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Scale-Up of a Recombinant Pig Liver Esterase-Catalyzed Desymmetrization of Dimethyl Cyclohex-4-ene-*cis*-1,2-dicarboxylate

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Supporting Information

ABSTRACT: A recombinant isoenzyme of pig liver esterase was used for the highly enantioselective desymmetrization of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate. The selected recombinant esterase showed a significant advantage in enantioselectivity over the commonly used esterase from the mammalian source. The process was scaled up to yield 265 g of product with a simplified pH control, and the target molecule was obtained with an enantiopurity of >99.5% ee.

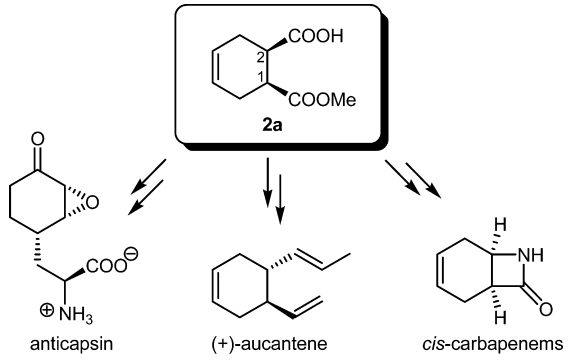
INTRODUCTION

The preparation of enantiopure building blocks gained significant relevance in the industrial production of important pharmaceuticals and agrochemicals during the past decades. For example, about 39% of drugs sold worldwide in 2002 were provided in an enantiomerically pure form.[1] Originally the separation of the enantiomers from the chemically synthesized racemic mixture was preferred, e.g., by preparative chromatography and (enantio)selective crystallization, which obviously limits the maximum yield to 50% and results in low atom efficiency and a significant waste problem. Asymmetric synthesis processes in contrast allow a maximum theoretical yield of 100%, and thus an impressive variety of chemical and biocatalytical processes have been developed over the past years.[2] Biocatalysis has proven multiple times that higher enantio- and regioselectivities are achieved at particularly mild reaction conditions, facilitating a suppression of unwanted side reactions, e.g., isomerization and racemization.[3−6] For this purpose the enzyme can be utilized in different forms: as (partially) purified enzymes, free or in an immobilized enzyme preparation, with cross-linking techniques, or even as whole cells. The eventually applied enzyme form strongly depends on the chosen specific process specifications.[7] In addition, protein stability, substrate specificity, and enantioselectivity of the biocatalyst itself can be improved by molecular biology techniques.[8]

From the available pool of biocatalysts various enzymes have repeatedly proven their value for the synthesis of chiral synthons, especially at industrial scale.[9−11] One major example is the esterase from porcine liver, typically referred to as pig liver esterase (PLE). PLE is a relatively low-cost enzyme that shows a good stability and hydrolyzes a large range of esters.[12,13] Examples include the synthesis of precursors of β3-receptor agonists, pyrethroids (from chrysanthemic acid esters), the growth hormone secretagogue, and the anti-HIV-drug (−)-FTC.[7,12,14]

Another major synthon is the target molecule (1*S*,2*R*)-1-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid **2a** ((1*S*,2*R*)-monoester), which is a valuable intermediate for the synthesis of many biologically active products, e.g., anticapsin, (+)-aucantene, and *cis*-carbapenems (Scheme 1).[15] Further

Scheme 1. Target molecule (1*S*,2*R*)-monoester 2a is a very useful intermediate for the production of various pharmaceutically relevant products



potential applications are the creation of ligands for DC-SIGN (useful in treatment of HIV infection), synthesis of β-polypeptides against cytomegalovirus (Herpes), and the synthesis of Tricyclic Core of Platencin (antibiotic to MRSA).[16−18]

Unfortunately, the classical preparation of pig liver esterase includes an extraction from the animal tissue, which yields varying mixtures of several isoenzymes.[19,20] All of these isoforms exhibit different enzyme characteristics, including different stabilities and pH-dependencies and may also result in opposite enantioselectivities, depending on the substrate.[21−23] In addition, differences in the isoenzyme ratio may occur

