Enantioselective Esterification of 2-Arylpropionic Acids Catalyzed by Immobilized *Rhizomucor miehei* Lipase

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A systematic study of the enzymatic activity of immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) in the enantioselective esterification of 2-arylpropionic acids has been carried out. The main variables controlling the process (enzyme amount, water amount, temperature, stirring speed, and type of organic solvent) were studied using factorial analysis. The negative effect of water amount is explained by means of water activity (a_w) considerations. A new and easy to calculation parameter (Enantiomeric Factor, EF) is defined for evaluating the enantioselectivity of the reaction. Influence of the alcohol and acid moieties is also considered. Lipozyme IM shows *S*-(+) enantiorecognition in all cases, except for (*R*,*S*)-Ketoprofen, where the *R*-(−) stereobias is confirmed using pure enantiomers ($V_R/V_S = 8$). An explanation for this different enantiopreference is suggested by means of MD calculus.

Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are able to catalyze esterification reactions in organic solvents displaying higher enantioselectivity than that showed in hydrolytic reactions.[1] These enzymes are very useful in synthetic organic chemistry because of their unique stability in nonpolar organic solvents and their conformational flexibility. Because of these properties, the use of lipases for preparation of optically-enriched compounds has become an interesting alternative to chemical asymmetric synthesis.[2]

Among the racemic drugs, 2-arylpropionic acids (APA's, the "profen" family) constitute an important group of nonsteroidal antiinflammatory drugs (NSAIDs), which are widely used as racemic mixtures to control the symptoms of arthritis and related connective tissue diseases.[3] However, it is well documented that only the *S*-(+) enantiomer is pharmacologically active, while only a certain portion of the *R*-(−) enantiomer could be transformed into the *S*-(+) isomer by *in vivo*[4] metabolic inversion.

In recent years, there have been many papers dealing with the lipase-catalyzed kinetic resolution of APA's, either by enantioselective hydrolysis of their esters[5] or by esterification in organic solvent.[6] The aim of this work is to study the influence of several experimental and structural factors on the activity of immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) in the enantioselective esterification of 2-arylpropionic acids. This biocatalyst is supplied by Novo-Nordisk, and it has been used for ester synthesis,[7] enantioselective transesterification (secondary alcohols,[8] and aromatic or heteroaromatic acids[9]), alcoholysis of esters of tertiary alcohols,[10] or diester crowns synthesis.[11] A factorial design is used in order to analyze the level of influence of the experimental variables on the synthetic activity of Lipozyme IM. The study of structural factors is carried out employing different organic solvents, alcohols and 2-arylpropionic acids.

Results and Discussion

Experimental Design. The nature of the main experimental variables that control the yield of the esterification reaction catalyzed by the immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) was the first point to be analyzed. This study was undertaken by factorial analysis, a multivariant method in which all the parameters are simultaneously changed in a suitable programmed manner.[12] The application of this method

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Table 1. Variables and Levels Used in the Factorial Design

variables	+1	center point	−1
x_A (mol/mol)	4:1	1:1	1:4
x_B (hours)	30	24	18
x_C (°C)	57	37	17
x_D (rpm)	700	500	300
x_E (mg of Lipozyme IM)	700	500	300
x_F (mL of cyclohexane)	450	300	150
x_G (mL of water)	0.6	0.3	0,00

Table 2. Factorial Design: Experimental Matrix

run	x_A	x_B	x_C	x_D	x_E	x_F	x_G	Y (%)
1	−	−	−	−	−	−	−	5.7
2	+	−	−	−	−	+	+	0
3	−	+	−	−	+	−	+	50.1
4	+	+	−	−	+	+	−	11.4
5	−	−	+	−	+	+	+	9.7
6	+	−	+	−	+	−	−	37.6
7	−	+	+	−	−	−	+	0
8	+	+	+	−	−	+	−	30.0
9	+	−	−	+	+	+	−	21.1
10	−	−	−	+	+	−	+	13.2
11	−	+	−	+	−	+	+	0
12	+	+	−	+	−	−	−	9.1
13	+	−	+	+	−	−	+	3.2
14	−	−	+	+	−	+	−	24.4
15	−	+	+	+	+	−	−	69.9
16	+	+	+	+	+	+	+	3.1
17	0	0	0	0	0	0	0	35.9
18	0	0	0	0	0	0	0	46.9
19	0	0	0	0	0	0	0	47.9

requires the appropriate selection of response, factors, and levels. The esterification of Ibuprofen ((*R*,*S*)-2-(isobutylphenyl)propionic acid) (0.125 M) with 1-butanol (0.125 M) in cyclohexane was chosen as the reaction test. In this case the selected response was the ester yield (Y) and was shown by a polynomial function of seven experimental variables (eq 1):

$$Y = b_0 + \sum b_i x_i + \sum b_{ij} x_i x_j \qquad (1)$$

x_A = acid/alcohol molar ratio (mol/mol) x_B = time (h)
x_C = temperature (°C) x_D = stirring speed (rpm)
x_E = amount of immobilized lipase (mg) x_F = solvent amount (mL)
x_G = water amount (mL).

Selection of the levels was carried out considering working condition limits of the lipase. The maximum (+1) and minimum (−1) levels of each factor are shown in Table 1. The experiments were randomly performed according to a 2^4 factorial design. The results (ester yield) obtained in the standard reaction with different combinations of maximum and minimum values of each variable (entries 1−16) and the center point values (entries 17−19) are shown in Table 2. The statistical analysis of this factorial design is summarized in Table 3.

Daniel's method[13] was used as the significance test (Figure 1). In this methodology, the points that are not fitted to the statistical probability model are the variables that have some influence on the esterification process (points with higher statistic coefficients). The most significant factors were enzyme amount ($b_E = 17.96$) and water amount ($b_G = -25.93$). Furthermore, three other variable interactions must be considered as significant: catalyst amount × water amount ($b_{EG} = -10.57$), time × solvent amount ($b_{BF} = -10.23$), and temperature × solvent amount ($b_{CG} = -9.41$). The use of analysis and factorial design of the experiments allows us to see the analyzed response as a polynomial model of the significant factors as follows:

$$\text{yield (\%)} = 18.03 + 17.96x_E - 25.93x_G - 10.57x_E x_G - 10.23\, x_B x_F - 9.41x_C x_G \qquad (2)$$

The nonsignificance of acid/alcohol ratio (x_A) permits us to reject the presence of diffusional restrictions for both the acid and the alcohol, because otherwise this parameter would be strongly significant.

