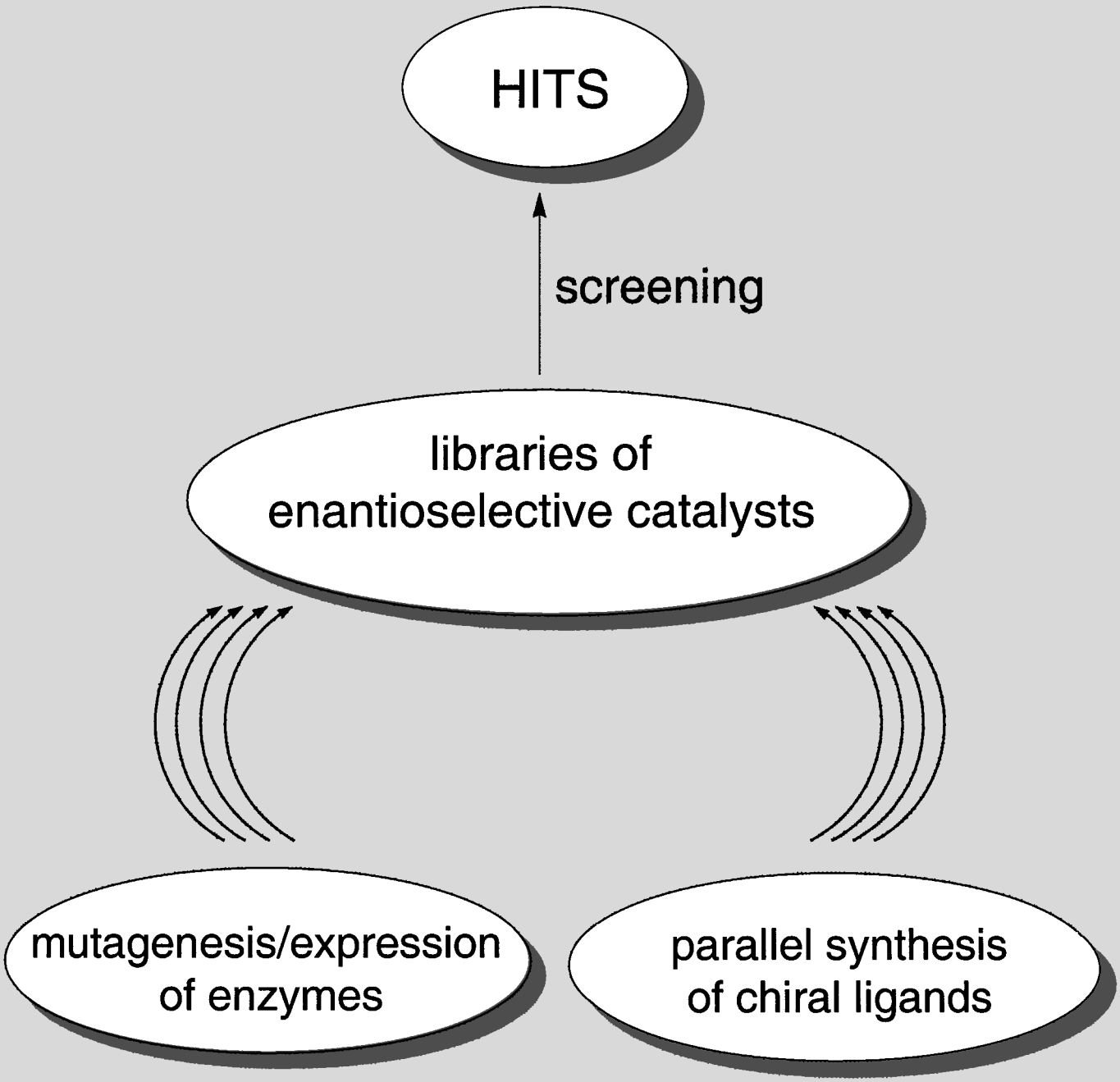

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Combinatorial and Evolution-Based Methods in the Creation of Enantioselective Catalysts

Manfred T. Reetz*

Dedicated to Professor K. Barry Sharpless on the occasion of his 60th birthday

Combinatorial methods in the development of enantioselective homogeneous catalysts constitute a new branch of catalysis research. The goal is to prepare libraries of potential asymmetric catalysts, rather than choosing the traditional one-catalyst-at-a-time approach. Several conceptional advancements have been reported in the parallel preparation of chiral ligands. Currently the most meaningful systems constitute modularly constructed ligands on solid supports, which allow high degrees of structural diversity and thus the maximum probability of finding enantioselective catalysts or even new types of ligands for asymmetric catalysis. Search strategies have been developed which amongst other things, lead to catalysts not likely to have been discovered by traditional methods. Genuine application of such strategies involve thousands of catalysts and require high-throughput screening systems capable of assaying enantioselectivity. The first high-throughput *ee*-screening systems were in fact developed for use in the directed evolution of enantioselective enzymes, a process based on "evolution in the test tube" in which the appropriate methods of random mutagenesis, gene expression, and *ee* assays are combined. Since no screening system is likely to be universal, different approaches are necessary. Thus far these include assays based on UV/Vis, fluorescence, circular dichroism, mass spectrometry, and even modified gas chromatography as well as special forms of capillary electrophoresis. One of the most efficient systems involves the concept of the mass-spectrometric detection of deuterium-labeled pseudo-enantiomers and pseudo-prochiral compounds with which about 1000 exact *ee* determinations can be achieved per day, although the assay is restricted to kinetic resolution and/or reactions of prochiral compounds bearing enantiotopic groups. Super-high-throughput screening for enantioselectivity is possible in many cases by making use of chirally modified capillary array electrophoresis in a parallel step. Accordingly, 7000 to 30000 *ee* determinations can be carried out per day. These and other analytical developments are expected to stimulate further research in the combinatorial search for asymmetric homogeneous catalysts and in the directed evolution of enantioselective enzymes for use in organic chemistry.

Keywords: analytical methods · asymmetric catalysis · combinatorial chemistry · directed evolution · enzyme catalysis · high-throughput screening

1. Introduction

The importance of enantiomerically pure or enriched organic compounds in pharmaceutical, agricultural, synthetic organic, and natural products chemistry is steadily growing.[1] This is also reflected by the so-called "chiral market" of industrial pharmaceutical products which in 1999 amounted to more than $80 billion and which is expected to continue to grow.[1d,e] Although many of these products are not prepared synthetically in the laboratories of organic chemists, nor are they ever likely to be so, many chiral target compounds or intermediates are in fact amenable to synthesis by organic chemists. Although classical separation of enantiomers coupled with racemization of the undesired stereoisomer is still the preferred industrial process,[1d] in the future the economically and ecologically most attractive strategy is likely to be enantioselective catalysis. Indeed, a good part of basic research in organic chemistry during the last three decades has been devoted to the development of methods in the area of asymmetric synthesis,[2] but more efforts are necessary. The two options are man-made catalysts, such as transition metal complexes,[3] on the one hand and biocatalysts on the other.[4] In the former case one considers a known transition metal catalyzed reaction and modifies the catalyst by introducing chiral ligands. Although application of such a catalyst in a given reaction necessarily leads to diastereomorphic transition states with formation of the two possible enantiomeric

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products, the respective energy difference $|\Delta G_R^* - \Delta G_S^*|$ may be so small that no appreciable enantioselectivity is observed. Indeed, more than 2000 chiral phosphorus-containing ligands have been prepared, yet only a handful are truly enantioselective ($ee \geq 95\,\%$), and application is generally restricted to certain classes of substrates or even specific substitution patterns.[3] As a result of ligand tuning, second and *n*th generation catalysts continue to be developed in a process that requires experience, intuition, knowledge of the reaction mechanism, the ability to apply molecular modeling and other theoretical techniques, as well as a great deal of trial and error (Figure 1 a). In almost all cases not only do steric and

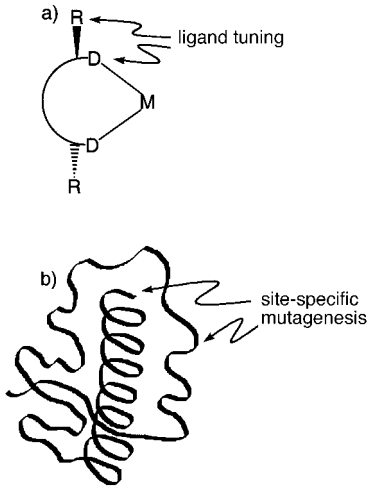

Figure 1. a) Schematic representation of ligand tuning in the design of a chiral transition metal (M) catalyst (the C_2 symmetry is arbitrarily shown); the arrows symbolize points of potential structural variation and D denotes donor atom. b) Schematic representation of a de novo design of an enantioselective enzyme; the arrows symbolize the exchange of amino acids on the basis of site-specific mutagenesis.

electronic properties of the chiral ligand need to be considered, but also the nature of the metal, temperature, solvent, and chiral or achiral additives. Since each catalyst is optimized one by one, an enormous amount of costs and labor is involved. Stereoselectivity is routinely ascertained by measuring the enantiomeric excess (*ee*) of a given product using conventional techniques such as gas chromatography (GC),[5] high-performance liquid chromatography (HPLC),[6] or (less often) capillary electrophoresis (CE),[7] all with chiral stationary phases. A few dozen samples can be assayed per day, which is certainly not the slow step in the overall process of developing an enantioselective catalyst in a traditional way. If biocatalysis is the option, as many commercially available enzymes as possible are tested until success emerges.[4] If enantioselectivity remains unacceptable for a given reaction of interest, then a process analogous to ligand tuning in transition metal catalysis can be attempted, namely the exchange of the amino acid present at a given position against one of the remaining 19 naturally occurring amino acids using site-directed mutagenesis[8] (Figure 1 b). Unfortunately, as a consequence of the structural complexity of the enzyme and the lack of appropriate theoretical techniques, very few successful cases have been reported so far.

In addition to enantioselectivity, catalyst performance includes such parameters as activity, stability, and ability to be recycled. The challenges in preparing active and enantioselective transition metal catalysts or engineering enzymes for real use in asymmetric transformations are thus reminiscent of (but not identical to) the problems that chemists encountered in traditional therapeutic drug development prior to the advent of combinatorial chemistry, namely, when compounds were synthesized and tested sequentially one by one in a labor-intensive manner. Combinatorial methods in the discovery of pharmaceuticals and other biologically active compounds constitute tools with which large libraries of compounds can be synthesized and screened within a short period of time.[9] Two different strategies can be used, namely, the iterative split-and-mix (also called split-and-pool) or parallel synthesis. The advantage of the split-and-mix synthesis, which is performed on polymer resin beads, relates to the enormously high number of compounds that are theoretically possible within a short period of time as a result of the permutational possibilities provided by the number of synthetic steps and reaction components. However, the identification of the members of the library requires time and effort as a consequence of deconvolution or encoding.[9g] In parallel synthesis all reactions are performed in separate vessels using robotic equipment, with the products being lower in number, but available in defined arrays. Such spatially addressable libraries of compounds are readily amenable to high-throughput screening for biological properties, with many methods being based on fluorescent-active or radiolabeled receptors. Although a consensus concerning terminology has not been

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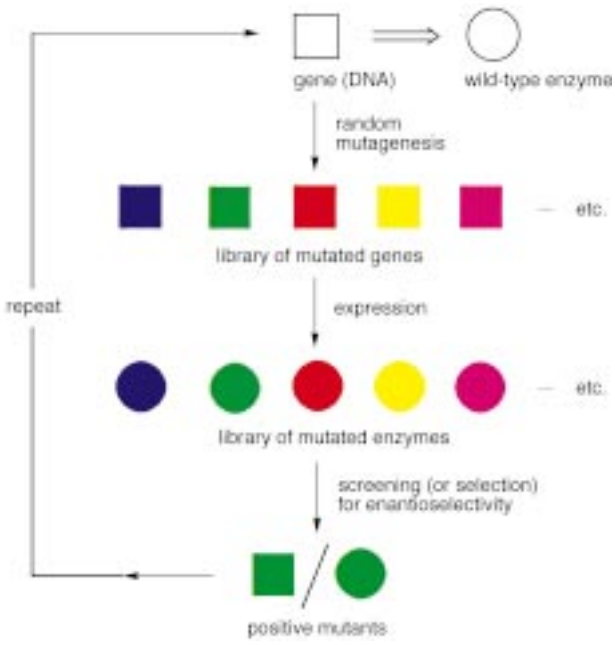
reached,[10] in a strict sense "combinatorial" was originally reserved for designating split-and-mix methods.[9] Indeed, parallel methods in solution or on a solid phase are not basically new, except that automation and robotics allow for high throughput on a small scale by using miniaturized equipment such as microtiter plates. Perhaps the simple definition of combinatorial chemistry by Combs reflects the opinion of the majority of researchers in the area:[11] "the applied use of technologies and automation for the rapid chemical syntheses of relative large numbers of compounds". It has been noted that in drug discovery there is increasing emphasis on parallel synthesis, although future trends are difficult to predict.[9]

The enormous success of combinatorial methods in pharmaceutical[9] and subsequently in material science[12] research has stimulated chemists to apply the techniques to catalysis.[12] Rather than synthesizing and testing catalysts individually in a time-consuming process as, for example, in the famous Haber–Bosch production of ammonia, in which thousands of catalysts or catalyst formulations had to be prepared and tested one by one, the idea is to create and evaluate large numbers of catalysts all at once (or nearly so).[12, 13] Efforts concerning this fascinating prospect were hampered by two factors, one real—because the appropriate techniques were lacking—the other psychological: critics maintained that any strategy lacking structural and/or mechanistic aspects is not only ill-suited for intellectual reasons (lack of creativity!), but also doomed to fail. However, it has become abundantly clear that the use of combinatorial and traditional methods in catalysis research are in fact complementary. Therefore one method will never displace the other.

Research in the combinatorial development of enantioselective transition metal catalysts involves two distinct problems: 1) devising strategies and methods for the preparation of large libraries of chiral ligands and/or catalysts displaying high degrees of structural diversity; and 2) developing methods for the high-throughput screening of such catalysts. Screening for catalyst activity and selectivity involves the study of kinetic properties. It may thus be more difficult than developing assays for biological properties based on binding phenomena. The problem of identifying chiral selectors in large libraries of potential candidates for use in chromatographic enantiomer separation[14] is also different from the challenge of devising high-speed assays for asymmetric catalysts. Although significant progress in developing high-speed detection systems for achiral homogeneous and heterogeneous catalysts has been made since the mid 1990s,[12, 13] analogous research in the area of enantioselective catalysts was slow to start.

