Communications

High-Throughput Method for Determining the Enantioselectivity of Enzyme-Catalyzed Hydroxylations Based on Mass Spectrometry**

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Catalytic asymmetric synthesis has attracted great attention because of the importance of enantiopure compounds in the preparation of pharmaceuticals, fine chemicals, and agrochemicals.[1] Recently, the discovery of enantioselective catalysts for such syntheses has focused on the generation and screening of libraries of chemical catalysts[2] and enzymes.[3,4] While huge catalyst libraries can be quickly created by combinatorial synthesis or molecular biotechnology, such as the error-prone polymerase chain reaction (epPCR) and DNA shuffling,[5] the analysis of the enantioselectivities of these catalysts is the main bottleneck.

Many high-throughput methods for determining the enantioselectivity of catalysts have been developed.[6] While the catalyst enantioselectivity for kinetic resolutions can be quickly estimated based on the different reaction rates of the two enantiomers of the substrates, the determination of catalyst enantioselectivity for asymmetric transformations from an achiral substrate has to rely on the measurement of product *ee* values except for some reversible transformations, the back reactions of which could be studied as kinetic resolutions.[7] As the general and conventional methods for the analysis of product *ee* values by GC or HPLC with a chiral column suffer from long analysis times and other drawbacks, the quick determination of the *ee* values of some special products can be achieved by GC/GC with a chiral column,[8a] HPLC with CD/UV[8b,c] or optical rotation/refractive index unit detection,[8c] chirally modified capillary electrophoresis,[8d] electrospray ionization tandem mass spectrometry (MS),[8e] color indicators based on doped liquid crystals,[8f] or competitive enzyme immunoassays.[8g]

More complicated high-throughput methods requiring further conversion of the product have also been developed: product *ee* values can be estimated by exploiting kinetic resolution effects on the product,[9a] by using mass- or fluorescence-tagged quasienantiomeric mixtures of acylating agents with MS[9b,c] or fluorescence[9d] detection; with known product concentration, the product *ee* value can be deter-

mined by using an enzyme to catalyze a further transformation of the product detected by UV spectroscopy[10a] or IR thermography;[10b] the product *ee* value can also be established by using two enantioselective enzymes to modify the product with UV detection of NAD(P)H formation.[11] Nevertheless, the application of these relatively complicated methods in catalyst discovery depends on many factors and has to be evaluated on a case-by-case basis.

Enantioselective hydroxylations are important reactions for the preparation of chiral alcohols, which are useful and valuable pharmaceutical intermediates and fine chemicals. While chemical hydroxylations often suffer from poor chemo-, regio-, and enantioselectivity,[12] enzymatic hydroxylations have received great attention.[13] Many microorganisms have been discovered and developed for a number of useful biohydroxylations.[14] Several monooxygenases have been purified, cloned, and genetically engineered for more efficient biohydroxylations.[15] In many cases, the enantioselectivity of enzymatic hydroxylations needs to be further improved, and some success has been made by directed evolution of monooxygenases, such as P450 BM-3 hydroxylase.[16] However, none of the known high-throughput enantioselectivity assays has been applied in such evolutions. The screening of enzymes was based on activity-assay and GC or HPLC analysis with a chiral column of the selected active mutants. The lack of practical high-throughput enantioselectivity assays may limit the chance of success in discovering appropriate enzymes with desired enantioselectivity.

We are interested in developing a practical high-throughput enantioselectivity assay for biohydroxylations. MS-based assays are known to be simple and to have high throughput, such as the use of isotopically labeled quasienantiomeric or *meso* substrates[17] to determine the catalyst enantioselectivity. However, this approach cannot be extended to asymmetric transformations of a chiral non-*meso* substrate. Herein, we report a different strategy and new principle for high-throughput *ee* determination for biohydroxylations by the use of an optically active, isotopically labeled substrate with MS detection.