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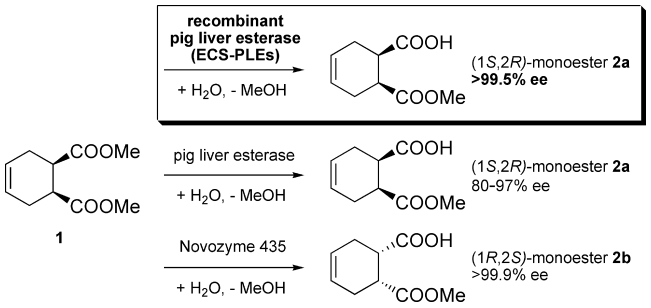
between PLE production batches. Consequently the observed enantiomeric excess of a reaction represents only a sum parameter and is not always easily reproducible, which can cause problems in large scale applications. The very unattractive presence of several different PLE isoenzymes was ultimately overcome by cloning and recombinant expression in safe non-mammalian producer strains.[24] Various authors reported the expression in *P. pastoris* and in *E. coli*, which allows the use of the full potential of selected isoenzymes, including high enantioselectivity, but without side activities and regulatory issues.[25−28] In this contribution we report the successful application of a recombinant pig liver esterase for the desymmetrization reaction of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate **1** (Scheme 2). From the available six recombinant

Scheme 2. Comparison of differing enzyme preparations for the desymmetrization of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate 1



pig liver esterases (ECS-PLE01−06) the most valuable isoenzyme and the optimal reaction conditions were chosen. The characteristic properties of the recombinant biocatalysts were compared with their natural equivalent from the porcine liver extract. Finally, the reaction was transformed into 250-g scale, proving again the strong potential and large scale applicability of recombinant pig liver esterases. Furthermore, a simplified pH-control concept was used for this reaction, which requires no additional equipment, e.g., titrator and pH electrode. This self-regulating concept requires only standard laboratory equipment, thus keeping overall costs low.

RESULTS AND DISCUSSION

Desymmetrization of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate **1** allows the synthesis of two different enantiomers. (1*R*,2*S*)-Monoester **2b** can be achieved with the lipase B from *Candida antarctica* as immobilized form Novozym 435 with excellent enantioselectivity (>99.9%) and a yield of up to 99.8%.[29] The other enantiomer, (1*S*,2*R*)-monoester **2a**, was repeatedly synthesized via a pig liver esterase-catalyzed reaction. Unfortunately the reaction is not entirely enantioselective, and a lower enantiomeric excess of 80−97% ee was obtained (Scheme 2).[12,30−36] The reduced enantioselectivity results from the presence of several parallel hydrolytic reactions, each independently catalyzed by different isoenzymes of pig liver esterase. The products of highly enantioselective isoenzymes are hereby mixed with products from less selective enzymes. Thus, the use of a single, recombinantly expressed PLE isoenzyme allows a highly enantioselective synthesis of **2a** (>99.5% ee).

Choice of Optimal PLE Isoenzyme. The available six PLE isoenzymes (ECS-PLEs from Enzymicals AG) were screened for the production of (1*S*,2*R*)-monoester **2a** via desymmetrization from diester **1**. As expected, strong differences in activity and selectivity were found between the isoenzymes (Figure 1).

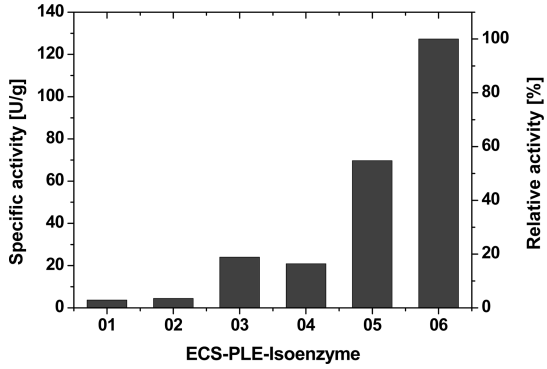


Figure 1. Comparison of the specific activity towards dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate **1** (hydrolysis reaction). Conditions: 25−50 mg PLE isoenzyme (lyophilizate), 0.2 mmol **1**, 40 °C, 5 mL 5 mM phosphate buffer, pH 8, titration with 50 mM NaOH solution.

