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Using a Lipase as a High-Throughput Screening Method for Measuring the Enantiomeric Excess of Allylic Acetates

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This report describes a high-throughput method for measuring the enantiomeric excess of allylic acetates. Such methods are useful tools for screening libraries of potential catalysts for enantioselective reactions. This technique, which is called EMDee for an enzymatic method for determining enantiomeric excess, uses the lipase from *Pseudomonas cepacia* to hydrolyze the (*R*) enantiomer of an allylic acetate, while the (*S*) enantiomer does not react. The rate of the reaction is monitored by measuring the acetic acid that is produced during the hydrolysis reaction with a pH indicator. Using the Michaelis−Menten equation, the rate of the reaction can be correlated with the concentration of the (*R*) enantiomer. This method can process 88 samples in less that 30 min.

Introduction

Combinatorial methods have become important tools in several disciplines including molecular biology, materials science, and pharmaceutical and synthetic chemistry. A number of investigators have been applying these methods to the discovery of new catalysts for asymmetric reactions.[1,2] Combinatorial techniques are useful in this area because they have the potential for increasing the efficiency by which chiral catalysts are discovered and for optimizing the stereoselectivity of a catalyst for a particular substrate of interest. Using these techniques, it is generally straightforward to synthesize thousands of potential catalysts. However, it is time-consuming to screen this number of catalysts for enantioselectivity using standard methods such as chiral HPLC or GC. With these types of serial analytical methods, the analysis time increases linearly with the size of the library. In addition, these methods often require that the products from the catalyzed reaction be purified before they are analyzed.

To overcome some of these limitations, new high-throughput methods for measuring enantiomeric excess are being developed.[3] Many of these new methods rely on parallel, rather than serial, analyses and thus can be used to screen large libraries of catalysts. These high-throughput methods fall into four general categories: (1) kinetic resolution reactions in which an alcohol or amine that is to be analyzed is coupled with a chiral carboxylic acid to give a mixture of diastereomers,[4] (2) parallel chromatographic methods that are followed by analysis with UV, fluorescence or CD spectroscopies,[5] (3) comparison of the reaction rates of the purified enantiomers of a chiral starting material,[6] and (4) biological methods that rely on antibodies or enzymes.[7]

We have recently developed a new high-throughput method for screening enantiomeric excess that falls into the fourth category. This method is called EMDee, for enzymatic method for determining enantiomeric excess.[8] In its most general form, the EMDee screen uses an enzyme as an analytical tool to measure the % ee of a sample. The enzyme is introduced into a sample of unknown stereochemical composition, and it catalyzes a reaction using only one of the enantiomers as a substrate. The rate of the enzyme-catalyzed reaction is monitored by standard methods, and this rate can be correlated to the % ee of the sample as long as the total concentration

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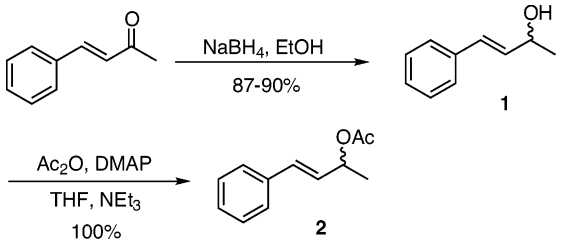
SCHEME 1



of the substrate plus its enantiomer is known. Two important features of the EMDee method are its high stereoselectivity, since enzymes typically have a strong specificity for one enantiomer of a substrate, and the ability to perform the screen in a 96- or 384-well format using a UV or fluorescence plate reader.

We first demonstrated the EMDee assay using an alcohol dehydrogenase to oxidize one enantiomer of a chiral benzylic alcohol.[8] This alcohol was obtained through the catalytic asymmetric addition of diethylzinc to benzaldehyde. In this paper, we broaden the scope of EMDee by showing that a lipase can be used to measure the enantiomeric excess of chiral allylic acetates.

