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High-Throughput Synthesis and Analysis of Acylated Cyanohydrins

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Abstract: The yields and optical purities of products obtained from chiral Lewis acid/Lewis base-catalysed additions of α-ketonitriles to prochiral aldehydes could be accurately determined by an enzymatic method. The amount of remaining aldehyde was determined after its reduction to an alcohol, whilst the two product enantiomers were analysed after subsequent hydrolysis first by the (*S*)-selective *Candida antarctica* lipase B and then by the unselective pig liver esterase. The method could be used for analysis of products obtained from a number of aromatic aldehydes and aliphatic ketonitriles. Microreactor technology was successfully combined with high-throughput analysis for efficient catalyst optimization.

Keywords: asymmetric synthesis · cyanohydrins · enantioselectivity · enzymes · high-throughput screening · microreactors

Introduction

Efficient techniques by which large catalyst diversity can be achieved have been developed in recent years and have found applications in asymmetric synthesis.[1] Examples include the use of combinatorial techniques for modular synthesis of catalyst libraries,[2] the use of achiral and chiral additives to affect the reactivity and selectivity of the catalyst,[3] and the combination of different ligands.[4] Microreactors have been recognised as versatile tools for optimization of reaction conditions and have been found to be particularly useful in combination with high-throughput methods.[5] We have recently established that a microreactor can be used for reaction optimization in asymmetric metal catalysis.[6] Major advantages of micro-fluidic systems are the lower reagent consumption and the potential for continuous operation.

Along with the development of high-throughput screening in asymmetric catalysis, the need for rapid and reliable methods for *ee* analysis has increased. Chromatographic techniques such as GC and HPLC are at present the most frequently used methods, but the drawbacks of these are that they are time-consuming and require serial analyses. Several new techniques intended to solve this problem have indeed been developed;[7] they include the use of biocatalytic methods combined with UV/Vis spectroscopy,[8] capillary electrophoresis,[9] colour tests based on liquid crystals,[10] IR thermography,[11] circular dichroism,[12] mass spectroscopy,[13] and fluorescence.[14] None of these methods is general and new methods are required to allow analysis of a wider range of substrates. It would be particularly attractive to combine microreactor technology both with high-throughput synthesis and with analysis. This has been achieved in a single case recently reported by Reetz and co-workers, who developed an integrated micro-fluidic system for enantioselective biocatalysis and analysis.[15]

Enzymatic methods for the determination of enantiomeric excesses (EMD*ee*)[8b] have been used in a few cases. The principle of EMD*ee* is to convert a mixture of enantiomers into a mixture of chemically different species by means of a selective enzymatic transformation, thereby enabling measurement of the *ee* by conventional chemical analyses. Along such lines, selectivity in kinetic resolutions of epoxides was analysed by Reetz, who used an enzyme for the selective acetylation of one product enantiomer, the amount of which was determined by IR thermography.[11] Abato and Seto employed enzymes for *ee* determination of secondary alcohols formed by the addition of diethylzinc to benzalde-

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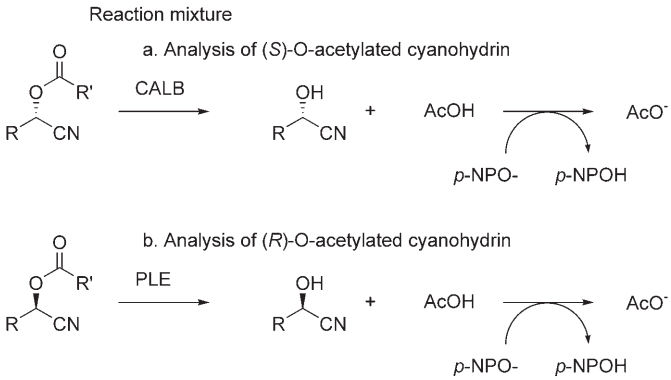
hyde,[8b] monitoring the rate of selective oxidation of one enantiomer by spectroscopic detection of NADPH. To distinguish reactions providing high conversion and low stereoselectivity from those proceeding with low conversion and high selectivity, an enzyme with opposite stereoselectivity was used in a second set of assays. Two different enzymes were also employed by Li and co-workers for enantioselective oxidation of enantiomeric alcohols, enabling determination of the concentration of each enantiomer and thus providing both *ee* values and yields.[8e] Two dehydrogenases with different enantiomeric preferences were used in parallel by Berkowitz to provide information relating to enantioselectivity and relative rates in hydrolytic kinetic resolutions of racemic propylene oxide.[8f] In another study, scalemic allylic acetates and other esters were subjected to enzymatic enantioselective hydrolysis whilst the amount of acetic acid produced was determined by use of a pH indicator.[8d] Finally, it was found that the enantiomeric excesses of chiral sulfoxides could be determined by measuring the inhibition of oxidation of ethanol catalysed by alcohol dehydrogenase.[8g]

