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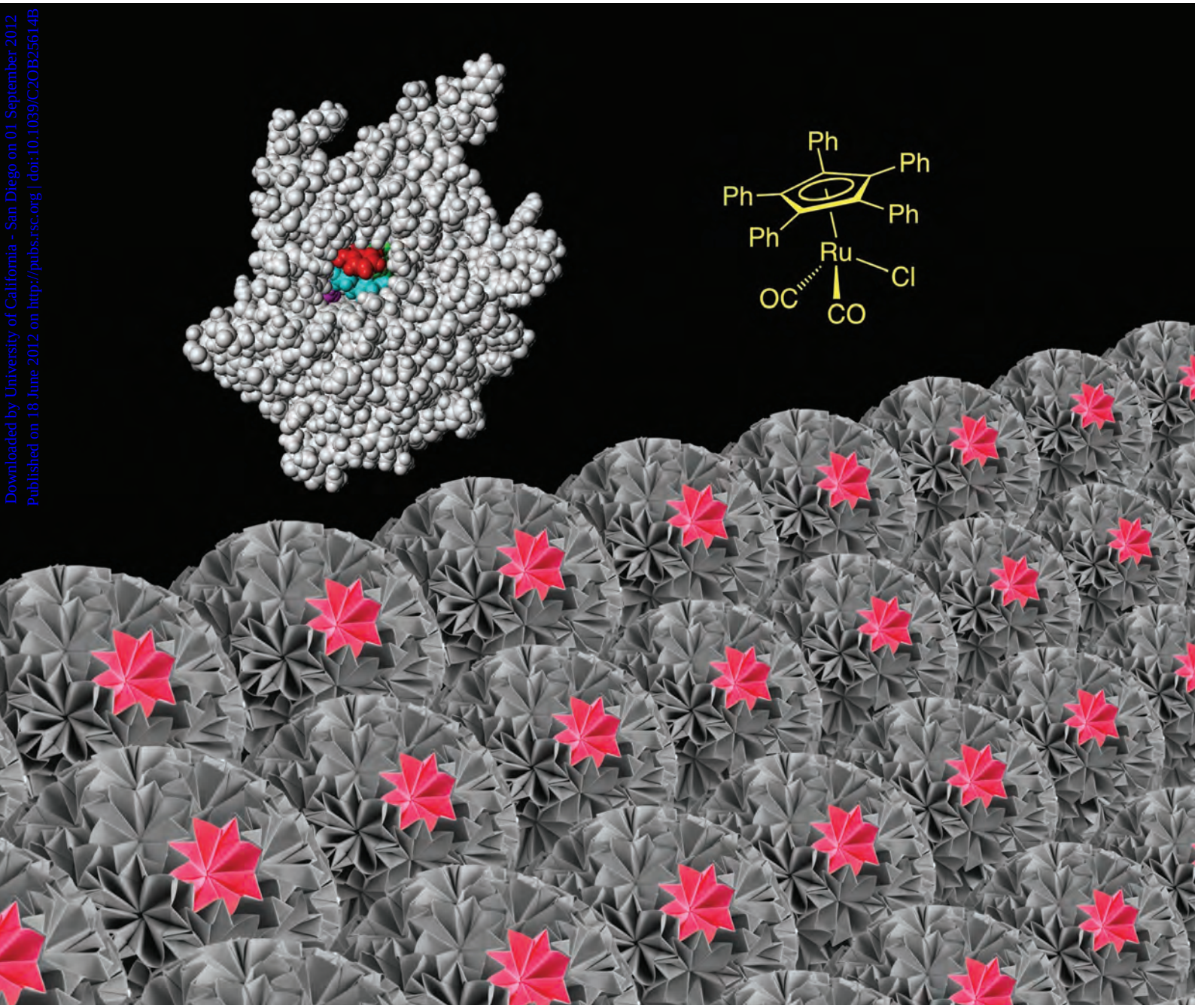

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PAPER

Redesign of enzyme for improving catalytic activity and enantioselectivity toward poor substrates: manipulation of the transition state†

Tadashi Ema,* Yasuko Nakano, Daiki Yoshida, Shusuke Kamata and Takashi Sakai

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Secondary alcohols having bulky substituents on both sides of the hydroxy group are inherently poor substrates for most lipases. In view of this weakness, we redesigned a *Burkholderia cepacia* lipase to create a variant with improved enzymatic characteristics. The I287F/I290A double mutant showed a high conversion and a high *E* value (>200) for a poor substrate for which the wild-type enzyme showed a low conversion and a low *E* value (5). This enhancement of catalytic activity and enantioselectivity of the variant resulted from the cooperative action of two mutations: Phe287 contributed to both enhancement of the (*R*)-enantiomer reactivity and suppression of the (*S*)-enantiomer reactivity, while Ala290 created a space to facilitate the acylation of the (*R*)-enantiomer. The kinetic constants indicated that the mutations effectively altered the transition state. Substrate mapping analysis strongly suggested that the CH/π interaction partly enhanced the (*R*)-enantiomer reactivity, the estimated energy of the CH/π interaction being −0.4 kcal mol^{-1}. The substrate scope of the I287F/I290A double mutant was broad. This biocatalyst was useful for the dynamic kinetic resolution of a variety of bulky secondary alcohols for which the wild-type enzyme shows little or no activity.