Results and Discussion

Choice of the Substrate. We have chosen chiral allylic acetates as substrates for this ee screen because these compounds and their related chiral allylic alcohols are important intermediates in synthetic chemistry.[9] However, this method will be applicable to other esters and is not limited to allylic acetates in particular. There are a variety of catalytic asymmetric methods that are available for synthesizing chiral allylic acetates. These include palladium-catalyzed allylic substitution reactions[10] and the kinetic resolution of racemic alcohols.[11] We have selected compound **2** (Scheme 1) as the test substrate for this lipase-based EMDee assay because it is a common component of a wide diversity of allylic substitution reactions,[12] it is a useful starting point for the synthesis medicinally important compounds including potential glycosyltransferase inhibitors,[13] and it can be synthesized in chiral form using Fu's chiral acylation catalyst[14] and via dynamic kinetic resolution.[15]

Synthesis of the Substrate. To perform the enzyme assays, we needed to synthesize the allylic acetates (*R*)-**2** and (*S*)-**2** and the corresponding alcohols (*R*)-**1** and (*S*)-**1** in enantiomerically pure form. As shown in Scheme 1, compounds **1** and **2** were prepared in racemic form by reducing 1-phenyl-1-buten-3-one with sodium borohydride to give alcohol **1**, which was subsequently acylated with acetic anhydride and catalytic DMAP to give allylic acetate **2**.

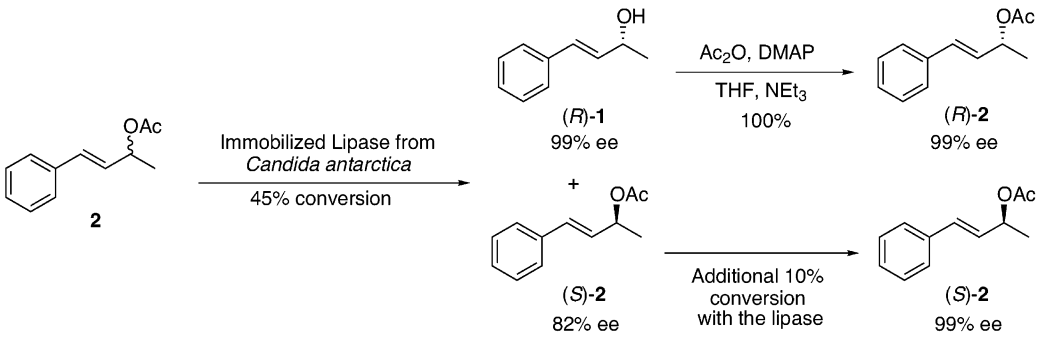
Compound **2** was subjected to a stereoselective hydrolysis using an immobilized lipase from *Candida antarctica* as shown in Scheme 2. After 45% conversion, the reaction was stopped and the product alcohol was isolated to give (*R*)-**1** in 99% ee as determined by chiral HPLC. This alcohol was acylated with acetic anhydride and catalytic DMAP to give (*R*)-**2**, also in 99% ee. The remaining starting material from the enzymatic reaction, (*S*)-**2** in 82% ee, was subjected to an additional 10% conversion with the immobilized lipase. After the product alcohol was removed by flash chromatography, the remaining starting material was re-isolated to give (*S*)-**2** in 99% ee. Finally, alcohol (*S*)-**1** was obtained in 99% ee by base-promoted hydrolysis of (*S*)-**2** as shown in Scheme 3.

Design of the Assay. We have selected the lipase from *Pseudomonas cepacia* for the EMDee assays of allylic acetates. This lipase has a very broad substrate specificity and generally shows excellent stereoselectivity with a wide variety of substrates.[16] The general scheme for the assays is shown in Figure 1. A sample of allylic acetate **2** of unknown stereochemical composition is treated with the lipase from *Pseudomonas cepacia*, and the enzyme catalyzes the hydrolysis of the (*R*) enantiomer. The rate of the enzymatic reaction can be correlated with the concentration of (*R*)-**2** using the Michaelis–Menten equation. Since the starting concentration of the allylic acetate is known, the % ee of the sample can be calculated.

To monitor the rate of the enzyme-catalyzed reaction by UV spectroscopy, we used *p*-nitrophenol as a pH-sensitive indicator according to the method of Kazlauskas and co-workers.[17] As the enzymatic hydrolysis occurs, acetic acid is liberated and protonates the indicator. Since *p*-nitrophenol and the BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffer in the reaction have the same pK_a, production of acetic acid is accompanied by a linear change in absorbance at 405 nm.

