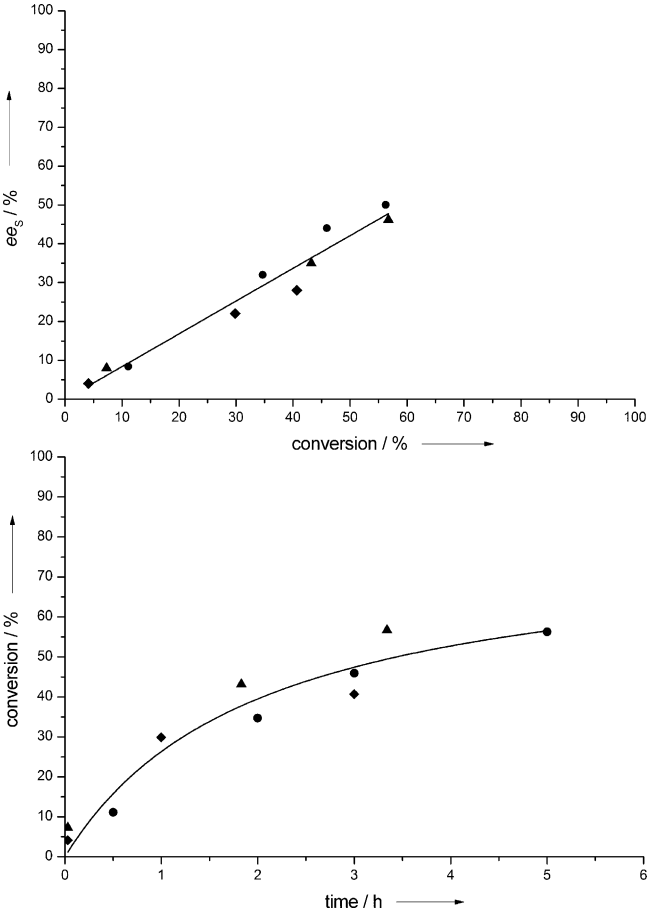

Figure 3. Hydrolysis of 3-hydroxy fatty acid esters **2**(●), **3**(▲), and **4**(◆) by Novozym 435 dissolved in a 2% DABCO solution in hexane; a) reaction rate; b) enantiomeric excess versus conversion.

The stereospecific catalyzed hydrolysis was not limited to the 3-hydroxy fatty acid esters but could be realized with diverse chiral esters.

Alpha-hydroxy acid esters like ethyl mandelate **7** and ethyl lactate **8** were suitable substrates for the reaction. The reaction with ethyl mandelate had $E = 3$ (30% conversion; 20% *ee*) and the conversion reached 60% after three days. The reaction with ethyl lactate in which the commercially available (*S*)-lactate was employed, reached 84% conversion after three days.

Finally, we investigated the resolutions of 2-methyloctanoic acid methyl ester **9**, 2-ethyloctanoic acid methyl ester **10** and 3-methyloctanoic acid methyl ester **11** dissolved in **1**. Whereas the α- and β-methylated esters **11** and **9** were hydrolyzed within 7 and 17 h, respectively, the 2-ethyl ester **10** only had 13% conversion within four days. Unfortunately this hydrolysis also showed poor selectivity, $E \approx 2$, which is similar to the buffered aqueous reaction that we conducted for comparison.

Obviously the use of a tertiary amine leads to accelerated reaction rates for non-aqueous enzymatic ester cleavage. In some cases improved selectivities are found, compared with aqueous buffered reactions. Synthetically, it might be a great advantage not to work under aqueous conditions but to resolve the ester in an organic medium. The acid is bound by the base and after filtration of the enzyme it can be washed out easily.

One of the remaining questions is how the amine activates the reaction with the enzyme. We discount the interaction of **1** with the ester as a key step because this would mean the formation of an activated intermediate in which the amine had replaced the alcohol. This is improbable, as CAL-B is, in contrast to CAL-A, not known to accept such tertiary structures, that is, tertiary butyl esters.[22,23] At present we are systematically testing various amines to clarify the underlying mechanisms.

For future synthetic work we will examine the possibility of replacing water by other nucleophiles, for example, alcohols or sulfides; transesterification experiments with ethanol have shown encouraging results. The improvement of the reaction conditions is still ongoing as rate and stereoselectivity depend on both the nucleophile concentration and the additive amount.

Experimental Section

Unless otherwise stated, all materials used were of analytical grade and bought from Merck, Darmstadt. Novozym 435 Batch No. LC2 00009 was provided by Novo Nordisk A/S.

The 3-hydroxy fatty acid methyl esters were synthesized as reported previously in Ref. [11].

The respective fatty acid esters **2**–**5** (5 μL, 30–20 μmol) were dissolved in **1** (1 mL) or one of the other bases applied. In the case of **6**, a solution in hexane (2%) was used. The solution was then measured for a blank value and subsequently Novozym 435 (23–25 mg) was added. GC analysis of the reaction mixture was done at regular time intervals to determine the diminution of the ester.

HP 6890 GC, with an autosampler 7683, split/splitless injector, mass selective detector (MSD) 5973, and Cyclosil-B (30 m, ID 0.25 mm, 0.25 μm film) from Agilent were used, with an He flow rate of 1 mL min^{-1}. All separations were performed isothermally.

Retention times data: 2 (140 °C) [a: 9.6 min, b: 9.9 min]; **3** (150 °C) [a: 15.9 min, b: 16.4 min]; **4** (160 °C) [a: 25.7 min, b: 26.4 min]; **5** (160 °C) [a: 63.3 min, b: 65.3 min]; **6** (160 °C) [a: 159.7 min, b: 164.4 min]; **7** (140 °C) [a: 14.7 min, b: 15.2 min]; **8** (70 °C) [5.4 min]; **9** (50 °C); [a: 181.0 min, b: 184.1 min] **10** (90 °C); [a: 30.7 min, b: 34.7 min] **11** (60 °C); [a: 76.7 min, b: 78.3 min].

As confirmed for **5** the first component is the (*R*) enantiomer in the cases of the hydroxy acid esters.

For the semi-preparative synthesis, **4** (500 μl; 526 mg; 2.1 mmol) was dissolved in **1** (50 mL) or alternatively in a DABCO solution in hexane (2%). Novozym 435 (2 g) was added to the mixture and stirred for 20 h. GC-reaction control showed 75% conversion and 77% *ee* of the remaining ester. The enzyme was filtered off and the solvent was evaporated. The residue was dissolved in hexane and washed with 2M HCl (50 mL). The phases were separated, the hexane phase dried with sodium carbonate, filtered and then evaporated to leave (*R*) - **4** (120 mg, 0.52 mmol) ($ee = 77\%$). $[\alpha]_D = -12^\circ$ ($c = 2$; CH_3Cl).

The enzyme was washed with 2M HCl (50 mL) and diethylether (50 mL), the aqueous phases were combined and buffered with KH_2PO4 to pH 7.2. The aqueous solution was extracted twice with diethyl ether (75 mL) and the combined ether extract dried with sodium sulfate. The salt was filtered off and the ether was evaporated to leave crystallized 3-hydroxy dodecanoic acid (290 mg, 1.34 mmol) ($ee = 25.5\%$) . Found: MS (EI) 70 eV: m/z (%): 216. This was recorded on a Finnigan MAT 95.

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Keywords: chiral resolution · enzymes · fatty acids · hydrolysis · solvent effects

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