It is desirable to be able to determine both the yield (and/or conversion) and the enantioselectivity of a catalytic reaction simultaneously; however, only a few of the high-throughput methods described so far fulfil this requirement.[8e] It has been accomplished in the case of antibodies: evaluation of yield and enantioselectivity was achieved by the combined use of one antibody that did not discriminate between the two enantiomers of hydroxy acids and one that bound selectively to one enantiomer.[8c]

We have previously reported an enzymatic method based on the analysis of benzaldehyde through the use of NADH.[16] With this method, yield and enantiomeric excess could be simultaneously determined from a single sample. In this full account we describe the extension of the method to a variety of acylated cyanohydrins. We also report the use of a combination of the enzymatic method and a microreactor for reaction optimization.

Results and Discussion

EMD*ee* with a pH indicator: We decided to use a combination of a selective enzyme and an unselective enzyme for *ee* determination in *O*-acylated cyanohydrins. One product enantiomer was first hydrolysed by treatment with *Candida antarctica* lipase B (CALB), an enantioselective—in this case (*S*)-specific—enzyme.[17] This was followed by hydrolysis with the unselective pig liver esterase (PLE). In order to analyse the amounts of the two alcohols obtained, a suitable detector system was needed. Seto and co-workers had used a pH indicator for *ee* analysis of chiral esters.[8d] In this method one of the enantiomeric esters was hydrolysed by a selective enzyme and the acid formed was quantified by use of a pH indicator. By studying the rate of hydrolysis, obtained by recording the colour change spectrometrically, the enantiomeric excess was accessible. A method based on pH measurements was also first developed for our reaction (Scheme 1). Each step produces acetic acid, which was titrated with yellow *p*-nitrophenolate to yield colourless *p*-nitrophenol. Through spectrometric recording of the colour



Scheme 1. Determination of *ee* after protonation of *p*-nitrophenolate (*p*-NPO^-) at 405 nm.

change over the two steps, the enantioselectivity was accessible.

This method worked very well in cuvettes, giving a linear relationship with *ee* values determined by conventional analytical methods (GC). Slightly lower values were observed when the enzymatic method was employed, but the accuracy was still high (Figure 1). Attempts to perform the screening on microtitre plates, however, gave results with low reproducibility. The reason for this could have been that the reaction buffer has to be weak to produce a measurable change

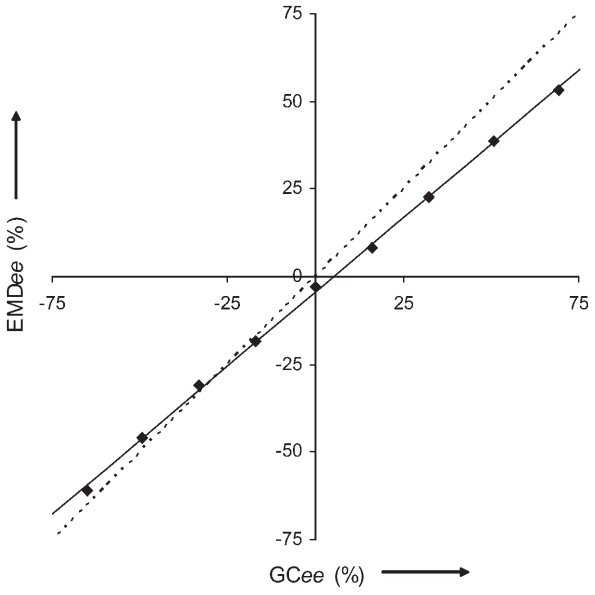


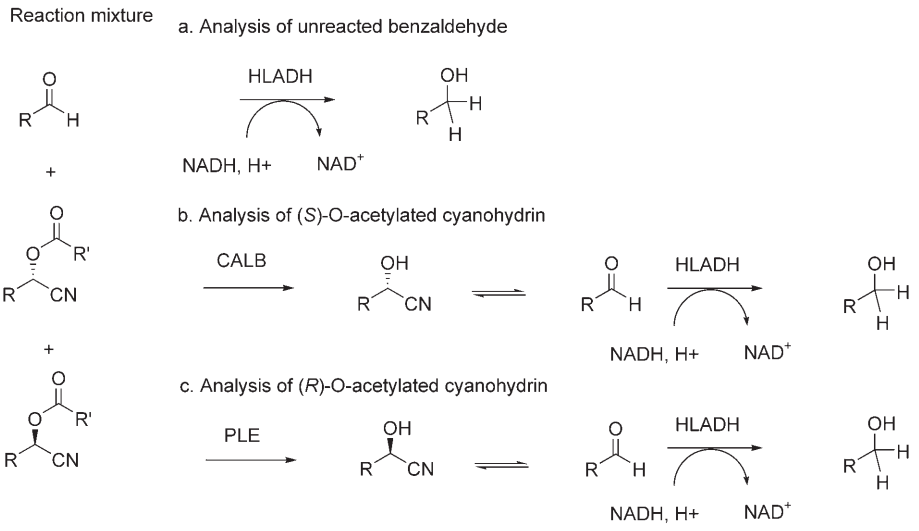
Figure 1. Example of enzymatic determination of enantiomeric excess plotted as a function of the corresponding values determined by GC. Positive *ee* values correspond to an excess of the *R* enantiomer, whereas negative values denote an excess of the *S* enantiomer. The dotted line corresponds to EMD*ee* = GC*ee*.

