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Lipase-catalyzed enantioselective resolution of (*R*,*S*)-N-2-methylalkanoyl-3-(2-pyridyl)pyrazoles in organic solvents

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ABSTRACT

The lipase-catalyzed resolution of (*R*,*S*)-pyrazolides containing a 2-aryl substituent to the α-chiral center has been successfully extended to (*R*,*S*)-N-2-methylalkanoyl-3-(2-pyridyl)pyrazoles (**1**–**4**) containing different alkanoyl-chain lengths. The best reaction condition for CALB-catalyzed hydrolysis of (*R*,*S*)-N-2-methylheptanoyl-3-(2-pyridyl)pyrazole (**1**) in water-saturated MTBE at 35 °C is selected, leading to an excellent enantioselectivity ($V_R/V_S > 100$) with improved initial specific activities in comparison with that of (*R*,*S*)-N-2-phenylpropionyl-3-(2-pyridyl)pyrazole. The thermodynamic analysis for the hydrolysis of **1** demonstrates great influences of water content and solvent hydrophobicity on varying the ehthalpic and entropic contributions in water-saturated and anhydrous MTBE and IPE, and leads to an excellent enthalpy–entropy compensation relationship $\Delta\Delta S = 3.113\Delta\Delta H + 33.86$ ($r^2 = 0.999$). Moreover, a thorough kinetic analysis for all substrates indicates that a critical valeroyl-chain length for obtaining the enantiomer discrimination and improved lipase activity for the fast-reacting (*R*)-pyrazolide is needed.

1. Introduction

Lipases as versatile biocatalysts have been employed for the preparation of chiral alcohols, amines, carboxylic acids, or their derivatives for synthesizing a variety of fine chemicals including pharmaceuticals [1,2]. In order to obtain the fine-tuning of enzyme active-site structure to enhance the enantiomer discrimination toward targeted racemates, various approaches of using substrate engineering [3–6], medium engineering [7,8], enzyme engineering [9–11], or their combinations have been proposed. Recently, a kinetic resolution process 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 [12–14]. The results indicated that the activity and enantioselectivity of an immobilized *Candida antarctica* lipase B (CALB) in water-saturated or anhydrous methyl *tert*-butyl ether (MTBE) greatly increased, if (*R*,*S*)-pyrazolides containing a leaving 3- or 3,4-substituted-pyrazole moiety were employed for preparing optically pure carboxylic acids or esters containing an 2-aryl group to the α-chiral center. This may be attributed to the unique pyrazolide 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 in the rate-limiting acylation step [14,15].

If the rationale of obtaining improved enzyme performance is valid for the (*R*,*S*)-pyrazolides containing an aryl substituent to the α-chiral center, one may wonder if the resolution platform can be extended to other substrates without containing the aryl moiety mainly causing the enantiomer discrimination when comparing with the other substituent such as a methyl, ethyl, bromo, or methoxy group in the α-chiral center [14]. By assigning (*R*,*S*)-N-2-methylalkanoyl-3-(2-pyridyl)pyrazoles as the substrates, it is aimed to investigate the alkanoyl-chain length on affecting the lipase activity and enantioselectivity in water-saturated or anhydrous organic solvents. The resultant (*R*)- or (*S*)-2-methylalkanoic acid products have been used as drugs, pesticides, flavors and fragrances, sweeteners, and synthetic building blocks [16]. They can be prepared mainly via *Candida rugosa* lipase-catalyzed hydrolysis of (*R*,*S*)-2-methylalkanoyl alkyl esters, esterification or transesterification of the corresponding acids or vinyl esters by alcohol in alkane solvents [16–24]. Depending on the alkanoyl-chain length and reaction conditions, low to good enantioselectivity with in general low enzyme activity due to the substrate inhibition from acid or alcohol was found.

