Chiral Chemistry

High-Throughput Measurement of the Enantiomeric Excess of Chiral Alcohols by Using Two Enzymes**

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Dedicated to Professor Karl Schlögl on the occasion of his 80th birthday

The development of enantioselective catalytic processes has attracted increasing attention as a result of the importance of enantiomerically pure organic compounds in pharmaceutical, agricultural, and fine chemical syntheses.[1] Attempts to discover new enantioselective catalysts have recently focused on the screening of libraries of chemical catalysts[2] created by combinatorial synthesis, and enzymes and enzyme mutants[3,4] generated by molecular biotechnology techniques such as the error-prone polymerase chain reaction and gene shuffling.[5] Since the catalyst libraries are huge, analysis of the catalyst enantioselectivity is the bottleneck in such approaches. Many methods have been developed for high-throughput enantioselectivity analysis.[6] The enantioselectivity factor E for kinetic resolution can be estimated quickly by measuring separately the reaction rates of the *R* and *S* enantiomers of the substrate.[7] This technique can also be applied to the reversible asymmetric reaction of a prochiral substrate by examining separately the reverse reactions of the *R* and *S* products.[7d] On the other hand, high-throughput analysis of the product enantiomeric excess (*ee*) can be used for the evaluation of the catalyst enantioselectivity in asymmetric transformations. The product *ee* can be determined by GC-GC on a column with a chiral stationary phase,[8a] HPLC with CD/UV,[8b,c] or by using optical rotation/refractive index (OR/RIU) detection[8c], chirally modified capillary electrophoresis,[8d] electrospray ionization tandem MS,[8e] colour indicators based on doped liquid crystals,[8f] or competitive enzyme immunoassays.[8g] The concept developed by Schoofs and Horeau[9a] allows the *ee* value of the product to be established by exploiting kinetic resolution effects and using mass-tagged or fluorescence-tagged pseudo-enantiomeric mixtures of acylating agents, with mass spectrometry (MS)[9b,c] or fluorescence[9d] detection. Elegant approaches for determination of the *ee* values also include the use of isotopically labeled pseudo-enantiomeric or pseudo-*meso* substrates and MS[10a,b] or NMR detection.[10c,d] When the product concentration is known, the *ee* value of the product can be determined by using an enzyme to catalyze a further reaction that can be followed by UV spectroscopy[11a] or infrared thermography.[11b] In the former method, known as EMDee (enzymatic method for determining enantiomeric excess), an enantiospecific alcohol dehydrogenase is used to oxidize one enantiomer of an alcohol at a known concentration, the oxidation rate is determined by following the formation of NAD(P)H (NAD, nicotinamide-adenine dinucleotide), and the *ee* value of the alcohol is calculated by referring to a standard curve of rate versus the *ee* value established at the same alcohol concentration. This method is high-throughput and sensitive and has been successfully used to determine the *ee* value of the alcohol generated from a chemical catalysis in which 100% conversion is achieved. However, the general application of such a method in high-throughput screening of enantioselective catalysts is rather limited since conversion is often below 100%, which means that the product concentration must be determined by another high-throughput method before analysis of the *ee* value. Herein, we describe a method for determining the *ee* value based on the new concept of using two enantioselective enzymes to modify the product. This method allows the determination of the *ee* value of the product independent of concentration. In contrast to the concept of Schoofs and Horeau, which applies to pseudo-enantiomeric mixtures of reagents in one reaction, our analysis of the *ee* value involves two separate enzymatic reactions. Our method allows sensitive, accurate, high-throughput measurement of the enantiomeric excess of a chiral alcohol. In addition, the alcohol concentration can be estimated within the same process.