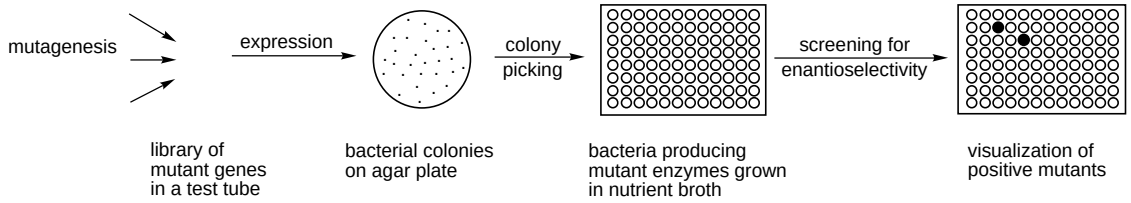
In 1995 my research group encountered the need to develop high-throughput screening systems for assaying the enantioselectivity of thousands of biocatalysts.[15] We had just initiated a project with the aim of exploiting the tools of directed evolution[16] in the creation of enantioselective enzymes for use in organic synthesis.[17] Accordingly, the proper combination of molecular biological methods for random mutagenesis[16, 18] and gene expression with appropriate high-throughput screening systems formed the basis of our strategy (Scheme 1).[17, 19]



Scheme 1. Directed evolution of an enantioselective enzyme.[19]

The starting point for evolving an enantioselective catalyst for a given reaction (A → B) is a wild-type enzyme (that is, occurring in nature) which catalyzes the chemical transformation, but not with an acceptable level of enantioselectivity. The gene that encodes the enzyme is subjected to random mutagenesis using the error-prone polymerase chain reaction (epPCR)[16a,b] or recombinant methods such as DNA shuffling.[16c] Following expression in a suitable bacterial host, thousands of mutant enzymes are produced which then have to be screened for enantioselectivity in the reaction of interest (A → B).[17] Since conventional techniques for determining the *ee* value of a reaction (for example, GC or HPLC)[5, 6] are not suitable for handling such huge numbers of samples within a reasonable span of time, new methods had to be developed.[15] Although no such screening systems were available in 1995, we at least did not have any problems regarding deconvolution or encoding. The reason for this is simple. Upon plating the biological material out on agar plates, each bacterial colony produces a single mutant enzyme because it originates from a single cell. Thus, upon harvesting the bacterial colonies individually using a robotic colony picker and placing them in the wells of microtiter plates containing nutrient broth, arrays of thousands of potentially enantioselective catalysts become spatially accessible for testing (Scheme 2).[15, 17, 19]

At this point the method has the character of combinatorial catalysis. However, the overall process goes far beyond combinatorial chemistry because the evolutionary nature now comes into play. Following the identification of the most enantioselective enzyme, the corresponding mutant gene is subjected once more to mutagenesis and screening, and the process is repeated as often as is needed until enantioselectivity has reached the desired level.[17, 19] Since the inferior enzymes/genes are discarded and only the most enantioselective one is considered, a type of "evolution in the test tube" is at work. This approach to enantioselectivity is radically different from all other strategies such as ligand tuning in



Scheme 2. Individual steps in the directed evolution of an enantioselective enzyme.[19]

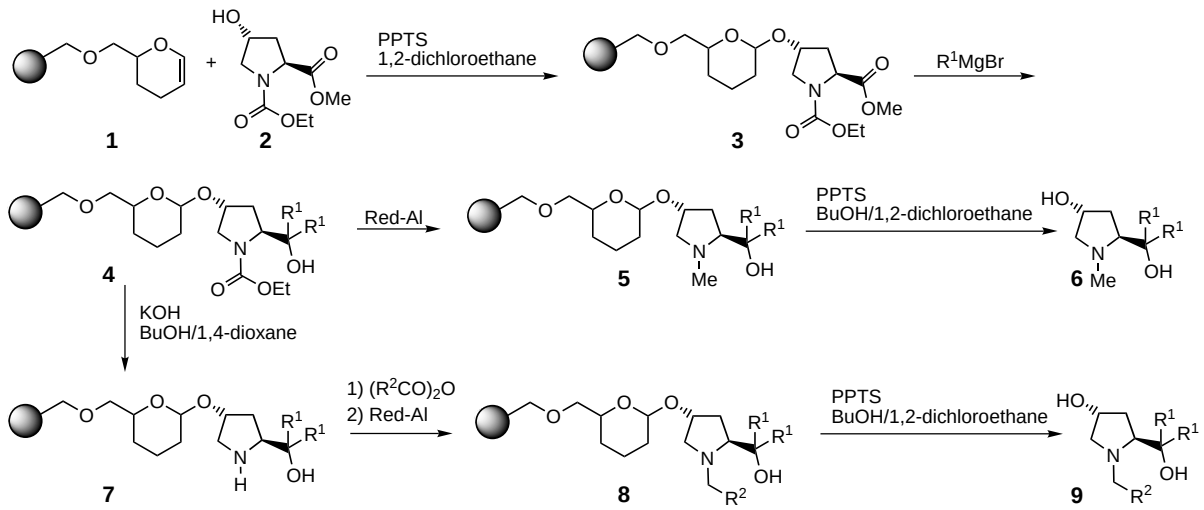
homogeneous metal catalysis,[3] de novo design of enzymes,[8] or formation of catalytic antibodies[20] because it is independent of any structural or mechanistic considerations. In a certain sense such an evolution-based strategy is strictly rational. Indeed, proof of principle was first presented in 1997 (Section 3.1.),[17] and further advancements have been achieved.[19, 21]

Most high-throughput *ee*-screening systems designed for assaying enzyme-catalyzed reactions are expected to work for asymmetric transition metal catalyzed processes as well. For this reason many recent efforts in developing such assays have attracted a great deal of attention, and in fact this research is ongoing. Before presenting and evaluating the screening systems that we and others have described in the literature thus far (Section 3), a short summary of the key developments in the combinatorial preparation of enantioselective transition metal catalysts is appropriate (Section 2). It will become apparent that these seminal contributions constitute the beginnings of a fascinating new research area, but that the actual goal of preparing and evaluating truly large numbers of potentially enantioselective catalysts has not been reached, primarily because of a lack of screening systems.

2. Strategies in the Combinatorial Design and Preparation of Enantioselective Transition Metal Catalysts

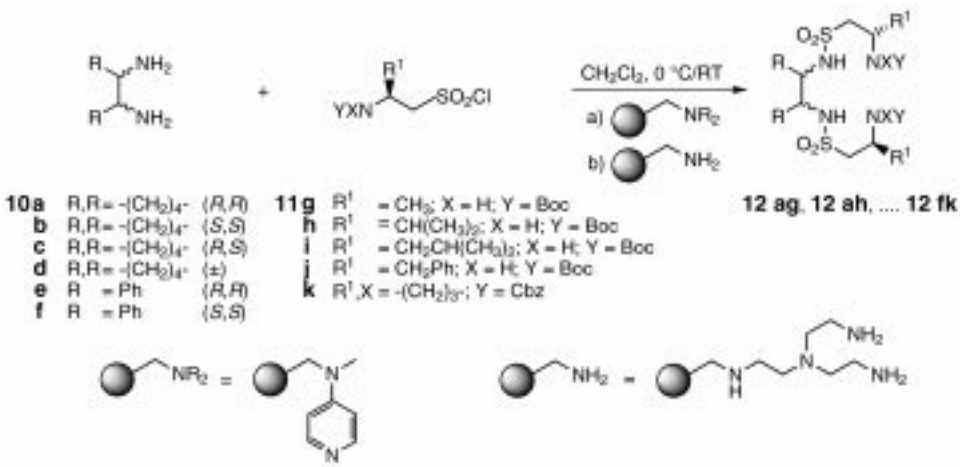
Early studies concerning combinatorial methods in enantioselective transition metal catalysis resulted in the emergence of two basic approaches: 1) the preparation of analogues of known types of ligands, preferably using a modular building block strategy to incorporate diversity with respect to steric and electronic effects at the metal binding site, and 2) optimization of reaction conditions in a given catalytic asymmetric transformation, including solvent and temperature, in conjunction with known ligands. Since solid-phase chemistry has been shown to be ideal for the preparation of libraries of potential therapeutic drugs,[9] the advantages involved are also likely to pertain in the preparation of chiral ligands for asymmetric catalysis. Specifically, parallel synthesis, as opposed to the split-and-pool methodology, allows spatial access to each ligand, which means that catalysts can be screened individually. Of course, theoretically this limits diversity and therefore the size of the libraries, which raises the question whether the method is only applicable to the optimization of known ligands, or whether new types of chiral ligand systems can also be discovered combinatorially by parallel synthesis. We tend to favor the latter prediction, provided that search systems are set up creatively.

Based on previous reports involving the use of chiral β-amino alcohols as catalytically active ligands in the enantioselective addition of Et_2Zn to aldehydes, Ellman and coworkers reported in 1995 the parallel solid-phase synthesis of a small library of substituted 2-pyrrolidine methanol derivatives **6** and **9** (Scheme 3).[22] *trans*-4-Hydroxy-L-proline methyl ester (**2**) was first anchored onto a polystyrene (Merrifield) resin **1** through a cleavable tetrahydropyranol linker. Addition of various Grignard reagents followed by protective-group manipulation at the nitrogen atom afforded ten differ-



Scheme 3. Solid-phase synthesis of chiral 2-pyrrolidinemethanol ligands.[22] PPTS = pyridinium-*p*-toluene sulfonate.

ent immobilized ligands **5** and **8**, which on an optional basis could be released from the resin by an acid-induced hydrolytic cleavage to form **6** and **9**, respectively. They were then tested one by one in the addition of Et_2Zn to various aldehydes using classical methods, including *ee* determination by conventional GC on a chiral stationary phase. On-bead performance in the best cases (for aromatic and aliphatic aldehydes up to 94 and 85% *ee*, respectively) was also studied and turned out to be slightly inferior relative to the enantioselectivity of the corresponding free ligand in solution. This early contribution demonstrates the feasibility of the solid-phase parallel synthesis of new members of a specific ligand class, but it did not include any hint as to how high-throughput screening might be achieved in real applications. Indeed the authors did not claim that the concept of high-throughput preparation and screening of new enantioselective catalysts had been put into practice. It is conceivable that the diversity of the library and therefore the probability of finding even better catalysts could be increased by using hundreds of different Grignard reagents and *N*-protective groups. Automation would make larger ligand libraries available within a short period of time.

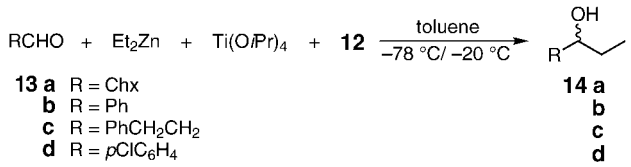


Scheme 4. Solid-phase synthesis of chiral sulfonamide ligands.[25] Boc = *tert*-butoxycarbonyl; Cbz = benzyloxycarbonyl.

In another effort Gilbertson and Wang applied the Geysen polyethylene pin technique to synthesize a 63-member library of phosphane-modified peptides as ligands in the enantioselective Rh-catalyzed hydrogenation of methyl-2-acetamidoacrylate.[23] Individual on-bead screening in a parallel 24-vial reactor was performed conventionally using GC. Although the *ee* values did not exceed 18%, correlations between peptide sequence and enantioselectivity were observed. The actual chemistry is novel, but it does not go deeply into combinatorial catalysis because truly large libraries of ligands as well as the appropriate high-throughput screening systems were not considered. Nevertheless, this research is a noteworthy first step because phosphanes constitute a very important class of ligands. Indeed, it remains a challenge to devise an efficient combinatorial approach to the preparation and use of chiral phosphanes. Gilbertson and Wang also described a similar strategy for optimizing Pd catalysts for use in asymmetric allylic substitution (up to 85% *ee*).[24]

Gennari et al. went one step further in developing new enantioselective catalysts by parallel synthesis, although again genuine high-throughput screening was not part of the effort.[25] New members of a known family of chiral ligands based on sulfonamides were constructed in a modular manner using solid-phase extraction (SPE) techniques. Vicinal diamines **10** were treated with an excess of sulfonyl chlorides **11** to ensure complete conversion (Scheme 4). To avoid laborious purification of the sulfonamide products **12** the reactions were run in dichloromethane in the presence of polymer-bound dimethylaminopyridine, which not only catalyzes the coupling reaction but also scavenges the liberated HCl. The excess sulfonyl chloride was then removed by reaction with solid-phase-bound tris(2-aminoethyl)amine. Thus, modular preparation and smooth separation of the "side products" from the "pure" ligands were possible in a simple protocol.

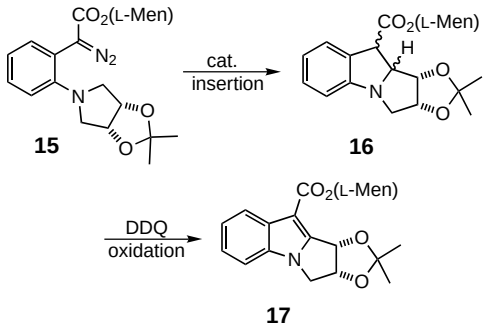
The disulfonamide library was tested in the $Ti(OiPr)_4$-mediated addition of Et_2Zn to aldehydes **13** (Scheme 5),[25] a reaction first described by Yoshioka, Ohno, and co-workers using the bis-trifluoromethylsulfonamide of 1,2-diaminocyclohexane.[26] The tests were run in 30 different reaction



Scheme 5. Ti-promoted enantioselective addition of Et_2Zn to aldehydes in the presence of catalytic amounts of chiral sulfonamide ligands.[25] Chx = cyclohexyl.

vessels in a parallel format. In order to increase throughput each vessel was charged with four different aldehydes, and the enantioselectivities of the four reaction products were determined by conventional GC analysis of the crude mixtures. The idea of using a given catalyst to screen the reactions of different substrates combinatorially had previously been proposed by Kagan in a different context.[27] In the present case each mixture required about one hour to be screened, which means that about 96 *ee* determinations of products **14** could be performed per day. A total of 120 results were collected and the best enantioselectivities were greater than 90% *ee*. More recently, a similar approach was taken by Liskamp and co-workers, who used solid-phase synthesis to prepare a small library of peptidosulfonamides as chiral ligands for the same reaction (up to 66% *ee*).[28] Again, about 96 *ee* determinations per day were supposedly possible by GC, but only a few dozen were actually carried out.

Based on earlier work by Lim and Sulikowski concerning enantioselective Cu-catalyzed intramolecular C-H insertions of metal carbenoids,[29] Burgess et al. devised an interesting system for optimizing the proper combination of ligand, transition metal, and solvent for the reaction of the diazo compound **15** (Scheme 6).[30] The reaction parameters were

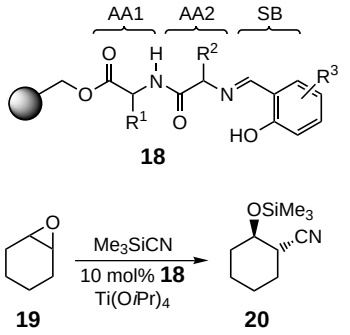


Scheme 6. Catalytic stereoselective intramolecular carbene insertion.[30] L-Men = L-menthyl; DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

varied systematically on a standard 96-well microtiter/filtration plate. In total five different ligands, seven metal precursors, and four solvents were tested in an iterative optimization mode. Standard HPLC was used to monitor the stereoselectivity following a DDQ-induced oxidation. This type of catalyst search led to the discovery that Ag^I ions catalyze the process, a finding that had no precedence in the literature. Moreover, by using a Pfaltz-type ligand[31] the diastereoselectivity in the formation of the final product **17** increased from the original 2.3:1 (at 64% yield) to 3.9:1 (at 75% conversion) and to 5.9:1 (at 23% conversion).[30] Here again it would be of interest to see if truly high-throughput screening of a much greater number of reactions would lead to even better results. Such proof of principle would really illustrate the power of combinatorial techniques relative to the traditional approach based on the sequential study of individual catalysts under different conditions.

Burgess and Porte also studied the Pd-catalyzed asymmetric allylic substitution using seven different phosphanyloxazoline ligands at various ligand-to-metal ratios.[32] An aluminum block containing 27 wells was placed in a dry-box in which the reactions were carried out in parallel. Analyses were performed by conventional GC on a column with a chiral stationary phase and equipped with an autosampler. Such a setup would allow about 33 catalyst evaluations per day. Apparently, only a few dozen were carried out in the study, and resulted in the identification of a catalyst showing an *ee* value of 74% in the reaction of 4-acyloxy-2-pentene with malonate. It is not clear whether further ligand diversification would lead to a ligand more selective than the record set in this case by the Trost catalyst (92% *ee*).[33]

An important development concerning the optimization of asymmetric catalysts was described by Snapper, Hoveyda, and co-workers in 1996.[34] They pioneered, amongst other things, diversity-based procedures in the Ti-mediated enantioselective ring-opening reaction of *meso* epoxides such as **19** with cyanotrimethylsilane (TMSCN) to nitrile **20** (Scheme 7). Peptides composed of three independently variable subunits, namely Schiff base (SB), amino acid 1 (AA1), and amino



Scheme 7. Ti-catalyzed ring-opening of **19** in the presence of modular ligands **18**.[34]

acid 2 (AA2) were chosen as the catalysts. The modular nature of this system allows for a high degree of diversity and is therefore ideally suited for combinatorial catalyst research. For example, a simple calculation shows that the use of 20 different aldehydes for the Schiff bases and 20 natural amino acids would give rise to 8000 different chiral ligands. However, the lack of high-throughput screening systems at the time of this seminal study contributed to the decision *not* to generate such large libraries. Rather, only a small library of ligands was prepared. All of them were detached from the respective beads and screened in the Ti-catalyzed reaction of cyclohexene oxide (**19**) with TMSCN in solution by conventional GC. Later a similar study was performed on the catalytically active beads themselves.[35a]

Snapper, Hoveyda, and co-workers utilized a search strategy which in their opinion makes possible the "identification of effective ligands without examination of all possibilities".[34, 35a–c] Accordingly, each of the three modular subunits was successively optimized while the other two subunits were kept constant (Figure 2). The best catalyst from a total of only 60 was identified as inducing an enantioselectivity of 89% *ee* for the model reaction. As the authors emphasize, positional scanning of this type is based on the assumption that additivity but not cooperativity between the three subunits pertains. Although this evaluation cannot be

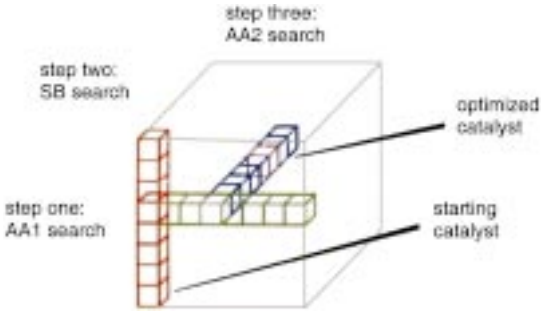
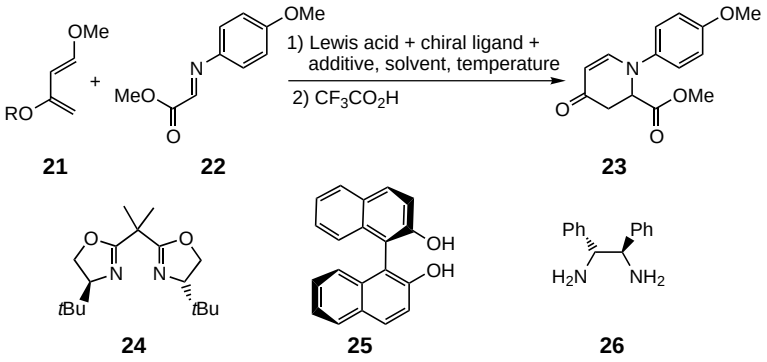


Figure 2. Search strategy for screening enantioselective modularly constructed catalysts.[34, 35] AA = amino acid; SB = Schiff base.

