

Selected Paper

How Does Lipase Flexibility Affect Its Enantioselectivity in Organic Solvents? A Possible Role of CH···π Association in Stabilization of Enzyme–Substrate Complex

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For lipase-catalyzed reactions of 2-(4-substitued phenoxy)propionic acids with alcohols in organic solvents containing a small amount of water, the increase of the lipase flexibility brought about by addition of water is found to be favorable for the induced-fit motion of the lipase for the correctly binding enantiomer of the substrate used, thus resulting in the improvement of the lipase enantioselectivity. In particular, for the reaction of the substrate with rich π electron density on its aromatic ring, the enantioselectivity was much more sensitive to the change of the lipase flexibility. Thus, in the induced fit motion, the CH···π association between amino acids side chains around the lipase's active site and the aromatic ring of the correctly binding enantiomer is assumed to accelerate the accommodation of the substrate into the lipase's active site and the stabilization of the complex between the enzyme and the substrate. This assumption is also supported by a discussion based on the value of the Michaelis constant obtained. Furthermore, on the basis of a model concerning the acyl-enzyme structure for the incorrectly binding enantiomer, the long alkyl chain alcohols as a nucleophile are found to improve the lipase enantioselectivity markedly.

The demand for enantiomerically pure compounds in the syntheses of pharmaceutical and agricultural chemicals has been increasing, in terms of displaying biological activity for only one of the enantiomers. Recently, biocatalyses are often employed as a useful method for the optical resolution, because of their ability to discriminate between enantiomers. In particular, lipases have been established as effective and cheaply available catalysts in organic solvents.[1] For lipase-catalyzed reactions in organic solvents, however, the enantioselectivity and/or activity of lipase are not always high, because the rigid conformation of lipases in organic solvents may prevent their active site from accepting the substrate.

As one of the classical manners to overcome this problem, it has been generally accepted that the addition of a small amount of water to organic solvents improves the enantioselectivity and/or activity in lipase-catalyzed reactions.[2] The mechanistic details for the improvement of the enantioselectivity, however, are little understood,[3] although the increase of the conformational flexibility brought about by addition of water is assumed to be mainly responsible for the improvement of the enantioselectivity.[2a–c,4] The question of whether the flexibility of enzymes plays an important role in the stability of the enzyme–substrate complex is essential with regard to the enhancement of their enantioselectivities.

In this paper, we report that the increase of the conformational flexibility of lipase brought about by addition of water is favorable for the association between the correctly binding enantiomer of the substrate and the lipase through its induced fit motion, thus leading to the improvement of the enantioselectivity for lipase-catalyzed esterification in organic solvents. The conformational flexibility of lipase was estimated by the ESR measurements. In this induced fit motion, a possible role of the CH···π association between the aromatic ring of the substrate and the methyl group of leucines around the lipase active site will be discussed in connection with the stabilization of the enzyme–substrate complex. Furthermore, on the basis of the model for the acyl-enzyme structure, the alcohol bearing the bulkier alkyl chain is found to serve as a better nucleophile to improve the enantioselectivity.

Results and Discussion

Effect of the Conformational Flexibility of Lipase on the Enantioselectivity for Lipase-Catalyzed Esterification in an Organic Solvent Containing a Small Amount of Water. For the esterification of 2-(4-substituted phenoxy)propionic acids catalyzed by *Candida rugosa* lipase MY with 1-butanol in hexane, two substrates bearing the substituents of similar size and contrasting electronic nature, CH_3 and CF_3, were chosen for the investigation of the effect of water as an additive on the enantioselectivity (Scheme 1). Lipase MY catalyzes the preferentially *R* enantiomer of the substrates used in our model reaction. Table 1 summarizes the results of the variation of the enantioselectivity (*E* value) in the esterification of the substrates **1** (X = CH_3) and **2** (X = CF_3) by changing an amount of water from 0 to 0.5 vol %. As is seen in Table 1, when a small amount of water was added to the reaction medium, the enantioselectivity

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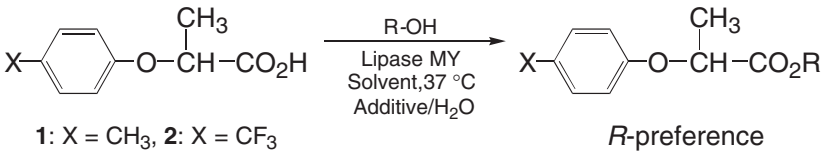


Scheme 1. Lipase-catalyzed esterification of 2-(4-substituted phenoxy)propionic acid with alcohol in organic solvents containing a small amount of water.

