New Methods for the High-Throughput Screening of Enantioselective Catalysts and Biocatalysts

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The importance of asymmetric catalysis is undisputed, as evidenced, amongst other things, by the 2001 Nobel Prize for Chemistry to K. Barry Sharpless, Ryoji Noyori, and William S. Knowles. The two major options available to chemists are homogeneous transition metal catalysts[1] and biocatalysts.[2] In the former case ligand tuning based on intuition, knowledge of the reaction mechanism, molecular modeling as well as some degree of trial and error is the key to success.

In the 1990s a new technique emerged which has been loosely called combinatorial asymmetric catalysis.[3] This involves time-saving parallel synthesis and the testing of large numbers of chiral catalysts. The challenges in this interesting new area of asymmetric catalysis are twofold, which center around stragegies for the modular synthesis of chiral ligands and on developing high-throughput assays for determining the enantiomeric excess (*ee*).[4] So far, the size of the catalyst libraries has usually been limited to less than a hundred or so catalysts, which were often analyzed conventionally.

The potential of combinatorial asymmetric catalysis has not been exploited to its full extent. Successfully finding hits is likely to increase upon expanding the size of the libraries. In the case of enzyme catalysis, the idea of applying the molecular biological methods of directed evolution in the creation of enantioselective enzyme mutants (variants) has been put into practice recently.[5, 6] The underlying concept goes beyond combinatorial catalysis, since it is based on repeating rounds of mutagenesis/screening which create an evolutionary pressure without the need to know the structure or mechanism of the enzymes. The size of each library of mutant enzymes produced by error-prone polymerase chain reaction or DNA shuffling typically amounts to several thousand members, which poses a truly difficult analytical problem if each of them is to be tested for enantioselectivity in a given reaction.

It is clear that both approaches to asymmetric catalysis require the availability of high-throughput *ee* screening systems. The first *ee* assay designed to handle a reasonably large number of samples was a rather crude UV/Vis-based screening system for the lipase-catalyzed kinetic resolution of chiral *p*-nitrophenol esters. The *R* and the *S* esters were tested separately pairwise on a 96-well microtiter plate using a simple UV/Vis-based plate reader.[5a] The concept of testing *R* and *S* substrates separately in kinetic resolution has some disadvantages.[4] Nevertheless, it forms the basis of other tests as well,[4] including an interesting recent development using coupled enzymatic transformations.[7] Several more general screening systems have been devised, such as methods based on the mass spectrometric analysis of deuterium-labeled substrates that allow for 1000 *ee* determinations per day (which has now been extended to 10000 by using an eight-channel, multiplexed sprayer system),[8] the use of capillary array electrophoresis (in some cases up to 30000 *ee* determinations per day),[9] and the use of HPLC/circular dichroism (CD) spectoscopy (typically 1000 samples per day).[10]

These and other methods were reviewed last year.[4] Nevertheless, continued efforts in this fascinating area of research are necessary because no single assay is universal. van Delden and Feringa have recently reported an ingenious color test for enantioselectivity based on chirality-dependent color generation in doped films of liquid crystals.[11] It remains to be seen if this interesting system can be modified into a high-throughput *ee* assay.

Recently, several new approaches to the high-throughput analysis of enantioselective catalysts have been described. A novel technique reported by Shair and co-workers makes use of DNA microarrays.[12] This type of technology had previously been used to determine relative levels of gene expression on a genome-wide basis, as measured by the ratio of fluorescent reporters.[13] In the newly developed *ee* assay, the goal was to measure the enantiopurity of chiral amino acids.[12] One can imagine that such *R*/*S* mixtures could be produced by Rh-catalyzed hydrogenation of the corresponding *N*-acylamino acrylate by using a library of combinatorially prepared catalysts. In the model study, mixtures of an *R* and *S* amino acid were first subjected to acylation at the amino function to form *N*-Boc-protected derivatives (Boc = *tert*-butoxycarbonyl). Samples were then covalently attached to amine-functionalized glass slides in a spatially arrayed manner (Figure 1). In a second step the uncoupled surface amino functions were acylated exhaustively. The third step involved complete deprotection to afford the free amino function of the amino acid. Finally, in a fourth step two pseudo-enantiomeric