ECS-PLE06 exhibits the highest enzymatic activity for the desymmetrization of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate, indicated as 100% relative activity. A reduced activity of approximately 55% was found for ECS-PLE05, which is still considerably high for the favored conversion of **1**. The activities of ECS-PLE03 and 04 are significantly lower, and they were therefore excluded due to economic reasons. The remaining isoenzymes ECS-PLE01 and 02 showed hardly any activity towards **1** and were not favored as well. The enantioselectivity of the available isoenzymes and thus the resulting synthetic potential were additionally investigated with conversion experiments on small scale (Figure 2).

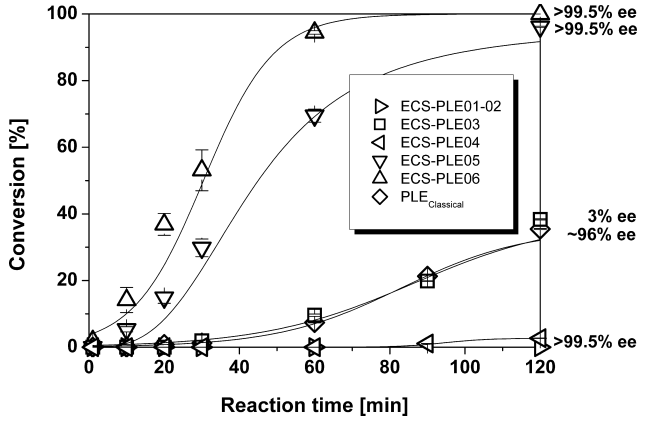


Figure 2. Desymmetrization of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate **1** to (1*S*,2*R*)-monester **2a** depending on the used pig liver esterase isoenzyme. Conditions: 23 °C, 100 mM TRIS buffer pH 7.5, 10 mmol/L substrate; 4 U/mL recombinant PLE (according to the standard activity assay).

In analogy to the results from the apparent reactivity the ECS-PLE06 exhibited the highest reactivity in the conversion experiments. After 120 min full conversion together with an excellent enantiomeric excess of >99.5% was obtained. A slightly lower rate of conversion was found for ECS-PLE05, which is also in accordance with the previous results, Figure 1. However, almost full conversion was also achieved after 120

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min, and an enantioselectivity of >99.5% was identified, which is identical to that of ECS-PLE06. Interestingly, based on the enzyme activity assay with *p*-nitrophenyl acetate, a similar amount of the classical pig liver esterase ($PLE_{Classical}$) yields a significantly reduced reaction rate in comparison to that of ECS-PLE05 and ECS-PLE06. After 60 min only 15% conversion was possible, which was increased to only about 30% after 120 min. Most importantly, an enantiomeric excess of nearly 96% ee was observed for the production of **2a** in this reaction, which is of course lower than the results obtained with ECS-PLE05 and ECS-PLE06. This fact illustrates conclusively the high synthetic advantage of recombinant PLEs. Furthermore, the other recombinant pig liver esterases showed no synthetic value. The use of ECS-PLE03 yields only an almost racemic product (3% ee) with 30% conversion after 120 min, and the other recombinant PLEs showed no significant conversion. Additional experiments with low amounts of water-miscible solvents, which are known to alter the characteristics of recombinant PLEs, were not successful for the improvement of this catalytic reaction.[37]

In conclusion, recombinant PLEs show impressive advantages in comparison to the classical pig liver esterase preparation. The highest reactivities and excellent enantioselectivities were found for ECS-PLE06, which was consequently chosen for the intended large scale-production of **2a**. The lower reactivity of the classical PLE is explainable by the presence of different isoenzymes, as mentioned above. Here the recombinant PLEs 1, 2, and 4 diminish the synthetic performance and the presence of PLE 3, which yields only a racemic product, causes a loss in enantioselectivity. Thus, only a lowered reactivity and enantiomeric excess can be obtained with the classical pig liver esterase.[12]

Choice of Reaction Conditions. The main enzyme characteristics of recombinant PLEs were already reported earlier in the literature.[20,22,25] Unfortunately, specific process data for the desymmetrization of **1** to **2a** were not available prior to this study. Thus, additional experiments were conducted to define efficient process conditions for the ECS-PLE06-catalyzed synthesis of **2a**. In particular reaction temperature and pH of the aqueous phase were of interest for the attempted scale-up. The influence of reaction temperature is exemplarily shown for ECS-PLE06 in Figure 3.