Once this method is defined, the most important operational variables were exhaustively studied. Not only did we consider the yield of the biocatalyzed reaction, but also the enantioselectivity. The most common parameter to determine the enantioselectivity of a kinetic resolution is the enantiomeric ratio, E, reported by Sih et al.[14] This paramter, as it was defined, is only valid for first (or pseudo-first)-order kinetics, and in this esterification, as the maximum acid/alcohol molar ratio used was 4/1 (see Table 1), the higher ratio in one substrate in respect to the other is not enough to ensure the first-order kinetic. Nevertheless, the fitting of the

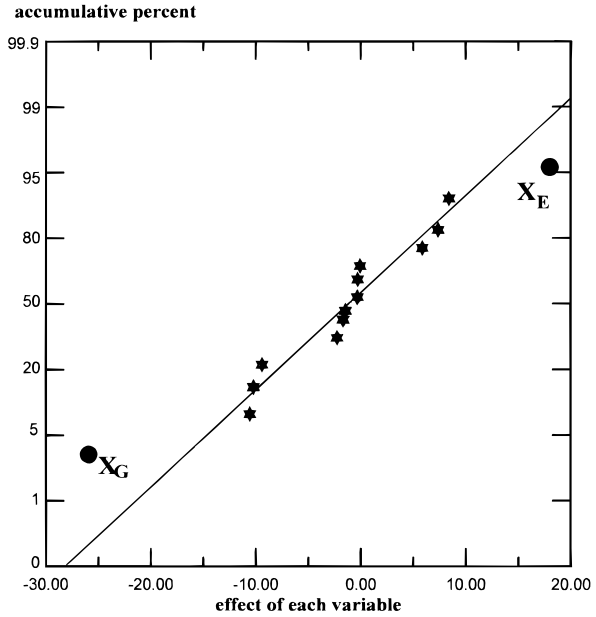

Figure 1. Error estimation: Daniel's method.

Table 3. 2^4 Factorial Design: Statistical Analysis

number of experiments: 16
degrees of freedom: 15
results of statistical analysis:
$b_O = 18.03$ $b_{AB} = b_{CF} = b_{EG} = -10.57$
$b_A = -1.69$ $b_{AC} = b_{DG} = b_{BF} = -10.23$
$b_B = 7.35$ $b_{BC} = b_{DE} = b_{AF} = -0.30$
$b_C = 8.40$ $b_{AD} = b_{CG} = b_{EF} = -9.41$
$b_D = -0.07$ $b_{BD} = b_{CE} = b_{FG} = -2.27$
$b_E = 17.96$ $b_{CD} = b_{BE} = b_{AG} = 5.86$
$b_F = -1.48$ $b_{AE} = b_{BG} = b_{DF} = -0.34$
$b_G = -25.9$
significance test (Student's t): center point analysis
confidence level: 95%
$Y_m = 43.5\%$
$t_2\ (\alpha = 0.05) = 2.9$
$S_x = 6.6$
confidence range = ±13.7

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progress curve of the reaction to first-order kinetics by using SIMFIT program[15] was good enough in all cases (with R^2 values around 0.95). Various methods to simplify the E determination have been described recently.[16] We propose a new parameter, easier to calculate, to quantify the enantioselectivity of a reaction, regardless of its kinetic order, specially when the resolution of the racemic substrate is the aim of the process.

This new parameter of enantioselectivity, enantiomeric factor (EF), is defined as the correlation between the experimental (ee_E) and the theoretical enantiomeric excess (ee_T) of a reaction.

$$EF = ee_E/ee_T \quad (3)$$

In this esterification, the experimental enantiomeric excess (ee_E) is the one measured (for the remaining substrate) at a defined reaction time. The theoretical enantiomeric excess (ee_T) is the value which would be obtained at the same reaction time with the same measured yield, *only if the fast-reacting enantiomer is transformed*. Therefore, to calculate this value, we must simply use eq 4.

$$ee_T = [\text{yield}/(100 - \text{yield})] \times 100 \quad (4)$$

Thus, the maximum EF value (EF = 1) would indicate that the enzyme is acting exclusively on one enantiomer. When the reaction yield is lower than 50%, an EF value of 0.95 ensures a good resolution, ranging from $E = 39$ at 10% yield to $E > 100$ for a 50% conversion. Taking into account kinetic resolution of racemates, when the reaction yield is higher than 50% (the enzyme converts both enantiomers), the EF value must be calculated considering $ee_T = 100$, a value which would indicate the maximum enantioselectivity reached at 50% of the yield. In this case, an EF value of 0.99 for conversions higher than 50% represents a very active enzymatic system, with a moderate enantioselectivity.

Influence of the Technical Variables on the Enantioselectivity of the Process. From the factorial analysis we deduced that the water amount is the most influential factor on the activity of Lipozyme IM for the synthesis of butyl ester of Ibuprofen, displaying a strong negative effect ($b_G = -25.9$). A more detailed study requires us to explore if this effect is related to the removal of the adsorbed protein from the solid support or to the denaturation of the protein, or it is caused by an interaction with the support. Therefore, in order to increase our knowledge of this effect we used a simple method based on water activity measurements. For this purpose, the water adsorption isotherms for Lipozyme IM and crude lipase (Lipozyme 10000L), in air and cyclohexane (standard solvent) at 25 °C, were obtained (Figure 2). As can be observed from that figure, the isotherms of the crude enzyme are very different from those of the immobilized lipase. Native enzyme needs more water molecules to achieve the same a_w value than immobilized enzyme, so that the solid support must play an important role in the water adsorption of the biocatalyst. Valivety et al.[17] showed that the isotherm of Lipozyme IM was very similar to that of the support (Duolite ES-568) alone, suggesting that most of the water is probably retained by the support rather than by the protein. Considering the organic solvent, the water adsorption isotherms of Lipozyme IM in air and cyclohexane show distinct behavior from that obtained for the native enzyme: in cyclohexane, the obtained isotherm of Lipozyme IM is placed above that of the native enzyme, therefore suggesting that both the dry cyclohexane and the biocatalyst must retain some amount of water, reaching a balance between all species capable of retaining water. Due to the lower hydrophilicity of Lipozyme IM compared to native enzyme, the excess of water molecules (not retained by the catalyst or dissolved by the solvent) will create a discrete aqueous phase.

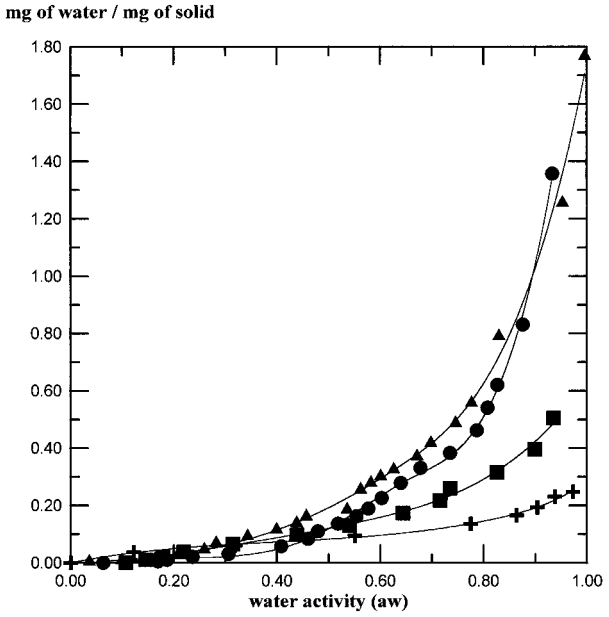

Figure 2. Water adsorption isotherms: crude enzyme (Lipozyme 10000L) in air (▲) and in cyclohexane (●). Immobilized enzyme (Lipozyme IM) in air (+) and in cyclohexane (■).