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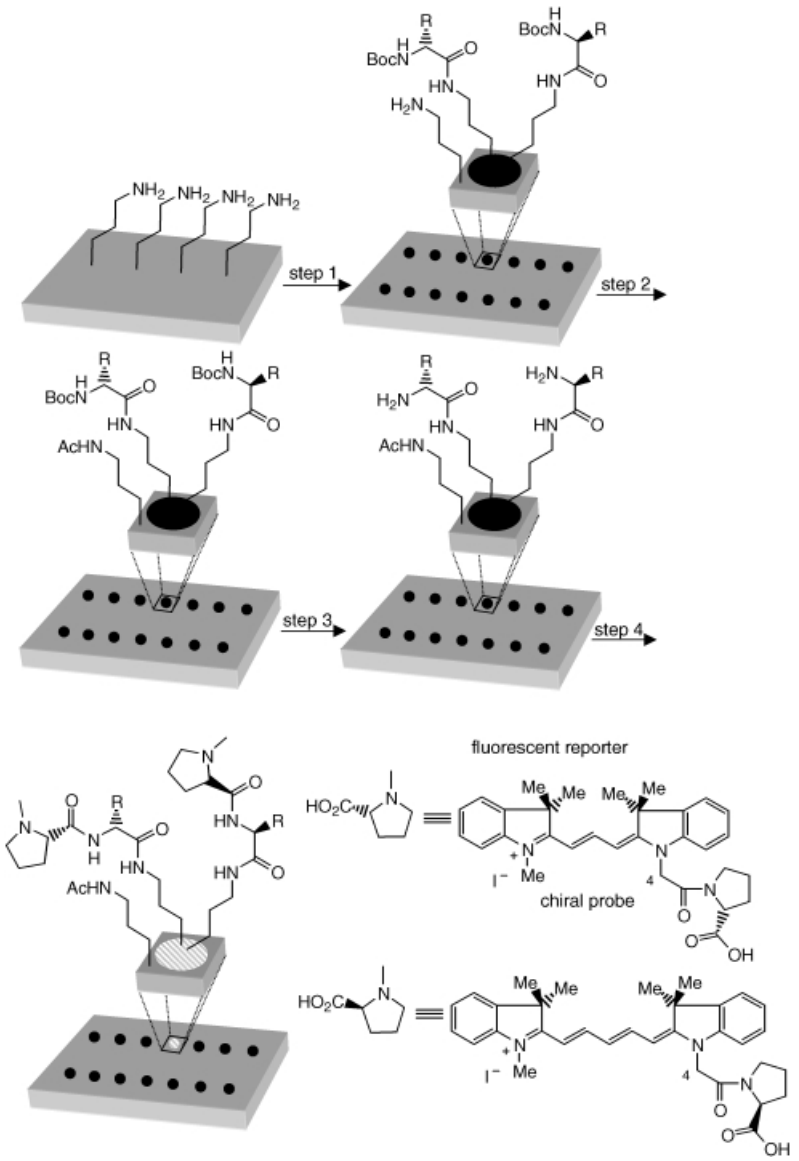
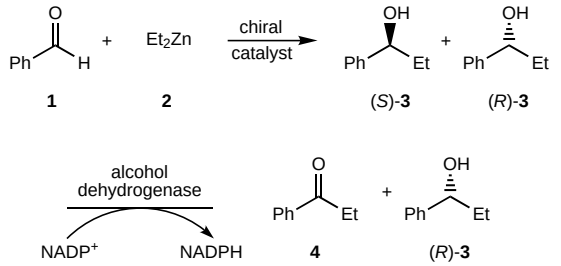


