Continuous Flow Enzymatic Kinetic Resolution and Enantiomer Separation using Ionic Liquid/Supercritical Carbon Dioxide Media

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Abstract: The combination of kinetic resolution in ionic liquids (IL) and selective extraction with supercritical carbon dioxide ($scCO_2$) provides a new approach for the separation of enantiomers as exemplified by the lipase-catalyzed esterification of chiral secondary alcohols. Excellent enantioselectivities are achieved upon conversion of alcohols **1a–e** to the corresponding acetates **4a–e** or laureates **5a–e** using various modifications of the lipase from *Candida antarctica* (CaL-B) in imidazolium-based ionic liquids. The anion of the ionic liquid has a significant influence on the performance of the bio-catalyst with bis(trifluoromethanesulfonamide) [BTA] giving the best results. The acetates **4a–e** can be extracted from the reaction mixture preferentially over the alcohols **1a–e** with $scCO_2$ under certain conditions, but preparatively useful selectivities would require advanced multi-step extraction procedures. In contrast, efficient separation is possible with relatively simple equipment if alcohols **1a–e** are extracted preferentially from their corresponding laureates **5a–e**. A "green" continuous process for the resolution of racemic alcohols without the use of organic solvents was devised on the basis of these findings.

Keywords: ionic liquids; kinetic resolution; lipases; supercritical carbon dioxide; supercritical fluids

Introduction

Efficient methodologies for the production of enantiomerically pure or enriched compounds are of great academic and industrial importance, (bio)catalytic kinetic resolution being an attractive option.[1,2] Lipase-catalyzed acylation of chiral alcohols is of particular interest as a preparative tool and as a benchmark reaction.[1,3] The main disadvantage for practical application results from the necessity to use chromatography for separation of the alcohol and ester, requiring the use and recycling or disposal of large volumes of organic solvents. Of course, if the particular system is amenable to dynamic kinetic resolution, a separation problem does not arise.[4] If this is not possible, alternative strategies need to be developed, and indeed, several innovative suggestions have been made recently. For example, scavenging of the unreacted alcohol on a solid support was used to enable separation by subsequent filtration.[5] In a different approach, batch-wise separation by multistep liquid/liquid extraction was achieved in certain cases through the use of succinic anhydride as the acylating agent.[6] In this case, the semi-ester formed in the lipase-catalyzed reaction is soluble in the aqueous phase, while the unreacted antipode remains in the organic phase, allowing for convenient separation. In yet another approach preferential partitioning of ester and alcohol was achieved by using a highly fluorinated acylating agent followed by separation in an organic/fluorous biphasic system.[7] We introduce here a new methodology based on the combination of an ionic liquid (IL) and supercritical carbon dioxide ($scCO_2$) as the solvent system for reaction and separation.

Several groups have demonstrated the feasibility of employing ionic liquids (ILs)[8] as solvents in enzymatic catalysis,[9] including the use of lipases in the kinetic resolution of chiral alcohols.[10] According to these reports, suspensions of lipases in ionic liquids can have several advantages such as high enzyme stability and sometimes even enhanced activity and stereoselectivity. However, although the actual kinetic resolution was performed in ionic liquids, organic solvents were in fact used to remove the mixture of ester and non-reacted alcohol from the ionic liquid phase and further separa-

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tion required conventional, chromatographic techniques. Obviously, this defeats part of the original goal as defined by the criteria of green chemistry.[11] Stimulated by pioneering work of Beckman and Brennecke on the phase behavior of IL/CO_2,[12] we recently developed a new and environmentally benign protocol for lipase-catalyzed reactions in ionic liquids which makes use of supercritical carbon dioxide ($scCO_2$)[13] as a means to extract the products in a completely solvent-free form.[14,15] This approach allows the enzyme/ionic liquid mixture to be recycled and re-used in batch-wise or continuous-flow operations. Independently, Lozano et al. described the combination of ionic liquids and $scCO_2$ in a lipase-catalyzed transesterification, whereby the starting ester was injected into the system in the form of a hexane solution.[16a] In other work the influence of compressed CO_2 on the transesterification performance of α-chymotrypsin in ionic liquids was studied by Laszlo et al.[16b]