in absorbance, making the system very sensitive to variations in pH caused by, for example, contamination by carbon dioxide from the atmosphere.

This analytical method, however, has one important advantage over previously described enzymatic methods. Whereas the earlier methods rely on analysis of reaction rates, this method determines the amounts of the two enantiomers by measurement of the relative end points, making it less sensitive to enzyme inhibition and pipetting precision. We therefore decided to focus on alternative detection methods, still employing one selective and one unselective enzyme, but avoiding pH measurements.

EMD*ee* with NADH: To circumvent problems associated with detection of protons, a method based on the analysis of benzaldehyde with the aid of NADH was developed. In the catalytic reaction a scalemic mixture of the chiral O-acylated cyanohydrin was obtained together with various amounts of unreacted aldehyde. The first step in the EMD*ee* method was to reduce the remaining benzaldehyde with horse liver alcohol dehydrogenase (HLADH) and NADH (Scheme 2). Enzymatic hydrolysis with CALB gave the free cyanohydrin of the *S* product, which exists in equilibrium with benzaldehyde and so could be analysed in the same way as unreacted aldehyde. Hydrolysis with PLE finally afforded the free cyanohydrin of the second enantiomer, which was again reduced and analysed with the aid of NADH.

We had previously demonstrated the successful use of this method for analysis of the acetylated cyanohydrin obtained from benzaldehyde (Figure 2, **1a**),[16] and the tech-



Scheme 2. Determination of *ee* after NADH reduction at 340 nm.

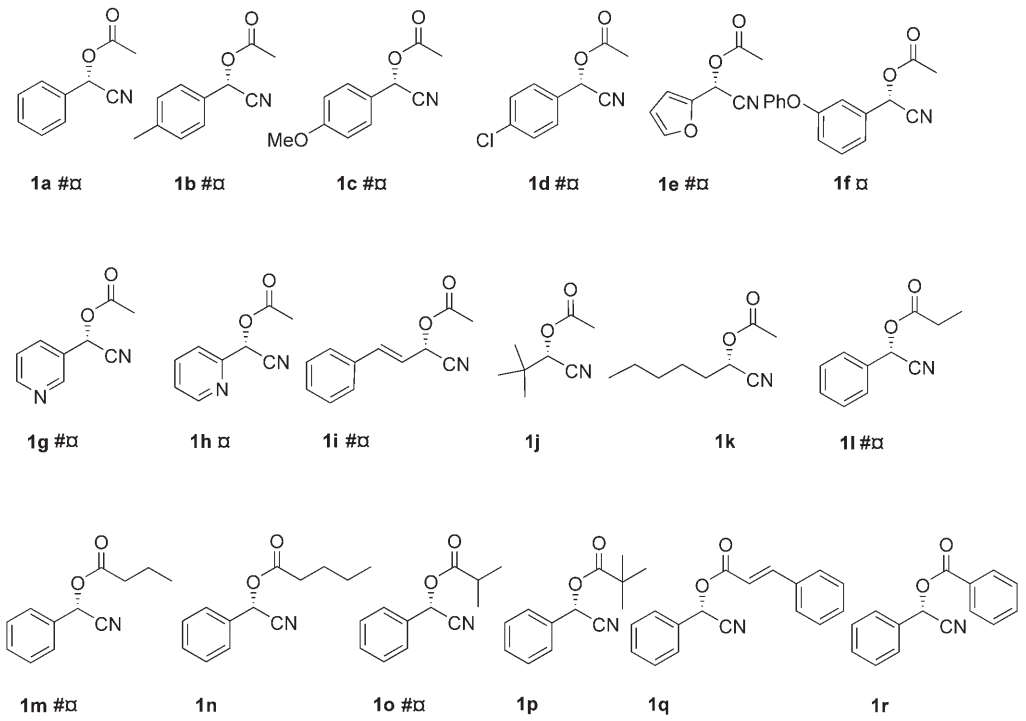


Figure 2. Structurally different cyanohydrin esters. Accurate determination of *ee* values could be achieved for compounds labelled with #, and levels of conversion for compounds labelled with ¤.

nique has now been extended to a variety of structurally different acylated cyanohydrins (**1a**–**1r**). The cyanohydrins were synthesised by our dual Lewis acid/Lewis base-catalysed addition of ketonitriles to aldehyde.[18] In total, 18 different substrates were tested, including cyanohydrins formed from 11 different aromatic and aliphatic aldehydes and eight different α-ketonitriles. For each substrate a racemic sample and two samples exhibiting excess of the *R* and the *S* enantiomer, respectively, were used. For each substrate, measurements were made on a solution giving a total absorbance change of 0.4–0.6 and on a sample obtained by twofold dilution of the initial solution.

