(*R,S*)-2-Chlorophenoxyl Pyrazolides as Novel Substrates for Improving Lipase-Catalyzed Hydrolytic Resolution

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ABSTRACT The best reaction condition of *Candida antartica* lipase B as biocatalyst, 3-(2-pyridyl)-pyrazole as leaving azole, and water-saturated methyl *t*-butyl ether as reaction medium at 45°C were first selected for performing the hydrolytic resolution of (*R,S*)-2-(4-chlorophenoxyl) azolides (**1–4**). In comparison with the kinetic resolution of (*R,S*)-2-phenylpropionyl 3-(2-pyridyl)-pyrazolide or (*R,S*)-α-methoxyphenylacetyl 3-(2-pyridyl)pyrazolide at the same reaction condition, excellent enantioselectivity with more than two order-of-magnitudes higher activity for each enantiomer was obtained. The resolution was then extended to other (*R,S*)-3-(2-pyridyl)-pyrazolides (**5–7**) containing 2-chloro, 3-chloro, or 2,4-dichloro substituent, giving good ($E > 48$) to excellent ($E > 100$) enantioselectivity. The thermodynamic analysis for **1**, **2**, and **4–7** demonstrates profound effects of the acyl or leaving moiety on varying enthalpic and entropic contributions to the difference of Gibbs free energies. A thorough kinetic analysis further indicates that on the basis of **6**, the excellent enantiomeric ratio for **4** and **7** is due to the higher reactivity of (S)-**4** and lower reactivity of (R)-**7**, respectively. *Chirality 24:60–66, 2012.*

KEY WORDS: (*R,S*)-2-chlorophenoxyl 3-(2-pyridyl)pyrazolides; lipases; hydrolysis; kinetic and thermodynamic analysis

INTRODUCTION

Lipases have proved the capability for the preparation of optically pure alcohols, amines, and carboxylic acids for synthesizing a variety of pharmaceuticals and agrochemicals.[1] To enhance the enzyme performance, the substrate engineering approach of using activated or irreversible acyl donors or acceptors,[2] medium engineering strategy of varying temperature, reaction media, or additives,[3] as well as enzyme engineering approach of using site-directed mutations, evolutions, or chemical modifications[4,5] have been proposed for the fine-tuning of enzyme active-site structure. Recently, an enzymatic resolution process using (*R,S*)-azolides but not their corresponding ester, thioester, or normal amide analogs as the substrate was reported for preparing optically pure carboxylic acids containing a 2-aryl group to the α-chiral center.[6–8]

(*R,S*)-2-Chlorophenoxypropionic acids (CPAs) and their esters are widely used as herbicides with the biological activity mainly residing on the (*R*)-isomers. In addition, (*R*)-2-(4-chlorophenoxy)propionic acid can lower the level of serum cholesterol to prevent platelet aggregation, whereas the (*S*)-antipode inhibits the chloride channel in muscles.[9] Optically pure CPAs have been prepared via lipase-catalyzed hydrolysis or esterification of their racemates. In general, low enantioselectivity for crude *Candida rugosa* lipases (CRL), moderate to high enantioselectivity but with low reactivity for *Carica papaya* lipase (CPL) were reported.[10–12] Many efforts have been made to improve CRL activity and enantioselectivity, for example, use of purified or isopropanol (IPA)-treated lipase,[13–18] addition of dimethyl sulfoxide (DMSO), carbon tetrachloride, or benzene,[17,19–21] ionic liquids as the reaction medium,[22] and organosilicon alcohols as the acyl acceptor.[11,12,23] Nevertheless, considerations of solvent toxicity, biocatalyst recycle and downstream separation capability, and cost effectiveness because of low reactivity have hindered such methods practicably used in industry.

It is, therefore, aimed to use the resolution platform of using (*R,S*)-azolides as novel substrate for improving the preparation of optically pure CPAs (Fig. 1). The best reaction conditions for the hydrolysis of (*R,S*)-2-(4-chlorophenoxy)-propionyl azolides (**1–4**) in water-saturated solvents is first selected and then extend to other (*R,S*)-2-chlorophenoxypropionyl 3-(2-pyridyl)pyrazolides (**5–7**). Moreover, the thermodynamic and kinetic analysis is also performed for elucidating the favorable results.

EXPERIMENTAL

Materials

Lipase MY (30,000 U g^{-1} using olive oil emulsion as substrate at 37°C and pH 7.0) from *Candida rugosa*, Novozym 435 as an immobilized lipase (7000 PLU g^{-1} using lauric acid and 1-propanol as substrates at 60°C) from *Candida antartica* lipase B (CALB), and partially purified CPL (26,700 U g^{-1} using olive oil emulsion as substrate at 40°C and pH 8.5) were provided by Meito Sangyo (Tokyo, Japan), Novo Nordisk (Bagsvaerd, Denmark), and Challenge Bioproducts (Yun-Lin Hsien, Taiwan), respectively. 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: (*R,S*)-2-(3-chlorophenoxy)propionic acid and (*R,S*)-2-(2,4-dichlorophenoxy)propionic acid from TCI (Tokyo, Japan); (*R,S*)-2-(2-chlorophenoxy) propionic acid and 4-methypyrazole from Acros (Geel, Belgium); 4-bromopyrazole, (*R,S*)-2-(4-chlorophenoxy)propionic acid, and N,N′-carbon-