In the present paper, we describe in detail the application of our new IL/$scCO_2$ system for lipase-catalyzed kinetic resolution of chiral secondary alcohols. In particular, we report how this approach allows us to combine (bio)catalytic esterification with highly efficient enantiomer separation in a continuous flow operation avoiding the use of organic solvents completely. The underlying principle of this approach is outlined in Figure 1. The racemic alcohol and the acylating agent are transported into the reactor using $scCO_2$ as the mobile phase. There, one of the enantiomers is esterified selectively by the lipase in the ionic liquid and the mixture of products is continuously extracted with the $scCO_2$ stream. Ester and unreacted alcohol are then separated downstream by controlled density reduction *via* variation of temperature and/or pressure. Judicious choice of the acylating agent allows modification of the molecular structure of the ester to exhibit either higher or lower solubility in $scCO_2$ than the corresponding alcohol, which is crucial for efficient extractive separation. Vinyl laureate (**3**) was found to be a cheap acylation agent that renders the ester less soluble than the alcohol, which allows for efficient separation.

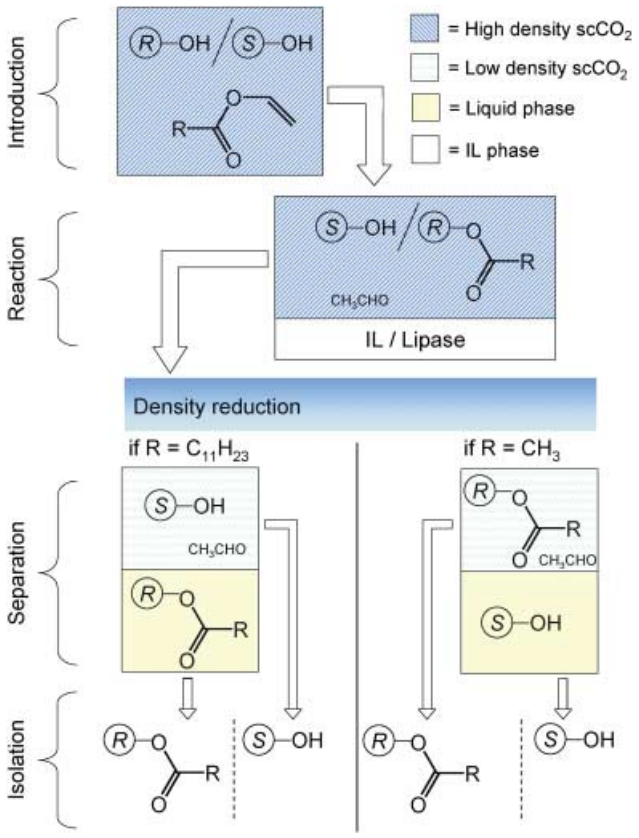


Figure 1. Strategies for enantiomer separation *via* (bio)catalytic kinetic resolution and selective supercritical extraction in an ionic liquid/$scCO_2$ continuous flow system.