Since each enzyme added to the reaction mixture caused a drop in absorbance, the relative amounts of the remaining aldehyde and of the *R* and the *S* enantiomers of the product could be measured; consequently, both enantiomeric excess and conversion could be determined from a single sample. An example is illustrated in Figure 3, in which the relationships between the enzymatic and the GC determinations of *ee* and conversion for compounds **1c** and **1i** are shown. The equations obtained by linear regression show acceptable agreements between GC and enzymatically determined values for both *ee* and conversion (r^2 values of 1.0, 0.98 (Figure 3a), 0.97 and 0.99 (Figure 3b)).

All substrates were analysed in the same way as substrates **1c** and **1i** (Figure 3). EMD*ee* could be accurately determined and expressed as a function of GC*ee* for ten of the substrates (**1a**–**e**, **1g**, **1i**, **1l**–**m** and **1o**, as indicated in Figure 2). Linear regressions of the functions obtained for these ten substrates gave r^2 values varying between 0.96 and 1.0 for the different substrates, and thus excellent correspondence between *ee* determination performed enzymatically and by GC. Moreover, use of samples with different substrate concentrations caused small variations in EMD*ee* (±5% units). The slopes varied between 0.63 and 0.97 depending on the substrate, and the intercepts varied between −20 and 5.1.

The calculated averages of both slopes and intercepts indicate an excess of the *S* enantiomer relative to the GC determinations. This discrepancy might be due to several factors. Background hydrolysis of both *R* and *S* enantiomers will affect the readout in favour of the *S* enantiomer. The *ee* then becomes dependent on the relationship of the reaction rates of the enzyme-catalysed and the spontaneous ester hydrolysis. Poor enantioselectivity for CALB towards the substrates and substrate racemization are other possible explanations for overestimation of the *S* enantiomer. In samples of substances that incorrectly show high excess of the *R* enantiomer, the *S* enantiomer is most probably underestimated. Poor activity of CALB for the substrate may give a false impression of depletion of the *S* enantiomer, resulting in addition of PLE at a too early stage. The values obtained for slopes and intercepts for the different substrates can be used for calibration of the method. The r^2 values demonstrate that the method is useful for accurate determination of the enantiomeric excess.

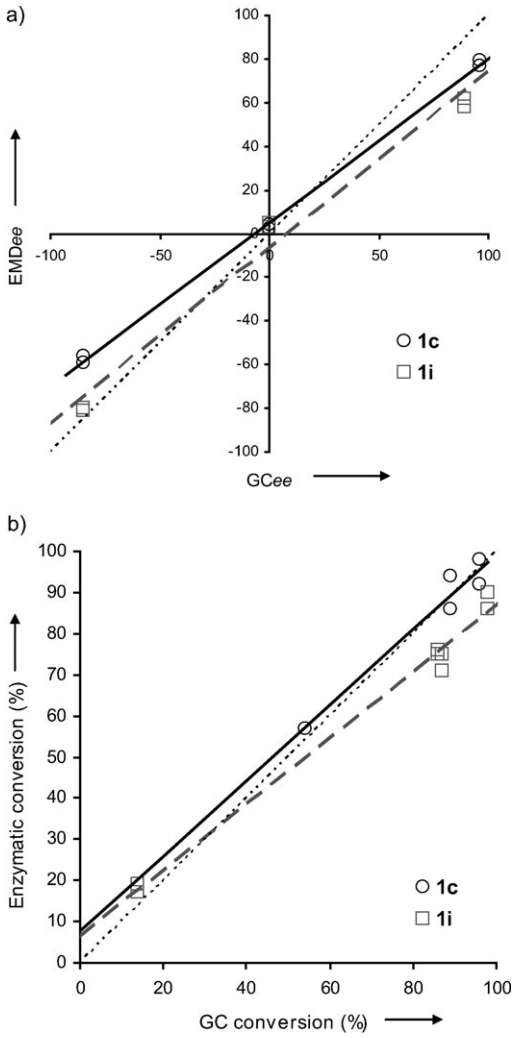


Figure 3. Example of enzymatic determination of *ee* (a) and conversion (b) plotted as a function of the corresponding values determined by GC for **1c** (○) and **1i** (□). Positive *ee* values correspond to an excess of the *R* enantiomer, negative to an excess of the *S* enantiomer. The dotted lines correspond to EMD value = GC value, whilst the continuous lines are linear regressions of results from **1c** (EMD*ee* = 0.75 GC*ee* + 5.1, r^2 = 1.0 and Enz−conv = 0.92 GC−conv + 7.6, r^2 = 0.97) and the dashed lines are linear regressions of results from **1i** (EMD*ee* = 0.81 GC*ee* − 6.6, r^2 = 0.98 and Enz−conv = 0.80 GC−conv + 6.3, r^2 = 0.99).