Chiral alcohols **4** were used as model compounds to demonstrate the principle of our approach. These alcohols represent an important class of intermediates for the synthesis of pharmaceuticals and fine chemicals[1] and can be prepared by various enantioselective catalytic transformations, such as biohydroxylation of hydrocarbons **1**, chemical or enzymatic reduction of ketones **2**, or enzymatic hydrolysis of esters **3** (or the reverse reaction, namely formation of an ester; Scheme 1). To analyze the *ee* value of **4**, enantioselective $NAD(P)^+$-dependent alcohol dehydrogenases A and B were each used to oxidize alcohol **4**. The reaction rates for enzyme A (v^a) and enzyme B (v^b) were determined by following the formation of NAD(P)H through its absorption at 340 nm. The enantiomers of chiral alcohols are generally competing substrates for enantioselective alcohol dehydrogenases so the reaction rates v^a and v^b can be expressed by Equations (1) and (2):[12]

$$v^a = \frac{V^a_{max,S} K^a_{m,R}[S] + V^a_{max,R} K^a_{m,S}[R]}{K^a_{m,R}[S] + K^a_{m,S}[R] + K^a_{m,S} K^a_{m,R}} \quad (1)$$

$$v^b = \frac{V^b_{max,S} K^b_{m,R}[S] + V^b_{max,R} K^b_{m,S}[R]}{K^b_{m,R}[S] + K^b_{m,S}[R] + K^b_{m,S} K^b_{m,R}} \quad (2)$$

V_{max} is the maximum reaction velocity, K_m is the Michaelis constant, a and b refer to the two enzymes, and *S* and *R* refer to the alcohol enantiomers. Equations (1) and (2) lead to Equations (3) and (4):

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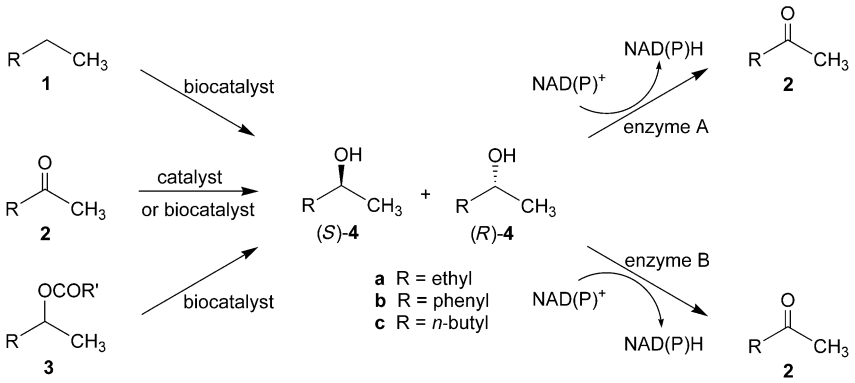

Scheme 1. Synthetic routes to alcohols **4** and catalysis of the oxidation of these alcohols with alcohol dehydrogenases A and B.

$$[S] = \frac{\frac{V^{a}_{max,R}{}^{-\nu^{a}}}{K^{a}_{m,R}{}^{\nu^{a}}} - \frac{V^{b}_{max,R}{}^{-\nu^{b}}}{K^{b}_{m,R}{}^{\nu^{b}}}}{\frac{(V^{a}_{max,R}{}^{-\nu^{a}})(V^{b}_{max,S}{}^{-\nu^{b}})}{K^{a}_{m,R}{}^{\nu^{a}} K^{b}_{m,S}{}^{\nu^{b}}} - \frac{(V^{b}_{max,R}{}^{-\nu^{b}})(V^{a}_{max,S}{}^{-\nu^{a}})}{K^{b}_{m,R}{}^{\nu^{b}} K^{a}_{m,S}{}^{\nu^{a}}}} \quad (3)$$

$$[R] = \frac{\frac{V^{b}_{max,S}{}^{-\nu^{b}}}{K^{b}_{m,S}{}^{\nu^{b}}} - \frac{V^{a}_{max,S}{}^{-\nu^{a}}}{K^{a}_{m,S}{}^{\nu^{a}}}}{\frac{(V^{a}_{max,R}{}^{-\nu^{a}})(V^{b}_{max,S}{}^{-\nu^{b}})}{K^{a}_{m,R}{}^{\nu^{a}} K^{b}_{m,S}{}^{\nu^{b}}} - \frac{(V^{b}_{max,R}{}^{-\nu^{b}})(V^{a}_{max,S}{}^{-\nu^{a}})}{K^{b}_{m,R}{}^{\nu^{b}} K^{a}_{m,S}{}^{\nu^{a}}}} \quad (4)$$