To study the influence of the amount of water on the activity of Lipozyme IM in organic medium, we carried out the standard esterification reaction with different degrees of humidity of the immobilized lipase: (a) dehydrated at vacuum in presence of P_2O_5 for 48 h at room temperature; (b) with 0.3 mL of added water; (c) commercial preparation without any treatment. The obtained results are shown in Figure 3. The results are different depending on the humidity conditions of the biocatalyst.

Dry Lipozyme IM ($a_w = 0.107$) requires a certain time (lag-time) to reach an optimum state of hydration, which is achieved by the water produced during the reaction progress. Taking into account the adsorption isotherms of the enzymatic derivative (Figure 2), both the enzyme and support must be dehydrated, so that only a small percentage of enzyme molecules would be able to catalyze the esterification reaction. Halling et al.[18] recommended the value of $a_w = 0.50-0.55$ as the optimum for developing esterifications with this enzyme. Looking at the

(15) SIMFIT V. 4.0. A computer package for simulation, curve fitting, statistical analysis and graph plotting using life science models. W. G. Bardsley, University of Manchester, 1994.

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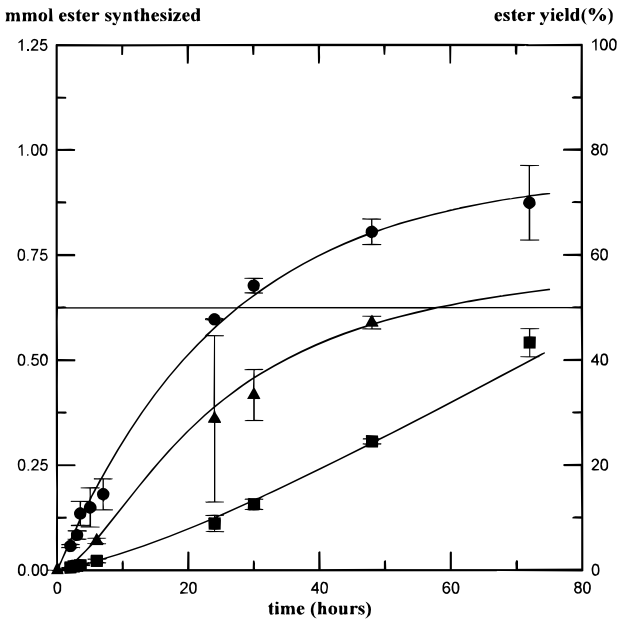

Figure 3. Influence of the water amount of Lipozyme IM in the esterification of (±)-Ibuprofen with 1-butanol in cyclohexane. 0.125 M Ibuprofen + 0.125 M 1-butanol in 10 mL of cyclohexane; $T = 37$ °C. (■) Dehydrated catalyst ($a_w = 0.107$); (●) comercial preparation (10% water, w/w, $a_w = 0.34$); (▲) fully hydrated ($a_w = 1$).

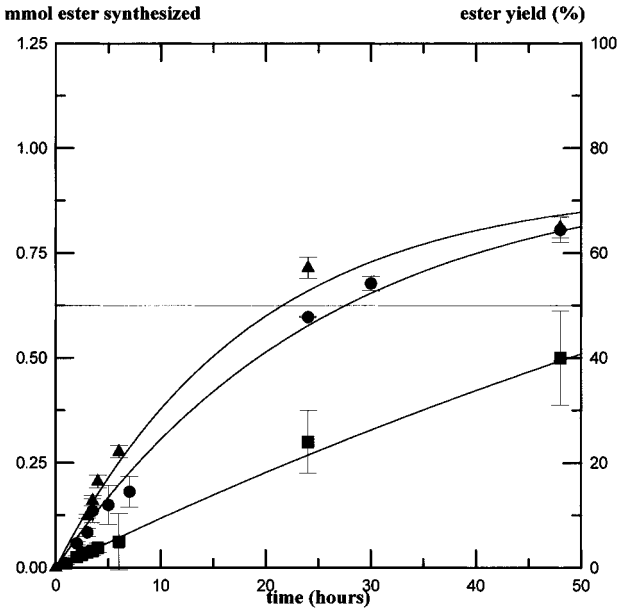

Figure 4. Influence of the amount of immobilized derivative Lipozyme IM in the esterification of (±)-Ibuprofen with 1-butanol in cyclohexane. 0.125 M Ibuprofen + 0.125 M 1-butanol in 10 mL of cyclohexane; $T = 37$ °C. (■) 150 mg; (●) 300 mg; (▲) 450 mg.

adsorption isotherm of Lipozyme IM in cyclohexane, we can observe how that value corresponds to the theoretical point of generation of free water into the solution.[19] As this system is far from the optimum a_w value, the reaction rate is low. As the reaction is progressing, the water molecules which are being produced would rehydrate the enzyme, increasing the reaction rate.

When the enzyme is saturated with water, the extent of conversion is lower. Vázquez-Lima et al.[7c] described a similar effect for water-saturated Lipozyme IM in the esterification of lauric acid with geraniol. This fact is attributed to the formation of a discrete aqueous phase, which would disturb the movement of substrates toward the immobilized lipase active site.[20] On the other hand, as described by Vázquez-Lima et al.,[7c] the accumulation of water on the support surface produces the aggregation of biocatalyst particles when the amount of water fluctuates between 0.32 and 0.44 mg of water/mg of Lipozyme IM ($a_w = 1$, Figure 2). This amount of water is lower than the concentration in this saturated system (1.1 mg of water/mg of Lipozyme IM), so this aggregation phenomenon will cause the poor reaction rate.

The best results are obtained with the commercial immobilized lipase, (abut 10% (w/w) of water, according to the supplier, ($a_w = 0.34$)). If we calculate the amount of water produced (from the stoichiometry of the reaction), at a determined reaction time, and we interpolated that value in the water adsorption isotherm (Figure 2), we could determine the value of a_w at the end of the reaction. In the reaction catalyzed by commercial preparation, after 72 h, the value of a_w would be 0.56, when there is still no free water in the system. As a consequence, the reaction yield was the best, because all the generated water was captured by Lipozyme IM, and there is no need to add any water externally, as Gandhi et al.[7d] described for the Lipozyme-catalyzed synthesis of butyl laurate, because the resin may maintain the correct hydration state in the immediate vicinity of the enzyme.

The positive influence of the amount of biocatalyst ($b_E = 17.96$, Table 3) is shown in Figure 4, where we can observe that amounts of derivative greater than 300 mg should not be used because productivity of the catalyst, measured as the specific activity (μmol of Ibuprofen esterified × (mg of Lipozyme IM)$^{-1}$ × (h)$^{-1}$, Table 4) decreases. This saturation effect is best observed considering not only the initial rate but also the ester yield at 48 h. On the other hand, considering the enantioselectivity of these esterifications shown in Table 4 by means of E and EF parameters, the best results were obtained with 300 mg of Lipozyme IM. If the amount of biocatalyst is increased, the enantioselectivity goes down. From this table, these results improve those previously described employing some other lipases for the same reaction: using lipase from *Candida antarctica*, Arroyo and Sinisterra[6d] reported E values always less than 4.4 in the esterification of Ibuprofen (*R*-(−)stereopreference) with 1-propanol, while Gradillas et al.[21] described the same esterification, adding benzo-[18]-crown-6-*meso*-tetraphenylporphyrin, yielding 79% of the *R*-ester with 53% ee ($E = 2.04$). Also, when using lipase from *Candida rugosa*, de la Casa et al.[22] obtained a smaller yield and a similar enantioselectivity (at a higher reaction time) than that shown in Table 4; although, Mustranta,[6c] using *C. rugosa* lipase and amyl alcohol in hexane, and Rantakylä and Aaltonen,[6b] using Lipozyme IM and 1-propanol in