Table 1. The Effect of Water as an Additive on the Enantioselectivity (*E* value) in Lipase-Catalyzed Esterification of **1** and **2** with 1-Butanol in Hexane

Substrate	Substituent	vol %	Time/h	Convn./%	ee/%	*E*
1	CH_3	0	118	11	6.0	1.1
		0.1	27	14	65	5.2
		0.2	3	40	93	52
		0.3	6	45	94	75
		0.4	9	41	95	78
		0.5	20	44	90	40
2	CF_3	0	118	11	4.5	1.1
		0.1	27	8.9	59	4.1
		0.2	10	39	90	34
		0.3	14	41	88	29
		0.4	23	41	89	32
		0.5	49	41	85	22

of lipase was significantly affected by the amount of water added. In particular, upon the addition of an optimum amount of water (0.4 vol % of water for **1**, 0.2 vol % of water for **2**), lipase displayed the highest enantioselectivity, which of **1** or **2** was ca.71 or 31 times higher than that for no additive conditions, respectively. A decrease in the *E* value, however, was produced by addition of an excess amount of water (Table 1). This can be explained by assuming that an excess of water in the reaction medium causes the hydrolysis (the reverse reaction) of the corresponding ester product, thus leading to the loss of the enantioselectivity.

The addition of water to organic solvents is empirically known to increase the enzyme flexibility through the hydrogen bond formation between the enzyme and water added, thus leading to the improvement of the enzyme's enantioselectivity.[2a–c] In practice, in order to investigate the change of the conformational flexibility of lipase brought about by addition of water, the mobility of a spin label (4-(ethoxyfluorophosphoryloxy)-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO-4-EPF)) bound to the active site of lipase was examined by the ESR measurements in hexane with water changing from 0 to 0.5 vol %. The introduction of the spin-label into the lipase's active site was confirmed by the loss of the enzymatic activity in our model reaction (see experimental section). Figure 1 shows the typical ESR spectrum (0 or 0.5 vol % of water), in which two parts of the spectrum are arbitrarily labeled Ha and Hi, respectively. By monitoring the change in the ratio of the peak height of Hi to (Ha + Hi), the degree of the flexibility of lipase can be roughly estimated,[5] because each peak of the Ha and Hi represents the anisotropy and isotropy, respectively. Table 2 summarizes the variation of the Hi/(Ha + Hi) value by the amount of water added to hexane. As can be judged from the result that the addition of water produced the increase of the Hi/(Ha + Hi) value, the conformation around the lipase's active site becomes

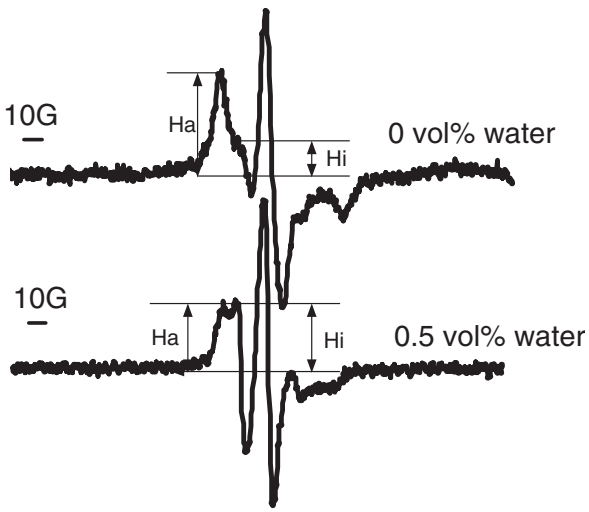


Fig. 1. Typical ESR spectrum of the spin-labeled lipase MY in hexane; (A) no additive condition, (B) 0.5 vol % of water.

