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Kinetic resolution of (*R*,*S*)-pyrazolides containing substituents in the leaving pyrazole for increased lipase enantioselectivity

Pei-Yun Wang, Chia-Hui Wu, Jyun-Fen Ciou, An-Chi Wu, Shau-Wei Tsai*

Institute of Biochemical and Biomedical Engineering, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-Shan Tao-Yuan 33302, Taiwan

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ABSTRACT

With hydrolysis of (*R*,*S*)-azolides in water-saturated methyl *tert*-butyl ether (MTBE) via *Candida antarctica* lipase B (CALB) as the model system, (*R*,*S*)-pyrazolides containing a leaving 3-, 4- or 3,4-substituted-pyrazole moiety are selected as the best substrates for preparing various optically pure carboxylic acids containing an α-chiral center. Great improvements of enzyme activity for the (*R*)-enantiomers with excellent enantioselectivity ($V_R/V_S > 100$) are obtainable, if (*R*,*S*)-pyrazolides containing a leaving 3- or 3,4-substituted-pyrazole moiety are employed for the hydrolysis or alcoholysis by methanol in anhydrous MTBE. A detailed kinetic analysis for (*R*,*S*)-*N*-2-phenylpropionylpyrazoles indicates that a bulky 3-substituent such as 3-(3-bromophenyl) or 3-(2-pyridyl) in the leaving pyrazole moiety has profound effects on decreasing the nucleophilic attack and proton transfer of catalytic serine for the slow-reacting enantiomer in anhydrous MTBE, as well as that and substrate affinity for both enantiomers in water-saturated MTBE. The resolution platform is also successfully applied to the hydrolysis of (*R*,*S*)-pyrazolides in water-saturated cyclohexane via *Candida rugosa* lipase (Lipase MY) having opposite enantioselectivity to CALB.

1. Introduction

Lipases are widely employed as versatile biocatalysts for preparing a variety of chiral alcohols, amines, carboxylic acids, or their derivatives for synthesizing pharmaceuticals and fine chemicals [1,2]. In comparison with the resolution of secondary alcohols and amines, the enzyme enantioselectivity toward carboxylic acids is usually low to modest [3–6]. Therefore, various approaches of using substrate engineering [7–10], medium engineering [11,12], enzyme engineering [5,13–15], or their combinations have been proposed for the fine-tuning of enzyme active-site structure to enhance the enantiomer discrimination toward targeted racemates. Recently, a new resolution process of using (*R*,*S*)-azolides, i.e. (*R*,*S*)-*N*-acylazoles, but not their corresponding ester, thioester, or normal amide analogs as the substrate via lipase-catalyzed hydrolysis or alcoholysis in organic solvents was reported [16,17]. The results indicate that by altering the simple leaving azole moiety from imidazole, pyrazole to 1,2,4-triazole, the enzyme activity and enantioselectivity can be improved. One possible explanation is due to the unique azolide structure that contains an unshared electron pairs on the acyl-substituted nitrogen *N*(1), making the carbonyl carbon atom more electrophilic and susceptible to nucleophilic attack, especially when accepting an proton from the imidazolium of catalytic histidine and increasing the number of nitrogens of the leaving azole [18–21].

If the rationale of obtaining the improved enzyme performance is valid for the (*R*,*S*)-azolides containing a simple leaving azole moiety, the question is then how one can further increase the substrate affinity to the enzyme to enhance the reactivity, stabilize the transition state for formation of tetrahedral adduct of the fast-reacting enantiomer, destabilize that of the slow-reacting enantiomer, or use their combinations for enhancing the enantioselectivity. Apparently, the substrate engineering approach of using (*R*,*S*)-azolides with a leaving azole moiety containing substituents is a possible and easy way to obtain the answer. This implies that the substituent may exert non-covalent bonding to the amino acid residues in the active site, leading to good substrate affinity, enantiomer discrimination, and/or higher electrophilicity of the carbonyl carbon atom for better nucleophilic attack. It is therefore aimed to investigate the substituent effects on affecting the enzyme activity and enantioselectivity by using (*R*,*S*)-azolides containing substituents in the leaving moiety as the substrate in organic solvents.

Although *N*-acetylimidazole is 22 times more reactive than the corresponding N-acetylpyrazole in water [20], this is not the case for lipase-catalyzed hydrolysis or alcoholysis of *N*-acylazoles [16,17]. By considering the variety of substituent allocation in the leaving azole moiety, background stability, enzyme activity and enantioselectivity, we mainly focus on the kinetic resolution of

* Corresponding author. Tel.: +886 3 2118800x3415; fax: +886 3 2118668.
E-mail address: tsai@mail.cgu.edu.tw (S.-W. Tsai).

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Nomenclature

ee_s	enantiomeric excesses for the substrate
E	enantiomeric ratio, defined as $k_{2R}K_{mS}/k_{2S}K_{mR}$
(E_t)	enzyme concentration (mg/ml)
k_{2i}	kinetic constants, $i = R$ or S for (R)- or (S)-enantiomer (mmol/g h)
K_{mi},	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 $[X_R + X_S]/2$

(R,S)-pyrazolides but not (R,S)-imidazolides and (R,S)-triazolides. The CALB-catalyzed hydrolysis of (R,S)-*N*-2-phenylpropionylazoles in anhydrous and water-saturated MTBE is first selected as the model system for studying the substrate structure and water content on the enzyme performance (Scheme 1). The kinetic constants for different (R,S)-*N*-2-phenylpropionylpyrazoles are then estimated and compared. Moreover, the resolution for other (R,S)-pyrazolides via CALB and Lipase MY is addressed, showing the promise of present resolution platform for preparing various (R)- or (S)-carboxylic acids containing an α-chiral center.