Results from the enzymatic method were also used to calculate conversion. Expression of the values determined enzymatically as a function of those determined by GC is shown in the example displayed in Figure 3b. Enzymatic determination of conversion could be accomplished with an average difference of ±9% units from the reference values obtained by GC analysis for 13 of the substrates (**1a**–**1i**, **1l**–**m** and **1o**). Again, the variations in the enzymatic analysis of conversion caused by different substrate concentrations were within an acceptable range (±6% units).

A comparison between GC and enzymatically determined data shows that the enzymatic method tends to underestimate conversion. Underestimations may be caused by overestimation of remaining aldehyde from background hydroly-

sis. Another possible reason for the measurement of low conversion value is the underestimation of the amount of product. This may be the result of a slow equilibrium between the unprotected cyanohydrin obtained from ester hydrolysis and the aldehyde.

For substrates **1f** and **1h**, only the conversion could be determined. The enzymatic measurements showed high excesses of the *S* enantiomers for samples of these substrates, regardless of the *ee*s measured by GC, which may be the result of substrate racemization during the analysis. The rates of hydrolysis with both CALB and PLE were severely reduced with substrates **1j**, **1k** and **1n** in comparison with the other tested substrates, which complicated measurements of *ee* and conversion. One possible reason for this might be poor solubility of the products of hydrolysis in the reaction buffer, which might strengthen interactions between the product and the active site and thereby cause inhibition. Another reason could be slow formation of aldehyde due to increased stability of the unprotected cyanohydrin. No activity of CALB was observed for compounds **1p**, **1q** and **1r**. This is probably due to the large acyl groups, which might not fit in the pocket of the active site of CALB.

High-throughput screening: For the synthesis of **1a** we used a T-shaped microreactor made in glass (Figure 4). The liquid was mobilised by application of a pressure flow. Different Lewis bases were screened and two different reaction times were used, providing 20 different entries (Figure 5).

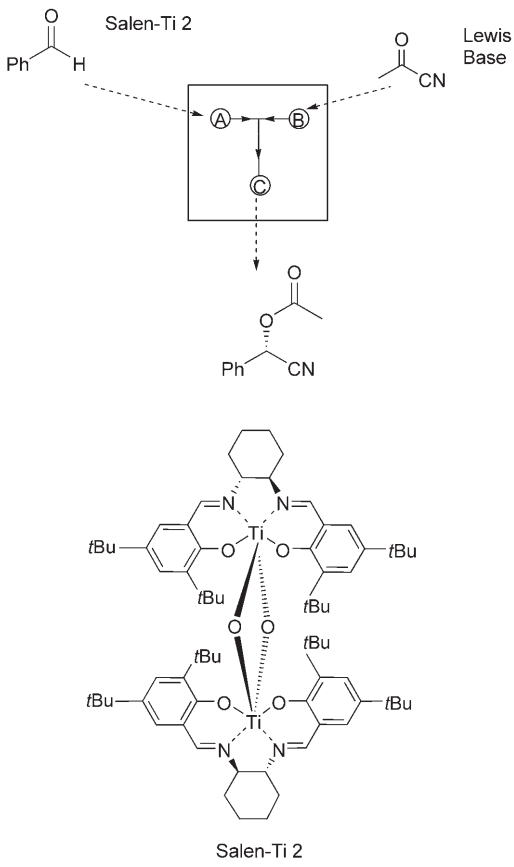


Figure 4. A schematic view of the microreactor, consisting of two inlets (A and B) and one outlet (C).

The crude reaction samples were analysed enzymatically on microtitre plates in order to evaluate the robustness of the method. The different Lewis bases and catalysts used for synthesis of the synthesis of acylated cyanohydrins (Figure 5) did not affect the enzymatic determination either of *ee* values or of conversion (Figure 6), so the method is insensitive to alterations of these synthesis parameters.

