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# Lipase-catalyzed regioselective hydrolysis of 3(5)-methylpyrazole-*N*-carboxylates in water-saturated organic solvents

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ABSTRACT

Pure *N*-substituted-azole regioisomers containing substituents on the *C*-atom of azole ring may exert different biological activities, just like pure enantiomers do in comparison with their antipodes. It is therefore highly desirable that they can be effectively synthesized from their precursors or separated from regioisomeric mixtures. With lipase-catalyzed hydrolysis of (*R*)-1-phenylethyl 3(5)-methylpyrazole-*N*-carboxylate mixture (**1a** and **1b**) in water-saturated organic solvents as the model system, excellent regioselectivity ($V_{1a}/V_{1b} > 100$) for 3-methylpyrazole-*N*-carboxylate (**1a**) is obtainable when selecting Novozym 435 from *Candida antartica* lipase B, but not Lipase MY-30 from *Candida rugosa* and Lipase PS-D from *Pseudomonas cepacia*, as the biocatalyst. Increasing of solvent hydrophobicity and temperature, or decreasing of water content, may enhance the enzyme activity but not regioselectivity. A change of the 1-phenylethyl moiety to an isopropyl or straight alkyl chain is longer than propyl for other substrates also results in excellent regioselectivity. The biocatalysis is successfully extended to the hydrolysis of (*R*)-1-phenylethyl 3(5)-methyl-4-bromopyrazole-*N*-carboxylate mixture (**8a** and **8b**) with $V_{8a}/V_{8b} > 100$ for preparing pure 5-methyl-4-bromopyrazole-*N*-carboxylate.



## 1. Introduction

Heterocyclic compounds containing more than one nitrogen atom in the molecule represent a very important group of molecules exhibiting significant biological activity including antimicrobial and pharmacological effects [1,2]. For example, pyrazole derivatives with a variety of alkyl, aryl or other substituents at the carbon or nitrogen atoms may act as anticancer, antidepressants, antipsychotic and anti-inflammatory agents, and herbicides [3–8], not to mention that they also play a central role in the coordination chemistry as ligands for preparing components in crystalline network structures, gels, and liquid crystals [9].

Azoles such as imidazole, pyrazole, 1,2,3-triazole, and 1,2,4-triazole often exhibit tautomeric equilibria in solution and the solid-state, owing to the intra- and inter-molecular proton transfer [10–12]. Moreover as the annular prototropic exchange processes in 1-H pyrazole derivatives are too fast on the NMR time-scale, special techniques *via* NMR or vibration circular dichroism spectroscopy have been employed for estimating the tautomeric equilibrium and hence the activation barrier of the exchange process [13]. For example by replacing the tautomeric proton with an acyl, methyl, phenyl, or trimethylsilyl moiety, the 1,2-migration rate may decrease such that the properties of resultant individual tautomers may be regarded as those of the "blocked" derivatives by doing a correction for the substituent effect [14–16]. Other functional groups such as benzyloxyl, 1,1-diethoxyethyl, *p*-methoxybenzyl, tetrahydropyran, *p*-toluenesulfonyl, 2-(trimethylsilyl)ethoxyl-methyl, triphenylmethyl, vinyl, $SO_2NMe_2$, and $SO_2Ph$ have also been used to protect the pyrazole nitrogen for further synthesizing a variety of regioisomers [17–19], and studying their regioisomeric or tautomeric influence on inhibiting enzymes associated with the diseases [20–24]. It is therefore aimed to the combination of *N*-substituted-pyrazole chemistry and lipase regioselectivity on developing a hydrolytic process for preparing pure *N*-substituted-pyrazole regioisomers.

Lipases as versatile biocatalysts have been widely applied for preparing chiral pharmaceuticals, structured lipids, and alkyl glucosides [25–29]. To the best of our knowledge, there is still no report on employing hydrolases as a reliable biocatalyst for preparing pure *N*-protected-pyrazole regioisomers. Therefore, the hydrolysis of 3(5)-methylpyrazole-*N*-carboxylate regioisomers in organic solvents is first selected as the model system (Scheme 1) for studying effects of solvent, temperature, lipase sources, *N*-protected group, and water content on varying the enzyme activity and regioselectivity. The results are then extended to the hydrolysis of 3(5)-methyl-4-bromopyrazole-*N*-carboxylate regioisomers, showing the promise of using the present hydrolytic platform for preparing pure 4,5-disubstituted-pyrazole-*N*-carboxylate and