2. Materials and methods

2.1. Materials

Lipase MY from *Candida rugosa* and Novozym 435 from *Candida antartica* lipase B were provided by Meito Sangyo (Tokyo, Japan) and Novo Nordisk (Bagsvaerd, Denmark), respectively. Dimethyl sulfoxide-d_6 (DMSO-d_6) containing 1% v/v tetramethylsilane (TMS) for ^{1}H NMR analysis was from Cambridge Isotope Laboratories (Andover, MA). Other chemicals of analytical grade were commercially available: 3,5-dimethyl-1H-pyrazole, 4-methyl-1H-pyrazole and pyrazole from Acros (Geel, Belgium); (R,S)-α-bromophenylacetic acid, 1,1′-carbonylbis(2-methylimidazole) and 4-bromo-1H-pyrazole from Aldrich (Milwaukee, WI); 3-amino-1H-pyrazole, 3-(3-bromophenyl)-1H-pyraozle, 3-methyl-1H-pyrazole, 3-(2-pyridyl)-1H-pyraozle, and 3-methyl-4-bromo-1H-pyrazole from Alfa Aesar (Ward Hill, MA); 1H-benzotriazole, N,N′-carbonyldi-1,2,4-triazole (CDT) and (R,S)-2-phenylpropionic acid from Fluck (Buchs, Switzerland); 4-nitro-1H-pyrazole from Matrix Scientific (Columbia, SC); (R,S)-2-ethyl-phenylacetic acid, (R,S)-ketoprofen (i.e. (R,S)-2-(3-benzoylphenyl)-propionic acid), (R,S)-α-methoxyphenylacetic acid and (R,S)-naproxen (i.e. (R,S)-2-(6-methoxy-2-naphthyl)-propionic acid) from TCI (Tokyo, Japan); (R,S)-flurbiprofen (i.e. (R,S)-2-fluoro-α-methyl-4-biphenylacetic acid) from Sigma (St. Louis, MO); cyclohexane, isopropanol (IPA), hexane, methanol and MTBE from Tedia (Fairfield, OH). Anhydrous MTBE was prepared by adding calcium hydride from Riedel-de Haen (Seelze, Germany) to MTBE for 24 h.

2.2. General procedures for substrates synthesis

To 5 ml benzene was added 1 mmol racemic acid and 1.5 mmol CDT and the mixture was stirred at 55 °C for 2 h. The resultant mixture was filtered and evaporated under reduced pressure, giving the desired (R,S)-*N*-acyl-1,2,4-triazole (**13**, **16**, **19**, **22**). Moreover to 1.25 ml of a benzene solution containing 3.25 mmol thionyl chloride were added dropwise a mixture consisting of 6.0 ml benzene, 2.50 mmol 4-bromo-1H-pyrazole, 3.25 mmol racemic acid, and 10 mmol triethylamine. The resultant solution was stirred for 2 h at room temperature. After being quenched in succession with 0.1 M HCl solution (3 × 10 ml), 0.1 M NaOH solution (3 × 10 ml) and 0.1 M NaCl solution (3 × 10 ml), the organic phase was separated, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure, giving the desired (R,S)-*N*-acyl-4-bromopyrazoles (**11**, **18**, **21**, **23**–**25**). With 4-bromo-1H-pyrazole replaced by another azole, similar procedures were carried out to prepare other (R,S)-azolides except for (R,S)-*N*-phenylpropionyl-3-aminopyrazole (**3**).

To 2.0 ml pyridine was added 1.0 mmol (R,S)-phenylpropionic acid, 3.0 mmol triethylamine, and 1.0 mmol thionyl chloride with stirring for 1 h at room temperature. The resultant mixture was then added to 0.5 ml pyridine containing 1.0 mmol 3-aminopyrazole and stirred for 2 h. After removing pyridine under reduced pressure, the residue was dissolved in 20 ml benzene, washed in succession with 0.1 M HCl solution (3 × 20 ml) and deionized water (20 ml). The organic phase was separated, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure, giving the desired substrate (**3**). All the synthesized substrates were confirmed from the retention time in HPLC analysis and ^{1}H NMR spectra recorded at 400 MHz on Brucker AC-400 spectrometer in DMSO-d_6 solution with TMS as an internal standard.

2.3. Analysis

Hydrolysis or alcoholysis of (R,S)-azolides was monitored by HPLC using a chiral column from Daicel (OD-H or OJ-H; Tokyo, Japan) or Regis ((*S*,*S*)-whelk-01; Morton Grove, IL) that is capable of separating the internal standard of benzene, 2-nitrotoluene, or *p*-nitrophenol. UV detection at 220 or 270 nm was employed for quantification at the room temperature. Detailed analytical conditions are represented in Table S1 of the Supporting Information.