Kinetics. We have used this assay system to measure the kinetics of hydrolysis of (*R*)-**2** by the lipase. Figure 2 shows a plot of the rate of the enzymatic reaction as a function of the concentration of (*R*)-**2**. These data were used to calculate a K_M value for this substrate of 2.6 mM. We have also determined that (*S*)-**2** is neither a substrate nor an inhibitor of the lipase up to the limits of solubility. In addition, further control experiments show that the corresponding alcohols (*R*)-**1** and (*S*)-**1** do not inhibit the

SCHEME 2



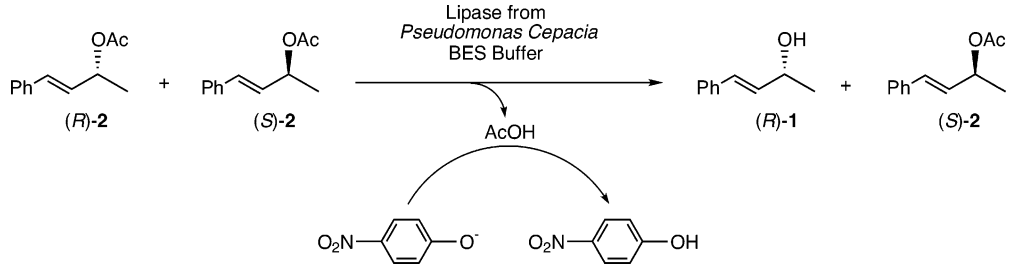


FIGURE 1. EMDee assay of allylic acetate **2** using the lipase from *Pseudomonas cepacia*.

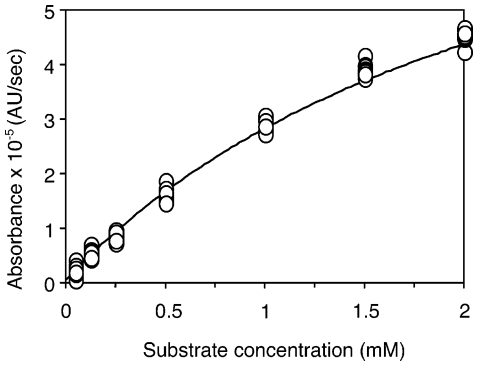


FIGURE 2. Plot of the rate of the lipase-catalyzed reaction as a function of the concentration of (*R*)-**2**.

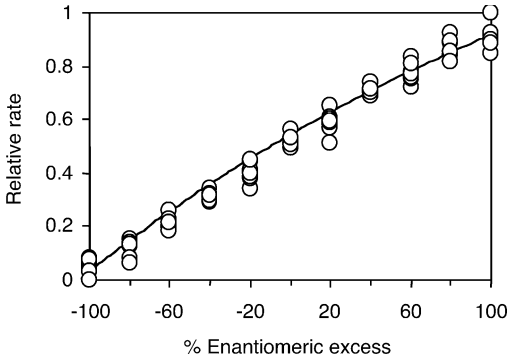
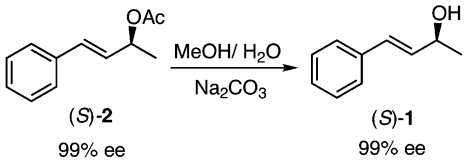


FIGURE 3. Analysis of 88 samples of compound **2** that range in ee from 100% (*S*) to 100% (*R*) using the lipase from *Pseudomonas cepacia*.

SCHEME 3



lipase and that there is no significant background hydrolysis of compound **2** under the assay conditions in the absence of enzyme.

To demonstrate that the lipase can be used to measure the % ee of compound **2**, we prepared 88 samples of allylic acetate **2** that varied in ee from 100% (*S*) to 100% (*R*). The ee of these samples were confirmed by chiral HPLC analysis prior to the assays. These samples, along with eight control reactions that did not contain substrate, were analyzed in a 96-well formal using a UV plate reader. The total concentration of compound **2** in each well was 1 mM. The assay solutions also contained 10% acetonitrile to improve solubility of the substrate. Data were collected over 25 min and are shown in Figure 3.