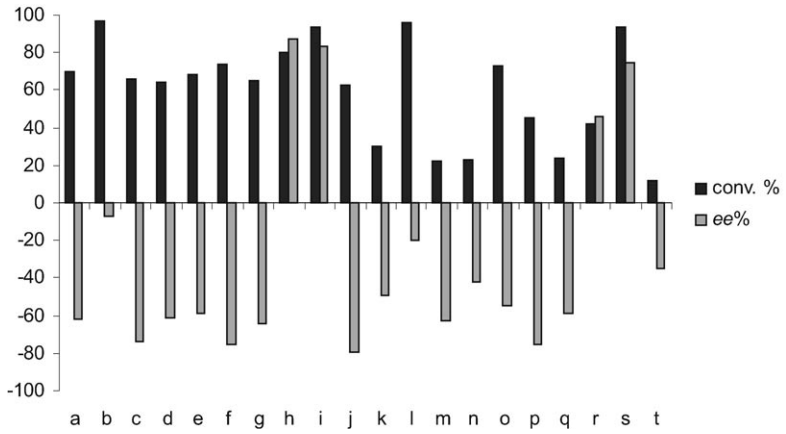


Figure 5. The influence of Lewis bases and reaction time on the enantioselectivity and conversion. All reactions were carried out in dichloromethane at room temperature for 40 min (a–j) or 20 min (k–t) with acetyl cyanide (2 equiv), 5 mol% of either **2** (a–g, j, k–q, t) or *ent*-**2** (h, i, r, s) and Lewis base (10 mol%). a) Et_3N, b) DBU, c) cinchonidine, d) quinine, e) DIPEA, f) DMAP, g) DEA, h) cinchonidine, i) quinine, j) DABCO. k) Et_3N, l) DBU, m) cinchonidine, n) quinine, o) DIPEA, p) DMAP, q) DEA, r) cinchonidine, s) quinine, t) DABCO. Levels of conversion and *ee* values were determined by GC. Positive *ee* values denote an excess of the *R* enantiomer whereas negative *ee* values denote an excess of the *S* enantiomer.

Variations in reaction time, Lewis base and catalyst afforded different levels of conversion of **1a** (Figure 5). Enzymatic analysis of conversion was adequately correlated with values obtained by GC (Figure 6b). Furthermore, EMD*ee* could be performed at all tested levels of conversion (Figure 6a), although lowering of the levels of conversion caused increased standard deviations in EMD*ee*. The average standard deviation of the EMD*ee* values obtained for samples with levels of conversion higher than 50% was 5 (EMD*ee*% units), whereas the corresponding value for samples with levels of conversion lower than 50% was 11 (EMD*ee*% units). The measuring range in the absorbance was limited (0–0.8) for the enzymatic method. Low levels of conversion consumed

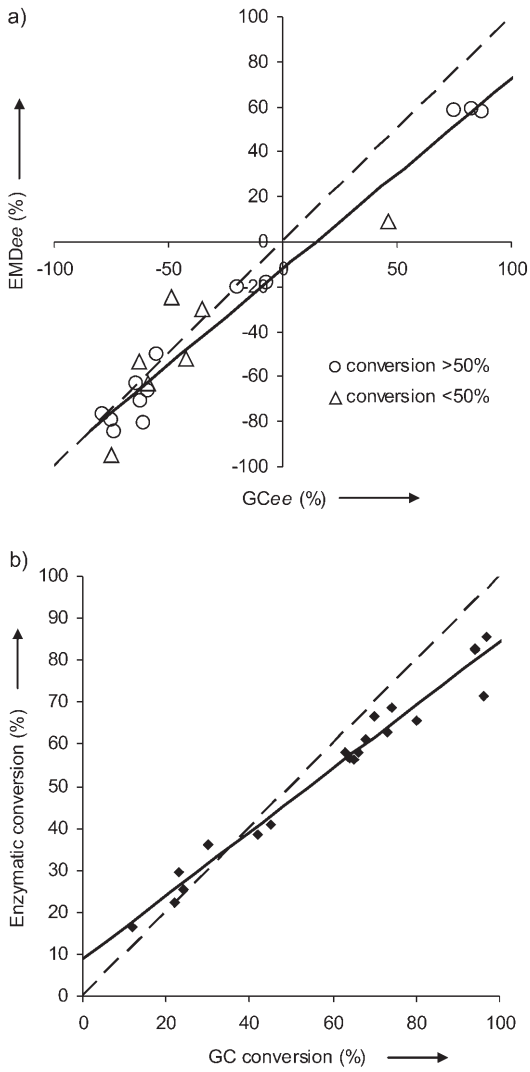


Figure 6. a) Average EMD*ee* from quadruplicate measurements of samples from mandelonitrile acetate prepared with various Lewis bases, plotted as a function of *ee* determined by GC. Positive *ee* values correspond to an excess of the *R* enantiomer, whereas negative values denote an excess of the *S* enantiomer. Determination of *ee* values were made on samples with an enzymatically determined conversion higher than 50% (○) and lower than 50% (△). b) Average levels of conversion from the same measurements as in a), plotted against the corresponding values obtained by GC. The continuous lines are linear regressions of the results and the dashed lines correspond to EMD value = GC value.

too much of the dynamic range to allow accurate estimations of the two enantiomers, which increases the error of the *ee* determination. Linear regression using all measurement points in Figure 6a and b gave the equations EMD*ee* = 0.85 GC*ee* − 12 and EMD*c* = 0.76 GCc + 8.8. As discussed above, the enzymatic measurements underestimate both enantiomeric excess and level of conversion in relation to the corresponding values obtained by GC. Consequently, substrate-specific calibration of the enzymatic method is necessary for accurate measurements of *ee* and yield. The method is highly suitable, however, for initial screening of enantiomeric purity and yield.