2.4. Effects of substrate structure

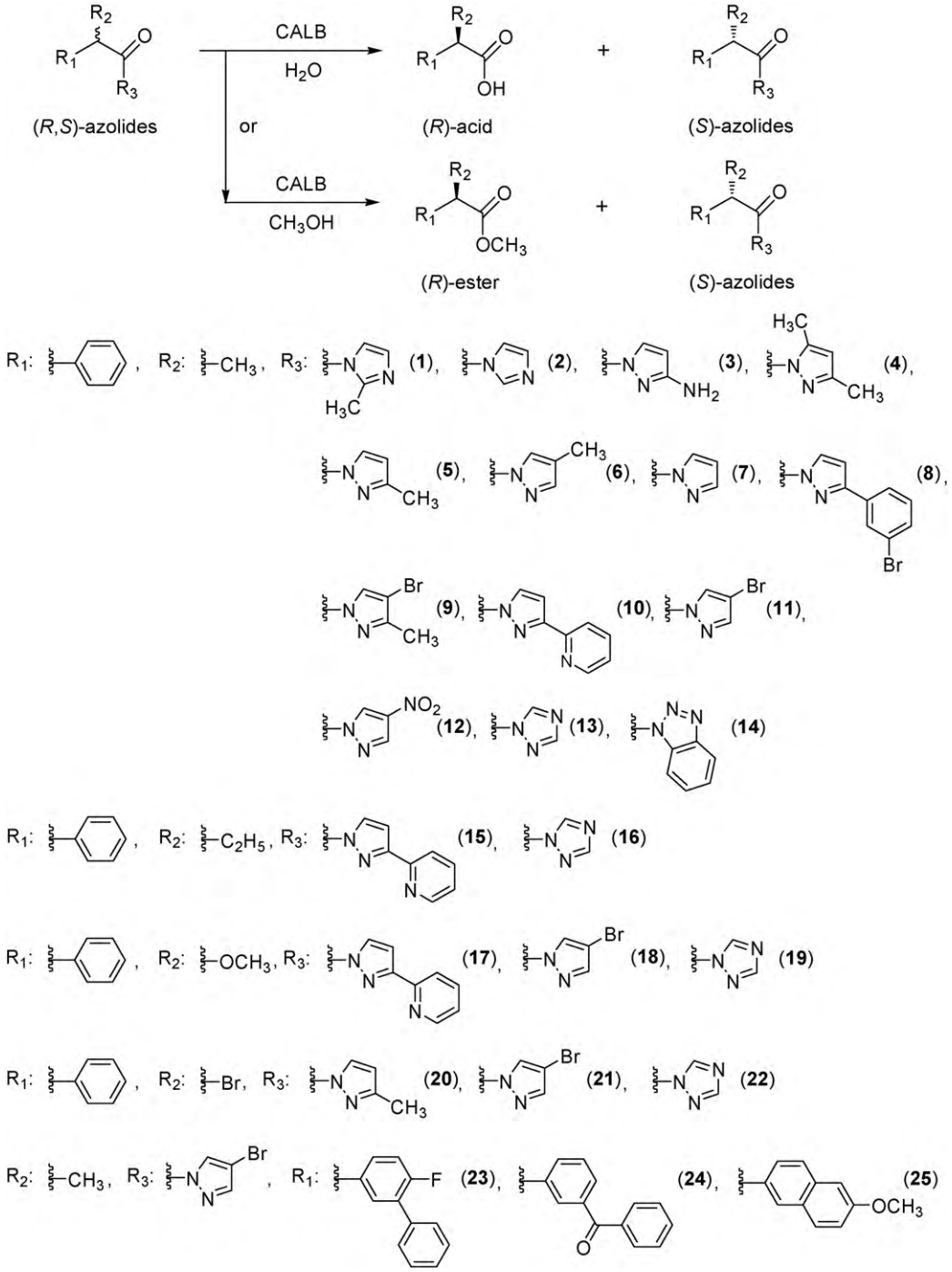
To 10 ml water-saturated MTBE containing 3 mM of different (R,S)-azolide was added a specific amount of CALB for performing the hydrolytic resolution. The resultant solution was stirred with a magnetic stirrer at 45 °C. Samples were removed from the reaction medium at different time intervals for HPLC analysis, from which the time-course conversions X_R and X_S, initial rates for both enantiomers V_R and V_S, racemate conversion X_t, and enantiomeric excess for the substrate ee_s were determined. Similar experiments were performed in anhydrous MTBE via CALB, as well as in water-saturated cyclohexane via Lipase MY having opposite enantioselectivity to CALB and low tolerance to polar organic solvents. The alcoholytic resolution was also carried out via CALB, except that 100 mM methanol was further added to 10 ml anhydrous MTBE containing 3 mM (R,S)-azolide.

In order to compare the kinetic behaviors at 45 °C, the hydrolytic reactions by varying the concentration of **7**, **8**, **10**, and **11** in anhydrous or water-saturated MTBE were carried out. Details for estimating the kinetic constants K_{mS} and k_{2S}, K_{mR} and k_{2R}, and hence E value (i.e. $k_{2R}K_{mS}/k_{2S}K_{mR}$) were described in the Supporting Information.

3. Results and discussion

3.1. Hydrolysis in water-saturated MTBE

Table 1 demonstrates effects of leaving azole moiety and acyl part of (R,S)-azolides on changing the initial specific activities $V_R/(E_t)$ and $V_S/(E_t)$, enantioselectivity in terms of V_R/V_S, racemate



Scheme 1. CALB-catalyzed hydrolysis or alcoholysis of (*R*,*S*)-azolides in organic solvents.

conversion X_t, and enantiomeric excess for the substrate ee_s at a specified reaction time in water-saturated MTBE. In comparison with **2**, an introduction of 2-methyl substituent to the leaving imidazole moiety of **1** mainly decreases the reactivity of fast-reacting (*R*)-enantiomer and hence the enantioselectivity. By comparing with **13**, a benzene group fused into the leaving 1,2,3-triazole moiety of **14** also greatly decreases the enzyme activity and enantioselectivity. One may attribute the former to the high pK_a of 2-methylimidazole on decreasing the electrophilicity of carbonyl carbon atom for nucleophilic attack, and the latter to the steric hindrance of leaving benzotriazole moiety for substrate affinity to the active site. However for **6**, **11**, or **12** containing a 4-methyl, 4-bromo, or 4-nitro substituent in the leaving pyrazole moiety, the enantioselectivity increases with the penalty of slight decreasing of $V_R/(E_t)$ in comparison with **7**. The strongest electro-withdrawing ability of 4-nitro substituent of **12**, giving the lowest pK_a of the resultant 4-nitropyrazole, should have benefits for the nucleophilic attack of catalytic serine for formation of tetrahedral adduct and then breakdown to release 4-nitropyrazole. Yet, only 3 times difference of $V_R/(E_t)$ with 5.04 units pK_a change of the leaving pyrazole between (*R*)-**6** and (*R*)-**12** is shown, implying that there must exist additional attractions or repulsions between the 4-substituent and amino acid residues of the active site. These interactions may yield minute distortion of the substrate orientation in the active site, such that the distance between and carbonyl carbon atom and catalytic serine increases to relax the electro-withdrawing ability of

Table 1
Effect of substrate structure on pK_a of leaving azole, specific initial rates, V_R/V_S, X_t and ee_s for CALB-catalyzed hydrolysis in water-saturated MTBE.