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Table 4. Productivity of Lipozyme IM in the Esterification of Ibuprofen with 1-Butanol[a]

amount of Lipozyme IM (mg)	initial rate[b]	productivity[c]	yield (48 h)	ee (%)[d] (48 h)	*E* (48 h)	EF (48 h)
150	12.0 ± 0.5	0.080 ± 0.003	40 ± 3	47 ± 2	9.1	0.92
300	37.1 ± 0.4	0.124 ± 0.001	64 ± 2	98 ± 2	14.6	0.98
450	50.4 ± 0.8	0.112 ± 0.002	65 ± 2	51 ± 1	2.8	0.51

[a] See Figure 4 for conditions. [b] μmol of ester synthesized $\times$ h^{-1}. [c] μmol of ester synthesized $\times$ h^{-1} $\times$ mg^{-1} of derivative. [d] Remnant acid.

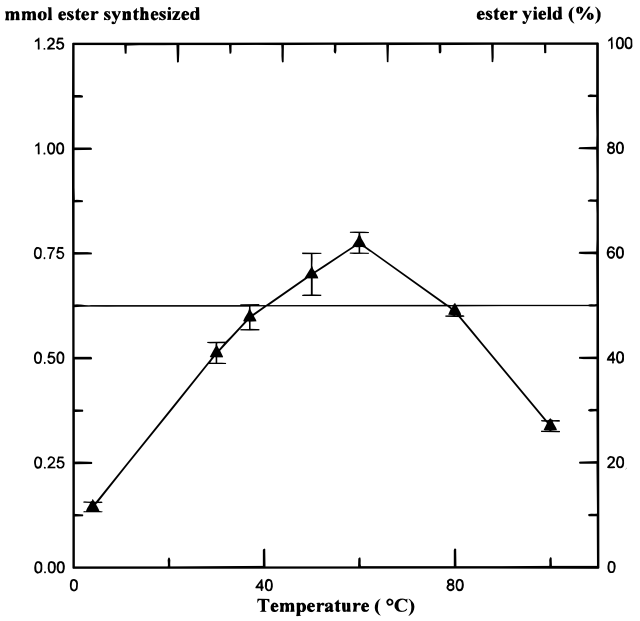

Figure 5. Influence of temperature in the esterification yield of (±)-Ibuprofen with 1-butanol in catalyzed by Lipozyme IM in cyclohexane. 0.125 M Ibuprofen + 0.125 M 1-butanol in 10 mL of cyclohexane. Reaction time, 24 h.

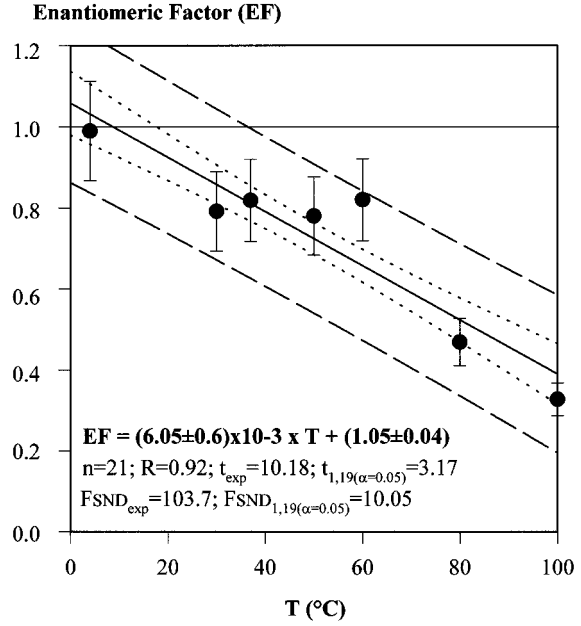

Figure 6. Influence of temperature in the enantioselectivity of esterification of (±)-Ibuprofen with 1-butanol catalyzed by Lipozyme IM in cyclohexane. 0.125 M Ibuprofen + 0.125 M 1-butanol in 10 mL of cyclohexane. Reaction time = 24 h.

supercritical CO_2, reported somewhat better enantioselectivities, their results must be carefully considered, because ee values and conversion percentages do not seem to be compatible.[23]

Although reaction temperature was not a very significant factor (b_E = 8.4, Table 1) on the activity of immobilized lipase from *Rh. miehei*, we analyzed this variable because it usually influences the enzyme activity. The range of temperature analyzed varied from 4 to 100 °C, wider than that analyzed in the factorial design (17 to 57 °C). The results of the activity obtained are shown in Figure 5. We can observe that the highest yield was obtained at 60 °C, decreasing at higher temperature. This result agrees with the described behavior for the Lipozyme-catalyzed synthesis of wax esters[24] and butyl laurate.[7d] Furthermore, the supplier of this enzymatic preparation (Novo-Nordisk) describes the optimum temperature for the development of synthetic activity of this enzyme between 60 and 70 °C.[25] However, Knez et al.[26] have observed that Lipozyme beads can be used up to 90 °C for the synthesis of *n*-butyl oleate, and Ergan et al.[27] could carry out triolein synthesis at temperatures as high as 80 °C without any deactivation. These authors state that the presence of butanol is responsible for the decrease in the maximum operational temperature. We have also studied the influence of temperature on the enantioselectivity of Lipozyme IM. The results obtained are shown in Figure 6. We can observe an inverse relationship between the enantioselectivity factor (EF) and the reaction temperature. The loss of enantioselectivity at higher temperature may be due to the deformation of the active center, which could produce an enhancement in the flexibility of the substrate recognition site. In order to avoid the evaporation of the organic solvent and to obtain a higher degree of enantioselectivity the optimum temperature in the esterification of Ibuprofen was fixed at 37 °C.

Influence of the Solvent Nature. In modern literature there are many references about the activity of lipases in organic media, in which they catalyzed reactions of esterification and transesterification.[1,2] The nature of organic solvent has been the subject of many papers,[28] and it has a great effect on the stability of the biocatalyst. To quantify the hydrophobicity/hydrophilicity of organic solvents, we use the parameter log *P*, the partition coefficient of the solvent between 1-octanol and

(23) Mustranta[6b] reported 42% yield and 99% enantiomeric excess as the best results for ibuprofen esterification: at that conversion, the maximum feasible enantiomeric excess should be 72.4% (if EF = 1); similar conclusions can be inferred from the data of Rantakylä and Aaltonen,[6c] which are again incompatible: 15% yield, 70% ee (maximum enantiomeric excess at that conversion, 17.6%).