Conclusion

We have developed an enzymatic method for the determination of yields and enantiomeric excesses of O-acylated cyanohydrins obtained by Lewis acid/Lewis base-catalysed addition of ketonitriles to aldehydes. The crude reaction mixtures were treated with NADH and horse liver alcohol dehydrogenase (HLADH), causing reduction of remaining starting aldehyde, followed by treatment with *Candida antarctica* lipase B (CALB), which selectively hydrolyses the *S* product, and finally with pig liver esterase (PLE), which hydrolyses the remaining ester. The hydrolysed cyanohydrins exist in equilibrium with the corresponding aldehydes, which are reduced by NADH/HLADH. After the addition of each enzyme, the amount of alcohol was determined by spectrometric measurement of the amount of remaining NADH. Since a single sample is used for the analyses, precise knowledge about concentrations or measurement of volumes is not required, which makes the method suitable for downscaling.

The new method was tested on 18 products, obtained from 11 different aldehydes and eight different ketonitriles. Compounds obtained from benzaldehyde, 4-methyl-, 4-methoxy-, and 4-chlorobenzaldehyde, furan-2-carbaldehyde, pyridine-3-carbaldehyde, and cinnamaldehyde and linear or branched aliphatic ketonitriles containing up to four carbon atoms could be accurately analysed. Close to linear relationships between values determined by the enzymatic method and by GC were observed and the method was insensitive to variations in substrate concentration. Even though the amount of *S* product was overestimated for some products, accurate *ee* determinations could be made after calibration.

The analytical procedure was combined with use of a microreactor for high-throughput optimization. The method is highly suitable for initial screening of yields and enantioselectivities without any purification of the reaction mixture. When successful catalytic systems have been identified, the performance of these can be evaluated more precisely by conventional analytic methods (GC and HPLC). A fully integrated system for synthesis and analysis remains a challenge for the future.

Experimental Section

General: Compounds **1a**–**1r** were prepared by our previously published procedure.[18] CALB was obtained from in-house cultivations and was purified by hyrophobic interaction chromatography (HIC).[19] PLE was purchased from BioChemika as lyophilised powder (130 U mg^{-1}). HLADH was purchased from Sigma as lyophilised powder (1.33 U mg^{-1} solid, 1.8 U mg^{-1} protein). Sodium dihydrogen phosphate was analysis grade, purchased from Merck. Sodium hydroxide was >98% pure, purchased from Merck. NADH was 98% pure, purchased from Sigma.

Enzymatic determination of enantiomeric excess by use of a pH indicator: Detection with a pH indicator was performed on samples of **1a** exhibiting *ee* values varying from 69% of the *R* enantiomer to 65% of the *S* enantiomer. The reaction samples were purified by column chromatography and concentrated by evaporation prior to analysis. The samples were diluted 15000 times into a reaction buffer containing *p*-nitrophenol

(0.08 mM), MOPS (4 mM, pH 7.2) and acetonitrile (0.7%). The absorbance was recorded at 405 nm with a Cary Bio spectrophotometer in 1.5 mL semi-micro UV cuvettes from Plastibrand. Analyses were carried out with 1.2 mL of the solution in each cuvette. The absorbance was measured continuously during the different reaction steps. A starting value of the absorbance was recorded over 10 minutes to verify a stable level of absorbance. Afterwards, CALB solution (10 mg mL^{-1}, 12 μL, 5 mM MOPS, pH 7.2) was added. The reaction time for stabilization of the absorbance level, corresponding to depletion of the *S* enantiomer, was 30 minutes. For the second reaction step, PLE solution (10 mg mL^{-1}, 12 μL, 5 mM MOPS, pH 7.2) was added and the reaction time for stabilization of the absorbance level, corresponding to depletion of the remaining *R* enantiomer, was 30 minutes. A control sample, not containing the reaction mixture, was used for subtraction of any background decrease in absorbance.