Introduction

Enzymes are powerful biocatalysts that can accelerate chemical reactions enormously.[1] The k_{cat} values, known as turnover numbers, range from 10^2 to 10^6 s^{-1} for natural substrates.[2,3] Considerable stabilization of transition states accounts for such high turnover numbers.[3] In contrast, enzymatic reactions for unnatural substrates are much slower, and in some cases they are poor substrates with little or no reactivity. If alteration of the enzyme structure can stabilize the transition state, such a poor substrate may become a good substrate. Another important aspect of enzymatic reactions is high enantioselectivity for natural substrates. However, enzymatic reactions do not necessarily attain high enantioselectivity for unnatural substrates. The most straightforward way of solving this problem is to create a mutant enzyme with an improved catalytic function.[4]

There are two major ways of creating variants: directed evolution and rational design. Directed evolution, which uses random mutagenesis and high-throughput screening of a large number of variants, can improve an enzymatic property without knowledge of the enzyme structure and reaction mechanism.[5–7] In contrast, once the reaction mechanism has become clear, rational design, which uses site-directed mutagenesis, is also useful.[8–11] Comparison of the random and rational approaches reveals that the latter seems to be more difficult and inefficient than the former, and therefore directed evolution has recently become the most popular method. Nevertheless, rational approaches are also becoming more attractive as a mechanistic understanding of biocatalysis is enhanced.[8–11]

Lipases are synthetically useful biocatalysts that can show high enantioselectivity and broad substrate specificity in both aqueous and nonaqueous media.[1] However, the kinetic resolution of secondary alcohols bearing bulky substituents on both sides of the hydroxy group remains difficult. Alteration of the enzyme structure may overcome this drawback. We have previously used mechanistic knowledge to successfully control (both increase and decrease) the enantioselectivity of a *Burkholderia cepacia* lipase with a single mutation.[10a] In that study, we controlled enantioselectivity by modulation of steric repulsion between the enzyme and the slower-reacting (*S*)-enantiomer. More recently, we have created a variant that enhances the reactivity of the (*R*)-enantiomer and that suppresses the reactivity of the (*S*)-enantiomer.[10b] Here we report in detail the rational creation of mutant lipases that display remarkably enhanced catalytic activity and enantioselectivity for poor substrates bearing bulky substituents on both sides of the hydroxy group. Among several variants, the I287F/I290A double mutant was the best biocatalyst, being useful not only for the kinetic resolution

Division of Chemistry and Biochemistry, Graduate School of Natural Science and Technology, Okayama University, Tsushima, Okayama 700-8530, Japan. E-mail: ema@cc.okayama-u.ac.jp; Fax: +81-86-251-8092; Tel: +81-86-251-8091

†Electronic supplementary information (ESI) available: Site-directed mutagenesis, synthesis of **1**, lipase-catalyzed reactions of **1**, and copies of NMR spectra. See DOI: 10.1039/c2ob25614b

but also for the dynamic kinetic resolution (DKR) of bulky substrates for which the wild-type enzyme showed little or no activity. The kinetic study demonstrated that the mutations effectively manipulated the transition state of the enzymatic reaction.

Results and discussion

Theoretical basis and working hypothesis

We have proposed a transition-state model to explain the enantioselectivity of lipases for secondary alcohols (Fig. 1a shows an expanded version).[12] Enantioselectivity results principally from the conformational requirements and repulsive interactions in the transition state, and no attractive interactions between the enzyme's pockets and the substrate's substituents are involved. In other words, the (*R*)-preference of lipases results from a suppression mechanism working on the (*S*)-enantiomer in the transition state. This mechanism has been supported by kinetic and thermodynamic analyses,[12a,c] and the use of a large secondary alcohol, 5-[4-(1-hydroxyethyl)phenyl]-10,15,20-triphenylporphyrin.[12b]

Improvement of the catalytic activity and enantioselectivity of an enzyme for a poor substrate may be achieved by a scenario that involves careful alteration of the enzyme structure (Fig. 2). Removal of steric hindrance or introduction of attractive interactions may stabilize the (*R*)-enantiomer in the transition state, while introduction of steric hindrance may destabilize the (*S*)-enantiomer in the transition state. As the mutation sites, we selected the following three amino acid residues, all of which are in proximity to the catalytic residues: Ile287, Ile290, and Gln292 (Fig. 1a). Although His86 was also a candidate for mutation, we found this amino acid residue to be crucial for enzymatic activity as predicted previously (data not shown).[12a] We therefore left His86 unchanged in this study.

We employed 1-phenyl-1-hexanol (**1a**) as a poor substrate to perform a docking experiment with respect to the transition-state model (Fig. 1a). The wild-type enzyme has Ile287, which appears to conflict with the alkyl chain of (*R*)-**1a** (Fig. 1b). We hypothesized that the I287F mutation might realize the strategy shown in Fig. 2 if Phe287 had the following two roles in the transition state: an attractive interaction with (*R*)-**1a** and a repulsive interaction with (*S*)-**1a**. More specifically, Phe287 appears to make favorable contact with the alkyl chain of (*R*)-**1a** (Fig. 1c), which would accelerate the acylation of (*R*)-**1a**, while Phe287 is likely to come into unfavorable contact with the phenyl group of (*S*)-**1a**, which would hinder the acylation of (*S*)-**1a** (not shown).[10a] On the other hand, Ile290 is the second mutation site, which also seems to conflict with the alkyl chain of (*R*)-**1a** (Fig. 1c). We hypothesized that the I290A mutation would create a space to accommodate (*R*)-**1a** nicely in the transition state (Fig. 1d), leading to higher catalytic activity. Gln292 is the mutation site farthest from the active site. We hypothesized that the Q292A mutation would also create a larger space for (*R*)-**1a** (Fig. 1e).

Rational creation of mutant enzymes

We conducted the recombinant lipase-catalyzed kinetic resolutions of **1a** and **1b** with vinyl acetate in dry *i*-Pr_2O at 30 °C

