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Improvements of enzyme activity and enantioselectivity in lipase-catalyzed alcoholysis of (*R*,*S*)-azolides

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ABSTRACT

With *Candida antarctica* lipase B (CALB)-catalyzed alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide at the optimal conditions (i.e. anhydrous MTBE as the solvent, and methanol as the acyl acceptor at 45 °C) as the model system, the enzyme enantioselectivity in terms of V_R/V_S = 105.8 and specific activity for the fast-reacting (*R*)-azolide $V_R/(E_t)$ = 0.979 mmol/(h g) were greatly improved in comparison with V_R/V_S = 8.0 and $V_R/(E_t)$ = 0.113 mmol/(h g) of using (*R*,*S*)-naproxenyl 2,2,2-trifluoroethyl ester as the substrate. The resolution strategy was successfully extended to other (*R*,*S*)-profenyl 1,2,4-triazolides and lipases from *Candida rugosa* (Lipase MY) and *Carica papaya* (CPL) having opposite enantioselectivity to CALB. Moreover, the kinetic constants were estimated, compared with those obtained via hydrolysis, and employed for modeling time-course conversions of (*R*,*S*)-naproxenyl 1,2,4-triazolide in anhydrous MTBE. The advantages of easy substrate preparation, high enzyme reactivity and enantioselectivity, as well as easy product separation from the remaining substrate via reactive extraction demonstrate merits of using (*R*,*S*)-azolides but not the corresponding esters for the alcoholytic resolution.

1. Introduction

Lipases as versatile biocatalysts are widely applied for preparing a variety of pharmaceuticals and fine chemicals containing a chiral center. Their active site consisting of a Ser-His-Asp/Glu catalytic triad catalyzes the hydrolysis or synthesis by following an acylation–deacylation displacement mechanism. In comparison with the resolution of secondary alcohols and amines [1–4], the enantioselectivity toward carboxylic acids is usually low to modest [5]. Therefore, various approaches of using substrate engineering [6–8], medium engineering [9–11], enzyme engineering [12–17], or their combinations have been proposed for improving the enzyme performance.

In comparison with esterification of (*R*,*S*)-carboxylic acids or hydrolysis of the corresponding (*R*,*S*)-esters, fewer studies relevant to transesterification, aminolysis and ammonolysis of (*R*,*S*)-esters are reported in the literature [7,18–23]. Apparently, this is due to at least an additional synthetic step for preparing ester substrates or hydrolytic step for obtaining acid products, as well as the difficulty of separating resultant ester or amide products from remaining ester substrates. In order to manipulate the enzyme active-site structure for increasing the enantiomer discrimination and reactivity, acyl donors containing an achiral activated or irreversible leaving group have been utilized [24,25]. Recently, (*R*,*S*)-azolides, i.e. N-acylazoles, as novel substrates for lipase-catalyzed hydrolysis in water-saturated organic solvents were reported [26]. The good enzyme performance was attributed to the unique azolide structure that contains an unshared electron pairs on the acyl-substituted nitrogen N(1), making the carbonyl carbon more electrophilic and susceptible to nucleophilic attack [27]. It is therefore aimed to extend the previous resolution process to the alcoholysis of (*R*,*S*)-azolides in anhydrous organic solvents for preparing optically pure carboxylic acids or their derivatives.

Candida antarctica lipase B (CALB), showing low to modest enantioselectivity toward (*R*,*S*)-profens via esterification and (*R*,*S*)-profenyl esters via hydrolysis and transesterification [7,20,22], was employed as the model lipase for resolving (*R*,*S*)-profenyl azolides via alcoholysis in anhydrous organic solvents (Scheme 1). Effects of solvent, temperature, alcohol, and substrate structure on varying the enzyme activity and enantioselectivity were firstly studied. The resolution was then extended to Lipase MY and CPL having opposite enantioselectivity to CALB. Moreover, the kinetic constants estimated and compared with those obtained via hydrolysis, as well as separation of ester products from the remaining substrate via reactive extraction were addressed.

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Nomenclature

ee_p, ee_s	enantiomeric excesses for product and substrate, respectively
E	enantiomeric ratio, defined as $k_{2S}K_{mR}/k_{2R}K_{mS}$
(E_t)	enzyme concentration (mg/ml)
G	parameter defined in Eq. (3)
k_{2i}, k_{4i}	kinetic constants, $i = R$ or S for (*R*)- or (*S*)-enantiomer (mmol/(g h))
K_I	inhibition constant (mM)
K_{mi}, K_{m3i}	kinetic constants, $i = R$ or S for (*R*)- or (*S*)-enantiomer (mM)
(S_i)	substrate concentration, $i = R$ or S for (*R*)- or (*S*)-enantiomer (mM)
$(S_i)_0$	initial concentration, $i = R$ or S for (*R*)- or (*S*)-enantiomer (mM)
V_i	initial rate, $i = R$ or S for (*R*)- or (*S*)-enantiomer (mM/h)
X_i, X_t	enantiomer conversion defined as $[1-(S_i)/(S_i)_0]$, $i = R$ or S; racemate conversion defined as $[X_R + X_S]/2$