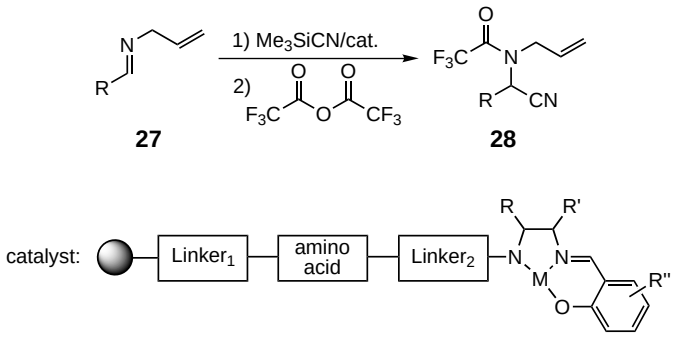
proven or disproven without scrutinizing larger libraries, it appears reasonable. A similar search strategy has been applied by the authors in the Ti-catalyzed enantioselective addition of cyanide to imines.[35d,e] Nevertheless, finding and optimizing modular catalyst systems which *do* show synergistic effects between subunits may be an intriguing goal for the future.

In the quest to find optimal conditions for an enantioselective Lewis acid catalyzed aza-Diels–Alder reaction (**21** + **22** → **23**; Scheme 8), Whiting and co-workers varied several parameters in parallel.[36] Good literature precedent for a potential catalyst was not so clear, although it was known that many different Lewis acids, including $Yb(OTf)_3$ (Tf = trifluoromethanesulfonyl) catalyze Diels–Alder reactions. Three different chiral ligands (**24**–**26**) known in the literature were tested in conjunction with $Yb(OTf)_3$, $Cu(OTf)_2$, MgI_2, and $FeCl_3$ as Lewis acids, with a limited number of additives and solvents also being evaluated. By using an autosampler and a conventional HPLC instrument equipped with a column having a chiral phase, 144 approximate yields and *ee* values were obtained in about one week, which means that in this system approximately 20 evaluations of catalyst systems for a given reaction are possible per day. Four of the best hits were then applied in scaled-up reactions. The optimal catalyst $Yb(OTf)_3$/**26**, resulted in cycloadduct **23** with 97% *ee* and in 64% yield (Scheme 8). Although quite successful in this particular case, it is not clear how effective this type of search really is, since ligand diversity was hardly considered. In principle, it can be expected that libraries composed of modular chiral ligands or of simple chiral ligands in conjunction with a high diversity of achiral additives are more meaningful in the combinatorial search for enantioselective catalysts.



Scheme 8. Hetero-Diels–Alder reaction catalyzed by Lewis acids in the presence of ligands **24**, **25**, or **26**.[36] R = trimethylsilyl.

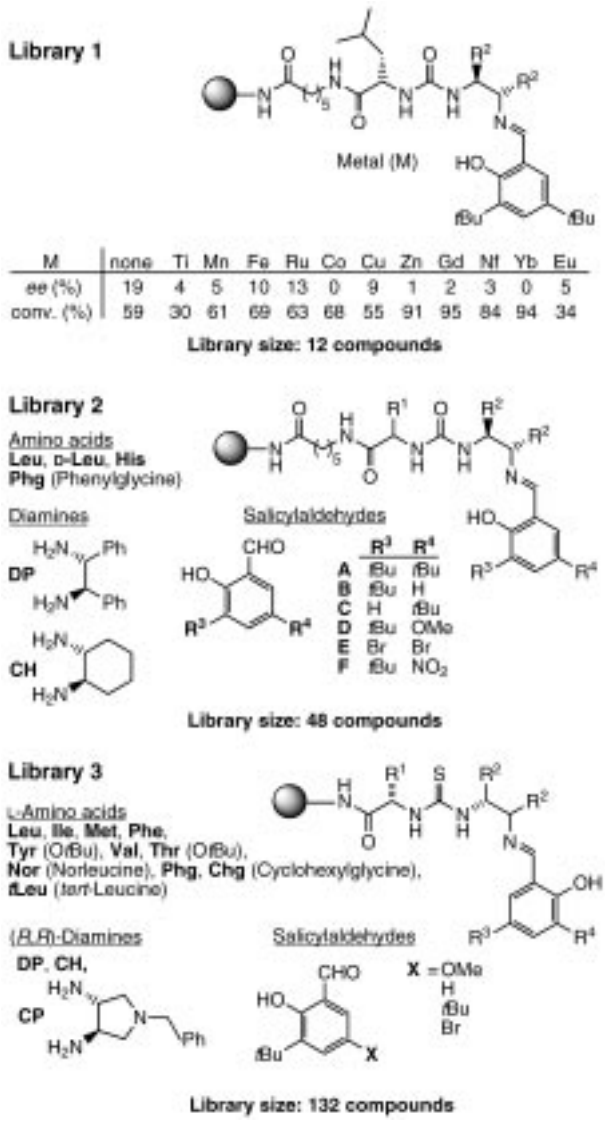
In 1998 Sigman and Jacobsen published a seminal paper in which they demonstrated that a combinatorial diversity-based method leads to the discovery of highly enantioselective catalysts that are not readily available by the traditional one-at-a-time approach to catalyst optimization.[37] A strategy for the parallel synthesis of a certain class of modularly constructed ligands, specifically for use in the catalytic asymmetric Strecker reaction of imines **27** (Scheme 9) was described. As in the Snapper, Hoveyda, and co-workers case,[34, 35] the



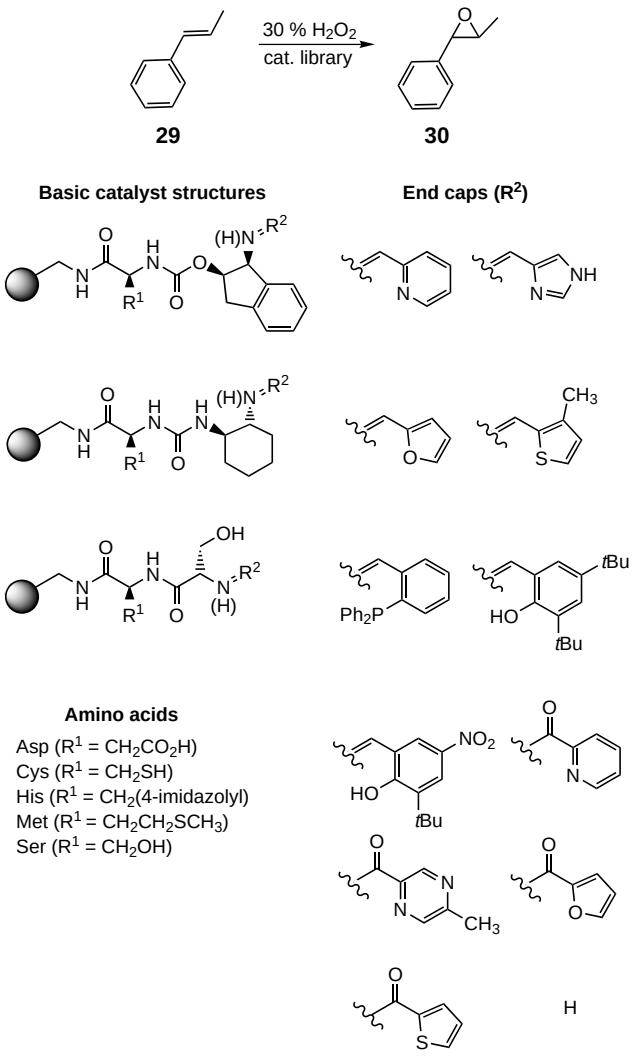
Scheme 9. Asymmetric Strecker reaction of aldimines **27** catalyzed by modular metal complexes.[37]

first step in the parallel preparation of a catalyst library is the selection of a ligand system amenable to solid-phase synthesis and systematic structural variation.[37] In addition to the requirement of high-yield reactions in the modular construction of the ligands, an unobtrusive site for the attachment to the solid support is necessary. A number of traditional C_2-symmetric ligands would require several tedious synthetic steps. In contrast, tridentate Schiff base compounds, which are known to be ligands for a variety of catalytic reactions, are in fact suitable for solid-phase synthesis.[37] These systems are normally comprised of three units: a chiral amino alcohol, a salicylaldehyde derivative, and a metal ion. In the above study the amino alcohol unit was replaced by chiral diamines, with the two linkers also contributing to structural diversity (Scheme 9).

Initially, a given ligand system was prepared on the solid support and a small collection of catalysts (library 1) generated by adding 11 different metals and in one case no metal (Scheme 10). The best *ee* value (19%) was in fact obtained in the metal-free system. Based on this observation a parallel ligand library of 48 members (library 2) was prepared using two different chiral 1,2-diamines and a variety of amino acids and Schiff bases. The combination of L-leucine and an (*R*,*R*)-diamine was shown to be optimal, the substitution pattern in the salicyclaldehyde also playing a crucial role. Thereafter, the linker elements were optimized by a traditional one-catalyst-at-a-time approach, which resulted in the emergence of the more-selective catalysts having thiourea linkers. Finally, these results were used to prepare library 3 containing 132 thiourea derivatives (Scheme 10). The enantioselectivity in the addition of HCN to a variety of imines turned out to be greater than 80%, with the best case being 91% *ee* at $-78\,^{\circ}C$ in a lab-scale experiment.[37] The *ee* determinations were performed using conventional HPLC with a chiral stationary phase. The actual throughput, however, was not defined. Nevertheless, the importance of this contribution concerns the fact that it touches on the question of the complexity arising from interrelated variables in catalytic processes. Indeed, the diversity-based method led to synergistic effects between catalyst components which were unexpected and which do not correspond to "normal" intuition.



Scheme 10. Catalyst libraries for the asymmetric Strecker reaction.[37] DP = 1,2-diphenyl-1,2-ethanediamine; CH = 1,2-cyclohexanediamine; CP = *N*-benzyl-3,4-diaminopyrrolidine.



Scheme 11. Ligand libraries for the asymmetric metal-catalyzed epoxidation of olefin **29**.[38]

The next important conceptional advancement, reported by Francis and Jacobsen, addresses the question whether combinatorial methods can be used to discover new *types* of chiral ligands.[38] In this case efforts concentrated on the combinatorial search for a chiral ligand/metal system which catalyzes the H_2O_2-mediated enantioselective epoxidation of *trans*-β-methylstyrene (**29**) (Scheme 11). First, the solid-phase synthesis of modularly constructed ligands composed of 1) amino acids, 2) 1-amino-2-indanol, *trans*-1,2-diaminocyclohexane, or serine, and 3) end groups was performed on a Wang resin. End-capped salicylamine was also attached to the Wang resin so as to include types of ligands known to be active in metal-catalyzed epoxidations. The combined library of 192 ligands was then treated with 30 different metal salts, which theoretically would result in 5760 possible metal–ligand complexes. On the basis of earlier experience concerning the application of metal-binding combinatorial libraries for the identification of coordination complexes[39] it was possible to show by visual color assays and by qualitative inorganic color tests that about 80 % of the 5760 possible metal–ligand complexes had been generated.[38]

A type of deconvolution strategy had to be developed to identify the most active and stereoselective bead. For this purpose a mixture of all 192 ligands was treated individually with a single metal source, and each group of catalysts was tested in the model epoxidation reaction. Conventional GC analysis of these 30 experiments showed that the two groups derived from $VOSO_4$ and $FeCl_2$ were the most active. Since it was observed that $VOSO_4$ alone is also catalytically quite active, the rest of the efforts focused on $FeCl_2$. Accordingly, 12 ligand libraries, each containing the 16 basic structures and one of the various end groups, were prepared and treated with $FeCl_2$. The usual screening procedure showed that the most active catalysts were those which contained a pyridine-type end group. It was then logical to test the 16 ligands with the most active end groups. However, since an active catalyst might theoretically be overlooked by such a restrictive procedure, further encoding was not carried out.[38] Rather, all $FeCl_2$ complexes of the original 192 ligands were screened

for activity. Pyridine-capped ligands turned out to be most active. This small group was then assayed with respect to enantioselectivity, and culminated in the identification of three $FeCl_2$/ligand complexes which led to *ee* values of 15–20% for product **30** in the model reaction. Although the enantioselectivities are modest, this study shows for the first time that it is possible to discover new lead ligand structures for use in asymmetric catalysis. Nevertheless, a combinatorial search system based on a strict analogy to the split-and-mix strategy used in therapeutic drug discovery has yet to be devised. One of the challenges would be the question of high-throughput screening.

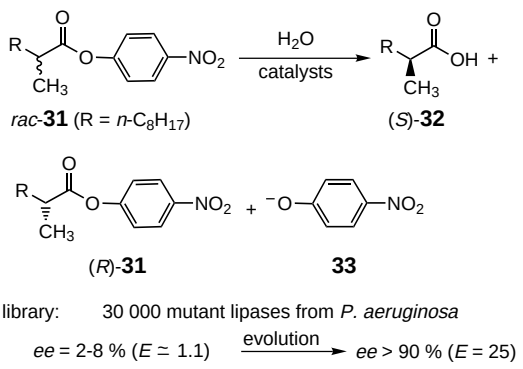
In summary, the overview presented in this section clearly shows that a number of important conceptional advancements have been made with regard to the synthesis of libraries of chiral ligands and catalysts. Several systems were designed to make possible a relatively high diversity of ligands, although in practice the actual number of ligands prepared was not pushed anywhere near the theoretical limits. Further examples have appeared.[40] The problems associated with the second part of putting asymmetric combinatorial catalysis into genuine practice, namely high-throughput screening systems for assaying enantioselectivity of truly high numbers of catalysts, were hardly addressed in these studies.

3. High-Throughput Screening Systems for Assaying Enantioselective Catalysts and Enzymes

In the 1990s several systems were developed for the high-throughput screening of the activity of enzymes[16] and of transition metal catalysts[12, 13] in reactions that do not involve enantioselectivity. Color tests or mass spectrometric (MS) assays were usually used. Moreover, many of the assays were rather crude, but sufficiently effective to identify particularly active catalysts out of large libraries. Once identified, these "hits" were subjected to conventional kinetic studies to ascertain precise data concerning their activity. Unfortunately, the extension of these systems to enantioselective processes was not straightforward which meant that new concepts had to be developed. Indeed, prior to 1997 not a single high-throughput *ee*-screening system existed.

3.1. UV/Vis-Based Systems

As delineated in Section 1 we were first confronted with the problem of developing a high-throughput *ee*-screening system in our studies relating to the directed evolution of enantioselective enzymes (Scheme 1).[17] Specifically, the goal was to create a highly enantioselective enzyme for the hydrolytic kinetic resolution of racemic 2-methyl decanoate (**31**, Scheme 12). The starting point was the lipase from *Pseudomonas aeruginosa*, which catalyzes the hydrolysis of the substrate, but only with a marginal degree of enantioselectivity (selectivity factor $E \simeq 1.1$, reflecting the relative reaction rates of (*S*)- and (*R*)-**31** and corresponding to *ee* values of 2–8% *ee* at 40–50% conversion) with a slight preference for the (*S*)-acid **32**.



Scheme 12. Kinetic resolution of ester **31** catalyzed by mutant lipases produced in the process of directed evolution.[17, 19, 21]

The *p*-nitrophenol rather than the methyl ester was used in the study because the appearance of the yellow-colored *p*-nitrophenolate (**33**) in buffered medium provides a simple means to monitor the reaction by measuring the absorbance at 410 nm as a function of time. Since the racemate would only afford kinetic information concerning total activity, the (*R*)- and (*S*)-esters **31** were synthesized and tested *separately* pairwise for each mutant lipase. Thus, using a standard 96-well microtiter plate, 48 mutants could be screened within a few minutes. Two typical experimental plots are displayed in Figure 3. The top one shows a lipase with almost no stereoselectivity (in this case the wild-type) and the bottom one indicates the improved enantioselectivity of the best mutant identified in the first library of approximately 1500 members.[17]

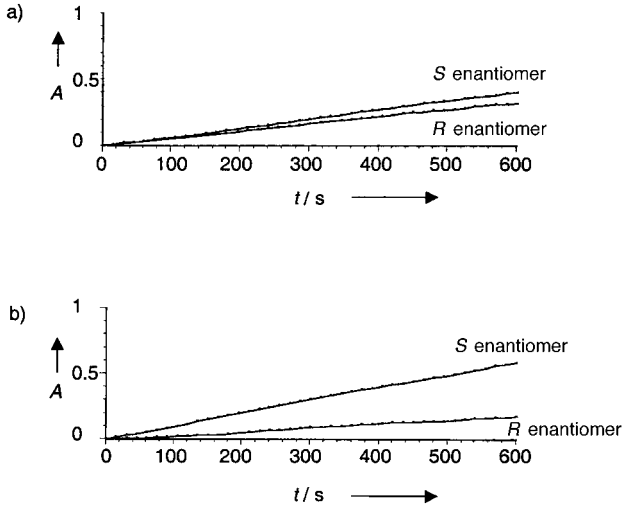


Figure 3. Course of the lipase-catalyzed hydrolysis of the (*R*)- and (*S*)-**31** as a function of time.[17] a) Wild-type lipase from *P. aeruginosa*, b) improved mutant in the first generation.