Enzymatic determination of enantiomeric excess and conversion by use of NADH: Compounds **1b–1r** were tested by conventional spectrophotometry. For each of the substances **1b–1i**, **1l–1m** and **1o**, a racemic sample and two samples exhibiting high *ee* values, one of the *R* and one of the *S* enantiomer, were tested. Racemic samples were tested for the substances **1k**, **1n** and **1p–1r**. The measurements were performed at 340 nm in 1.5 mL semi-micro UV cuvettes from Plastibrand in a Cary 300 Bio spectrophotometer. Two different dilutions were made for each sample, one giving a total change in absorbance of 0.4–0.6, and one giving a total absorbance change of 0.2–0.3. Typically, these were 600-fold and 1200-fold dilutions of the samples from the reaction mixture, which also contained NADH (0.17 mM), $Na_xH_{3-x}PO_4$ (83 mM, pH 7.0) and acetonitrile (0.8%). Analysis was carried out with a volume of 1.2 mL of the solution in each cuvette. The absorbance was measured continuously during the different reaction steps. A starting value of the absorbance was recorded over 10 minutes to verify a stable level of absorbance. Afterwards, HLADH solution (10 mg mL^{-1}, 12 μL, 100 mM $Na_xH_{3-x}PO_4$, pH 7.0) was added to the sample solution. The reaction time necessary to obtain a stable absorbance level, corresponding to depletion of unconverted aldehyde, was typically 20 minutes. In the following reaction step, CALB solution (10 mg mL^{-1}, 12 μL, 100 mM $Na_xH_{3-x}PO_4$, pH 7.2) was added. The reaction time for stabilization of the absorbance level, corresponding to depletion of the *S* enantiomer, was typically 45 minutes. For the last reaction step, PLE solution (10 mg mL^{-1}, 12 μL, 100 mM $Na_xH_{3-x}PO_4$, pH 7.0) was added and the reaction time for stabilization of the absorbance level, corresponding to depletion of the remaining *R* enantiomer, was typically 45 minutes. Variations in reaction times were less than 10 minutes, depending on the activity for the enzymes towards the different substrates. A control sample, without the reaction mixture, was used for subtraction of any background decrease in absorbance.

General procedure for microreactor-based reactions: A T-shaped microreactor design with three reservoirs, two inlets (A and B) and one outlet (C), with approximate channel dimensions of 100×50 μm and outer dimensions of 20×20×25 mm, was used.[10,20] Two standard solutions, S1 and S2, were prepared:

S1: Salen-Ti **2** (Figure 4, 60 mg, 0.049 mmol, 5 mol%), Lewis base (10 mol%) and benzaldehyde (100 μL, 0.98 mmol) were dissolved in dry dichloromethane (1 mL) and the solution was cooled to 0 °C.

S2: Pyruvonitrile (140 μL, 1.98 mmol) was dissolved in dry dichloromethane (1 mL) and the solution was cooled to 0 °C.

The channels of the microreactor were primed with dry dichloromethane prior to the addition of standard solutions S1 and S2 (50 μL of each) to reservoirs A and B. A pressure generating a flow of 1 μL min^{-1} was applied. Reactions were performed at room temperature over a 20 or 40 min period. The microreactor was washed with dry dichloromethane prior to each experiment to remove any residue from the system.

High-throughput screening: The screening was performed on a Greiner bio-one PS microplate (96 well microtitre plate) in a FLUOstar OPTIMA plate reader fitted with two separate injection pumps (pumps 1 and 2). Absorbance was recorded at 340 nm. The following solution was prepared individually for each of the 20 samples: 600-fold dilution of reaction mixture in NADH (0.17 mM), $Na_xH_{3-x}PO_4$ (83 mM, pH 7.0) and acetonitrile (0.8%). Analysis was carried out with a volume of 300 μL of this solution in each well. Measurement of absorbance was carried out in cycles. Initially a starting level of absorbance was recorded for 27 minutes. Addition of 3 μL of HLADH solution (10 mg mL^{-1}, 100 mM $Na_xH_{3-x}PO_4$, pH 7.0) was performed automatically from pump 1. The absorbance was measured for 46 minutes to ensure depletion of unreacted aldehyde. After completion of the first reaction step, pump 1 was washed with buffer and filled with CALB solution (10 mg mL^{-1}, 100 mM $Na_xH_{3-x}PO_4$, pH 7.2), whilst pump 2 was filled with PLE solution (10 mg mL^{-1}, 100 mM $Na_xH_{3-x}PO_4$, pH 7.0). Absorbance was recorded for 8 minutes (two cycles) to compensate for any differences between measurements. Addition of CALB solution (3 μL) was then performed from pump 1. The hydrolysis of the *S* enantiomer was monitored for 40 minutes, after which PLE solution (3 μL) was added from pump 2. The hydrolysis of remaining *R* enantiomer was observed after 44 minutes of reaction time. The last cycle of absorbance measurements after each reaction step was used for calculations of *ee* and conversion. The samples were run in quadruplicate. The mean value from 16 control samples, identical to the reaction mixture but without substance **1a**, was used for subtraction of any background decrease in absorbance.

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