

A Great Improvement of the Enantioselectivity of Lipase-Catalyzed Hydrolysis and Esterification Using Co-Solvents as an Additive

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Addition of co-solvents such as tetrahydrofuran resulted in a great improvement of the enantioselectivity of lipase-catalyzed hydrolysis of butyl 2-(4-substituted phenoxy)propanoates in an aqueous buffer solution. On the other hand, lipase lyophilized from an aqueous solution containing the co-solvents catalyzed highly enantioselective esterification of 2-(4-substituted phenoxy)propionic acids, 2-(4-isobutylphenyl)propionic acid (ibuprofen), and 2-(6-methoxy-2-naphthyl)propionic acid (naproxen) in an organic solvent. An increase in the *E* value up to two orders of magnitude was observed for some substrates. The origin of the enantioselectivity enhancement caused by the co-solvent addition was mainly attributed to a significant deceleration in the initial reaction rate for the incorrectly binding enantiomer, as compared with that for the correctly binding enantiomer. From the results of FT-IR, CD, and ESR spectra, the co-solvent addition was also found to bring about a partial destruction of the tertiary structure of lipase.

Lipase-catalyzed transformations play an important role in organic synthesis to fulfill the rapidly growing demand for enantiomerically pure compounds such as pharmaceuticals, agrochemicals, and natural products.[1] Native lipases, however, do not always show satisfying performance in terms of activity and most importantly enantioselectivity, because the structures of these synthetic targets are widely different from those of the triacylglycerols of their natural substrates. Enantioselectivity is one of the key features of lipases making them attractive for biocatalysis. For this reason, an applied study of the enantioselectivity enhancement of lipase is highly desirable.[2] In general, the enantioselectivity of lipase may be determined by a special relationship between the substrate structure and the origin of lipase, that is, the stereochemical environment of its active site. A traditional approach for this problem is to screen a large number of commercial lipases in the hope of finding a more enantioselective one. This procedure, however, seems to be somewhat tedious for organic chemists. Among several strategies to improve the enantioselectivity of lipase-catalyzed reactions, the method of additives seems to be attractive in view of its simplicity of use. In fact, various types of additives have been successfully applied to improve the outcome of the reactions.[3]

Here, we wish to report that the enantioselectivity for lipase-catalyzed hydrolysis is markedly improved by addition of co-solvents such as tetrahydrofuran to an aqueous buffer solution as the reaction medium (Scheme 1). On the other hand, lipase lyophilized from an aqueous solution containing the co-solvents also enhances the enantioselectivity of lipase-catalyzed esterification in an organic solvent (Scheme 1). Furthermore, the origin of the enhanced enantioselectivity of lipase caused by the co-solvent addition will be discussed briefly on the basis of the initial rate obtained for each enantiomer of the substrate used and the structural change of lipase estimated from the spectral results.

Results and Discussion

Effects of Co-Solvents on the Enantioselectivity of Lipase-Catalyzed Hydrolysis. The hydrolysis of butyl 2-(4-substituted phenoxy)propanoates **1**–**7**, butyl 2-(4-isobutylphenyl)propanoate **8** (butyl ester of ibuprofen), and butyl 2-(6-methoxy-2-naphthyl)propanoate **9** (butyl ester of naproxen) was catalyzed by *Candida rugosa* lipase Type VII in an aqueous buffer solution containing 0–30 vol % tetrahydrofuran (THF) (Scheme 1). Lipase Type VII favors the *R* enantiomers of **1**–**7** and the *S* enantiomers of **8** and **9**, however, these enantiomers have similar shapes, because of changes in priorities of the substituents. Some products from the hydrolysis of **1**–**7**, the *R* enantiomer of 2-aryloxypropanoic acids, are well-known herbicides and have other biological activities.[4] Furthermore, the *S* enantiomers of **8a** and **9a** belong to an important class of non-steroidal anti-inflammatory drugs.[5] Results of hydrolysis are listed in Table 1. The hydrolysis of these compounds without addition of THF gave low enantioselectivities (*E* values were less than 5), as was expected from the previously reported hydrolysis of methyl 2-aryloxypropanoates with *Candida rugosa* lipase (Table 1).[6] On the contrary, the addition of THF in the reaction mixture increased the enantioselectivities of the hydrolysis (Table 1). Thus, the reaction proceeded enantioselectively (*E* value > 100) when 20 vol % of THF was added to substrates **1**–**7**. For **8** and **9**, the reaction rate was too late to obtain a reliable *E* value.