Figure 1. Reaction microarrays in high-throughput *ee* determination. Reagents and conditions:[12] step 1) BocHNCHRCO$_2$H, PyAOP, *i*Pr$_2$NEt, DMF; step 2) Ac$_2$O, pyridine; step 3) 10% CF_3CO_2H and 10% Et_3SiH in CH_2Cl_2, then 3% Et_3N in CH_2Cl_2; step 4) pentafluorophenyl diphenylphosphinate, *i*Pr$_2$NEt, 1:1 mixture of the two fluorescent proline derivatives, DMF, $-20\,^{\circ}C$. PyOAP = 7-azabenzotriazol-1-yloxytris(pyrrolidino) phosphonium hexafluorophosphate.

fluorescent probes were attached to the free amino groups on the surface of the array.

An appreciable degree of parallel kinetic resolution in the process of amide coupling is a requirement for the success of the *ee* assay,[12] which is similar to a mass spectrometric based system previously devised by Finn and co-workers.[14] In the present case the *ee* values are accessible by measuring the ratio of the relevant fluorescent intensities. It was reported that 8000 *ee* determinations are possible per day, with precision amounting to $\pm 10\%$ of the actual value. It remains to be seen whether DNA microarray technology can be modified in an analogous manner in the case of other types of substrates.[19]

Abato and Seto have described an enzymatic method for determining enantiomeric excess (EMDee).[15] The method is based on the idea that an appropriate enzyme can be used to selectively process one enantiomer of a product from a catalytic reaction. The well-known catalytic addition of diethylzinc (**2**) to benzaldehyde (**1**) was chosen as a test-bed for demonstrating EMDee. The reaction product, 1-phenylpropanol (**3**), can be oxidized to ethyl phenyl ketone (**4**) using the alcohol dehydrogenase from *Thermoanaerobium sp.*, this process being completely *S* selective (Scheme 1). It was possible to measure the rate of this enzymatic oxidation by monitoring the formation of NADPH by UV spectroscopy at 340 nm.



Scheme 1. EMDee in the case of 1-phenylpropanol produced by asymmetric addition of diethylzinc to benzaldehyde.[15]

Decisive for the success of the assay is the finding that the rate of oxidation constitutes a direct measure of the *ee* value (Figure 2).[15] High-throughput was demonstrated by analyzing 100 samples in a 384-well format by using a UV/fluorescence plate reader. Each sample contained 1 μmol of 1-phenylpropanol (**3**) in a volume of 100 μL. The accuracy of the *ee* value amounts to $\pm 10\%$, as checked by independent GC determinations. About 100 samples can be processed in 30 minutes,[15] which calculates as 4800 *ee* determinations per day.

As noted by the author, EMDee does not distinguish between processes that proceed with low enantioselectivity but high conversion and high enantioselectivity but low conversion. EMDee was

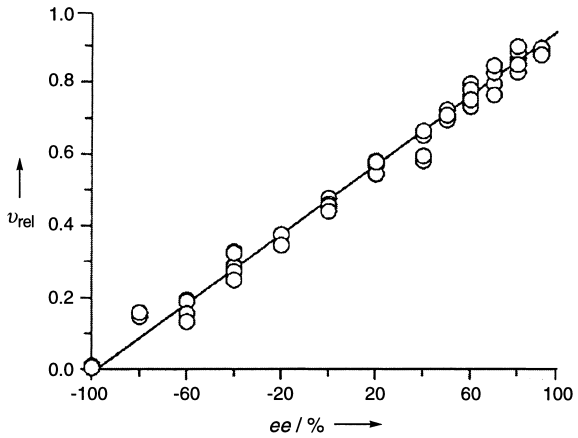


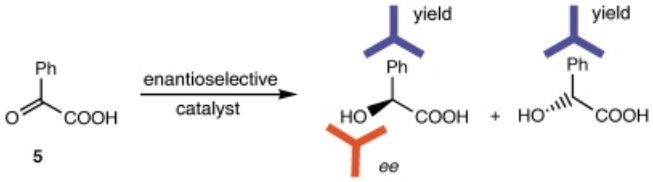
Figure 2. Plot of the initial rate of the enzyme-catalyzed oxidation of 1-phenylpropanol as a function of the *ee* value (the solid line represents a fit of the data to the Michaelis–Menten formalism for competitive inhibition where [S] = [(*S*)-**3**] and [I] = [(*R*)-**3**]).[15] The total alcohol concentration was maintained constant at 10 mM.