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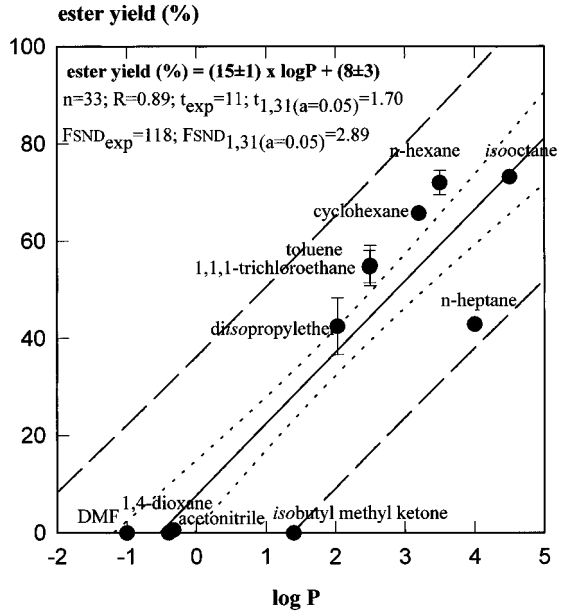

Figure 7. Influence of the nature of the solvent in the esterification yield (72 h) of (±)-Ibuprofen with 1-butanol catalyzed by Lipozyme IM. 0.125 M Ibuprofen + 0.125 M 1-butanol in 10 mL of organic solvent. T = 37 °C.

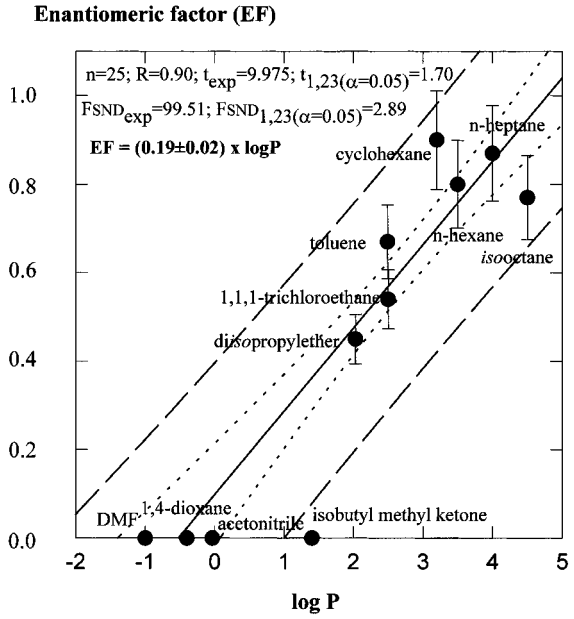

Figure 8. Influence of the nature of the solvent in the enantioselectivity esterification (72 h) of (±)-Ibuprofen with 1-butanol catalyzed by Lipozyme IM. 0.125 M Ibuprofen + 0.125 M 1-butanol in 10 mL of organic solvent. T = 37 °C.

water.[29] Generally, the catalytic activity of lipases is carried out in organic solvents with log P > 2, because the catalytic efficiency of enzymes decreases as the hydrophilicity of the solvent increases.[28c]

We studied the effect of different organic solvents on the esterification of Ibuprofen with 1-butanol catalyzed by Lipozyme IM. In Figure 7 we show the ester yield obtained in each reaction versus the log P of the organic solvents. As we can see, there is a fair linear correlation between the log P of the organic solvents and the reaction yield.

Lipozyme IM was active only with organic solvents with log P > 2. We can observe the immobilized enzyme was inactive with hydrophilic solvents (DMF (log P = −1.0); 1,4-dioxane (log P = −0.4); acetonitrile (log P = −0.33); isobutyl methyl ketone (log P = 1.4)), because these solvents remove the essential water from enzymes,[30] water which plays an important role in the maintenance of native conformation of the enzyme. On the other hand, high esterification rates were obtained with hydrophobic solvents. Similar results with Lipozyme IM were reported by Manjón et al.[31] and Miller et al.[32] in the synthesis of ethyl butyrate and propyl myristate in different organic solvents and by Kamińksa et al.[33] in transesterification of 1-(2-furyl)ethanol with vinyl acetate. Furthermore, Mustranta[6a] and Arroyo and Sinisterra[6d] described similar effects upon esterification of Ibuprofen with lipases from *C. rugosa* and *C. antarctica*. The influence on the enantioselectivity of Lipozyme IM with different solvents is shown in Figure 8. In this case we can also observe a moderate linear correlation between the enantioselectivity and log P of organic solvent. As Carrea et al.[28c] have reported, the nature of the organic solvent clearly influences enzyme enantioselectivity, although correlation with log P is not always achieved. We chose cyclohexane (log P = 3.2) as the organic solvent for the standard esterification reaction because of the high yield and enantioselectivity obtained.

Influence of the Structural Variables. Influence of the Alcohol Moiety. It is recognized that lipase from *Rh. miehei* works better in esterification of primary alcohols, whereas its activity is lower with secondary alcohols and is inactive with tertiary alcohols.[34] Nevertheless, Sonnet[35] described esterification of secondary alcohols with octanoic and hexanoic acid catalyzed by Lipozyme IM. Meanwhile, when using this enzyme for transesterification of enol esters, good activities and enantioselectivities can be reached with secondary alcohols,[8] and Barnier et al.[10] described the Lipozyme-catalyzed resolution of esters of tertiary alcohols. If we assume the formation of an acyl−enzyme intermediate as the crucial step during esterification, the final reaction yield will depend on the accessibility of the nucleophile (alcohol, in this case) to the acyl−enzyme complex, which is unique for each acylation reagent. This fact would create a specific geometry around the active site, which would determine the nature of the best recognized nucleophile. Due to all these factors the nature of alcohol moiety must play an important role in the development of these reactions.

With the aim of studying the effect of alcohol nature on our standard esterification reaction, we employed different alcohols in the esterification of (*R*,*S*)-Ibuprofen. The results are shown in Figure 9. In this study we analyzed linear primary alcohols with different chain lengths (1-butanol and 1-octanol), branched primary alcohols (3-methyl-1-butanol), linear secondary alcohols (2-propanol), and cyclic secondary alcohols (cyclohexanol).

(29) Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81−87.
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(35) Sonnet, P. E. *J. Org. Chem.* **1987**, *52*, 3477−3479.

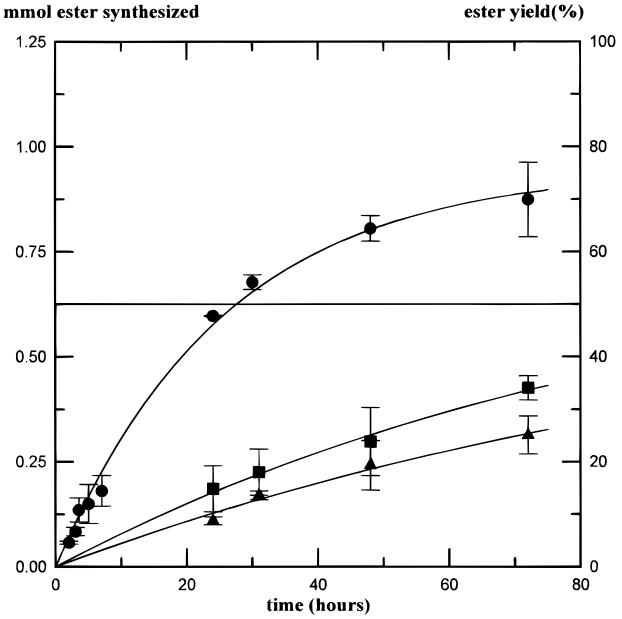

Figure 9. Influence of the alcohol moiety in the esterification of (±)-Ibuprofen catalyzed by Lipozyme IM. 0.125 M Ibuprofen + 0.125 M 1-butanol in 10 mL of cyclohexane. $T = 37$ °C. (●) 1-Butanol; (■) n-octanol; (▲) 3-methyl-1-butanol.