The principle of our method is illustrated in Scheme 1. Enantioselective biohydroxylation of ethyl benzene (**1**) to the corresponding alcohol (*S*)-**2** or (*R*)-**2** was selected as a target, since it represents a benzylic hydroxylation and the hydroxylation product is a useful synthetic intermediate. An enantioselective enzyme could catalyze the hydroxylation to give (*S*)-**2** and (*R*)-**2** in a concentration ratio of *x* [Eq (1)]. To
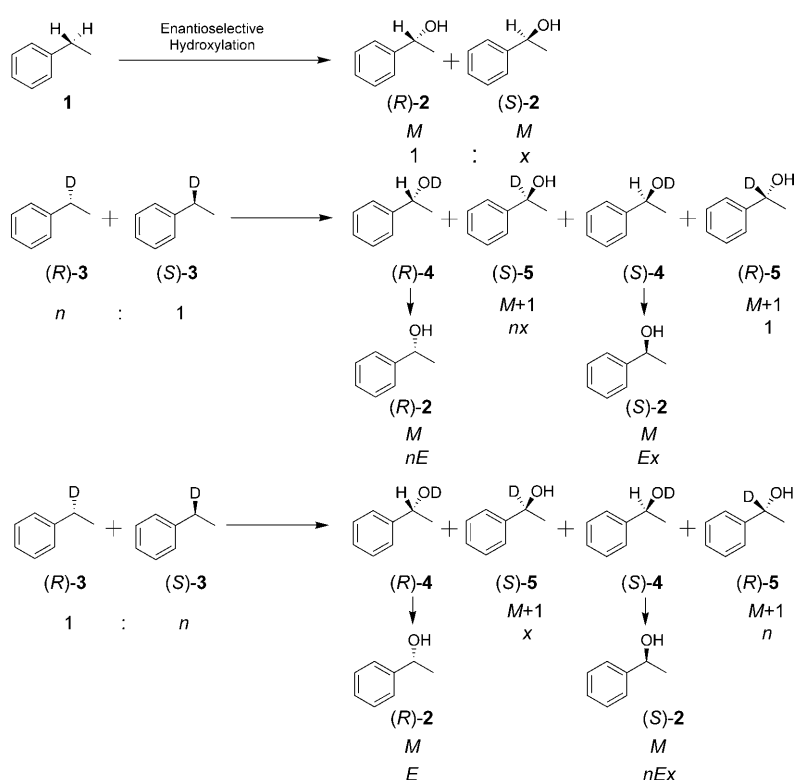
$$x = [S]/[R] \tag{1}$$

establish *x* and *ee*, enantiopure deuterated substrates (*R*)- and

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Enantioselective Hydroxylation

1 → (R)-**2** + (S)-**2**
M : M
1 : x

(R)-**3** + (S)-**3**
n : 1

(R)-**4** + (S)-**5** + (S)-**4** + (R)-**5**
M+1 M+1
nx 1

(R)-**2** (S)-**2**
M M
nE Ex

(R)-**3** + (S)-**3**
1 : n

(R)-**4** + (S)-**5** + (S)-**4** + (R)-**5**
M+1 M+1
x n

(R)-**2** (S)-**2**
M M
E nEx

Scheme 1. Principle of a high-throughput enantioselectivity assay for biohydroxylation based on the use of enantiomerically enriched deuterated substrates and MS detection. The symbols are explained in the text.

(S)-[D$_1$](1-phenylethane) (**3**) can be used to catalyze the biohydroxylation with the same enzyme, respectively. It is also possible to use enantiomerically enriched (R)-**3** and (S)-**3** as the hydroxylation substrate.

