CHIRALITY 12:479–482 (2000)

Circular Dichroism as a Detection Method in the Screening of Enantioselective Catalysts

MANFRED T. REETZ,* KLAUS M. KÜHLING, HEIKE HINRICHS, AND ALFRED DEEGE
Max-Planck-Institut für Kohlenforschung, Mülheim/Ruhr, Germany

ABSTRACT The combination of liquid chromatography (HPLC), UV/Vis-spectroscopy and circular dichroism (CD) can be used to construct a high-throughput screening system to determine the enantioselectivity of enzyme- or metal-catalyzed reduction of acetophenone with formation of (*S*)- and (*R*)-1-phenylethanol. Prerequisite for the viability of this system is the experimental finding that the anisotropy factor *g* is linearly related to the enantiomeric excess (ee) and that it is independent of concentration, thereby excluding possible aggregation effects. Chirality 12:479–482, 2000.

KEY WORDS: high-throughput screening; circular dichroism; enantioselectivity; combinatorial catalysis; enzyme catalysis; directed evolution

INTRODUCTION

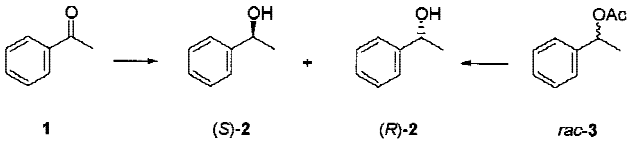
The world market for enantiopure compounds in the area of pharmaceuticals, plant-protecting agents, and fragrances is rapidly expanding.[1–3] From an economic and ecological viewpoint the most efficient way to prepare such compounds (or building blocks thereof) is enantioselective catalysis. Accordingly, the practicing organic chemist has two options, namely, the use of enantioselective transition metal catalysts[4,5] or biocatalysts.[6,7] Progress in metal catalysis is based on intensive efforts incorporating experience, intuition, molecular modeling, and a great deal of trial and error. Combinatorial methods in the development of enantioselective transition metal catalysts may turn out to speed up these efforts.[8] In the case of biocatalysis, new enzymes continue to be isolated and tested in organic synthesis, but substrate specificity imposes restrictions.[6,7] We recently described an alternative approach to the development of enantioselective catalysts which is based on the use of directed evolution of enzymes.[9,10] Accordingly, the proper combination of random mutagenesis, gene expression, and screening is applied in repeating cycles, knowledge of the structure of the enzyme not being necessary. The major challenge here and in combinatorial enantioselective homogeneous catalysis[8] is the lack of general high-throughput screening methods. Methods based on UV/Vis-spectroscopy[9–11] or fluorescence[12] are limited to relevant substrates. IR-thermography has also been applied successfully, although quantification still needs to be accomplished.[13] Recently, mass spectrometric high-throughput methods to determine the ee values of about 1,000 samples per day have been described,[14,15] although again certain limitations with respect to the type of substrates pertain.

In principle, it should be possible to use HPLC 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, enantiomeric excess (ee) then being determined by circular dichroism (CD). Indeed, this principle was first established by Mason and co-workers[16] in 1980 and developed by Salvadori et al.[17] and Mannschreck[18] on a broad basis. Recently, in an interesting development Mikami and co-workers[19] have shown that the method can be applied in the combinatorial search for enantioselective catalysts by using a JASCO-CD-995-instrument. Details concerning the number of ee determinations per day were not provided. The general method is based on the use of sensitive detectors for high performance liquid chromatography (HPLC) which determine in a parallel manner both the circular dichroism ($\Delta\varepsilon$) and the absorption (ε) of a sample at a fixed wavelength in a flow-through system. 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. Upon normalizing the CD-value with respect to absorption, the so-called anisotropy factor *g* is obtained:

$$g = \frac{\Delta\varepsilon}{\varepsilon}$$

For a mixture of enantiomers it is thus possible to determine the ee value without recourse to complicated calibration.[16–19] The fact that the method is theoretically valid

Dedicated to Prof. Ryoji Noyori, a pioneer in enantioselective catalysis.
*Correspondence to: Manfred T. Reetz, Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, D-45470 Mülheim/Ruhr, Germany.
E-mail: reetz@mpi-muelheim.mpg.de
Received for publication 8 December 1999; Accepted 31 January 2000



Scheme 1.

only if the *g* factor is independent of concentration and if it is linear with respect to ee has been emphasized several times.[16–19] However, we wish to point out that this need not be the case 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 not yet been reported, it is mandatory that this possibility is checked in each new system studied.

We had the need to develop fast and efficient ways to determine the enantiomeric purity of aryl-substituted alcohols of the type **2**, which can be produced either by reduction of the prochiral ketone **1**, e.g., using enzymes such as mutant reductases, or by kinetic resolution of *rac*-**3**, e.g., by mutant lipases. In both systems the CD approach is theoretically possible. In the former case an LC-column would have to separate any educt **1** from the product **2**, whereas in the latter case **2** would have to be separated from **3** (Scheme 1).