The best reaction condition for lipase-catalyzed hydrolysis of (*R*,*S*)-N-2-methylheptanoyl-3-(2-pyridyl)pyrazole in water-saturated solvents is first selected and extends to the alcoholysis by methanol in anhydrous solvents. The thermodynamic anal-

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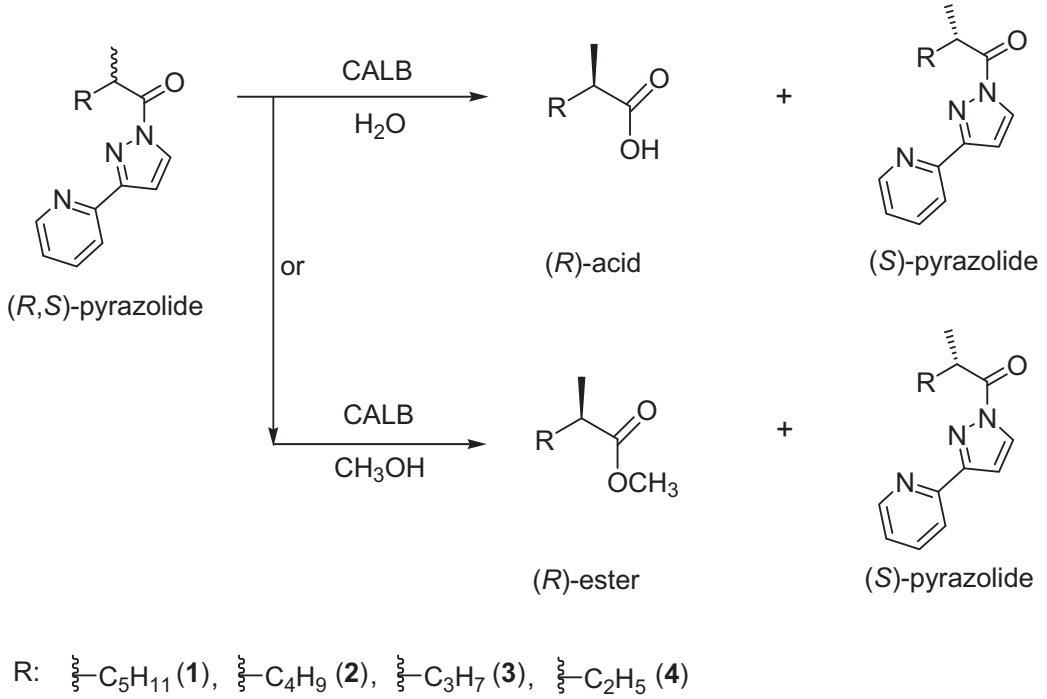

Scheme 1. Lipase-catalyzed enantioselective resolution of (*R*,*S*)-N-2-methylalkanoyl-3-(2-pyridyl)pyrazoles.

ysis for each enantiomer is then carried out. The hydrolytic resolution is also applied to other (*R*,*S*)-N-2-methylalkanoyl-3-(2-pyridyl)pyrazoles containing a shorter alkanoyl-chain length for doing the comparison from the kinetic analysis (Scheme 1).

2. Materials and methods

2.1. Materials

Lipase MY from *Candida rugosa* and Novozym 435 as a *Candida antartica* lipase B (CALB) immobilized on acrylic resins 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: (*R*,*S*)-2-methylbutyric acid and (*R*,*S*)-2-methylvaleric acid from TCI (Tokyo, Japan); (*R*,*S*)-2-methylheptanoic acid from Acros (Geel, Belgium); (*R*,*S*)-2-methylhexanoic acid and 3-(2-pyridyl)-1H-pyraozle from Alfa Aesar (Ward Hill, MA); benzene, cyclohexane (CYC), dipropylether (IPE), hexane (HEX), isopropanol (IPA), isooctane (ISO), and methyl *t*-butyl ether (MTBE) from Tedia (Fairfield, OH); calcium hydride and methanol from Riedel-deHaen (Seelze, Germany). Anhydrous IPE and MTBE were prepared by adding calcium hydride to both solvents for 24 h.