In order to investigate the effect of co-solvent on the enantioselectivity of the reaction, other co-solvents such as acetonitrile, acetone, and dioxane were tested using **1** as the substrate. Figure 1 shows that all solvents tested had the ability to in-

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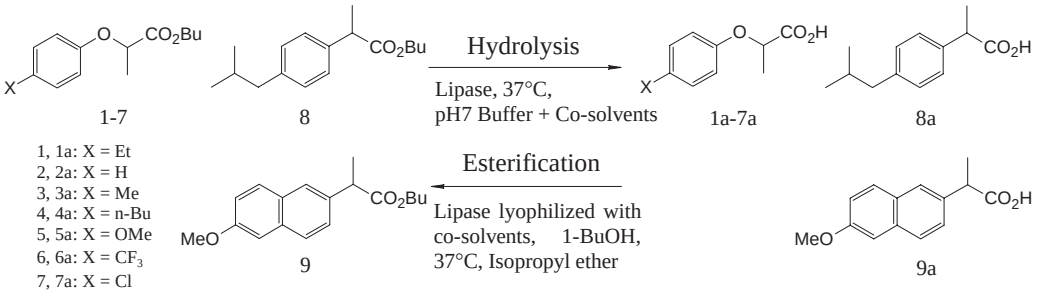

Scheme 1. Lipase-catalyzed hydrolysis of **1–9** and esterification of **1a–9a**.

Table 1. Enhancement of the Enantioselectivity of the Lipase Type VII-Catalyzed Hydrolysis of **1–7** by Addition of THF (0–30 vol %) to pH 7 Buffer at 37 °C

Substrate	THF/vol %	Time/min	Convn./%	ee/%	*E*
1	0	6	42	36	3
(X = Et)	10	25	43	48	4
	15	70	42	79	15
	20	220	44	96	112
	25	1440	19	97	91
	30	1440	4	95	37
2	0	35	47	13	1
(X = H)	10	100	41	53	5
	15	540	40	72	10
	20	1440	8	73	7
	25	21600	3	73	7
	30	1440	3	29	2
3	0	6	37	52	4
(X = Me)	10	30	42	55	5
	15	60	45	88	33
	20	150	37	97	138
	25	1440	16	99	171
	30	1440	2	80	9
4	0	15	37	35	3
(X = *n*-Bu)	10	50	46	27	2
	15	180	31	68	7
	20	1440	23	92	31
	25	1440	7	90	21
	30	1440	4	86	14
5	0	5	32	56	5
(X = OMe)	10	30	43	67	8
	15	110	43	86	25
	20	1440	42	97	129
	25	1440	14	98	105
	30	1440	1	81	10
6	0	8	41	38	3
(X = CF_3)	10	55	49	35	3
	15	195	44	54	5
	20	8640	9	88	18
	25	7200	7	87	9
	30	1440	5	30	2
7	0	5	33	30	2
(X = Cl)	10	90	38	81	15
	15	180	41	81	17
	20	1440	21	94	44
	25	1440	4	96	46
	30	1440	0	—	—

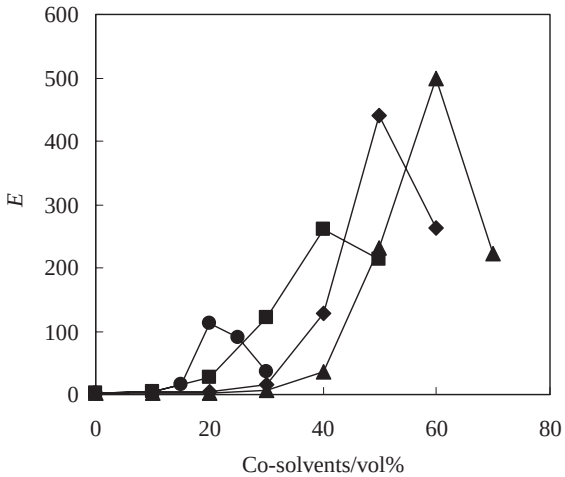

Figure 1. Effects of co-solvents (THF: ●, acetonitrile: ■, acetone: ◆, and dioxane: ▲) on the enantioselectivity of the lipase Type VII-catalyzed hydrolysis of **1** in pH 7 buffer at 37 °C. Reaction times at the maximum *E* value are 220 min (●), 1440 min (■), 260 min (◆), and 1440 min (▲), respectively.