At 10 °C only an insufficient reactivity with an unsatisfactory conversion of <10% after 30 min was observed. A temperature increase to 25 °C partially improves the apparent enzyme activity to a conversion of 47%. A further increase of the reaction temperature to 40 °C yields full conversion after 30 min, which includes a fully enantioselective product formation. Enzyme reactivity can be further improved with an increase of reaction temperature to 60 °C. However, such high temperatures are not recommended for applications with higher substrate concentrations and longer reaction times, because the recombinant PLEs are less stable at >50 °C and above.[25] Consequently, 40 °C was chosen as the optimal process temperature. Noteworthy, excellent enantiomeric excesses (>99.5% ee) were found within all investigated reactions, which represent a robust selectivity of the enzyme.

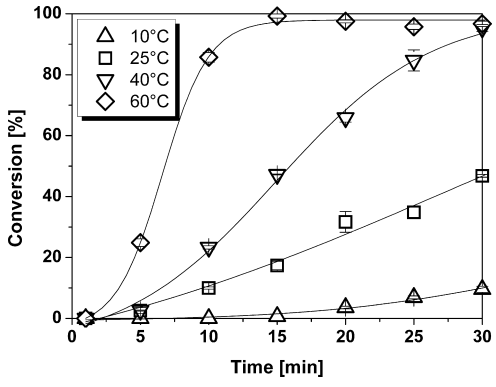

Figure 3. Effect of reaction temperature on the ECS-PLE06-catalyzed conversion of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate. Conditions: 50 mM phosphate buffer pH 7.5, 10 mM substrate; 4 U/mL recombinant PLE (according to the standard activity assay).

Aside temperature, the pH of the reaction mixture is a relevant parameter and therefore important in terms of productivity and obtained enantiomeric excess. A selection of representative experiments at different pH levels are shown in Figure 4.

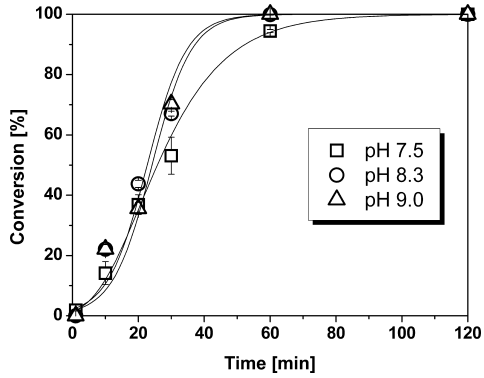

Figure 4. Effect of pH on the ECS-PLE06-catalyzed conversion of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate. Conditions: 50 mM phosphate buffer, 23 °C, 10 mM substrate; 4 U/mL enzyme (according to standard activity assay).

It is clearly shown that the process is very robust with respect to pH. No significant differences were found between results at pH 7.5, 8.3, and 9.0. Full conversion with excellent enantiomeric excess (>99.5% ee) were achieved at pH 8.3 and 9.0 after 60 min, and only a slightly longer reaction time was required at pH 7.5. Similar results with a broad pH optimum between 7.5 and 9.5 were reported by Lange et al. with a recombinant pig liver esterase for the hydrolysis of *p*-nitrophenyl acetate.[25] Thus, a relatively stable pH control at pH 8–9 will be sufficient for a large scale synthesis of **2a**.