2.2. General procedures for substrates synthesis

To 2.5 ml benzene containing 1 mmol racemic acid, 1.2 mmol 3-(2-pyridyl)pyrazole, and 4 mmol triethylamine was added dropwise a mixture containing 0.5 ml benzene and 1 mmol thionyl chloride at 0 °C with stirring for 2 h. 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 oily products. All the synthesized substrates were confirmed from the ^{1}H NMR spectra recorded at 400 MHz on Brucker AC-400 spectrometer in DMSO-d_6 solution with TMS as an internal standard.

(*R*,*S*)-N-2-methylheptanoyl-3-(2-pyridyl)pyrazole (**1**); ^{1}H NMR (DMSO-d_6/TMS) δ: 0.83 (3H, t), 1.23–1.53 (11H, m), 3.87 (1H, q), 7.15 (1H, d), 7.43–7.46 (1H, q), 7.91–7.94 (1H, m), 8.0 (1H, d), 8.47 (1H, d), 8.66 (1H, d). The abbreviations d, q, m and s were the peak multiplicities of doublet, quartet, multiplet and single, respectively.

(*R*,*S*)-N-2-methylhexanoyl-3-(2-pyridyl)pyrazole (**2**); ^{1}H NMR (DMSO-d_6/TMS) δ: 0.85 (3H, t), 1.24–1.55 (9H, m), 3.88 (1H, q), 7.15 (1H, d), 7.44–7.46 (1H, q), 7.92–7.95 (1H, m), 8.0 (1H, d), 8.47 (1H, d), 8.67 (1H, d).

(*R*,*S*)-N-2-methylvaleroyl-3-(2-pyridyl)pyrazole (**3**); ^{1}H NMR (DMSO-d_6/TMS) δ: 0.88 (3H, t), 1.25–1.57 (11H, m), 3.91 (1H, q), 7.17 (1H, d), 7.45–7.47 (1H, q), 7.93–7.95 (1H, m), 8.0 (1H, d), 8.49 (1H, d), 8.68 (1H, d).

(*R*,*S*)-N-2-methylbutanoyl-3-(2-pyridyl)pyrazole (**4**); ^{1}H NMR (DMSO-d_6/TMS) δ: 0.91 (3H, t), 1.24 (3H, t), 1.59 (2H, s), 3.82 (1H, q), 7.15 (1H, d), 7.44–7.47 (1H, q), 7.92–7.95 (1H, m), 8.0 (1H, d), 8.49 (1H, d), 8.67 (1H, d).

2.3. Analysis

The hydrolysis or alcoholysis of (*R*,*S*)-pyrazolides was monitored by HPLC using a chiral column from Daicel (OJ-H; Tokyo, Japan) that was capable of separating the internal standard of benzene (BEN). UV detection at 270 nm was employed for quantification at the room temperature. The mobile phase composition (HEX:IPA:acetic acid, v/v/v) were 99.8:0.1:0.1 for **1**, 98:2:0 for **2** and **3**, and 99.4:0.1:0.5 for **4**. The retention time for the internal standard and enantiomers were BEN:(*S*)-**1**:(*R*)-**1** = 2.3:8.5:10.0 min, BEN:(*S*)-**2**:(*R*)-**2** = 2.2:3.5:3.8 min, BEN:(*R*)-**3**:(*S*)-**3** = 2.2:3.5:3.8 min, and BEN:(*S*)-**4**:(*R*)-**4** = 2.6:10.7:12.4 min. Optical rotations of the acid product dissolved in ethanol were determined at 589 nm on a Atago AP-100 polarimeter.

Table 1
Effect of lipase source, solvent, and temperature on enantioselective resolution of (*R*,*S*)-N-2-methylheptanoyl-3-(2-pyridyl)pyrazole.