crease enantioselectivity. Each of the co-solvents had its own optimum concentration for the maximum *E* value toward **1**. An increase in the *E* value up to two orders of magnitude was achieved by addition of these co-solvents (Figure 1). In particular, acetone and dioxane gave an excellent *E* value ($E > 400$), thus leading to almost complete resolution of the enantiomers of **1**. Several investigations have been undertaken on the influence of the co-solvents on the enantioselectivity of lipase-catalyzed reactions.[7] Although many methods have been reported to increase the enantioselectivity of lipase-catalyzed reactions,[2] the use of co-solvent in the reaction mixture is probably the most convenient and useful method.

Furthermore, to test the generality of this co-solvent effect, hydrolysis of **1** catalyzed by other readily available lipases was also carried out in the presence of THF. Figure 2 shows the variation of *E* values of different lipases as a function of concentration of THF. As can be seen from Figure 2, this co-solvent effect is not limited to lipase Type VII. The *E* value observed for lipases AY and OF from *Candida rugosa* was very sensitive to the addition of THF, which was accompanied by a marked improvement of their enantioselectivity, as well as lipase Type VII. Lipase AH from *Burkholderia cepacia* also

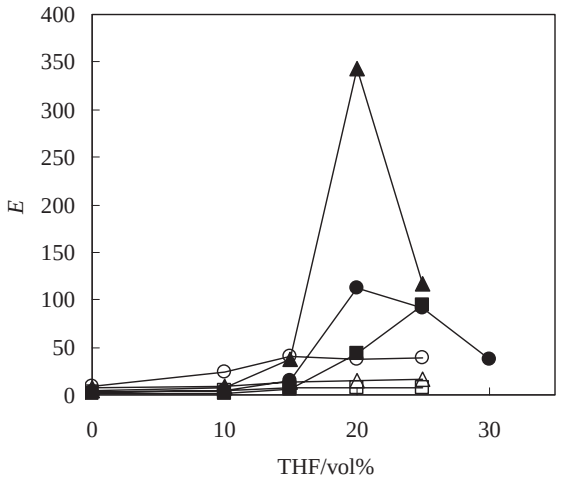

Figure 2. Variation of the enantioselectivity of different lipases (Type VII: ●, AY: ▲, OF: ■, AH: ○, AK: △, and PS: □) for the lipase-catalyzed hydrolysis of **1** by addition of THF to pH 7 buffer at 37 °C.

moderately increased the E value while the corresponding increase was small for lipase PS from *Burkholderia cepacia* and lipase AK from *Pseudomonas fluorescence* (Figure 2).

Effects of Co-Solvents on the Enantioselectivity of Lipase-Catalyzed Esterification. It is certainly of value to see if the co-solvent approach is valid in lipase-catalyzed esterification in an organic solvent. First, lipase Type VII-catalyzed esterification of **1a** (Scheme 1) was carried out in isopropyl ether containing THF as the co-solvent additive. In the presence of THF (10–30 vol %), however, the enantioselectivity of the esterification of **1a** decreased with an inhibition of the lipase activity (data not shown). It is logical to suppose that some of the essential waters, maintaining the lipase's structure and function, are stripped out by addition of water miscible co-solvent, THF.

Next, lipase Type VII was lyophilized from aqueous-THF (0–70 vol %) mixed solvents. Interestingly, this procedure increased the enantioselectivities of the esterification of **1a** in isopropyl ether compared to those using un-treated lipase. This treated lipase exhibited "solvent memory" as was observed in the hydrolysis of esters catalyzed by the crude lipase treated with organic solvents (Scheme 1 and Table 2).[8] As is seen in Table 2, the maximum E value ($E = 67$) for **1a** was obtained around 30 vol % THF, the E value of which was about 13 times as large as that for 0 vol % THF ($E = 5$). Therefore, the lipase Type VII lyophilized in the presence of 30 vol % THF was subjected to esterification using the other substrates **2a–9a** (Scheme 1 and Table 3); the enantiopreferences for **1a–9a** are the same as those in the hydrolysis. Inspection of the data summarized in Table 3 reveals that the remarkable enhancement of the E value brought about by the action of THF was observed for all the substrates studied here.