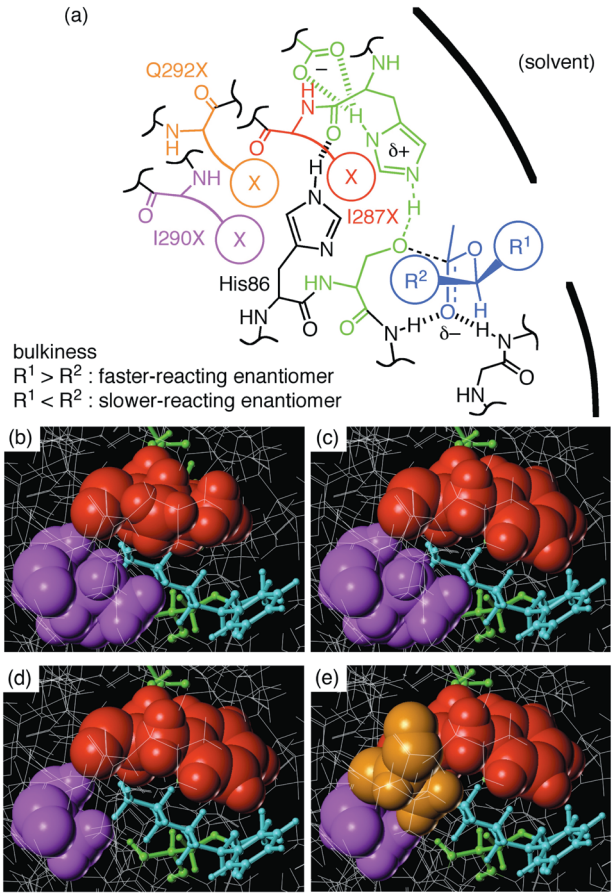


Fig. 1 (a) The transition-state model to rationalize the enantioselectivity in the lipase-catalyzed kinetic resolution of secondary alcohols (residues 287, 290, and 292 are added to the original version). (i) The C–O bond of the substrate takes the *gauche* conformation with respect to the breaking C–O bond, which is due to the stereoelectronic effect. (ii) The hydrogen atom attached to the stereocenter in the substrate is *syn*-oriented toward the carbonyl oxygen atom to minimize the torsional strain. Enantioselectivity is explained by the conformational requirements and repulsive interactions and/or strains caused in the transition state. The catalytic triad residues, the ester being produced, residue 287, residue 290, and residue 292 are shown in green, blue, red, magenta, and orange, respectively. Typically, the (*R*)-enantiomer reacts faster because, in this favorable conformation shown in blue, the larger substituent (R^1) can be directed toward external solvent without severe strain and/or steric hindrance. (b)–(e) The active sites of (b) the wild-type enzyme, (c) the I287F mutant, (d) the I287F/I290A double mutant, and (e) the I287F/I290A/Q292A triple mutant. (*R*)-**1a** connected to Ser87 in the tetrahedral intermediate is shown in light blue. Gln292 is omitted for clarity in (b)–(d).

(Scheme 1) and evaluated enantioselectivity on the basis of *E* values.[13] The results are summarized in Table 1. Because of the low activity of the wild-type enzyme for **1a**, high catalyst loading and a long reaction time were required (entry 1). To our delight, the conversion and *E* value of the I287F mutant for **1a** were twice and six times, respectively, the corresponding values of the wild-type enzyme (entry 2). Homolog **1b** exhibited the same trend (entries 10 and 11). In contrast, the I287A, I287W, and I287Y mutations resulted in lower conversions of **1a** (entries 3–5). These results suggest that only the I287F mutant had a

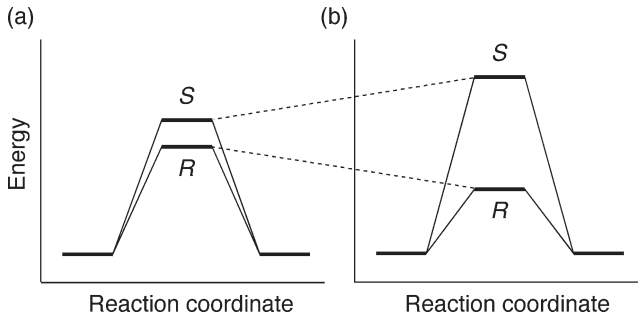
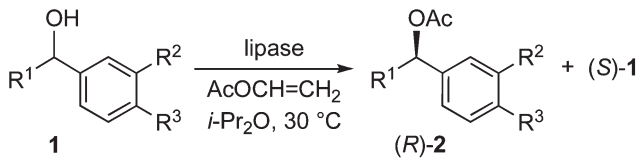


Fig. 2 (a) Energy diagram for an enantioselective enzymatic reaction toward a poor substrate. (b) Improvement of catalytic activity and enantioselectivity by stabilization of the (*R*)-enantiomer and destabilization of the (*S*)-enantiomer in the transition state.



a–q	R^1	R^2	R^3
a	*n*-C_5H_{11}	H	H
b	*n*-C_6H_{13}	H	H
c	$(CH_2)_4CF_3$	H	H
d	$(CH_2)_2(CF_2)_2CF_3$	H	H
e	CH_2OMOM	H	H
f	$(CH_2)_3CH{=}CH_2$	H	H
g	$(CH_2)_4OMOM$	H	H
h	$(CH_2)_5Ph$	H	H
i	$(CH_2)_3$*i*-Pr	H	H
j	*n*-C_5H_{11}	H	CF_3
k	*n*-C_5H_{11}	H	OMe
l	*n*-C_5H_{11}	OMOM	H
m	*n*-C_5H_{11}	-$(CH)_4$-	
n	*n*-Bu	H	H
o	*n*-Pr	H	H
p	Et	H	H
q	Me	H	H

Scheme 1 Lipase-catalyzed kinetic resolution of **1**. Only **2e** has the (*S*)-configuration according to the Cahn–Ingold–Prelog (CIP) priority system for nomenclature.

specific mechanism to enhance the reactivity of (*R*)-**1a**, such as an attractive interaction between Phe287 and the alkyl chain of (*R*)-**1a**, which was supported by kinetic measurements and substrate mapping analysis (*vide infra*). Steric bulkiness of Trp287 and Tyr287 seems to hinder the acylation, although we expected the same enhancement effect as that produced by Phe287.

We next examined a second-generation biocatalyst, the I287F/I290A double mutant. Surprisingly, this variant exhibited much higher activity and enantioselectivity for **1a** and **1b** (Table 1, entries 6 and 12). In both cases the conversions reached almost 50% within a few hours, and the *E* values exceeded 200. These results, which were beyond our expectation, strongly suggest that the effects of the double mutations were cooperative. In sharp contrast with the I287F/I290A double mutant, the I287F/I290F double mutant showed very poor activity and enantioselectivity for **1a** (entry 7). The I290A mutation had a positive effect on catalytic activity and enantioselectivity (entry 8). These results strongly suggest that the second mutation (I290A) enhanced the reactivity of (*R*)-**1a** and (*R*)-**1b** by eliminating steric hindrance.