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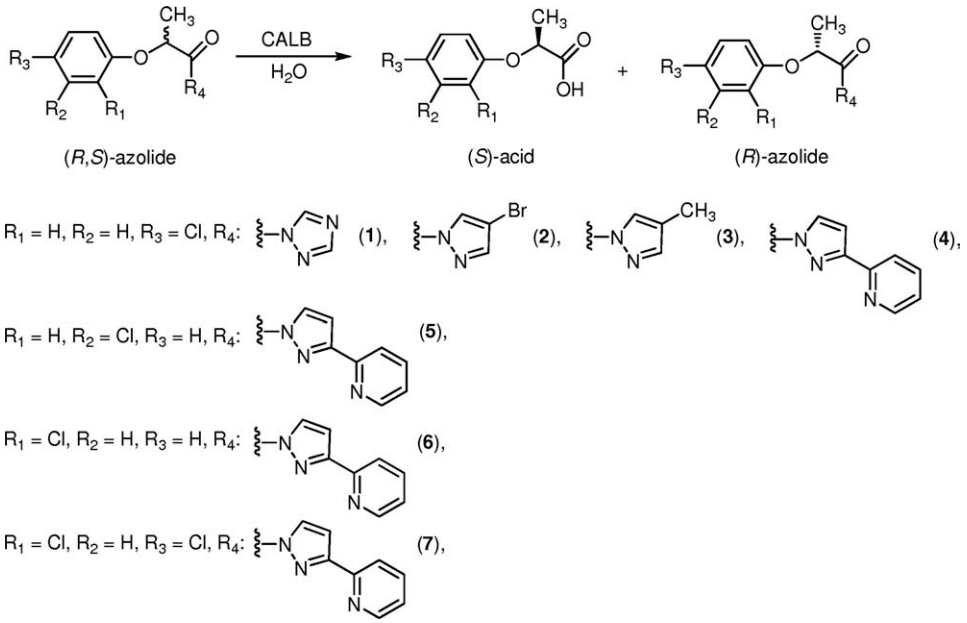

Fig. 1. Lipase-catalyzed hydrolytic resolution of (*R*,*S*)-2-chlorophenoxypropionyl azolides.

yldi(1,2,4-triazole) (CDT) from Sigma-Aldrich (Milwaukee, WI); 3-(2-pyridyl)-1H-pyraozle from Alfa Aesar (Ward Hill, MA); benzene, cyclohexane (CYC), dipropylether (IPE), hexane, IPA, and methyl *t*-butyl ether (MTBE) from Tedia (Fairfield, OH).

Substrates Synthesis

To 2.5 ml of benzene containing 1.3 mmol CPA, 1.0 mmol pyrazole, and 4 mmol triethylamine, a mixture containing 0.5 ml of benzene and 1.3 mmol thionyl chloride was added dropwise with stirring for 2 h at 0°C. The resultant mixture was filtered, 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 $MgSO_4$ for 12 h, filtered, and concentrated under reduced pressure, giving the desired (*R*,*S*)-pyrazolides. Moreover, to 2.5 ml of benzene, 1 mmol (*R*,*S*)-2-(4-chlorophenoxy)propionic acid and 1.5 mmol CDT were added and stirred for 2 h at 55°C. The resultant mixture was filtered and evaporated under reduced pressure, giving the desired (*R*,*S*)-2-(4-chlorophenoxy)propionyl-1,2,4-triazolide. All the synthesized substrates were confirmed from the ^{1}H NMR spectra recorded at 500 MHz on Brucker Avance DRX 500 spectrometer in DMSO-d_6 solution with TMS as an internal standard (IS).

(R,S)-2-(4-Chlorophenoxy)propionyl 1,2,4-triazolide (1). ^{1}H NMR (DMSO-d_6/TMS) δ: (DMSO-d_6/TMS) δ: 1.73 (3H, d), 5.95–6.00 (1H, q), 6.94–7.05 (2H, m), 7.22–7.29 (1H, m), 7.42–7.48 (1H, m), 8.46 (1H, s), 9.42 (1H, s). The abbreviations d, q, m, and s were the peak multiplicities of doublet, quartet, multiplet, and single, respectively.

(R,S)-2-(4-Chlorophenoxy)propionyl 4-bromopyrazolide (2). ^{1}H NMR (DMSO-d_6/TMS) δ: 1.66 (3H, d), 6.04–6.09 (1H, q), 6.85–6.87 (1H, m), 6.94–7.03 (2H, m), 7.27–7.31 (1H, m), 8.17 (1H, s), 8.76 (1H, s).

(R,S)-2-(4-Chlorophenoxy)propionyl 4-methylyrazolide (3). ^{1}H NMR (DMSO-d_6/TMS) δ: 1.66 (3H, d), 2.19 (3H, s), 5.99–6.04 (1H, q), 6.88–6.92 (2H, m), 7.29–7.36 (2H, m), 7.88 (1H, s), 8.24 (1H, s).

(R,S)-2-(4-Chlorophenoxy)propionyl 3-(2-pyridine)pyrazolide (4). ^{1}H NMR (DMSO-d_6/TMS) δ: 1.73 (3H, d), 6.16 (1H, d), 6.96–6.98 (2H, m), 7.23 (1H, d), 7.32–7.34 (2H, m), 7.47–7.49 (1H, q), 7.93–7.96 (1H, m), 8.10 (1H, d), 8.55 (1H, d), 8.70 (1H, d).

