ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 9 (2000) 49–56

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

# Enantioselective esterification of racemic ketoprofen in non-aqueous solvent under reduced pressure

Grégory De Crescenzo, Amélie Ducret, Michael Trani, Robert Lortie *

*Microbial and Enzyme Technology Group, Bioprocess Sector, Biotechnology Research Institute, National Research Council, 6100 Royalmount Ave., Montreal, QC, Canada, H4P 2R2*

Received 16 December 1998; received in revised form 21 July 1999; accepted 21 July 1999

**Abstract**

The enantioselective esterification of (*R,S*)-ketoprofen catalyzed by *Candida antarctica* lipase (type B) has been performed with dodecanol in solvent media under reduced pressure. The nature of the solvent, either aromatic, ether or ketone, affects the activity of the lipase and its enantioselectivity, defined as the ratio of the initial rates of reaction for each enantiomer. Faster reactions are obtained in hydrophobic solvents whereas the enantioselectivity remains constant across all solvents, except for ketones where the enantioselectivity increases with a decrease of the Log *P*. When the same reaction is performed with 1-propanol in xylenes, an inhibition by the nucleophile is observed, which is not detected when using dodecanol under the same conditions. 

*Keywords:* Enzymatic esterification; Resolution; Solvent; Non-aqueous; *Candida antarctica* lipase; Ketoprofen

## 1. Introduction

The stereoselectivity of enzymes is well documented [1,2] and has been used for industrial production of single enantiomers since the early 1970s [3]. The first generation processes were based on the hydrolysis of acylated amines or esterified alcohols and acids. The progresses made in recent years in the structural and mechanistic understanding of enzymes such as serine proteases, esterases and lipases has allowed a better comprehension and sometimes some prediction about their enantioselectivity in hydrolysis [4,5]. Apart from hydrolysis, hydrolases can catalyze other reactions and enantioselective synthesis and transesterification performed in organic media have been extensively studied and described [6,7]. These reactions may have some advantages over hydrolysis: solubility problems can be overcome more easily than in aqueous solution, and the number of steps can be reduced, since prior derivatization can be avoided. The increased stability of enzymatic activity in organic solvents has also been reported [8,9], as well as an increased stereoselectivity in the synthetic mode [10].

The use of organic media introduces two new parameters not present in aqueous solutions: the nature of the solvent and the hydration of the enzyme [11,12].

It is still difficult to explain, and even more difficult to predict, the effect of solvents on enzyme-catalysed reactions from the value of

* Corresponding author. Tel.: +1-514-496-6158; fax: +1-514-496-6144; E-mail: robert.lortie@nrc.ca

1381-1177/00/$ - see front matter 
PII: S1381-1177(99)00083-1

some easily determined parameter. The most frequently used is Log *P*, where *P* is the partition coefficient of the solvent between 1-octanol and water [13]. It has been shown in the case of lipase-catalyzed esterification of ibuprofen that the rate of reaction correlates well with the Log *P* of the solvent, which in turns is related to the solubility of ibuprofen in the solvent [11].

In the case of water, it is now quite clear that the water activity is the parameter to take into account to quantify the amount and influence of water in these systems [14]. However, some hydration hysteresis may be present which makes the catalytic activity of the enzyme depend not only on the water activity but also on the hydration history of the enzyme [15].

In any kinetic resolution process, enantioselectivity comes from the ratio of the rates of reaction with the two enantiomers present. It is then undeniable that these two factors, nature of the solvent and water content, may have an effect on the enantioselectivity, since the two reactions may be affected differently. Indeed, changes in stereoselectivity, and even stereoselectivity reversal, caused by change in solvent have been reported [16,17], and the effect of water activity on the resolution of ibuprofen has been described [11,18].

In the study presented herein, the enzymatic esterification of 2-(3-benzoyl-phenyl)-propionic acid (ketoprofen) was performed in non-aqueous solvent. A process previously described which allows the removal of the by-product water under reduced pressure was used [19,20]. The enzyme used was the *Candida antarctica* lipase (type B), which is well known for its enantioselectivity [21].

