Enantiodivergent Synthesis of Halofuginone by Candida antarctica Lipase B (CAL-B)-Catalyzed Kinetic Resolution in Cyclopentyl Methyl Ether (CPME)

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Abstract The synthesis of both enantiomers of a key intermediate in the synthesis of halofuginone was accomplished by a Candida antarctica lipase B (CAL-B)-catalyzed kinetic resolution of the corresponding racemate. When the resolution was carried out in the versatile solvent cyclopentyl methyl ether (CPME) using p-chlorophenylbutyrate (PCPB) as the acylating reagent, the highest enantiomeric ratio (E) values were measured, and highly enantiomERICally enriched (95% ee) compounds could be obtained in a single iteration. As an example, one of the two enantiomers was used as a starting material to prepare (+)-halofuginone in a three-step procedure.

Key words biocatalysis, lipase, kinetic resolution, halofuginone

Febrifugine (1, Figure 1) is a naturally occurring compound first isolated from the leaves of the Asian medicinal plant Dichroa febrifuga, traditionally used to reduce fever from malaria infection.1 However, translation of febrifugine into a medicine failed because of serious side effects such as nausea, vomiting, and liver damage.2 For this reason analogues were synthesized to improve the activity/toxicity profile. Amongst these, halofuginone (2, Figure 1), in which the metabolically vulnerable quinazoline protons in 1 were replaced by chlorine and bromine atoms, besides demonstrating effective antiprotozoal activity against several species of Eimeria in poultry,3 proved, in its racemic form, to possess a broad spectrum of pharmacological activities such as antifibrinosis,4 antitumor,5 and growth-promoting functions.6 As reports on the biological activity of the isolated enantiomers of 2 are rather limited,7 in the recent decades many efforts have been devoted to the enantioselective synthesis of halofuginone, and febrifugine as well, with the N-protected 3-hydroxypiperidine derivatives 3 (Figure 1), possessing (2R,3S) absolute configuration, being the key target intermediates.8–10

More recently, strategies explored for the enantioselective synthesis of 3a (R = Cbz) were based on the use of starting materials from the chiral pool such as d-arabinose11 and the installation of new stereocenters by organocatalysis.12,13 Despite the tremendous efficacy of enzymes in providing enantiopure compounds, to our knowledge only Evans has reported on the enantiodivergent asymmetric synthesis of febrifugine, halofuginone, and their hemiketals based on kinetic resolution (KR) of a linear alcohol 4, Scheme 1, a catalyzed by Candida antarctica lipase B.14 However, this approach required two iterations to provide one of the enantiomers (alcohol (+)-4) with high optical purity (98% ee) but in low (27%) overall yield. Encouraged by our recent results in the lipase-catalyzed KR of 3-hydroxypiperidine derivatives,15a the report by Yoshimura et al. on the efficient lipase-catalyzed KR of 3-hydroxypipelicolic acid derivative 6
(Scheme 1, b), structurally similar to compound 3, and in continuation of our studies on the enantioselective synthesis of (poly)hydroxypiperidine alkaloids by enzyme and transition-metal catalysis, we decided to establish a practical synthetic protocol for the synthesis of both enantiomers of 3a with high optical purity by lipase-catalyzed kinetic resolution (EKR) of the corresponding racemate (Scheme 1, c). Moreover, looking at a possible industrial application of our approach to 3a, we decided to study this enzymatic process in both green and nonconventional media, in particular focusing on cyclopentyl methyl ether (CPME). This is a versatile solvent, suitable for applications in biotechnology and biorefineries due to its properties such as low peroxide formation rate, stability under basic and acidic conditions, relatively high boiling point, and low water solubility and water content.17 To the same end, for the synthesis of racemic 3a, we also slightly modified the shortest known route from a commercial achiral precursor in order to reduce the number of chromatographic purifications. In this way we could establish an efficient protocol of possible industrial interest, useful for rapid preparation of both enantiomers of halofuginone for comparative bioassays.