(R,S)-2-(3-Chlorophenoxy)propionyl 3-(2-pyridine)pyrazolide (5). ^{1}H NMR (DMSO-d_6/TMS) δ: 1.72 (3H, d), 6.20 (1H, d), 6.90–6.92 (1H, q), 7.02–7.04 (1H, m), 7.06–7.07 (1H, m), 7.22 (1H, d), 7.29–7.32 (1H, m), 7.46–7.48 (1H, m), 7.92–7.95 (1H, m), 8.09 (1H, d), 8.55 (1H, d), 8.68–8.69 (1H, m).

(R,S)-2-(2-Chlorophenoxy)propionyl 3-(2-pyridine)pyrazolide (6). ^{1}H NMR (DMSO-d_6/TMS) δ: 1.77 (3H, d), 6.20–6.24 (1H, q), 6.95–7.04 (2H, m), 7.22–7.27 (2H, m), 7.44–7.48 (2H, m), 7.90–7.94 (1H, m), 8.03 (1H, d), 8.55 (1H, d), 8.68–8.69 (1H, m).

(R,S)-2-(2,4-Dichlorophenoxy)propionyl 3-(2-pyridine)pyrazolide (7). ^{1}H NMR (DMSO-d_6/TMS) δ: 1.78 (3H, d), 6.19–6.23 (1H, q), 7.01 (1H, d), 7.22 (1H, d), 7.34–7.37 (1H, m), 7.47–7.49 (1H, m), 7.60 (1H, d), 7.91–7.99 (1H, m), 8.00 (1H, d), 8.55 (1H, d), 8.69–8.70 (1H, q).

Analysis

The hydrolysis (*R*,*S*)-azolides was monitored by high performance liquid chromatography (HPLC) using a chiral column from Daicel (OJ-H or OD-H; Tokyo, Japan) or Regis [(S,S)-Whelk-O 1; Morten Grove, IL], that is, capable of separating the IS and substrates. UV detection at 220, 240, or 270 nm was used for quantification at room temperature. Detailed conditions, such as the mobile phase composition and retention time for each compound, are given in Table 1. The optical rotation of (*R*)-2-(2,4-dichlorophenoxy)propionic acid dissolved in ethanol was determined at 589 nm on a Atago AP-100 polarimeter.

Effects of Lipase Sources, Temperature, and Substrate Structure

To 10 ml of water-saturated MTBE containing 3 mM of **1–4**, a specific amount of CALB was added for performing the hydrolysis in a batch reactor 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 (i.e., $[1 - (S_R)/(S_{R0})]$, with (S_{R0}) as the initial (*R*)-enantiomer concentration) and X_S (i.e., $[1 - (S_S)/(S_{S0})]$, with (S_{S0}) as the initial (*S*)-enantiomer concentration), initial rates for both enantiomers V_R and V_S based on several conversion determinations, racemate conversion X_t [i.e., $0.5(X_R + X_S)$], and enantiomeric excess for the substrate ee_s were determined. Similar experiments were performed in water-saturated CYC for CRL or CPL. To study the temperature effect on CALB performances, the reaction was also performed in MTBE at 25 and 35°C.

More experiments were performed for investigating the acyl moiety in **5–7** on enzyme activity and enantioselectivity. To compare the kinetic behaviors, the hydrolysis of varying initial substrate concentrations (S_R)

TABLE 1. HPLC analytic conditions

Entry	Column	Wave length (nm)	Flow rate (ml min^{-1})	Mobile phase	Retention time (min)		
					IS	(*R*)-Azolide	(*S*)-Azolide
1	OD-H	220	2.0	90:10:0	3.1 (Nitrotoluene)	14.1	12.2
2	OD-H	240	1.5	93:7:0	2.6 (Benzene)	3.7	3.3
3	OD-H	270	2.0	90:10:0	2.0 (Benzene)	6.7	5.7
4	OD-H	270	2.0	99:1:0	2.0 (Benzene)	10.5	9.3
5	(S,S)-Whelk-O 1	270	2.0	90:10:0	2.5 (Nitrotoluene)	5.8	7.1
6	OJ-H	270	2.0	90:10:0	2.1 (benzene)	18.5	13.7
7	OD-H	570	2.0	98.5:1:0.5	2.0 (benzene)	14.9	11.8

Mobile phase composition in HEX:IPA:glacial acetic acid (v/v/v); IS as the internal standard.

and (S_S) of **5–7** in MTBE at 45°C was performed, with which the specificity constants k_{2R}/K_{mR} and k_{2S}/K_{mS}, and hence enantiomeric ratio (i.e., $E = k_{2R}K_{mS}/k_{2S}K_{mR}$) were estimated from the experimental data coupled with $V_R = k_{2R}(S_R)(E_t)/\{K_{mR} + (S_R)[1 + K_{mR}/K_{mS}]\}$ and $V_S = k_{2S}(S_S)(E_t)/\{K_{mS} + (S_S)[1 + K_{mS}/K_{mR}]\}$ at the initial stage. The kinetic constants, k_{2R}, k_{2S}, K_{mR}, and K_{mS}, using Michaelis–Menten kinetics for both enantiomers were previously defined.[8]

Products Separation

After terminating the hydrolysis and removing the lipase by filtration, the resultant solution of X_t 42.5% for **7** was collected, added to 0.1 M NaOH solution (2 × 10 ml) in succession, and stirred for 20 min. The aqueous solutions were collected, acidified to pH 2 by adding 0.3 ml of HCl solution (37%, w/w), and added in succession (2 × 10 ml) to benzene with stirring for 20 min. The organic phase was collected, dried over anhydrous $MgSO_4$ for 12 h, concentrated under reduced pressure, and gave the acid product. From the optical rotations of $[\alpha]_D^{20} = -28.0$ (*c* 0.5, EtOH) (Lit. $[\alpha]_D^{20} = -28.6$ (*c* 1.6, EtOH) for (*S*)-**7**),[17] the (*S*)-preference for all lipases was determined.

