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The enantioselectivity of lipase PS in chlorinated solvents increases as a function of substrate conversion

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Abstract: The enantiomeric ratio E of lipase from *Pseudomonas cepacia* increased markedly as a function of substrate conversion in the resolution of (±)-6-methyl-5-hepten-2-ol (sulcatol) when methylene chloride was used as reaction medium. Such behavior was observed, even though less pronounced, also in dichloroethane. Instead, when cyclohexane and toluene were used as reaction medium or when 3-methyl-2-cyclohexen-1-ol was employed as substrate, enzyme enantioselectivity remained constant. No explanation for the phenomenon, which can have practical implications for resolutions carried out in organic solvents, has been found yet. © 1997 Elsevier Science Ltd

Introduction

Resolution of racemic alcohols and acids is one of the most interesting applications of hydrolases in organic synthesis and, indeed, it has been extensively investigated by numerous researchers.[1] One of the advantages of hydrolases, which makes them particularly attractive, is their wide and easy applicability in both aqueous and organic media. Concerning organic media, it is also possible, by simply changing the reaction solvent, to modify the enantioselectivity or even induce a reversal of the enantiopreference of a hydrolase for a given substrate.[2,3]

Enzyme enantioselectivity can be quantitatively described by the enantiomeric ratio (E), which can be calculated for both reversible and irreversible enzyme-catalyzed reactions by equations formulated by Sih and coworkers,[4,5] after determination of the degree of conversion and the enantiomeric excess of either the product or the remaining substrate. According to this approach, under constant reaction conditions and even if changes in the overall enzyme activity occur as the reaction proceeds, the E value remains constant during the whole reaction course. Nevertheless, if the enzymatic kinetic mechanism hypothesized for the catalysis is not correct, or effects such as product inhibition occur, the E value does not follow the theoretical trend.[6,7] Besides these factors, problems in the determination of the E value can be encountered when reactions supposed to be irreversible are not really irreversible, as reported by Lundh *et al.* for some transesterifications with vinyl acetate.[8] They found that the E value was much higher when calculated for conversions below 50% than for conversion above 50%. It should be emphasized that all these factors tend to cause a decrease of calculated E values as substrate conversion increases.

In the present study, that was carried out in the context of continuing research on the effects of organic solvents on enzyme enantioselectivity, we found that the enantioselectivity of lipase from *Pseudomonas cepacia* increased as a function of substrate conversion when it was used for the resolution of (±)-6-methyl-5-hepten-2-ol (sulcatol) in methylene chloride. This surprising observation prompted us to investigate in more detail the phenomenon, testing other solvents (chlorinated and not) and substrates in order to find out a possible explanation for such behavior.

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Experimental section

Materials

Lipase from *Pseudomonas cepacia* (lipase PS, 30 units/mg solid using sesame oil) was purchased from Amano. Novozym 435 (immobilized *Candida antarctica* lipase) was a gift from Novo Nordisk. All the substrates and reagents were purchased from Aldrich except vinyl butyrate which was obtained from Fluka. The solvents used were of analytical grade.

Preparation of (R)- and (S)-sulcatol butyrate

(*R*)-Sulcatol butyrate was prepared by enantioselective esterification of a 0.016 M solution of (±)-sulcatol in hexane, using vinyl butyrate (0.032 M) and 0.5 mg/ml of immobilized *Candida antarctica* lipase, under shaking, at 25°C. The reaction was stopped at 43% conversion (ee(P) 98%) and the ester (*R*)-sulcatol butyrate purified by flash chromatography. (*S*)-Sulcatol was prepared from (±)-sulcatol using the same reaction conditions but pushing the reaction up to 56% conversion (ee(*S*) 96%). (*S*)-Sulcatol was purified by flash chromatography and then chemically esterified to (*S*)-sulcatol butyrate using butyric anhydride.