Table 1 Kinetic resolution of **1** with the wild-type and mutant enzymes[a]

Entry	**1**	Lipase	Time (h)	c^b (%)	E^c
1	**1a**	Wild-type	41	23	5
2	**1a**	I287F	41	46	32
3	**1a**	I287A	41	20	1.4
4	**1a**	I287W	41	17	5
5	**1a**	I287Y	41	10	13
6	**1a**	I287F/I290A	2.5	50	>200
7	**1a**	I287F/I290F	41	10	4
8	**1a**	I290A	41	41	79
9	**1a**	I287F/I290A/Q292A	6	45	52
10	**1b**	Wild-type	41	39	9
11	**1b**	I287F	22	47	71
12	**1b**	I287F/I290A	4	47	>200
13	**1c**	Wild-type	41	34	14
14	**1c**	I287F	22	47	55
15	**1c**	I287F/I290A	4	50	>200
16	**1d**	I287F/I290A	75	19	10
17	**1e**	Wild-type	41	45	5
18	**1e**	I287F	41	42	16
19	**1e**	I287F/I290A	3	43	>200

[a] Conditions: immobilized lipase (700 mg, 0.5% (w/w) enzyme/Toyonite-200M), **1** (0.50 mmol), vinyl acetate (1.0 mmol), molecular sieves 3A (three pieces), dry *i*-Pr_2O (5 mL), 30 °C. [b] Conversion calculated from c = ee(**1**)/(ee(**1**) + ee(**2**)). [c] Calculated from $E = \ln[1 - c(1 + \text{ee}(\mathbf{2}))]/\ln[1 - c(1 - \text{ee}(\mathbf{2}))]$.

We next tested a third-generation biocatalyst, the I287F/I290A/Q292A triple mutant. We unexpectedly found this variant to be less active and enantioselective than the I287F/I290A double mutant (entry 9). This result, however, suggests an important role of Gln292, which is located at the outer side of Phe287 and Ala290 (Fig. 1a). The Q292A mutation, which locally creates a space to facilitate the acylation of (*R*)-**1a** (Fig. 1e), may bring about fluctuation of the adjacent Phe287, which may decrease catalytic activity and enantioselectivity. Of all the variants that we prepared (Table 1), the I287F/I290A double mutant was the best biocatalyst for the bulky substrates examined.

The total turnover number (TTN) and the turnover frequency (TOF) provide a rough estimate of the extent of catalytic activity. TTN is defined as the number of substrate molecules converted by one enzyme molecule, while TOF is the turnover number per unit time. These values can be calculated from the data in Table 1. For example, the TTN of the wild-type enzyme for **1a** (entry 1) is 1080 in 41 h, and the TOF is 26 h^{-1}.[14] In contrast, the TTN of the I287F/I290A double mutant for **1a** (entry 6) is 2350 in 2.5 h, and the TOF is 940 h^{-1}. Clearly, the double mutation has remarkably improved enzymatic activity for this substrate, although the values of the I287F/I290A double mutant

for **1a** are still smaller than the corresponding values of the wild-type enzyme for an inherently good substrate, 1-phenylethanol (TTN = 7800 in 4.5 h; TOF = 1700 h^{-1}).[10a]

Kinetic measurements

We measured time courses to compare the relative activities of recombinant lipases for each enantiomer of **1a**. A mixture of enantiomerically pure alcohol **1a** (50 mM), immobilized lipase (300 mg), and molecular sieves 3A (one piece) in dry *i*-Pr_2O (1.0 mL) was stirred at 30 °C for 30 min. We started the reaction by addition of vinyl acetate (1.0 M). At appropriate time intervals, we withdrew aliquots (10 μL) and added them to EtOAc (0.5 mL). After centrifuging the diluted solution, we filtered the supernatant through a syringe filter (pore size 0.45 μm). The filtrate was then analyzed by gas chromatography to determine the conversion rates (calibrated). The time courses are shown in Fig. 3. The use of enantiomerically pure alcohol **1a** was helpful for understanding the effect of each mutation on the enzymatic activity. Fig. 3 clearly showed the relative activity of the recombinant lipases for **1a** because all the reactions were conducted under the same conditions. The I287F mutant showed higher activity for (*R*)-**1a** and lower activity for (*S*)-**1a** than the wild-type enzyme. Furthermore, the I287F/I290A double mutant exhibited much higher activity for (*R*)-**1a** than the wild-type enzyme or the I287F mutant. These results are consistent with our working hypothesis described above.

To investigate the mutational effect more quantitatively, we used previously determined kinetic constants (Fig. 4).[10b] This reaction is heterogeneous because the immobilized enzyme powder is insoluble. The V_{max} value is normalized by the weight of the enzyme powder and corresponds to the k_{cat} value in a homogeneous system. The V_{max} value is therefore related to the transition state, while the K_m value is related to the substrate-binding step. Fig. 4 indicates that enantioselectivity originated from the difference in V_{max} values (in other words, from differences in the transition state), as demonstrated previously.[12a,c] Importantly, the mutations caused the following changes in the V_{max} values: the I287F mutant had a V_{max} value for (*R*)-**1a** that was approximately three times that of the wild-type enzyme, whereas the I287F mutant had a V_{max} value for (*S*)-**1a** that was half that of the wild-type enzyme. These results clearly support our hypothesis concerning the two roles of Phe287: enhancement of the reactivity of (*R*)-**1a** and suppression of the reactivity of (*S*)-**1a**. Furthermore, the I287F/I290A double mutant had a V_{max} value for (*R*)-**1a** that was approximately five times that of the I287F mutant. Obviously the second mutation (I290A) enhanced the reactivity of (*R*)-**1a**. These kinetic constants are consistent with our expectation that the mutations would successfully manipulate the transition state.