Scale-Up. Based on the initial experiments, the scale of the reaction was gradually increased to facilitate the desymmetrization of **1** at higher substrate concentrations. Unfortunately, such an increase yields the formation of a stoichiometric amount of **2a**, which is an acid and lowers the pH in the aqueous solution. Thus, an efficient pH-adjustment technique is essential to maintain optimal reaction conditions for the biocatalyst throughout the reaction, typically by an external addition of a base solution. Table 1, entries 1–4, shows exemplarily the results via external pH controller (pH-stat titration using NaOH solution). Reaction volumes from 20 to 630 mL with substrate concentration of 80–100 mM were tested, and high conversions with excellent enantioselectivities were obtained. Experiments at smaller scale (20–40 mL) demonstrated clearly that the reaction allows full conversion

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Table 1. Desymmetrization of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate at larger scale with pH-adjustment by an external base solution or the acid scavenger $NaHCO_3$

entry	T [°C]	V [L]	substrate [mM]	reaction time [h]	activity[a] [U/mL]	conversion [%]	STY [g/(L·h)]	isolated yield [%]
					pH-stat			
1	25	0.02	100	10.0	3.8	>99	1.8	
2	25	0.02	100	3.9	5.7	>99	4.7	83
3	40	0.04	80	2.3	3.0	>99	6.4	83
4	40	0.63	80	8.0	4.8	91	1.8	75
					$NaHCO_3$			
5	25	0.04	80	6.5	3.0	>99	2.3	
6	40	0.04	80	1.2	6.1	>99	12.3	91
7	40	0.04	200	1.2	15.2	>99	30.7	74
8	40	8.80	200	4.0	5.5	>99	9.2	82

[a]Used volumetric enzyme activity; calculated from specific activity [U/mg] according to standard activity assay and enzyme amount [mg] per volume [mL].

after 3.9 h at 25 °C, which can be further decreased to 2.3 h at 40 °C, representing a maximum space-time yield of 6.4 g/(L·h). Unfortunately the application of larger volumes (0.63 L) requires also larger amounts of NaOH for pH-adjustment, which may result in a decrease of enzyme stability due to local high-pH hot spots. For example only 91% conversion was obtained at 630-mL scale, even with a prolonged reaction time of 8 h. Apparently an insufficient mixing of the reaction solution with the aqueous base for pH-adjustment at larger scale causes strong fluctuation of pH and thus local enzyme denaturation with a loss of enzyme activity. An adjustment of the reactor geometry and base dosing may minimize this problem but will require further investigations and still needs higher and therefore uneconomic enzyme loadings to overcome these limitations. The fluctuation of pH, especially at the early stage of the reaction, is exemplarily shown in Figure 5.

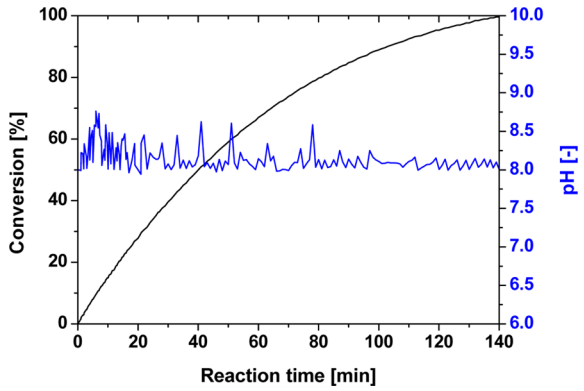

Figure 5. pH-dependent addition of a base solution causes fluctuations of the pH, depending on the reactor geometry, especially during the beginning of the reaction. Conditions: total volume 0.04 L, 80 mM substrate, 40 °C, pH control with 0.5 M NaOH solution, pH measurement near the buret; conversion was calculated from NaOH consumption.

As an alternative other techniques for internal pH control were evaluated, to overcome the limitations based on pH fluctuation. High concentration buffers were excluded since very high buffer concentrations would be required, which is impractical due to costs and limited salt solubility. Fortunately, weak bases such as $CaCO_3$ or $NaHCO_3$ were found to be compatible with the enzymatic reaction. The highest potential was demonstrated for sodium hydrogen carbonate ($NaHCO_3$), which exhibits a sufficient solubility in aqueous systems and maintains an optimal pH slightly above 8.[38] Formed monoester **2a**, a monocarboxylic acid, is rapidly neutralized by sodium hydrogen carbonate. The formation of gaseous carbon dioxide (CO_2) can be visualized with a bubble counter and represents also a rudimental reaction control. With this technique optimal reaction conditions are easily maintained without any further addition of secondary reactants (base solution). As shown in Table 1, entries 5−8, higher substrate concentrations of up to 200 mM were successfully applied and allowed full conversion at even shorter reactions times in comparison to the classical technique. A maximum space-time yield of 30.7 g/(L·h) was found, which is a 5.6-fold increase over the best example with a classical pH-adjustment via addition of a base solution.