The organic phase after the extraction via the NaOH solution was separated, added to 0.1 M HCl solution (20 ml), and stirred for 2 h. The organic phase was collected, dried over anhydrous $MgSO_4$ for 12 h, concentrated under reduced pressure, and gave the remaining substrate. The aqueous phase was added to 10 ml of benzene containing 0.2 M NaOH solution (0.2 ml), and stirred for 20 min. The organic phase was separated, dried over anhydrous $MgSO_4$ for 12 h, and concentrated under reduced pressure to give 3-(2-pyridyl)pyrazole product.

RESULTS AND DISCUSSION

Effects of Lipase Sources, Solvent, Temperature, and Substrate Structure

Table 2 demonstrates effects of lipase sources, solvent, temperature, and substrate structure on the hydrolysis of (*R*,*S*)-2-chlorophenoxypropionyl azolides on changing the initial specific activities $V_R/(E_t)$ and $V_S/(E_t)$, enantioselectivity in terms of initial V_R/V_S (or V_S/V_R), X_t, and ee_s at a specified reaction time. In comparison with the bad CPL performance for **1**, a moderate enantioselectivity with an opposite stereopreference for CRL and CALB is found. A decrease of temperature results in lowering $V_R/(E_t)$ and $V_S/(E_t)$, but with a slight increase of enantioselectivity for CALB. By replacing the leaving 1,2,4-triazole with 4-bromopyrazole for **2**, only slight changes of $V_R/(E_t)$ and $V_S/(E_t)$, and hence V_R/V_S, for CRL and CPL are shown. Yet for CALB at 45°C, more than fourfold and 11-fold decreases of $V_R/(E_t)$ and $V_S/(E_t)$, respectively, lead to an enhancement of V_S/V_R from 17.9 to 40.4. Therefore, there should have been noncovalent bonds between the 4-bromo substituent and amino acid residues, when considering the pK_a of 0.63 for 4-bromopyazolium lower than 2.19 for 1,2,4-triazolium. This bonding may induce a minute change of the orientations of substrate and catalytic triads on decreasing the nucleophilic attack and proton transfer (i.e., k_{2R} and k_{2S}) but not substrate affinity to the active site (K_{mS} and K_{mR}), as previously reported for the hydrolysis of (*R*,*S*)-2-phenylpropionyl azolides.[8]

By further changing the substrate to **3** containing a leaving 4-methylpyrazole, the enzyme activity for each enantiomer increases, yielding a slight increase of initial V_S/V_R to 47.7. This again provides an indirect evidence of the noncovalent bonding between 4-methyl substituent and amino acid residues, when considering the higher pK_a of 3.04 for 4-methylpyazolium that is disadvantageous for the nucleophilic attack to the carbonyl carbon atom, and leaving of 4-methylpyrazole in nonenzymatic reactions. On the contrary, decreasing of temperature to 25°C is a more effective way to increase V_S/V_R to 73.2 for **2**, yet with the penalty of fourfold lower of $V_S/(E_t)$ for the fast-reacting enantiomer.

It has been shown that, a bulky 3-(2-pyridyl) or 3-(3-bromophenyl) substituent to the leaving pyrazole is beneficial for giving excellent enantioselectivity for Novozym 435.[8] Using **4** containing a leaving 3-(2-pyridine)pyrazole as the substrate, excellent enantioselectivity of $V_S/V_R = 220.0$ with $V_S/(E_t) = 3.96$ mmol h^{-1} g^{-1} at 45°C is obtainable. In comparison with the hydrolysis of (*R*,*S*)-2-phenylpropionyl 3-(2-pyridyl)pyrazolide at the same reaction condition (i.e., $V_R/(E_t) = 1.27 \times 10^{-2}$ mmol h^{-1} g^{-1}, $V_S/(E_t) = 2.63 \times 10^{-5}$ mmol h^{-1} g^{-1}, and $V_R/V_S = 482$), more than 311- and 684-fold enhancements of specific activity for the fast- and slow-reacting enantiomers, respectively, are estimated. These corresponds to free energy contributions of about 15.2 and 17.2 kJ mol^{-1} (i.e., $RT\ln(311)$ and $RT\ln(684)$, respectively, at 45°C) well within the range of a weak hydrogen bond between the 4-chlorophenoxy oxygen and amino acid residues or adsorbed water in a hydrophobic environment. The inductive effect due to electronegative 4-chlorophenoxy moiety at the 2-position of acyl donor can be ruled out, when considering the lowest reactivity of **7** containing a more electronegative 2,4-dichlorophenoxy moiety.