2. Materials and methods

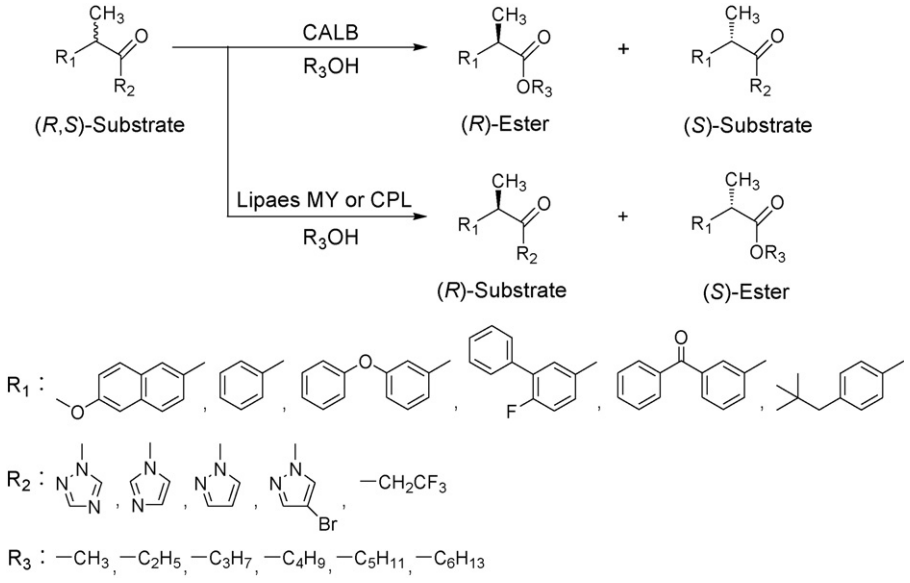
2.1. Materials

Novozym 435 (CALB) from *Candida antartica* lipase B and Lipase MY from *Candida rugosa* were provided by Novo Nordisk (Bagsvaerd, Denmark) and Meito Sangyo Industries Ltd. (Tokyo, Japan), respectively. A *Carica papaya* lipase (CPL) partially purified from the aqueous-insoluble aggregate of crude papain was kindly donated from Challenge Bioproducts (Yun-Lin Hsien, Taiwan). Other chemicals of analytical grade were commercially available: pyrazole from Acros (Geel, Belgium); 4-bromopyrazole, N,N-carbonyldiimidazole (CDI), N,N-carbonyldi-1,2,4-triazole (CDT) and 2,2,2-trifluoroethanol from Aldrich (Milwaukee, WI); (*R*,*S*)-2-phenylpropionic acid from Fluck (Buchs, Switzerland); (*R*,*S*)-ketoprofen (i.e. (*R*,*S*)-2-(3-benzoylphenyl)-propionic acid), (*R*,*S*)-naproxen (i.e. (*R*,*S*)-2-(6-methoxy-2-naphthyl)-propionic acid) and (*S*)-naproxen from TCI (Tokyo, Japan); (*R*,*S*)-flurbiprofen (i.e. (*R*,*S*)-2-fluoro-α-methyl-4-biphenylacetic acid), (*R*,*S*)-ibuprofen (i.e. (*R*,*S*)-2-(4-isobutylphenyl)-propionic acid) and (*R*,*S*)-fenoprofen (i.e. (*R*,*S*)-2-(3-phenoxyphenyl)-propionic acid) from Sigma (St. Louis, MO); dichloromethane, diisopropyl ether (IPE), ethanol, hexane, isooctane (ISO), isopropanol, methanol, methyl *tert*-butyl ether (MTBE), propanol, butanol, pentanol, hexanol, *tert*-butyl alcohol (TBA) and triethylamine from Tedia (Fairfield, OH). Anhydrous solvent was prepared by adding calcium hydride from Riedel-de Haen (Seelze, Germany) to the organic solvent for 24 h.

2.2. Substrate preparations

The general procedures for preparing (*S*)- or (*R*,*S*)-profenyl azolides from the acylation of azoles with acid chloride or via direct activation of carboxylic acid by CDI or CDT were previously described [26]. For example, to 5 ml benzene was added 1 mmol (*R*,*S*)-naproxen and 1.5 mmol CDT and stirred at 55 °C for 2 h. The resultant mixture was filtered and evaporated under reduced pressure, giving the desired (*R*,*S*)-naproxenyl 1,2,4-triazolide. Thionyl chloride of 13 mmol in 5 ml benzene was added dropwise to a mixture consisting of 10 mmol 4-bromopyrazole, 13 mmol (*R*,*S*)-naproxen, and 40 mmol triethylamine. The resultant solution was stirred for 2 h at room temperature. After being quenched in succession with 0.1 M HCl solution, 0.1 M NaOH solution and 0.1 M NaCl solution each for three times (3× 10 ml), the organic phase was separated, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure, giving the desired (*R*,*S*)-naproxenyl 4-bromopyrazolide.