Previous literature reports on EKR and the approach reported herein

![Scheme 1](https://example.com/scheme1)

This work

Thus, commercially available, N-Cbz-protected 1,2,3,4-tetrahydropyridine 8 (Scheme 2) was treated with Oxone® (2 equiv) in acetone/water and in the presence of K2CO3 (2 equiv) to obtain hemiaminal 9 (96% yield) that, as the crude product, was subjected to Horner–Wadsworth–Emmons (HWE) olefination by reaction with phosphonate to give alcohol 11 with E geometry. Treatment of crude 11 with BFs2O in anhydrous acetonitrile eventually provided racemic 3a contaminated with furan derivative 12 (15% by 1H NMR analysis). The advantage of the HWE over the Wittig olefination of the same compound is that only one chromatography of crude 3a on silica gel is necessary to separate this product from both 12 and residual unreacted phosphonate. In this way, pure (+)-3a could be obtained in 50% yield over the three steps and the synthesis could be rapidly carried out, usually on a 4 mmol scale, whenever 3a was needed.

With racemic 3a in hand, we studied its enzymatic kinetic resolution (EKR) via lipase-catalyzed esterification in solvents with low water content, focusing on the use of the commercially available immobilized enzyme, iCALB (Candida antarctica lipase B immobilized on acrylic resin, Novozym® 435). This choice stemmed from the fact that we had successfully previously used iCALB for the kinetic resolution of hydroxylated piperidine derivatives. This enzyme was used as provided by the supplier without any treatment prior use but we measured its esterification activity of 1-octanol in CPME using vinyl acetate as acylating reagent at 30 °C. At a substrate/enzyme ratio of 1:10 (0.19 mmol of substrate (S) and 1.9 mg of immobilized enzyme (Enz)), substrate concentration 0.75 M, and 3.5 equiv of vinyl acetate, the conversion into product after 5 min was 49%, corresponding to an activity value of 9.8 × 10^-6 mol(S)/[min × mg(Enz)]. The results of this study are reported in Tables 1 and 2.

Starting with vinyl butyrate as the acylating agent we then screened different solvents (Table 1), such as THF (logP 0.46), to compare with the standard solvents we had used in our previous studies. On the basis of very recent literature on the use of Deep Eutectic Solvents (DES) as media for biocatalyzed transformations, we also employed two DES obtained by mixing choline chloride with urea and glycerol, respectively. The reactions were monitored by GLC and stopped when the conversion was in the 40–50% range. The first experiments (Table 1, entries 1–3) were carried out with solvents dried by distillation over Na/benzophenone. In all cases, the iCALB lipase preferentially catalyzed esterification of the (2S,3R) enantiomer as determined by the
sign of the optical rotation values (for 13a after hydrolysis), with comparable E (enantiomeric ratio) values; although in CPME (and to a lesser extent in 2-MeTHF) the reaction was much faster (7 h). As interfacial activation is not known for this lipase, the higher activity in the more lipophilic CPME could be related to the increased hydrophobicity of the hydration layer, which facilitates the access of the substrate to the enzyme surface, and is in line with the increased activity (and selectivity) observed in this solvent in other CALB-catalyzed resolution processes. As the reaction was faster in CPME with a high E value, we continued our study with this solvent because of its specific properties as mentioned earlier.

![Diagram](image_url)

Table 1  
ICALB-Catalyzed Kinetic Resolution of (+)-3a in Different Solvents

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
<th>t (h)</th>
<th>c (%)</th>
<th>(+)-13a ee (%)</th>
<th>(–)-3a ee (%)</th>
<th>E²</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>THF</td>
<td>25</td>
<td>45</td>
<td>94</td>
<td>88</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>2-MeTHF</td>
<td>7.5</td>
<td>39</td>
<td>97</td>
<td>64</td>
<td>127</td>
</tr>
<tr>
<td>3</td>
<td>CPME</td>
<td>7</td>
<td>46</td>
<td>94</td>
<td>84</td>
<td>86</td>
</tr>
<tr>
<td>4</td>
<td>CPME</td>
<td>8</td>
<td>48</td>
<td>92</td>
<td>87</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>CPME</td>
<td>8.5</td>
<td>37</td>
<td>95</td>
<td>68</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>CHCl/urea (1:2)</td>
<td>118</td>
<td>16</td>
<td>93</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>CHCl/urea (1:2) + CPME (10%)</td>
<td>120</td>
<td>21</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>CHCl/glycerol (1:2)</td>
<td>22</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