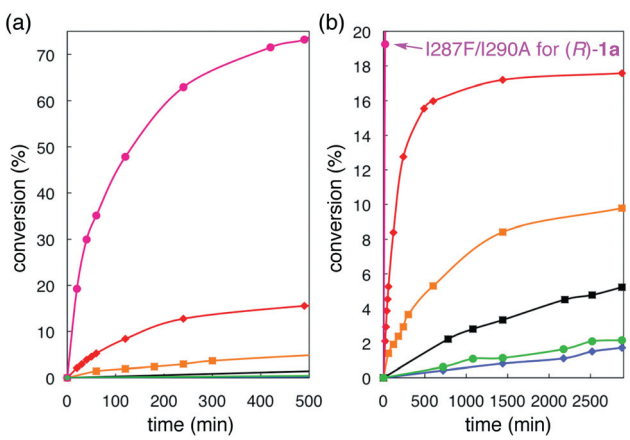


Fig. 3 Time courses of the lipase-catalyzed acylations of **1a**. The same data are plotted in (a) and (b), but the scales of the *x*- and *y*-axes are adjusted to clarify the difference in the reaction rate. Conditions: immobilized lipase (300 mg, 0.5% (w/w) enzyme/Toyonite-200M), **1a** (0.050 mmol), vinyl acetate (1.0 mmol), molecular sieves 3A (one piece), dry *i*-Pr_2O (1.0 mL), 30 °C. Orange square: wild-type enzyme toward (*R*)-**1a**; red diamond: I287F mutant toward (*R*)-**1a**; magenta circle: I287F/I290A double mutant toward (*R*)-**1a**; black square: wild-type enzyme toward (*S*)-**1a**; blue diamond: I287F mutant toward (*S*)-**1a**; green circle: I287F/I290A double mutant toward (*S*)-**1a**.

Attractive interaction in the transition state

We considered the possibility of a CH/π interaction as an attractive interaction between the phenyl group of Phe287 and the alkyl chain of (*R*)-**1a** (Fig. 1c and d).[15–17] We decided to use substrate mapping analysis to specify and characterize the attractive interaction. Alcohols **1c–e** (Scheme 1) were selected

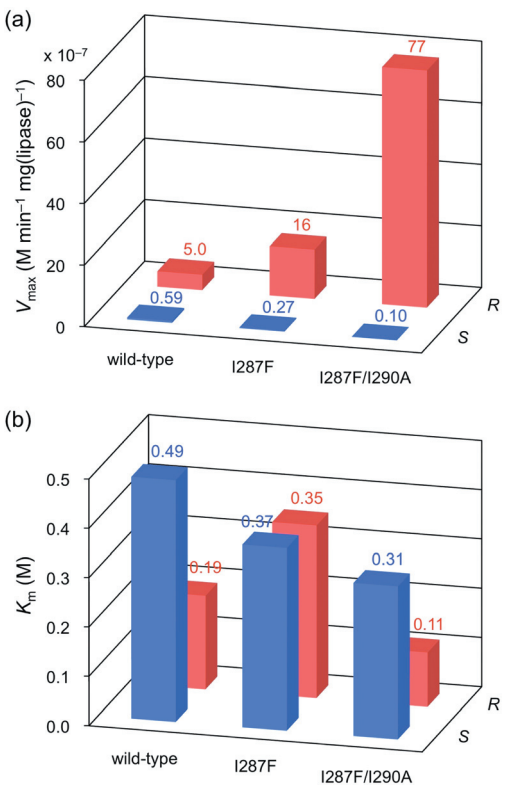


Fig. 4 Kinetic constants for the lipase-catalyzed acylations of **1a**. (a) V_{max} values. (b) K_m values. Because of the heterogeneous reaction, the nonlinear least-squares method was applied to the Michaelis–Menten type of equation: $v_0 = V_{max}(E)_{mg}[S]_0/(K_m + [S]_0)$, where V_{max} is normalized by the weight of the immobilized enzyme powder $(E)_{mg}$.

because they are almost isosteric to **1a** and because a CH/π interaction cannot take place at the fluorine or oxygen atoms in **1c–e**.

As shown in Table 1, the enzymatic activity and enantioselectivity for **1c** decreased in the following order: the I287F/I290A double mutant (entry 15) > the I287F mutant (entry 14) > the wild-type enzyme (entry 13). This trend is quite similar to that for **1a**, which rules out the possibility that the terminal methyl group of **1a** participated in attractive interactions. In sharp contrast, the fact that the I287F/I290A double mutant showed poor activity and enantioselectivity for **1d** (entry 16) strongly suggests that the fluorinated methylene moiety of (*R*)-**1d** was subject to severe steric repulsion (Fig. 5b). The conformation shown in Fig. 1c suggests that the hydrogen atoms at the C5 and C3 positions of (*R*)-**1a** participate in the CH/π interaction with the phenyl group of Phe287 (Fig. 5a).

On the basis of this model (Fig. 5a), we considered that **1e** could be used to quantify the energy of the CH/π interaction stabilizing (*R*)-**1a** in the transition state. Because (*S*)-**1e** cannot participate in a CH/π interaction (Fig. 5c), the increased activity and enantioselectivity of the enzyme variant for **1a** compared to **1e** can be ascribed to the additional CH/π interactions. The results of the lipase-catalyzed kinetic resolution of **1e** shown in Table 1 (entries 17–19) reveal that the I287F mutant converted **1e** a little more slowly than the wild-type enzyme (entries 17 and 18), whereas the I287F/I290A double mutant converted **1e** much faster than the wild-type enzyme (entries 17 and 19). This strongly suggests that the I290A mutation but not the I287F mutation enhanced the catalytic activity of the I287F/I290A double mutant toward (*S*)-**1e** by reducing steric hindrance. The *E* value for the I287F mutant toward **1e** was ~3 times that for the wild-type enzyme (entries 17 and 18), whereas that for the I287F/I290A double mutant toward **1e** was more than 40 times that for the wild-type enzyme (entries 17 and 19). Although **1a** and **1e** exhibited a similar trend, a clear difference between **1a** and **1e** was apparent in the I287F mutation: the conversion and *E* value of the I287F mutant for **1a** were twice and six times, respectively, the corresponding values of the wild-type enzyme (entries 1 and 2), whereas the conversion and *E* value of the I287F mutant for **1e** were comparable and three times, respectively, the corresponding values of the wild-type enzyme (entries 17 and 18). On the basis of the *E* values for the wild-type and I287F mutant enzymes toward **1a** and **1e**, we estimated the free energy of the CH/π interaction to be −0.4 kcal mol^{-1} according to the following equation: $\Delta_{1a-1e}\Delta_{F-I}\Delta_{R-S}\Delta G^{\ddagger} = -RT\ln(E_F/E_I)_{1a} + RT\ln(E_F/E_I)_{1e} = -RT\ln[(32/5)/(16/5)]$.[18]