To 20 ml benzene containing 27 mmol thionyl chloride and 15 mmol (*R*,*S*)-naproxen was refluxed for 1.5 h at 80 °C. After evaporating the solvent and remaining thionyl chloride, 30 ml benzene containing 15 mmol pyridine and 27 mmol 2,2,2-trifluoroethanol was added and refluxed for 4 h at 80 °C. The resultant solution was quenched in succession with NaOH solution (pH = 12.5, 4× 50 ml) and deionized water (2× 100 ml). After purification in silica gel chromatography with the mobile phase of hexane/ethyl acetate (2/1, v/v) and concentrated under reduced pressure, the desired (*R*,*S*)-naproxen 2,2,2-trifluoroethyl ester in white powder was obtained. The synthesized substrates were confirmed from the retention time in HPLC analysis and ^{1}H NMR spectra [8,26].



Scheme 1. Lipase-catalyzed alcoholysis of (*R*,*S*)-profenyl azolides and (*R*,*S*)-naproxenyl 2,2,2-trifluoroethyl ester in anhydrous organic solvents.

Table 1
Effects of solvent on specific initial rates $V_R/(E_t)$ and $V_S/(E_t)$, V_R/V_S, X_t and ee_s for CALB-catalyzed alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide.

Solvent (*log P*)	$V_R/(E_t)$ (mmol/(h g))	$V_S/(E_t)$ (mmol/(h g))	V_R/V_S	Time (h)	X_t (%)	ee_s (%)
ISO (4.50)	4.95E−1	2.51E−2	19.7	1.0	66.6	>99.8
IPE (1.90)	6.03E−1	1.25E−2	48.3	1.5	53.0	>99.8
MTBE (1.43)	9.79E−1	9.25E−3	106	2.2	53.3	>99.8
TBA (0.79)	1.33E−1	3.50E−3	38.1	7.0	53.9	>99.8

Conditions: 10 ml anhydrous solvent containing 3 mM racemate (but 1 mM racemate in ISO), 100 mM methanol, and 6 mg/ml lipase at 45 °C and 400 rpm. Symbols E−1 and *P* for solvent as 10^{-1} and partition coefficient of the solvent between water and octanol, respectively.

2.3. Analysis

Alcoholysis of (*R*,*S*)-naproxenyl 4-bromopyrazolide was monitored by HPLC using a chiral (*S*,*S*)-whelk-01 column from Regis (Morton Grove, IL) that was capable of separating the internal standard of 2-nitrotoluene, (*S*)- and (*R*)-azolides with the retention time of 2.2, 3.0, 4.1 min, respectively. The mobile phase was a mixture of n-hexane/isopropanol (90/10, v/v) at a flow rate of 2.0 ml/min. UV detection at 270 nm was employed for quantification at the room temperature. For the analysis of (*R*,*S*)-naproxen 2,2,2-trifluoroethyl ester, the composition of mobile phase was changed to n-hexane/isopropanol/acetic acid glacial (80/20/0.5, v/v/v), giving the retention time for the internal standard of acetopheone, (*R*)- and (*S*)-esters as 2.4, 3.2, 4.0 min, respectively. Detailed analytical conditions for other (*R*)- and (*S*)-profenyl azolides were reported elsewhere [26]. The products of (*R*)- and (*S*)-naproxen methyl esters were monitored at 270 nm by using a Daicel chiral OD-H column (Tokyo, Japan) that was capable of separating the internal standard of 2-nitrotoluene, (*R*)- and (*S*)-ester with the retention time of 2.7, 3.4, 3.8 min, respectively. The mobile phase was a mixture of n-hexane/isopropanol (96.5/3, v/v) at a flow rate of 2.0 ml/min.

2.4. Effects of solvent, temperature, alcohol, substrate structure, and lipase source

To 10 ml anhydrous ISO, IPE, MTBE or TBA containing 3 mM (*R*,*S*)-naproxenyl 1,2,4-triazolide and 100 mM methanol at 45 °C was added 6 mg/ml CALB. The resultant solution was stirred with a magnetic stirrer, and samples were removed at different time intervals for the HPLC analysis, from which the time-course conversions X_R and X_S with the analytical error less than ±1.3%, initial rates for both enantiomers V_R and V_S based on several conversion determinations, racemate conversion X_t, and enantiomeric excess for the substrate ee_s were determined. Similar experiments were performed except that anhydrous MTBE was employed as the solvent at 35 and 55 °C. In order to investigate effects of acyl acceptor on the lipase performance, more experiments in anhydrous MTBE containing 100 mM of ethanol, propanol, butanol, pentanol or hexanol were carried out at 45 °C.