## 2. Materials and methods

### *2.1. Materials*

Novozym 435 (immobilized *C. antarctica* type B lipase) and Lipozyme IM20 (immobilized *Mucor miehei* lipase) were a gracious gift from Novo Industries (Bagsvaerd, Denmark). Racemic ketoprofen (2-(3-benzoylphenyl)propionic acid) was purchased from Sigma (St. Louis, MO, USA). Optically pure (*R*)- and (*S*)-ketoprofen were a generous gift from Laboratorios Menarini (Badalona, Spain). All the solvents used were always of the highest grade available and were used without further purification. 1-Dodecanol, 1-propanol, *t*-butylmethylether, mesitylene, 4-methyl-2-pentanone, 2-methyl cyclohexanone, *n*-octane and decane were purchased from Aldrich (Milwaukee, WI, USA). Dibutylether, diisopropylether and cyclohexanone were purchased from BDH (Montreal, Quebec, Canada). HPLC grade water was purchased from Anachemia (Montreal, Quebec, Canada). Benzene, toluene, xylenes, heptane, HPLC grade hexane, acetone and acetonitrile, as well as molecular sieves (3 Å) were purchased from Fisher Scientific (Nepean, Ontario, Canada). Diethyl ether was purchased from ACP Chemicals (Montreal, Quebec, Canada). Silica gel (4 μm) used for the esters purification was furnished by J.T. Backer (Phillipsburg, NJ, USA).

### *2.2. Equipment*

All HPLC analyses were performed using a Waters Maxima 820 liquid chromatography system purchased from Waters Scientific (Mississauga, Ontario, Canada) and equipped with two Waters M590 solvent delivery systems, two Waters WISP sample processors: 710B and 712, one Waters M481 (UV) spectrophotometer, one refractive index detector (RI) 1037A (Hewlett-Packard, Palo Alto, CA, USA), two Croco-Cil™ column heaters, and a NEC PowerMate 386/25 computer.

### *2.3. C-18 HPLC analysis*

Separation of the compounds was accomplished with a HPLC system equipped with a reversed-phase column: CSC-S, Spherisorb

ODS-2, 5 μm (25 cm × 4.6 mm) (CSC, Montreal, Quebec, Canada) connected to a RI detector. Each sample was dissolved in the mobile phase (acetone/acetonitrile/1 mM acetic acid 50/50/1, v/v/v). The flow rate was maintained constant at 1.1 ml/min and the column temperature at 20°C. 25 μl of each sample was injected. The retention times of total ketoprofen, dodecanol and total dodecyl ketoprofenate were 2.29, 4.30 and 5.47 min, respectively.

### 2.4. Chiral HPLC analysis

Chiral resolution of the ketoprofen enantiomers was accomplished with a HPLC system equipped with a chiral column: Chiralcel OJ (25 cm × 4.6 mm) (Chiral Technologies, Exton, PA, USA) connected to a UV detector. After being previously evaporated to dryness, each sample was dissolved in the mobile phase (hexane/2-propanol/acetic acid 9/1/0.05, v/v/v). The flow rate was maintained constant at 1.0 ml/min and the column temperature at 20°C. All HPLC chromatograms were monitored at 254 nm. 10 μl of each sample was injected. The retention times of the unresolved ester, (*R*)-ketoprofen and (*S*)-ketoprofen, were 4.48, 14.10 and 19.77 min, respectively.

### 2.5. Synthesis without any solvent under reduced pressure

In a 30-ml jacketed flask, 5 mmol (0.932 g) of 1-dodecanol was added to 5 mmol (1.271 g) of racemic ketoprofen. The reactor was brought to 75°C with stirring. When all substrates had liquefied, the biocatalyst (Novozym 435 or Lipozyme IM20) was added and vacuum applied ($P < 1$ mbar) in order to remove water formed during the reaction. Periodically, an aliquot of the reaction media was taken for HPLC analysis.

The same process has been used for the production of dodecyl ketoprofenate standard to calibrate both HPLC systems. Racemic ketoprofen has been used as starting material, since it was not necessary to have enantiopure (*R*)- and (*S*)-esters which are not separated in the achiral HPLC analysis. Purification of the resulting ester by chromatography on silica gel using an eluent solvent of hexane/ether (70/30) has led to a purified yield of dodecyl ketoprofenate of 2.35 g (65.6%).