In this article, we concentrate on the possibility of utilizing the CD method for screening enantioselectivity in the transformation **1** → **2**.

RESULTS AND DISCUSSION

Since acetophenone **1** has a considerably higher extinction coefficient than 1-phenylethanol **2** at the same wavelength (e.g., 260 nm), separation of starting material from product was absolutely necessary. This was accomplished using a relatively short HPLC-column based on a reversed-phase system. A mixture of methanol/water (35:65) was employed as the eluant. In preliminary experiments using enantiomerically pure product **2** the maximum value of the CD-signal was determined. Mixtures of **2** having different enantiomer ratios (and therefore ee values) were prepared and analyzed precisely by gas chromatography based on a chiral phase. The same samples were studied by CD, resulting in the compilation of *g* values. Upon plotting the *g* against the ee values, a linear dependency was in fact observed with a correlation factor of $r = 0.99995$, which translates into a simple equation for enantioselectivity (Fig. 1).

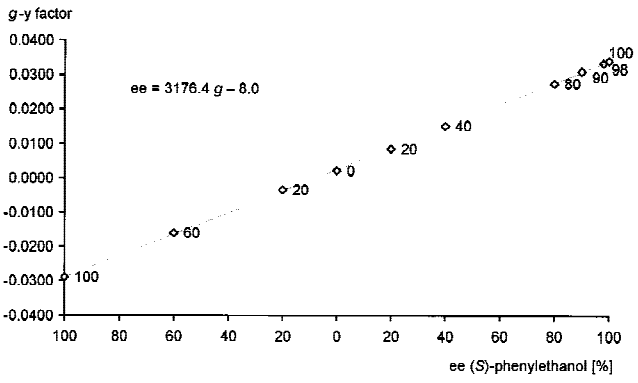


Fig. 1. Linear dependency of the *g* factor on the ee of a sample of 1-phenylethanol **2** at a concentration of 10 mg ml^{-1}.

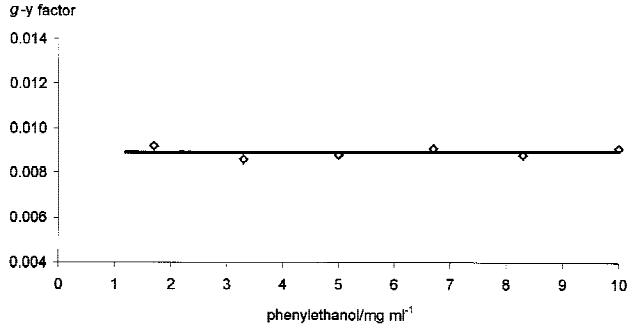


Fig. 2. Dependency of the *g* factor on concentration of a sample of 1-phenylethanol **2** enriched in the (*S*)-enantiomer (20% ee).

We then studied the possible dependency of the *g* factors on concentration. A mixture of (*S*)- and (*R*)-**2** corresponding to ee = 20% was prepared at a concentration of 10 µl ml^{-1} in acetonitrile, which was then successively diluted. Figure 2 clearly shows no dependency of *g* on concentration (standard deviation = 2.6%). Thus, possible aggregation due to hydrogen bonding between two or more molecules of the substrate (*S*)- and (*R*)-**2** in this medium which could lead to artifacts is not involved, 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 question of efficient and fast separation of **1** from **2**. Although complete optimization was not carried out at this point, an efficient separation system using reversed phase silica as the column material and methanol/water (47:53) as the eluant was developed. In view of

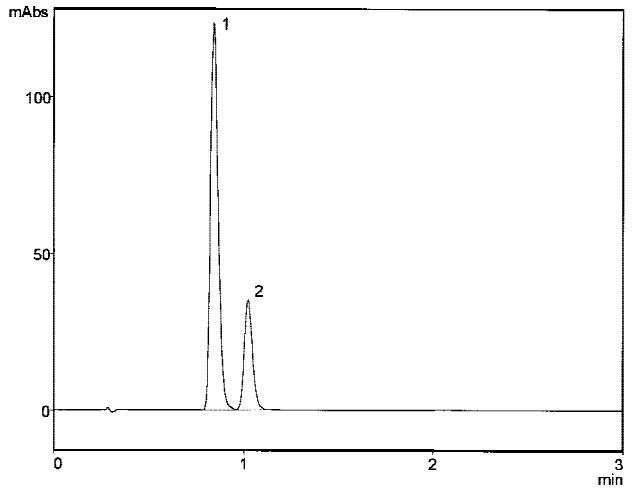
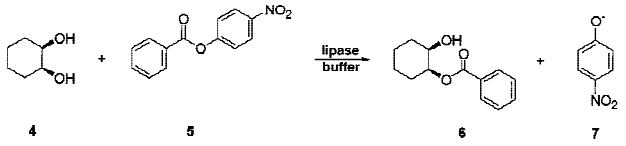