In the next three rounds of random mutagenesis, about 2000–2400 mutants were screened in each generation. The most selective mutant ("hit") was subsequently studied by performing the kinetic resolution of the racemate, with the assay being conventional GC. On average about 6–7 improved variants were observed per library of 2000–2400 mutant enzymes. In each case the gene of the best

variant was used in subsequent mutagenesis. After four rounds of mutagenesis a mutant lipase displaying a selectivity factor of $E = 11.3$, which corresponds to an *ee* value of 81 % at 30 % conversion, in favor of (*S*)-**32** was identified.[17] Improved methods based on the combination of error-prone PCR and saturation mutagenesis for exploring protein sequence space with respect to enantioselectivity led to the creation of a small family of mutant lipases, all showing *E* values of 20–25 ($ee > 90-93$ %).[19, 21] The total effort for this achievement involved about 30000 mutants which were all screened by the above assay. Moreover, by inverting the direction of the evolutionary pressure, that is, by screening for *R*-selective mutants, it was possible to reverse the sense of the enantioselectivity.[19]

In the early phase of this study many of the steps were performed manually, for example, colony picking and pipetting, which limited the number of mutants which could be screened.[15, 17, 41] Nevertheless, about 500 catalysts were assayed per day. This was later increased to about 1000 mutants per day, primarily by introducing a pipette robot. The use of complete automation including a colony picker and a robotic workstation increased throughput to about 1500 mutants per day.[42] Moreover, since the process of random mutagenesis is expected to create a certain percentage of mutant enzymes showing no lipase activity whatsoever ("dead variants"), we recently introduced a rough pre-screening based on the tributyrin test.[42] Accordingly, the agar plates containing the bacterial colonies (Scheme 2) were charged with glyceryl tributyrate (tributyrin), which is a well-known substrate for lipases. The plates have a milky appearance because of the insolubility of tributyrin in the medium. A catalytically active mutant lipase causes hydrolysis of tributyrin with formation of water-soluble products, which results in clear spots around the respective colony (Figure 4). Thus, the absence of such clear spots signals an absence of catalytic activity. These bacterial colonies need not be harvested, which leads to a reduced number of mutants that have to be scrutinized by the actual screening system.

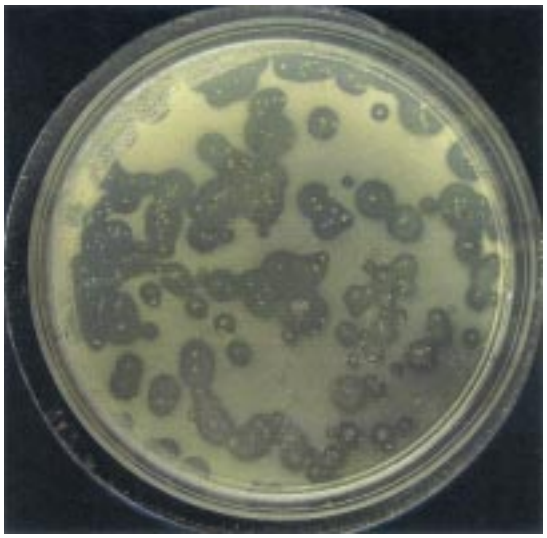
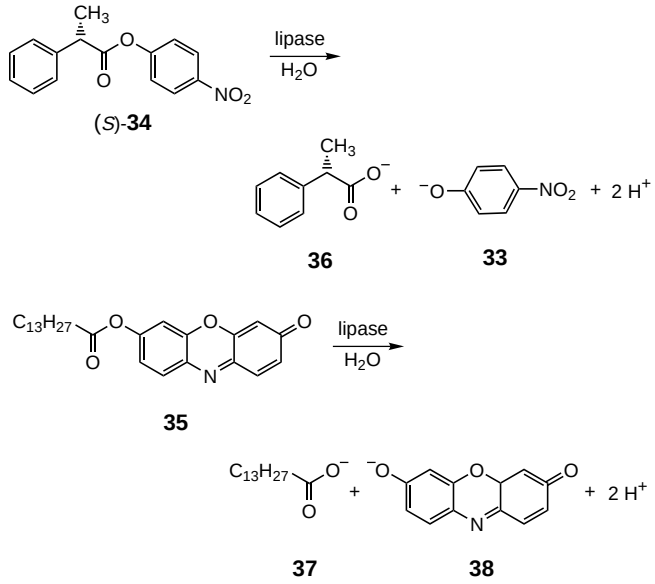


Figure 4. Typical tributyrin test for lipase activity in which the agar plate is placed on a black background for visualization.[42] White dots represent bacterial colonies; those having no (clear) black background contain inactive mutants.

Although the UV/Vis-based method constitutes the first example of high-throughput screening for enantioselectivity, it suffers from two limitations. Since (*R*)- and (*S*)-**31** need to be screened separately, substrate competition with respect to the enzyme is not ensured. Normal enzymatic conditions do not pertain. Therefore, the kinetic diagrams in Figure 3 cannot be used to derive reliable *E* or *ee* values, which means that the assay is simply a convenient way to identify positive "hits". Problems arise at high *ee* values. A more serious disadvantage has to do with the fact that evolution-based optimization is performed on the *p*-nitrophenol ester **31** and not on a more practical candidate such as the analogous methyl ester.

James and Kazlauskas have also developed a colorimetric assay for testing the enantioselectivity of lipases or esterases in ester hydrolyses.[43] The so-called Quick-*E*-Test was developed to simulate the state of competitive conditions of an enzymatic process. In this test a mixture of the *p*-nitrophenol ester of one enantiomeric form of a chiral ester **34** and a resorufin ester **35** is subjected to enzyme-catalyzed hydrolysis, with the latter taking on the "role" of the enantiomer (Scheme 13). The two hydrolyses are monitored by recording



Scheme 13. Quick-*E*-Test for the enantioselectivity of lipases or esterases.[43]

the UV/Vis absorption of the two products **33** and **38** at two distinctly different wavelengths (410 and 570 nm, respectively). The same is performed with (*R*)-**34**/**35**. Although this makes a more precise determination of *E* values possible, the method suffers from the same disadvantage as noted for our system, namely the necessity of employing the *p*-nitrophenol ester of the chiral acid.[43] Nevertheless, appropriate automation should allow a throughput of several thousand samples per day.

Following this report, Kazlauskas and co-workers devised a second test based on the notion that hydrolysis leads to a change in acidity, which should be measurable by an appropriate pH indicator.[44] A linear correlation between the amount of acid generated and the degree of protonation of the indicator was established when a buffer (*N*,*N*-bis(2-

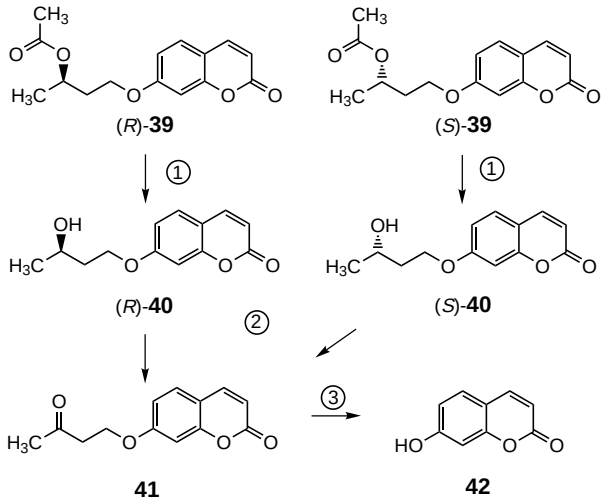
hydroxyethyl)-2-(aminoethanesulfonic acid) and a pH indicator (*p*-nitrophenol) having the same pK_a value were used. In this case the two enantiomeric esters were studied separately pairwise in a similar manner to our assay,[17] with the color change upon protonation in each case being monitored colorimetrically. Currently it is not quite clear how general and how precise this method actually is, since it was later observed that appreciable discrepancies exist in some cases between the *E* value obtained and the *E* value measured conventionally in control experiments.[45]

Several other related colorimetric high-throughput assays for enantioselective enzymes have been described recently.[46] These and the systems summarized here are not restricted to the evaluation of enantioselective enzymes, since any enantioselective catalyst should be amenable to such screening. Nevertheless, most of the systems require the incorporation of some kind of a chromophoric moiety, which means that the evolutionary creation of an enzyme or the combinatorial optimization of a transition metal catalyst focuses on reactions of chromophorically modified substrates and not on "normal" compounds of interest in laboratory- or industrial-scale reactions.

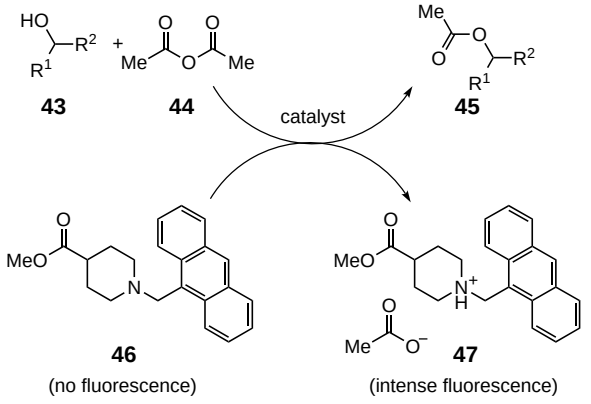
3.2. Fluorescence-Based Systems

A major advantage of assays based on fluorescence is the high degree of sensitivity, which allows one to use very dilute substrate concentrations and extremely small amounts of catalysts.[47] This is not always necessary, but in some situations the conditions cannot be modified, for example, when assaying catalytic antibodies in cell cultures. Klein and Reymond have developed a simple and practical fluorogenic assay for the hydrolytic kinetic resolution of chiral acetates, such as **39** (Scheme 14).[48] It is based on a sequence of two coupled enzymatic steps that converts a pair of enantiomeric alcohols formed by the asymmetric hydrolysis under study (for example, (*R*)- and (*S*)-**40**) into a fluorescent product (for example, **42**). In the first step (*R*)- and (*S*)-**39** are subjected separately to hydrolysis in reactions catalyzed by a mutant enzyme (lipase or esterase), a catalytic antibody, or in principle a synthetic catalyst compatible with the system. The goal of the assay is to measure the enantioselectivity of this kinetic resolution. The relative amount of (*R*)- and (*S*)-**40** produced after a given reaction time is a measure of the enantioselectivity and can be ascertained rapidly, but not directly. Two subsequent chemical transformations are necessary. First the enantiomeric alcohols (*R*)- and (*S*)-**40** are oxidized separately by horse-liver alcohol dehydrogenase (HLDH) to the ketone **41**, from which the fluorescent final product umbelliferone (**42**) is then released in each case by the catalytic action of bovine serum albumin (BSA). Thus, by measuring the fluorescence of **42** for the *R* and *S* substrate separately, the relative amounts of (*R*)- and (*S*)-**40** can be determined. The authors tested 30 different esterases and lipases and followed the rate of release of **42** in the wells of standard microtiter plates by fluorescence.[48] Control experiments ensured that the apparent rate of umbelliferone release is directly proportional to the rate of acetate hydrolysis. The predicted and observed *E* and *ee* values (as checked by standard HPLC assay on a chiral phase) were found to lie within $\pm 20\,\%$. Only in one case was a larger discrepancy observed, a result that was believed to be caused by the occurrence of an unusually low Michaelis constant K_M for one of the enantiomers. Thus, since the test can be carried out on 96-well microtiter plates, high-throughput should be possible. Of course, the inherent disadvantage noted earlier for the colorimetric tests also applies here, namely the fact that the optimization of a potential catalyst is focused on a specific substrate **39** modified by incorporation of a probe, in this case the fluorogenic moiety **42**.

More recently Copeland and Miller described a novel fluorescence-based method for assaying the *activity* of catalysts in acylation reactions.[49] Since in principle it should be possible to extend it to include the determination of enantioselectivity, we describe it here in detail. The underlying idea is to use a molecular sensor which fluoresces upon formation of a product (Scheme 15). In exploratory experiments isopropanol (**43**; $R^1 = R^2 = CH_3$) was treated with acetic acid anhydride (**44**) in the presence of the nonfluorescent chemosensor **46**. No fluorescence was observed in the absence of catalysts. In contrast, known nucleophilic acylation



Scheme 14. Fluorescence assay for the determination of enantioselectivity of catalytic ester hydrolysis.[48]



Scheme 15. Fluorescence screening system for assessing the activity of acylation catalysts.[49]

catalysts such as dimethylaminopyridine (DMAP) or piperidinopyridine (PPY) led to intense fluorescence as a result of the fact that the chemosensor is protonated by the acetic acid to form **47**. The study was then extended to include these and other catalysts such as the modularly constructed tetrapeptides previously reported by the authors in the acylation of a racemic chiral alcohol.[49] Seven different catalysts were added to solutions of the substrate in toluene in the wells of a 96-format microtiter plate at three different loadings, each reaction being replicated three times for a total of 63 experiments. The reaction progress was monitored within 30–40 minutes by a fluorescence plate reader, and the results were in excellent agreement with control experiments based on standard kinetics. Interestingly, in the case of chiral catalysts based on various tetrapeptides shown previously to be enantioselective, a correlation was observed between the absolute rate and degree of asymmetric induction determined by traditional assays.[49] In this sense the system can be viewed as an *ee*-detection assay. However, the generalization of this assumption to include new substrates and/or other catalysts is not justified. In contrast, *E* and *ee* determinations should be possible simply by performing the tests on *R*- and *S*-configurated substrates separately on the microtiter plates, as in our UV/Vis-based system[17] (Section 3.1).

In an extension of their work Copeland and Miller showed that their activity assay can be applied to the real-time simultaneous screening of one bead/one compound combinatorial libraries.[49] In this interesting variant, the aminomethylanthracene sensor and modularly prepared tetrapeptide catalyst were attached to a support by solid-phase synthesis. Upon exposing sets of beads to the reaction conditions, the relative fluorescence intensities as measured by fluorescence microscopy were shown to correlate with the relative rates of reaction previously determined in solution using standard techniques. This approach, if modified and extended appropriately, may have considerable potential in the application of high-throughput screening of enantioselective acylation catalysts.

Reymond and co-workers recently continued their studies of fluorogenic assays by developing a catalyst screening system using antibody sensors.[50] Although not yet adapted to enantioselective processes, this system also seems promising. It is based on an idea first developed by Green and co-workers,[51] who showed that catalytic activity can be monitored by an immunoassay in the same way as in a regular enzyme-linked immunosorbent assay (ELISA). The catELISA assay, as it is now called, was used by Reymond and co-workers to monitor the hydrolysis of an ester on a solid support,[50] a procedure that was also used and developed further by MacBeath and Hilvert in their study of Diels–Alder reactions catalyzed by antibodies.[52] The substrate is first bound to a solid support which is then exposed to a solution containing potential catalysts. After sufficient reaction time the test solution is washed away and the product assayed quantitatively by ELISA using a product-specific polyclonal rabbit antibody. Whereas a chromophoric group was required in the original catELISA, Reymond and co-workers used an antibody sensor consisting of a product-specific antibody tightly bound to a product analogue which is covalently labeled with a sensitive fluorescent tag (acridone).[50] The system is compatible with 96- or 384-well plastic microtiter plates and allows for high throughput. It will be interesting to see if the concept can be extended to include the quantitative assay of enantioselective processes.

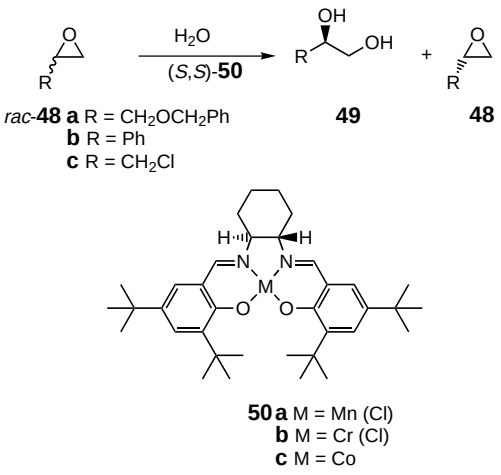
A different approach to fluorescence-based *ee* assays is theoretically possible by developing fluorescent-active sensors capable of distinguishing between enantiomers. The principle has been known for some time, although the effects involved, namely high fluorescence intensity (or quenching) *and* efficient enantiomeric recognition, turned out to be rather small.[53] Nevertheless, a relationship between fluorescent properties and enantiomeric purity was established. An example was described by Iwanek and Mattay who used 2,2′-dihydroxy-1,1′-binaphthol (BINOL, **25**; see Scheme 8) as a weakly fluorescent sensor in the chiral discrimination of amines which cause fluorescence quenching upon interaction.[53a] We have prepared a highly fluorescent chiral sensor capable of unusually efficient enantiomeric recognition, namely the helical compound [2,15]-dihydroxyhexahelicene.[54] Fluorescent quenching studies show that chiral discrimination between enantiomeric amines and amino alcohols is pronounced. It remains to be seen if a high-throughput *ee*-screening system can be developed. Finally, it needs to be pointed out that fluorescence can be used as a detection system in chromatography (see Section 3.6).