### 2.6. Synthesis in solvent media under reduced pressure

The enzymatic reactions in non-aqueous solvent under reduced pressure were carried out according to the same process previously used to perform enantioselective resolution of ibuprofen [20]. A total of 3.25 mmol (0.826 g) of (*R,S*)-ketoprofen and amount ranging from 1.63 to 9.75 mmol (0.304–1.817 g) of 1-dodecanol were dissolved in 50 ml of the chosen solvent and the reaction carried out at 60°C with 1.5 g of Novozym 435. This process allows the continuous removing of the water generated by the reaction by vaporizing the solvent rich in water under reduced pressure and recycling the dry solvent in the reaction media after passing it through a water trap. Periodically, a 0.5-ml aliquot was removed and filtered for HPLC analysis. The initial reaction rates were determined by non-linear regression over 5–7 points with SigmaPlot software (version 2.01) using rate equation first order with respect to ketoprofen and zero order with respect to alcohol.

The same process has been used for ketoprofen esterification with 1-propanol (details will be given below in Section 3). Propyl ketoprofenate standard for the HPLC analysis has been obtained by performing the esterification during 4 days directly in 1-propanol as solvent, leading to a final yield of 0.558 g (29.0%) after purification by chromatography on silica gel.

### 2.7. Solubility measurements

The solvents were first dried with molecular sieves for at least 3 days. To determine the solubility of ketoprofen in each solvent, racemic

ketoprofen was first dissolved at 40°C. The solution was allowed to cool overnight at 21°C, during which excess ketoprofen came out of solution. This was removed by filtration and aliquots of the remaining saturated solvent (0.3–1.0 ml) were evaporated to dryness to measure the weight of ketoprofen.

## 3. Results and discussion

Since it has been shown that lipases can catalyse with good yields the resolution of racemic ibuprofen in solventless media [22,23], the esterification of ketoprofen with dodecanol was first carried out without any solvent at 75°C, simply by mixing the starting materials together with the biocatalyst (Novozym 435 or Lipozyme IM20) under reduced pressure in order to displace the equilibrium of the reaction towards ester synthesis by the continuous elimination of water. The melting point of ketoprofen (94°C) imposed a minimum reaction temperature of 70°C for the mixture. However, even if non-negligible chemical esterification is observed, no enzymatic reaction was detected, neither with Novozym 435 nor Lipozyme IM20. Indeed, the addition of different amounts of both biocatalysts does not increase the amount of ester formed (results not shown). Even if some thermoinactivation of the catalyst occurred, it could not be the sole reason for the lack of an enzymatic reaction. Indeed, as mentioned by Novo Ind., Novozym 435 is a very heat-tolerant product with a maximum activity in the range 70°–80°C [24,25]. In addition, esterification of ibuprofen, which is related in structure to ketoprofen, with dodecanol has been reported at 70°C with Novozym 435 leading to good yields [23] and the optimum temperature for the esterification of ibuprofen with 1-propanol is 80°C [26]. For these reasons, it is more plausible that enzyme inhibition by an excess of substrate may account for the lack of reactivity, the concentration of each substrate being around 1 M.

To circumvent this limitation, the enzymatic esterification of ketoprofen was performed in non-aqueous solvent media under reduced pressure according to a process initially developed for the enzymatic production of sucroesters [19] and successfully adapted for the enzymatic resolution of ibuprofen [20]. Using these conditions, the concentrations of both substrates were decreased by a factor of 10. It was first verified that no reaction occurred in the absence of enzyme within 6 h. The time-course of a typical reaction performed in xylenes is given in Fig. 1. As can be seen, the enzyme shows some activity and both enantiomers react with different rates. In order to study the influence of the dodecanol concentration on the kinetics, various reactions were performed in xylenes, fixing the initial ratio of alcohol/ketoprofen between 0.5 and 3, at a constant ketoprofen concentration (Fig. 2). The initial rates obtained for both enantiomers stayed the same for all the ratios studied, and as a result, the enantioselectivity observed remained around 3.5. No inhibition by the nucleophile is detected under these conditions.