Results and Discussion

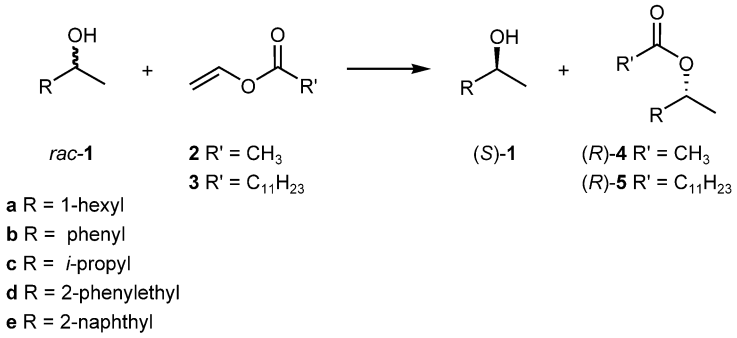
Our study focused on lipase B from *Candida antarctica* (CaL-B) as a transesterification catalyst known to exhibit high activities and selectivities for a broad range of substrates in organic solvents.[1,3] Exploratory experiments to select the optimum combination of ionic liquid and lipase modification were carried out using 2-octanol (**1a**) and vinyl acetate (**2**) as standard substrates under conventional conditions (Scheme 1, Table 1). All ionic liquids examined here resulted in high enzyme activity, substantiating previous findings that ILs with lipophilic non-coordinating anions are the best choice for enzymatic catalysis.[9] Even within this class of ILs, the anion has a significant influence on the performance of the bio-catalyst. With 1-butyl-3-methylimidazolium [BMIM] as cation, the bis(trifluoromethanesulfonamide) anion [BTA] led to the highest selectivity with almost perfect discrimination between the two enantiomers. The selectivity was largely unaffected by the choice of the enzyme modification. Surprisingly,[17] the activity was higher with the commercial lyophilized biocatalyst as compared to the supported enzyme either in form of Novozyme SP 435 or as a sol-gel immobilizate.[18] Nevertheless, the immobilized enzymes were chosen for subsequent studies because they did not result in significant transesterification beyond 50% conversion even after prolonged reaction times.

Initial experiments directed towards the selective separation of ester and unreacted alcohol with $scCO_2$ were carried out for the kinetic resolution of 1-phenylethanol (**1b**) with vinyl acetate (**2**) using the apparatus depicted in Figure 2. The system comprises a window-equipped 10-mL stainless steel reactor that is heated, and agitation is ensured by a magnetic stirring bar. Initially, the reactor is charged with the suspension of the immobilized enzyme in the ionic liquid followed by the

Table 1. Influence of solvent and lipase-preparation on kinetic resolution of alcohol **1a**.

Entry	Reaction Medium	Biocatalyst	ee (**1a**)[a] [%]	ee (**4a**)[a] [%]	Activity [μmol min^{-1} g^{-1}]	Conversion [%]		
						15 min	18 h	68 h
1	Isooctane	Lyophilized Cal-B	>99.9	88.6	–	53.0	82.2	–
2	[BMIM][PF_6]	Lyophilized Cal-B	94.9	89.8	–	51.4	100	–
3	[BMIM][BF_4]	Lyophilized Cal-B	>99.9	85.7	–	53.9	70.8	–
4	[BMIM][BTA]	Lyophilized Cal-B	99.2	98.7	–	50.1	65.2	–
5[b]	[BMIM][BTA]	Lyophilized Cal-B	98.4	97.8	5800	41.6	–	57.0
6[b]	[BMIM][BTA]	Novozyme SP 435	>99.9	98.2	1190	11.8	–	50.4
7[b]	[BMIM][BTA]	Sol-gel immobilizate	>99.9	99.6	1250	10.4	–	50.9

Quantities: **1a** = 0.5 mmol; **2** = 1 mmol; biocatalyst = 5 mg; reaction medium = 1 mL. Reaction conditions: thermo mixer 1000 rpm; T = 45 °C.
[a] Measured at approximately 50% conversion.
[b] Biocatalyst = 2.5 mg.



Scheme 1. Stereoselective acylation of chiral alcohols.