Furthermore, the effect of the lyophilized lipase on the enantioselectivity of the esterification of **1a** was examined using the other co-solvents and lipases, as in the case of the hydrolysis. As is seen from the plot in Figure 3, lipase Type VII lyophilized with various concentrations of the co-solvents (THF, acetonitrile, acetone, and dioxane) also produced

Table 2. Enantioselectivity of the Lipase-Catalyzed Esterification of **1a** in Isopropyl Ether at 37 °C by Use of Lipase Type VII Lyophilized with THF (0–70 vol %)

THF/vol %	Time/h	Convn./%	ee/%	E
0	30	42	55	5
10	30	38	73	10
15	30	38	80	15
20	30	38	87	24
25	30	38	91	35
30	30	34	95	67
40	48	40	85	23
50	48	39	89	30
60	48	39	88	27
70	48	41	82	17

Table 3. Enhancement of the Enantioselectivity of the Lipase-Catalyzed Esterification of **1a–9a** in Isopropyl Ether at 37 °C by Use of Lipase Type VII Lyophilized with THF (0 and 30 vol %)

Substrate	THF/vol %	Time/h	Convn./%	ee/%	E
1a	0	30	42	55	5
(X = Et)	30	30	34	95	67
2a	0	29	40	41	3
(X = H)	30	94	37	82	16
3a	0	29	41	55	5
(X = Me)	30	69	37	93	48
4a	0	44.5	38	43	3
(X = *n*-Bu)	30	94	35	82	16
5a	0	29	44	64	7
(X = OMe)	30	45	38	91	38
6a	0	44.5	30	58	5
($X = CF_3$)	30	94	36	83	17
7a	0	44.5	48	34	3
(X = Cl)	30	94	37	85	21
8a	0	170	9	94	36
	30	170	12	99	259
9a	0	167	12	94	35
	30	167	11	99	189

marked enhancement of the E value, although this effect was largest for THF.

Figure 4 represents the variation in the enantioselectivity of the esterification of **1a** catalyzed by various lipases lyophilized in the presence of 30 vol % THF. The E value for lipases Type VII, AY, and OF from *Candida rugosa* was found to be largely improved by lyophilization with THF. For instance, the E value of lipase OF was raised from $E = 3$ to $E = 355$. In contrast to this, the E value remained essentially unchanged for lipases AH and PS from *Burkholderia cepacia* and lipase AK from *Pseudomonas fluorescence* (Figure 4).

Effects of Co-Solvents on the Initial Rate of Each Enantiomer of the Substrate and the Structure of Lipase. In order to gain an insight into the origin of the enantioselectivity enhancement caused by the co-solvent addition, we investigated the initial rate of each enantiomer of **1** for the hydrolysis catalyzed by lipase Type VII in the presence of 20 vol % THF (Table 4) and that of **1a** for the esterification catalyzed by lipase Type VII lyophilized in the presence of 30 vol %

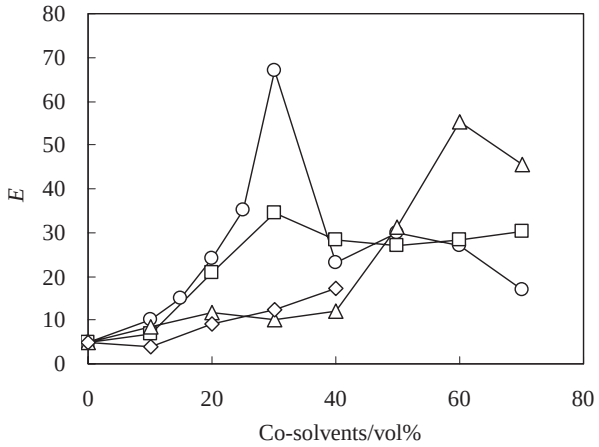

Figure 3. Effects of lyophilization of lipase Type VII with THF (○), acetonitrile (□), acetone (◇), and dioxane (△) on the enantioselectivity of the lipase-catalyzed esterification of **1a** in isopropyl ether at 37 °C. Reaction times at the maximum E value are 30 h (○), 25 h (□), 25 h (◇), and 28.5 h (△), respectively.