The solubility of ketoprofen has been determined in various solvents and plotted as a function of their Log *P*, defined as the logarithm of the partition coefficient between *n*-octanol and

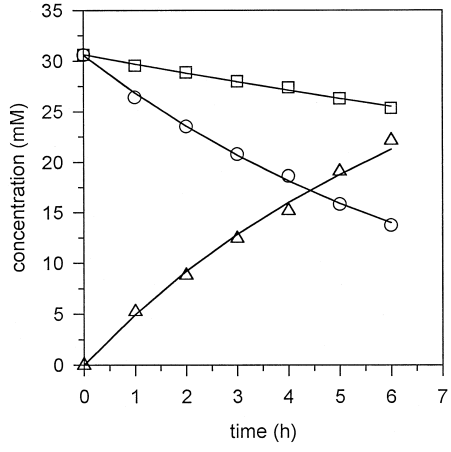


Fig. 1. Time course reaction for resolution of ketoprofen in xylene. Conditions as described in Section 2. (□: (*R*)-ketoprofen, ○: (*S*)-ketoprofen, △: (*R*,*S*)-ester.)

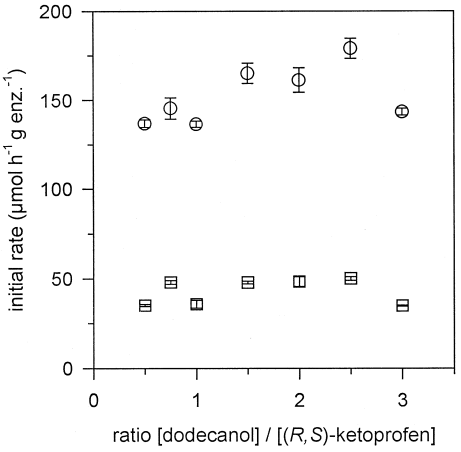


Fig. 2. Influence of the initial dodecanol/ketoprofen ratio on the initial rates for both ketoprofen enantiomers. Conditions as described in Section 2. (○: (*R*)-ketoprofen, □: (*S*)-ketoprofen.)

water [13]. As can be seen in Fig. 3, the solubility decreases drastically when Log *P* increases, ketoprofen being insoluble in alkanes. Compared to ibuprofen, the addition of a second aromatic ring reduces significantly the solubility of ketoprofen. This solubility profile limits the number of solvents, and, thus, the range of Log *P*, which can be used for the esterification of ketoprofen, especially when it is generally recognized that enzymatic catalysis in non-aqueous media is favored in less polar solvents [27,28]. The most common explanations for this are that non-polar solvents are usually less harmful to the structural integrity of the enzyme, they lead to a decrease in the ground state stabilization of the substrate due to a modification of the substrate partition between the bulk phase and the micro-aqueous environment of the enzyme, and they limit the water stripping from the enzyme. This last point, however, is not relevant when the reactions are performed at fixed water activity [11]. Ten solvents from the three families of ketones, ethers and aromatics were chosen and the esterification rates for each enantiomer were measured. The results are presented in Fig. 4. For all the solvents chosen, the enzyme showed

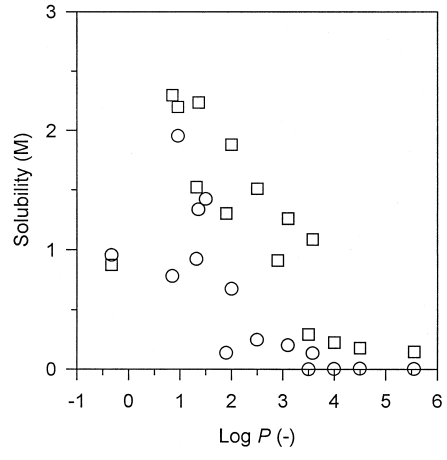


Fig. 3. Solubility of ketoprofen (○) and ibuprofen (□) as a function of Log *P* in various solvents. Solubility of ibuprofen in various solvents have been determined previously [11]. Solvents tested with the corresponding Log *P* values calculated according to Rekker and de Kort [33] are: acetonitrile (−0.33), diethylether (0.85), cyclohexanone (0.96), 4-methyl-2-pentanone (1.32), *t*-butylmethylether (1.36), 2-methyl-cyclohexanone (1.50), diisopropylether (1.90), benzene (2.00), toluene (2.50), dibutylether (2.90), xylene (3.10), hexane (3.50), mesitylene (3.58), heptane (4.00), *n*-octane (4.50), decane (5.55).