3.3. IR-Thermographic Assays

All objects emit infrared radiation (black body radiation), a process that can be detected by modern photovoltaic IR cameras equipped with focal plane array (FPA) detectors.[55] The picture obtained thereby provides a two-dimensional thermal image which is nothing but a spatial map of the temperature and emissivity distribution of all objects in the picture. It is customary to use different colors in the pictures to visualize different photon intensities of the detected infrared radiation, for example, red areas indicate "hot spots" and blue areas denote "cold spots". The technique was first used by Pawlicki and Schmitz[56] to monitor the dynamics of reactions on solid surfaces and was extended by Sermon and co-workers[57] to obtain temperature profiles of exothermic gas-phase reactions catalyzed by SiO_2-supported platinum particles. The first case of parallel testing of the activity of the members of a small library of catalysts was reported by Moates et al. in 1996.[58] Two years later Taylor and Morken demonstrated that the activity of acylation catalysts on beads, prepared by the split-and-pool method, could be detected by IR thermography provided the beads swim on the surface of the solution containing the alcohol and the acylation agent.[59] Only then is detection possible. At about the same time Maier and co-workers reported another conceptional advancement,[60] namely emissivity-corrected IR thermography of large libraries of heterogeneous catalysts, a technique that requires only very small amounts of catalysts (< 200 μg). The main object of this study was to visualize temperature differences arising solely from the catalytic activity of the catalysts. This was achieved by applying a linear correction to

the detector response and subtracting the IR image of the library just before the start of the reaction, namely, the background (offset), from the images during catalytic experiments. This process means that local emissivity differences are no longer visible, and the heat evolution arising from the catalytic reactions on a microtiter plate can then be reliably detected. This technique was successfully applied to large libraries of heterogeneous catalysts in gas-phase hydrogenation and oxidation.[60] However, quantification still needs to be achieved here and in the other IR-thermographic studies.

In our own efforts directed towards developing high-throughput screening systems for assaying enantioselectivity, we envisioned the use of IR thermography in the kinetic resolution of chiral substrates.[61] However, it was not certain that IR thermography could actually be applied to homogeneous solutions since this question had never been posed previously. We set out to test the concept by studying the hydrolytic kinetic resolution of chiral epoxides **48** catalyzed by the Jacobsen catalysts **50**[62] in homogeneous solutions of moist toluene by using an AIM-256² IR camera equipped with a PtSi-FPA detector and a germanium lens (Scheme 16).[61]



Scheme 16. Hydrolytic kinetic resolution of epoxides **48** catalyzed by the Jacobsen catalysts **50**. Screening was carried out by IR thermography.[61]

After some experimentation with regard to the optimal materials,[63] we developed a special microtiter plate which allows for efficient heat transfer and which could be shaken so as to ensure sufficient mixing. A commercially available Eppendorf-Thermomixer was modified such that the top was replaced by an aluminum plate. Holes were then drilled into the plate and cylindrical glass reaction vessels about 8 mm in diameter and 35 mm in height placed therein (Figure 5). Thus, the whole microtiter plate can be shaken so as to ensure agitation of the reaction contents in each well.

Since the use of a racemate would deliver information regarding only the overall catalyst activity, the *S* and *R* enantiomers were tested pairwise in separate wells of the microtiter plate. This is the same trick that we used in our original UV/Vis-based screening system (Section 3.1).[17] In the present case we also included the racemate. Following placement of moist toluene solutions of (*S*,*S*)-**50** in the wells of the microtiter plate, (*R*)- and (*S*)-epichlorohydrin ((*R*)- and

Figure 5. Commercially available Eppendorf shaker with a modified aluminum plate on which the microtiter plate is placed (for better visualization half of the Al plate is uncovered).[61,63]

(*S*)-**48c**) were added and the temperature calibrated in the range of 24–39 °C. The reaction was then initiated by the addition of water. After a specified amount of time the shaking was interrupted for 10 s, 500 IR-thermographic pictures taken, and the average of these printed out as an image. The time-resolved results (Figure 6) show for the first time that IR thermography can be used to detect heat evolution caused by a reaction occurring in homogeneous solution, and that a highly enantioselective catalyst can indeed be identified.[61] Thus, the cobalt complex (*S*,*S*)-**50c** was found to exhibit the highest catalyst activity and selectivity; it reacts enantioselectively with the epoxide (*S*)-**48c**. The chromium catalyst (*S*,*S*)-**50b** is also selective, although less active, whereas the manganese complex (*S*,*S*)-**50a** displays no significant activity. This is in line with results obtained by Jacobsen and co-workers using benzoic acid as the nucleophile in lab-scale reactions.[62] It was also seen that heat generation after seven minutes is so pronounced in the case of the cobalt complex for the reaction of (*S*)-**48c** and *rac*-**48c** that no significant difference in the visualization using the applied temperature window (1 °C) could be detected. Therefore, the temperature scale covered by the window was increased to a total of 10 °C, which resulted in the clear identification of the "hottest" reaction (compare Figure 6e and 6f).[61]

Moreover, using the cobalt catalyst (*S*,*S*)-**50c** it was possible to perform substrate screening so that the chiral epoxides **48a–c** could be studied in parallel. It was found that (*S*)-**48a** is the most reactive substrate followed by (*S*)-**48c** and (*R*)-**48b**. The relative reactivity of (*R*)-**48b** and (*S*)-**48c** corresponds to that reported by Jacobsen and co-workers for lab-scale reactions,[62] whereas the reaction of (*S*)-**48a**, which is most rapid, had not been described in the literature previously. Thus, the IR-thermographic images contain an interesting chemical prediction.

In another series of experiments time-resolved detection of an enantioselective enzyme-catalyzed kinetic resolution was

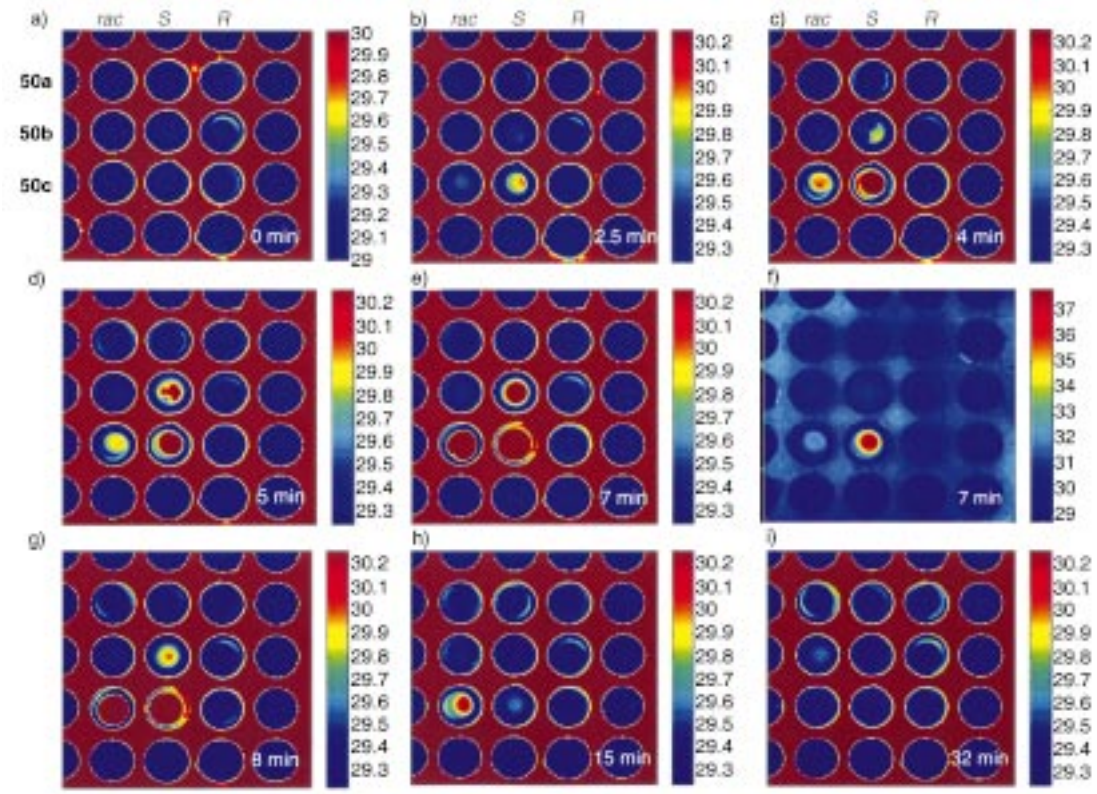
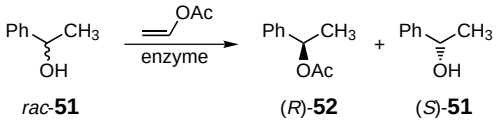


Figure 6. Time-resolved IR-thermographic imaging of the enantioselective hydrolysis of epoxide **48c** catalyzed by (*S*,*S*)-**50a–c** after a) 0, b) 2.5, c) 4, d) 5, e) 7, g) 8, h) 15, and i) 32 min.[61] In f) the same image is shown as in e), except that the temperature window ranges over 10 K. The bars on the far right of each image is the temperature/color key of the temperature window used [°C].

also demonstrated.[61] In this case the enzyme (lipase from *Candida antarctica*) was added to the wells of the microtiter plate in immobilized form, that is, the reaction was catalyzed by a heterogeneous catalyst. It was demonstrated using (*R*)- and (*S*)-1-phenylethanol ((*R*)- and (*S*)-**51**) as the substrate and vinyl acetate as the acylating agent that the reaction is highly *R*-selective, namely, hot spots appeared above the wells of the microtiter plate containing (*R*)-**51** (Scheme 17). This is



rac-**51** (*R*)-**52** (*S*)-**51**

Scheme 17. Enzyme-catalyzed kinetic resolution of alcohol **51**. Screening was carried out by IR thermography.[61]

in perfect agreement with the literature data, which shows that the *ee* value of the acylated form at 50% conversion is >99% in favor of (*R*)-**52**.[64] More recently we have studied this particular assay in more detail using an IR camera with even higher resolution and likewise obtained good time-resolved images.[65]

In conclusion, IR thermography is an excellent tool in the high-throughput identification of highly active and enantioselective catalysts. The method allows one to distinguish such "hits" from other members of a library of catalysts which are much less active or less enantioselective. However, quantification has yet to be achieved. This means that small differences in enantioselectivity, as usually observed in sequential rounds of enzyme mutagenesis (Scheme 1), cannot (yet) be picked up by IR-thermographic assays. Another limitation of the method, which by nature is expected to persist, pertains to the fact that in the absence of special effects, such as gas evolution, thermoneutral reactions cannot be assayed. This stands in contrast to endothermic processes which in fact show "cold spots" in the IR images.[66] Although this intriguing effect has not been demonstrated in an enantioselective process, the method was shown to work quite well in the search for active olefin-metathesis catalysts, the most active catalysts being identified on the microtiter plates as "cold spots".[66]

3.4. Circular Dichroism Assays

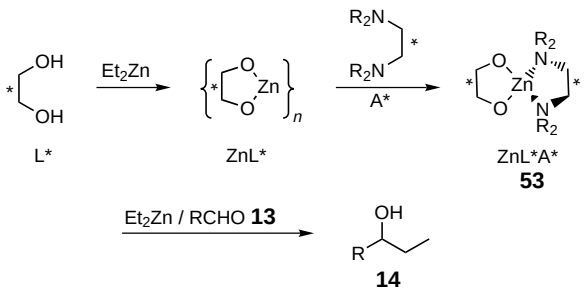
In principle it should be possible to use HPLC[6] in the high-throughput determination of enantiomeric purity provided that efficient separation of enantiomers by chirally modified columns is possible within a reasonable amount of time. Since rapid separation of this kind is a formidable challenge, an alternative approach is to use normal columns which simply separate the starting materials from the enantiomeric products. The enantiomeric excess (*ee*) of the mixture of enantiomers is then determined by circular dichroism (CD) spectroscopy. Indeed, this principle was first established by

Mason and co-workers[67] in 1980 and developed on a broad basis by Salvadori et al.[68] and Mannschreck.[69] Recently, Mikami and co-workers have shown that the method can be applied in the screening of combinatorially prepared enantioselective catalysts.[70] The method is based on the use of sensitive detectors for HPLC which determine in a parallel manner both the circular dichroism ($\Delta\varepsilon$) and the UV absorption (ε) of a sample at a fixed wavelength in a flow-through system.[67–70] The CD signal depends only on the enantiomeric composition of the chiral products, whereas the absorption relates to their concentration. Thus, only short HPLC columns are necessary.[67–69] Upon normalizing the CD value with respect to absorption, the so-called anisotropy factor g [Eq. (1)] is obtained.[67]

$$g = \frac{\Delta\varepsilon}{\varepsilon} \quad (1)$$

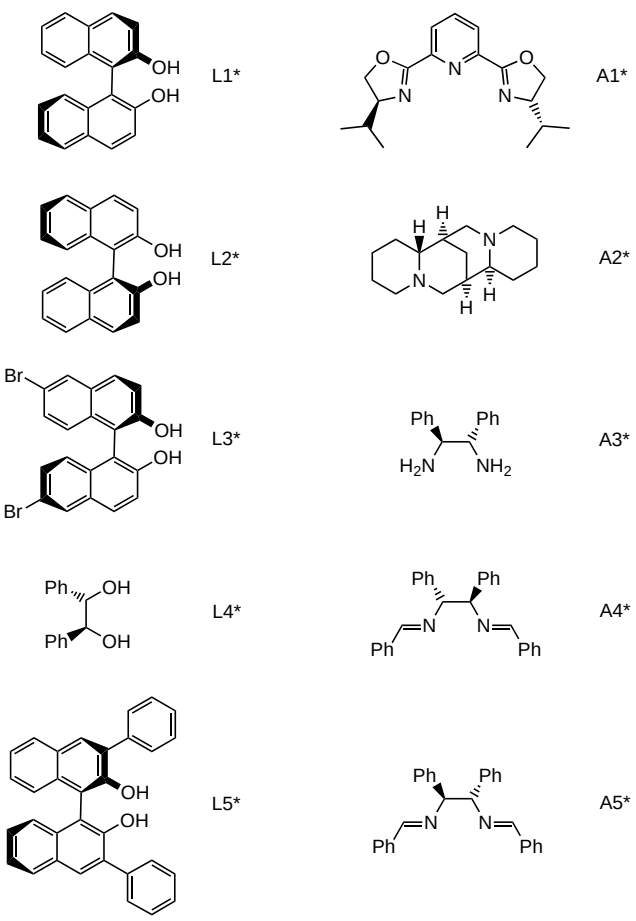
For a mixture of enantiomers it is thus possible to determine the *ee* value without recourse to complicated calibration. The fact that the method is theoretically valid only if the g factor is independent of concentration and if it is linear with respect to the *ee* value has been emphasized repeatedly.[67–70] However, it needs to be pointed out that these conditions may not hold if the chiral compounds form dimers or aggregates, because such enantiomeric or diastereomeric species would give rise to their own particular CD effects. Although such cases have yet to be reported, it is mandatory that this possibility be checked in each new system under study.