This value (−0.4 kcal mol^{-1}) is reasonable when compared with the data in the literature.[15–18] For example, Hunter and co-workers have determined the energy of the CH/π interaction in synthetic host–guest complexes to range from −0.3 to −1.1 kcal mol^{-1},[18a,b] depending on aromatic–aromatic combinations. Shimizu and co-workers have used molecular balances to determine that the energy of the Me–arene CH/π interaction is −0.95 kcal mol^{-1}.[16f] Fersht and co-workers have reported that the energetic contribution of the Tyr–Tyr CH/π interaction to protein stabilization is −1.3 kcal mol^{-1}.[18c] The energy of the CH/π interaction between carbohydrate and the aromatic side chain of a protein has been determined to be −0.8 kcal mol^{-1}.[18d]

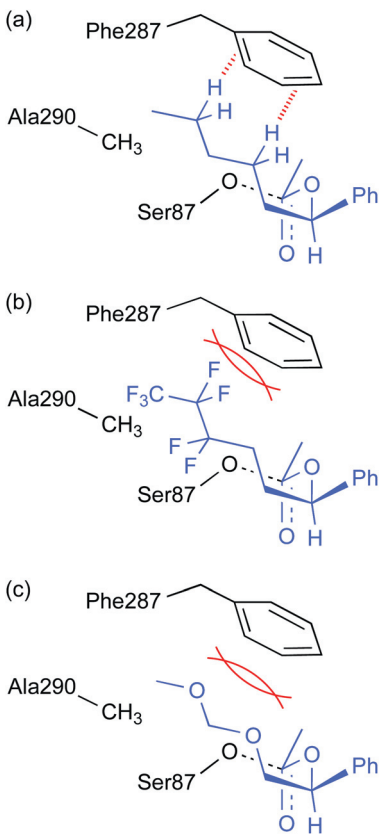
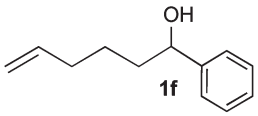
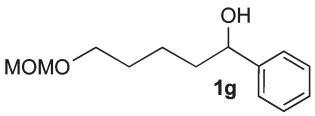
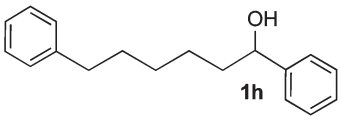
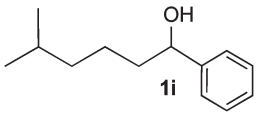
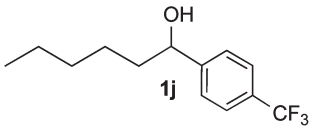
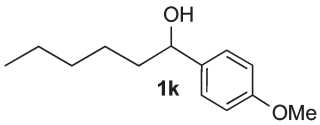
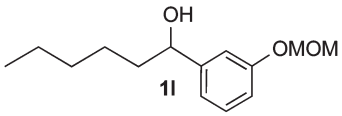
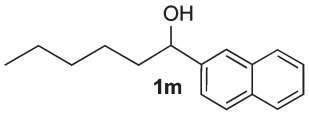
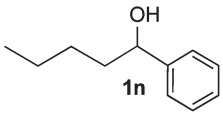
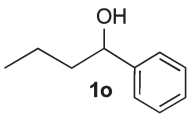
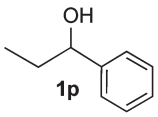
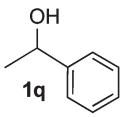


Fig. 5 Proposed interactions in the transition state of the I287F/I290A double mutant-catalyzed reactions. (a) The CH/π interactions for (*R*)-**1a**. (b) The steric repulsion for (*R*)-**1d**. (c) No attractive interactions for (*S*)-**1e** (absolute configuration based on the CIP priority system for nomenclature).

Substrate scope of the I287F/I290A double mutant

We next investigated the substrate scope of the I287F/I290A double mutant (Scheme 1). The results of the kinetic resolutions of **1f–q** are summarized in Table 2. Clearly, this variant showed high activity and enantioselectivity for various bulky alcohols. For example, alcohols **1f** and **1i**, which contain an olefin and a branched chain, respectively, in the aliphatic substituent, reacted smoothly with high *E* values (entries 1 and 4). Although the reaction of **1g**, bearing a methoxymethyl ether group at the terminus of the alkyl group, took a long time, the *E* value was high (entry 2). In contrast, the acylation of **1h**, which has a phenyl group at the terminus of the alkyl group, proceeded rapidly with a high *E* value (entry 3). The fact that the reactivity of **1j** was only modest (entry 5) was probably due to a decrease of the nucleophilicity of the hydroxy group that was caused by the trifluoromethyl group or due to the steric bulkiness of the trifluoromethyl group, whereas **1k** and **1l**, both of which contain an ether group in the aromatic substituent, were very reactive (entries 6 and 7). Alcohol **1m**, which contains a naphthyl group, also showed good reactivity (entry 8). Interestingly, the reaction of **1n**, with a shorter alkyl chain, took more time (entry 9). The TTN values for the I287F/I290A double mutant toward **1f–n** (entries 1–9) reached a practical level, 5770–7590.[14] In sharp

Table 2 Substrate scope of the I287F/I290A double mutant and wild-type enzyme[a]

Entry	Substrate **1**	Time (h)	I287F/I290A		Wild-type	
			c^b (%)	E^c	c^d (%)	E^c
1	 **1f**	7	39	>200	0	—
2	 **1g**	22	46	143	6	—
3	 **1h**	6.5	41	>200	3	—
4	 **1i**	9	35	147	0	—
5	 **1j**	54	45	>200	4	—
6	 **1k**	7	41	>200	2	—
7	 **1l**	7	42	>200	3	—
8	 **1m**	7	41	>200	0	—
9	 **1n**	24	41	199	5	—
10	 **1o**	50	42	>200	18[b]	19
11	 **1p**	50	36	>200	38[b]	113
12	 **1q**	3	50	>200	45[b]	68