Table 2. The Variation of the Hi/(Ha + Hi) Value as a Direct Measure of the Lipase Flexibility in Hexane with Water Changing from 0 to 0.5 vol % by the ESR Measurement

Water/vol %	Hi/(Ha + Hi)
0	0.114
0.1	0.266
0.2	0.387
0.3	0.455
0.4	0.542
0.5	0.548

Table 3. Kinetic Parameters in Lipase-Catalyzed Esterification of **1** and **2** with 1-Butanol in Hexane

Substrate	Substituent	Config.	Water	K_m	V_{max}	V_{max}/K_m
			vol %	mM	μmol h^{-1}	10^{-7} h^{-1}
1	CH_3	*R*	0	270	0.22	8.1
		R	0.4	160	4.5	281
		S	0	550	0.23	4.2
		S	0.4	390	0.56	14
2	CF_3	*R*	0	440	0.29	6.6
		R	0.4	440	5.2	118
		S	0	530	0.14	2.6
		S	0.4	370	0.53	14

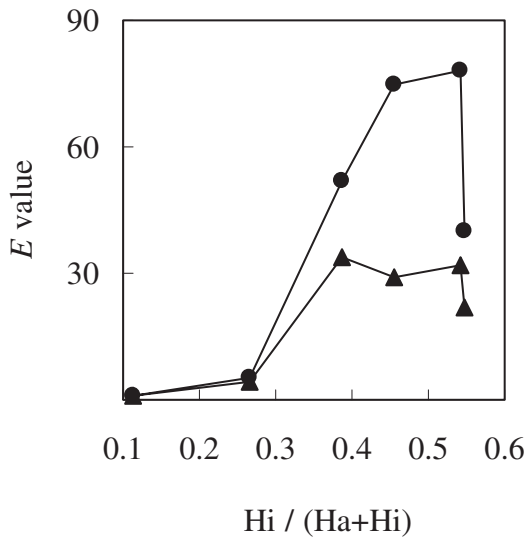


Fig. 2. Variation of the enantioselectivity (*E* value) as a function of the lipase's flexibility (Hi/(Ha + Hi) value) in lipase-catalyzed esterification of **1** (●) and **2** (▲) in hexane.

more flexible with the increase of water in the reaction medium.

Next, in order to clarify the effect of the lipase flexibility brought about by addition of water upon the enantioselectivity in changing the substrates from **1** (X = CH_3) to **2** (X = CF_3), we plot the *E* values for **1** and **2** as a function of the Hi/(Ha + Hi) value in Fig. 2. As is seen in Fig. 2, the enantioselectivity of **1** is found to be much more sensitive to the change of the flexibility around the lipase's active site than that of **2**; lipase displays the optimum flexibility to produce the maximal enantioselectivity toward the substrates, **1** and **2**. Taking into consideration the opposite electronic nature of the substituents, CH_3 and CF_3, one can speculate that the effect of the lipase flexibility on the variation of the enantioselectivity for **1** should be attributed to the electron-donating factor of the substituent (X = CH_3) far remote from the stereocenter of the substrate. In other words, the combined effects of the large lipase's flexibility and the increased π electron density of the aromatic ring by the electron-donating group such as CH_3 may be responsible for the marked improvement of the enantioselectivity of **1**.

A Possible Role of the CH···π Association in Stabilization of the Complex between the Substrate and Lipase. In order to elucidate the mechanistic details about the combined effects on the enantioselectivity, we investigated the Michaelis constant (K_m) and the maximal velocity (V_{max}) for lipase-catalyzed esterification of each enantiomer of **1** and **2** in hexane with 0 or 0.4 vol % of water (Table 3). For the correctly binding *R* enantiomer, the addition of water (0.4 vol %), corresponding to the optimum flexibility of lipase toward the substrate **1**, displayed more significant enhancement in the V_{max}/K_m value for **1** ($(V_{max}/K_m)_{0.4vol\%}/(V_{max}/K_m)_{0vol\%} = 35$) than for **2** ($(V_{max}/K_m)_{0.4vol\%}/(V_{max}/K_m)_{0vol\%} = 18$), although the values for the incorrectly binding *S* enantiomers of **1** and **2** were less sensitive to the increase of the lipase flexibility ($(V_{max}/K_m)_{0.4vol\%}/(V_{max}/K_m)_{0vol\%} = 3.3$ for **1** and $(V_{max}/K_m)_{0.4vol\%}/(V_{max}/K_m)_{0vol\%} = 5.9$ for **2**).