Lipase	Solvent	Temp. (°C)	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_R/V_S or V_S/V_R	Time (h)	X_t (%)	ee_s (%)
Hydrolysis in water-saturated solvents								
Lipase MY	CYC	25	3.12E−5	4.20E−4	13	21.6	3.1	3.6
CALB	CYC	25	7.69E−2	5.02E−4	153	3.3	48.1	92.8
CALB	ISO	25	3.92E−1	2.08E−3	188	1.0	56.6	100.0
CALB	IPE	25	2.20E−2	7.39E−5	298	8.5	47.1	86.8
CALB	MTBE	25	3.19E−2	1.65E−4	193	8.5	48.6	94.5
CALB	MTBE	35	5.58E−2	3.90E−4	143	3.4	43.7	85.9
CALB	MTBE	45	1.17E−1	1.40E−3	84	2.3	46.1	95.2
CALB	IPE	35	4.88E−2	3.90E−4	125	3.4	43.3	81.8
CALB	IPE	45	1.14E−1	1.71E−3	66	1.5	43.8	83.2
Alcoholysis by 100 mM methanol in anhydrous solvents								
CALB	MTBE	25	3.13E−1	2.36E−3	132	5.4	53.7	96.9
CALB	MTBE	35	6.66E−1	9.00E−3	74	2.1	54.0	100.0
CALB	MTBE	45	9.21E−1	1.44E−2	64	1.9	51.5	98.1
CALB	IPE	25	1.08E−1	1.38E−3	78	4.7	50.3	92.6
CALB	IPE	35	2.60E−1	4.26E−3	61	2.4	49.6	89.4
CALB	IPE	45	6.16E−1	1.08E−2	57	1.9	51.2	91.7

Reaction conditions: 10 ml solvent containing 3 mM racemate at 400 rpm; 20 mg/ml lipase employed for hydrolysis; 4 mg/ml CALB for alcoholysis except for 10 mg/ml in IPE at 25 and 35 °C. Symbol E−1 as 10^{-1}. The temperature-variation of water content in water-saturated organic solvents can be found from [26–28], e.g. 467.7 mM and 110.6 mM in water-saturated MTBE and IPE, respectively, at 35 °C. It is reasonable to assume no water dissolving in anhydrous solvents after adding calcium hydride into the solvent for 24 h.

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

To 10 ml water-saturated CYC containing 3 mM of **1** was added 20 mg/ml of Lipase MY or CALB for performing the hydrolysis at 25 °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 based on several conversion determinations, racemate conversion X_t, and enantiomeric excess for the substrate ee_s were determined. Similar experiments were carried out for CALB in water-saturated ISO, IPE or MTBE. In order to study the temperature effect on CALB performances, the same reaction was carried out in water-saturated IPE or MTBE at 35 or 45 °C. The alcoholytic resolution via CALB was also performed at 25–45 °C, except that 100 mM methanol as an acyl acceptor was added to 10 ml anhydrous IPE or MTBE containing 3 mM of **1**.

More experiments were carried for investigating the substrate structure on the enzyme activity and enantioselectivity, in which the alkanoyl-chain length was reduced from 7 to 4. In order to compare the kinetic behaviors at 35 °C, the hydrolysis of varying the substrate concentration of **1**–**4** in water-saturated MTBE was carried out. From the time-course conversions X_R and X_S, and hence the specific initial rates $V_R/(E_t)$ and $V_S/(E_t)$, the kinetic constants k_{2R}/K_{mR}, k_{2S}/K_{mS}, and E value (i.e. $k_{2R}K_{mS}/k_{2S}K_{mR}$) were estimated [14].