- Reaction carried out on 0.2 mmol of substrate at 30 °C; substrate concentration: 0.76 M; enzyme (mg)/substrate (mmol) ratio: 100 mg mmol⁻¹; 3.5 equiv of acylating agent.
- Reaction monitored by GLC; conversion determined by ¹H NMR spectroscopy.
- Determined by HPLC analysis on a HPLC Lux® 5 μm Amynose-1 (250 × 4.6 mm) column; for (+)-13a after hydrolysis to alcohol (–)-3a.
- E was calculated by using the formula E = ln[(1 – eeS)/(1 + eeS/eeP)]/ln [(1 + eeS/eeP)] as reported in ref. 26.
- Distilled over Na/benzophenone.
- Water content: 255 ppm.
- Commercial material; water content: 1330 ppm.
- Dried over 4 Å MS and reaction was carried out in the presence of 4 Å MS (130 mg mmol⁻¹).
- Carried out at 30 °C.
- Carried out at a c = 0.25 M.

Disappointing results were obtained in the two DES we investigated (entries 6–8), in which reaction rates were unacceptably low despite several attempts at changing concentration, stirring rate, temperature, quantity of acylating agent, using crushed lipase or adding a co-solvent (data not shown). On the other hand, successful iCALB-catalyzed transesterification was observed in the two DES we investigated using a large excess of alcohol to convert vinyl laurate.

These negative results are consistent with those obtained by Petrenz et al. who attempted a lipase-catalyzed KR of benzoin in DES with vinyl butyrate and found a much lower activity (10% conversion after 48 h) than in CPME and 2-MeTHF, most likely due to mass-transfer limitations as a result of high DES viscosity.

We evaluated CPME as provided by the supplier, too (entry 4), observing just a slight decrease of the E value (68) but not of the reaction rate. We measured the content of water in CPME before (1330 ppm) and after distillation (255 ppm) over Na/benzophenone by Karl Fischer titration. Our results show that the water content in commercial CPME is not sufficiently high to affect the reaction rate, for example, by competing with the substrate, at least under our conditions, but has instead a small influence on the enantioselectivity. The reaction rate was instead slightly lower when carried out in the presence of 4 Å molecular sieves (MS) and in CPME dried over 4 Å MS for two days (entry 5).

The next experiments were carried out in CPME distilled over Na/benzophenone, changing the acylating agent, in the attempt to further improve the E value (Table 2, entries 1–7). In all cases, the reaction was slower than with vinyl butyrate (Table 2, entry 1) but we were very satisfied to observe that, with PCPB as the acylating agent, the E value was very high (290) and the reaction was still reasonably fast (24 h to reach 46% conversion). In general, the reaction was much faster when carried out with vinyl butyrate (Table 2, entries 2, 6, 7) than with esters such as TFEB and TCEA (Table 2, entries 2 and 5). Reducing the amount of enzyme to a quarter (25 mg per mmol of substrate) the reaction rate was, as expected, much lower (Table 2, entries 8 and 9), without any significant effect on the enantiomeric ratio E. When reducing the amount of acylating agent, while we did not observe any variation in the reaction rate with vinyl butyrate (cf. Table 2, entries 9 and 11), the reaction did slow down significantly when we used PCPB (cf. Table 2, entries 3 and 10). Despite this, we opted for the latter conditions for a larger scale KR of (±)-3a (and the subsequent synthesis of enantiopure halofuginone) to avoid using a relatively large excess of the acylating agent, the residual amounts of which can nevertheless be separated by chromatography and recycled.

For the synthesis of (+)-halofuginone (Scheme 3) we first resolved 2 mmol of (±)-3a using iCALB under the conditions of Table 2, entry 10. Given the high enantioselectivity of the enzyme, only one iteration was necessary to obtain in good yield highly enantioenriched compounds (–)-3a (45%, 95% ee) and (+)-13a (44%). Hydrolysis of (+)-13a, carried out in the presence of MeONa in anhydrous methanol, then furnished alcohol (+)-3a (95% ee) in 89% yield. The next steps of the synthesis were carried out as reported by α-bromination of (–)-3a with N-bromosuccinimide.
When the resolution was carried out in the CPME solvent, using p-chlorophenylbutyrate (PCPB) as the acylating reagent, the highest enantiomeric ratio (E) values were measured, providing almost enantiopure (95% ee) compounds in a single iteration of the kinetic resolution. Since the racemic material can be quickly obtained in three steps from a commercially available piperidine derivative, with only one chromatographic purification needed, our approach is an efficient and sustainable synthesis of halofuginone for further investigating the biological properties of both enantiomers of this biologically active compound. As an illustration, one of the two enantiomers was converted into enantiopure (+)-halofuginone according to a literature procedure.