The solid line in the plot in Figure 3 represents the theoretical curve that is generated using the Michaelis–Menten equation with the constraints that the total concentration of compound **2** remains constant at 1 mM, that (*R*)-**2** is the substrate, and that (*S*)-**2** is neither a substrate nor an inhibitor for the lipase. The good correlation between the experimental data and the theoretical curve demonstrates that the rate of the lipase-catalyzed reaction can be used to estimate the stereochemical composition of samples of compound **2**. In addition, this is a fast, parallel analysis in which 88 samples can be analyzed in less than 30 min.

Analysis of Crude Acylation Products. We have also investigated the possibility of using this lipase-based EMDee assay on crude reaction products. Fu and coworkers have used a planar-chiral DMAP derivative to

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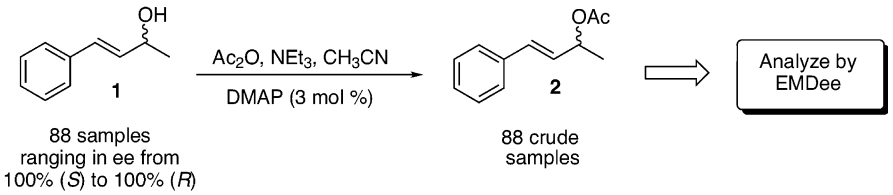

FIGURE 4. Preparation and EMDee analysis of 88 samples of crude allylic acetate **2**.

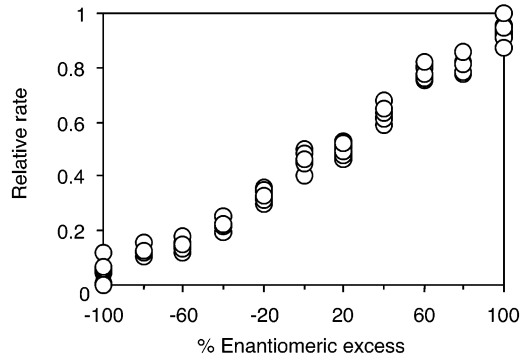

FIGURE 5. Lipase-based screen of 88 samples of compound **2** that range in ee from 100% (*S*) to 100% (*R*). These samples are crude products from the acylation reaction shown in Figure 4 and were analyzed without isolation or purification.

selectively acylate one enantiomer of a racemic mixture of a variety of allylic alcohols.[14] To model the products from these types of enantioselective acylation reactions, we prepared 88 samples of allylic alcohol **1** that varied in ee from 100% (*S*) to 100% (*R*). These samples were acylated with 1.2 equiv of acetic anhydride and catalytic DMAP in acetonitrile as shown in Figure 4. The crude allylic acetates were then directly subjected to the EMDee assay without prior removal of the solvent, workup, or purification. The buffer concentration in the enzyme assays was high enough to ensure that the excess acetic acid that is generated during the acylation reactions did not interfere with the functioning of the pH indicator.

Figure 5 shows the data from the assay of these crude samples. There is a strong correlation between the % ee of the allylic alcohol starting material and the rate of the enzymatic reaction with the crude allylic acetate products. These results demonstrate that the lipase-based EMDee screen is a robust method that can reliably measure the enantiomeric excess of products from an acylation reaction without the need for isolation or purification of the allylic acetates.

Application to Other Esters. One potential limitation of the EMDee method is that many enzymes are highly specific for a particular substrate and do not tolerate significant variations in substrate structure. To address this issue, we have examined a second allylic acetate, indene derivative (*R*)-**4** (Figure 6), as a potential substrate for the lipase. Chiral derivatives of indene are useful substructures in medicinal chemistry, and have been used in the synthesis of a number of inhibitors for HIV protease.[18] Compound (*R*)-**4** was synthesized in 99% ee from racemic inden-1-o1[19] using a procedure similar to that shown in Scheme 2.[20] We have found that (*R*)-**4** is a substrate for the lipase with a K_M value of 5.2 mM,

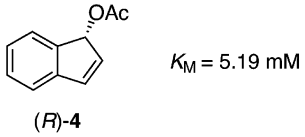

FIGURE 6. Indene derivative (*R*)-**4** is a substrate for the lipase.