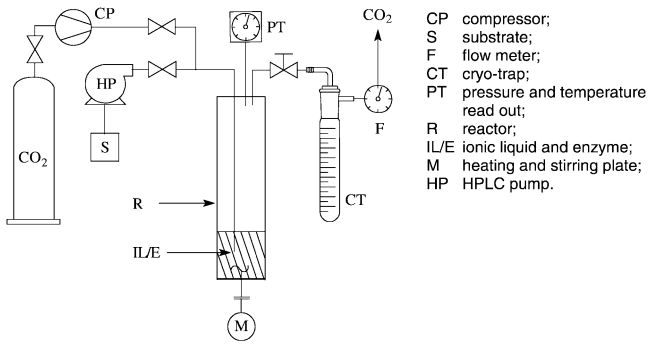


Figure 2. Reaction/separation system for batch-wise combination of enzymatic kinetic resolution and enantiomer separation using an ionic liquid/$scCO_2$ medium.

liquid substrate. After heating for the desired reaction time, CO_2 extraction is carried out using a compressor and a capillary tube dipping into the IL phase. After complete extraction, new substrate can be introduced by an HPLC pump without depressurization.

As reported previously, batch-wise extraction with $scCO_2$ at 40 °C and 110 bar allows the complete recovery of a 1:1 *mixture* of (*R*)-**4b** and (*S*)-**1b**, each in high enantiomeric purity amounting to 95 and 99.6% ee, respectively.[14] In agreement with the generally higher CO_2 solubility of acetates as compared to the corresponding alcohols in $scCO_2$,[19] we found that an enrichment of ester (*R*)-**4b** can be achieved when extraction from the IL is carried out at lower CO_2 densities. A maximum enrichment of (*R*)-**4b**:(*S*)-**1b** of 1.4:1 was achieved in the CO_2 stream at 60 °C and 90 bar in a single extraction step. After extraction of about 65% of the total ester formed, an alcohol rich fraction [(*R*)-**4b**:(*S*)-**1b** = 1:3] was obtained upon increasing the CO_2 pressure to 150 bar. Similarly, moderate levels of enrichment could be achieved with 1-(2-naphthyl) ethanol (**1e**) as substrate using this methodology [(*R*)-**4e**:(*S*)-**1e** = 1.9:1 at 60 °C/110 bar; (*R*)-**4e**:(*S*)-**1e** = 1:4.2 at 50 °C/185 bar].

In agreement with findings of other researchers, these data suggest that a preparatively useful separation of alcohols and acetates obtained from enzymatic kinetic resolutions requires the use of advanced multi-step extraction procedures.[20] However, it appeared intriguing to us to improve the separation efficiency by *molecularly engineering* the relative solubility of alcohol and ester in $scCO_2$ (see Figure 1). The solubility of a compound in $scCO_2$ depends critically on polarity, vapor pressure, cohesive energy, and the presence of special "CO_2-philic" groups.[21,22] In the present case, we decided

to concentrate on vapor pressure as the variable because it is readily influenced by variation of the chain length of the carboxylate group in the acylating agent. If the chain is long enough, the low volatility of the molecule dominates over the influence of polarity and the ester should become less soluble in $scCO_2$ than the corresponding alcohol. Gratifyingly, a cheap and readily accessible long-chain acylating agent is available in he form of vinyl laureate (**3**) which is produced on a large technical scale as a co-monomer for vinyl polymers.

Figure 3 demonstrates the viability of this concept for the lipase-catalyzed kinetic resolution of 2-octanol (**1a**) coupled with a simple isobaric extraction at 60 °C and 90 bar. In the early fractions, the alcohol (*S*)-**1a** is extracted with high selectivity ((*R*)-**5a**:(*S*)-**1a** = 1:18), whereas the lauryl ester (*R*)-**5a** is obtained with high purity in the later fractions [(*R*)-**5a**:(*S*)-**1a** = 21:1).[23] In order to speed up the extraction for the ester-rich fraction, it is more convenient to increase the CO_2 density after most of the alcohol has been isolated. This simple and non-optimized procedure yields efficient levels of separation for a variety of secondary alcohols **1** and their corresponding lauryl esters **5** (Table 2). For example, in the case of 3-methyl-2-butanol (**1c**) the enrichment reaches a ratio of (*R*)-**5c**:(*S*)-**1c** = 1:20 in the low density fractions (90 bar/60 °C) and (*R*)-**5c**:(*S*)-**1c** = 14:1 in the high density fractions (200 bar/45 °C). This corresponds to 95.2% alcohol selectivity and 93.5% ester selectivity, each with high levels of enantiomeric purity of > 99.5% for (*S*)-**1c** and 92.8% for (*R*)-**5c**, respectively (Table 2).