  1433-7851/02/4108-1336 $ 20.00+.50/0 *Angew. Chem. Int. Ed.* **2002**, *41*, No. 8

therefore extended to provide information regarding both the *ee* value and conversion.[15] Accordingly, in a second set of assays the *R*-selective alcohol dehydrogenase from *Lactobacillus kefir* was used to quantify the amount of (*R*)-**3** present in the mixture. The conversion can be calculated since the amounts of (*R*)-**3** and (*S*)-**3** are known.

The question arises as to the generality of the EMDee assay in the case of other chiral alcohols which do not show such high enantioselectivity in the oxidation catalyzed by alcohol dehydrogenase. Theoretically, the test should still work, although accuracy in such cases would have to be demonstrated. It may be easier to use a different, more selective alcohol dehydrogenase, and indeed a large number of such enzymes are commercially available. In summary, EMDee constitutes an interesting way to determine the *ee* value of alcohols in a high-throughput manner by using standard instrumentation. Of course, the assay has to be optimized for each new chiral alcohol under study.

A third recent development, reported by Wagner, Mioskowski, and co-workers, concerns high-throughput screening of enantioselective catalysts by enzyme immunoassays,[16] a technology that is routinely applied in biology and medicine. The new assay was illustrated by analyzing *R*/*S* mixtures of mandelic acid prepared by the enantioselective Ru-catalyzed hydrogenation of benzoyl formic acid (**5**; Scheme 2). By employing an antibody that binds both enantiomers it was possible to measure the concentration of the reaction product, thereby allowing the yield to be calculated. The use of an *S*-specific antibody then makes the determination of the *ee* value possible.



Scheme 2. High-throughput screening of enantioselective catalysts by competitive enzyme immunoassays.[16] The antibody marked blue recognizes both enantiomers, whereas the antibody marked red is *S* specific, thus making the determination of yield and *ee* value possible.

Although this procedure may sound complicated to some organic chemists, and in fact details need to be consulted, antibodies can be raised to almost any compound of interest. Moreover, simple automated equipment comprising a plate washer and plate absorbance reader is all that is necessary. About 1000 *ee* determinations are possible per day, with precision amounting to $\pm 9\,\%$.[16]

In summary, several novel high-throughput *ee* assays have been reported recently,[7, 12, 15, 16] thus extending the list of previously developed systems.[4] Yet another *ee* assay has been devised in the author's laboratories which utilizes NMR detection, and makes possible at least 1000 *ee* determinations per day with surprisingly high accuracy.[17] Perhaps one of the advantages of this system has to do with the fact that organic chemists are well acquainted with NMR spectroscopy. The decision as to which *ee* screening system to use also depends upon the particular analytical problem. If medium-throughput is sufficient for a given purpose, that is, 300 – 700 samples per day, even classical gas or liquid chromatography in properly modified forms may suffice. Such a system comprising two GC columns in separate ovens, a single prep-and-load sample manager, and one PC has been shown to function well for certain substrates (500 – 700 *ee* determinations per day).[18] The precision of the *ee* determination is another criterion which needs to be considered, and depends upon the particular analytical problem. Screening for hits in combinatorial asymmetric transition metal catalysis or in the early phases of directed evolution of an enantioselective enzyme may not require maximum precision in the *ee* value. However, in the late stages of a directed evolution project, for example, when attempting to increase the *ee* value in a stepwise manner from typically 90 % to higher values, high analytical precision does in fact become important.

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