In order to gain further insights into the effect of lipase flexibility on the V_{max}/K_m and K_m values, we investigated the molecular arrangement to accommodate each enantiomer into the lipase's active site on the basis of the molecular modeling (Mac Spartan Pro) using the X-ray structure of *Candida rugosa* lipase obtained from the Brookhaven Data Bank.[6] Figure 3 displays the acyl-enzyme structure for the correctly binding *R* enantiomer of **1**. In the modeling for the acyl-enzyme structure of the correctly binding *R* enantiomer (overlapping van der Waals radius was avoided), we found that the aromatic ring of the substrate is accommodated well in the active site model and that two leucines are placed around the aromatic ring with almost van der Waals contact (Fig. 3). The latter finding suggests a possible role of CH···π association[7] between the methyl groups of the leucines and the π electrons of the substrate in stabilizing the enzyme–substrate complex (see discussion below). Figures 4a and 4b also show the space-fitting models for the acyl-enzyme structure of the *R* and *S* enantiomers viewed from the direction above the entrance to the tunnel of lipase, respectively. For the *R* enantiomer, the aromatic ring of the substrate lies in the hydrophobic area surrounding the active site; the yellow coloration corresponding to the carbonyl carbon of the substrate is placed in the entrance to the tunnel (Fig. 4a). In contrast to the *R* enantiomer, the situation for the incorrectly binding *S* enantiomer is just opposite location in the active site model; the entrance of lipase is assumed to be shielded by the presence of the aromatic ring of the *S* enantiomer (Fig. 4b).

Taking into account the model shown in Fig. 3, we suggest that one of the possible explanations for the different sensitivity for the flexibility effect on the V_{max}/K_m and K_m values between the substrates **1** and **2** is as follows. On the basis of the concept of the induced fit motions of the enzyme toward the substrate,[8] the larger flexibility of lipase is assumed to favor the preferred

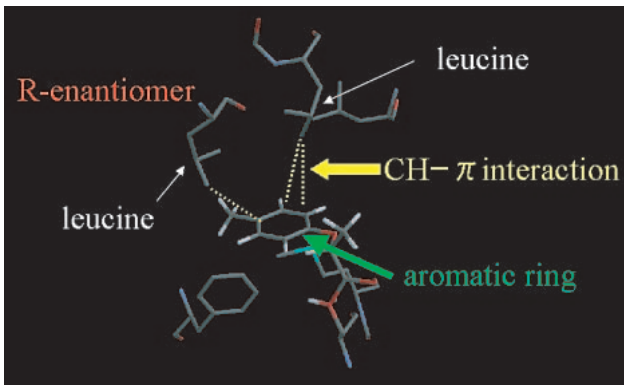
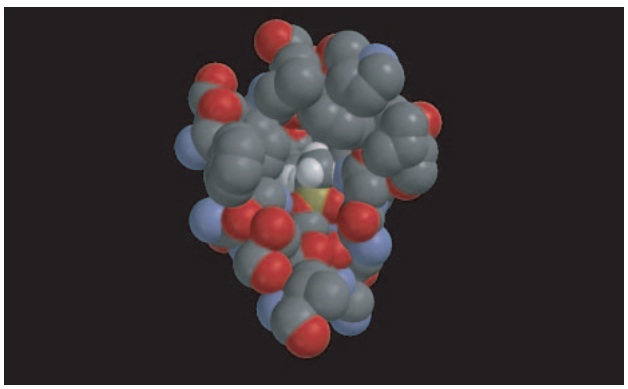


Fig. 3. Acyl-enzyme structure for the correctly binding *R* enantiomer of **1**.