Effects of substrate structure on the enzyme activity and enantioselectivity were studied, in which the alcoholysis of methanol with (*R*,*S*)-naproxen 2,2,2-trifluoroethyl ester or (*R*,*S*)-naproxenyl azolides containing a leaving imidazole, pyrazole, or 4-bromopyrazole was performed in anhydrous MTBE at 45 °C. Similar experiments of using other (*R*,*S*)-profenyl 1,2,4-triazolides as the acyl donor were carried out, and the results were compared with those via hydrolysis in anhydrous and water-saturated MTBE. In order to test if the resolution process could be applied to other lipases having opposite enantioselectivity to CALB, the alcoholysis of (*R*,*S*)-naproxenyl azolides by methanol via Lipase MY or CPL in anhydrous isooctane at 45 °C was also performed.

2.5. Kinetic analysis

The alcoholysis was performed in 10 ml anhydrous MTBE consisting of 6 mg/ml CALB, methanol and (*R*,*S*)-naproxenyl 1,2,4-triazolide of different concentrations at 45 °C, from which the time-course conversions and initial rates for each enantiomer were estimated. Similar experiments of only using (*S*)-naproxenyl 1,2,4-triazolide as the acyl donor were carried out. An irreversible ping-pong Bi–Bi mechanism by considering alcohol inhibition (see Supplementary Information) was employed for deriving the rate equations as follows:

$$V_R = \frac{-d(S_R)}{dt} = \frac{k_{2R}(S_R)(E_t)/K_{mR}}{G} \quad (1)$$

$$V_S = \frac{-d(S_R)}{dt} = \frac{k_{2S}(S_S)(E_t)/K_{mS}}{G} \quad (2)$$

$$G = 1 + \frac{(S_R)[1 + k_{2R}/k_{4R}]}{K_{mR}} + \frac{(S_S)[1 + k_{2S}/k_{4S}]}{K_{mS}} + \frac{(M)}{K_I} + \left[\frac{k_{2R}K_{m3R}(S_R)}{k_{4R}K_{mR}} + \frac{k_{2S}K_{m3S}(S_S)}{k_{4S}K_{mS}}\right]\left[\frac{1}{(M)}\right] \quad (3)$$

Notations (E_t), (M), (S_R) and (S_S) denoted the concentrations of enzyme, alcohol, (*R*)- and (*S*)-azolides in the solvent, respectively. All kinetic constants defined in the Nomenclature were estimated and employed for modeling the time-course conversions of the substrates.

2.6. Reactive extraction for separating the product

In order to simplify the synthesis procedures, 62.0 mM (*R*,*S*)-naproxenyl 1,2,4-triazolide was prepared in 10 ml anhydrous MTBE but not benzene via direct activation of (*R*,*S*)-naproxen by CDT at 55 °C for 12 h. After centrifuging the precipitate, to 8 ml of the solution was added 6 mg/ml CALB and 50 mM methanol at 45 °C for 2.5 h, giving X_t = 51.5%, ee_p = 94.1% and ee_s = 100% from the HPLC analysis. As an example for the easy product separation, to 5 ml of the resultant solution after removing CALB via centrifugation was added 5 ml sodium carbonate buffer (pH = 9, 300 mM) at 45 °C with stirring for 7 h. The methyl ester products of 38.1 mM and ee_p = 91.5% without the traces of (*S*)-azolide in the organic phase was found from HPLC analysis. Apparently, the hydrolysis of (*S*)-naproxenyl 1,2,4-triazolide in the aqueous phase was more rapidly than that of (*R*)- or (*S*)-naproxenyl methyl ester. Yet in order to enhance the optical purity of (*S*)-naproxen in the aqueous phase, other extraction conditions for decreasing the ester hydrolysis should be found.

3. Results and discussion

3.1. Effects of solvent, temperature and alcohol

Table 1 demonstrates the effects of anhydrous organic solvent on the initial specific activities $V_R/(E_t)$ and $V_S/(E_t)$, and enan-

Table 2
Effects of temperature on specific initial rates $V_R/(E_t)$ and $V_S/(E_t)$, V_R/V_S, X_t and ee_s for CALB-catalyzed alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide.

Temperature (°C)	$V_R/(E_t)$ (mmol/(h g))	$V_S/(E_t)$ (mmol/(h g))	V_R/V_S	Time (h)	X_t (%)	ee_s (%)
35	4.82E−1	4.00E−3	120	3.0	52.4	>99.8
55	1.30	1.63E−2	80.0	2.0	55.2	>99.8

Conditions: 10 ml anhydrous MTBE containing 3 mM racemate, 100 mM methanol, and 6 mg/ml lipase at 400 rpm. Symbol of E−1 as 10^{-1}.

Table 3
Effects of alcohol on specific initial rates $V_R/(E_t)$ and $V_S/(E_t)$, V_R/V_S, X_t and ee_s for CALB-catalyzed alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide.