As shown in Scheme 1, biohydroxylation of (R)-**3** gives (R)-**4** and (S)-**5**, and biohydroxylation of (S)-**3** gives (S)-**4** and (R)-**5**. The ratio of (R)-**4**, (S)-**5**, (S)-**4**, and (R)-**5** in the product depends on the ratio (n) of the two enantiomers in the starting substrate **3**, enzyme enantioselectivity, and the deuterium effect (E) on hydroxylation [Eq (2); k_H and k_D are the rate constants of the hydroxylation of the C−H and C−D bonds, respectively]. A key innovation in this method is that both

$$E = k_H/k_D \tag{2}$$

(R)-**4** and (S)-**4** have an O−D bond that can be quickly exchanged to O−H in the biotransformation medium, thus giving rise to (R)-**2** and (S)-**2**, respectively. While (R)-**2** and (S)-**2** have a mass M, (R)-**5** and (S)-**5** have a mass $M + 1$. The ratio of the intensities of M and $M + 1$ peaks in the mass spectra can be determined, and corresponds to the values a in Equation (3) and b in Equation (4) for the two cases shown in

$$a = [M]/[M+1] = (nE + Ex)/(nx + 1) \tag{3}$$

$$b = [M]/[M+1] = (E + nEx)/(n + x) \tag{4}$$

Scheme 1, respectively. Based on Equations (3) and (4), the x value can be expressed by Equation (5). Since n is known,
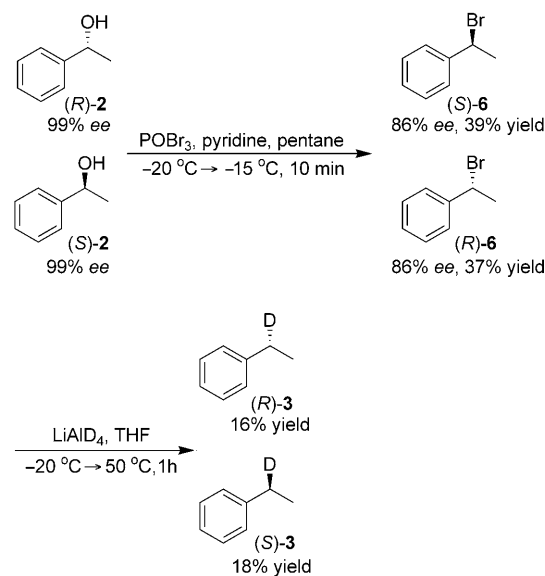
$$x = \frac{n\sqrt{b/a} - 1}{n - \sqrt{b/a}} \tag{5}$$

and a and b can be quickly determined by MS from the two separate biohydroxylations, the x value can be easily calculated. Finally, the *ee* value of bioproduct **2** can be established by using Equation (6).

$$ee = \frac{[R] - [S]}{[R] + [S]} 100\% = \frac{1 - x}{1 + x} 100\% \tag{6}$$

To prove the concept, the deuterated substrates (R)-**3** and (S)-**3** were prepared according to the route shown in Scheme 2, starting with commercially available enantiopure (R)-**2** and (S)-**2**.[18] Although the *ee* values of (R)-**3** and (S)-**3** cannot be directly determined, we assume that (R)-**3** and (S)-**3** have the same *ee* value as (S)-**6** and (R)-**6** (86%), as the configuration cannot be changed during deuteration.

The synthesized enantioenriched (R)-**3** and (S)-**3** were used for biohydroxylation, and our recently discovered strain *Pseudomonas monteilii* TA-5[19] was chosen as the biocatalyst. The biohydroxylations were performed with resting cells (10 g cdw L^{-1}; cdw = cell dry weight) of the TA-5 strain and 10 mM substrate in 100 mM potassium phosphate buffer (pH 7.0) at 30 °C

(R)-**2** 99% ee
(S)-**2** 99% ee

POBr$_3$, pyridine, pentane
−20 °C → −15 °C, 10 min

(S)-**6** 86% ee, 39% yield
(R)-**6** 86% ee, 37% yield

LiAlD$_4$, THF
−20 °C → 50 °C, 1h

(R)-**3** 16% yield
(S)-**3** 18% yield

Scheme 2. Synthesis of deuterated (R)-**3** and (S)-**3**.

for 15 minutes. The products were extracted with ethyl acetate and analyzed by GC/MS. Typical spectra are shown in Figure 1, and a and b values were easily obtained from the intensities of the signals at *m/z* 122 (M) and 123 ($M + 1$). The *ee* value was calculated from Equations (5) and (6) as 82% (R; Table 1, entry 1).