Table 5. Esterification of (*R,S*)-Ibuprofen with Different Alcohols Catalyzed by Lipozyme IM after 72 Hours

alcohol	yield in ester (%)	ee of remaining acid (%)	EF	*E*
1-butanol	66 ± 5	99 ± 6	0.99	14.5
3-methyl-1-butanol	25 ± 1	32 ± 1	0.97	67.0
1-octanol	34 ± 1	50 ± 3	0.98	>100
2-propanol	0	–	–	–
cyclohexanol	0	–	–	–

The studied primary alcohols possess more than four atoms of carbon because the lipase from *Rh. miehei* presents a low activity with shorter polar alcohols, like ethanol, which are able to dehydrate the enzyme.[30] Lipozyme IM did not act on any of the studied secondary alcohols, for this specific acylation reagent. The reaction rate is also influenced by the length of the alcohol moiety. The highest ester conversion is obtained with the shortest primary alcohol (1-butanol). Although 3-methyl-1-butanol is shorter than 1-octanol, the ester yield was lower because the methyl radical in position 3 may impede the approach of this alcohol to the acyl−enzyme complex, as described by Miller et al.[32] in the esterification of different primary alcohols with myristic acid. These authors stated that, due to the hydrophobic nature of the active site of this enzyme, hydrophobic alcohols would also be the most appropriate for enzyme recognition. Nevertheless, not only the lipophilicity but also the geometry of the alcohols must be considered.

We have also studied the influence of alcohol moiety on the enantioselectivity of the esterification. The results are shown in Table 5, where we can observe a remarkably high enantioselectivity for 1-octanol. This fact agrees with the literature,[34] where large linear alkyl chains are recommended for the good enantiodiscrimination of Lipozyme IM in the esterification of octanoic acid with different chiral alcohols in hexane. On the other hand, the stereobias (*S*-(+)-preference) is the same for all the nucleophiles tested.

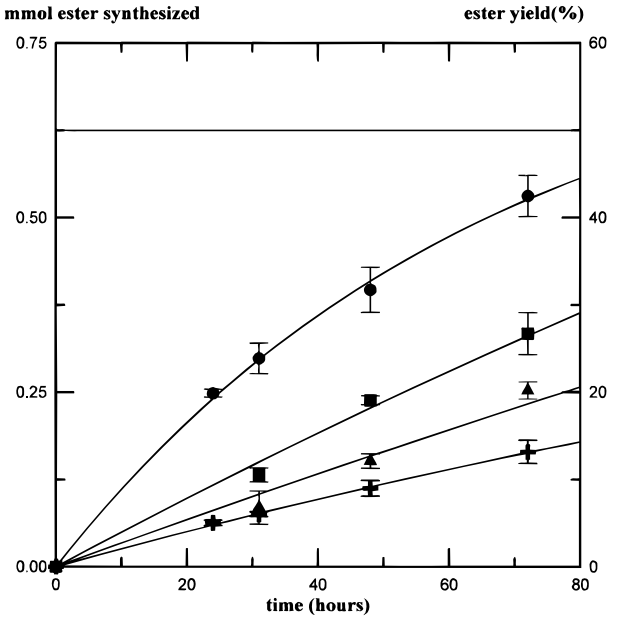

Figure 10. Influence of the acid moiety in the esterification of (±)-2-arylpropionic acids with 1-butanol catalyzed by Lipozyme IM. 0.125 M racemic acid + 0.125 M 1-butanol in 10 mL of diisopropyl ether. (●) (±)-Ibuprofen; (■) (±)-2-(6-methoxy-2-naphthyl)propionic acid; (▲) (±)-Ketoprofen; (+) (±)-2-phenylpropionic acid.

Influence of the Acid Moiety. Finally, we studied how the nature of (±)-2-arylpropionic acid influences the activity of Lipozyme IM. The results obtained in the standard esterification of 2-arylpropionic acids with 1-butanol are represented in Figure 10, using diisopropyl ether as solvent because of the poor solubility of some of the APA's studied in cyclohexane. Table 6 shows the enantioselectivity obtained by means of the EF parameter. The higher yield is acquired using Ibuprofen (the most hydrophobic substrate), maybe because of interface considerations: the more hydrophobic the substrate, the more stable the interface and the more active the biocatalyst. In all cases, Lipozyme IM reacts better on the S(+) enantiomer of (*R,S*)-2-arylpropionic acids, except for Ketoprofen. In order to confirm the change of enantioselectivity observed using Ketoprofen, we carried out the esterification of each enantiomer of Ketoprofen separately (Table 7), corroborating the stereobias observed. Conflictive references can be found in the literature concerning the enantiodiscrimination of *Rh. miehei* lipase on this type of racemic acids: Sih et al.[36] noted a enantioselective preference of this lipase for the *R*-(−) enantiomer of (*R,S*)-2-(6-methoxy-2-naphthyl)propionic acid by hydrolysis of its methyl ester, and Palomer et al.[37] described similar stereobias (*R*-(−) recognition) on both enantiomers of Ketoprofen. On the contrary, Mustranta[6a] observed a enantiopreference for the *S*-(+) enantiomer of Ibuprofen in the esterification with 3-methyl-1-butanol, and Rantakylä and Aaltonen[6b] described similar stereobias in the esterification of Ibuprofen with 1-propanol. All these authors worked with *Rh. miehei* lipase from Novo-Nordisk, except Sih et al.[36] and Palomer et al.,[37] who used the lipase from Amano.

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Table 6. Enantiomeric Factor Obtained in the Esterofication of 2-Arylpropionic Acids Catalyzed by Lipozyme in Diisopropyl Ether[a]

substrate	log P^b	product configuration	ester yield (72 h)	ee[c] (%)	EF	E
Ibuprofen	3.74	*S*-(+)	42	32	0.44	3.5
(*R,S*)-2-(6-methoxy-2-naphthyl)propionic acid	2.83	*S*-(+)	27	7	0.19	1.6
Ketoprofen	2.66	*R*-(-)	20	9.5	0.38	2.4
(*R,S*)-2-phenyl propionic acid	1.84	*S*-(+)	13	8.5	0.57	4.0

[a] See Figure 10 for conditions. [b] Calculated from molar fragments[40]. [c] Remnat acid.

Table 7. Yield of the Esterification Reaction of R(−)- and S(+)-Ketoprofen with 1-Butanol, Catalyzed by Liposome IM, in Diisopropyl Ether

time (h)	yield of R(−)-Ketoprofen(%)	initial rate,[a] V_{0_R}	yield of S(+)-Ketoprofen (%)	initial rate,[a] (V_{0_S})	V_{0_R}/V_{0_S}
167	48 ± 2	5.73×10^{-3}	7.3 ± 0.3	7.13×10^{-4}	8.0
263	60 ± 2		15.0 ± 0.7		

[a] μmol of ester synthesized $\times$ h^{-1}.