Alcohol	$V_R/(E_t)$ (mmol/(h g))	$V_S/(E_t)$ (mmol/(h g))	V_R/V_S	Time (h)	X_t (%)	ee_s (%)
Ethanol	6.73E−1	7.13E−3	94.4	2.2	54.0	>99.8
Propanol	6.15E−1	5.86E−3	105	4.1	52.9	>99.8
Butanol	5.75E−1	6.50E−3	88.4	4.1	54.8	>99.8
Pentanol	7.86E−1	7.65E−3	102	2.2	53.5	>99.8
Hexanol	7.81E−1	7.33E−3	106	2.2	53.0	>99.8

Conditions: 10 ml anhydrous MTBE containing 3 mM racemate, 100 mM alcohol, and 6 mg/ml lipase at 45 °C and 400 rpm. Symbol of E−1 as 10^{-1}.

tioselectivity in terms of V_R/V_S for CALB-catalyzed alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide by methanol at 45 °C. In general, the specific activity for $V_S/(E_t)$ but not $V_R/(E_t)$ increased with the solvent hydrophilicity in terms of the *log P* value, yet it reversed if the value further increased from 1.43 of MTBE to 1.90 of IPE. An optimal $V_R/(E_t)$ for the fast-reacting enantiomer and $V_R/V_S = 105.8$ in anhydrous MTBE was thus obtained. In comparison with $V_R/(E_t) = 0.113$ mmol/(h g) and $V_R/V_S = 81.8$ for the lipase-catalyzed hydrolysis in water-saturated MTBE [26], the better enzyme activity and enantioselectivity via alcoholysis was apparent.

Table 2 shows effects of temperature on varying the enzyme activity and enantioselectivity in anhydrous MTBE. A liner relationship of $ln(V_R/(E_t))$, $ln(V_S/(E_t))$ or $ln(V_R/V_S)$ with inverse of the absolute temperature was found (data not shown), implying that the lipase was thermally stable at 55 °C. Yet, 45 °C was selected as the best temperature by considering a compromise between the enzyme activity and enantioselectivity.

Table 3 further demonstrates the effects of alcohol on $V_R/(E_t)$, $V_S/(E_t)$, and V_R/V_S for CALB-catalyzed alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide in anhydrous MTBE at 45 °C. In general, the carbon-chain length of the acyl acceptor had minute influences on the enzyme activity and enantioselectivity, implying that the rate-limiting step for the alcoholysis was the acylation step. By considering the lowest cost, methanol was selected as the best acyl acceptor for the following experiments.

3.2. Effects of substrate structure and lipase source

In order to demonstrate that (*R*,*S*)-azolides acted as versatile substrates for the kinetic resolution in anhydrous organic solvents, Table 4 indicates the alcoholysis of (*R*,*S*)-naproxenyl azolides and (*R*,*S*)-naproxenyl 2,2,2,-trifluoroetheyl ester by methanol in anhydrous MTBE. The 172-fold enhancement of $V_R/(E_t)$ for (*R*,*S*)-1,2,4-triazolide in comparison with that for (*R*,*S*)-imidazolide led to the rate-limiting acylation step for all fast-reacting enantiomers as they had the same acyl-enzyme intermediate for performing the deacylation step. By increasing the number of nitrogens from 2 of leaving imidazole or pyrazole to 3 of 1,2,4-triazole, the specific activity for each enantiomer increased and resulted in maximum enantioselectivity of $V_R/V_S = 179.3$ for (*R*,*S*)-naproxenyl pyrazolide. One may attribute the high reactivity of azolides to the unshared electron pairs on the acyl-substituted nitrogen N(1) that are part of the cyclic π-system of the azole units. This unique structure can lead to a partial positive charge on N(1) that interfaces with the normal carboxamide resonance and exerts an electron-withdrawing effect on the carbonyl groups. Therefore, the carbonyl carbon is more electrophilic and susceptible to nucleophilic attack, especially when the number of nitrogens of leaving azoles increases [26,27].

The enantioselectivity further increased to $V_R/V_S = 205.5$ when employing (*R*,*S*)-naproxenyl 4-bromopyrazolide as the substrate. A comparison of $pK_a = 0.64$ for the conjugated acid of 4-bromopyrazole (i.e. 4-bromopyrazolium) with 7.0 of imidazolium, 2.52 of pyrazolium, and 2.19 of 1,2,4-triazolium [28] indicated that the leaving 4-bromopyrazole moiety might exert the strongest electron-withdrawing effect on the carbonyl carbon atom, making it the most electrophilic and susceptible to nucleophilic attack. Yet, the specific activity $V_R/(E_t)$ of the 4-bromopyrazolide was only slightly higher than that of (*R*,*S*)-naproxenyl pyrazolide and even lower than that of (*R*,*S*)-naproxenyl 1,2,4-triazolide, indicating that extra interactions between 4-bromo substituent and the amino acid residues of enzyme active site might exist to weaken the electron-withdrawing ability of the substituent. This can be verified from comparing the extent of interactions by docking different azolides into the active site via molecular modeling techniques.