In order to increase the separation, an additional separation chamber can be introduced between the reactor and the cryo-trap of the apparatus depicted in Figure 2. This was applied to the kinetic resolution of *rac*-**1b**. After transesterification with Novozyme SP 435 in [BMIM][BTA] at 45 °C for seven hours, the resulting mixture of (*S*)-**1b** and (*R*)-**5b** was extracted at 60 °C and 105 bar into the separation chamber where the pressure was reduced to 80 bar before the CO_2 stream was finally vented through cryo-traps that were changed periodically (total extraction time approximately 90 h). With this simple procedure, 66% of the theoretical amount of (*S*)-**1b** was isolated from the first four traps with an enantiomeric purity > 99.9% and less than 0.5% contamination with (*R*)-**5b**. The next two traps contained small amounts of a mixed fraction, after which the pressure in the reaction and extraction chamber was increased stepwise to 200 bar, yielding a total of 89% of the theoretical amount of (*R*)-**5b** (ee > 99.9%) with less than 1% contamination with alcohol (*S*)-**1b**.

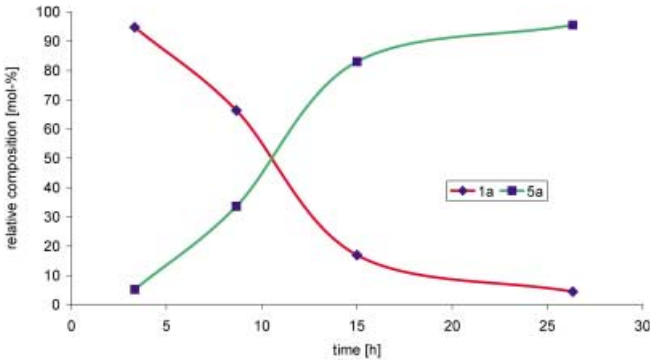


Figure 3. Separation of (*S*)-2-octanol [(*S*)-**1a**] and the lauryl ester of the (*R*)-enantiomer [(*R*)-**5a**] obtained from lipase-catalyzed kinetic resolution in [BMIM][BTA] by isobaric extraction with $scCO_2$ (60 °C/90 bar).

Following the CO_2 extraction, the mixture of ionic liquid and biocatalyst remaining in the reactor can be reused without apparent loss of activity and selectivity.[14] This remarkable stability is independent of the alcohol used. For *rac*-**1c**, nine consecutive batches were run with no significant changes in the enzyme's performance, and separation efficiencies of > 90% for alcohol and esters were achieved in the selective extractions in each case. It was also possible to carry out several sequential trans-

Table 2. Results of kinetic resolution and extraction.

Substrate	Extraction Conditions	Selectivity for **1** [%]	Selectivity for **4** [%]	ee (**1**) [%]	ee (**4**) [%]
1a[a]	90 bar, 60 °C	94.8	95.5	> 99.5	85.2
1b[b]	90 bar, 60 °C/200 bar, 45 °C	93.5	66.3	98.9	> 99.5
1c[c]	75 bar, 60 °C/200 bar, 45 °C	95.2	93.5	> 99.5	92.8
1d[d]	100 bar, 60 °C/200 bar, 45 °C	95.4	72.5	65.0	88.2
1e[e]	110 bar, 60 °C/200 bar, 45 °C	75.8	69.2	98.2	75.0