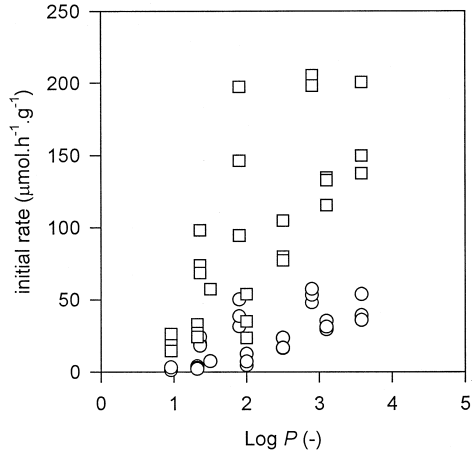


Fig. 4. Esterification rates of (*R*)-ketoprofen (□) and (*S*)-ketoprofen (○) with dodecanol: 65 mM racemic ketoprofen, 65 mM dodecanol, 1.5 g Novozym 435, 50 ml solvent, 60°C, reduced pressure allowing solvent refluxing. Solvents used and Log *P*: methyl *t*-butylether (1.36), diisopropylether (1.90), dibutylether (2.90), 2-methyl-cyclohexanone (1.50), 4-methyl-2-pentanone (1.32), cyclohexanone (0.96), benzene (2.00), toluene (2.50), xylene (3.10), mesitylene (3.50). A one-way analysis of variance has shown that *F*, the ratio between the variance between the solvents and the variance for one solvent is equal to 37.9 for the (*S*)-isomer and 25.3 for the (*R*)-isomer (with $P < 0.00001$ in both cases), showing that the nature of the solvent has a strong influence on the reaction rate. The unicity of the variance has been previously verified with a Bartlett test.

the same preference towards the (*R*)-enantiomer but there is some scattering in the data obtained for each solvent. This can come from the process itself. Because the solvent is being continuously evaporated, recondensed, and dried in a Soxhlet extractor before returning to the reaction media, the reaction volume, and, thus, the concentrations of the substrates, products and biocatalyst, fluctuate slightly during individual reactions and the pattern may not be the same for each experiment.

As noticed before [11,13], there is a general trend allowing faster reactions in hydrophobic solvents. Looking at each individual family, the trend follows quite a linear pattern within the studied range of Log *P*. We must bear in mind that $K_M$ varies from solvent to solvent, for instance, during the esterification of dodecanoic acid and dodecanol with the *R. arrhizus* lipase at fixed water activity, the apparent $K_M$ for dodecanol at fixed decanoic acid concentration decreased as the hydrophobicity of the solvent increased [29]. Data presented here have been obtained for a fixed substrate concentration, and as the $K_M$ value has not been determined in each solvent studied, the measured initial rates can vary from $V_{max}[S]/K_M$ (first order reaction) to $V_{max}$ (zero order reaction) [30].

If the results are expressed in terms of $v_{(R)}/v_{(S)}$, it gives the observed enantioselectivity for the experimental conditions used, and in the case where the reactions are in the first order region, it is a measure of E, the enantiomeric ratio [31]. It appears that the enzyme always displays some selectivity (Fig. 5). With our reaction conditions, for the ether and aromatic families where the rates were the highest, the selectivity does not change with the Log *P* and stays around 4, whereas for the ketone family, the enantioselectivity increases significantly with a decrease of the Log *P*.