The results of using (*R*,*S*)-naproxenyl 2,2,2-trifluoroethyl ester as the substrate was also tabulated (Table 4). About 8.6-fold and 13.2-fold lower of $V_R/(E_t)$ and V_R/V_S, respectively, were perceived when comparing with those for (*R*,*S*)-naproxenyl 1,2,4-triazolide (Table I). By further considering the difficulty of separating methyl ester from remaining 2,2,2-trifluoroethyl ester, the benefit of using (*R*,*S*)-azolides but not (*R*,*S*)-esters as the substrate was evident. The analysis was then extended to other (*R*,*S*)-profenyl 1,2,4-triazolides (Table 5), showing very good enzyme performances similar to those for (*R*,*S*)-naproxenyl 1,2,4-triazolide. In general the more bulky was the acyl group; the lower enzyme activity for

Table 4
Effects of leaving moiety on specific initial rates $V_R/(E_t)$ and $V_S/(E_t)$, V_R/V_S, X_t and ee_s for CALB-catalyzed alcoholysis of (*R*,*S*)-naproxenyl azolides.

Leaving moiety	$V_R/(E_t)$ (mmol/(h g))	$V_S/(E_t)$ (mmol/(h g))	V_R/V_S	Time (h)	X_t (%)	ee_s (%)
Imidazole	5.69E−3	1.81E−3	3.1	7.1	29.2	18.6
Pyrazole	3.51E−1	1.96E−3	179	8.2	57.0	>99.8
4-Bromopyrazole	4.26E−1	2.08E−3	205	3.0	51.2	>99.8
1,2,4-Triazole	9.79E−1	9.25E−3	105	2.2	53.3	>99.8
Trifluoroethanol	1.13E−1	1.41E−2	8.0	7.0	64.2	86.8

Conditions: 10 ml anhydrous MTBE containing 3 mM racemate, 100 mM methanol, and 6 mg/ml lipase at 45 °C and 400 rpm. Symbol of E−1 as 10^{-1}.

Table 5
Effects of alcohol on specific initial rates $V_R/(E_t)$ and $V_S/(E_t)$, V_R/V_S, X_t and ee_s for CALB-catalyzed alcoholysis of (*R*,*S*)-profenyl 1,2,4-triazolides.

Acyl moiety and alcohol	$V_R/(E_t)$ (mmol/(h g))	$V_S/(E_t)$ (mmol/(h g))	V_R/V_S	(E_t) (mg/ml)	Time (h)	X_t (%)	ee_s (%)
2-Phenylpropionyl							
Methanol	9.46	2.15E−1	43.9	2.0	0.6	60.0	>99.8
Ethanol	7.32	1.28E−1	57.1	2.0	0.3	53.5	>99.8
Propanol	6.33	1.41E−1	44.8	2.0	0.5	55.0	>99.8
Butanol	6.52	1.50E−1	43.4	2.0	0.3	56.2	>99.8
Pentanol	8.66	1.50E−1	57.6	2.0	0.5	57.4	>99.8
Hexanol	6.16	1.43E−1	43.1	2.0	1.0	60.0	>99.8
Fenoprofenyl							
Methanol	4.94	4.94E−2	100	2.0	2.0	55.0	>99.8
Hexanol	6.62	6.98E−2	94.9	2.0	0.7	53.4	>99.8
Flurbiprofenyl							
Methanol	1.08	1.31E−2	82.5	6.0	3.0	56.1	>99.8
Hexanol	7.53E−1	7.38E−3	102	6.0	1.0	51.0	>99.8
Ibuprofenyl							
Methanol	1.29	1.13E−2	114	2.0	8.0	52.1	>99.8
Hexanol	4.77E−1	4.80E−3	99.4	2.0	9.0	60.0	>99.8
Ketoprofenyl							
Methanol	3.96	6.25E−2	63.4	2.0	1.0	51.4	>99.8
Hexanol	4.61	5.24E−2	88.0	2.0	1.0	50.9	>99.8

Conditions: 10 ml anhydrous MTBE containing 3 mM racemate and 100 mM alcohol at 45 °C and 400 rpm. Symbol of E−1 as 10^{-1}.

Table 6
Effects of lipase-catalyzed alcoholysis of (*R*,*S*)-naproxenyl azolides on specific initial rates $V_R/(E_t)$ and $V_S/(E_t)$, V_S/V_R, X_t and ee_s.

Lipase and leaving azole	$V_R/(E_t)$ (mmol/(h g))	$V_S/(E_t)$ (mmol/(h g))	V_S/V_R	(E_t) (mg/ml)	Time (h)	X_t (%)	ee_s (%)
Lipase MY							
Pyrazole	2.40E−4	9.16E−3	38.2	50.0	23.5	58.8	>99.8
4-Bromopyrazole	4.95E−4	6.21E−2	125	10.0	10.0	51.5	>99.8
1,2,4-Triazole	2.04E−2	1.67E−1	8.2	6.0	4.0	60.5	>99.8
CPL							
4-Bromopyrazole	1.35E−4	6.71E−3	49.7	10.0	22.9	32.7	45.6
1,2,4-Triazole	3.44E−2	3.82E−2	1.1	6.0	5.0	50.1	2.7

Conditions: 10 ml anhydrous isooctane containing 100 mM methanol and 3 mM racemate (but 1 mM (*R*,*S*)-naproxenyl 1,2,4-triazole) at 45 °C and 400 rpm. Symbol of E−1 as 10^{-1}.

the fast-reacting enantiomer but with better enantioselectivity was obtainable.