The *ee* value can thus be calculated by using Equation (5):

$$ee = \frac{[R]-[S]}{[R]+[S]} = \frac{\frac{V^{b}_{max,S}{}^{-\nu^{b}}}{K^{b}_{m,S}{}^{\nu^{b}}} - \frac{V^{a}_{max,S}{}^{-\nu^{a}}}{K^{a}_{m,S}{}^{\nu^{a}}} - \frac{V^{a}_{max,R}{}^{-\nu^{a}}}{K^{a}_{m,R}{}^{\nu^{a}}} + \frac{V^{b}_{max,R}{}^{-\nu^{b}}}{K^{b}_{m,R}{}^{\nu^{b}}}}{\frac{V^{b}_{max,S}{}^{-\nu^{b}}}{K^{b}_{m,S}{}^{\nu^{b}}} - \frac{V^{a}_{max,S}{}^{-\nu^{a}}}{K^{a}_{m,S}{}^{\nu^{a}}} + \frac{V^{a}_{max,R}{}^{-\nu^{a}}}{K^{a}_{m,R}{}^{\nu^{a}}} - \frac{V^{b}_{max,R}{}^{-\nu^{b}}}{K^{b}_{m,R}{}^{\nu^{b}}}} \quad (5)$$

If two enantiospecific alcohol dehydrogenases are used, Equation (5) can be simplified. Suppose enzyme A specifically oxidizes (*R*)-**4** and enzyme B specifically oxidizes (*S*)-**4**. In this case, $V^{a}_{max,S}=0$, $K^{a}_{m,S}=K^{a}_{I}$, $V^{b}_{max,R}=0$, $K^{b}_{m,R}=K^{b}_{I}$, where K_I is the inhibition constant (see the Experimental Section). Therefore,

$$ee = \frac{[R]-[S]}{[R]+[S]} = \frac{\frac{V^{b}_{max,S}{}^{-\nu^{b}}}{K^{b}_{m,S}{}^{\nu^{b}}} + \frac{1}{K^{a}_{I}} - \frac{V^{a}_{max,R}{}^{-\nu^{a}}}{K^{a}_{m,R}{}^{\nu^{a}}} - \frac{1}{K^{b}_{I}}}{\frac{V^{b}_{max,S}{}^{-\nu^{b}}}{K^{b}_{m,S}{}^{\nu^{b}}} + \frac{1}{K^{a}_{I}} + \frac{V^{a}_{max,R}{}^{-\nu^{a}}}{K^{a}_{m,R}{}^{\nu^{a}}} + \frac{1}{K^{b}_{I}}} \quad (6)$$

The kinetic constants $V^{a}_{max,R}$, $V^{a}_{max,S}$, $K^{a}_{m,R}$, $K^{a}_{m,S}$, $V^{b}_{max,R}$, $V^{b}_{max,S}$, $K^{b}_{m,R}$, $K^{b}_{m,S}$, K^{a}_{I}, and K^{b}_{I} can be quickly established by separate oxidation of (*R*)-**4**, (*S*)-**4**, and mixtures of (*R*)- and (*S*)-**4** in known ratios with enzymes A and B, respectively. We used an enzyme preparation with a constant concentration to establish the kinetic constants and analyze unknown samples. The *ee* values could then be calculated by applying Equation (5) or (6). The use of V_{max} is advantageous because this approach does not require accurate determination of the enzyme concentration in each experiment.

To illustrate this method, three commercially available enzymes were used for the oxidations: the alcohol dehydrogenases from *Lactobacillus kefir* (LKADH),[13a] *Thermoanaerobium brockii* (TBADH, a thermostable enzyme),[13b] and *Rhodococcus erythropolis* (READH).[13c] These enzymes are relatively stable and their stock solutions, when kept at 4 °C, can be used for analysis for at least 10 h without loss of activity. LKADH and TBADH were used for the oxidation of **4a**, whereas LKADH and READH were used for the oxidation of the alcohols **4b** and **4c**. Bioconversions were performed with a 200-μL solution containing buffer, substrate, NAD(P)$^+$, and enzyme in a deep well microtiter plate and the UV absorption at 340 nm of the produced NAD(P)H was measured for 5 min. The kinetic data for the enzyme-catalyzed oxidation of (*S*)-**4** and (*R*)-**4** were established for each enzyme in separate experiments and are summarized in Table 1.