2.5. Product separation for determining lipase enantioselectivity

After removing the lipase employed in the kinetic analysis, the resultant solutions of X_t about 70% were collected, added to a biphasic medium containing 5 ml benzene and 10 ml aqueous solution (0.1 N NaOH), and stirred for 20 min. The aqueous phase was separated, added to 10 ml benzene containing 0.45 ml HCl solution (37%), and stirred for 20 min. The organic phase was then separated, dried over anhydrous $MgSO_4$ for 12 h, filtered and concentrated under reduced pressure to give the acid product. From the optical rotations of $[\alpha]_D = -9.1$ (*c* 0.11, EtOH) for (*R*)- and (*S*)-2-methylvaleric acid mixture (Lit. $[\alpha]_D^{20} = -20.1$ (*c* 5.5, Et_2O) for the (*R*)-acid) [17], $[\alpha]_D = -13.0$ (*c* 0.38, EtOH) for (*R*)- and (*S*)-2-methylhexanoic acid mixture (Lit. $[\alpha]_D^{20} = -20.2$ (*c* 5.3, Et_2O) for the (*R*)-acid) [17], and $[\alpha]_D = -9.7$ (*c* 0.31, EtOH) for (*R*)- and (*S*)-2-methylheptanoic acid mixture (Lit. $[\alpha]_D = -15.6$ (*c* 0.55, EtOH) for (*R*)-acid) [25], one might determine the (*R*)-preference for CALB and (*S*)-preference for Lipase MY.

3. Results and discussion

3.1. Effects of lipase source, solvent, and temperature

Table 1 demonstrates effects of lipase source, solvent, and temperature on the kinetic resolution of (*R*,*S*)-N-2-methylheptanoyl-3-(2-pyridyl)pyrazole on changing the initial specific activities $V_R/(E_t)$ and $V_S/(E_t)$, enantioselectivity in terms of V_R/V_S or V_S/V_R, X_t, and ee_s at a specified reaction time. In comparison with Lipase MY-catalyzed esterification of (*R*,*S*)-2-methylheptanoic acid with 1-hexadecanol in CYC at the room temperature ($E = 23$, $X_t = 15.1\%$, $ee_s = 16.1\%$ at 5.4 h) [19], a better enzyme activity and enantioselectivity ($V_R/V_S = 153$, $X_t = 48.1\%$, $ee_s = 92.8\%$ at 3.3 h) for CALB is shown for the hydrolysis of **1** in water-saturated CYC at 20 °C, regardless of giving an opposite stereo-preference to Lipase MY. A change of the reaction medium to ISO but not IPE or MTBE results in 5-fold enhancements of $V_R/(E_t)$ and slight increasing of the enzyme enantioselectivity. However, by considering the low water solubility in ISO and CYC that is disadvantageous for carrying the hydrolytic resolution, IPE and MTBE are selected for the temperature screening.

An increase of reaction temperature to 45 °C has resulted in enhancements of $V_R/(E_t)$ and $V_S/(E_t)$, but leading to decreasing of the enantioselectivity, in water-saturated IPE and MTBE. By considering the higher water solubility and enzyme activity with V_R/V_S value higher than 100, CALB-catalyzed hydrolysis in water-saturated MTBE at 35 °C is selected as the best reaction condition. In comparison the hydrolysis of (*R*,*S*)-N-2-phenylpropionyl-3-(2-pyridyl)pyrazole in water-saturated MTBE at 45 °C ($V_R/(E_t) = 1.27 \times 10^{-2}$ mmol/h g, $V_S/(E_t) = 2.63 \times 10^{-5}$ mmol/h g, and $V_R/V_S = 482$) with that of **1** [14], the lower CALB activity with higher enantioselectivity may be attributed to the more hindered 2-phenyl substituent for decreasing the substrate affinity and proton transfer in the active site.

In comparison with the hydrolytic resolution, the methanolysis in anhydrous MTBE or IPE has yielded enhanced $V_R/(E_t)$ and $V_S/(E_t)$, but leading to decreasing of the enantioselectivity, especially at low temperature. For example for the alcoholysis in anhydrous MTBE at 25 °C, 9.8- and 14.3-fold higher of $V_R/(E_t)$ and $V_S/(E_t)$, respectively, and hence 1.4-fold lower of V_R/V_S, were estimated from Table 1. These are very similar to the kinetic behaviors by using (*R*,*S*)-N-

Table 2
Thermodynamic analysis for CALB-catalyzed hydrolysis of (*R*,*S*)-N-2-methylheptanoyl-3-(2-pyridyl) pyrazole.