Transesterification reactions

In a typical experiment, 50 mg of lipase PS were added to 1 ml of solvent containing 0.079 M of alcohol ((±)-sulcatol or (±)-3-methyl-2-cyclohexen-1-ol) and 0.12 M of vinyl ester (vinyl acetate or vinyl butyrate). Previous to mixing, organic solvents, vinyl esters, enzymes and substrates were separately adjusted to the desired water activity a_w in sealed containers for at least 2 days, at 25°C, with the vapour phase of saturated salt solutions of known a_w: LiCl (a_w 0.11), $Mg(NO_3)_2 \cdot 6H_2O$ (a_w 0.53).[9]

When the influence of (*R*)- or (*S*)-sulcatol butyrate on the transesterification of (±)-sulcatol with vinyl acetate was studied, they were added to the reaction system before adding the enzyme at a 0.09 M concentration. The same procedure and concentration were used when the influence of acetic acid or acetaldehyde was tested.

In all cases the reaction mixtures were shaken in an orbital shaker at 250 rpm and 45°C. Periodically, aliquots were withdrawn and assayed by chiral GLC.

Determination of enantiomeric excess and conversion degree

In all cases investigated, both the conversion degree of the alcohol into the correspondent ester and the ee(P) were determined using a CP-Cyclodextrin-β-2,3,6-M-19 column (50 m, 0.25 mm ID, Chrompack) under the following conditions: 90°C (initial time 15 min) to 130°C with a heating rate of 1.5°C/min for the reaction with (±)-sulcatol[2] and isothermal at 80°C for the reaction with 3-methyl-2-cyclohexen-1-ol,[10] H_2 as carrier gas.

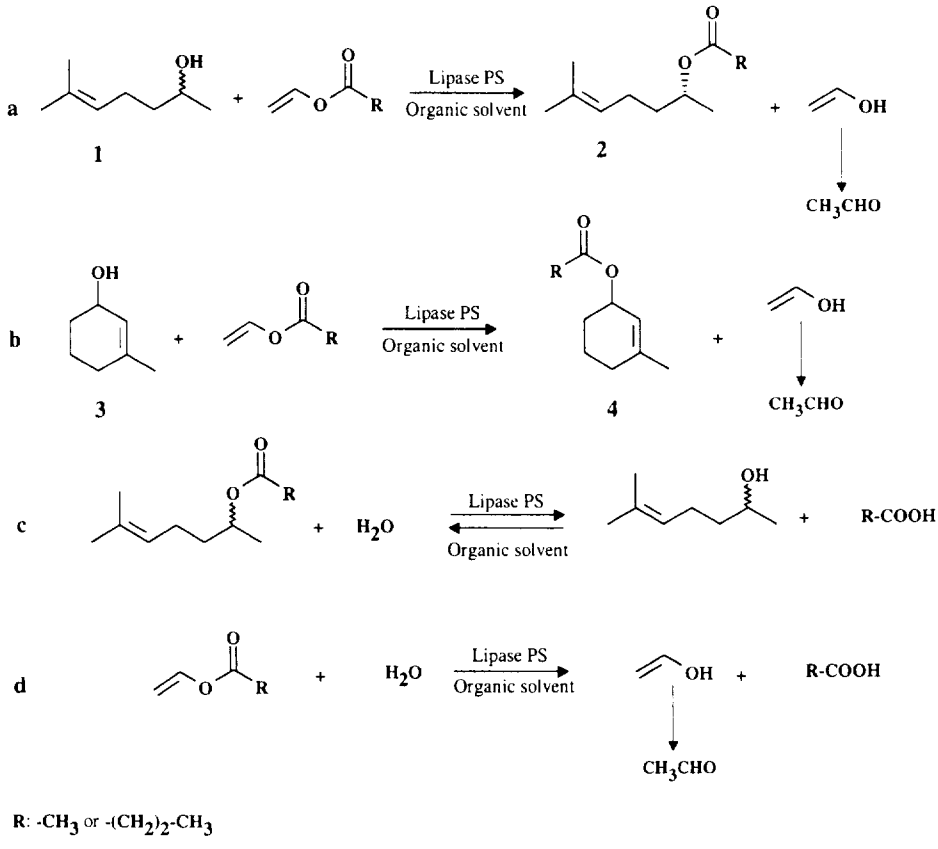
Calculation of the enantiomeric ratio E

In all cases the value of E was calculated from the enantiomeric excess of the product ee(P) and the conversion degree (c), obtained in the same chromatographic analysis, using the following formula:[4]
$E = \ln[1-c(1+ee(P))]/\ln[1-c(1-ee(P))]$.