Entry	pK_a	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_R/V_S	(E_t) (mg/ml)	Time (h)	X_t (%)	ee_s (%)
1	7.85[a]	3.77E−3	3.70E−3	1.0	30.0	20.0	29.9	1.0
2	7.00[a]	2.88E−2	4.76E−3	6.1	20.0	4.3	51.8	58.8
3	6.00[b]	6.57E−2	3.35E−4	196	20.0	5.0	51.4	100.0
4	4.06	N	N	N	60.0	118.0	N	N
5	3.27	9.05E−2	5.62E−4	161	20.0	4.0	51.5	100.0
6	3.04	5.28E−1	4.73E−3	111	20.0	1.0	50.5	100.0
7	2.52[a]	2.62	3.23E−2	81	2.0	1.0	53.5	100.0
8	1.84[b]	3.62E−3	1.76E−5	291	40.0	48.0	51.1	100.0
9	1.43	1.32E−2	5.00E−5	264	60.0	18.0	51.8	100.0
10	1.33[b]	1.27E−2	2.63E−5	482	40.0	24.0	50.7	100.0
11	0.63	5.56E−1	3.55E−3	156	12.0	2.0	50.8	100.0
12	−2.00	1.61	6.62E−3	243	20.0	0.3	50.1	100.0
13	2.19[a]	4.38	4.58E−2	95	2.0	0.7	55.6	100.0
14	1.60	2.10E−4	1.35E−5	1.6	20.0	72.0	9.5	1.7
15	1.33[b]	8.85E−4	N	N	40.0	147.5	48.5	94.2
16	2.19	4.25E−1	2.25E−3	188	10.0	2.0	51.5	100.0
17	1.33[b]	5.92E−3	1.73E−5	342	20.0	48.0	51.8	100.0
18	0.63	1.39E−1	1.41E−2	9.9	20.0	0.8	50.5	68.3
19	2.19	1.88	2.54E−1	7.4	2.0	1.3	68.3	100.0
20	3.27	2.41E−2	9.75E−5	247	20.0	9.5	50.8	100.0
21	0.63	6.04E−2	2.63E−3	23	20.0	12.0	50.3	78.9
22	2.19	1.15	9.05E−2	12	2.0	3.0	64.4	100.0
23	0.63	1.79E−2	8.63E−5	207	40.0	24.0	54.7	100.0
24	0.63	2.15E−1	8.25E−4	260	20.0	2.0	50.5	100.0
25[c]	0.63	2.31E−2	2.10E−4	110	40.0	19.5	57.5	100.0

[a] From Wang et al. [16].
[b] Estimated by pK_a = 2.289 − 5.642σ_p (R^2 = 0.988) correlated from known pK_a and σ_p of **5**, **7**, **11** and **12**; Hammett *para*-substituent constants σ_p for 3-amino, 3-(2-pyridyl), and 3-(3-bromophenyl) as −0.66, 0.08 and 0.17, respectively, from Hansch et al. [22].
[c] From Wu et al. [17]. Reaction conditions: 10 ml water-saturated MTBE containing 3 mM racemate at 45 °C and 400 rpm. Symbol E−1 as 10^{-1}; notation N as too low to be determined.

4-bromo substituent in (*R*)-**11** and 4-nitro substituent in (*R*)-**12**, and hence decreases the enzyme activity as elucidated from the kinetic analysis.

More improvements in enantioselectivity are perceived for **3**, **5**, **8**, and **10** containing a 3-amino, 3-methyl, 3-(3-bromophenyl), or 3-(2-pyridyl) substituent in the leaving pyrazole moiety. Yet, no correlations between log($V_R/(E_t)$) or log($V_S/(E_t)$) and pK_a of the leaving 3-substitited-pyrazole were found, indicating that more complicated interactions among the 3-substituent, amino acid residues of the enzyme, and water adsorbed in the active site should occur. In comparison with **5** and **11**, the introduction of a 3-methyl-4-bromo substituent to the leaving pyrazole moiety of **9** can further enhance the enantioselectivity but decreases the reactivity for each enantiomer. However no reactivity of **4** containing a leaving 3,5-dimethylpyrazole moiety was detected at 118 h by employing 60 mg/ml of CALB, indicating that the lipase did not accommodate (*R*)- and (S)-azolides of **4** and **14** containing a 5-substituent in the leaving azole moiety.

In order to demonstrate the merit of using (*R*,*S*)-azolides for preparing various carboxylic acids containing an α-chiral center, the α-methyl moiety of **1**–**14** was replaced by α-ethyl moiety of **15** and **16**. In comparison with 480-fold enhancement of $V_R/(E_t)$ with excellent enantioselectivity for **16** containing a leaving 1,2,4-triazole moiety, very low reactivity of (*R*)-**15** without detecting (*S*)-**15** reactivity at 147.5 h was found. Apparently, the α-ethyl moiety might impede the substrate affinity to the active

Table 2
Effect of substrate structure on pK_a of leaving azole, specific initial rates, V_R/V_S, X_t and ee_s for CALB-catalyzed hydrolysis in anhydrous MTBE.