Quantities: Novozyme SP 435 = 100 mg; [BMIM][BTA] = 3 mL; (*rac*)-**1** = 4 mmol; **3** = 6 mmol. Reaction conditions:
[a] 65.5 h; $T = 60$ °C; $p = 100$ bar (CO_2).
[b] 65.5 h; $T = ca.$ 20 °C; atmospheric pressure.
[c] 61 h; $T = ca.$ 20 °C; atmospheric pressure.
[d] 15 h; $T = ca.$ 20 °C; atmospheric pressure.
[e] 65.5 h; $T = 60$ °C; $p = 110$ bar (CO_2).

esterifications with different alcohols using the same IL/enzyme mixture. Encouraged by the long-term stability of the biocatalytic system, we envisaged continuous-flow operation of the combined kinetic resolution and separation procedure.[24]

The apparatus applied for continuous-flow processes is depicted in Figure 4. A cascade of two reactors of different geometries was used in order to ensure conversion close to the theoretical maximum of 50% without the necessity for detailed optimization of substrate and CO_2 flow rates. The separation part consisted of two high pressure chambers connected with a recirculating pump and the possibility to introduce additional CO_2 independently of the exit flow of the reactor. Recycling of CO_2 at the end of the process was not implemented at this stage of the investigations, but is theoretically possible. The kinetic resolution of racemic **1b** was used as a model reaction to demonstrate the principle features of the continuous reaction/separation sequence.

The mixture of *rac*-**1b** and **3** was passed through the reactor part in a stream of high density $scCO_2$ as the mobile phase and transesterification occurred upon contact with the biocatalyst suspended in the ionic liquid [BMIM][PF_6]. The CO_2 stream was expanded into the first separation chamber where a liquid phase was formed due to the density reduction and concomitant lowering of the solvent power of CO_2. Extraction was continued with additional CO_2 to the second chamber associated with another density reduction. The liquid phase formed in the second chamber was recycled periodically into the first vessel. The chiral alcohol (*S*)-**1b** was separated continuously from the final CO_2 stream upon venting through a cryo-trap, whereas the ester (*R*)-**5b** accumulated in the first separation chamber.

After continuous operation for 112 h, substrate delivery was stopped and extraction continued until no more alcohol was contained in the CO_2 stream. A total of 5.1 g (81% of theory) of (*S*)-**1b** was collected in the cryo-trap with an ee of $> 97\%$ containing less than 0.1% of ester (*R*)-**5b**. From the first extractor, (*R*)-**5b** (15.2 g, 97% of theory) was obtained with an ee $> 97\%$ and $< 0.5\%$ of (*S*)-**1b**. After resuming the substrate flow, the process started again with identical activity and selectivity, proving again the remarkable long-term stability of the biocatalyst under the reaction and extraction conditions.

Conclusions

In summary, we have developed a new method for enantiomer separation based on a combination of (bio)catalytic kinetic resolution and supercritical fluid extraction using an ionic liquid/$scCO_2$ system. The interplay of molecular engineering and process optimization makes this approach highly flexible as demon-