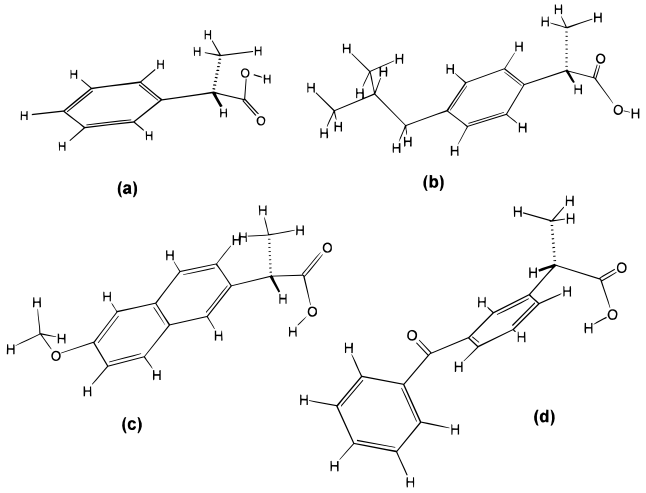

Figure 11. Minimum energy conformers of *S*-(+)-2-phenylpropionic acid (a), *S*-(+)-Ibuprofen (b), *S*-(+)-2-(6-methoxy-2-naphthyl)-propionic acid (c) and *R*-(−)-Ketoprofen (d).

It stands to reason that the different origins of the lipases (from different laboratories) could be the cause of these contradictory results, as Sonnet[35] demonstrated: he arrived to these conclusions by checking the enantioselectivity of the *Rh. miehei* lipase produced by three different laboratories in the esterification of (±)-2-octanol with octanoic acid in hexane, and he attributed the results to slight alterations of the protein during genetic manipulation. In order to explain the behavior of lipase from *Rh. miehei* and to increase our knowledge of the enzyme−substrate interaction, a study of the molecular dynamics and the molecular mechanics of each enantiomer of the 2-arylpropionic acids was carried out employing HYPERCHEM program.[38]

The minimum energy conformers of *S*-(+)-2-phenylpropionic acid, *S*-(+)-Ibuprofen, *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid, and *R*-(−)-Ketoprofen are depicted in Figure 11. Because the conformer of *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid is the bulkiest of the studied substrates, we used its structure as the reference for the overlapping of these conformers (Figures 12a and 12b). The point for the overlapping of these structures was the stereogenic carbon and its bond to the aromatic ring. The overlaped conformers of *S*-(+) enantiomers are very similar, except for *S*-(+)-Ketoprofen (Figure 12a). We can observe that the benzoyl group of *S*-(+)-Ketoprofen is located far away from the aromatic area of *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid, which was very similar to the other conformers. When overlapping the conformers of *R*-(−)-Ketoprofen, *S*-(+)-Ibuprofen, and *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid (Figure 12b), it is noticeable that the benzoyl group is located closer to the aromatic area of *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid, though hydrogen and methyl are located in opposite sides in both cases.

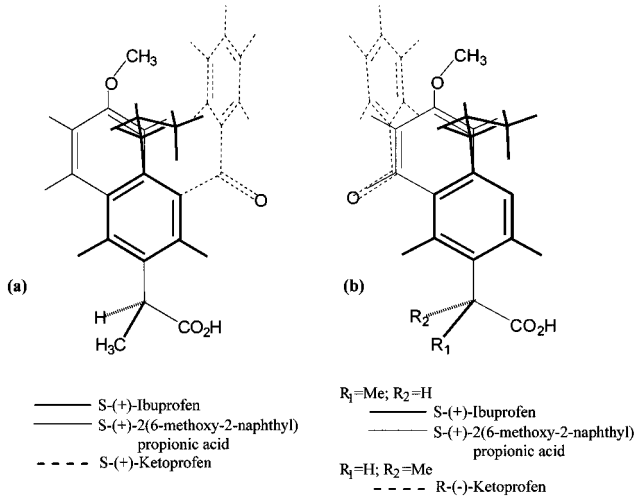

Figure 12. (a) Overlapped conformers of *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid, *S*-(+)-Ibuprofen and *S*-(+)-Ketoprofen. (b) Overlapped conformers of *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid, *S*-(+)-Ibuprofen, and *R*-(−)-Ketoprofen.

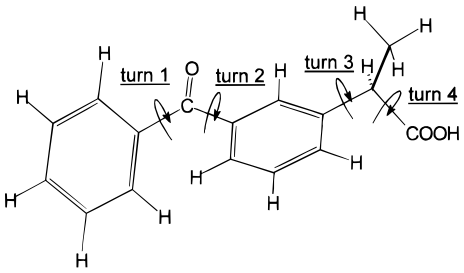

Figure 13. Spinning of *R*-(−)-Ketoprofen bonds.

The dimensions of the overlapped *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid and *S*-(+)-Ketoprofen conformers, shown in Figure 12a, confirm the biggest volume of *S*-(+)-Ketoprofen. By means of molecular dynamics, the Ketoprofen main bond spinning energy requirements (shown in Figure 13) were calculated in order to analyze its ability to adapt its structure to the active center of *Rh. miehei* lipase. The results are summarized in Table 8, where we can observe the high energy needed to turn the bonds around the carbonyl group of the benzoyl substituent of this molecule (turns 1 and 2, Figure 13). Similar studies were carried out for the other APA's (not shown), without obtaining any similar bond-turning

(38) HIPERCHEM V. 3.0 for Windows. Molecular modeling system. Hypercube, Inc. and Autodesk, Inc., 1993.

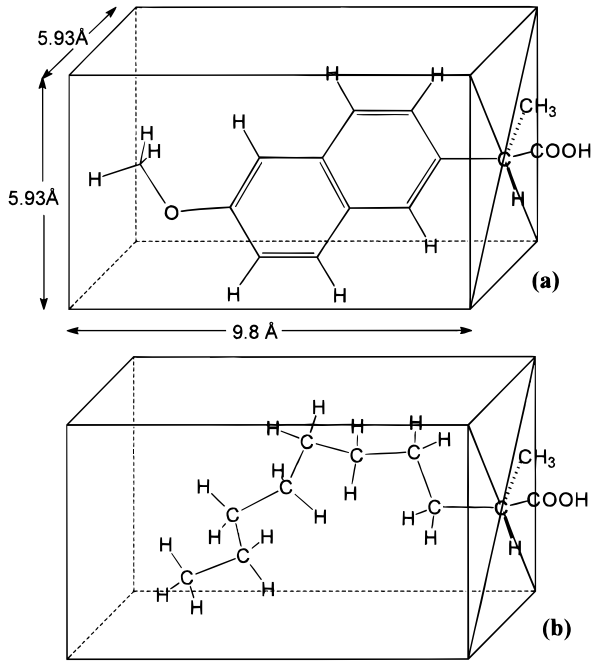

Figure 14. Dimensions of the "Ar" zone of *Rh. miehei* lipase, showing inside: (a) the minimum-energy conformer of *S*-(+)-2-(6-methoxy-2-naphthyl)propionic acid; (b) the minimum-energy conformer of 2-methyldecanoic acid.