[a] Conditions: immobilized lipase (200 mg, 0.5% (w/w) enzyme/Toyonite-200M), **1** (0.50 mmol), vinyl acetate (1.0 mmol), molecular sieves 3A (three pieces), dry *i*-Pr_2O (5 mL), 30 °C. [b] Conversion calculated from $c = ee(\mathbf{1})/(ee(\mathbf{1}) + ee(\mathbf{2}))$. [c] Calculated from $E = \ln[1 - c(1 + ee(\mathbf{2}))]/\ln[1 - c(1 - ee(\mathbf{2}))]$. [d] Conversion calculated from ^{1}H NMR.

contrast, the conversions of these bulky alcohols **1f–n** were much lower (<6%) when catalyzed by the wild-type enzyme.

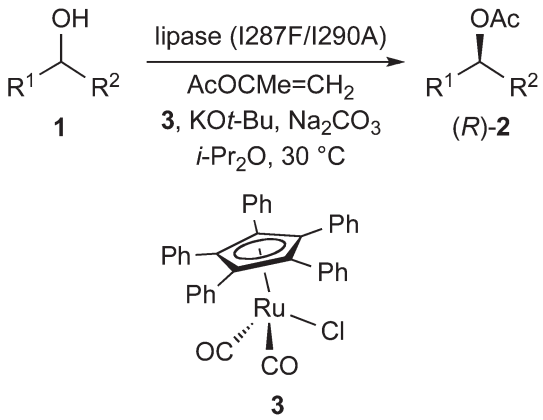
The reactivity of **1n–q** for the wild-type enzyme decreased in the following order: **1q** > **1p** > **1o** > **1n** (Table 2, entries 9–12). This result is quite reasonable because steric repulsion between the alkyl substituent of (*R*)-**1** and the wild-type enzyme decreases in the following order: **1n** > **1o** > **1p** > **1q** (Fig. 1a). In contrast, the reactivity of **1n–q** for the I287F/I290A double mutant varied as follows: **1q** > **1n** > **1o** > **1p** (entries 9–12). The reactivity of **1p** was much lower than that of **1q** (entries 11 and 12) because of steric hindrance around the ethyl group of (*R*)-**1p** (Fig. 1a). Further elongation of the alkyl chain of **1** led to an acceleration of the reaction catalyzed by the I287F/I290A double mutant (entries 9 and 10), a result that can be explained by an attractive interaction as described above. The I287F/I290A double mutant exhibited higher *E* values for **1n–q** than the wild-type enzyme (entries 9–12), which was partly due to the I287F mutation. Because phenylalanine is more bulky than isoleucine, the former hindered the acylation of (*S*)-**1n–q** more efficiently.

Hult and co-workers have created an (*S*)-selective variant of *Candida antarctica* lipase B that shows activity for bulky alcohols such as **1a**.[9f] In that study they examined only simple secondary alcohols having phenyl or alkyl groups, and their variant showed low enantioselectivity for **1q**. They proposed that their variant has a cavity to accommodate the (unsubstituted) phenyl group, which is responsible for the inversion of the enantiopreference. In contrast, the results in Table 2 demonstrate a wide range of applicability of the I287F/I290A double mutant. To the best of our knowledge, this is the first example of the successful enzyme-catalyzed kinetic resolution of bulky alcohols **1f–m**.

Dynamic kinetic resolution

DKR is a fascinating technology because a racemic substrate can be converted into an optically pure product in 100% yield at most.[19–23] Among various racemization catalysts reported so far, chlorodicarbonyl(1,2,3,4,5-pentaphenylcyclopentadienyl)-ruthenium(II) (**3**) is highly active and commercially available.[20] Although most DKRs with native lipases are successful only for secondary alcohols with a small substituent at the stereocenter, Bäckvall and co-workers have recently reported the DKR of bulky secondary alcohols by using an (*S*)-selective variant of *Candida antarctica* lipase B.[20j] We expected the I287F/I290A double mutant to be an excellent biocatalyst for the (*R*)-selective DKR of bulky secondary alcohols.

We conducted the DKR of **1** with the I287F/I290A double mutant, isopropenyl acetate, Ru complex **3**, KO*t*-Bu, and Na_2CO_3 in dry *i*-Pr_2O at 30 °C (Scheme 2). During optimization of the reaction conditions for **1a**, we observed the formation of a considerable amount of the corresponding ketone (data not shown), which was released from the catalytic cycle of the Ru-catalyzed racemization, probably because of the bulkiness of **1a**.[20a–c,j] The results obtained under optimized conditions are shown in Table 3. The simple bulky alcohols **1a**, **1b**, **1h**, **1i**, and **1m** and functionalized bulky alcohols **1c** and **1f**, which have trifluoromethyl and alkenyl groups, respectively, were converted successfully into the corresponding (*R*)-esters in good yields



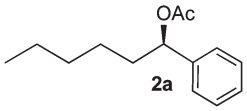
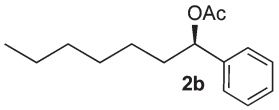
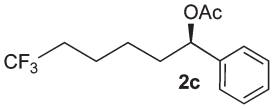
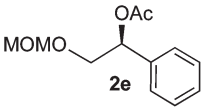
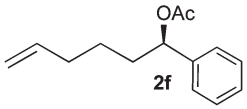
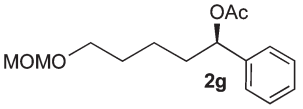
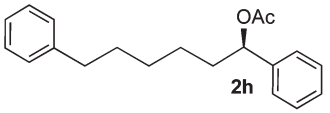
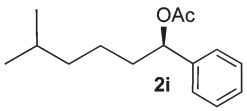
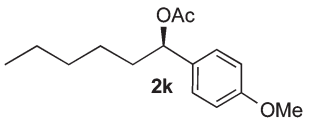
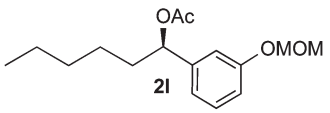
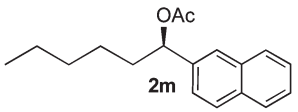
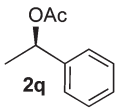
Scheme 2 Dynamic kinetic resolution of **1** using the I287F/I290A double mutant and Ru catalyst **3**.