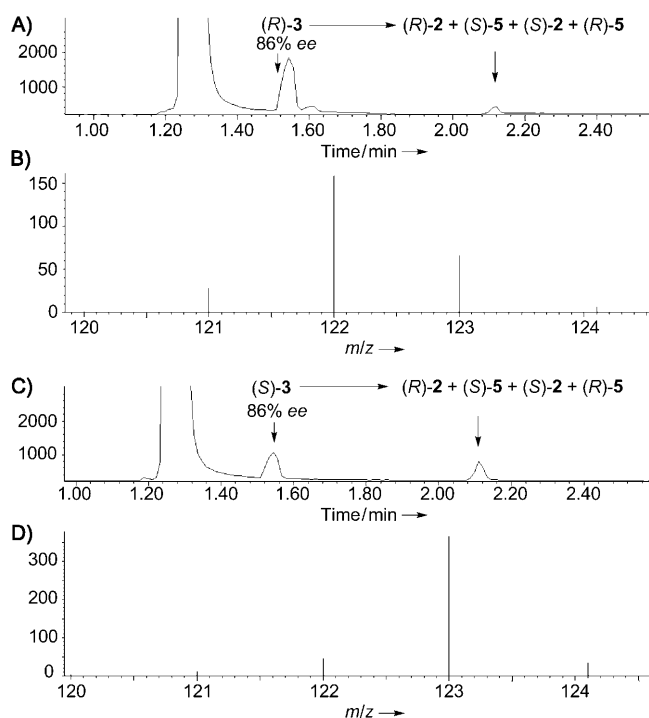**Figure 1.** GC/MS analysis of the products from biohydroxylation of (R)-**3** and (S)-**3** with *P. monteilii* TA-5. A,C) Gas chromatograms; B,D) mass spectra of the product peak at 2.12 min in GC.

Table 1: Product *ee* values for the biohydroxylation of **1** to **2** with *P. monteilii* TA-5 established by GC/MS-based assay using (R)-**3** and (S)-**3** as substrates.

Entry	Product conc.[a] [mM]	n	a	b	x	ee[b] [%]	ee[c] [%]
1	0.170	13.3	3.050	0.093	0.102	82 (R)	83 (R)
2	0.085	13.3	3.972	0.124	0.103	81 (R)	–
3	0.034	13.3	2.913	0.120	0.130	77 (R)	–

[a] Entry 1: original concentration; entries 2 and 3: diluted samples. [b] Determined and calculated based on GC/MS analysis using the new method. [c] Determined by chiral HPLC analysis of the product from biohydroxylation of **1** with *P. monteilii* TA-5, using a concentrated sample.

For comparison, the hydroxylation of ethyl benzene (**1**; 10 mM) was carried out with *P. monteilii* TA-5 under the same conditions for 15 minutes, and the product was extracted with ethyl acetate. The product was further concentrated for chiral HPLC analysis, which gave an *ee* value of 83% (R). Thus, our new method gave an accurate *ee* value, with an error of only about 1% *ee*. The high sensitivity of the method was also demonstrated: analysis of diluted samples with product concentrations of 0.085 and 0.034 mM gave product *ee* values of 81% (R) and 77% (R), respectively, with only a slight difference from the real value. The GC/MS analysis of each sample took about 2.2 minutes at 160°C (Figure 1). The analysis time was further reduced to 1.5 minutes at 180°C, with the same analytical accuracy. Theoretically, 960 samples could be determined per day.

To further demonstrate the generality of this method, enantioselective biohydroxylation of *N*-benzylpyrrolidine (**7**)

at the 3-position was selected as another example. This represents an enantioselective hydroxylation of a nonactivated carbon atom, a symmetric substrate with equal C–H bonds at the 3- and 4-positions, and a useful transformation to prepare the corresponding product (S)-**8** or (R)-**8**, which is a key synthetic intermediate for several pharmaceuticals.[20]