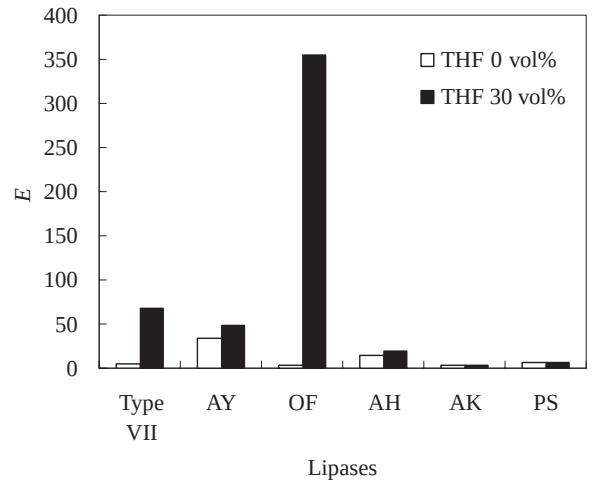

Figure 4. Variation of the enantioselectivity of lipases Type VII, AY, OF, AH, AK, and PS lyophilized with THF (0 and 30 vol %) for the lipase-catalyzed esterification of **1a** in isopropyl ether at 37 °C.

THF (Table 4). As is seen from the data summarized in Table 4, the initial rates of the R and S enantiomers for both hydrolysis and esterification in the presence of THF were decelerated, and its deceleration for the slower reacting (the incorrectly binding) S enantiomer is much more serious than that for the faster reacting (the correctly binding) R enantiomer. Thus, in the presence of THF, the larger value of the quotient of the initial rates (V_R/V_S) listed in Table 4, arising from the larger difference in the initial rates between the two enantiomers, is responsible for the significant enhancement of the enantioselectivity.

Then, a possible conformational change of lipase Type VII caused by the addition of THF was investigated on the basis of the results of FT-IR, CD, and ESR spectra. To estimate the secondary structure of the lipase, FT-IR measurements in the amide I region of lipase Type VII in D_2O containing THF

Table 4. Effects of THF on the Initial Rate of Lipase Type VII-Catalyzed Hydrolysis and Esterification of Each Enantiomer of **1** and **1a** in pH 7 Buffer and Isopropyl Ether, Respectively at 37 °C

Reaction (Substrate)	THF/vol %	Initial rate/μmol h^{-1} mg^{-1}		V_R/V_S	E
		V_R	V_S		
Hydrolysis	0	20.4	0.792	25.8	3
(**1**)	20	0.648	0.00288	225	112
Esterification	0	2.16	0.0912	23.7	5
(**1a**)	30	1.60	0.00580	275	67

Table 5. Effects of THF on the Secondary Structure of Lipase Type VII Determined by FT-IR in the Amide I Spectral Region

THF/vol %	Position/cm^{-1}	Assignment	Area/%
0	1621	β-aggregates	14
	1631	β-sheet	16
	1640	irregular	17
	1650	α-helix	33
	1659	α-helix	
	1669	β-turn	12
	1679	β-aggregates	8
20	1622	β-aggregates	15
	1631	β-sheet	16
	1641	irregular	17
	1650	α-helix	33
	1659	α-helix	
	1669	β-turn	12
	1680	β-aggregates	8

Table 6. Effects of Lyophilization with THF on the Secondary Structure of Lipase Type VII Determined by FT-IR in the Amide III Spectral Region

THF/vol %	Position/cm^{-1}	Assignment	Area/%
0	1229	β-sheet	13
	1240	random	
	1250	random	51
	1262	random	
	1276	random	
	1291	α-helix	
	1305	α-helix	37
	1319	α-helix	
30	1230	β-sheet	15
	1243	random	
	1257	random	51
	1269	random	
	1280	random	
	1292	α-helix	
	1306	α-helix	34
	1319	α-helix	

and in the amide III region of lipase Type VII lyophilized in the presence of THF were carried out. All the IR bands in the amide I and III regions were assigned to individual secondary structural elements (Tables 5 and 6), according to a known method of Gaussian curve fitting.[9] As is seen from

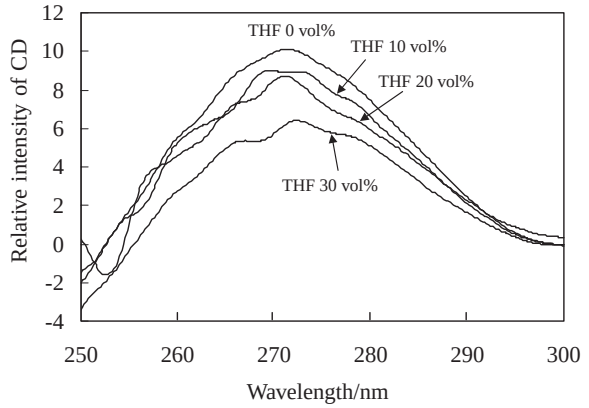

Figure 5. CD spectra of lipase Type VII dissolved in water containing THF (0, 10, 20, and 30 vol %).