Results and discussion

In the course of a study on the effects of organic solvents on enzyme enantioselectivity, it occurred to us to investigate the influence of methylene chloride on the selectivity of lipase from *Pseudomonas cepacia*. The reaction investigated was the kinetic resolution of (±)-sulcatol (compound **1** in Scheme 1) using vinyl acetate as acyl donor, at a water activity (a_w) of 0.11.

To our surprise, we found that the ee values of the product, i.e. (*R*)-sulcatol acetate, did not decrease as a function of conversion as expected, but slightly increased (Figure 1). The E value calculated[4] for the point at the lowest degree of conversion (3.65%), and considering the transesterification reaction as irreversible, was 26. At this point a plot of ee(P) as a function of conversion was computer generated



Scheme 1.

using the E value of 26 (Figure 1). If we compare the ee(P) values experimentally determined with the computer generated curve, we can see that the deviation of the experimental points from the theoretical ones was quite remarkable.

The results appeared even more impressive if the E values obtained from the experimental data were plotted against conversion (Figure 2). An approximate 3-fold increase of the enantiomeric ratio was observed moving from a conversion of about 4% to a conversion of about 42%. It should be emphasized that, theoretically, the enantiomeric ratio should remain constant from 0 to 100% conversion.[4]

Such behavior was evident, even though less pronounced, also when dichloroethane was used as solvent. Instead, when cyclohexane and toluene were used in place of the above mentioned chlorinated solvents or when 3-methyl-2-cyclohexen-1-ol (**3** in Scheme 1, part b) was used as substrate instead of sulcatol, the enantioselectivity remained constant as the conversion increased (Figure 2). It is worth mentioning that in all cases investigated, only *c* values below 0.5 were taken into account, because in this region possible small errors in the determination of the degree of conversion scarcely affect the determination of the E value.[11]

Since the commercial preparation of *Pseudomonas cepacia* lipase does not contain other detectable contaminant hydrolase activities,[12] we can rule out the possibility that the above described phenomenon is due to a progressive inactivation of some enzyme with lower or even opposite enantioselectivity respect to lipase PS. Moreover, it should be emphasized that the use of vinyl esters as acyl donors makes the reactions under investigation virtually irreversible (Scheme 1, part a and b) and that the increase of E as function of conversion occurs only when (±)-sulcatol resolution is carried out in the above mentioned chlorinated solvents. Therefore, it should be the particular reaction medium employed