Mikami and co-workers studied various libraries of chiral ligands and activators in the addition of Et_2Zn to aldehydes (Scheme 18).[70] First the primary combinatorial library of

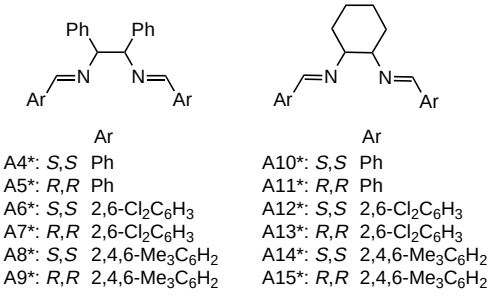


Scheme 18. Addition of Et_2Zn to aldehydes **13** catalyzed by chiral Zn-diolates in the presence of chiral diamine additives[70] and screened by CD.

chiral ligands L1*–L5* and chiral activators A1*–A5* (Scheme 19) were studied in order to optimize the lead structure of the next generation of chiral ligands and activators. The best combinations were found to be L5*/A4* and L5*/A5*, with *ee* values of up to 65% being observed in the Et_2Zn addition to benzaldehyde. Based on these results a new library composed of chiral diimines as activators was scrutinized (Scheme 20).[70] The best combination turned out to be L5*/A9* (*ee* = 90% at room temperature and 99% at $-78\,^{\circ}C$ with benzaldehyde and *ee* = 92–99% with other aldehydes). Although only a few dozen reactions were monitored by a JASCO-CD-995 instrument, the CD-based



Scheme 19. Chiral diols as ligands and diamino compounds as additives in the addition of Et_2Zn to aldehydes **13**.[70]

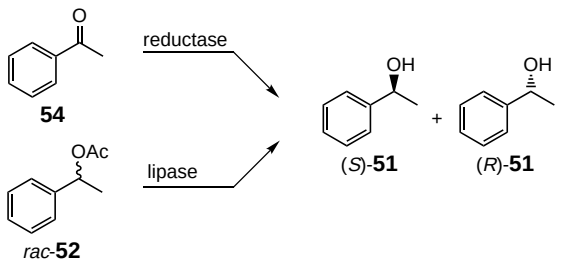


Ar		Ar	
A4*: *S,S*	Ph	A10*: *S,S*	Ph
A5*: *R,R*	Ph	A11*: *R,R*	Ph
A6*: *S,S*	2,6-$Cl_2C_6H_3$	A12*: *S,S*	2,6-$Cl_2C_6H_3$
A7*: *R,R*	2,6-$Cl_2C_6H_3$	A13*: *R,R*	2,6-$Cl_2C_6H_3$
A8*: *S,S*	2,4,6-$Me_3C_6H_2$	A14*: *S,S*	2,4,6-$Me_3C_6H_2$
A9*: *R,R*	2,4,6-$Me_3C_6H_2$	A15*: *R,R*	2,4,6-$Me_3C_6H_2$

Scheme 20. Chiral dialdimines as additives in the addition of Et_2Zn to aldehydes **13**.[70]

assay is theoretically amenable to high-throughput screening of enantioselective catalysts.

Following the publication of Mikami and co-workers[70] we reported our independent results in the assay of related chiral alcohols.[71,72] In our work concerning the directed evolution of enantioselective enzymes we needed to develop fast and efficient ways to determine the enantiomeric purity of these compounds, which can be produced enzymatically either by reduction of the prochiral ketone (for example, **54**) using reductases or by kinetic resolution of *rac*-acetates (for example, *rac*-**52**) by lipases (Scheme 21). In both systems the CD assay is theoretically possible. In the former case liquid chromatography would have to be used to separate the



Scheme 21. Reductase-catalyzed reduction of ketone **54** and lipase-catalyzed kinetic resolution of acetate **52**. Screening was carried out by CD.[71]

starting material **54** from the product (*S*)/(*R*)-**51**, whereas in the latter case (*S*)/(*R*)-**51** would have to be separated from (*S*)/(*R*)-**52**.

Since acetophenone (**54**) has a considerably higher extinction coefficient than 1-phenylethanol (**51**) at about 260 nm, the separation of starting material from product was absolutely necessary and was accomplished on a relatively short reversed-phase HPLC column. The maximum value of the CD signal was determined in preliminary experiments using enantiomerically pure product **51**.[71] Mixtures of **51** having different enantiomer ratios (and therefore *ee* values) were prepared and analyzed precisely by GC on a chiral stationary phase. The same samples were studied by CD and resulted in the compilation of *g* values. A linear dependency with a correlation factor of $r = 0.99995$ was in fact observed upon plotting the *g* against the *ee* values, which translates into a simple equation for enantioselectivity [Eq. (2)].

$$ee = 3176.4g - 8.0 \quad (2)$$

We then studied the possible dependency of the *g* factors on concentration.[71] A mixture of (*S*)- and (*R*)-**51** with $ee = 20\,\%$ was prepared at a concentration of 10 μL mL^{-1} in acetonitrile, which was then successively diluted. Figure 7 clearly shows

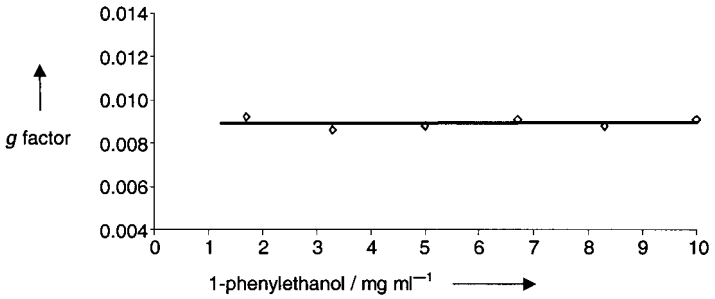


Figure 7. Dependency of the *g* factors on concentration in a mixture of (*S*)- and (*R*)-**51** ($ee = 20\,\%$).[71]

there is no dependency of *g* on concentration (standard deviation = 2.6 %). Thus, possible aggregation as a consequence of hydrogen bonding between two or more molecules of the product (*S*)- and (*R*)-**51** in this medium which could lead to artifacts is not involved, thus making the system amenable to CD analysis.

The question whether high-throughput screening of the above reaction system can in fact be put into practice was now reduced to the problem of the efficient and fast separation of **54** from (*S*)/(*R*)-**51**. Although complete optimization was not carried out, separation was in fact accomplished using reversed-phase silica as the column material and methanol/water (47/53) as the eluant. In view of the results concerning the dependency of the *g* factor on concentration (see above), aggregation can be excluded in this protic medium. Figure 8 shows the corresponding HPLC chromatogram in which the mixture is fully separated within less than 1.5 minutes. Thus, it is possible to perform about 700 – 900 exact *ee* determinations per day by using the JASCO-CD-1595 instrument in conjunction with a robotic autosampler.[71, 72]

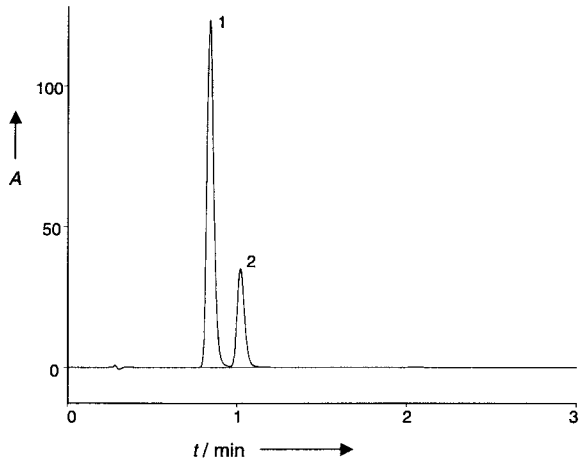
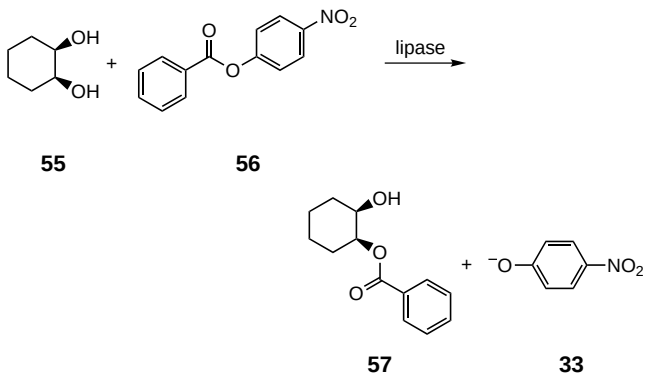


Figure 8. HPLC chromatogram of a mixture of **54** (peak 1) and (*S*)/(*R*)-**51** (peak 2).[71]

Finally, we wish to point out the possibility that reliable *ee* determinations using CD-based assays are theoretically possible in certain systems even though no liquid chromatographic separation is performed whatsoever.[71–73] Prerequisite is a prochiral substrate (for example, a *meso* compound) as well as a UV-active product (chromophore) which is formed as the enantioselective reaction proceeds. The absorption maximum of the chromophore formed has to differ considerably from that of the desired chiral product. This new principle is illustrated by the lipase-catalyzed enantioselective acylation of the *meso*-diol **55** by benzoic acid *p*-nitrophenyl ester (**56**) with formation of the chiral product **57** and the yellow-colored *p*-nitrophenolate (**33**) which has a characteristic UV/Vis-absorption at 410 nm (Scheme 22). Upon measuring the *g* value of the absorption maximum of **57** and the additional UV absorption of **33**, all information necessary to determine the conversion and enantiopurity is available without the need to perform any liquid chromatographic separation. The advantage of this novel approach has to do with ease of performance and the clear prospect of higher throughput.[71–73]

In conclusion, on the basis of these and previous studies HPLC-UV-CD or UV-CD alone may well constitute a viable high-throughput screening system for enantioselective (bio)-catalysts in a given situation. Success will depend upon the particular substrate under study. Moreover, the precautions as delineated above need to be considered.



Scheme 22. Lipase-catalyzed asymmetric acylation of *meso*-diol **55**. Screening was carried out by CD.[71–73]

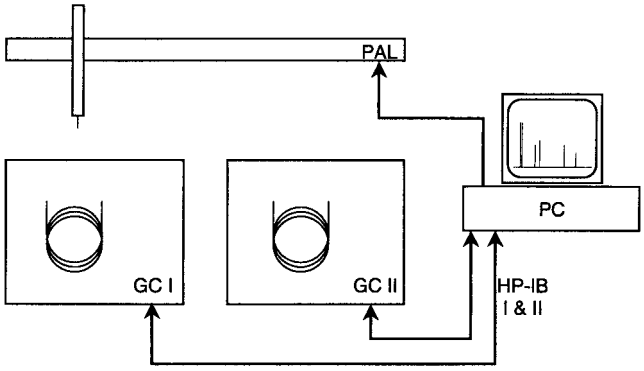
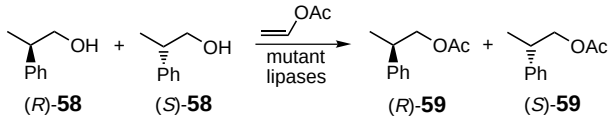


Figure 9. Schematic representation of a GC screening system comprising two GC instruments.[74]

3.5. Chromatographic Assays

Conventional GC or HPLC based on the use of chiral stationary phases can only handle a few dozen *ee* determinations per day.[5, 6] However, we have recently demonstrated that GC can be modified so that in certain cases about 700 exact *ee* and *E* determinations are possible per day.[74] The case study concerns the lipase-catalyzed kinetic resolution of the chiral alcohol (*R*)- and (*S*)-**58** with formation of the acylated forms (*R*)-**59** and (*S*)-**59** (Scheme 23). Thousands of mutants of the lipase from *Pseudomonas aeruginosa* were created by error-prone PCR[17] for use as catalysts in the model reaction.[72]



Scheme 23. Lipase-catalyzed kinetic resolution of alcohol **58**. Screening was carried out by GC.[72, 74]

The idea for accomplishing a reasonably high throughput was to somehow combine two GC columns in one configuration. The initial approach concerned the use of two columns in a single GC oven.[72, 74] However, this turned out to have a number of disadvantages. The successful construction consists of two GC instruments,[75] one prep-and-load sample manager (PAL),[76] and a PC (Figure 9). The instruments are connected to the PC through a standardized data bus (HP-IB)[75] which controls pressure, temperature, etc. and handles other data such as that from the detector. A wash station as well as a drawer system with a maximum of eight microtiter plates were included. Using a special construction developed in-house, the sample manager was attached to the unit in such a way as to reach both injection ports. Since the sample manager can inject samples from 96- or 384-well microtiter plates, over 3000 samples can be handled without manual intervention. The software (Chemstation)[77] enables additional programs (macros) to be applied before and after each analytical run. Such a macro controls the sample manager, with each position on the microtiter plate being labeled by the sequence table. Another macro ensures that the analysis following each sample is carried out in a specified manner, namely, the peaks of the chiral compounds **58** and **59** are analyzed quantitatively. The analytical data are transferred to an Excel spreadsheet by DDE (Dynamic Data Exchange)[78] in a table format or in microtiter-format, which allows for a rapid overview. Finally, since the carrier gas is hydrogen the setup includes H_2 guards which monitor the H_2 concentration in the ovens; at concentrations exceeding 1% (potentially explosive at $>4\%$ H_2) the systems responds and automatically switches to nitrogen as the carrier gas.[72, 74]

Using a stationary phase based on a β-cyclodextrin derivative (2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-CD) complete separation of (*R*)- and (*S*)-**58** (but not of (*R*)/(*S*)-**59**) was achieved within 3.9 minutes.[72, 74] About 700 exact *ee* determinations of (*R*)/(*S*)-**58** are possible per day with the two simultaneously operating GC units shown in Figure 9. Moreover, the corresponding values for the conversion and the selectivity factor *E* are likewise automatically provided in microtiter format. A typical example is shown in Figure 10 in which the data corresponding to the most selective mutant enzymes are shown in gray boxes ($E \geq 2.4$).[74] Mutants displaying 0% conversion imply complete lack of enzyme activity. Negative *ee* values indicate reversal of enantioselectivity.

Figure 10. Excel spreadsheet of GC data in microtiter plate (MTP) format showing values for conversion *c* [%], *ee* value [%], and selectivity factor *E* for mutant lipases catalyzing the hydrolytic kinetic resolution of alcohol **58**.[74]

Thus, contrary to common belief, it is possible to utilize GC in the high-throughput screening of enantioselectivity in appropriate cases. This type of GC setup should also be useful in the screening of achiral transformations. Moreover, it is sometimes possible to increase throughput even further by injecting samples at proper times which are shorter than the total time span of the actual chromatogram, thus enabling the maximum use of time between runs (interlocking chromatograms).[72, 74] Major advantages relative to the employment of two totally separate GC units include optimal use of laboratory space and the utilization of a single sampler and computer system, which results in high instrumental and economical efficiency. Although optimization needs to be performed for each new chiral compound to be tested, it can be anticipated that in appropriate cases 600 – 800 samples can easily be handled per day. It remains to be seen if a similar adaptation of HPLC can be developed to suit the requirements of a given analytical problem. However, it is unlikely that truly high-throughput *ee* determinations, that is, many thousands of samples per day, can be achieved in a general way on the basis of GC or HPLC. Of course, depending upon the particular problem at hand, a throughput of 600 – 800 *ee* determinations per day may suffice.

A related question concerns the high-throughput screening of enantioselectivity based on thin-layer chromatography (TLC).[72] It is known that enantiomer separation is possible using chirally modified stationary phases and that the relative size of the "spots" for the *R* and *S* isomers correlate with enantiomeric purity (*ee*).[79] Günther has applied this technique in the determination of the *ee* values of chiral amino acids and hydroxycarboxylic acids,[79b] although the authors did not strive for high throughput. This can principally be achieved by applying appropriate computer image processing. One possibility that we suggest is the routine MORFO of the commercially available IMAGIC-5 software package which recognizes and "integrates" spots on a given surface.[80, 81] In the case of kinetic resolution, the relevant TLC plate would have the schematic form shown in Figure 11.[72] It is easy to imagine that hundreds of these TLC plates can be scanned rapidly. The real challenge is to find efficient chiral selectors which result in sufficient enantiomer separation.

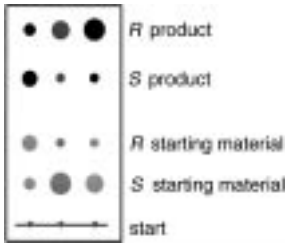


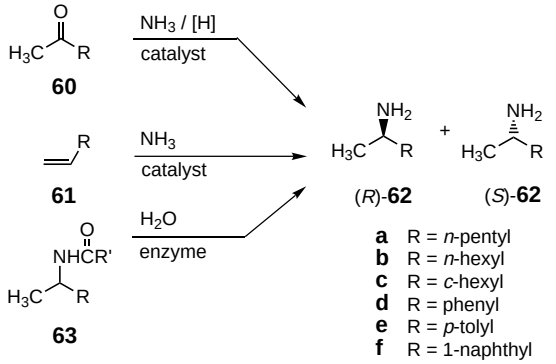
Figure 11. Schematic representation of a TLC plate showing the result of kinetic resolution of a *rac*-starting material.[72]

3.6. Parallel Chirally Modified Capillary Electrophoresis

As mentioned in Section 1 the determination of enantiomeric purity is sometimes carried out by capillary electrophoresis (CE) in which the electrolyte contains chiral selectors such as cyclodextrin (CD) derivatives.[7] Unfortunately the conventional forms of this analytical technique allows for only a few dozen *ee* determinations per day. However, as a consequence of the analytical demands arising from the Human Genome Project CE has been revolutionized in recent years so that efficient techniques for instrumental miniaturization are now available, which make the super-high-throughput analysis of biomolecules possible for the first time.[82, 83] Two different approaches have emerged, namely capillary array electrophoresis (CAE)[82] and CE on microchips (also called CAE on chips).[83] Both techniques can be used to carry out DNA sequence analyses and/or to analyze oligonucleotides, DNA-restriction fragments, amino acids, or PCR products. Many hundreds of thousands of analytical data points can be accumulated per day.[82, 83] We therefore thought that chiral modification of such techniques would allow for the first time the super-high-throughput analyses of enantiomeric purity (*ee*).[84]

In the case of CAE, commercially available instruments have been developed which contain a high number of capillaries in parallel, for example, the 96-capillary unit MegaBACE which consists of 6 bundles of 16 capillaries.[85] The system can therefore address a 96-well microtiter plate. Each capillary is about 50 cm long.