In order to test if the resolution strategy could be applied to other lipases having opposite enantioselectivity to CALB, the alcoholysis of (*R*,*S*)-naproxenyl azolides by methanol in anhydrous isooctane at 45 °C via *C. rugosa* and *C. papaya* lipases was carried out. As shown in Table 6, good to excellent enantioselectivity for CPL and Lipase MY was obtained when selecting (*R*,*S*)-naproxenyl 4-bromopyrazolide as the substrate. All the results represented in Tables 4–6 really imply that for a custom-make (*R*)- or (*S*)-carboxylic acid, one may find an efficient azole to prepare the corresponding (*R*,*S*)-azolide for carrying out the alcoholysis.

3.3. Kinetic analysis

As azolides might act as activated acyl donors for carrying out the lipase-catalyzed alcoholysis, an irreversible ping-pong Bi–Bi mechanism by considering alcohol as a competitive inhibitor was employed for deriving the rate equations. Variations of initial $V_R/(E_t)$ and $100\,V_S/(E_t)$ with enantiomer and methanol concentrations, respectively, for CALB-catalyzed alcoholysis of (*R*,*S*)-naproxen 1,2,4-triazolide in anhydrous MTBE were illustrated in Figs. 1 and 2. As described in Supplementary Information, the kinetic constants $k_{2R} = 181$ mmol/(h g), $k_{2S} = 0.43$ mmol/(h g), $K_{mR} = 86.8$ mM, $K_{mS} = 33.3$ mM, $K_I = 34.7$ mM, $k_{4R}K_{mR} \gg k_{2R}K_{m3R}$, and $k_{4S}K_{mS} \gg k_{2S}K_{m3S}$, and then $k_{2R}/K_{mR} = 2.08$ L/(h g), $k_{2S}/K_{mS} = 1.29 \times 10^{-2}$ L/(h g), and $E = k_{2R}K_{mS}/k_{2S}K_{mR} = 161$, were estimated, showing that the acylation step was rate-limiting in the whole reaction steps. By substituting theses kinetic constants into Eqs. (1)–(3), the time-course (S_R) and (S_S), and hence X_R and X_S, were solved by using a fourth-order Runge–Kutta method. Some experimental data in agreement with the theoretical predictions were represented in Fig. S1.

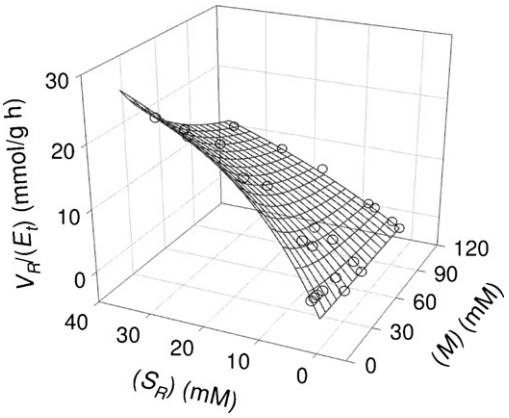


Fig. 1. Variations of specific initial rate $V_R/(E_t)$ (○) with (*R*)-naproxenyl 1,2,4-triazolide and methanol concentrations in anhydrous MTBE consisting of 6 mg/ml CALB at 45 °C. (—) Best-fit results.

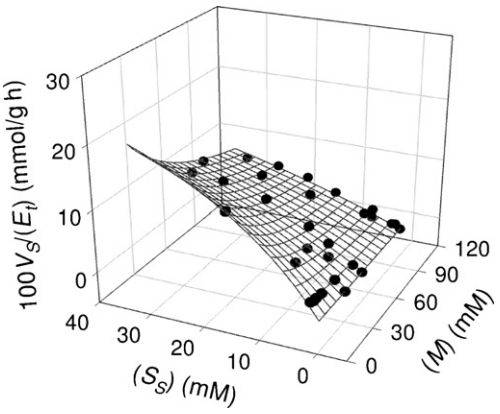


Fig. 2. Variations of specific initial rate $100\,V_S/(E_t)$ (●) with (*S*)-naproxenyl 1,2,4-triazolide and methanol concentrations in anhydrous MTBE consisting of 6 mg/ml CALB at 45 °C. (—) Best-fit results.