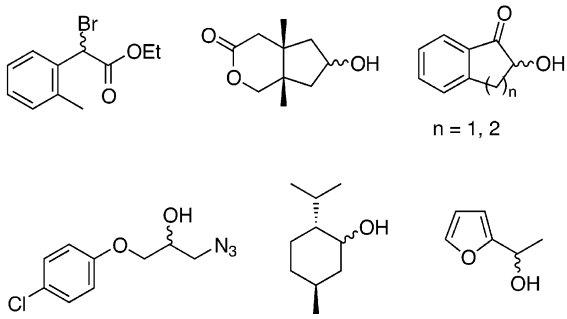

FIGURE 7. Structures of several substrates for the lipase from *Pseudomonas cepacia*.

while its enantiomer (*S*)-**4** is not a substrate or an inhibitor of the enzyme.

To further probe the potential scope of this ee screen, we have found other alcohols and esters reported in the literature that are substrates for the lipase from *Pseudomonas cepacia*. Seven of these structures are shown in Figure 7. These particular compounds were chosen to highlight the ability of this enzyme to accept substrates with a diversity of structures.[16] The lipase catalyzes either the esterification or hydrolysis of all of these substrates with high stereoselectivity. Thus, our own experiments with compounds (*R*)-**2** and (*R*)-**4**, along with previous studies of this enzyme suggest that the lipase-based EMDee screen should be applicable to a wide variety of allylic acetates and other esters that are of interest to the synthetic community.

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(19) Hoppe, I.; Marsch, M.; Harms, K.; Boche, G.; Hoppe, D. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2158. WARNING: This procedure for the synthesis of compound **3** (inden-1-ol) involves the preparation of indene 1-peroxide as an intermediate. We have performed this procedure a number of times, using appropriate safety precautions including the use of a blast shield, without mishap. However, on one occasion while the reaction mixture was being quenched with a solution of NaI, a serious explosion occurred that resulted in a small fire and injury from flying glass. We have not determined the exact cause of this explosion, but recommend that all appropriate safety precautions be used when carrying out this procedure.

(20) For the details of the synthesis of racemic **4**, (*R*)-**3**, (*R*)-**4**, and (*S*)-**4** see the Supporting Information.

In our current studies, we have analyzed samples in which the total concentration of allylic acetate is known. However, if this technique is used to analyze a combinatorial library of asymmetric catalysts, there are two factors that will influence the rate that is observed in the EMDee assay; the % ee of the sample, and the extent of conversion of the catalyzed reaction. We believe that the EMDee assay will be most useful as a preliminary screening technique to identify promising catalysts from large libraries that give both high yields and that show high stereoselectivity. These catalysts will give the highest rates in the EMDee assay. Conversely, catalysts that give low conversion or that have low stereoselectivity will give low rates. If it becomes necessary to identify catalysts that have low conversion but good stereoselectivity, these compounds could be identified using a second EMDee assay, as we have shown previously.[8] This requires two enzymes with opposite stereoselectivity that are used to quantitate each of the two enantiomers of the product.

Conclusions

In summary, we have developed an EMDee screen that uses the lipase from *Pseudomonas cepacia* to measure the enantiomeric excess of allylic acetates in a high-throughput manner. This method can accommodate samples that range in stereochemistry from 100% (*S)* to 100% (*R)*, and is able to process 88 samples in approximately 25 min. Because the lipase has a very broad substrate specificity, this assay procedure should be useful for screening potential enantioselective catalysts for a variety of synthetic transformations.