A preparative synthesis at larger scale was performed at 350 g substrate scale (1.77 mol) with the prior defined optimal reaction conditions, but with a slightly more economic enzyme usage (entry 8). The required pH control was herein facilitated with the presented simplified $NaHCO_3$ protocol and allowed full conversion of **1** to **2a** after approximately 240 min (Figure 6).

Interestingly, initially produced **2a** was obtained with lower enantiomeric excess and slowly reached an excellent enantiomeric excess of >99.5% ee when full conversion was achieved. The initial presence of small amounts of racemic product **2**, from a nonenzymatic hydrolysis prior the enzymatic reaction experiment, reduces the initial enantiomeric excess to ∼91% (30 min). With the continuous progression of the fully enantioselective biocatalytic reaction the enantiomeric excess slowly increased to >99.5 ee.

The pH remained stable slightly above 8 throughout the whole experiment. Formed CO_2 from the acid-induced decomposition of $NaHCO_3$ was captured and visualized with a gas bubble counter. After the full conversion was achieved, the reaction was stopped with an excess of hydrochloric acid and subjected to work-up. In total 265 g (82% yield) of enantiopure (1*S*,2*R*)-monoester **2a** was obtained, which corresponds to a space time yield of 9.2 g/(L·h).

■ CONCLUSION

A novel and efficient process for the recombinant pig liver esterase-catalyzed desymmetrization of **1** to (1*S*,2*R*)-1-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid **2a** was presented. For this purpose six commercially available recombinant isoenzymes of pig liver esterase were investigated within this study, and the most potent isoenzyme ECS-PLE06 was chosen. Optimal reaction conditions were determined and

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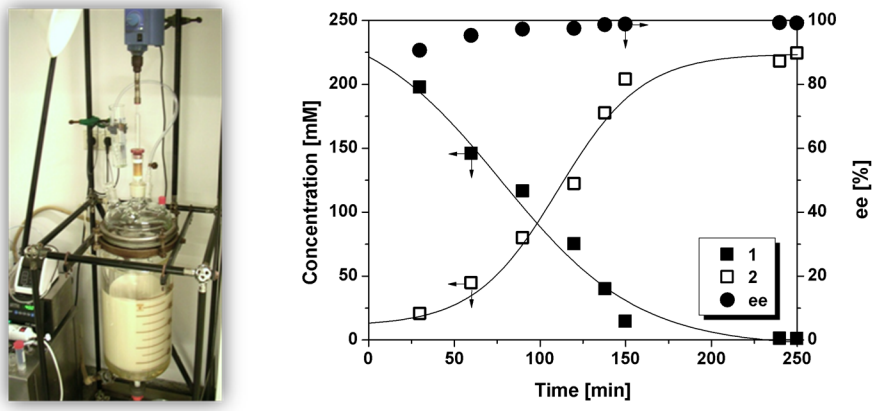

Figure 6. Scaled-up desymmetrization of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate. (Left) Temperature-controlled bioreactor with a total capacity of 10 L. (Right) Time course of concentration of reagent **1** (■), concentration of product **2** (□), and ee (●) as determined by GC analysis. Conditions: 200 mM substrate, 5.5 U/mL ECS-PLE06, total volume 8.8 L, stirrer speed 300 rpm, 40 °C, pH control via acid scavenger $NaHCO_3$.

eventually transferred for scale-up enabling very high space-time yield. A simplified pH control, applying $NaHCO_3$ as an acid scavenger, was used, which stabilizes the pH at slightly above 8 and requires no further equipment for dosing of a base. A large scale synthesis yielded 265 g of enantiopure (1*S*,2*R*)-1-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid **2a**. Further optimization may include even higher substrate concentrations or enhanced enzyme formulation. In addition, the presented pH regulation concept with sodium hydrogen carbonate $NaHCO_3$ can be easily transferred to similar processes.

As shown in this study, recombinant pig liver isoenzymes can be easily used at larger scales with high substrate concentrations. The enhanced enantiomeric excess of the target substance **2a** shows a significant advantage over the use of classical preparations of pig liver esterases from mammalian sources.