(a)



(b)

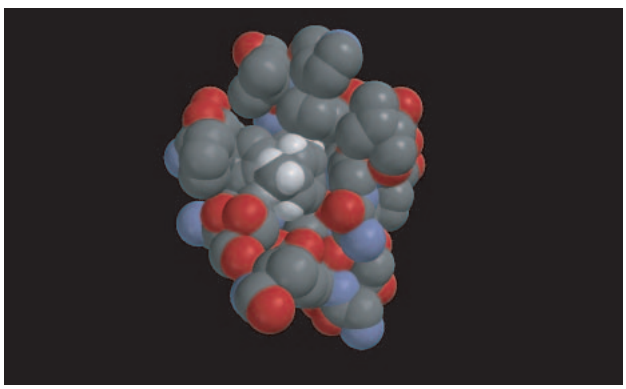


Fig. 4. Acyl-enzyme structure of (a) the *R* enantiomer and (b) the *S* enantiomer of **1** viewed from the direction above the entrance to the tunnel of lipase.

orientations of its amino acids side chains to associate effectively with the aromatic ring of the substrates used. For the correctly binding *R* enantiomer, the CH···π association between the methyl groups of the leucines and the π electrons of the substrate may bring about the more effective induced-fit motion for the correctly binding *R* enantiomer of **1**, as compared with that of **2**. In other words, the suitable CH···π association can accelerate the accommodation of the *R* enantiomer of **1** into the lipase's active site and the stabilization of the complex between the substrate and the lipase's active site, because the increase of the π electron density on the aromatic ring can be favorable for the CH···π association. In fact, as is seen from K_m value of the *R* enantiomer in Table 3, the increased lipase flexibility causes the more serious decrease in the K_m value of **1** with electron donating methyl group on the aromatic ring in contrast to the unchanged K_m value of **2** with electron withdrawing trifluoromethyl group.

Interestingly, the obtained V_{max} value displayed the different sensitivity to the lipase flexibility between the *R* and *S* enantiomers of the substrates **1** and **2** (Table 3). As is seen from V_{max} value in Table 3, the addition of 0.4 vol % of water, corresponding to the increased lipase flexibility, produced the larger V_{max} value for the correctly binding *R* enantiomer of **1** (20-fold) and **2** (18-fold) than that for no additive conditions, although the V_{max} value for the incorrectly binding *S* enantiomer is not appreciably increased by the flexibility effect (2.4-fold for **1**, 3.8-fold for **2**). One of the possibilities that reasonably explains our finding is a favorable attack of the nucleophile to the acyl-enzyme formed. One can see from Fig. 3 that, for the *R* enantiomer, the nucleophile such as 1-butanol can readily attack the carbonyl group, while the carbonyl group in the acyl-enzyme for the incorrectly *S* enantiomer is unlikely to be attacked by the nucleophile, due to the steric hindrance by the aromatic ring oriented toward the entrance of lipase. Consequently, for the *R* enantiomer, the large flexibility of lipase can allow easier access for the nucleophile and can accelerate the accommodation of the nucleophile into the active site, thus resulting in the increased V_{max} value. In sharp contrast to the *R* enantiomer, for the incorrectly binding *S* enantiomer, the serious steric difficulty in attacking the acyl-enzyme would cause the small sensitivity of the V_{max} value to the lipase flexibility. In addition, no additive conditions, corresponding to the rigid conformation of lipase, produced the small V_{max} value for both enantiomers of **1** and **2** (Table 3), because the rigid conformation of lipase would prevent its active site from accepting the nucleophile.

Furthermore, to test the above model for the acyl-enzyme structure, we investigated the effect of the long alkyl chain alcohol as a nucleophile on the enantioselectivity for lipase-catalyzed esterification of **1** in isopropyl ether containing a small amount of water (1.0 vol %) (Table 4). The idea is based on the view that for the incorrectly binding *S* enantiomer, the steric difficulty encountered by the nucleophile would be more serious than that for the *R* enantiomer. Isopropyl ether is chosen as a useful solvent, because it displays the good solubility for the most substrates. As is seen in Table 4, the reaction using the longer alkyl chain alcohol produced the improvement of

Table 4. Substituent Effect of Alcohol on the Enantioselectivity (*E* value) in Lipase-Catalyzed Esterification of **1** in Isopropyl Ether Containing a Small Amount of Water (1.0 vol %)