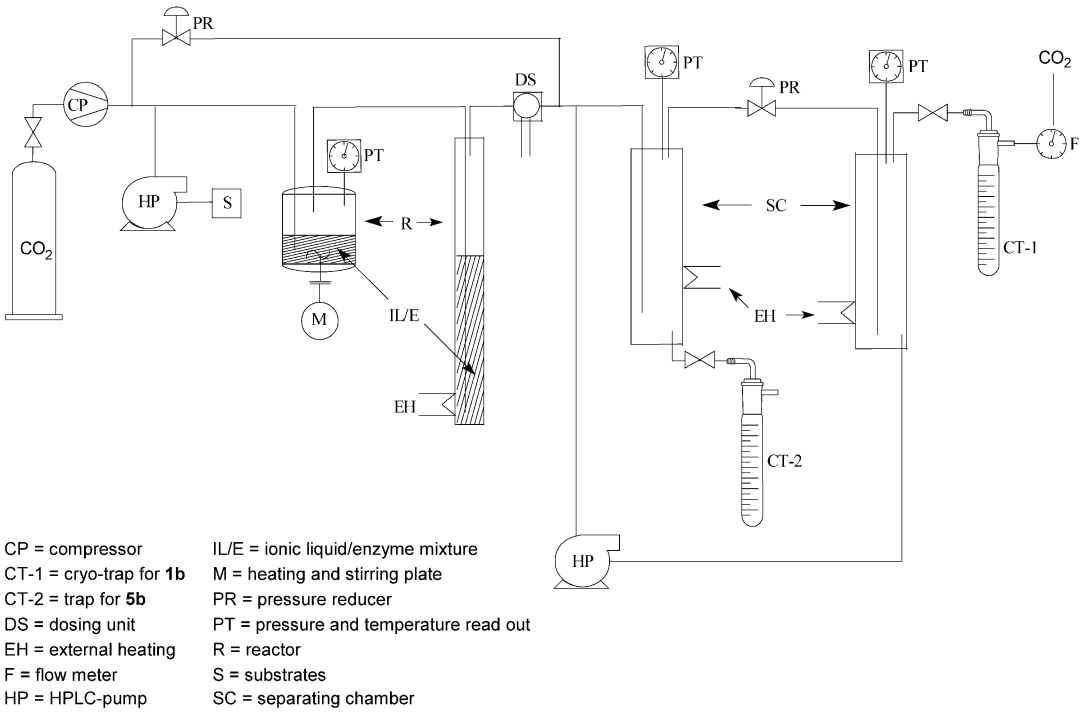


Figure 4. Reaction/separation system for continuous-flow combination of enzymatic kinetic resolution and enantiomer separation using an ionic liquid/$scCO_2$ medium.

strated for the lipase-catalyzed esterification of racemic secondary alcohols. Preparatively useful separation efficiencies can be achieved in single step extractions when vinyl laureate is used as cheap acylating agent which reduces the CO_2 solubility of the ester relative to the alcohol. Excellent enantiomer separation, high enantioselectivity and pronounced long-term stability of the lipase are key features of the process. At the same time, the methodology avoids the use of organic solvents completely and allows for continuous flow operation. We therefore believe that this novel approach provides a promising basis for the development of environmentally benign methodologies for the practical production of enantiomerically pure or enriched compounds.

Experimental Section

Caution

The handling of pressurized gases must be carried out only using suitable equipment under appropriate safety conditions.

Materials and Analysis

The biocatalysts used in this study were obtained from Roche (lyophilized CaL-B: Chirazyme L-2) and Novo Nordisk (immobilized CaL-B: Novozyme SP 435) or prepared according to known procedures (sol-gel immobilized CaL-B).[18] The ionic liquids were purchased from Solvent Innovation, Germany,[25] and used after filtration over a short path of silica using methylene chloride as the mobile phase. The secondary alcohols **1a–e**, vinyl acetate (**2**) and vinyl laureate (**3**) were commercial products and used as received. Analysis of the product mixtures for conversion and composition was achieved by ^{1}H NMR spectroscopy of $CDCl_3$ solutions using a Bruker DPX 300 or AMX 400 spectrometer. The enantiomeric purity was determined by GC or HPLC in the Department of Chromatography at the Max-Planck-Institut für Kohlenforschung.[26]

Batchwise Kinetic Resolution and Subsequent Extraction with $scCO_2$

Experiments were carried out using the apparatus depicted in Figure 2. A suspension of immobilized CaL-B (Novozyme SP 435; 100 mg) in [BMIM][BTA] (3 mL) was added in a window-equipped stainless-steel high pressure reactor (V = 10 mL) equipped with a magnetic stirring bar, a thermocouple, a pressure sensor and an inlet and outlet valve. The racemic alcohol (4 mmol) and acylating agent (6 mmol) were then introduced. The reaction mixture was stirred at the desired temperature for a given time as indicated in Table 2.