Solvent	ΔH_R (kJ/mol)	ΔH_S (kJ/mol)	$A+\Delta S_R$ (J/mol K)	$A+\Delta S_S$ (J/mol K)	$-\Delta\Delta H$ (kJ/mol)	$-\Delta\Delta S$ (J/mol K)	$-\Delta\Delta G$ (kJ/mol)
Hydrolysis in water-saturated solvents							
MTBE	51.1	83.9	142.6	209.0	32.8	66.0	12.5
IPE	73.0	130.4	211.6	347.1	57.3	145.4	12.5
Alcoholysis in anhydrous solvents							
MTBE	42.7	71.6	134.0	191.3	28.8	56.6	11.3
IPE	68.6	81.1	211.6	217.6	12.4	5.4	8.9

Reaction conditions given in Table 1; $-\Delta\Delta G$ calculated at 35 °C.

2-phenylpropionyl-3-(2-pyridyl)pyrazole as the substrate, and can be attributed to the water adsorbed in the active site near the bulky 3-(2-pyridyl)pyrazole moiety on impeding the substrate affinity in water-saturated MTBE [14]. Apparently, more studies are needed for determining whether the hydrolysis or the alcoholysis is the best resolution platform.

3.2. Thermodynamic analysis

The transition state theory relates the microscopic rate constant of an elementary step to the Gibbs free energy difference between reactant's ground state and the activated transition state [29]. By assuming Michaelis–Menten kinetics for each enantiomer in the acylation step, the equation relating the kinetic constants k_{2i} and K_{mi} and the difference of Gibbs free energies between ground and transition states ΔG_i is expressed as $k_{2i}/K_{mi} = (\kappa k_B T/h)\exp(-\Delta G_i/RT)$ with h, k_B, R, T, κ as Plank's constant, Boltzmann's constant, gas constant, absolute temperature, transmission coefficient, respectively, and hence $ln(E) = -\Delta\Delta G/RT = -\Delta\Delta H/RT + \Delta\Delta S/R$ with $-\Delta\Delta G$, $-\Delta\Delta H$, and $-\Delta\Delta S$ as 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 enantiomers. For the temperature varying in a narrow range, the equation is approximated as $ln(k_{2i}/K_{mi}) = -\Delta H_i/RT + (A + \Delta S_i)/R$, with $A = R\,ln(\kappa k_B T/h)$ regarded as a constant.

Good liner relationships between $ln(V_R/V_S)$ and $ln(k_{2i}/K_{mi})$ and T^{-1}, $i = R$ and S, in water-saturated or anhydrous solvents in Table 1 were found, from which ΔH_i, $(A+\Delta S_i)$, $-\Delta\Delta H$, and $-\Delta\Delta S$ were estimated and represented in Table 2. The thermodynamic parameter ΔH_R or ΔH_S increases by replacing the anhydrous solvent by water-saturated ones, implying that the stabilization effect due to interactions of more dissolved water with substrate, solvent, and enzyme molecules at the ground state is higher than that at the transition state, especially for ΔH_S of the slow-reacting enantiomer in water-saturated IPE. On the contrary, the unfavorable enthalpic contribution due to desolvation of polar water (or methanol) at the transition state should be compensated from the favorable enthalpic gain of the polar group with water (or methanol) and solvent, leading to a lower ΔH_R (or ΔH_S) in the more polar water-saturated (or anhydrous) MTBE relative to IPE. In general, it is difficult to elucidate the variations of ΔS_R and ΔS_S and hence $\Delta\Delta S$ with the reaction condition, owing to the complicated entropic contributions from interactions between water, solvent, substrate, and enzyme molecules on changing the number of degrees of freedom. Yet in Table 2, one can still obtain the enthalpy–entropy compensation relationships $\Delta S_R = 2.900\Delta H_R + 4.187 - A$ ($r^2 = 0.959$) and $\Delta S_S = 2.704\Delta H_S - 6.982 - A$ ($r^2 = 0.988$).