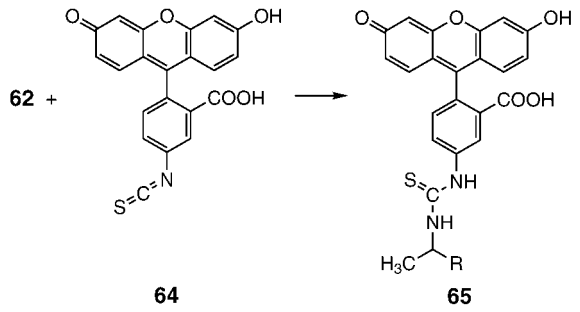
We therefore set out to adapt this system as a super-high-throughput analytical tool for *ee* determination.[86] In this initial study chiral amines of type **62**, which are of importance in the synthesis of pharmaceutical and agrochemical products,[87] were used as the model substrates. They are potentially accessible by catalytic reductive amination of ketones **60**, Markovnikov addition of ammonia to olefins **61**, or enzymatic hydrolysis of acetamides **63** (the reverse reaction also being possible; Scheme 24).



Scheme 24. Catalytic formation of chiral amines **62**. Screened was carried out by CAE.[86]

In exploratory experiments the conditions for a conventional CE assay of the amines **62** were first optimized using various α- and β-CD derivatives as chiral selectors.[86] The amines were first derivatized by conventional reaction with fluorescene isothiocyanate (**64**) to give the fluorescence-active compounds **65** (Scheme 25) that would enable a sensitive detection system, specifically laser-induced fluorescence detection (LIF), to be used. Although extensive optimization was not carried out (only six CD derivatives were tested), satisfactory baseline separation was accomplished in all cases (Table 1).

The next step involved the use of compounds **62c**/**65c** as the model substrates for CAE analysis using a MegaBACE type



Scheme 25. Derivatization of chiral amines **62** for LIF detection in CAE screening.[86]

Table 1. Results of CE-based enantiomeric separation of amine derivatives **65** as a function of the electrolyte.[a][86]

Buffer, cyclodextrin used[b]	**65a**	**65b**	**65c**	**65d**	**65e**	**65f**
40 mmol CHES, 40 mmol γ-CD + 15% CH_3CN	B	B	–	n.m.	–	–
20 mmol borate, 20 mmol DM-β-CD	–	–	–	–	–	–
40 mmol CHES, 20 mmol DM-β-CD	–	–	–	–	–	–
40 mmol CHES, 5 mmol DM-β-CD	–	–	–	P	–	–
40 mmol CHES, 40 mmol HE-β-CD	–	–	–	–	–	–
40 mmol CHES, 20 mmol HE-β-CD	P	–	–	P	P	–
40 mmol CHES, 10 mmol HE-β-CD	P	–	–	P	P	–
40 mmol CHES, 5 mmol HE-β-CD	aB	P	P	aB	aB	–
40 mmol CHES, 2.5 mmol HE-β-CD	aB	P	P	P	aB	–
40 mmol CHES, 5 mmol HP-α-CD	–	–	–	–	–	–
40 mmol CHES, 20 mmol HP-β-CD	–	–	–	P	–	–
40 mmol CHES, 5 mmol HP-β-CD	P	–	P	aB	P	–
40 mmol CHES, 20 mmol HP-β-CD + 15% CH_3CN	P	–	–	P	–	P
40 mmol CHES, 25 mmol HP-γ-CD	–	–	P	–	–	–
40 mmol CHES, 6.25 mmol HP-γ-CD	–	–	B	–	P	aB
40 mmol CHES, 25 mmol HP-γ-CD + 15% CH_3CN	–	–	B	–	–	–
40 mmol CHES, 10 mmol NH_2-β-CD	–	–	–	–	–	–
40 mmol CHES, 10 mmol β-CD	–	–	–	–	–	–

[a] P: Partial separation; B: basisline separation; aB: almost basisline separation; –: no separation; n.m.: not measured. [b] DM: heptakis(2,6-di-*O*-methyl); HE: hydroxyethyl; HP: hydroxypropyl; NH_2: 6^A-amino-6^A-deoxy; CHES: 2-(*N*-cyclohexylamino)ethanesulfonic acid.

of instrument. Known enantiomeric mixtures of the amine **62c** were transformed into the fluorescence-active derivative **65c**. The latter samples were then analyzed by CAE. Unfortunately, the results of the conventional single capillarly system could not be reproduced in the CAE experiments because of unstable electrophoretic runs. The problem was solved by developing a special electrolyte having a higher viscosity. It is composed of 40 mM 2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES; pH 9.1) and 6.25 mM γ-CD (5/1) diluted with a buffer containing linear polyacrylamide. The MegaBACE instrument was operated at a voltage of -10 kV (at 8 μA) per capillary and a sampling voltage of -2 kV (9 s). Under these conditions baseline separation was excellent (Figure 12). The agreement between the *ee* values of mixtures of(*R*)- and (*S*)-**65c** determined by CAE and those of the corresponding mixtures of (*R*)- and (*S*)-**62c** as measured by GC turned out to be excellent.[86]

The enantiomer separation of (*R*)/(*S*)-**65c** on the MegaBACE instrument required about 19 minutes. This means that even though the conditions are far from optimized the automated 96-array system can provide more than 7000 *ee* determinations in a single day.[86] In related cases optimization resulted in shorter analysis times for the separation of the enantiomers, so that a daily throughput of 15000 to 30000 *ee* determinations is realistic. Such super-high-throughput screening for enantioselectivity is not possible by any other currently available technology. In view of the possibility of chiral selector optimization and the fact that CAE has many advantages such as the use of extremely small amounts of samples, essentially no solvent consumption, the absence of high pressure pumps and valves, as well as high durability of columns, we believe that our CAE assay is ideally suited for high-speed *ee* determination.

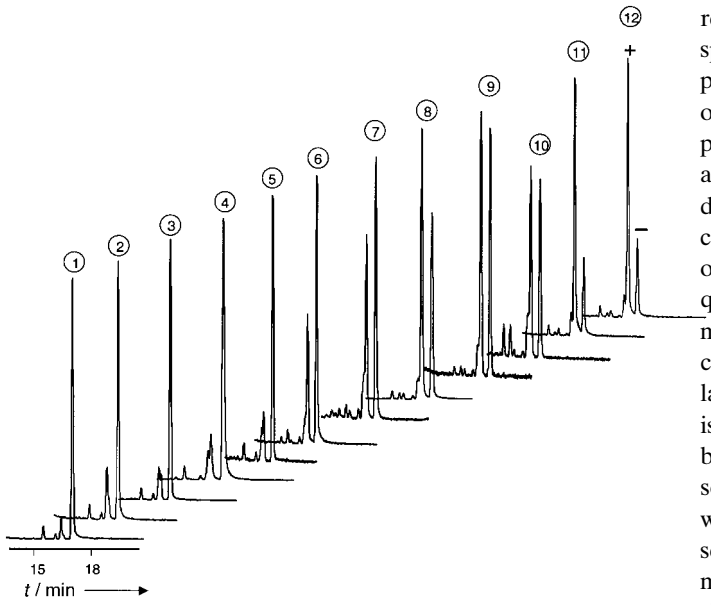


Figure 12. CAE antipode separation of the enantiomers of **65c** in a representative sample.[86]

In 1997 we described a variation of the above method, namely the possibility of high-throughput *ee* screening of chiral organic compounds, by utilizing capillary electrophoresis on microchips.[84] The general procedure for CE (or more specifically CAE) on microchips (typically 10×10 cm) had previously been developed by Manz and co-workers[83a] and others[83c–h] for the analysis of biomolecules. Traditional photolithographic techniques were used to produce capillary arrays on plastic or glass microchips.[83] In our work we discovered that the enantiomer separation of organic molecules on plastic microchips is not generally feasible as a result of the chemical instability of such systems. The situation is quite different in the case of glass chips.[86] In such a modification the separation of enantiomers, for example, of compound **65c**, is possible with a detection system based on laser induced fluorescence (LIF). Automation using robotics is currently being optimized, which means that a second CAE-based assay for super-high-throughput *ee* determination will soon emerge. In view of the recent reports by Mathies and co-workers[83c] and Li and co-workers[83h] concerning enantiomer separation of aqueous solutions of amino acids on CAE microchips, we expect that this type of assay will play a significant role in the future.[88] Of course, for a given analytical problem, derivatization and antipode separation

need to be efficient, which means that universal generality cannot be claimed.

In summary, the two forms of capillary array electrophoresis constitute powerful methods for the determination of the enantiomeric purity of chiral compounds in a truly high-throughput manner. Various modifications are possible, for example, detection systems based on UV/Vis, MS, or electrical conductivity. Moreover, chiral selectors in the CE electrolyte are not even necessary if the mixture of enantiomers is first converted into diastereomers, for example, using chiral fluorescent-active derivatization agents.[86b]

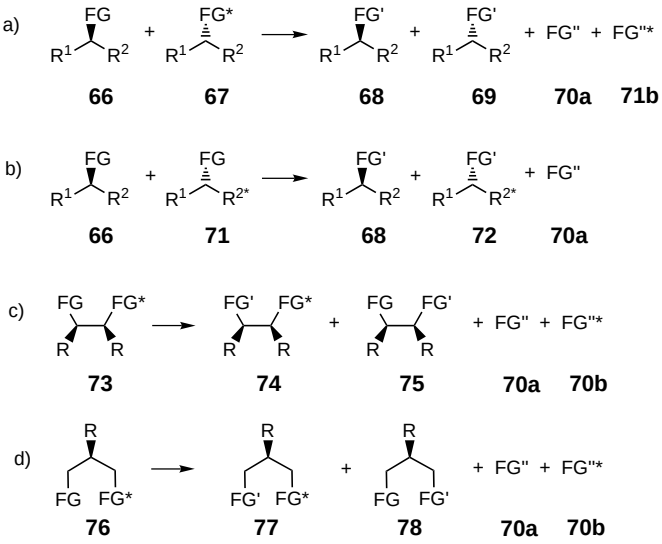
3.7. Mass-Spectrometric Assays

Although several mass-spectrometric (MS) assays have been developed for use in combinatorial catalysis,[12, 13b, 13d, 13g, 13j] their application to the screening of enantioselective catalysts is not straightforward simply because the *R* and *S* forms of a chiral compound show identical mass spectra. We have nevertheless devised an MS-based system which makes possible the determination of enantioselectivity (*ee* and/or *E* value) without any chromatographic separation or diastereomer formation. Typically about 1000 catalyst evaluations are possible per day.[89] Ionization can be accomplished by a number of standard methods, including electrospray ionization (ESI) and matrix-assisted laser-desorption ionization (MALDI).[63] Two basically different stereochemical processes can be monitored by our method: the kinetic resolution of racemates and the asymmetric transformation of prochiral substrates with enantiotopic groups.[89, 90]

The underlying principle is based on the use of isotopically labeled substrates in the form of pseudo-enantiomers or pseudo-prochiral compounds (Scheme 26). The course of the asymmetric transformation, that is, the relative amounts of reactants and/or products, are detected by ESI-MS.

In the case of kinetic resolution, compounds **66** and **67**, which differ in their absolute configuration and in their labeling at the functional group FG*, need to be prepared in enantiomerically pure forms and then mixed in a 1:1 manner to simulate a racemate (Scheme 26a). Following asymmetric functional group transformation (in an ideal kinetic resolution there is 50 % conversion), the true enantiomers **68** and **69** are formed together with the nonlabeled and labeled achiral products **70a** and **70b**, respectively. The ratios of the total intensities of **66**/**67** and **70a**/**70b** in the mass spectra (*m*/*z* intensities of the quasi-molecular ions) allow for the determination of enantiomeric purity and therefore enantioselectivity of a catalyst. In some cases it may be advantageous to use an internal standard to determine the extent of conversion.[89]

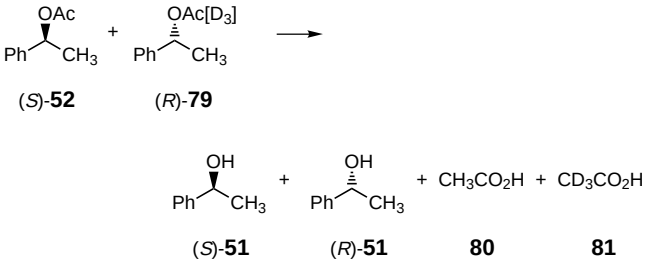
As a variation of this theme, kinetic resolution of the pseudo-enantiomers **66** and **71** which are labeled at residue R^2 affords a new pair of pseudo-enantiomers **68** and **72** (Scheme 26b). Based on the *m*/*z* intensities of the quasi-molecular ions of **66**/**71** and **68**/**72**, the conversion, enantioselectivity (*ee*), and selectivity factor (*E*) can be obtained. An internal standard is not necessary.[89]



Scheme 26. a) Asymmetric transformation of a mixture of pseudo-enantiomers involving cleavage of the functional groups FG and labeled functional groups FG*. b) Asymmetric transformation of a mixture of *pseudo*-enantiomers involving either cleavage or bond formation at the functional group FG; isotopic labeling at R^2 is indicated by the asterisk. c) Asymmetric transformation of a pseudo-*meso* substrate involving cleavage of the functional groups FG and labeled functional groups FG*. d) Asymmetric transformation of a pseudo-prochiral substrate involving cleavage of the functional groups FG and labeled functional groups FG*.[89]

In the case of prochiral substrates having enantiotopic groups, for example, *meso* compounds (Scheme 26c), the synthesis of a single pseudo-*meso* compound, such as **73**, suffices, since the stereodifferentiating reaction of interest delivers a mixture of two MS-detectable pseudo-enantiomers **74** and **75**. The same applies to other pseudo-prochiral substrates of type **76** (Scheme 26d).

The first system to be tested concerned the kinetic resolution of racemic 1-phenylethyl acetate (**52**) (Scheme 27).[89] For this purpose the pseudo-enantiomers (*S*)-**52** and (*R*)-**79** were prepared in enantiomerically pure



Scheme 27. Kinetic resolution of pseudo-enantiomers (*S*)-**52** and (*R*)-**79**. Screening was carried out by ESI-MS.[89]

form. These two compounds were mixed in various ratios and the resulting mixtures analyzed by GC in order to ascertain the exact pseudo-*ee* values as a control. Thereafter the same samples were analyzed by ESI-MS. A typical ESI-mass spectrum is shown in Figure 13. Since the deuterium labeling results in the sodium adducts of (*S*)-**52** and (*R*)-**79** appearing at different *m*/*z* values, integration is a simple manner. A total of 17 control samples were studied, and the agreement

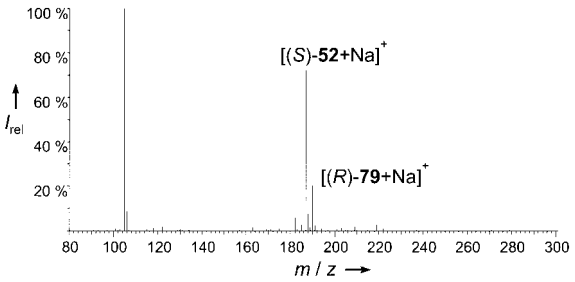
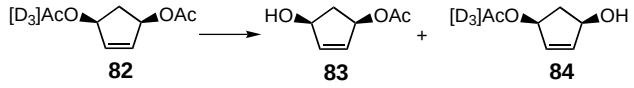


Figure 13. ESI-mass spectrum of a sample containing (*S*)-**52** and (*R*)-**79**.[89]

between the *ee* values determined by GC and ESI-MS was excellent (± 5%).[89] In contrast to a number of other methods which suffer from the fact that *R*- and *S*-configurated substrates are tested separately as pairs on microtiter plates, the present system utilizes 1:1 mixtures of pseudo-enantiomers in kinetic resolutions. Moreover, analogous reactions on the solid phase, if necessary, should pose no problems.

An experimental setup capable of high-throughput screening of enantioselective reactions was then devised. This was achieved by combining an automated liquid sampler for microtiter plates (96-format) with an ESI-MS system, both commercially available (Figure 14).[89, 90] This unit allows about 1000 determinations of the *ee* value and the conversion (and thus *E*) of such transformations as the above model reaction to be made in a single day. As shown in Scheme 26, deuterium labeling can be performed at any position of the substrate. It is advisable to perform a quick kinetic study of labeled and nonlabeled substrates in order to exclude possible secondary isotope effects.

As an example of an asymmetric transformation of a prochiral substrate, we considered the known desymmetrization of *cis*-1,4-diacetoxycyclopentene.[89] In this case the pseudo-prochiral compound **82** was prepared (Scheme 28). The products of the asymmetric transformation are compounds **83** and **84**, each having two stereogenic centers. Since



Scheme 28. "Desymmetrization" of a pseudo-prochiral substrate **82**. Screening was carried out by ESI-MS.[89]

they are pseudo-enantiomers they can easily be distinguished by ESI-MS. Indeed, we are currently studying the directed evolution of an enantioselective lipase for this transformation, with the screening being possible by the unit shown in Figure 14.