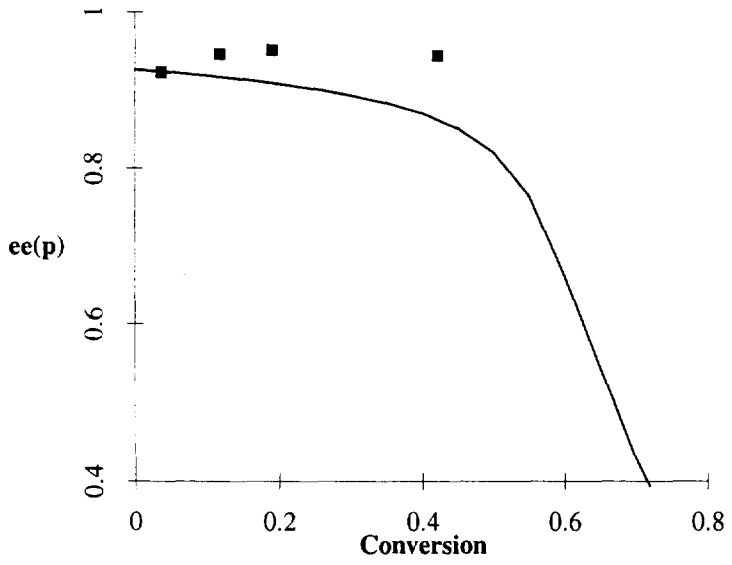


Figure 1. Effect of the degree of conversion on the ee of the product ((*R*)-sulcatol acetate) in the lipase PS-catalyzed resolution of (±)-sulcatol using vinyl acetate as acyl donor and methylene chloride as solvent, at a_w 0.11 (■). The points were averages of at least two determinations. The continuous line was computer generated from an E value of 26.

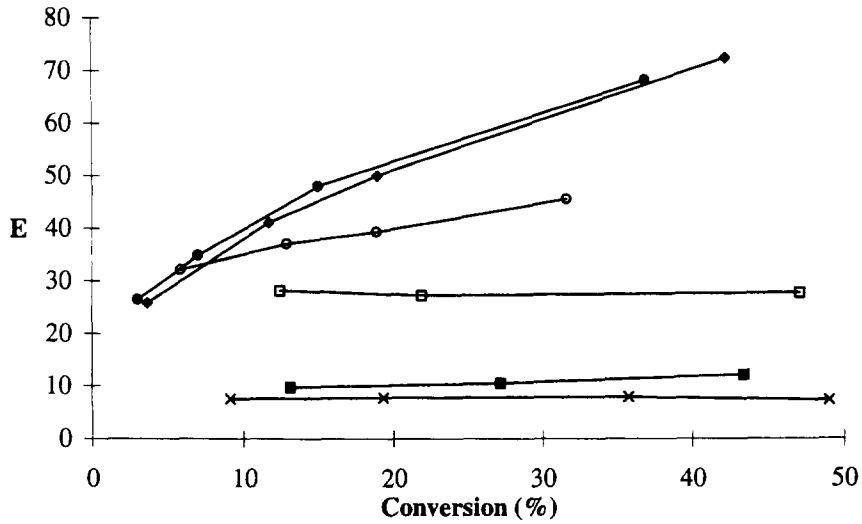


Figure 2. Influence of the degree of conversion on the enantiomeric ratio E of lipase PS in the resolution of different substrates in different solvents. Transesterification of (±)-sulcatol with vinyl acetate in dichloromethane (◆), dichloroethane (◯), toluene (□), cyclohexane (■). Transesterification of (±)-sulcatol with vinyl butyrate in dichloromethane (●). Transesterification of (±)-3-methyl-2-cyclohexen-1-ol with vinyl acetate in dichloromethane (×). Water activity was 0.11 in all cases. The points were averages of at least two determinations.

and not the inadequacy of the equation used for the calculation of the E value, or a peculiar kinetic mechanism of this enzyme,[6,7] the reason for the observed phenomenon. Conformational modifications induced by the solvent can also be excluded because the same increase of E was observed when the enzyme was preincubated in methylene chloride for 1.5–6 h before addition of substrates (periods of time which allow conversion degrees of 5–20% when the substrates are present) (Figure 3).

A hypothesis we considered to explain the above described increase of the E value (Figure 2)