The excellent enthalpy–entropy compensation phenomena $\Delta\Delta S = 3.113\Delta\Delta H + 33.86$ ($r^2 = 0.999$) might be attributed to the narrow range of parameters investigated, i.e. similar solvents and acyl acceptors of water and methanol. It is interesting to find the increase of $-\Delta\Delta H$ from 12.4 kJ/mol of anhydrous IPE to 28.8 kJ/mol of MTBE, implying that the polar MTBE is favorable for enhancing interactions between the fast-reacting (*R*)-enantiomer at the transition state and enzyme residues to lower the enthalpy. Apparently, the addition of water in water-saturated IPE is advantageous for giving the interactions mainly for (*R*)-enantiomer, yielding $-\Delta\Delta H$ of 57.3 kJ/mol, as some water will bind to the active site. However, more water added and hence adsorbed in water-saturated MTBE also contributes the enthalpic gain for both (*R*)- and (*S*)-enantiomers at the transition state, leading to slight increasing of $-\Delta\Delta H$ from 28.8 to 32.8 kJ/mol. More data on using other solvents and substrates are needed in the future in order to confirm the elucidation and obtained empirical relationships. By further comparing $-\Delta\Delta H$ and $-\Delta\Delta G$ at 35 °C for each case, the enantiomer discrimination is found to be enthalpy-driven, especially for the hydrolysis in water saturated IPE.

3.3. Kinetic analysis

The best reaction condition for CALB-catalyzed hydrolysis of **1** in water-saturated MTBE is extended to other (*R*,*S*)-N-2-methylalkanoyl-3-(2-pyridyl)pyrazoles containing a shorter alkanoyl-chain length. Some results are tabulated in Table 3, in which very similar enzyme performances with excellent enantioselectivity are demonstrated when using **1**–**3** as the substrates. However by using **4** as the substrate, more than 20-fold increases of $V_S/(E_t)/(S_S)$ and 6-fold decreases of $V_R/(E_t)/(S_R)$ in comparison with those for **1** are obtainable and yield no enantioselectivity. This can be attributed to the slight difference of ethyl and methyl moieties to the α-chiral center on discriminating (*R*)-**1** and (*S*)-**1**. However, when the ethyl moiety is replaced by a propyl group for **3**, about 1.5-fold enhancements of $V_R/(E_t)$ with 49.6-fold decreases of $V_S/(E_t)$, and hence giving V_R/V_S as 74.

In order to shed insights into effects of alkanoyl-chain length on the hydrolytic resolution, the kinetic analysis based on Michaelis–Menten mechanism was carried out. Fig. 1 illustrate

Table 3
Effect of substrate structure on CALB-catalyzed hydrolysis at 35 °C.

Entry	(S_R) or (S_S) (mM)	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_R/V_S	Time (h)	X_t (%)	ee_s (%)
1	4.4	1.78E−1	1.41E−3	126	3.0	59.7	100.0
2	5.5	2.01E−1	1.52E−3	132	4.0	51.4	87.8
3	2.0	4.11E−2	5.54E−4	74	4.1	50.7	78.0
4	4.2	2.75E−2	2.75E−2	1	4.2	45.1	0.0

Reaction conditions: 10 ml water-saturated MTBE at 400 rpm. Symbol E−1 as 10^{-1}. From the liner relationships in Fig. 1, one can compare CALB activity for each enantiomer via $V_R/(E_t)/(S_R)$ or $V_R/(E_t)/(S_R)$.