Entry	pK_a	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_R/V_S	(E_t) (mg/ml)	Time (h)	X_t (%)	ee_s (%)
3	6.00	2.51	1.92E−2	130	2.0	0.9	6.0	100.0
4	4.06	9.50E−5	2.50E−5	3.8	60.0	118.0	23.2	14.4
5	3.27	4.96	1.91E−2	259	2.0	1.0	53.8	100.0
6	3.04	2.04	3.88E−2	52	6.0	0.7	54.4	100.0
7	2.52	5.60	1.82E−1	30	2.0	5.0	54.2	100.0
8	1.84	3.43	5.89E−3	582	2.0	1.3	50.6	100.0
9	1.43	3.20	7.73E−3	414	2.0	5.0	52.1	100.0
10	1.33	7.13	1.84E−2	387	2.0	2.0	52.5	100.0
11	0.63	4.24	7.91E−2	53	2.0	0.8	53.4	100.0
12	−2.00	4.27	1.31E−1	32	6.0	0.7	63.5	100.0
13	2.19	1.08E+1	7.23E−1	14	2.0	0.2	58.0	100.0
14	1.60	1.08E−3	1.47E−4	7.3	20.0	72.0	34.2	34.0
15	1.33	8.28E−1	1.10E−3	752	4.0	2.0	50.6	100.0
16	2.19	3.91	1.45E−2	269	2.0	0.8	50.7	100.0
17	1.33	2.83	4.43E−3	638	2.0	1.0	52.5	100.0
18	0.63	5.63	1.14E−1	49	2.0	0.8	55.0	100.0
19	2.19	7.05	1.61	4.4	2.0	0.2	51.4	46.8
20	3.27	1.09	3.38E−3	322	2.0	3.5	50.3	100.0
22	2.19	6.06	3.08E−1	19	2.0	0.3	58.7	63.2
24	0.63	3.70	2.95E−2	125	2.0	1.0	50.2	100.0

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

site [23], when comparing $V_R/(E_t)$ or $V_S/(E_t)$ for **13** and **16**, and especially those for **10** and **15** containing a bulky 3-(2-pyridyl) substituent in the leaving pyrazole moiety. When the α-methyl moiety was changed to α-methoxy of **17**–**19** containing a leaving 1,2,4-triazole, 4-bromopyrazole, or 3-(2-pyridyl)pyrazole moiety, similar enzyme performances as those for **10**, **11**, and **13** were observed, except for giving very low enantioselectivity for **18** and **19**. This really indicates the complicated interactions among the substituent, α-methoxy moiety, and amino acid residues mainly affecting $V_S/(E_t)$ of slow-reacting substrates. If the α-methyl moiety was further replaced by α-bromo of **20**–**22** containing a leaving 3-methylpyrazole, 4-bromopyrazole, or 1,2,4-triazole moiety, only **20** gave excellent enantioselectivity. However, when employing (*R*,*S*)-*N*-profenyl-4-bromopyrazoles of **23**–**25** as the substrate, increased enantioselectivity with the penalty of slight deceasing of $V_R/(E_t)$ was perceived when comparing with the previous results of using (*R*,*S*)-*N*-profenyl-1,2,4-triazoles as the substrate [16].

All the results in Table 1 demonstrate that a minute change of the α-substituent of acyl part or leaving pyrazole moiety may greatly affect CALB activity and enantioselectivity. However, a guarantee of giving excellent enantioselectivity may be made if (*R*,*S*)-pyrazolides containing a leaving 3-, 4- or 3,4-substituted-pyrazole moiety are selected as the substrate. This is especially true for **8**, **10**, and **17** containing a bulky 3-(2-pyridyl) or 3-(3-bromophenyl) substituent. In order to improve the reactivity of (*R*)-*N*-acylpyrazole containing a leaving 3, 4- or 3,4-substituented-pyrazole, the hydrolytic resolution with water originally adsorbed on CALB as the acyl acceptor was carried out in anhydrous MTBE.

3.2. Hydrolysis in anhydrous MTBE

In comparison with the reactivity of **13** in Table 1, about 2.5- and 15.8-fold enhancements of $V_R/(E_t)$ and $V_S/(E_t)$, respectively, are demonstrated, leading to lower enantioselectivity in anhydrous MTBE (Table 2). This indicates that water adsorbed in the active site might affect the substrate affinity and/or nucleophilic attack and proton transfer of catalytic serine for each enantiomer [16]. The very low CALB activity and enantioselectivity of **4** and **14** (Tables 1 and 2) really reflects the difficult substrate affinity in anhydrous or water-saturated MTBE. On the contrary, more than an order-of-magnitude higher of $V_R/(E_t)$ for (*R*)-**6**, (*R*)-**11**, and (*R*)-**12** as well as two order-of-magnitudes higher of $V_R/(E_t)$ for (*R*)-**5**, (*R*)-**8**, (*R*)-**9**, and (*R*)-**10** are shown when comparing with those in Table 1. This indicates that decreasing of water content in the reaction medium has profound effects on affecting the enzyme performance. The high reactivity associated with excellent enantioselectivity for **3**, **5**, and **8**–**10** containing a leaving 3- or 3,4-substituted-pyrazole in anhydrous MTBE may provide an indirect evidence that water adsorbed in the active site should allocate in the region near the 3-substituent in water-saturated MTBE.

Similar arguments for the improvement of enzyme activity and enantioselectivity can be applied to other (*R*)-azolides, especially **15**–**17**, **20**, and **24**. Therefore, the best strategy for increasing the (*R*)-enantiomer productivity with high optical purity is to employ (*R*,*S*)-*N*-acyl-3-substituted-pyrazole as the substrate in the reaction medium controlled at low water content in which water as the acyl acceptor can be added in a feed-batch mode.