Table 8. Energetic Increment of the Bond Turns of Ketoprofen

turn 1		turn 2		turn 3		turn 4	
angle	ΔE	angle	ΔE	angle	ΔE	angle	ΔE
α_0	0	α_0	0	α_0	0	α_0	0
$\alpha_0 + 70°$	336	$\alpha_0 + 70°$	330	$\alpha_0 + 120°$	43	$\alpha_0 + 90°$	−0.3
$\alpha_0 + 180°$	0.8	$\alpha_0 + 180°$	2.1	$\alpha_0 + 180°$	1.6	$\alpha_0 + 180°$	−0.6
$\alpha_0 + 250°$	340	$\alpha_0 + 250°$	301	$\alpha_0 + 240°$	8	$\alpha_0 + 270°$	5.7
$\alpha_0 + 310°$	4.5	$\alpha_0 + 320°$	4.8	$\alpha_0 + 310°$	40	$\alpha_0 + 360°$	0
$\alpha_0 + 360°$	0	$\alpha_0 + 360°$	0	$\alpha_0 + 360°$	0		

problems. Therefore, the molecule of Ketoprofen can be considered more rigid than the others because the spinning of bonds 1 and 2 (Figure 13) presents severe energetic impediments. Due to this fact it would be nearly impossible for the molecule of S(+)-Ketoprofen to rotate its bonds in order to be precisely recognized by the active center of *Rh. miehei* lipase. Thereby the lipase must "sacrifice" the recognition of the hydrogen and methyl groups, thought not to be the decisive factor for the enantioselection of APA's,[6d] and it must accept the "other" enantiomer allowing the entrance of the highly rigid benzoyl group of *R*-(−)-Ketoprofen in the large ("L") subsite of the active site, according to the well-established acyl-binding model of lipases.[39] Consequently, we can describe, at a qualitative level, the dimensions (Figure 14) of the "Ar" steric restriction zone able to accept the *S*-isomers of Ibuprofen, 2-(6-methoxy-2-naphthyl)propionic acid, and 2-phenylpropionic acid and the *R*-isomer of Ketoprofen. In this zone, the minimum-energy conformer of 2-methyldecanoic acid, the largest chiral substrate described for this enzyme,[40] would fit without any difficulty.

In conclusion we could explain the change of enantioselectivity of *Rh. miehei* lipase on Ketoprofen, as a consequence of its bulky and rigid structure and the active site flexibility.

Experimental Section

General. Lipozyme IM, lipase from *Rh. miehei* immobilized onto a porous granular weak base anion exchange resin (Duolite A568) (3.8 ± 0.4 LU/mg of protein), and crude lipase from *Rh. miehei* (Lipozyme 1000L) (88 ± 4 LU/mg of protein) were kindly donated by Novo Bioindustrias (Spain). Racemic 2-phenylpropionic acid was purchased from Fluka (Buchs, Switzerland). Racemic Ibuprofen ((*R,S*)-2-(4-isobutylphenyl)-propionic acid) was donated by Boots Pharmaceuticals (Nottingham, U.K.). Racemic and pure enantiomers of Ketoprofen ((*R,S*)-2-(3-benzoylphenyl)propionic acid) were kindly donated by Laboratorios Menarini S.A. (Badalona, Spain). (*R,S*)-2-(6-Methoxy-2-naphthyl)propionic acid was donated by Syntex Research (Palo Alto, CA). The alcohols were from Sigma (St. Louis, MO), and the organic solvents (with analytical grade) were from Merck (Darmstadt, Germany). Tributylthin was purchased from Aldrich (Steinhiem, Germany), and products of emulsification reagent (NaCl, KH_2PO_4, glycerin, and arabic gum) were from Sigma (St. Louis, MO).

Protein Determination. The protein content (64 ± 2 mg/mL) in the crude enzyme preparation (Lipozyme 10000L) was determined by the Biuret method[41] using bovine serum albumin as the standard. The protein content of the immobilized preparation (0.12 mg of protein/mg of derivative) was determined elsewhere.[7c]

General Procedure for Esterification. The standard reaction mixture was composed of organic solvent (10 mL), racemic 2-arylpropionic acid (0.125 M), and alcohol (0.125 M). The reaction was carried out at 37 °C by stirring in 25 mL flasks for a specified time. The reaction was started by adding some amount of immobilized lipase (Lipozyme IM). Then, aliquots of 0.1 mL were taken from the solution (at different times) and added to 1.4 mL of the same organic solvent; after microfiltration, they were analyzed by gas chromatography for calculating conversion. At the end of the reaction the mixture was filtered and analyzed by HPLC to determine the enantiomeric excess.

Gas Chromatography Analysis. This technique was performed in a Shimadzu GC-14A gas chromatograph equipped with FID detector, a split injector (1:2), and a SPB-1 sulfur column (15 m × 0.32 mm). Injector temperature was 300 °C and detector temperature was 350 °C; carrier gas was nitrogen. Different conditions for quantitative analysis were used depending on the compound: for 2-phenylpropionic acid the column temperature was 180 °C and a N_2 stream of 3 mL/min; for Ibuprofen the column temperature was 180 °C and the N_2 stream was 12 mL/min; for (*R,S*)-2-(6-methoxy-2-naphthyl)propionic acid and Ketoprofen the column temperature was 190 °C and the N_2 stream was 30 mL/min. An external standard method was employed to quantify the remnant acid and the formed ester.

HPLC Analysis. These analysis were performed using a Water-Millipore apparatus, Model 590, equipped with a chiral column of cellulose carbamate (25 cm × 0.46 cm) (Chiracel-OD; Daicel Chemical Industries Ltd.; Tokyo, Japan) capable of separating the *R*- and *S*-enantiomers of 2-arylpropionic acids. The mobile phase was different for each acid: the mobile phase composition for Ibuprofen was hexane/2-propanol/trifluoracetic acid (100/1/0.1) (v/v/v); for 2-phenylpropionic acid it was hexane/2-propanol/formic acid (98/2/1) (v/v/v), and for (*R,S*)-2-(6-methoxy-2-naphthyl)propionic acid it was hexane/2-propanol/acetic acid (97/3/1) (v/v/v). The flow of the mobile phase was 0.8 mL/min. The remnant acids were detected spectrophotometrically at 254 nm.

To analyze the enantiomeric excess of Ketoprofen another chiral column of cellulose ester (25 cm × 0.46 cm) was used (Chiralcel-OJ; Daicel Chemical Industries Ltd.; Tokyo, Japan). The mobile phase was (hexane/2-propanol/acetic acid (90/10/

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1) (v/v/v) (flo of mobile phase, 1 mL/min); the remnant acid was detected at 254 nm.

Measurement of Water Adsorption Isotherms. A 1 mL volume of crude lipase from *Rh. miehei* (Lipozyme 10000L) was frozen at −180 °C with liquid N_2. The samples (frozen crude lipase or immobilized lipase) were predried with P_2O_5. The a_w value of solid preparations was measured at 25 °C using a hygrometric sensor (Rotronic Hygroscopic D.T.) precalibrated with two saturated salt solutions at $a_w = 0.11$ and 0.98. The isotherms in cyclohexane were measured with the same amount of solids plus 1 mL of dried solvent.

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