Similar activity enhancements of CALB or *Pseudomonas cepacia* lipase, for the resolution of (*R*,*S*)-amines in organic solvents, have been reported using methyl methoxyacetate as a highly active acyl donor.[24,25] Apparently, an extra hydrogen bond between the methoxy oxygen and amine hydrogen previous proposed does not apply to the present case. It is also interesting to note the low CALB activity (i.e., $V_R/(E_t) = 5.29 \times 10^{-3}$ mmol h^{-1} g^{-1} and $V_S/(E_t) = 1.73 \times 10^{-5}$ mmol h^{-1} g^{-1}) for hydrolyzing (*R*,*S*)-α-methoxyphenylacetyl 3-(2-pyridyl)pyrazolide in water-saturated MTBE.[8] Although the 2-position of acyl part also contains methoxy oxygen, no formation of an extra hydrogen bond is perceived. This may

TABLE 2. Effect of lipase sources, solvent, temperature, and substrate on hydrolysis of 1–7

Entry Lipase	Solvent	Temp. (°C)	$V_R/(E_t)$ (mmol h^{-1} g^{-1})	$V_S/(E_t)$ (mmol h^{-1} g^{-1})	V_R/V_S or V_S/V_R	(E_t) (mg ml^{-1})	Time (h)	X_t (%)	ee_s (%)
1									
CRL	CYC	45	1.64	8.36 E 2	19.6	2	3.8	49.2	28.3
CPL	CYC	45	1.87 E −1	1.58 E −1	1.2	2	8.3	82.1	13.1
CALB	MTBE	45	6.06 E −1	1.09 E +1	17.9	1	0.8	61.7	100.0
CALB	MTBE	35	3.69 E −1	6.99	18.9	1	0.7	57.5	100.0
CALB	MTBE	25	2.22 E −1	4.86	21.9	2	1.0	64.2	100.0
2									
CRL	CYC	45	9.48 E −1	4.22 E −2	22.5	2	4.5	37.1	20.9
CPL	CYC	45	2.95 E −2	1.58 E −2	1.9	2	10.5	51.3	12.3
CALB	MTBE	45	5.50 E −2	2.22	40.4	2	3.5	63.0	100.0
CALB	MTBE	35	3.65 E −2	1.52	41.6	2	4.0	57.0	100.0
CALB	MTBE	25	7.65 E −3	5.60 E −1	73.2	2	26.0	60.9	100.0
3									
CALB	MTBE	45	1.42 E −1	6.78	47.7	1	8.7	78.7	100.0
4									
CALB	MTBE	45	1.80 E −2	3.96	220.0	2	2.5	52.3	100.0
CALB	MTBE	35	9.30 E −3	2.39	257.0	2	9.0	56.4	100.0
CALB	MTBE	25	4.20 E −3	1.19	283.3	6	3.1	50.2	100.0
5									
CALB	MTBE	45	1.94 E −1	5.08	26.2	10	4.1	60.9	100.0
CALB	MTBE	35	6.30 E −2	2.31	36.8	10	2.0	58.4	4.9
CALB	MTBE	25	2.90 E −2	1.38	48.1	5	1.0	55.7	100.0
6									
CALB	MTBE	45	2.73 E −2	1.13	41.4	12	0.5	53.2	100.0
CALB	MTBE	35	1.22 E −2	7.03 E −1	57.6	12	1.0	53.2	83.2
CALB	MTBE	25	4.44 E −3	3.29 E −1	74.1	12	3.5	56.6	100.0
7									
CRL	CYC	45	1.31 E −3	5.68 E −3	4.3	12	4.3	54.7	30.3
CPL	CYC	45	5.05 E −3	1.11 E −1	22.0	12	10.5	63.8	83.2
CALB	CYC	45	3.79 E −3	7.86 E −1	207.4	12	5.0	59.6	100.0
CALB	IPE	45	6.91 E −3	1.49	215.6	12	5.0	65.7	100.0
CALB	MTBE	45	4.24 E −3	1.08	254.7	12	1.0	55.9	100.0
CALB	MTBE	35	2.01 E −3	6.40 E −1	318.4	12	7.1	56.2	100.0
CALB	MTBE	25	1.00 E −3	3.74 E −1	374.0	12	30.0	54.8	100.0

Reaction conditions: 10 ml solvent containing 3 mM racemate at 400 rpm. Symbol E-1 as 10^{-1}.

be attributed to the largest pocket of active site originally occupied by the methoxy oxygen of methyl methoxyacetate or chlorophenoxy oxygen of **1–7**, being now occupied by 2-phenyl moiety to lose the capability of forming a hydrogen bond with the methoxy oxygen.

Table 2 further demonstrates the results for **5–7** containing a leaving 3-(2-pyridine)pyrazole moiety. More than 10-fold increases of specific activity for the slow-reacting (*R*)-**5**, but not (*S*)-**5**, at 45°C were obtained and led to great decreasing of the enantioselectivity. A decrease of temperature to 25°C is advantageous to improve V_S/V_R from 26.2 to 48.1, yet with the penalty of 3.6-fold decreasing of initial $V_S/(E_t)$. On the contrary, a decrease of specific activity for each enantiomer yields a slight enhancement of V_S/V_R to 41.1 at 45°C, when using **6** containing a 2-chlorophenoxy moiety as the substrate. This can be improved to 74.1 by lowering the temperature to 25°C.

Using **7** containing a 2,4-dichlorophenoxy moiety as the substrate, it is interesting to find the change of stereopreference of CRL and CPL, but not CALB. Similar kinetic behaviors of giving the (*S*)-preference for crude CRL but not the IPA-treated preparation have been reported,[17] and were attributed to the isoenzymes having an opposite stereopreference. The excellent enantioselectivity of CALB is shown in Table 2, for the hydrolysis of **7** in MTBE at 45°C. Moreover, changing of the solvent to IPE or CYC has caused minor influences on varying the enzyme activity and enantioselectivity. Yet the enantioselectivity improves by decreasing temperature to 25°C. On the basis of above results, the best condition of CALB as the biocatalyst, water-saturated MTBE as the reaction medium, 45°C for **4** and **7** and 25°C for **5** and **6** is concluded. To shed insights into effects of substrate structure on the hydrolytic resolution, the thermodynamic and kinetic analysis for CALB was performed.