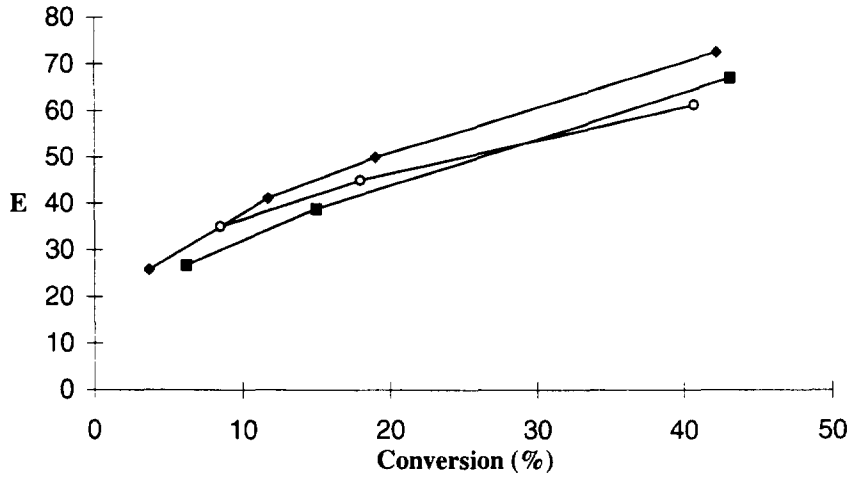


Figure 3. Effect of enzyme preincubation in dichloromethane on the enantiomeric ratio of lipase PS. Transesterification of (±)-sulcatol with vinyl acetate in dichloromethane without previous incubation (◆), and with preincubation for 1.5 h (■) and 6h (○) in the same solvent. Water activity 0.11. The points were averages of two determinations.

was that, in methylene chloride, one of the products of the reaction ((*R*)- and (*S*)-sulcatol acetate, acetaldehyde and acetic acid; Scheme 1) was able to strongly and specifically inhibit the esterification of the slow reacting enantiomer (*S*)-sulcatol, thus causing an increase of the enantiomeric excess of (*R*)-sulcatol acetate (Figure 1).

Since it was not possible to investigate the effects of sulcatol acetates on the resolution of sulcatol because their addition to the reaction mixture prevented the correct determination of c and ee(P), we circumvented this obstacle by employing sulcatol butyrates. In fact, we found that the value of the enantiomeric ratio E showed the same increase as a function of conversion when vinyl butyrate was used instead of vinyl acetate as acyl donor (Figure 2). Therefore, the (*R*)- and (*S*)-butyryl esters should behave as the analogous acetate esters. Consequently, the two butyryl esters were prepared with ee respectively of 98% and 96% and separately added to two parallel reactions where vinyl acetate was used as acyl donor (Scheme 1, part a). Figure 4 shows that also in the presence of the (*R*)- or (*S*)-butyryl ester there was the usual increase of the E value and, therefore, no inhibition effects on the synthesis of the slow reacting enantiomer, as above postulated, can be ascribed to sulcatol esters. Indeed, if an inhibition had been exerted on the esterification of the slow reacting enantiomer, a higher and constant E value should have been observed since the beginning of the reaction. The same result was obtained when acetaldehyde was added to the reaction system (Figure 4). Finally, the effect of acetic acid, which is formed because of the undesired hydrolytic reaction of both vinyl acetate and sulcatol butyrate (Scheme 1, part c and d) was also investigated but, in this case too, no effect was observed (Figure 4).This should also exclude that modifications of E values can be a consequence of modifications of the pH of enzyme environment which, for instance, have been shown to influence the enantioselectivity of alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* in aqueous media.[13]

Concerning water activity, it should be noted that all the experiments described so far were performed at a value of a_w 0.11. However, some experiments were also carried out at a_w 0.53 and they showed that the resolution of sulcatol with vinyl acetate in methylene chloride had the same trend observed at a_w 0.11 (Figure 5). At high a_w it was not possible to push the reaction beyond about 10% conversion because of the low rate of transesterification due to concomitant hydrolysis of the product and the acylating agent.