■ EXPERIMENTAL SECTION

General. All chemicals and solvents were purchased from Alfa Aesar (Ward Hill, USA), Sigma-Aldrich (St. Louis, USA), and Carl Roth GmbH & Co KG. (Karlsruhe, Germany) and used as received. Deionized water was used throughout this study.

Synthesis of Dimethyl Cyclohex-4-ene-*cis*-1,2-dicarboxylate 1. A 4 L round-bottom flask was charged with 500 g (2.9 mol) of *cis*-cyclohex-4-ene-1,2-dicarboxylic acid, 2.4 L of methanol, a magnetic stir bar, and a reflux condenser. Subsequently 125 mL of concentrated sulfuric acid (98%) was added dropwise to the stirred suspension. The reaction mixture was heated to reflux and stirred for 48 h. The resulting solution was partially evaporated under reduced pressure to volume of 1 L. The concentrate was extracted three times with 1 L of MTBE. The combined organic layers were washed with deionized water, neutralized with a concentrated aqueous sodium hydrogen carbonate solution, and washed again with deionized water. The ether was removed under reduced pressure to give 495 g (yield 85%) of the clear oil **1**. Details are given in the Supporting Information.

Enzymes. Recombinant pig liver esterases ECS-PLE01 to ECS-PLE06 are commercially available (as lyophilizate) from Enzymicals AG (Greifswald, Germany), and the esterase from porcine liver (mixture of isoenzymes as crude protein powder) was purchased from Sigma-Aldrich (St. Louis, USA).

Standard Activity Assay. The activity of PLEs was determined using *p*-nitrophenyl acetate (*p*NPA, 30 mM dissolved in DMSO) with spectrophotometric quantification of the *p*-nitrophenolate release over 100 s at 401 nm ($\varepsilon_{30\ °C}$ = 17 759 L/(mol·cm)) containing 850 μL of phosphate buffer (pH 7.5, 50 mM), 50 μL of enzyme solution, and 100 μL of 30 mM *p*-nitrophenyl acetate in DMSO. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenolate per minute under standard assay conditions. In order to detect changes in absorbance we used a UV−vis spectrometer (Specord 50, Analytic Jena AG, Jena and Helios Alpha UV−vis spectrophotometer, Thermo Scientific GmbH, Schwerte, Germany) equipped with a reference cell including equal solutions without enzyme. Data are reported as the mean ± standard deviation of triplicate measurements.

Isoenzyme Screening. Enzyme activity screening was executed with a TitroLine 7000 from SI-analytics (Mainz, Germany) in pH-Stat mode. A 100 mg portion of ECS-PLE01−06 (lyophilized) was suspended in 15 mL of 5 mM sodium phosphate buffer pH 8.0 at 40 °C. The reaction was started by adding 45 mg (0.2 mmol) of diester **1**. The activity of the enzyme was calculated on the basis of the consumption of 50 mM sodium hydroxide solution. One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 μmol of dimethyl cyclohex-4-ene-*cis*-1,2-dicarboxylate **1** per minute under the mentioned assay conditions.

Small scale desymmetrization experiments were carried out in 5 mL glass vials with 10 mg of the respective isoenzyme in 4 mL of a buffered solution (0.1 M phosphate buffer, pH 7−8) with 10 mM diester **1**. The enzymatic hydrolysis was conducted at 10, 23, and 40 °C. Samples (100 μL) were taken in intervals, acidified with 5 μL of 6 M HCl (pH 2), and extracted with EtOAc (200 μL). Conversion and enantiomeric excess of the enzymatic reactions were obtained from analysis with gas chromatography (see below).

Small-Scale Biocatalysis with ECS-PLE06 (pH-stat). A 100 mL round-bottom flask was charged with 40 mL of deionized water, ECS-PLE06, and a magnetic stir bar. The suspension was set to 40 °C and pretitrated to pH 8. The reaction was started by adding 0.6 g (3.2 mmol) of **1** to the reaction mixture containing 50 mM sodium phosphate buffer pH 8.0. The solution was kept constant at pH 8 by addition of 0.5 M sodium hydroxide solution via an autoburet. After the

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addition of the required NaOH solution, the reaction was stopped by addition of concentrated hydrochloric acid (adjusted to pH 1−2). The precipitated protein was spun down, and the aqueous layer extracted three times with 40 mL of ethyl acetate. The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give 0.56 g of **2** as yellowish oil (95% yield). After crystallization the product was isolated as 0.49 g of white crystals with 83% yield and excellent enantiomeric excess (>99.5% ee).