3.3. Kinetic analysis

In order to shed insights into effects of water content and substrate structure on the hydrolytic resolution, the kinetic analysis based on Michaelis–Menten mechanism was carried out. Figs. S1 and S2 of the Supplementary Information illustrate the initial rates V_R and V_S varied with substrate concentrations for **7**, **8**, **10**, and **11** in anhydrous and water-saturated MTBE, with which the kinetic constants represented in Table 3 are then estimated and compared. In anhydrous MTBE, all Michaelis constants K_{mR} and K_{mS} have the same order-of-magnitude. This is also perceived for all kinetic constants k_{2R} but not k_{2S}, indicating that the leaving 1,2,4-triazole or pyrazole moiety with or without containing a substituent has minor influences on varying the substrate affinity and nucleophilic attack to the carbonyl carbon atom of (*R*)-enantiomers but not their antipodes. Therefore, one obtains the same order-of-magnitude of k_{2R}/K_{mR} but not k_{2S}/K_{mS}, leading to excellent enantioselectivity for **8** and **10**, modest for **7** and **11**, but low for **13**. A detailed analysis of k_{2S}/K_{mS} gives the free energy difference between the ground and transient states for (*S*)-**8** to be 9.4 kJ/mol higher than that for (*S*)-**7**. Similarly, only 5.9 kJ/mol difference between those of (*S*)-**10** and (*S*)-**7**, as well as 1.3 kJ/mol between those of (*S*)-**11** and (*S*)-**7**, are estimated. Therefore, more interactions between the 3-(3-bromophenyl) substituent and amino acid residues of the active site, modest for the 3-(2-pyridyl) substituent, and low for the 4-bromo substituent, on impeding the nucleophilic attack and proton transfer for formation of (*S*)-tetrahedral adduct can be deduced.

Table 3
Effects of leaving azole on kinetic constants and *E* values for CALB-catalyzed hydrolysis in anhydrous and water-saturated MTBE.

Entry	**13**[a]	**7**	**11**	**8**	**10**
Anhydrous MTBE					
k_{2R} (mmol/h g)	2.01E+2	1.37E+2	9.47E+1	4.82E+1	1.00E+2
K_{mR} (mM)	2.54E+1	2.28E+1	1.62E+1	1.78E+1	1.72E+1
k_{2R}/K_{mR} (l/h g)	7.91	6.00	5.85	2.71	5.81
k_{2S} (mmol/h g)	2.07E+1	3.11	9.44E−1	6.40E−2	2.19E−1
K_{mS} (mM)	3.42E+1	2.16E+1	1.06E+1	1.56E+1	1.41E+1
k_{2S}/K_{mS} (l/h g)	6.05E−1	1.44E−1	8.91E−2	4.10E−3	1.55E−2
E	13.1	41.7	65.6	661	375
Water-saturated MTBE					
k_{2R} (mmol/h g)	1.33E+3	1.14E+3	1.35E+2		
K_{mR} (mM)	4.59E+2	5.34E+2	4.17E+2		
k_{2R}/K_{mR} (l/h g)	2.90	2.13	3.24E−1	2.33E−3	5.54E−3
k_{2S} (mmol/h g)	1.55E+1	1.25E+1	9.44E−1		
K_{mS} (mM)	5.31E+2	5.29E+2	4.76E+2		
k_{2S}/K_{mS} (l/h g)	2.92E−2	2.36E−2	1.98E−3	1.11E−5	1.58E−5
E	99.3	90.2	164	210	351

[a] From Wang et al. [16]. Reaction conditions: 10 ml MTBE at 45 °C and 400 rpm. Symbol E−1 as 10^{-1}.

Table 3 also demonstrates nearly the same K_{mR} and K_{mS} for **7**, **11** and **13** in water-saturated MTBE, which are an order-of-magnitude higher than those in anhydrous MTBE. Very similar kinetic behaviors giving almost the same k_{2R}/K_{mR} or k_{2S}/K_{mS} and hence *E* values for **7** and **13** are shown. In comparison with **7**, about an order-of-magnitude lower of k_{2R} and k_{2R}/K_{mR} (or k_{2S} and k_{2S}/K_{mS}), and then higher *E* value (i.e. $k_{2R}K_{mS}/k_{2S}K_{mR}$), for **11** containing a leaving 4-bromopyrazole moiety are obtainable. This indicates that water adsorbed in the active site has decreased the nucleophilic attack and proton transfer of catalytic serine but not the substrate affinity, and hence the reactivity of (*R*)- and (*S*)-**11**. For **8** and **10** containing a bulky 3-substitutent in the leaving pyrazole moiety, liner relationships between the initial rates and substrate concentrations are illustrated (Fig. S2). This indicates that the Michaelis constants K_{mR} and K_{mS} are too large to be determined, giving a strong support for the adsorbed water allocated in the active site near the 3-substituent on impeding the substrate affinity. In comparison with **7**, more than two order-of-magnitudes lower of k_{2R}/K_{mR} and k_{2S}/K_{mS} but with excellent enantioselectivity for **8** and **10** are shown, implying that the adsorbed water might also decrease the nucleophilic attack and proton transfer of the catalytic serine.