The principle of the method is illustrated in Scheme 3. An enantioselective enzyme could catalyze the hydroxylation of **7** to give (S)-**8** and (R)-**8** in a concentration ratio *y* [Eq (7)]. To establish the *y* value and thus the product *ee* value, a simplified approach is applied: instead of using an enantio-

$$y = [S]/[R] \qquad (7)$$
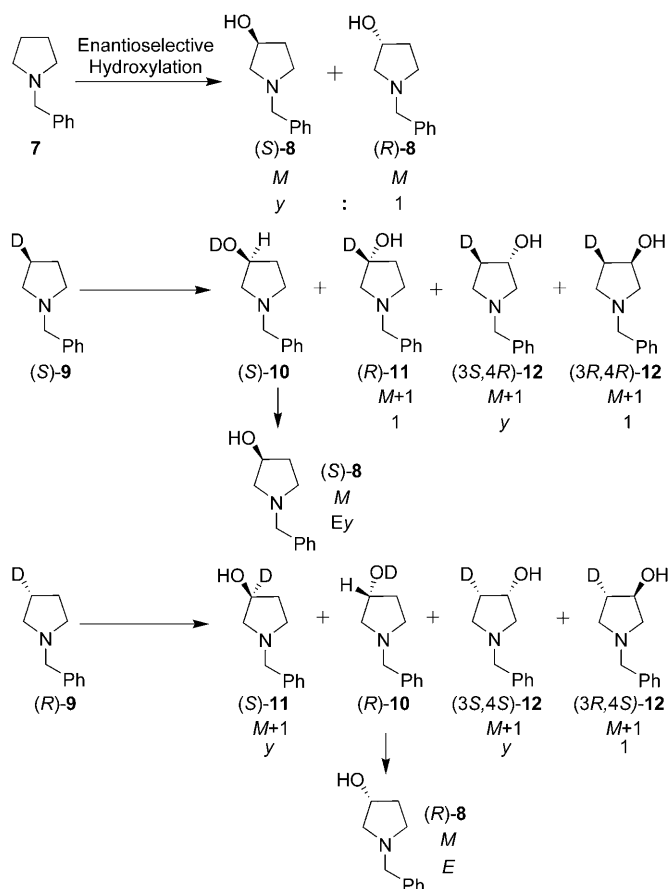
Scheme 3. Principle of a high-throughput enantioselectivity assay for biohydroxylation of a symmetric substrate based on the use of enantiopure deuterated substrates and MS detection.

merically enriched deuterated substrate, enantiopure [3-D](R)- and -(S)-1-benzylpyrrolidine (**9**) are used as substrates for the biohydroxylation with the same enzyme. Biohydroxylation of (S)-**9** gives (S)-**10**, (R)-**11**, (3S,4R)-**12**, and (3R,4R)-**12**, whereas biohydroxylation of (R)-**9** gives (S)-**11**, (R)-**10**, (3S,4S)-**12**, and (3R,4S)-**12**. The ratio of these compounds in the products depends on the enzyme enantioselectivity and

the deuterium effect (E) on hydroxylation [Eq (8)]. Compounds (S)-**10** and (R)-**10** have an O–D bond that is quickly

$$E = k_H/k_D \tag{8}$$

pounds (S)-**10** and (R)-**10** have an O–D bond that is quickly exchanged to O–H in the biotransformation medium, thus giving rise to (S)-**8** and (R)-**8**, respectively, with a mass M. All other products have a mass of $M+1$. The ratio of the intensities of M and $M+1$ peaks in the mass spectra can be determined, which corresponds to the a value in Equation (9)