Experimental Section

Procedure for Lipase Assays. Samples of compound **2** were used as substrates for Amano lipase PS from *Pseudomonas cepacia*. Protonation of the indicator *p*-nitrophenoxide by the acetic acid that is formed during the enzymatic reaction was monitored by observing the decrease in absorbance at 404 nm. Each well of the 96-well plate contained 300 μL of a solution of 1 mM substrate, 6.67 mM BES buffer (*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) at pH 7.20, 0.22 mM *p*-nitrophenol, 60 μL of enzyme stock solution, and 10% acetonitrile as a cosolvent. Samples of compound **2** varying in % ee composition were prepared using enantiomerically pure (*R*)-**2** and (*S*)-**2**. Enzyme stock solutions were prepared by dissolving the lipase (1 mg/mL) and BSA (bovine serum albumin, 1 mg/mL) in BES buffer. This solution was agitated with a shaker at room temperature for 30 min, then centrifuged twice to remove the insoluble particles. The reactions were initiated by the addition of enzyme stock solution, and absorbance data was collected over 25 min. These assays were performed under ambient atmosphere.

Procedure for Acylation of 11 Samples of Compound 1 and Analysis of the Resulting Crude Allylic Acetates Using the Lipase. Eleven samples of compound **1** ranging in % ee from 100% (*R*)-**1** to 100% (*S*)-**1** were prepared using stock solutions of pure (*R*)-**1** and (*S*)-**1** in acetonitrile. These samples were acylated using acetic anhydride (1.2 equiv), NEt_3 (1.2 equiv), and DMAP (3 mol %) in acetonitrile solvent at room temperature. The reactions were monitored by HPLC and shown to be complete in less than 1 h. Samples were adjusted to a final concentration of 10 mM in the allylic acetate product using acetonitrile, and eight aliquots of each reaction was subjected to analysis using the lipase as described above. The enzymatic analyses were performed on the crude samples, with no workup or purification of the products. The reactions and subsequent enzymatic analyses were performed multiple times with similar results.

Racemic Alcohol 1. (*E*)-3-Hydroxy-1-phenyl-1-butene **1** was prepared by reducing (*E*)-4-phenyl-3-buten-2-one (25 g, 171 mmol) with $NaBH_4$ (6.47 g, 171 mmol) in ethanol (250 mL). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 3 h. The reaction was quenched with 1 N HCl, and the ethanol was removed by rotary evaporation. The aqueous phase was extracted with ethyl acetate, and the organic phase was washed with water and brine and dried over $MgSO_4$. Purification by flash column chromatography (EtOAc/hexanes 1:4) gave a clear oil (22.8 g, 154 mmol, 90%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.40 (d, J = 6.4 Hz, 3H), 1.64 (s, 1H), 4.51−4.54 (m, 1H), 6.29 (dd, J = 15.9, 6.4 Hz, 1H), 6.60 (d, J = 15.9 Hz, 1H), 7.26 (t, J = 7.6 Hz, 1H), 7.34 (dd, J = 7.0, 1.7 Hz, 2H), 7.41 (d, J = 3.3 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 137.1, 134.0, 129.8, 129.0, 128.1, 126.9, 69.4, 23.8; HRMS (ESI) calcd for $C_{10}H_{12}O$ 148.0888, found 148.0892.

General Procedure for the Preparation of Compounds 2 and 4. Racemic **2**, racemic **4**, (*R*)-**2**, and (*R*)-**4** were prepared from the corresponding alcohols by DMAP-catalyzed acylation. In a typical procedure, the alcohol (100 mmol) was combined with THF (70 mL), acetic anhydride (120 mmol), triethylamine (180 mmol), and DMAP (0.5 mmol) at 0 °C. The reaction mixture was maintained at 0 °C for 1 h and then was allowed to stir at room temperature overnight. The solvent was removed by rotary evaporation, and the resulting material was washed with water, brine and dried over $MgSO_4$. Purification by flash column chromatography (EtOAc/hexanes 1:4) gave the desired product.

Racemic 2. Racemic **2** was prepared as described above from alcohol **1** (14.0 g, 95 mmol). Purification gave a clear oil (18.0 g, 95 mmol, 100%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.45 (d, J = 6.5 Hz, 3H), 2.11 (s, 3H), 5.55−5.58 (m, 1H), 6.22 (dd, J = 16.0, 6.8 Hz, 1H), 6.64 (d, J = 16.0 Hz, 1H), 7.29 (t, J = 4.3 Hz, 1H), 7.35 (m, 2H), 7.42 (d, J = 9.6 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.7, 136.8, 132.0, 129.2, 129.0, 128.3, 127.0, 71.4, 21.8, 20.8; HRMS (ESI) calcd for $C_{12}H_{14}O_2$ 190.0994, found 190.0986.