Fig. 3. Liquid chromatogram of a mixture of acetophenone **1** and racemic 1-phenylethanol **2**. The separation was performed at 308 K on a Purospher STAR RP18e-column (55 mm; 4.0 mm i.d.). The mobile phase consisted of a methanol/water mixture (47:53), the flow rate being 2 ml min^{-1}.

the results concerning the dependency of the *g* factor on concentration (see above), aggregation can be excluded in this protic medium. Figure 3 shows the corresponding HPLC-chromatogram in which the mixture is fully separated within less than 1½ min. Thus, using the JASCO-CD-1595 instrument in conjunction with a robotic autosampler, it is possible to perform about 700–900 exact ee determinations per day.

In conclusion, we have shown that reliable high-throughput screening of enantioselectivity is possible in the enzyme- (or metal-)catalyzed transformation **1** → **2**, the assay being based on the combination of liquid chromatography, UV-spectroscopy, and circular dichroism (HPLC-UV-CD). In doing so, it was important to demonstrate experimentally the linear correlation of the anisotropy factor *g* with ee, as well as the independence of the *g* factor on the concentration of the chiral product being examined.

Finally, we wish to point out the possibility that reliable ee determinations using CD are theoretically possible in certain systems even though no LC-separation is performed whatsoever. Prerequisite is a prochiral substrate (e.g., a *meso*-compound) as well as a UV-active product (chromophore) which is formed as the enantioselective reaction proceeds whose absorption maximum differs considerably from that of the desired chiral product. This new principle is illustrated by the lipase-catalyzed enantioselective acylation of the *meso*-diol **4** by benzoic acid *p*-nitrophenyl ester **5** with formation of the chiral product **6** and the yellow-colored *p*-nitrophenolate **7** having a characteristic UV/Vis-absorption at 410 nm. Upon measuring the *g* value of the absorption maximum of **6** and the additional UV-absorption of **7**, all information necessary to determine conversion and enantiopurity is available *without* the need to perform any LC-separation. The advantage of this approach has to do with higher throughput (Scheme 2).



Scheme 2.

TABLE 2. Concentration dependency of the *g*-y factors

Concentration/ µl (ml acetonitrile)$^{-1}$	*g*-y factor	Signal/noise ratio S/N
10	0.0091	17
8.3	0.0088	16
6.7	0.0091	13
5.0	0.0088	9.2
3.3	0.0086	6.1
1.7	0.0092	3.8

The signal/noise ratio was determined manually from the chromatograms.

On the basis of these and previous studies[16–19] we expect that HPLC-UV-CD or UV-CD alone will turn out to be a viable high-throughput screening system for enantioselective (bio)catalysts, provided the proper substrates are chosen and the necessary precautions are taken as described in this article.

EXPERIMENTAL

All experiments relating to CD were performed with a CD-1595 detector from Jasco Co. and a LC-10A HPLC-system from Shimadzu. In preliminary experiments concerning chiral separation of 1-phenylethanol **2**, the column Chiralcel OD-H (250 mm; 4.6 mm i.d.) was used in conjunction with *n*-heptane/2-propanol (90:10) as the eluant at 303 K, the flow rate being 0.5 ml min^{-1} at a pressure of 2.0 MPa. A sample of 5 µl of racemic **2** at a concentration of 10 µl ml^{-1} in 2-propanol was used. The separation of **1** from **2** was performed at room temperature on a Nucleosil 100-5-C_{18}-column (125 mm; 4.5 mm i.d.). The mobile phase consisted of a methanol/water mixture (35:65), the flow rate being 0.8 ml min^{-1}. Detection was set for 260 nm. Each sample required 11 min for detection.

For the linearity test (Fig. 1) various solutions of the alcohol **2** in methanol were prepared having different enantiomeric ratios. The exact ee values were first determined using chiral gas chromatography (column: IVADEX 7, 12.5 m, 0.25 mm i.d.) summarized in Table 1. The areas of the respective peaks in the HPLC-measurements were evaluated using the software Colachrom. The resulting integral areas were chosen as a measure for ε and $\Delta\varepsilon$, and

TABLE 1. Linearity test of the *g*-y factor

(*S*)-phenylethanol/ (*R*)-phenylethanol	ee (*S*)/% GC*	UV-absorption ε	CD-signal $\Delta\varepsilon$	*g*-y factor ($\Delta\varepsilon/\varepsilon$)
100:0	100	15,875,100	543,657	0.03425
99:1	98.1	15,655,181	525,604	0.03357
95:5	90.7	15,771,687	491,946	0.03119
90:10	81.1	15,623,660	432,669	0.02769
70:30	40.7	15,770,320	241,483	0.01531
60:40	19.9	15,553,147	133,366	0.00857
50:50	0.21	15,994,894	36,020	0.00225
40:60	–19.7	15,601,431	–54,208	–0.00347
20:80	–59.4	15,542,524	–249,007	–0.01602
0:100	–100	15,637,191	–452,150	–0.02892

The UV- and CD-signals are given in terms of peak areas.
*A negative sign of the ee y value means that the (*R*)-enantiomer is the major component.

from these the *g* factors were calculated. Regression analysis resulted in a correlation coefficient of $r = 0.99995$.