$$a = [M]/[M+1] = Ey/(y+2) \tag{9}$$

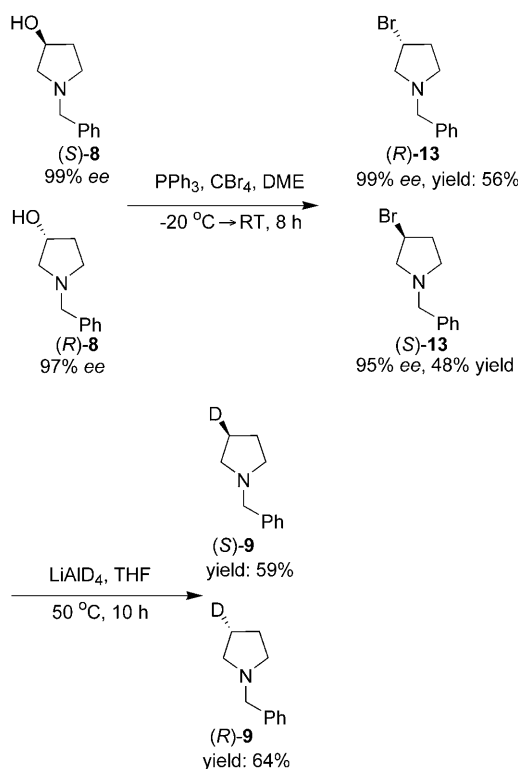and the b value in Equation (10) for the two cases shown in

$$b = [M]/[M+1] = E/(2y+1) \tag{10}$$

Scheme 3, respectively. Based on Equations (11) and (12), the y and ee values can be established.

$$y = \frac{-(b-a) + \sqrt{(b-a)^2 + 16ab}}{4b} \tag{11}$$

$$ee = \frac{[R]-[S]}{[R]+[S]}100\% = \frac{1-y}{1+y}100\% \tag{12}$$

To prove the concept, (S)-**9** and (R)-**9** were prepared for the first time from the commercially available enantiopure (S)-**8** and (R)-**8** according to the routes shown in Scheme 4. The ee value of **9** is expected to be the same as that of the

corresponding precursor **13**: (S)-**9** in 99% ee and (R)-**9** in 95% ee.

To evaluate the new method, (S)-**9** and (R)-**9** were used for the hydroxylation, and the potential of using LC/MS for the analysis was explored. At the beginning, *Sphingomonas* sp. HXN-200, a known biocatalyst for the hydroxylation of non-activated carbon atoms,[20] was selected as the catalyst. Biohydroxylation was performed with 5 mM (R)-**9** or (S)-**9** in a cell suspension (5.5 g cdw L^{-1}) in 50 mM potassium phosphate buffer (5 mL, pH 7.5) containing glucose 2% (w/v) at 30°C for 1 h. Samples were taken directly from the aqueous biotransformation mixtures and analyzed by LC/MS; typical chromatograms are shown in Figure 2. No column separation
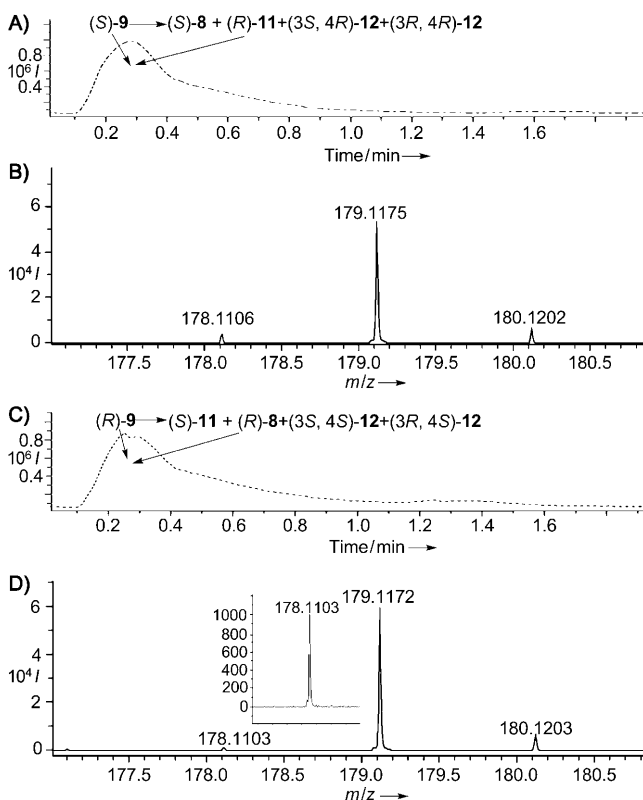

Figure 2. LC/MS analysis of the products from biohydroxylation of (S)-**9** and (R)-**9** with *Sphingomonas* sp. HXN-200. A,C) Liquid chromatograms; B,D) mass spectra of the peak between 0.2 and 0.3 min in LC.