Anhydrous solvents were prepared according to the standard techniques. Commercially available reagents were used without further purification. Chromatographic separations were performed under pressure on silica gel 60 (Merck, 70–230 mesh) by using flash column techniques; Rf values refer to TLC carried out on 0.25 mm silica gel plates (F254) with the same eluent indicated for column chromatography. 1H NMR (400 and 200 MHz) and 13C NMR (100.4 MHz) spectra were recorded on Varian Inova (400 MHz) and Mercury (400 and 200 MHz) spectrometers in the specified deuterated solvent at 25 °C. 1H and 13C NMR spectra, respectively. Mass spectrometric analyses were carried out by direct inlet of a 10 ppm solution in MeOH into an LCQ Fleet Ion Trap LC–MS system (Thermo Fisher Scientific) with an electrospray ionization (ESI) interface in the positive ion mode. HPLC analyses were carried out by a Dionex Ultimate 3000 instrument, using a Lux 5 μm Amyleose-1 (250 × 4.6 mm) column; substrate concentrations: 0.76 M; enzyme (mg)/substrate (mmol) ratio: 100 mg mmol–1; reaction carried out at 30 °C; Rf values refer to TLC carried out on 0.25 mm silica gel plates (F254) with the same eluent indicated for column chromatography. 1H NMR (400 and 200 MHz) and 13C NMR (100.4 MHz) spectra were recorded on Varian Inova (400 MHz) and Mercury (400 and 200 MHz) spectrometers in the specified deuterated solvent at 25 °C. 1H NMR (600 MHz) and 13C NMR (150 MHz) spectra were recorded on a Jeol ECZR600 spectrometer. Solvent references were set at δ = 7.26 and 77.0 ppm (CDCl3), 2.50 and 39.5 ppm (DMSO-d6) in the 1H NMR and 13C NMR spectra, respectively. Mass spectrometric analyses were carried out by direct inlet of a 10 ppm solution in MeOH into an LCQ Fleet Ion Trap LC–MS system (Thermo Fisher Scientific) with an electrospray ionization (ESI) interface in the positive ion mode. HPLC analyses were carried out by a Dionex Ultimate 3000 instrument, using a Lux 5 μm Amyleose-1 (250 × 4.6 mm) column and eluting at 0.5 mL min–1 flow rate in isocratic 30% IPA, 70% hexane. Signals were monitored at λ = 223 nm with a UV detector. Compound 8 is commercially available or can be prepared as reported.18 Compounds (+)-2HBr,14 (±)-3a,20 (–)-3a,14 (+)-3a,9 10 11 12 14 and (–)-1514 are known. Candida antarctica lipase B immobilized on acrylic resin (Novozym® 435) was purchased from Strem Chemicals, Inc.
(2-Oxopropyl)phosphonic Acid Diethyl Ester (10)\(^9\)

Chloroacetic acid (2 mL, 25 mmol) was added to a suspension of KI (4.6 g, 27.5 mmol) in anhydrous MeCN (10 mL) and anhydrous acetone (10 mL), and the reaction was stirred at room temperature for 1 h. Then triethylphosphite (4.34 mL, 25 mmol) was added dropwise over 10 min. After 16 h, the reaction mixture was quenched with water (20 mL) and extracted with EtOAc (3 × 40 mL). The combined organic layers were washed with water (2 × 60 mL), brine (60 mL), dried over Na\(_2\)SO\(_4\), and concentrated in vacuo. The yield of product 10 (80%) was calculated by \(^1\)H NMR analysis, using MeNO\(_2\) as internal standard. Crude 10 was then used without further purification.