Since Arroyo and Sinisterra [26] have shown that using Novozym 435 leads to good esterification activity between ketoprofen and a short chain length alcohol (1-propanol), performing this reaction under reduced pressure using 1-propanol as the nucleophile and the solvent at the same time can seem attractive from a process point of view (a large excess of alcohol can displace the equilibrium of the reaction towards synthesis). The same process as above has then been used, replacing the dodecanol by 1-propanol. As 1-propanol forms ternary azeotropes with water and most of the solvents (xylene in particular), the initial concentration of alcohol was set the same in the reaction flask (containing the reaction media) and in the Soxhlet (where the solvent is dried). Due to the high vapor pressure of the 1-propanol compared to alcohols with longer chain length, the condenser used was not powerful enough to recondense all the 1-propanol vaporized in the azeotrope and some loss of 1-propanol occurred. Thus, the reaction rates could not be calculated accurately in this case. For this reason, only the first 30 min of reaction have been taken into account and initial rates have been approximated using the following equation: $v_{init} = (n_{30\ min} - n_0)/(tm)$ with $n_{30\ min}$ the mole number of (*R*)- or (*S*)-ketoprofen at 30 min, $n_0$ the initial mole number of (*R*)- or (*S*)-ketoprofen, $t = 0.5$ h, and *m* the weight of biocatalyst. As with dodecanol, various reactions were performed in xylenes fixing

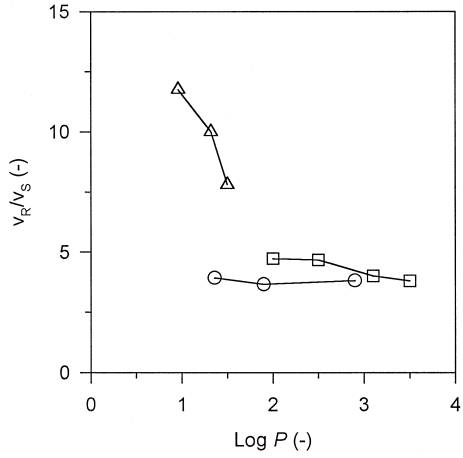


Fig. 5. Observed enantioselectivity of Novozym 435 expressed as the ratio of initial rate of (*R*)- and (*S*)-ketoprofen esterification as a function of solvent hydrophobicity. (△: ketones, ○: ethers, □: aromatics.)

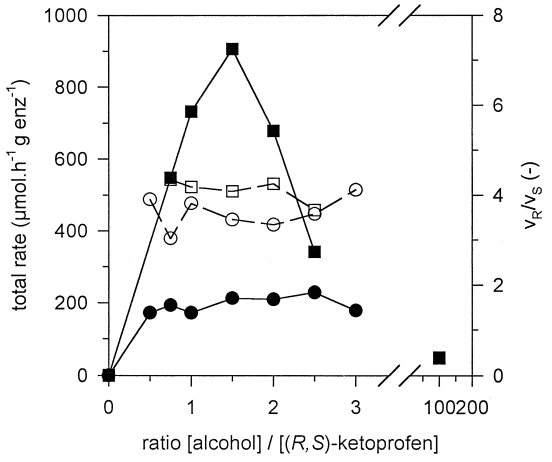


Fig. 6. Total esterification rate expressed as the sum of the initial rates of both enantiomers (filled symbols), and enantioselectivity of Novozym 435 expressed as the ratio of initial rate of (*R*)- and (*S*)-ketoprofen esterification (open symbols), as a function of the ratio alcohol/ketoprofen. (□: esterification with 1-propanol, ○: esterification with 1-dodecanol.)

the initial ratio alcohol/ketoprofen between 0.75 and 2.5. The rates are faster with 1-propanol than with dodecanol (Fig. 6). However, in this case, they are not constant for all the ratios studied and an inhibition by the nucleophile appears clearly when the concentration of 1-propanol is increased. This was not the case with a longer chain length alcohol. In addition, when performing the enzymatic preparation of pure propyl ketoprofenate for the calibration of the HPLC method, 1-propanol was used as substrate and solvent at the same time. Under these conditions, the ratio alcohol/ketoprofen was around 100 and the initial esterification rate estimated at 48 $\mu$mol $h^{-1}$ g $enz^{-1}$. Inhibition of *C. antarctica* lipase B in cyclohexane by short chain length alcohol has been recently reported by Garcia-Alles and Gotor [32], who observed that the competitive inhibition constant values obtained for different chain length alcohols correlate with the calculated activity coefficients of the alcohols, which suggests that the alcohol inhibition is highly related to the alcohol desolvation. The enantioselectivity determined as the ratio of $v_{(R)}/v_{(S)}$ remains constant around 4.1 for all the ratios, which is essentially the same as when the reaction was performed with dodecanol (around 3.6). Developing an enzymatic resolution process for the ketoprofen using an alcohol which is at the same time the nucleophile and the solvent could have been attractive, however, the inhibition by high 1-propanol concentration prevents use of this application even if the same enantioselectivity is maintained.