It has been reported that the water content in MTBE had profound influence on CALB-catalyzed hydrolysis of (*R*,*S*)-2-phenylpropionyl 1,2,4-triazolide, in which an order-of-magnitude higher K_{mR} and K_{mS}, as well as k_{2R} but not k_{2S}, were found when anhydrous MTBE as the reaction medium was replaced with water-saturated MTBE [26]. This implied that water molecules adsorbed in the enzyme active site not only impeded the substrate affinity to the binding pockets, but also played as lubricants for the easy proton transfer from catalytic serine to histidine to perform the nucleophilic reaction. However in comparison $k_{2R}/K_{mR} = 7.91\,l/(h\,g)$, $k_{2S}/K_{mS} = 0.605\,l/(h\,g)$ and hence $E = 13.1$ in anhydrous MTBE with $k_{2R}/K_{mR} = 2.90\,l/(h\,g)$, $k_{2S}/K_{mS} = 2.92 \times 10^{-2}\,l/(h\,g)$ and hence $E = 99.0$ in water-saturated MTBE, the higher enantioselectivity for the latter was offset by the lower enzyme reactivity for the fast-reacting (*R*)-enantiomer. Similarly by regarding $K_{mR} \gg (S_R)$ and $K_{mS} \gg (S_S)$ for the hydrolysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide, $k_{2R}/K_{mR} = 1.47\,l/(h\,g)$ and $k_{2S}/K_{mS} = 1.52 \times 10^{-2}\,l/(h\,g)$ in anhydrous MTBE (Table S1), as well as $k_{2R}/K_{mR} = 0.173\,l/(h\,g)$ and $k_{2S}/K_{mS} = 2.12 \times 10^{-3}\,l/(h\,g)$ in water-saturated MTBE [26], were estimated from $V_R/(E_t)/(S_R)$ and $V_S/(E_t)/(S_S)$, respectively. Therefore regardless of the acyl part of (*R*,*S*)-profenyl azolides, $V_R/(E_t)$ and $V_S/(E_t)$ as well as k_{2R}/K_{mR} and k_{2S}/K_{mS} in anhydrous MTBE should be greater than those in water-saturated MTBE, implying that one might regard water as a competitive enzyme inhibitor in deriving the rate equations [29].

A comparison of k_{2R}/K_{mR} (or k_{2S}/K_{mS}) values for the hydrolysis and alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide in anhydrous MTBE indicated that the presence of methanol had minute influences on varying the intrinsic kinetic behavior of CALB. As the water content at 45 °C in water-saturated MTBE was estimated as 472.7 mM [30], one might evaluate $k_{2R}/K_{mR}/[1 + (M)/K_I] = 0.142\,l/(h\,g)$ and $k_{2S}/K_{mS}/[1 + (M)/K_I] = 8.82 \times 10^{-4}\,l/(h\,g)$ if $(M) = 472.7$ mM was employed for the alcoholysis. By considering effects of water content on the enzyme performance via hydrolysis [26] as well as $k_{2R}/K_{mR} = 0.173\,l/(h\,g)$ and $k_{2S}/K_{mS} = 2.12 \times 10^{-3}\,l/(h\,g)$ in water-saturated MTBE that were comparable to those via alcoholysis in anhydrous MTBE, water playing the same role as methanol on inhibiting the lipase activity was deducible.

4. Conclusions

With CALB-catalyzed alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide in anhydrous solvents as the model system, the optimal conditions of 45 °C, MTBE as the solvent, and methanol as the acyl acceptor were screened. A change of the leaving azole moiety from 1,2,4-triazole to pyrazole and 4-bromopyrazole but not imidazole at the optimal conditions yielded enhancements of enzyme enantioselectivity but not specific activity for the fast-reacting (*R*)-enantiomer. In comparison with the enzyme performance of using (*R*,*S*)-naproxenyl 1,2,4-triazolide and (*R*,*S*)-2,2,2-trifluoroethyl ester as the substrate, about 8.6-fold and 13.2-fold higher of $V_R/(E_t)$ and V_R/V_S, respectively, for the former were obtained. The resolution strategy was then applied to other (*R*,*S*)-profenyl 1,2,4-triazolides, showing good to excellent enzyme enantioselectivity. Moreover, with a careful selection of reaction conditions (i.e. anhydrous isooctane as the solvent and 4-bromopyrazole as the leaving moiety), the strategy was successfully extended to Lipase MY and CPL having opposite enantioselectivity to CALB.

A thorough kinetic analysis for the alcoholysis of (*R*,*S*)-naproxenyl 1,2,4-triazolide at the optimal conditions led to the rate-limiting acylation step with the alcohol as a competitive inhibitor. The kinetic constants estimated were successfully employed for modeling the time-course conversions for the substrate. A comparison of k_{2R}/K_{mR} and k_{2S}/K_{mS} for the alcoholysis in anhydrous MTBE with those via hydrolysis in anhydrous and water-saturated MTBE implied that water also acted as an enzyme inhibitor. An alkaline buffer solution was further employed for performing the reactive extraction, in which the ester products in the organic phase can be easily separately from the remaining azolide substrate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2009.11.001.

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