In conclusion, the results described above indicate that the enantiomeric ratio of lipase PS increases

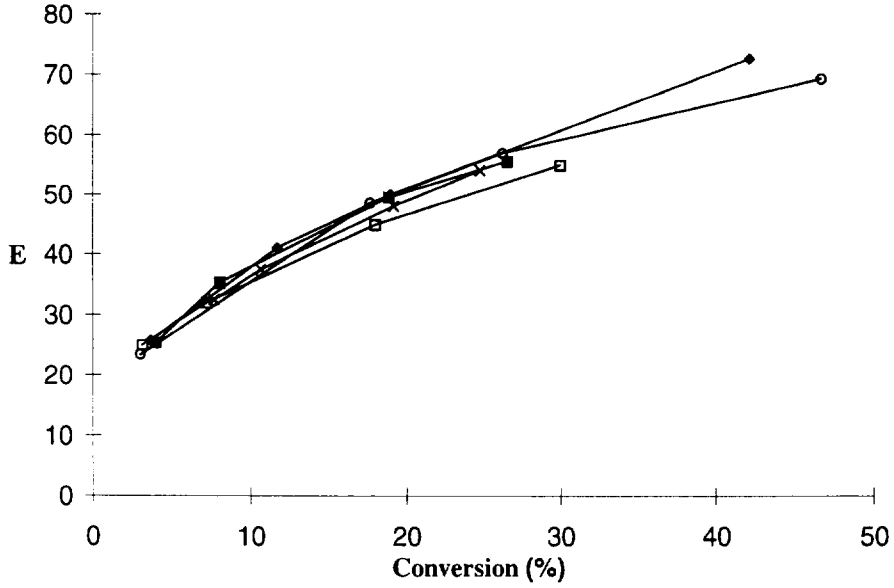


Figure 4. Influence of products on the enantiomeric ratio of lipase PS in dichloromethane. Transesterification of (±)-sulcatol with vinyl acetate in the absence (◆) or presence of 0.09 M (*R*)-sulcatol butyrate (■), (*S*)-sulcatol butyrate (◯), acetic acid (×) or acetaldehyde (□). Water activity 0.11. The points were averages of two determinations.

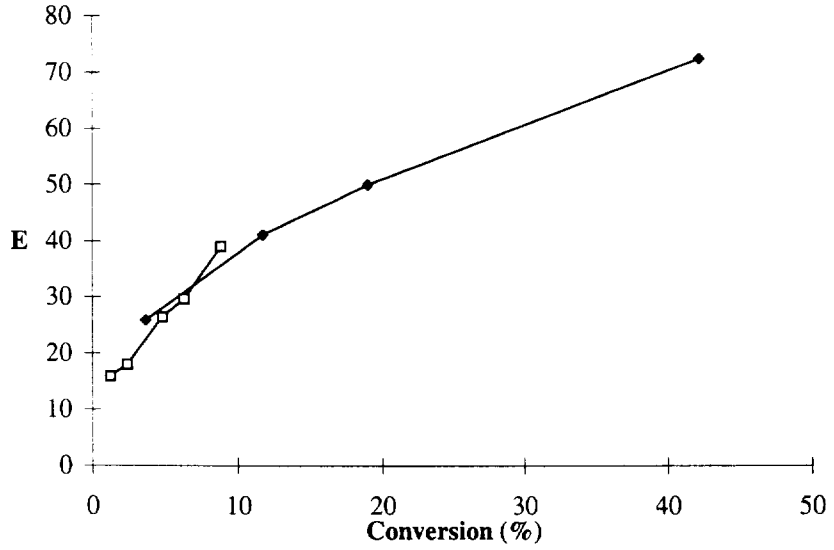


Figure 5. Effect of water activity on the enantiomeric ratio of lipase PS in dichloromethane. Transesterification of (±)-sulcatol with vinyl acetate at a_w 0.11 (◆) or a_w 0.5 (□).The points were averages of at least two determinations.

as a function of conversion when sulcatol is esterified in chlorinated solvents such as dichloromethane and, to a lesser extent, dichloroethane and, on the contrary, there is no increase of the E value when cyclohexane or toluene or another substrate such as 3-methyl-2-cyclohexen-1-ol are used. This behavior allows us to esclude that the mathematical approach[4] used for the calculation of the enantiomeric ratio is responsible for such increase. In fact, since we determined the enantiomeric ratio E identically in all the cases considered, if the formula we applied had been incorrect we would have observed an

analogous increase of E as function of conversion in all the solvents examined. On the other hand, it is likely that the Michaelis–Menten kinetic mechanism hypothesized[4] for lipase PS in toluene or cyclohexane is also applicable when the same enzyme is used to catalyze the identical reaction in a chlorinated solvent. Moreover, conformational modifications of the enzyme were ruled out and possible effects of the products were also taken into account and excluded.