Small-Scale Biocatalysis with ECS-PLE06 (Sodium Hydrogen Carbonate). A 100 mL round-bottom flask with an integrated CO_2-bubble counter was charged with 40 mL of saturated sodium hydrogen carbonate solution, ECS-PLE06, and a magnetic stirrer. The reaction was started by adding 0.6 g (3.2 mmol) of **1** to the reaction mixture and stirring at 40 °C. The process was monitored by TLC and monitored by a bubble counter. After 70 min (TLC analysis indicated full conversion) the reaction was stopped by adding concentrated hydrochloric acid to pH 1−2, precipitated protein was removed by centrifugation, and the resulting aqueous layer was extracted three times with 40 mL of MTBE. The combined organic phases were dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give 0.53 g of crystalline product (91% yield, >99.5% ee).

Large-Scale Biocatalysis with ECS-PLE06 (Sodium Hydrogen Carbonate). A stirred-tank reactor (STR) was charged with 8.5 L of deionized water, 500 g of sodium hydrogen carbonate, and ECS-PLE06. The suspension was heated to 40 °C, and the reaction was started by adding 350 g (1.77 mol) of **1** to the stirring solution. The pH was kept constant at 8 during the whole process. After 4 h the reaction mixture was heated to 95 °C to denature the protein. Afterwards the suspension was acidified with concentrated hydrochloric acid to pH 1. The precipitated protein was spun down, and the aqueous layer was extracted three times with 8.8 L of MTBE (Cryofuge 8500i, Thermo Scientific, Schwerte, Germany). The combined organic phases were dried over $MgSO_4$ and evaporated under reduced pressure to give 265 g crystalline monoester **2** (82% yield, >99.5% ee), mp 65 °C; $[\alpha]_D^{22}$ +12.5 (*c* 1.0, methanol). Analytical details are given in the Supporting Information.

Analytical Methods. Conversion and the corresponding enantiomeric excess of **2** were quantified by GC-FID using a CP3800 equipped with an autosampler CP8400 (all formerly Varian, now Agilent, Santa Barbara, USA) and a Chrompack Chiralsil Dex-CB column (25 m × 0.25 mm × 0.25 μm). The injector temperature was set to 220 °C, the detector temperature to 250 °C. Temperature gradient: 130 °C for 12 min, rise with 10 °C/min to 170 °C and hold for 20 min. Retention times: 23.9 min (1*R*,2*S*)-1-(methoxycarbonyl)-cyclohex-4-ene-1-carboxylic acid **2b** and 24.8 min (1*S*,2*R*)-1-(methoxycarbonyl)cyclohex-4-ene-1-carboxylic acid **2a**. The reactions were monitored by TLC, if required (cyclohexane/2-propanol, 95:5; R_f: diester **1**, 0.6; monoester **2**, 0.3; diacid, 0.2). Plates were stained with $KMnO_4$ solution (3 g $KMnO_4$, 20 g K_2CO_3, 5 mL 5% NaOH, 300 mL H_2O). Melting points were determined using a Mettler Toledo FP 90/82 HT with a Leitz microscope and are uncorrected. Highly sensitive differential scanning calorimetry (DSC, Mettler Toledo DSC 823e) was used for determination of oxidation stability and melting behavior of the crystals. Optical rotations for solutions in a 1 dm cell were obtained with an automatic polarimeter (Polar L-μP, IBZ).

NMR Analysis. ^1H NMR and ^{13}C NMR spectra were recorded with a Bruker AVANCE (250 II) in $CDCl_3$ or DMSO-d_6. Chemical shifts are reported in parts per million relative to the solvent peak or TMS as an internal reference. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TLC, thin layer chromatography; (−)-FTC, (−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; MRSA, methicillin-resistant *Staphylococcus aureus*

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