Alcohol	Time/h	Convn./%	ee/%	*E*
1-Propanol	18	39	69	8.2
2-Propanol	26	29	87	20
1-Butanol	8	41	87	26
1-Octanol	9	37	93	45

the enantioselectivity. The improvement of the enantioselectivity is mainly attributed to the lower reactivity of the *S* enantiomer for the alcohol bearing the longer alkyl chain, while the reactivity for the *R* enantiomer is increased by increase of the lipase flexibility brought about by addition of water. Thus, this result obtained also supports the model that for the *S* enantiomer, the serious steric difficulty in attacking the acyl-enzyme would cause the small sensitivity of the V_{max} value to the lipase flexibility. For lipase-catalyzed reactions, the choice of the alcohol as a nucleophile is found to be a useful method to improve the enantioselectivity.

Conclusions

For lipase-catalyzed reactions in organic solvents, the increase of the lipase flexibility brought about by addition of water is found to be favorable for the induced fit motion of the lipase for the correctly binding *R* enantiomer of the substrate **1**, thus resulting in the improvement of the lipase enantioselectivity. In the induced fit motion, the CH$\cdots\pi$ association between amino acids side chains around the lipase's active site and the aromatic ring of the *R* enantiomer is assumed to stabilize the complex between the enzyme and the substrate. Furthermore, on the basis of the model concerning the acyl-enzyme structure for the incorrectly binding enantiomer, the long alkyl chain alcohols that act as nucleophiles are found to improve lipase enantioselectivity markedly.

The success of the model should be of interest to organic chemists and useful for understanding the mechanism for the optimization of the lipase-catalyzed reactions in organic solvents.

Experimental

Materials. Lipase MY was supplied from Meito Sangyo Co., Ltd., Japan, and was semi-purified by dialyzing and lyophilizing from crude materials. Organic solvents were purchased from Wako Pure Chemical Industries, Ltd., Japan, and were dried over molecular sieves for more than 24 h before use. Racemic 2-(4-substituted phenoxy)propionic acids **1**–**2** were prepared by the reaction of the corresponding 4-substitiued phenol and ethyl 2-bromopropionate (Tokyo Kasei Kogyo Co., Ltd., Japan), followed by hydrolysis of the corresponding esters, according to a known method.[9]

Lipase-Catalyzed Esterification in Organic Solvent. The substrates **1**–**2** (0.18 mmol) and 1-butanol (1.08 mmol) were dissolved in hexane (2 mL). To the solution, a small amount of water was added, followed by ultrasonic dispersion, and then the semi-purified lipase (2 mg) was added. The suspension was shaken (170 strokes/min) at 37 °C. The *E* value was calculated from the enantiomeric excess (ee) for the butyl ester produced, according to the literature.[10] The conversion and ee were measured by HPLC on a chiral column (Chiralcel OK, from Daicel Chemical Industries Co., Ltd., Japan).

For the esterification of **1** with various alcohols, the substrate (0.36 mmol) and 1-butanol (1.08 mmol) were dissolved in isopropyl ether (2 mL). To the solution, 1.0 vol % of water was added, followed by ultrasonic dispersion, and then the crude lipase (30 mg) was added.

Michaelis–Menten Kinetic Parameters. The kinetic study was carried out by measuring the initial rates of the transesterification of each enantiomer of **1**–**2**. Each enantiomer was prepared by the lipase MY-catalyzed esterification, according to our method.[11] The concentration of each enantiomer was varied from 0.006–0.05 mmol and the concentration of 1-butanol was kept constant (0.15 mmol). Each enantiomer was submitted to the model reaction under the same additive conditions. At an appropriate time interval, aliquots were withdrawn and the supernatant was analyzed by HPLC on a chiral column to determine the initial rate at each substrate concentration. The values of K_m and V_{max} were obtained from the Michaelis–Menten equation. All the experimental data points obtained from the Lineweaver–Burk plot gave a straight line with the correlation coefficient >0.97. The values were reproducible to about $\pm5\%$ on repeated runs.