It is reasonable to assume that K_{mR} and K_{mS} for all substrates in Tables 1 and 2 are at least an order-of-magnitude higher than the employed substrate concentration. As an approximation for esti-

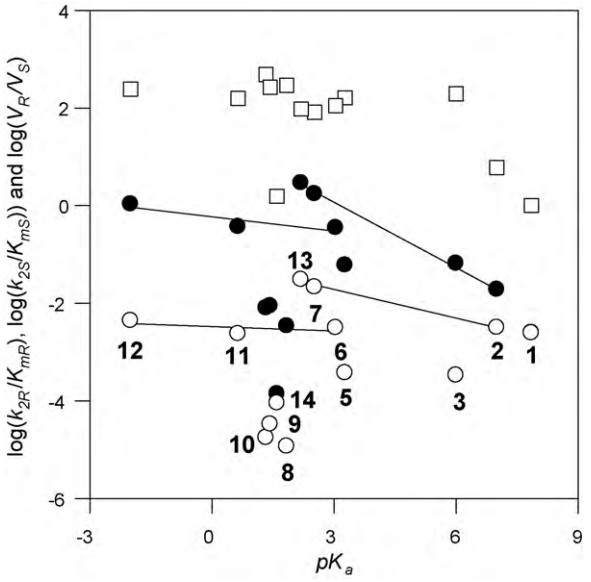


Fig. 1. Variations of $log(k_{2R}/K_{mR})$ (●), $log(k_{2S}/K_{mS})$ (○), and $log(V_R/V_S)$ (□) with pK_a of leaving azole for CALB-catalyzed hydrolysis in water-saturated MTBE. (—) Best-fit results for (*R*)- or (*S*)-enantiomers of **6**, **11** and **12**, and those of **2**, **7** and **13** from Wang et al. [16].

mating k_{2R}/K_{mR} from $V_R/(E_t)/(S_R)$ and k_{2S}/K_{mS} from $V_S/(E_t)/(S_S)$ in the hydrolysis, the structure-reactivity correlations in terms of pK_a of leaving azole in water-saturated MTBE are illustrated in Fig. 1. Good linear relationships for **2**, **7** and **13**, modest for **6**, **11** and **12**, but bad for **3**, **5** and **8**–**10** are shown. The unique azolide structure on the reactivity is nicely reflected from the Brønsted slope of −0.45 for the fast-reacting (*R*)-**2**, (*R*)-**7**, and (*R*)-**13**, and −0.20 of their antipodes [16]. However, the slopes drop to −0.09 and −0.03 for (*R*)- and (*S*)-enantiomers of **6**, **11** and **12**, respectively. This might provide an indirect evidence for the existence of interactions among the 4-substitent moiety, amino acid residues, and adsorbed water, such that the electro-withdrawing effect exerted by 4-bromo or 4-nitro substituent on enhancing the nucleophilic attack of catalytic serine and hence the enzyme activity is relaxed. No correlations for (*R*)- and (*S*)-enantiomers of **3**, **5** and **8**–**10** are perceived, reflecting the more complicated interactions of the 3-substituent moiety in the active site. As illustrated in Figs. S3 and S4 of the Supplementary Information, the above arguments in Fig. 1 can be applied to the hydrolysis and alcoholysis of (*R*,*S*)-*N*-2-phenylpropionylazoles in anhydrous MTBE, where the effect of adsorbed water on the non-covalent interactions in the active site is minimized.

3.4. Alcoholysis by methanol in anhydrous MTBE

In order to demonstrate the novelty of using (*R*,*S*)-pyrazolides containing substituents in the leaving pyrazole moiety for lipase-catalyzed resolution in anhydrous organic solvents, the alcoholysis of (*R*,*S*)-*N*-acylazoles by 100 mM methanol in anhydrous MTBE is represented in Table 4. Similar enzyme performances as those in Table 2 but with lower reactivity for each substrate are perceived, yet still leading to excellent enantioselectivity for (*R*,*S*)-pyrazolides containing a leaving 3- or 3,4-substitued-pyrazole moiety. The

Table 4
Effect of substrate structure on pK_a of leaving azole, specific initial rates, V_R/V_S, X_t and ee_s for CALB-catalyzed alcoholysis in anhydrous MTBE.

Entry	pK_a	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_R/V_S	(E_t) (mg/ml)	Time (h)	X_t (%)	ee_s (%)
3	6.00	1.06	3.05E−3	347	2.0	3.0	50.7	100.0
5	3.27	9.69E−1	3.63E−3	266	10.0	1.0	50.6	100.0
8	2.19	7.99E−1	1.08E−3	739	2.0	20.0	51.1	100.0
9	1.43	5.07E−1	1.82E−3	278	10.0	6.0	50.4	100.0
10	1.33	1.99	2.40E−3	829	2.0	3.4	50.8	100.0
11	0.63	2.98	3.79E−2	78	10.0	0.3	50.4	100.0
13	2.19	9.46	2.15E−1	43	2.0	0.6	60.0	100.0
15	1.33	1.56E−1	1.40E−4	111	20.0	2.0	50.2	100.0
16	2.19	1.63	1.43E−2	114	2.0	3.5	50.1	100.0
17	1.33	6.82E−1	2.76E−3	247	2.0	3.1	51.4	100.0
18	0.63	2.36	6.62E−2	35	2.0	1.3	54.4	100.0
19	2.19	5.60	1.09	5.1	2.0	0.3	64.8	72.1
20	3.27	3.07E−1	2.30E−3	133	10.0	3.5	50.6	100.0
22	2.19	3.60	1.13E−1	31	2.0	1.0	54.6	100.0
23	0.63	6.01E−1	4.58E−3	131	6.0	1.5	52.1	100.0

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

Table 5
Effect of lipase sources on pK_a of leaving azole, specific initial rates, V_S/V_R, X_t and ee_s for Lipase MY-catalyzed hydrolysis in water-saturated cyclohexane.