At present we have no explanation for the phenomenon which, being observed only with one of the two substrates investigated (sulcatol) and only with chlorinated solvents, could possibly be ascribed to the formation of complex interactions (in the medium or on the enzyme) among the particular substrate and solvent and two or more products (each single product was shown to be ineffective, see Figure 4) which, in some way, improve enzyme selectivity. Anyhow, the phenomenon, which from the synthetic point of view appears advantageous, is very striking and unusual since so far only decreases of E values as a function of conversion have been observed and rationalized.[6–8] Work is in progress to test if also other enzymes and substrates display such behavior in chlorinated solvents.

Acknowledgements

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References

1. For Reviews see, Jones J.B. *Tetrahedron* **1986**, *42*, 3351–3403; Klibanov, A.M. *CHEMTECH.* **1986**, *16*, 354–359; Sih, C.J.; Wu, S.-H. *Topics in Stereochemistry* **1989**, *19*, 63–125; Santaniello, E.; Ferraboschi, P.; Grisenti, P.; Manzocchi, A. *Chem. Rev.* **1992**, *92*,1071–1140; Drauz, K.; Waldmann, H. (Eds), *Enzyme Catalysis in Organic Synthesis*, VCH, Weinheim, **1995**.
2. Secundo, F.; Riva, S.; Carrea, G. *Tetrahedron: Asymmetry* **1992**, *3*, 267–280.
3. Hirose, Y.; Kariya, K.; Sasaki, J.; Kurono, Y.; Ebike, H.; Achiwa, K. *Tetrahedron Lett.* **1992**, *33*, 7157–7160; Wescott, C.R.; Klibanov, A.M. *Biochim. Biophys. Acta* **1994**, *1206*, 1–9; Carrea, G.; Ottolina G.; Riva, S. *Trends Biotechnol.* **1995**, *13*, 63–70; Wescott, C.R.; Noritomi, H; Klibanov, A.M. *J. Am. Chem. Soc.*, **1996**, *118*, 10365–10370.
4. Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C.J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299.
5. Chen, C.-S.; Wu, S.-H; Girdaukas, G.; Sih, C.J. *J. Am. Chem. Soc.* **1987**, *109*, 2812– 2817.
6. Straathof, A.J.J.; Rakels, J.L.L.; Heijnen,J.J. *Biocatalysis* **1992**, *7*,13–27.
7. van Tol, J.B.; Geerlof, A.; Jongejan, J.A.; Duine, J.A. *Ann. N.Y. Acad. Sci.* **1992**, *672*, 462–470.
8. Lundh, M.; Nordin, O.; Hedenstrom, E.; Hogberg, H.-E. *Tetrahedron: Asymmetry* **1995**, *6*, 2237–2244.
9. Valivety, R.H.; Halling, P.J.; Macrae, A.R. *Biochim. Biophys. Acta* **1992**, *1118*, 218–222.
10. Orrenius, C.; Norin, T.; Hult, K.; Carrea, G. *Tetrahedron: Asymmetry* **1995**, *6*, 3023–3030.
11. van Tol, J.B.; Jongejan, J.A.; Geerlof, A.; Duine, J.A. *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 255–262.
12. Sugiura, M.; Oikawa, T.; Hirano, K.; Inukai, T. *Biochim. Biophys. Acta*, **1977**, *488*, 353–358; Weissfloch A.N.E.; Kazlauskas, R.J. *J. Org. Chem.* **1995**, *60*, 6959–6969; Takami, K. Amano Pharmaceutical Co., personal communication.
13. Secundo, F.; Phillips, R.S. *Enzyme Microb. Technol.* **1996**, *19*, 487–492.

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