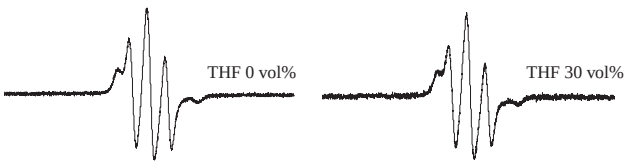

Figure 6. ESR spectra of the spin-labeled lipase Type VII lyophilized with THF (0 and 30 vol %).

the data listed in Tables 5 and 6, the contents of the α-helix (33–37%) and β-sheet (13–16%) calculated from the areas of the IR bands obtained are nearly identical to the X-ray crystallographic data (33% α-helix and 12% β-sheet).[10] Furthermore, it was found by comparison of the data in Tables 5 and 6 that both IR spectra in the presence and absence of THF showed almost the same secondary structure composition in the amide I and III regions. Thus, the native secondary structure of lipase Type VII seems not to be changed by the addition of THF.

On the other hand, the near-UV CD spectrum in water showed a marked decrease of the relative intensity of the positive band in the range from 260 to 290 nm, corresponding to the tertiary structure of lipase, upon addition of 0–30 vol % THF (Figure 5). From this observation, one could speculate that a partial destruction of the tertiary structure of lipase Type VII produces a more compact conformation or a local conformational change around its active site. In fact, the incorrectly binding *S* enantiomer causes a significant decrease in the initial rate, compared with the correctly binding *R* enantiomer (see also Table 4), probably because the steric difficulty encountered by the *S* enantiomer would become more serious in fitting into the more compact binding pocket.

In connection with a partial destruction of the tertiary structure of lipase, the ESR spectrum of lipase Type VII with a spin label was taken to estimate its conformational flexibility (Figure 6). In the previous study, we showed that the enantioselectivity of lipase in organic solvents is mainly controlled by its conformational flexibility estimated from the ESR spectrum.[11] As judged from the ESR spectrum in Figure 6, contrary to our expectations, the spectral lines were found to remain unchanged even in the presence of 30 vol % THF. In other words, THF provides no direct effect on the conformational flexibility of lipase.

Conclusion

The technique of co-solvent addition provided a simple and powerful tool to improve the enantioselectivity of lipase-catalyzed hydrolysis. Also, the lipase lyophilized with the co-solvent catalyzed nicely the esterification with high enantioselectivity. The enantioselectivity enhancement observed was found to be mainly attributed to the serious deceleration in the initial rate for the incorrectly binding *S* enantiomer, arising from a partial destruction of the tertiary structure of lipase.

Experimental

Materials. Lipase OF and lipases AY, PS, AH, and AK were generously provided by Meito Sangyo Co., Ltd. and Amano Pharmaceutical Co., Ltd., respectively. Lipase Type VII was purchased from Sigma Chemical. These lipases were used without further purification for the hydrolysis and esterification. For the spectral measurements, lipase Type VII was semi-purified by dialyzing and lyophilizing from crude material. The MALDI-TOF-MS spectrum of the semi-purified lipase Type VII showed a single parent peak, m/z 60.2 kDa, which is consistent with the molecular weight of *Candida rugosa* lipase. Ibuprofen and naproxen were purchased from Tokyo Kasei Kogyo Co., Ltd. All other chemicals were from commercial sources and of reagent grade.

Preparation of Substrates. According to a known method,[12] the substrates **1a–7a** (racemic carboxylic acids) were prepared from the corresponding 4-substituted phenols and ethyl 2-bromopropionate, followed by the hydrolysis of the esters. The products were purified by recrystallization from hexane. The substrates **1–7** (racemic butyl esters) were prepared from the corresponding substrates **1a–7a** and 1-butanol. The *R* or *S* single enantiomer of **1a** (ca. 98% ee) was prepared according to our method.[13]

Lipase-Catalyzed Hydrolysis. In a typical procedure, the substrates **1–9** (racemic butyl esters, 0.036 mmol) were added to pH 7 phosphate buffer solution containing the co-solvent such as tetrahydrofuran (2 mL), followed by the ultrasonic dispersion, and lipase (10 mg) was added. The suspension was shaken (170 strokes min^{-1}) at 37 °C until approximately 40% of the substrates had reacted. After the reaction, the product and residual substrate were extracted into hexane, and the filtered aliquot was used as the sample solution.