Thermodynamic Analysis

According to the transition theory, the specificity constant can be expressed as $\ln(k_{2i}/K_{mi}) = -\Delta H_i/\mathrm{RT} + (A + \Delta S_i)/R$, i = *R* or *S*. A constant $A = R\ln(\kappa k_B T/h)$, with h, k_B, R, T, κ as the Plank's constant, Boltzmann's constant, gas constant, absolute temperature, and transmission coefficient, respectively, can be assumed, when varying the temperature in a narrow range. From the enthalpic and entropic differences (i.e., ΔH_i and ΔS_i) between the transition and ground states for each enantiomer, one obtains $\ln(E) = -\Delta\Delta G/RT = -\Delta\Delta H/RT + \Delta\Delta S/R$, where $-\Delta\Delta G$, $-\Delta\Delta H$, and $-\Delta\Delta S$ represent the differences of Gibbs free energies (ΔG_R and ΔG_S), enthalpies (ΔH_R and ΔH_S), and entropies (ΔS_R and ΔS_S) between the transition states of both the enantiomers, respectively.

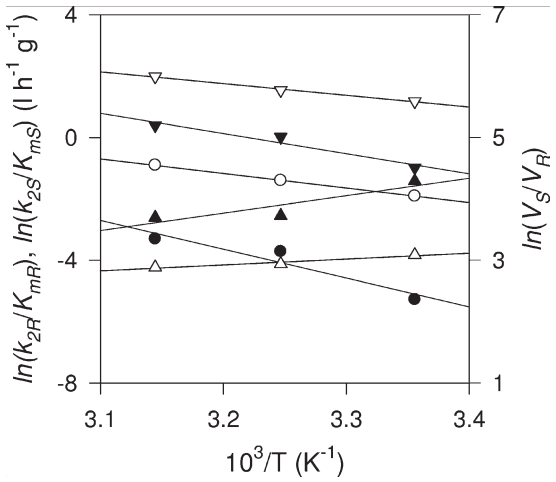

Fig. 2. Variations of $\ln(k_{2R}/K_{mR})$ (○, ●), $\ln(k_{2S}/K_{mS})$ (▽, ▼), and $\ln(E)$ (□, ▲) with inverse of absolute temperature for **1** (○, ▽, □) and **2** (●, ▼, ▲).

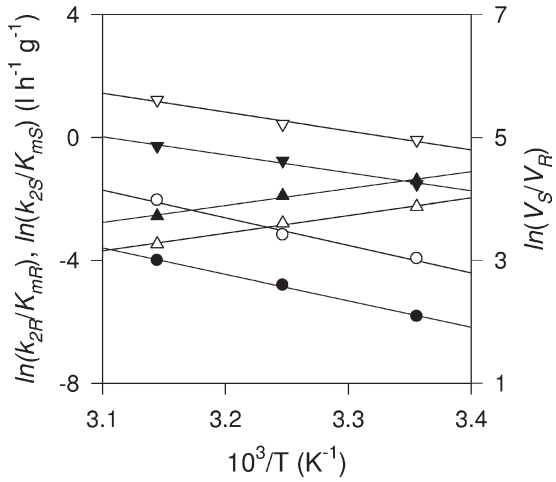

Fig. 4. Variations of $\ln(k_{2R}/K_{mR})$ (○, ●), $\ln(k_{2S}/K_{mS})$ (▽, ▼), and $\ln(E)$ (□, ▲) with inverse of absolute temperature for **5** (○, ▽, □) and **6** (●, ▼, ▲).

Good liner relationships between $\ln(E)$ and $\ln(k_{2i}/K_{mi})$ and the inverse of absolute temperature (Figs. 2–4) were found, in which ΔH_i, $(A + \Delta S_i)$, $-\Delta\Delta H$, and $-\Delta\Delta S$ were estimated and represented in Table 3. The thermodynamic parameter ΔH_S for **1**, **2**, or **4** increases when the leaving azole moiety contains a substituent, implying that the unfavorable enthalpic contribution due to desolvation of polar water at the transition state cannot compensate from the favorable enthalpic gain of the substituent with amino acid residues or adsorbed water in the active site. This is especially valid for ΔH_R of slow-reacting enantiomer (*R*)-**2** containing a 4-bromopyrazole moiety.

It is difficult to elucidate ΔS_R and ΔS_S varied with the substituent, owing to the complicated entropic contributions from interactions between water, solvent, substrate, and enzyme molecules on changing the number of degrees of freedom. Yet the enthalpy–entropy compensation relationships between ΔH_R and $(A + \Delta S_R)$, ΔH_S and $(A + \Delta S_S)$, and hence $-\Delta\Delta H$ and $-\Delta\Delta S$, still hold. Moreover, a comparison of $-\Delta\Delta G$ and $-\Delta\Delta H$ at 45°C indicates that the enantiomer discrimination is found to be mainly enthalpic-driven for **1**, with compensation from the entropic gain for **2**, and even with favor from the entropic loss for **4**.