The measurement of the *ee* value of **4a** is a typical example of an analysis using two enantioselective but not enantiospecific enzymes. In total, 54 samples with 37 different *ee* values and 22 different concentrations (0.4–3.5 mM) were analyzed in a microtiter plate by treatment with LKADH and TBADH, respectively. The results are shown in Figure 1, in which each point represents the average of two independent measurements. Figure 1a is a plot of the measured versus actual *ee* values. In every case (from −100% to 100% *ee*) but one (0% *ee*), the measured value falls within 9% *ee* of the true value. The analysis of the *ee* values is independent of sample concentration; nearly the same *ee* values were measured for samples with identical actual *ee* values but different concentrations.

The concentrations ($C=[R]+[S]$) of alcohol **4a** were also calculated from the results of the same experiments by using Equations (3) and (4). As shown in Figure 1b, the measured concentrations corresponded well to the actual concentra-

Table 1: Kinetic data for the oxidation of enantiopure secondary alcohols **4a–c** catalyzed by alcohol dehydrogenases LKADH, TBADH, and READH.

Substrate	Enzyme[a]	Enzyme conc. [mg mL^{-1}]	Enzyme conc. [U mL^{-1}][b]	NADP$^+$ [mM]	NAD$^+$ [mM]	K_m [μM]	K_I [μM]	V_{max} [μM min^{-1}]
(*R*)-**4a**	LKADH	0.10		1.0		365		19
(*S*)-**4a**	LKADH	0.10		1.0		663		5.4
(*R*)-**4a**	TBADH	0.015		1.0		900		37
(*S*)-**4a**	TBADH	0.015		1.0		1250		30
(*R*)-**4b**	LKADH	0.10		1.0		2600		20
(*S*)-**4b**	LKADH	0.10		1.0		–	3070	0
(*R*)-**4b**	READH		0.036		2.0	–	400	0
(*S*)-**4b**	READH		0.036		2.0	792		18
(*R*)-**4c**	LKADH	0.050		1.0		136		14
(*S*)-**4c**	LKADH	0.050		1.0		–	360	0
(*R*)-**4c**	READH		0.036		2.0	–	2630	0
(*S*)-**4c**	READH		0.036		2.0	326		27

[a] The enzymatic assays were carried out in tris(hydroxymethyl)aminomethane buffer (50 mM, pH 7.5) at 25 °C for LKADH and in potassium phosphate buffer (50 mM, pH 8.5) at 40 °C and 25 °C for TBADH and READH, respectively. [b] The specific activity U refers to the reduction of *p*-chloroacetophenone.