Lipase-Catalyzed Esterification. In a typical procedure, the substrates **1a–9a** (racemic carboxylic acids, 0.36 mmol) and 1-butanol (1.08 mmol) were dissolved in diisopropyl ether (2 mL). To the solution, 1.2 vol % of water (24 μL) was added, followed by ultrasonic dispersion, and lipase (30 mg) was added. The suspension was shaken (170 strokes min^{-1}) at 37 °C until approximately 40% of the substrates had reacted. After the reaction, the filtered aliquot was used as the sample solution.

Determination of the Enantiomeric Ratio (*E* Value). For measurements of the enantiomeric excess (ee) of the product, the sample solutions obtained from lipase-catalyzed hydrolysis and esterification were analyzed by HPLC (Shimadzu LC-10A) on a chiral column (CHIRALCEL OK or CHIRALCEL OD-H, Daicel Chemical Industries Co., Ltd.) using a mixture of hexane, 2-propanol, and trifluoroacetic acid (970:30:1) as a mobile phase. The enantiomeric ratio (*E* value) was calculated from the enantio-

meric excess (ee), according to the literature.[14]

Measurement of the Initial Rate for Lipase-Catalyzed Hydrolysis of Each Enantiomer of 1. In a typical procedure, (*R*)- or (*S*)-**1** (0.018 mmol) was added to pH 7 phosphate buffer solution containing the co-solvent (2 mL), followed by ultrasonic dispersion, and lipase (5 mg) was added. The suspension was shaken (170 strokes min^{-1}) at 37 °C. At appropriate times, the product and residual substrate were extracted into hexane, and the filtered aliquot was analyzed by HPLC conditions shown above to obtain the conversion. Several data points were collected to determine the initial rate.

Measurement of the Initial Rate for Lipase-Catalyzed Esterification of Each Enantiomer of 1a. In a typical procedure, (*R*)- or (*S*)-**1a** (0.18 mmol) and 1-butanol (0.50 mmol) were dissolved in diisopropyl ether (2 mL). To the solution, 1.2 vol % of water (24 μL) was added, followed by ultrasonic dispersion, and lipase (15 mg) was added. The suspension was shaken (170 strokes min^{-1}) at 37 °C. At appropriate times, the filtered aliquot was analyzed by HPLC conditions shown above to obtain the conversion. Several data points were collected to determine the initial rate.

FT-IR Measurements of Lipase Type VII and Secondary Structure Analysis. The FT-IR measurements were performed at room temperature on a BRUKER TENSOR 27 spectrometer purged with dry N_2. All the spectra were averaged over a total of 100 scans at 2 cm^{-1} resolution. For the secondary structure analysis of lipase, the IR spectra were taken as solutions of lipase Type VII (50 mg mL^{-1}) in D_2O containing 0, 30 vol % THF (corresponding to the hydrolysis) and as KBr tablets of lipase Type VII lyophilized in the presence of THF (corresponding to the esterification). The spectral data obtained were deconvoluted by using a BRUKER OPUS 5.0 curve-fitting module.

CD Measurements of Lipase Type VII and Tertiary Structure Analysis. The CD measurements were performed at room temperature on a JEOL MN-EX90A spectrometer. For the tertiary structure analysis of lipase, the CD spectra of solutions of lipase Type VII (4 mg mL^{-1}) in water containing 0–30 vol % THF were measured.

Spin-Labeling to the Lipase Type VII and ESR Measurements. The methionine residues on the surface of lipase Type VII were spin-labeled with 4-(2-indoacetamido)-TEMPO purchased from Aldrich Chemical, according to a procedure reported by Morrisett and Broomfield.[15] The spin-labeled lipase Type VII lyophilized from aqueous solution containing 0 and 30 vol % THF was subjected to the ESR measurements in diisopropyl ether with 0.3 vol % water. All the ESR measurements were carried out at room temperature on a BRUKER EMX081 spectrometer at X-band frequency.

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