Table 3 also demonstrates ΔH_R and ΔH_S varied with the acyl group for **4–7**. Apparently, the 4-chloro substituent in **4** or **7** especially for the slow-reacting (*R*)-enantiomer, but not the 3- or 2-chloro group in **5** or **6**, is capable of compensating the unfavorable enthalpic contribution due to desolvation of polar water at the transition state. The good enthalpy–entropy compensation relationships of $\Delta S_R = -113.5 + 4.315\Delta H_R - A$ ($r^2 = 0.97$) and $\Delta S_S = -40.41 + 4.074\Delta H_S - A$ ($r^2 = 0.91$), and hence $\Delta\Delta S = 61.43 + 4.487\Delta\Delta H$ ($r^2 = 0.98$) might be attributed to the similar acyl donors investigated here. It is interesting to compare the thermodynamic parameters for **4**, **6**, and **7**. The introduction of a 4-chloro substituent in **7** is more favorable on lowering ΔH_R and $(A + \Delta S_R)$, leading to $-\Delta\Delta H$ of 15.11 kJ mol^{-1} and $-\Delta\Delta S$ of 1.33 J mol^{-1} K^{-1}. Thus, the entropic compensation effect is nearly negligible at 45°C for obtaining an excellent CALB enantioselectivity. On the contrary, the introduction of a 2-chloro group in **4** mainly lowers ΔH_S and $(A + \Delta S_S)$, and leads to the enthalpic gain just compensated from the entropic gain, resulting in nearly the same $-\Delta\Delta G$, and hence enzyme enantioselectivity for **4** and **7**.

It envisages that a weak hydrogen bond should exist between the 4-chloro substituent of (*R*)-**4** and (*R*)-**7** and amino acid residues. This can not only lower ΔH_R but also decreases the number of degrees of freedom of the transition state, such as $\Delta S_R < \Delta S_S$ to obtain a negative $-\Delta\Delta S$ for **4**. Moreover, the 2-chloro substituent of (*S*)-**7** can, furthermore, contribute a weaker hydrogen bond to lower ΔH_S and $(A + \Delta S_S)$ in comparison with those for (*S*)-**4**. To confirm the elucidation, more experiments of varying the substituent to the 2-phenoxyl group or use of theoretical predictions via molecular modeling are needed.

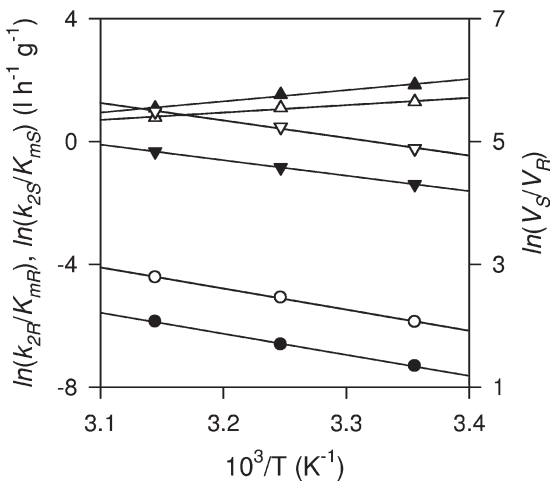

Fig. 3. Variations of $\ln(k_{2R}/K_{mR})$ (○, ●), $\ln(k_{2S}/K_{mS})$ (▽, ▼), and $\ln(E)$ (□, ▲) with inverse of absolute temperature for **4** (○, ▽, □) and **7** (●, ▼, ▲).

Kinetic Analysis

Figure 5 illustrates the specific activity varied with the initial enantiomer concentration (S_R) or (S_S) for **5–7**, with which the specificity constants k_{2R}/K_{mR} and k_{2S}/K_{mS} can be estimated and represented in Table 4. It is also reasonable to

TABLE 3. Thermodynamic analysis for CALB-catalyzed hydrolysis of 1, 2, and 4–7 in water-saturated MTBE

Entry	ΔH_R (kJ mol^{-1})	ΔH_S (kJ mol^{-1})	A + ΔS_R (J mol^{-1} K^{-1})	A + ΔS_S (J mol^{-1} K^{-1})	ΔG_R − AT (kJ mol^{-1})	ΔG_S – AT (kJ mol^{-1})	−ΔΔH (kJ mol^{-1})	−ΔΔS (J mol^{-1} K^{-1})	−ΔΔG (kJ mol^{-1})
1	39.58	31.80	116.8	116.3	2.42	−5.20	7.78	0.56	7.60
2	78.24	54.55	219.9	175.5	8.27	−1.28	23.69	44.45	9.55
4	57.42	47.47	143.8	157.5	11.67	−2.63	9.94	−13.66	14.28
5	74.76	51.10	217.4	170.3	5.59	−3.08	23.66	47.09	8.68
6	71.67	48.76	192.1	151.2	10.55	0.64	22.92	40.94	9.89
7	56.92	41.80	129.9	128.6	15.59	0.88	15.11	1.33	14.69

Reaction conditions as given in Tables 1–3; $-\Delta G_R$, $-\Delta G_S$ and $-\Delta\Delta G$ calculated at 45°C.

estimate the constants equal to $V_R/(E_t)/(S_R)$ and $V_S/(E_t)/(S_S)$ for **4** at the low substrate concentration of 3 mM in Table 2. Moreover, by further assuming the same enantiomer affinity to the active site for each racemate, one obtains $K_{mR} = K_{mS} = 38.5$ mM for **5**, 29.7 mM for **6**, and 120 mM for **7**. The highest Michaelis constants for **7** can be attributed to the dichloro substituent on hindering the substrate affinity to active site. In the hydrolysis of (*R*,*S*)-2-phenylpropionyl pyrazolides containing a bulky leaving 3-(2-pyridyl)pyrazole or 3-(3-bromophenyl)pyrazole,[8] the Michaelis constants K_{mR} and K_{mS} are too large to be determined, and were attributed to the adsorbed water allocated in active site near the 3-substituted substituent on impeding the substrate affinity. Apparently, this will relax if the 2-phenyl moiety is replaced by a chlorophenoxyl group in **5–7**. More data from experiments or theoretical predictions via molecular modeling are needed to elucidate the different behaviors from interactions between chlorophenoxyl oxygen atom and amino acid residues/adsorbed water for fine-tuning the active site.