Scheme 4. Synthesis of [3-D](R)- and -(S)-1-benzylpyrrolidine (**9**). DME = dimethyl ether.

was required, and the a and b values were easily obtained from the intensities of the signals at m/z 178 (M) and 179 ($M+1$). Thus, the y and ee values were calculated based on Equations (11) and (12).

As summarized in Table 2, entry 1, the product ee value for biohydroxylation of **7** with *Sphingomonas* sp. HXN-200 was estimated as 53% (S) from this method. The real product ee value for the biohydroxylation of **7** with this catalyst under the same conditions was established as 54% (S) by chiral HPLC analysis. This result demonstrates the high accuracy of the new method. Moreover, the use of LC/MS analysis does not require the extraction of product into organic solvent, thus allowing the direct use of aqueous samples taken from

Table 2: Product *ee* values for the biohydroxylation of **7** to **8** with different biocatalysts established by LC/MS-based assay using (*R*)-**9** and (*S*)-**9** as substrates.

Entry	Biocatalyst[a]	*t* [h]	Product conc.[b] [mM]	*a*	*b*	*γ*	*ee*[c] [%]	*ee*[d] [%]
1	*Sphingomonas* sp. HXN-200	1.0	0.023	0.080	0.017	3.25	53 (*S*)	54 (*S*)
2	*E. coli* BL21(DE3) 1AF4	0.5	0.005	0.049	0.179	0.372	46 (*R*)	42 (*R*)
3	*P. oleovorans* GPo1	12	0.009	0.014	0.061	0.324	51 (*R*)	57 (*R*)

[a] Enantioselective hydroxylations of 2–5 mM (*R*)-**9** and (*S*)-**9** were performed in cell suspensions (5 mL, 5–30 g cdw L^{-1}) of different biocatalysts at 30°C and 300 rpm. [b] Concentration refers to the analytical sample with 20 times dilution of the original aqueous sample. [c] Determined and calculated based on LC/MS analysis using the new method. [d] Determined by chiral HPLC analysis of the product from biohydroxylation of **7** with different biocatalysts under the same biotransformation conditions and using a concentrated sample.

the biotransformation mixtures. It also provides high sensitivity, with no problem of using a 20 times diluted sample containing 0.023 mM product. In comparison, chiral HPLC analysis required the extraction of the product from aqueous medium (5 mL) with ethyl acetate (5 mL) followed by concentration of the sample by evaporation to 30 μL. In fact, chiral HPLC analysis failed with a nonconcentrated sample because of the sensitivity limitation. In addition, LC/MS analysis took only 1.0 minute, which provided a theoretical analytical throughput of 1440 samples per day. In comparison, chiral HPLC analysis required about 30 minutes for each sample.

The method was further examined with other available biocatalysts for this hydroxylation. Recombinant *Escherichia coli* BL21 expressing the 1AF4 mutant of the P450 monooxygenase of *Sphingomonas* sp. HXN-200 catalyzed the biohydroxylation of **7** to give the corresponding (*R*)-**8** in 42% *ee* (Table 2, entry 2). This is the opposite enantioselectivity to that obtained with *Sphingomonas* sp. HXN-200. To obtain the product *ee* value by our new method, biotransformation of (*S*)-**9** and (*R*)-**9** with BL21 (DE3) 1AF4 was performed under the same conditions, and samples from aqueous buffer were analyzed by LC/MS without column separation. As shown in Table 2, entry 2, the product *ee* value was established as 46% (*R*) by this method, which has an error of only 4% *ee*. Also in this case, the *ee* determination is sensitive, with a product concentration of 0.005 mM, and fast, with an analysis time of 1.0 minute.