Entry	pK_a	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_S/V_R	(E_t) (mg/ml)	Time (h)	X_t (%)	ee_s (%)
3	6.00	5.14E−4	3.44E−2	66	40.0	5.0	53.2	100.0
5	3.27	1.16E−5	4.79E−3	41	40.0	72.0	42.8	68.0
6	2.19	6.78E−4	8.47E−2	124	20.0	5.0	52.8	100.0
9	1.43	3.45E−5	6.68E−3	193	40.0	47.0	46.1	82.9
11	0.63	8.10E−4	9.86E−2	121	20.0	5.0	54.8	100.0
12	−2.00	8.95E−3	4.61E−1	51	20.0	1.0	53.1	100.0
13	2.19	2.92E−2	3.94E−1	13	10.0	2.0	54.6	82.9
14	1.60	N	N	N	40.0	115.0	N	N
17	1.33	2.40E−4	8.92E−3	37	60.0	9.0	51.5	89.2
18	0.63	7.33E−2	1.08	14	10.0	0.6	58.1	96.2
19	2.19	5.30E−2	2.02E−1	3.8	2.0	15.4	44.2	31.5
20	3.27	1.89E−3	1.11E−1	58	40.0	2.0	53.0	100.0
21	0.63	1.69E−3	8.90E−2	52	20.0	4.0	53.4	100.0
23	0.63	1.16E−3	7.24E−2	62	20.0	20.0	59.5	100.0
25	0.63	2.37E−3	7.56E−2	31	10.0	22.5	62.6	87.5

Reaction conditions: 10 ml water-saturated cyclohexane containing 3 mM racemate at 45 °C and 400 rpm. Symbol E−1 as 10^{-1}; notation N as too low to be determined.

competitive enzyme inhibition by methanol has been proposed for elucidating the lower enzyme activity [17]. Based on the hydrolysis of (*R*)-*N*-naproxenyl-1,2,4-triazole in water-saturated MTBE, 8.5- and 3.7-fold increases of $V_R/(E_t)$ for the hydrolysis and alcoholysis, respectively, in anhydrous MTBE were estimated. These enhancements increase to 561 and 156 folds for (*R*)-**10**, 935 and 176 folds for (*R*)-**15**, and 478 and 115 folds for (*R*)-**17**, implying that water adsorbed in the active site does exert strong interactions to the amino acid residues of the active site and 3-(2-pyridyl) substituent. Therefore, the best strategy for increasing the (*R*)-ester productivity and optical purity is to employ (*R*,*S*)-*N*-acyl-3-substituted-pyrazole as the substrate in the reaction medium controlled at low methanol concentration in which methanol as the acyl acceptor can be added in a feed-batch mode.

3.5. Hydrolysis in water-saturated cyclohexane via Lipase MY

In order to test if the resolution strategy can be extended to lipases having opposite enantioselectivity to CALB, Lipase MY-catalyzed hydrolysis of (*R*,*S*)-azolides in water-saturated cyclohexane but not MTBE owing to the low enantioselectivity and stability in polar organic solvents was performed (Table 5). In comparison with **13** and **19** containing a leaving 1,2,4-triazole moiety, (*R*,*S*)-pyrazolides containing a substituent, especially a 4- or 3,4-substituent, in the leaving pyrazole moiety might yield good to excellent enantioselectivity. By considering the enzyme performance in the hydrolytic resolution, CALB is superior to Lipase MY regardless of the different optical preferences. All the results represented in Tables 1–5 really indicate that one may employ the present hydrolytic or alcoholytic resolution platform for preparing (*R*)- and (*S*)-carboxylic acid containing an α-chiral center.

4. Conclusions

With CALB-catalyzed hydrolytic resolution of (*R*,*S*)-*N*-2-phenylpropionylazoles (**1**–**14**) and other (*R*,*S*)-*N*-acylazoles (**15**–**24**) in water-saturated MTBE as the model system, (*R*,*S*)-pyrazolides containing a leaving 3-, 4- or 3,4-substituted pyrazole moiety are exploited as the best substrates, when comparing with **13** containing a leaving 1,2,4-triazole moiety. Great improvements of enzyme activity with excellent enantioselectivity ($V_R/V_S > 100$) for the fast-reacting enantiomers are also obtainable, if (*R*,*S*)-pyrazolides containing a leaving 3- or 3,4-substituted-pyrazole moiety are employed for the hydrolysis or alcoholysis by methanol in anhydrous MTBE. Therefore, the best strategy for carrying out the present resolution process is to use (*R*,*S*)-pyrazolides containing a leaving 3-substituted pyrazole moiety as the substrate, and keeps the reaction medium at low water (or methanol) contents by adding the acyl acceptor in a fed-batch mode.

A thorough kinetic analysis for (*R*,*S*)-*N*-2-phenylpropionylpyrazoles indicates that a bulky 3-substituent in the leaving pyrazole moiety has profound effects on decreasing the nucleophilic attack and proton transfer of catalytic serine for the slow-reacting enantiomer in anhydrous MTBE, as well as that and substrate affinity for both enantiomers in water-saturated MTBE. The good linear structure-reactivity correlations in water-saturated MTBE for **2**, **7** and **13**, modest for **6**, **11** and **12**, and bad for **3**, **5** and **8**–**10**, are regarded as indirect evidences for the strong interactions of 3-substituent (but modest for the 4-substitutent) with amino acid residues and adsorbed water in the active site. The resolution platform has also been extended to the hydrolysis of (*R*,*S*)-*N*-acylpyrazoles in water-saturated cyclohexane via Lipase MY having opposite enantioselectivity to CALB.

Supplementary information: Information on product characterization specifications (^{1}H NMR spectra and retention time in HPLC analysis), initial rates varied with substrate concentrations for CALB-catalyzed hydrolysis in anhydrous and water-saturated MTBE, and variations of $log(k_{2R}/K_{mR})$, $log(k_{2S}/K_{mS})$, and $log(V_R/V_S)$ with pK_a of leaving azole in anhydrous MTBE is available free of charge via the Internet at http://www.sciencedirect.com.

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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.2010.04.003.

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