ESR Measurement. The active site (serine) of the semi-purified lipase was spin-labeled with 4-(ethoxyfluorophosphoryloxy)-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO-4-EPF) purchased from SIGMA, according to the procedure reported by Morrisett and Broomfield.[12] It can be assumed that the spin label has attached to the active site, because the spin-labeled lipase showed a clear decrease in the enzymatic activity for the esterification of **1**. Typically, about 35% of the active sites are considered to be labeled, as calculated from the residual enzymatic activity. All the ESR measurements were carried out at room temperature (ca. 25 °C) on a Bruker EMX081 spectrometer at X-band frequency in organic solvents containing 0.3 vol % of water.

Molecular Arrangement to Accommodate Each Enantiomer into the Lipase's Active Site. The molecular arrangement to accommodate each enantiomer into the lipase's active site was carried out on the basis of the molecular modeling (Mac Spartan Pro) using the X-ray structure of *Candida rugosa* lipase obtained from the Brookhaven Data Bank.[6] In the modeling for the acyl-enzyme structure of the correctly binding *R* enantiomer (overlapping van der Waals radius was avoided), the aromatic ring of the substrates **1**–**2** was accommodated well in the active site model and two leucines are placed around the aromatic ring with almost van der Waals contact.

References

1 a) T. Sugai, *Curr. Org. Chem.*, **3**, 373 (1999). b) S. M. Roberts, *J. Chem. Soc., Perkin Trans. 1*, **1999**, 1. c) H. Stecher and K. Faber, *Synthesis*, **1999**, 1. d) G. Carrea and S. Riva, *Angew. Chem., Int. Ed.*, **39**, 2226 (2000). e) A. M. Klibanov, *Nature*, **409**, 241 (2001).

2 a) A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, **263**, 8017 (1988). b) H. Kitaguchi, I. Itoh, and M. Ono, *Chem. Lett.*, **1990**, 1203. c) K. Kawashiro, H. Sugahara, S. Sugiyama, and H. Hayashi, *Biotechnol. Lett.*, **17**, 1161 (1995). d) M. Zarevúcka, M. Rejzek, M. Hoskovec, A. Svatos, Z. Wimmer, B. Koutek, and M.-D. Legoy, *Biotechnol. Lett.*, **19**, 745 (1997). e) M. T. López-Belmonte, A. R. Alcántara, and J. V. Sinisterra, *J. Org. Chem.*, **62**, 1831 (1997). f) A. Sánchez, F. Valero, J. Lafuente, and C. Solà, *Enzyme Microb. Technol.*, **27**, 157 (2000).

3 F. Theil, *Tetrahedron*, **56**, 2905 (2000).

4 S. Ueji, M. Nishimura, R. Kudo, R. Matsumi, K. Watanabe, and Y. Ebara, *Chem. Lett.*, **2001**, 912.

5 a) K. Watanabe, T. Yoshida, and S. Ueji, *Chem. Commun.*, **2001**, 1260. b) S. Ueji, T. Taniguchi, T. Okamoto, K. Watanabe, Y. Ebara, and H. Ohta, *Bull. Chem. Soc. Jpn.*, **76**, 399 (2003).

6 P. Grochulski, Y. Li, J. D. Schrag, F. Bouthillier, P. Smith, D. Harrison, B. Rubin, and M. Cygler, *J. Biol. Chem.*, **268**, 12843 (1993).

7 M. Nishio, M. Hirota, and Y. Umezawa, "The CH/π Interaction," Wiley-VCH, New York (1998).

8 a) T. L. Rosenberry, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3834 (1975). b) C. M. Anderson, F. H. Zucker, and T. A. Steitz, *Science*, **204**, 375 (1979). c) D. Herschlag, *Bioorg. Chem.*, **16**, 62 (1988).

9 D. T. Witiak, C.-L. Ho, W. E. Corner, and R. E. Hackney, *J. Med. Chem.*, **11**, 1086 (1968).

10 C.-S. Chen, Y. Fujimoto, G. Girdaukas, and C. J. Sih, *J. Am. Chem. Soc.*, **104**, 7294 (1982).

11 T. Okamoto and S. Ueji, *Chem. Commun.*, **1999**, 939.

12 J. D. Morrisett and C. A. Broomfield, *J. Biol. Chem.*, **247**, 7224 (1972).