In order to study the possible concentration dependency (Fig. 2), a solution of the alcohol **2** was prepared in acetonitrile such that the (*S*)- to (*R*)-ratio is 60:40, corresponding to an ee value of 20% at a concentration of 10 µl ml^{-1}. This solution was diluted successively with acetonitrile and then subjected to the measurements. Table 2 lists the resulting *g* factors and the signal/noise ratios. The standard deviation for the *g* factor is 2.62%.

ACKNOWLEDGMENTS

We are grateful to Dr. A. Hütz, Jasco Deutschland GmbH, Groβ-Umstadt, for the opportunity to use the CD-1595 Circular Dichroism Detector of Jasco International Co., Ltd. Tokyo.

LITERATURE CITED

1. Collins AN, Sheldrake GN, Crosby J, editors. Chirality in industry: the commercial manufacture and applications of optically active compounds. Chichester, UK: Wiley; 1992. 409 p.
2. Collins AN, Sheldrake GN, Crosby J, editors. Chirality in industry. II. Developments in the commercial manufacture and applications of optically active compounds. Chichester, UK: Wiley; 1997. 411 p.
3. Sheldon RA. Chirotechnology: industrial synthesis of optically active compounds. New York: Dekker; 1993. 416 p.
4. Noyori R. Asymmetric catalysis in organic synthesis. New York: Wiley; 1994. 378 p.
5. Jacobsen EN, Pfaltz A, Yamamoto H, editors. Comprehensive asymmetric catalysis, vol. I-III. Berlin: Springer; 1999. 1500 p.
6. Drauz K, Waldmann H, editors. Enzyme catalysis in organic synthesis. Weinheim: VCH; 1995. 504 p.
7. Faber K. Biotransformations in organic chemistry, 3rd ed. Berlin: Springer; 1997. 402 p.
8. Jandeleit B, Schaefer DJ, Powers TS, Turner HW, Weinberg WH. Combinatorial materials science and catalysis. Angew Chem Int Ed 1999; 38:2494–2532.
9. Reetz MT, Zonta A, Schimossek K, Liebeton K, Jaeger K-E. Creation of enantioselective biocatalysts for organic chemistry by in vitro evolution. Angew Chem Int Ed Engl 1997;36:2830–2832.
10. Reetz MT, Jaeger K-E. Enantioselective enzymes for organic synthesis created by directed evolution. Chem Eur J 2000;6:407–412.
11. Janes LE, Löwendahl AC, Kazlauskas RJ. Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases. Chem Eur J 1998;4:2324–2331.
12. Klein G, Reymond J-L. Enantioselective fluorogenic assay of acetate hydrolysis for detecting lipase catalytic antibodies. Helv Chim Acta 1999;82:400–406.
13. Reetz MT, Becker MH, Kühling KM, Holzwarth A. Time-resolved IR-thermographic detection and screening of enantioselectivity in catalytic reactions. Angew Chem Int Ed 1998;37:2647–2650.
14. Reetz MT, Becker MH, Klein H-W, Stöckigt D. A method for high-throughput screening of enantioselective catalysts. Angew Chem Int Ed 1999b;38:1758–1761.
15. Guo J, Wu J, Siuzdak G, Finn MG. Measurement of enantiomeric excess by kinetic resolution and mass spectrometry. Angew Chem Int Ed 1999;38:1755–1758.
16. Drake AF, Gould JM, Mason SF. Simultaneous monitoring of light-absorption and optical activity in the liquid chromatography of chiral substances. J Chromatogr 1980;202:239–245.
17. Salvadori P, Bertucci C, Rosini C. Circular dichroism detection in HPLC. Chirality 1991;3:376–385.
18. Mannschreck A. On-line measurement of circular dichroism spectra during enantioselective liquid chromatography. Trends Anal Chem 1993;12:220–225.
19. Ding K, Ishii A, Mikami K. Super high throughput screening (SHTS) of chiral ligands and activators: asymmetric activation of chiral diolzinc catalysts by chiral nitrogen activators for the enantioselective addition of diethylzinc to aldehydes. Angew Chem Int Ed 1999;38:497–501.