P. oleovorans GPo1 contains a well-known membrane-bound alkB hydroxylase system and catalyzes the hydroxylation of a number of aliphatic compounds.[21] Biohydroxylation of **7** with this strain gave (*R*)-**8** in 57% *ee* (Table 2, entry 3). Similarly, the *ee* value was also established by using the new method through the separate biotransformation of (*S*)-**9** and (*R*)-**9** with *P. oleovorans* GPo1 and LC/MS analysis of the aqueous samples. As shown in Table 2, entry 3, the product *ee* value was established as 51% (*R*) by this method with an error of 6% *ee*. Once again, the analysis is sensitive and fast, being able to determine samples containing 0.009 mM product within 1.0 minute.

In summary, we have developed a new method for measuring the product *ee* value for enantioselective hydroxylation based on the use of enantiopure or -enriched deuterated substrates and MS detection. Our method has

several distinctive features. 1) The *ee* value can be determined with satisfactory accuracy, independent of the type of hydroxylation and the nature of the biocatalyst. 2) The analysis method is very sensitive. It can analyze samples with product concentration as low as 0.005 mM, thus being suitable for the screening of enantioselective enzymes for biohydroxylation, for which the product concentration is often low. 3) The analysis is based on MS and does not require separation. It takes only 1.0 minute for LC/MS analysis, thus providing high-throughput analysis of 1440 samples per day. 4) The analysis method is simple. It allows direct analysis of aqueous samples from biotransformation and does not require further reactions of the bioproduct, which is often necessary for many other assays. 5) The deuterated substrate can be easily prepared from the corresponding alcohol, and does not need to be enantiopure. Currently we are applying this method to the discovery of enantioselective enzymes for biohydroxylations through direct evolution of monooxygenases.

Experimental Section

General procedure for determining the *ee* value of bioproduct **2** from biohydroxylation of **1**: In two parallel experiments, enantiomerically enriched (*R*)-**3** and (*S*)-**3** were added to a cell suspension (10 g cdw L^{-1}) of *P. monteilii* TA-5 in 100 mM potassium phosphate buffer (1 mL, pH 7.0) to a substrate concentration of 10 mM. The mixture was shaken at 1000 rpm at 30°C for 15 min. The cells were removed by centrifugation, and the aqueous solution (0.4 mL) was mixed with ethyl acetate (0.4 mL). The organic phase was separated and analyzed by GC/MS. The ratio of the signals at *M* and *M* + 1 in the mass spectra was determined as 3.050 for *a* and 0.093 for *b*, respectively, and the values were used to calculate the *ee* value as 82% (*R*) for product **2** from the biohydroxylation of **1** with the same enzyme. Upon comparison of the product *ee* value, 83% (*R*), from the real biohydroxylation of **1** with the same enzyme and determined by chiral HPLC analysis, the GC/MS-based method gave an accurate *ee* value with an error of 1% *ee*. The analysis took 2.2 min for samples with a product concentration from 0.034 to 0.170 mM.

General procedure for determining the *ee* value of bioproduct **8** from biohydroxylation of **7**: In two parallel experiments, (*S*)-**9** or (*R*)-**9** was added, to a final concentration of 5.0 mM, to a suspension of cells of *Sphingomonas* sp. HXN-200 (5.5 g cdw L^{-1}) in 50 mM potassium phosphate buffer (5 mL, pH 7.5) containing 2% (w/v) glucose. The mixture was shaken at 300 rpm at 30°C for 1 h, and the cells were removed by centrifugation. The supernatant (50 μL) was diluted 20 times with methanol and used as the sample for LC/MS analysis. From the mass spectra, *a* and *b* values were obtained as 0.080 and 0.017, respectively, thus establishing the *ee* value of product **8** as 53% (*S*) from the biohydroxylation of **7** with *Sphingomonas* sp. HXN-200. As biohydroxylation of **7** with the same strain gave product **8** in 54% *ee* (*S*) determined by chiral HPLC analysis, the LC/MS based method is accurate, with an error of only 1%. The analysis took 1.0 min for samples with a product concentration of 0.023 mM.

Keywords: enantioselectivity · enzyme catalysis · high-throughput screening · hydroxylation · mass spectrometry

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