After the desired conversion was reached, the products were extracted by flushing CO_2 *via* a capillary through the ionic liquid. At the reactor exit, the CO_2 stream was depressurized using a heated needle valve and the organic materials were collected from the gas stream in cryo-traps at 0 °C, which were changed periodically during the extraction procedure. Generally, the extraction conditions were initially set at high temperature and low pressure (low density) and gradually changed to higher densities by increasing the pressure and/or lowering the temperature (see Table 2 and text for details). Typically, CO_2 flow rates were adjusted to $10-20\ L \cdot h^{-1}$ (measured as exit flow after venting).

Continuous-Flow Combination of Kinetic Resolution and Extraction with $scCO_2$

In the apparatus shown in Figure 4, a mixture of *rac*-**1b** and **3** (molar ratio 1:2) was introduced into a stream of high density $scCO_2$ (50 °C/200 bar) using a HPLC pump at a flow rate of $0.6\ mL \cdot h^{-1}$. The mobile phase passed through the cascade of two small reactors ($V = 10$ mL and 15 mL, respectively) that were filled with immobilized CaL-B as a suspension in the ionic liquid [BMIM][PF_6] (3 mL and 8 mL, respectively). A mixture of commercially available lipase immobilized Novozyme SP 435 and a sol-gel immobilizate[18] was used in a 1:2 ratio. Novozyme SP 435 floats on top of the ionic liquid, whereas the sol-gel immobilizate is dispersed more homogeneously. The total amount of immobilizate was 150 mg in the first and 600 mg in the second reactor.

The CO_2 stream was expanded into the first separation chamber ($V = 225$ mL, $T = 70$ °C, $p = 130$ bar) where a liquid phase was formed. A constant exit flow was achieved by using a gas dosing unit at the end of the reactor with a sampling volume of 0.02 mL at a frequency of $0.1\ s^{-1}$. Additional CO_2 was introduced after the dosing unit at the entrance of the first separation chamber and extraction was continued into the second chamber ($V = 100$ mL, $T = 100$ °C, $p = 100$ bar). The liquid phase formed in the second chamber was recycled periodically into the first vessel with an HPLC pump. After leaving the second chamber, the final CO_2 stream was vented through a cryo-trap kept at 0 °C. The contents of the cryo-trap and the first extraction chamber were collected for off line analysis.

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[22] To indicate the interplay of volatility (distillation) and solubility (extraction) Kurt Zosel coined the artificial word "Destraktion" (from Latin "destillare" and "extrahere") in his pioneering work on supercritical fluid extraction: K. Zosel, *Angew. Chem.* **1978**, *90*, 748 – 755; *Angew. Chem. Int. Ed. Engl.* **1978**, *17*, 702.

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[25] http://solvent-innovation.de.
[26] GC: 2-octanol (**1a**), 25 m LIPODEX A; 2-octyl laureate (**5a**), 25 m PER ME-BCD/OV 225/ FS 611; 1-phenylethanol (**1b**), 1-phenylethyl acetate (**4b**), and 3-methyl-2-butanol (**1c**), 25 m TBBCD/OV1701/ FS 610; 3-methyl-2-butyl laureate (**5c**), 25 m IVADEX 1/PS086; 1-(2-naphthyl)ethyl acetate (**4e**), 25 m IVADEX-7/OV-1701. HPLC: 1-phenylethyl laureate (**5b**) and 4-phenyl-2-butyl laureate (**5d)**, 250 mm; Chiralcel OD-H, 4.6 mm i. d.; 4-phenyl-2-butanol (**1d)**, 1-(2-naphthyl)ethanol (**1e**), and 1-(2-naphthyl)ethyl laureate (**5e**), 250 mm, Chiralcel OD-R, 4 mm i.d.