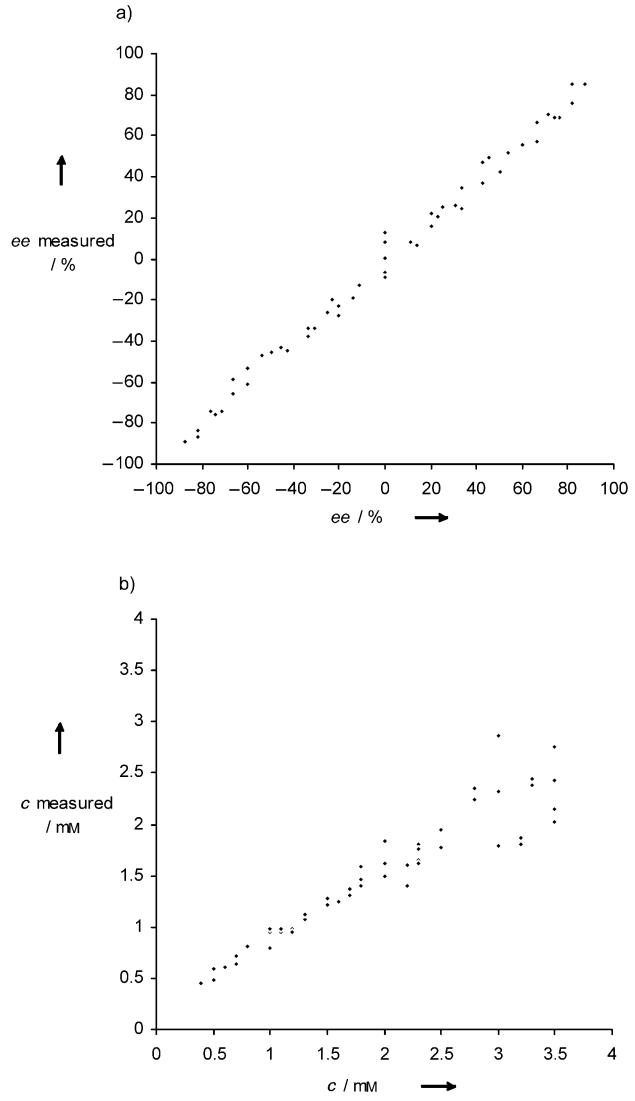

Figure 1. a) Plot of the actual *ee* values of **4a** versus those measured by using two enantioselective alcohol dehydrogenases, LKADH and TBADH. b) Plot of the actual concentrations (*c*) of **4a** versus those measured by using two enantioselective alcohol dehydrogenases, LKADH and TBADH.

tions from 0.4 to 2.0 mM, but large errors were observed at higher concentrations. The measured oxidation rate may not be very accurate when the concentration of **4a** is much higher than the value of K_m. According to Equations (3)–(5), small errors in v^a and v^b create much bigger errors in the concentration than in the *ee* value. Nevertheless, such concentration measurements could still be useful for qualitative estimates of the extent of conversion for catalyst screening. In the case of a high product concentration, the sample could be diluted several fold for analysis.

As a representative example of the use of two enantiospecific alcohol dehydrogenases for analysis of the *ee* value, an experiment was carried out with LKADH and READH. We tested 64 samples of **4b** with 35 different *ee* values and 29 different concentrations (0.4–10 mM), as well as 83 samples of **4c** with 51 different *ee* values and 33 different concentrations (0.2–5.0 mM). Figure 2 shows plots of the measured *ee* values versus the actual values for **4b** and **4c**. For **4b**, each point represents the average of three independent measurements and demonstrates the accuracy of the method: 60 of the 64 measured values show an error of less than 5% *ee* of the true

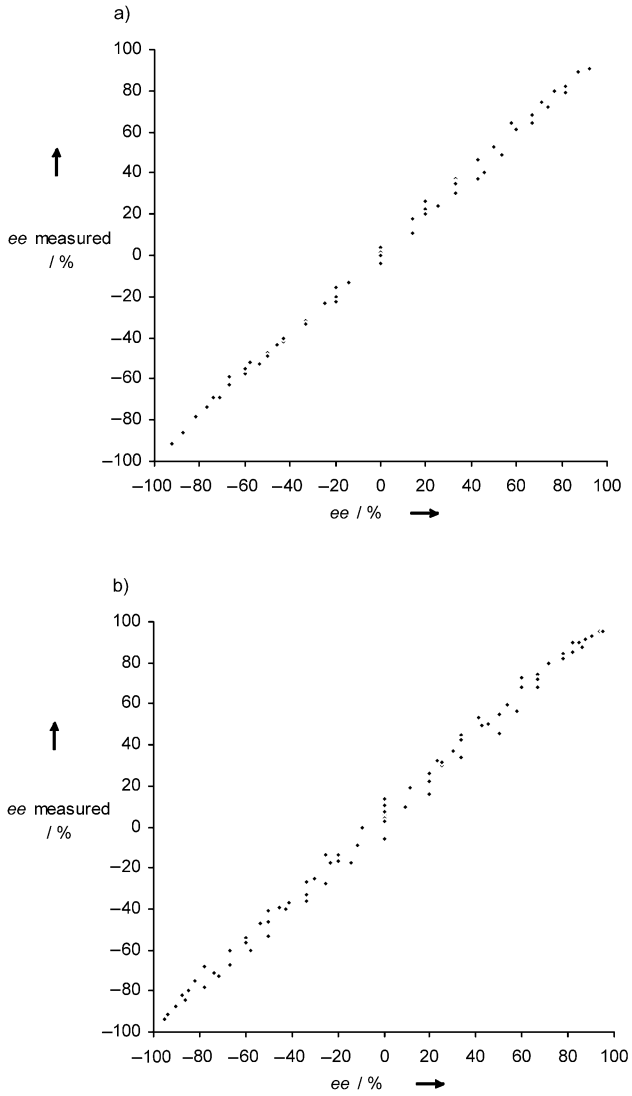

Figure 2. Plots of the actual *ee* values of **4b** (a) and **4c** (b) versus the *ee* values measured by using two enantiospecific alcohol dehydrogenases, LKADH and READH.

value, and the other four values have errors of 5–7% *ee*. For **4c**, only one measurement was made at each *ee* value. Of the 83 measured *ee* values, 79 are within 10% *ee* of the true value, and four results have an error of 10–12% *ee*.