\(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta = 4.22–4.07\) (m, 4 H), 3.08 (d, \(J = 22.8\) Hz, 2 H), 2.32 (s, 3 H), 1.34 (t, \(J = 7.0\) Hz, 6 H) ppm.

3-Hydroxy-2-(2-oxopropyl)piperidine-1-carboxylic Acid Benzyl Ester ((2)-3a)

To a solution of 8 (696 \(\mu\)L, 3.6 mmol) and K\(_2\)CO\(_3\) (1 g, 7.2 mmol) in acetonitrile (12 mL) and water (12 mL) at 0 °C was added dropwise a solution of Oxone\(^8\) (4.42 g, 7.2 mmol) in water (30 mL) over 5 min, and the reaction mixture was stirred at room temperature for 2 h. Then the mixture was poured into ice and water (40 mL) and, after separation of the layers, the aqueous one was extracted with EtOAc (3 × 40 mL). The combined organic layers were washed with brine (40 mL), dried over Na\(_2\)SO\(_4\), and concentrated under reduced pressure to afford the crude intermediate 9 in 96% yield as a colorless oil, which was used in the next step without further purification.\(^{18}\)

\(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta = 7.39–7.30\) (m, 5 H), 5.77–5.70 (m, 1 H, major), 5.60–5.54 (m, 1 H, minor), 5.14 (s, 2 H), 3.96–3.77 (m, 1 H, major), 3.68–3.51 (m, 1 H, minor), 3.19 (dd, \(J = 12.4\), 3.6 Hz, 1 H, minor), 3.04 (dd, \(J = 12.8\), 3.2 Hz, 1 H, major), 2.34–2.21 (m, 1 H, major), 2.08–1.98 (m, 1 H, minor), 1.97–1.42 (m, 4 H) ppm.

Crude 10 (1.67 g, 8.6 mmol) was dissolved in anhydrous THF (38 mL) and treated with NaH 60% (w/w) in mineral oil (344 mg, 8.6 mmol) at 0 °C. After 25 min, a saturated aqueous solution of NH\(_4\)Cl (34 mL) was added, the mixture stirred, and the progress of EKR process was monitored by GLC. After 72 h, the conversion reached 50%, and the reaction was stopped by filtration through a thin layer of Celite\(^8\) and the filter cake was washed with EtOAc (3 × 5 mL). After evaporation of the solvent, the crude product was purified by flash chromatography (n-hexane/EtOAc, 1:1) to give (+)-3a (582 mg, 44%; \(R_f = 0.40\) as a colorless oil (95% ee), and (+)-3a (262 mg, 45%; \(R_f = 0.18\) as a clear yellow oil.

Lipase-Catalyzed Kinetic Resolution of (5)-3a

iCAL-B (200 mg) was added to a solution of (2)-3a (582 mg, 2 mmol) in anhydrous CPME (2.6 mL) at 30 °C and, after 10 min, p-chlorophenyl butyrate (795 mg, 4 mmol) was added. The reaction mixture was stirred, and the progress of EKR process was monitored by GLC. After 72 h, the conversion reached 50%, and the reaction was stopped by filtration through a thin layer of Celite\(^8\) and the filter cake was washed with EtOAc (3 × 5 mL). After evaporation of the solvent, the crude product was purified by flash chromatography (n-hexane/EtOAc, 1:1) to give (+)-3a (318 mg, 44%; \(R_f = 0.40\) as a colorless oil (95% ee), and (−)-3a (262 mg, 45%; \(R_f = 0.18\) as a clear yellow oil.

3-Hydroxy-2-(2-oxopropyl)piperidine-1-carboxylic Acid Benzyl Ester ((−)-3a)

\([\alpha]_D^{20} = 19.9 (c 0.68, CHCl\(_3\)). Spectroscopic data are identical to those reported for (+)-3a.

3-Butyryloxy-2-(2-oxopropyl)piperidine-1-carboxylic Acid Benzyl Ester ((+)-13a)

\([\alpha]_D^{20} = 50.8 (c 0.7, CHCl\(_3\)). Spectroscopic data are identical to those reported for (−)-3a.