It is difficult to explain why the maximum specificity constants occur for **5** but not **4** containing the least hindered 4-chlorophenoxyl group for performing the nucleophilic attack by catalytic serine. However, by considering the lowest k_{2S} for (*S*)-**6**, a 3- or 4-chloro substituent to the phenoxyl group is advantageous to enhance the nucleophilic attack of catalytic serine to the carbonyl carbon atom and proton transfer to the leaving 3-(2-pyridyl)pyrazole of (*S*)-**5** or (*S*)-**7** but not (*S*)-**6**. On the contrary, a 2-chloro substituent in (*R*)-**6** or (*R*)-**7** but not (*R*)-**5** mainly decreases the nucleophilic attack and proton transfer when considering an order-of-magnitude higher k_{2R} for the later. Therefore, on the basis of **6**, the excellent enantiomeric ratio is attributed to the high k_{2S}/K_{mS} of fast-reacting enantiomer for **4** and lower k_{2R}/K_{mR} of slow-reacting antipode for **7**, as envisaged from changes of (ΔG_R – AT) and (ΔG_S – AT) at 45°C in Table 3.

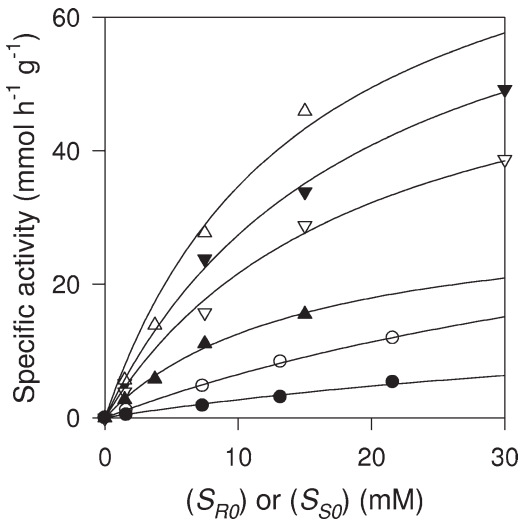

Fig. 5. Specific activity varied with initial enantiomer concentration (S_{R0}) or (S_{S0}) for CALB-catalyzed hydrolysis in water-saturated MTBE at 45°C: $V_S/(E_t)$ for (*S*)-**5** (▽), (*S*)-**6** (□), and (*S*)-**7** (○); $20V_R/(E_t)$ for (*R*)-**5** (▼), $100V_R/(E_t)$ for (*R*)-**6** (▲), and (*R*)-**7** (●). (—) Best-fit results.

CONCLUSION

The lipase-catalyzed hydrolytic resolution of (*R*,*S*)-2-chlorophenoxylpyrazolides was studied for preparing optically pure CPAs containing a 2-, 3-, 4-chloro, or 2,4-dichloro substituent to the 2-phenoxyl moiety. The best reaction condition of CALB as the biocatalyst, water-saturated MTBE as the reaction medium, 25°C for **5** and **6** and 45°C for **4** and **7** was selected, leading to good ($E > 48$) to excellent ($E > 100$) enantioselectivity and high reactivity for the fast-reacting enantiomer, when comparing the results for CRL or CPL. A detailed thermodynamic analysis for CALB-catalyzed hydrolysis of **1**, **2**, and **4–7** demonstrates profound influences of the acyl or leaving group on varying enthalpic and entropic contributions to the difference of Gibbs free energies ΔG_R, ΔG_S, and $-\Delta\Delta G$. The kinetic analysis also indicates that on the basis of **6**, the excellent enantiomeric ratio for **4** and **7** is

TABLE 4. Effects of substrate structure on kinetic constants for CALB-catalyzed hydrolysis at 45°C

Entry	**4**	**5**	**6**	**7**
k_{2R}/K_{mR} (l h^{-1} g^{-1})	1.20 E −2	1.64 E −1	2.10 E −2	3.16 E −3
K_{mR} (mM)	ND	3.85 E +1	2.97 E +1	1.20 E +2
k_{2R} (mmol h^{-1} g^{-1})	ND	6.32	6.25 E −1	3.79 E −1
k_{2S}/K_{mS} (l h^{-1} g^{-1})	2.64	4.16	1.16	7.57 E −1
K_{mS} (mM)	ND	3.85 E +1	2.97 E +1	1.20 E +2
k_{2S} (mmol h^{-1} g^{-1})	ND	1.60 E +2	3.44 E +1	9.09 E +1
E	220.0	25.3	55.1	239.5

Reaction conditions: 10 ml water-saturated MTBE at 400 rpm. Symbol E −1 as 10^{-1}. ND as not determined due to shortage of (*R*,*S*)-2-(4-chlorophenoxy)-propionic acid from Sigma–Aldrich.

attributed to the higher k_{2S}/K_{mS} of (*S*)-**4** and lower k_{2R}/K_{mR} of (*R*)-**7**.

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