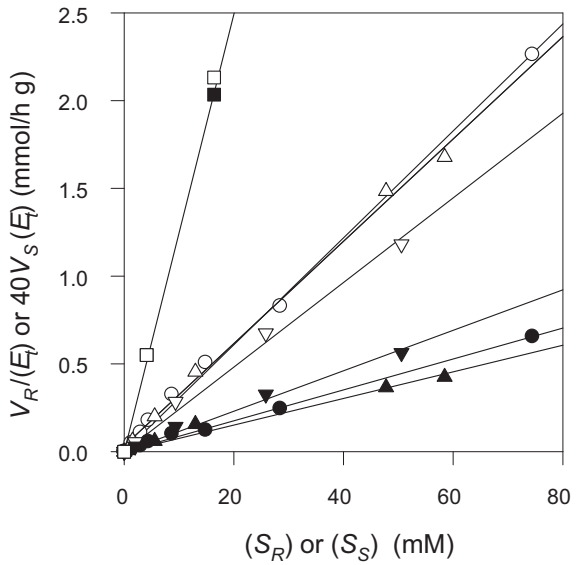

Fig. 1. Variations of $V_R/(E_t)$ and $40V_R/(E_t)$ with (S_R) and (S_S) for CALB-catalyzed hydrolysis in water-saturated MTBE for (*R*)-**1** (○), (*S*)-**1** (●), (*R*)-**2** (△), (*S*)-**2** (▲), (*R*)-**3** (▽), and (*S*)-**3** (▼); those of $20V_R/(E_t)$ and $20V_R/(E_t)$ for (*R*)-**4** (□) and (*S*)-**4** (■). (—) Best-fit results.

Table 4
Effects of substrate structure on the kinetic constants for CALB-catalyzed hydrolysis at 35 °C.

Entry	k_{2R}/K_{mR} (l/h g)	k_{2S}/K_{mS} (l/h g)	$E = k_{2R}K_{mS}/k_{2S}K_{mR}$
1	3.01E−2	2.20E−4	137
2	2.97E−2	1.86E−4	160
3	2.41E−2	2.88E−4	84
4	6.52E−3	6.17E−3	1

Reaction conditions: 10 ml water-saturated MTBE at 400 rpm. Symbol E−1 as 10^{-1}.

the initial specific activities $V_S/(E_t)$ and $V_R/(E_t)$ varied with the enantiomer concentration for **1**–**4** in water-saturated MTBE at 35 °C, with which the kinetic constant combinations k_{2R}/K_{mR} and k_{2S}/K_{mS} are estimated and represented in Table 4. Similar kinetic behaviors for the hydrolysis of (*R*,*S*)-N-2-phenylpropionyl-3-(2-pyridyl)pyrazole at 45 °C were reported [14], indicating that the Michaelis constants K_{mR} and K_{mS} are too large to be determined, and are attributed to the adsorbed water allocated in the active site near the 3-substituent on impeding the substrate affinity. The same order-of-magnitude of k_{2R}/K_{mR} (or k_{2S}/K_{mS}) for **1**–**3** but not **4** indicates that a critical change of the substrate structure can cause great influences on varying the lipase activity and enantioselectivity. It is reasonable to attribute the kinetic behavior to the different k_{2R} and k_{2S} (i.e. nucleophilic attack ability and proton transfer to the carbonyl carbon atom) but not K_{mR} and K_{mS} (i.e. affinity of both enantiomers to the enzyme), when comparing the similar size and electron-withdrawing ability of the propyl substituent for **3** and ethyl substituent for **4** to the α-chiral center.

4. Conclusions

With lipase-catalyzed kinetic resolution of (*R*,*S*)-N-2-methylheptanoyl-3-(2-pyridyl)pyrazole (**1**) as the model system, the best reaction condition of CALB-catalyzed hydrolysis in water-saturated MTBE at 35 °C is selected, in which an excellent enantioselectivity ($V_R/V_S > 100$) with improved enzyme activities is obtainable when comparing with $V_S/(E_t)$ and $V_R/(E_t)$ using (*R*,*S*)-N-2-phenylpropionyl-3-(2-pyridyl)pyrazole as the substrate. The thermodynamic analysis demonstrates the great influence of water content and solvent hydrophobicity on varying the ehthalpic and entropic contributions in water-saturated and anhydrous MTBE and IPE, and leads to an excellent enthalpy–entropy compensation relationship between $\Delta\Delta S$ and $\Delta\Delta H$. The resolution platform is successfully extended to other substrates of **2** and **3**, but not **4**. A thorough kinetic analysis for all substrates indicates that a critical valeroyl-chain length is needed for obtaining the enantiomer discrimination and improved lipase activity for the fast-reacting (*R*)-enantiomer.

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