(69–88%) with high enantiomeric purities (95–98% ee). In the DKR of **1g** and **1k**, we obtained considerable amounts of the corresponding ketones, which decreased the yields of **2g** and **2k** (entries 6 and 9). Bulky alcohols having an ether group (**1e**, **1g**, and **1k**), except for **1l**, reacted sluggishly. Even when the reaction required a very long time, TLC indicated that the reaction proceeded gradually, an indication that both the biocatalyst and the racemization catalyst were active for a long time. In contrast to the DKR of the bulky substrates, which required a larger amount of **3** (10 mol%) and longer reaction times (72–145 h) (entries 1–11), the DKR of the least bulky substrate **1q** with a smaller amount of **3** (4 mol%) was completed within 24 h without producing the corresponding ketone (entry 12). The TTN values for the I287F/I290A double mutant toward **1** reached 9070–14500 (entries 1–12).[14] These results clearly demonstrate that this variant is robust and can be used in the DKR of a wide range of bulky secondary alcohols.

Conclusions

Improvement of the catalytic function of enzymes is important from scientific and industrial viewpoints. Despite remarkable advances in enzyme science and technology, rational control of the enzymatic function remains difficult because of the complexity of reaction mechanisms. The fact that secondary alcohols having bulky substituents on both sides of the hydroxy group are inherently poor substrates for most lipases has been an unresolved weakness that has limited the usefulness of the biocatalysts. In this study the synergic effects of only two mutations (I287F/I290A) dramatically enhanced both catalytic activity and enantioselectivity for poor substrates. Phe287 contributed to both enhancement of the reactivity of the (*R*)-enantiomer and suppression of the reactivity of the (*S*)-enantiomer, and Ala290 made space to facilitate the acylation of the (*R*)-enantiomer. Kinetic constants indicated that the mutations effectively manipulated the transition state. Substrate mapping analysis strongly suggested that the reactivity of the (*R*)-enantiomer was partly enhanced by a CH/π interaction, of which the estimated energy was −0.4 kcal mol^{-1}. To the best of our knowledge, this is the first example of an introduction of a CH/π interaction in the transition state to promote a more enantioselective enzymatic

Table 3 Dynamic kinetic resolution of **1** with I287F/I290A double mutant and **3**[a]

Entry	Product (*R*)-**2**	% Yield[b] (% ee)	Ketone
1	 **2a**	88 (95)	2
2	 **2b**	69 (97)	9
3	 **2c**	87 (98)	2
4	 **2e**	70 (95)[c]	6
5	 **2f**	88 (95)	1
6	 **2g**	55 (96)	29
7	 **2h**	87 (95)	9
8	 **2i**	80 (95)	6
9	 **2k**	67 (94)	16
10	 **2l**	85 (97)	4
11	 **2m**	78 (98)	5
12	 **2q**	87 (96)	0

[a] Conditions: immobilized lipase (400 mg, 0.5% (w/w) enzyme/Toyonite-200M), **1** (1.0 mmol), isopropenyl acetate (1.5 mmol), **3** (10 mol% except for **1q** (4 mol%)), KO*t*-Bu (0.13 mmol except for **1q** (0.05 mmol)), Na_2CO_3 (1.0 mmol), molecular sieves 4A (20 pieces), dry *i*-Pr_2O (2 mL), 30 °C, 72 h except for **1g** (120 h), **1i** (145 h), and **1q** (24 h). [b] Isolated yield. [c] Only **2e** has the (*S*)-configuration according to the CIP priority system for nomenclature.

reaction. The substrate scope of the I287F/I290A double mutant was broad, and we were able to use this variant in the DKR of various bulky secondary alcohols for which the wild-type enzyme showed little or no activity. The I287F/I290A double mutant achieved a TTN up to 14500. We expect that this variant will be useful for the synthesis of chiral intermediates that cannot be prepared with other lipases. Although the structures of the variants that we have prepared and presented here have not yet been fully optimized, for example, by directed evolution, the impact of the mutations was nevertheless great. The present results clearly demonstrate the efficiency and power of rational design for the creation of excellent biocatalysts.

Experimental

General

^1H and ^{13}C NMR spectra were measured on Varian 600 or 400 MHz NMR spectrometers. IR spectra were recorded on a Shimadzu FTIR-8900 spectrometer. Silica gel column chromatography was performed using Fuji Silysia BW-127 ZH (100–270 mesh), and thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254}.

General procedure for dynamic kinetic resolution

Immobilized lipase (0.5% (w/w) enzyme/Toyonite-200M) was dried under reduced pressure overnight. A solution of KO*t*-Bu (0.5 M in THF, 250 μL) was added to a Schlenk flask, and THF was removed under reduced pressure. Immobilized lipase (400 mg), Ru catalyst **3** (chlorodicarbonyl(1,2,3,4,5-pentaphenylcyclopentadienyl)ruthenium(II)) (63.8 mg, 0.100 mmol), Na_2CO_3 (106 mg, 1.00 mmol), and molecular sieves 4A (20 pieces) were added to the flask. The flask was evacuated and filled with Ar. Dry *i*-Pr_2O (2 mL) was added, and the mixture was stirred at 30 °C for 15 min. Alcohol **1** (1.0 mmol) was added, and the mixture was stirred for 15 min. The reaction was started by addition of isopropenyl acetate (165 μL, 1.5 mmol). The mixture was stirred at 30 °C for 24–145 h. The reaction was stopped by filtration, and the mixture was concentrated. Purification by silica gel column chromatography gave ester **2**. In the case of **2g** and **2k**, the corresponding ketone was removed by silica gel column chromatography after conversion of **2g** and **2k** to the corresponding alcohols.

Acknowledgements

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