This approach constitutes a powerful method for high-throughput screening of enantioselective enzymes or synthetic asymmetric catalysts. Instead of deuterium labeling, mass-tagging (for example, methyl groups in place of hydrogen atoms) at remote positions in the molecule is also possible. However, the method has limitations because it is restricted to the analysis of the two classes of compounds having the symmetry properties delineated above (Scheme 26). This means that transformations involving prochiral compounds lacking enantiotopic groups cannot be screened (for example, reduction of acetophenone (**54**) to 1-phenylethanol (**51**); Scheme 21).

A different MS-based approach, specifically in the determination of *ee* values of alcohols, was described by Finn and co-workers[91] and can be viewed as an extension of Horeau's method.[92] It is based on derivatization using chiral mass-tagged acylation reagents. The method requires a measurable degree of kinetic resolution in the derivatization step.[91] The technique makes use of an equimolar mixture of pseudo-enantiomeric mass-tagged chiral acylating agents that differ in a substituent remote to the stereogenic center (for example, methyl versus hydrogen) in a way that the mass of the molecule correlates with its absolute configuration. In principle the reactions of enantiomers with chiral reagents can proceed with unequal rate constants ($k_f > k_s$; f = fast, s = slow). Scheme 29 shows the case of chiral alcohols (*R*)-OH and (*S*)-OH and mass-tagged enantiomerically pure acylating agents.[91] The enantiomeric alcohols (*R*)-OH and (*S*)-OH are first treated with the chiral mass-tagged acids A-CO_2H and B-CO_2H in the presence of 1,3-dicyclohexylcarbodiimide (DCC). The relative amounts of the product esters as measured by MS can then be used (after two calibration measurements) to determine the enantiomeric composition of the starting mixture (*R*)-OH/(*S*)-OH and therefore the *ee* value.

Finn and co-workers used the proline-derived mass-tagged acylating agents **85** and **86**.[91] The reagents need to be used in great excess, and for the system to work it is necessary that a small but measurable degree of kinetic resolution occurs. The sensitivity of the method was shown to be ±10% *ee*. The

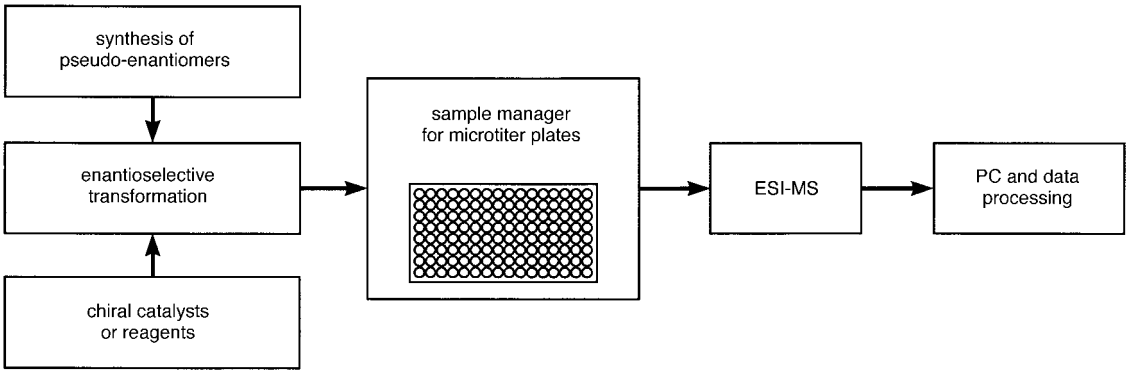
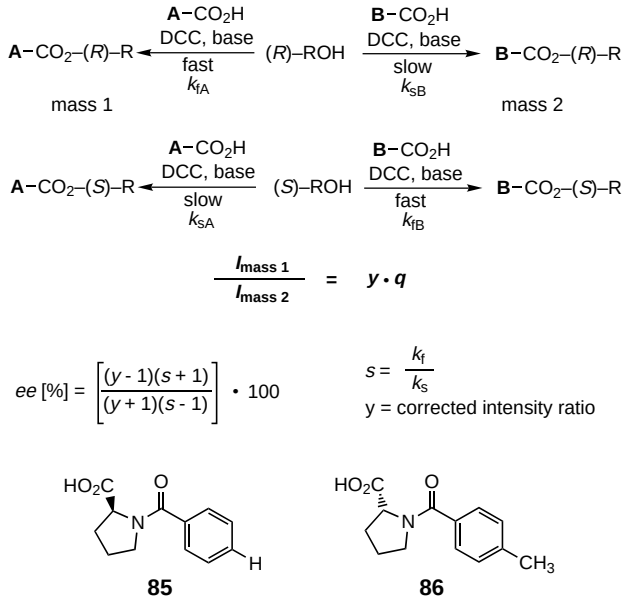


Figure 14. Experimental setup of an ESI-MS *ee*-screening system.[89]

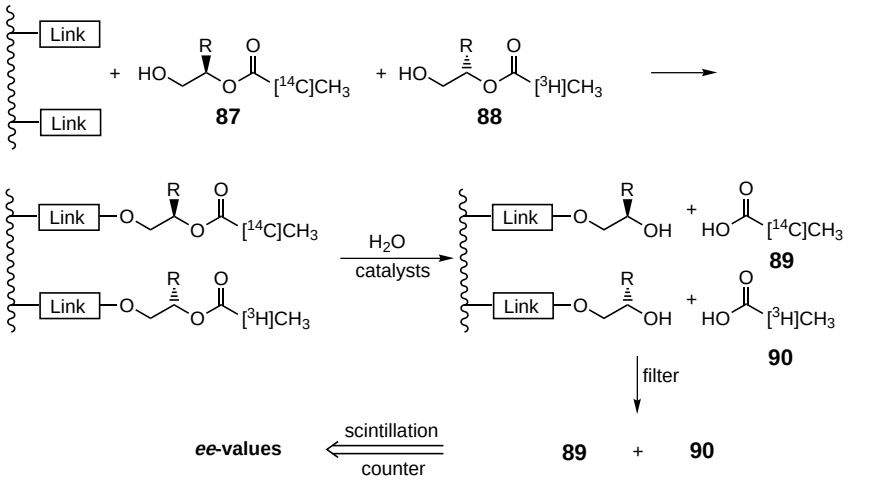


Scheme 29. Mass-spectrometric *ee* determination in the kinetic resolution of alcohols (*R*)-OH/(*S*)-OH.[91] *I* = peak intensity; *q* = correction factor for ionization; DCC = dicyclohexylcarbodiimide.

technique can also be reversed using mass-tagged chiral nucleophiles for measuring the *ee* values observed when using acylating agents. As in our own MS-based system,[89] chromatographic separation is not involved. Moreover, the authors have pointed out the possibility of a robotic high-throughput screening system using microtiter plates,[91] which should certainly work. Finally, instead of mass-tagging based on the use of methyl groups, it is also possible to carry out the same type of analyses using deuterium-labeling.[63]

3.8. Radioactivity Assays

In 1997 we pointed out the possibility of a detection system for the determination of *ee* values using radiolabeled substrates on a solid support and measuring the radioactivity of the solution containing the cleaved products.[84] For example, attachment of appropriate radiolabeled pseudo-enantiomers **87**/**88** is necessary in the case of the hydrolytic kinetic resolution of esters of the type $ROCH_2CH(R)OC(O)CH_3$ by the enantioselective formation of the corresponding *O*-protected diols $ROCH_2CH(R)OH$ (Scheme 30). With this system thousands of (bio)catalysts can then be screened. Following catalytic hydrolysis in the wells of microtiter plates, the solution is separated from the solid support and the relative amounts of radiolabeled acetic acids **89** and **90** are determined using a conventional scintillation counter. This provides the *ee* values at different conversions. In special cases this method may be advantageous, although automation using a robotic scintillation counter needs to be developed.

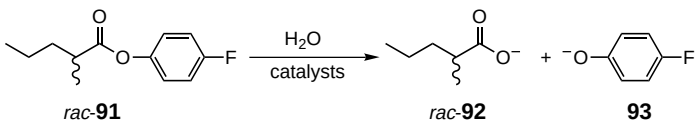


Scheme 30. Schematic representation of an *ee* assay based on radioactivity measurements.[84]

3.9. NMR and IR Assays

NMR spectroscopy is a relatively slow analytical procedure and therefore may not appear to be amenable to high-throughput analyses. However, progress has in fact been made in the utilization of NMR methods in combinatorial drug discovery processes both on solid supports and in solution.[93] Of course, one of the drawbacks is low sensitivity necessitating the use of high concentrations. Developments in the design of NMR flow probes and cryoprobes are expected to stimulate future progress in the use of NMR spectroscopy in combinatorial chemistry.

Since high throughput stands at the heart of combinatorial catalysis and because it is of advantage to carry out the measurements on a large number of samples simultaneously, we have considered the application of magnetic imaging (NMR tomography) for sample screening. This technique is used successfully in medicine to image tissues and organs. In our case the goal was to obtain tomograms of microtiter plates on which enantioselective reactions were occurring.[72] In exploratory experiments we decided to utilize the ^{19}F nucleus in order to monitor the hydrolysis of the *R*- and *S*-configured ester **91** (Scheme 31).[72, 94] In an initial measurement a 3:2



Scheme 31. Model reaction for an NMR-based screening system.[72, 94]

mixture of *rac*-**91** and *p*-fluorophenolate (**93**) in DMF and tris(hydroxymethyl)methylamine (TRIS) buffer (pH 7.5) in D_2O:H_2O (3:7) was studied. Separate ^{19}F NMR peaks of **91** and **93** were readily observed ($\delta = -117$ and -124, respectively).

In further exploratory experiments NMR tubes were charged with mixtures of **91** and **93** in ratios of 1:1.4 (sample A) and 1:9 (sample B). The samples were measured with a micro-5 micro-imaging probe head on a Bruker DMX300WB spectrometer.[94] The 3D chemical shift imaging

(CSI) allows two dimensions to be resolved spatially and the third is chemical shift sensitive (Figure 15).[94] It is easy to identify the resonance of the ester **91** (left signal) and that of *p*-fluorophenolate (**93**; right signal). In each case the traces along the *x*- and *y*-axes reflect the total absorption of the resonance frequency of *p*-fluorophenolate (in sample A) and that of the ester **91** (in sample B). Ester **91** is present in such a low concentration in the case of sample B that only a weak signal is discernable.

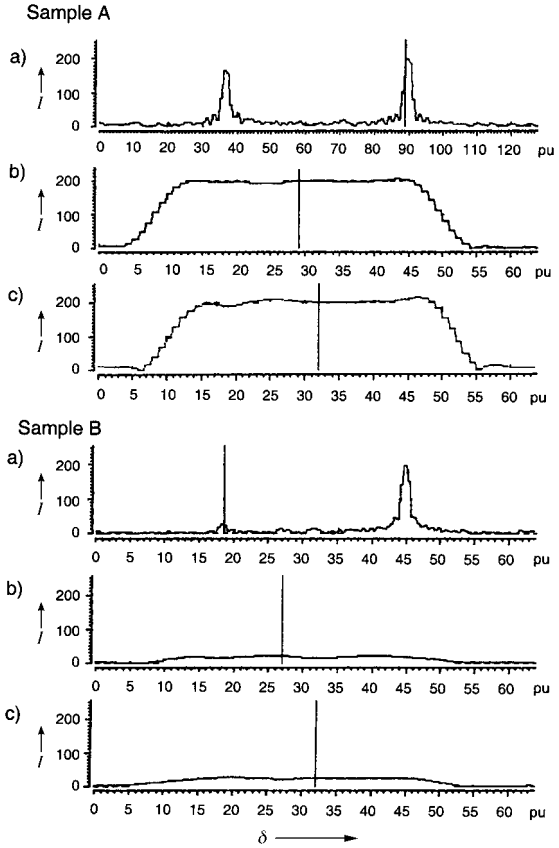


Figure 15. Chemical shift in two dimensionals using 3D-CSI.[72,94] Sample A: **91**:**93** = 1:1.4; Sample B: **91**:**93** = 1:9. In each case the chemical shift (a) as well as the signals along *x* (b) and *y* axes (c) are given; *I* = intensity; pu = pixel units.

The data of this type of 3D-CSI measurements can be presented visually in different ways. In addition to the traces shown in Figure 15, spatial positioning of the ^{19}F NMR resonance lines is possible, as shown by the images in Figure 16.[72, 94] The two discs represent the two-dimensional resolved distribution of the NMR resonance lines of sample A. Just as in an NMR tomogram these can be rotated in space. The intensities of the images correlate with the concentrations of the compounds in the samples (**93**: light disc, **91** dark disc corresponding to a 1.4:1 ratio). In a potential *ee* assay (*R*)- and (*S*)-**91** would be allowed to react separately pairwise with each catalyst on a microtiter plate.

On the basis of these experiments it should indeed be possible to image the enantiomeric purity of products in the wells of a microtiter plate by NMR tomography.[72] However, the instrumentation currently available is not yet sensitive

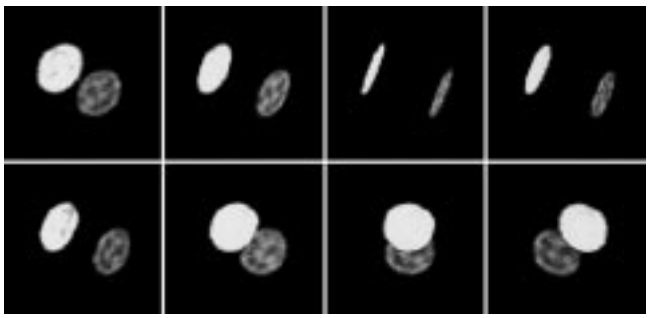


Figure 16. Different ways to present 3D-CSI measurements of sample A (**93**:**91** = 1.4:1).[72, 94]

enough for practical applications. We conclude that this method nevertheless has some potential in the determination of *ee* values (and other types of screening). Finally, van Leeuwen has shown that in some cases IR spectroscopy is useful in the evaluation of asymmetric reactions.[95]

4. Conclusion

Considerable advances in the parallel synthesis of chiral ligands for use in catalytic enantioselective reactions have been made during the last five years. At the same time strategies for exploiting the principles of combinatorial chemistry in the search for enantioselective catalysts have been proposed. It currently appears that the greatest prospects for successful applications are to be found in the study of modularly constructed chiral ligands because these offer the maximum degree of structural diversity and therefore maximize the prospect of finding enantioselective catalysts. This approach is also expected to pave one of the ways to the discovery of new types of chiral ligands for use in asymmetric catalysis, provided the experiments are devised intelligently. As one of the referees of this review has rightly pointed out, the perhaps most important message emerging from this research concerns the question: "How much have we missed over the years by *not* using diversity-based approaches to catalyst discovery and optimization?"

The systematic screening of achiral additives in appropriate systems which not only cause rate enhancement[96] but also influence the degree of enantioselectivity is also worthy of mention. Such additives are almost unlimited in number. Of particular academic and practical interest are those additives which are only formally achiral, for example, most *meso*-compounds, but which are in fact two rapidly interconverting enantiomers. These compounds form diastereomers upon interaction with the actual chiral ligand used in the catalytic transformation.[97] A variation of this theme is the covalent attachment of such compounds to the chiral ligand,[98] with combinatorial diversity again being possible.

Inspite of considerable conceptional progress, the actual goal of preparing truly large libraries of chiral ligands or catalysts has not been put into practice to date, in part this is a result of the lack of high-throughput screening systems. An exception is the application of directed evolution as a means to create enantioselective enzymes for use in organic chem-

istry.[17, 19] It is this area of endeavor which led to the development of the first high-throughput assays for enantioselectivity. Most of the more recent screening systems can also be used in the combinatorial search for asymmetric transition metal catalysts. This opens up completely new perspectives for the future, simply because now extremely large libraries can actually be screened rapidly. This means that the creative chemist can put more of his/her ideas into practice per unit of time. Hopefully, the application of genetic algorithms[99] and/or neural networks[100] can be of help in the combinatorial search for enantioselective catalysts.

Among the various approaches described for high throughput *ee*-screening systems, those based on mass spectrometry[89–91] and capillary array electrophoresis[86] currently appear to be most promising. However, no single system is expected to be universal, which means that the use and/or development of alternatives continue to be important. In the case of directed evolution of enantioselective enzymes, one such possibility is the application of phage display[101] as a selection system. Such systems would allow the evaluation of millions of mutant enzymes with respect to the enantioselectivity of a given reaction.

Whatever screening systems will be used in the future to assay enantioselective catalysts, data management needs to be considered as super-high-throughput emerges.[86] Thus, research in this general aspect of combinatorial catalysis is expected to gain in importance. Finally, the ideal screening system in any combinatorial catalyst system should include the possibility to detect and identify the unexpected result, which would provide the chemist with a means to harness the all important phenomenon of serendipity.[102]

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