3-Hydroxy-2-(2-oxopropyl)piperidine-1-carboxylic Acid Benzyl Ester ((+)-3a)

To a solution of (−)-3a (318 mg, 0.88 mmol) in anhydrous MeOH (6 mL) cooled to 0 °C was added MeONa (95 mg, 1.76 mmol), and the reaction was stirred at room temperature overnight. A saturated aqueous solution of NaHCl (880 mL) was then added and the reaction mixture concentrated under reduced pressure. Water (88 mL) and EtOAc (88 mL) were added to the residue, and, after separation of the phases, the aqueous phase was further extracted with EtOAc (3 × 50 mL) and the combined organic extracts were dried over Na\(_2\)SO\(_4\). After filtration and evaporation of the solvent, crude (+)-3a was purified by flash chromatography (n-hexane/EtOAc, 1:1; \(R_f = 0.18\), affording pure (−)-3a (227 mg, 89%) as a clear yellow oil (95% ee). \([\alpha]_D^{20} +23.2 (c 0.7, CHCl\(_3\)). Spectroscopic data are identical to those reported for (−)-3a.

2-[3-(7-Bromo-6-chloro-4-oxo-4H-quinazolin-3-yl)-2-oxopropyl]-3-hydroxyipiperidine-1-carboxylic Acid Benzyl Ester ((−)-N-Chloro-Protected Halofuginone, (−)-15)

DIPEA (502 mL, 2.88 mmol) and TMSOTf (521 mL, 2.88 mmol) were added to a solution of (−)-3a (262 mg, 0.90 mmol) in anhydrous DCM (16 mL), cooled to 0 °C (ice bath). After 20 min, NBS (224 mg, 1.26 mmol) was added, the ice bath removed, and, after 20 min, the reaction was stopped by dilution with DCM (16 mL). The organic solvent was washed with water (2 × 60 mL), brine (16 mL), and dried over Na\(_2\)SO\(_4\). After filtration and evaporation of the solvent, crude α-brominated intermediate 14 was obtained and used as such in the next step.
1H NMR (400 MHz, CDCl3): δ (mixture of rotamers) = 7.38–7.28 (m, 5 H), 5.11 (s, 2 H), 4.72 (t, J = 6.8 Hz, 1 H), 4.05 (br s, 1 H), 3.83 (br s, 2 H), 3.01–2.87 (m, 2 H), 2.84 (d, J = 8.0 Hz, 2 H, major), 2.80 (d, J = 8.0 Hz, 2 H, minor), 2.85–2.79 (m, 1 H), 1.89 (qt, J = 6.4 Hz, 1 H), 1.82–1.73 (m, 1 H), 1.72–1.63 (m, 1 H), 1.48–1.39 (m, 1 H) ppm.

Crude 14 was dissolved in anhydrous DMF (10 mL) and reacted with 7-bromo-6-chloro-4-quinazolin-3-yl)-2-oxopropyl]-3-hydroxyperyipendinum Bromide (+)-2 2HBr

(+-)N-Cbz-protected halofuginone (−)-15 (307 mg, 62%) as a white solid. Spectroscopic and analytical data are in accordance with those reported in the literature.14

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Supporting Information
Supporting information for this article is available online at https://doi.org/10.1055/a-1523-6428.

References and Notes
(9) For a review see: (a) Smullen, S.; McLaughlin, N. P.; Evans, P. Bioorg. Med. Chem. 2018, 26, 2199. See also: (b) Zhang, J.; Yao, Q.; Liu, Z. Molecules 2017, 22, 1086; and references therein.


(b) When we attempted a Wittig olefination, we found that two consecutive chromatographic separations were necessary to obtain pure 3a; one on silica gel to remove 12 and the excess of ylide, and a second one on alumina to remove triphenylphosphine oxide.


(26) The enantioselectivity in an enzymatic kinetic resolution process is expressed by the enantiomeric ratio E, which is the ratio between the specificity constants (\(k_{cat}/K_M\)) of the enzyme for the competing R and S enantiomers: \(E = (k_{cat}/K_M)_R/(k_{cat}/K_M)_S\). E was calculated by using the formula \(E = \ln[(1 - ee_s)/(1 + ee_s/eep)]/\ln[(1 + ee_s)/(1 + ee_s/eep)]\). See: Rakels, J. L. L.; Straathof, A. J. J.; Heijnen, J. Enzyme Microb. Technol. 1993, 15, 1051.