Alcohol (*R*)-1. (*R*)-**1** was synthesized from racemic acetate **2** by enantioselective hydrolysis using an immobilized lipase from *Candida antarctica*. To the racemic acetate **2** (60.3 mg, 0.317 mmol) were added acetonitrile (3 mL), water (3 mL), and the immobilized lipase (120.6 mg), and the reaction mixture was gently shaken at room temperature for 24 h under ambient atmosphere. After the reaction had reached 45% completion as determined by the ^{1}H NMR spectrum of an aliquot of the reaction mixture, the immobilized enzyme was removed by filtration and the acetonitrile was removed by rotary evaporation. Water (4 mL) was added, the product was extracted into ethyl acetate, and the organic phase was dried over $MgSO_4$. Purification by flash column chromatography (EtOAc/hexanes 1:4) yielded alcohol (*R*)-**1** (19.2 mg, 0.13 mmol, 91% of the theoretical yield) in 99% ee as judged by HPLC analysis (Chiralcel OD-H column, 90:10 hexane/2-propanol, 1 mL/min, 254 nm UV detection), t_R = 8.3 min for (*R*)-**1** and t_R = 12.5 min for (*S*)-**1**. The chromatographic purification also yielded (*S*)-**2** in 82% ee.

Acetate (*R*)-2. (*R*)-**2** was prepared from alcohol (*R*)-**1** as described above in the general procedure. 99% ee by HPLC analysis (Chiralcel OD-H column, hexane, 1 mL/min, 254 nm UV detection), t_R = 22.7 min for (*R*)-**2** and t_R = 26.5 min for (*S*)-**2**.

Acetate (*S*)-2. The (*S*)-**2** (82% ee) that was obtained during the synthesis of alcohol (*R*)-**1** was subjected to an additional 10% conversion with the immobilized lipase from *Candida antarctica*. After the desired conversion was attained as determined by the ^{1}H NMR spectrum of an aliquot of the reaction mixture, the immobilized enzyme was filtered and the acetonitrile was removed by rotary evaporation. Water (4 mL) was added, the product was extracted into ethyl acetate, and

the organic phase was dried over $MgSO_4$. Purification by flash column chromatography (EtOAc/hexanes 1:4) yielded acetate (*S*)-**2** (23 mg, 0.12 mmol, 85% of theoretical): 99% ee as judged by HPLC analysis (Chiralcel OD-H column, hexane, 1 mL/min, 254 nm UV detection), t_R= 22.7 min for (*R*)-**2** and t_R= 26.5 min for (*S*)-**2**.

Alcohol (*S*)-1. Alcohol (*S*)-**1** was synthesized from acetate (*S*)-**2** (99% ee) by basic hydrolysis. To the acetate (*S*)-**2** (700 mg, 3.68 mmol) were added methanol (30 mL), water (60 mL), and sodium carbonate (1.12 g), and the reaction mixture was stirred at room temperature for 10 h. The methanol was removed by rotary evaporation, the product was extracted into ethyl acetate, and the organic phase was dried over $MgSO_4$. Purification by flash column chromatography (EtOAc/hexanes 1:4) yielded (*S*)-**1** (491 mg, 3.32 mmol, 90%): 99% ee as judged by HPLC analysis (Chiralcel OD-H column, 90:10 hexane/2-propanol, 1 mL/min, 254 nm UV detection), t_R= 8.3 min for (*R*)-**1** and t_R= 12.5 min for (*S*)-**1**.

Supporting Information Available: Procedures for the synthesis of racemic **4**, (*R*)-**3**, (*R*)-**4**, and (*S*)-**4**; ^1H and ^{13}C NMR spectra for compounds **1**, **2**, and **4**; HPLC traces for (*R*)-**1**, (*S*)-**1**, (*R*)-**2**, (*S*)-**2**, (*R*)-**3**, (*R*)-**4**, and (*S*)-**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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