Our method for the fast determination of the enantiomeric excess of secondary alcohols by using two alcohol dehydrogenases has several distinctive features: 1) The *ee* can be determined with satisfactory accuracy, independent of the concentration. 2) The enzymes do not have to be specific to, or highly active towards the alcohol. A large number of alcohol dehydrogenases with broad substrate ranges are now available and it is thus easy to find appropriate alcohol

dehydrogenases for analysis of the *ee* value of a given alcohol. 3) The analysis method is very sensitive. The technique can be used to determine the *ee* value of a sample with a concentration as low as 200 μM and is therefore particularly useful for biocatalyst screening, where product concentration is often low. 4) The analysis is performed with UV spectroscopy; no special instrument is required. 5) Experiments are performed in a 96-well microtiter plate, which allows analysis of the *ee* values of 48 samples within 5 min. Given a preparation time of 5 min, about 288 samples can be analyzed in an hour, which is a high enough throughput for most practical applications. 6) The method can be extended to measure the *ee* values of other types of compounds. Two enzymes of another type may be applied, coupled with detection by UV spectroscopy, MS, or even HPLC or GC for short analysis times. 7) The method also provides the possibility to measure concentration. For high concentrations, samples may be diluted and then analyzed, which makes the method useful for screening catalyst activities.

Further investigations will focus on the application of this method to the discovery of enantioselective biological and/or chemical catalysts. The scope and possible limitations of the method will also be explored.

Experimental Section

Enzymes: LKADH was obtained from Fluka as a lyophilized powder with an activity of 0.4 U mg^{-1} for the reduction of acetophenone. TBADH was purchased from Aldrich as a lyophilized powder with an activity of 4.7 U mg^{-1} for the oxidation of 2-propanol. READH was obtained from Juelich Fine Chemicals as a solution with an activity of 144 U mL^{-1} for the reduction of *p*-chloroacetophenone. The enzyme stock solutions were freshly prepared each day, kept in aqueous buffer at 4 °C, and used for measurement of the kinetic data and for the analysis of the *ee* value.

General procedure for measurement of the *ee* value: A mixture of buffer, alcohol **4**, NAD(P)$^+$, and enzyme with a total volume of 200 μL was pipetted into individual deep wells in a microtiter plate and the absorption of the produced NAD(P)H at 340 nm was measured for 5 minutes at 25 or 40 °C on a Spectra Max Plus photometer with an optical density resolution of 0.001. Data points were recorded every 12 s, which gave 26 points for every measurement. The absorption data were used to calculate the NAD(P)H concentration from a previously established standard curve. The initial reaction velocity was calculated by linear regression as the slope of a plot of [NAD(P)H] versus time.

The kinetic constants were quickly obtained by oxidizing (*R*)-**4**, (*S*)-**4**, and mixtures of the enantiomers at various concentrations by treatment with a single enzyme on a microtiter plate. The average velocities calculated from three measurements and the alcohol concentrations were used as input for the program Enzfit, which was used to calculate the values of K_m and V_{max}. To determine the inhibition constant K_I, Lineweaver–Burk (L-B) plots of v^{-1} versus $[S]^{-1}$ were generated for various inhibitor concentrations. A graph of the slopes of the L–B plots versus the inhibitor concentration was created. The value of K_I was determined by dividing the intercept by the slope of this plot.

To measure the *ee* value, samples containing the two enantiomers in various ratios and at a range of concentrations were prepared in microtiter plates and separately oxidized with each of two enzymes. The oxidation velocities were measured and used, together with kinetic data, to calculate the *ee* values from Equation (5) or (6). The measured *ee* values were plotted against the actual values.

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