## Acknowledgements

The authors wish to thank Mr. Adrien Nonn for the determination of the solubility values.

## References

[1] E. Santaniello, P. Ferraboschi, P. Grisenti, A. Manzocchi, Chem. Rev. 92 (1992) 1071.
[2] J.B. Jones, Tetrahedron 42 (1986) 3351.
[3] I. Chibata, in: I. Chibata (Ed.), Immobilized Enzymes, Research and Development, Kodansha, Tokyo, 1978, pp. 168–178.
[4] R.J. Kazlauskas, Trends Biotechnol. 12 (1994) 464.
[5] J. Zuegg, H. Hönig, J.D. Schrag, M. Cygler, J. Mol. Catal. B: Enzym. 3 (1997) 83.
[6] A.M. Klibanov, Acc. Chem. Res. 23 (1990) 114.
[7] A.L. Margolin, Enzyme Microb. Technol. 15 (1993) 266.
[8] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 3194.
[9] D.B. Volkin, A. Staubli, R. Langer, A.M. Klibanov, Biotechnol. Bioeng. 37 (1991) 843.
[10] C.S. Chen, C.J. Sih, Angew. Chem. Int. Ed. Engl. 28 (1989) 695.
[11] A. Ducret, M. Trani, R. Lortie, Enzyme Microb. Technol. 22 (1998) 212.
[12] R. Lortie, Biotechnol. Adv. 15 (1997) 1.
[13] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81.
[14] G. Bell, P.J. Halling, B.D. Moore, J. Partridge, D.G. Rees, Trends Biotechnol. 13 (1995) 468.
[15] J. Partridge, G.A. Hutcheon, B.D. Moore, P.J. Halling, J. Am. Chem. Soc. 118 (1996) 12873.
[16] P.A. Fitzpatrick, A.M. Klibanov, J. Am. Chem. Soc. 113 (1991) 3166.
[17] S. Tawaki, A.M. Klibanov, J. Am. Chem. Soc. 114 (1992) 1882.
[18] P. Pepin, R. Lortie, Biotechnol. Bioeng. 63 (1999) 502.
[19] A. Ducret, A. Giroux, M. Trani, R. Lortie, Biotechnol. Bioeng. 48 (1995) 214.
[20] F. Roure, A. Ducret, M. Trani, R. Lortie, J. Chem. Technol. Biotechnol. 69 (1997) 266.

[21] E. Rogalska, V. Cudrey, F. Ferrato, R. Verger, Chirality 5 (1993) 24.
[22] F. Ergan, M. Trani, R. Lortie, Ann. N. Y. Acad. Sci. 750 (1995) 228.
[23] A. Ducret, P. Pepin, M. Trani, R. Lortie, Biotechnol. Lett. 17 (1995) 1095.
[24] Product Sheet Novozym 435, Novo Nordisk, B 606c-GB 200, 1992.
[25] M. Arroyo, J.M. Sanchez-Montero, J.V. Sinisterra, Enzyme Microb. Technol. 24 (1998) 3.
[26] M. Arroyo, J.V. Sinisterra, J. Org. Chem. 59 (1994) 4410.
[27] Z. Yang, A.J. Russell, in: A.M.P. Koskinen, A.M. Klibanov (Eds.), Enzymatic Reactions in Organic Media, Blackie Academic and Professional, Glasgow, 1995, pp. 43–69.
[28] L. Kvittingen, Tetrahedron 50 (1994) 8253.
[29] E. Wehtje, P. Adlercreutz, Biotechnol. Bioeng. 55 (1997) 798.
[30] A.R. Fersht, Enzyme Structure and Mechanism, W.H. Freeman, New York, 1985.
[31] K. Faber, Enantiomer 2 (1997) 411.
[32] L.F. Garcia-Alles, V. Gotor, Biotechnol. Bioeng. 59 (1998) 163.
[33] R. Rekker, H.M. de Kort, Eur. J. Med. Chem. Chim. Ther. 14 (1979) 479.