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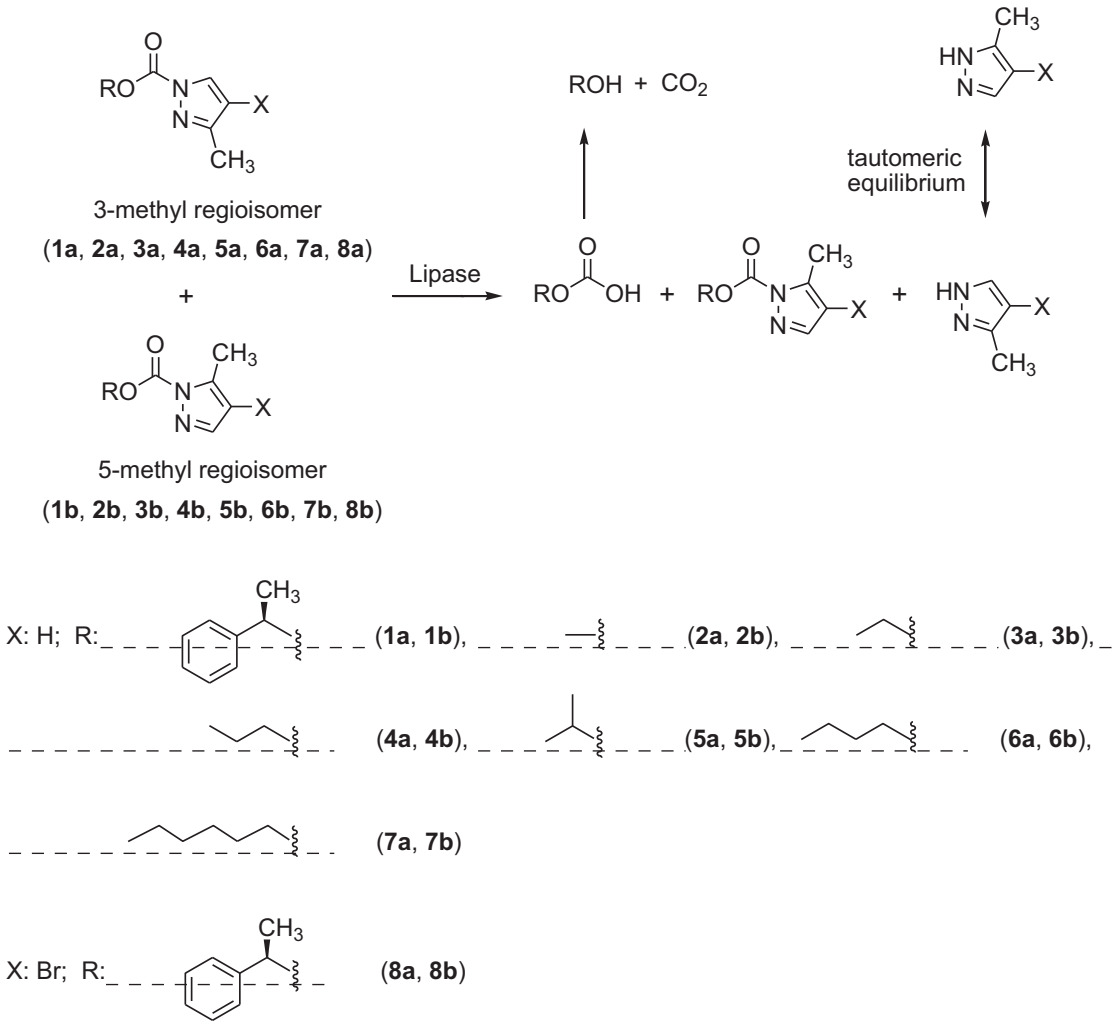


**Scheme 1.** Lipase-catalyzed hydrolysis of 3(5)-methylpyrazole-*N*-carboxylates and 3(5)-methyl-4-bromopyrazole-*N*-carboxylates.

5-substituted-pyrazole-*N*-carboxylate, as well as other regioisomers with medicinal values in the future.

## 2. Materials and methods

### 2.1. Materials

Novozym 435 (7000 PLU/g using lauric acid and 1-propanol as substrates at 60 °C; with 1–2% (w/w) water content) from *Candida antartica* lipase B, Lipase MY-30 (30,000 U/g using olive oil emulsion as substrate at 37 °C and pH 7.0) from *Candida rugosa*, and Lipase PS-D Amano I from *Pseudomonas cepacia* were provided by Novo Nordisk (Bagsvaerd, Denmark), Meito Sangyo Industries Ltd. (Tokyo, Japan), and Wako (Osaka, Japan), respectively. Other chemicals of analytical grade were commercially available: 3(5)-methylpyrazole from Acros (Geel, Belgium); diphosgene and 3(5)-methyl-4-bromopyrazole from Alfa Aesar (Ward Hill, MA); methanol from Riedel-deHaen (Seelze, Germany); (*R*)-1-phenylethanol from Wako (Tokyo, Japan); cyclohexane (CYC), diisopropyl ether (IPE), *n*-hexane (HEX), isopropanol (IPA), isooctane (ISO), methyl *tert*-butyl ether (MTBE), and triethylamine from Tedia (Fairfield, OH). Chloroform-d ($CDCl_3$) containing 1% (v/v) tetramethylsilane (TMS) for $^1H$ and $^{13}C$ NMR analysis was from Cambridge Isotope Laboratories (Andover, MA). Anhydrous MTBE was prepared by adding calcium hydride from Riedel-de Haen (Seelze, Germany) to the organic solvent for 24 h.

### 2.2. Substrate preparations and analysis

To 5 ml benzene containing 1 mmol 3(5)-methylpyrazole (or 3(5)-methyl-4-bromopyrazole) and 0.5 mmol diphosgene was added dropwise 1 mmol triethylamine at the room temperature with stirring for 1 h. To the resultant mixture was further added 1 mmol triethylamine and 0.5 mmol (*R*)-1-phenylethanol or IPA with stirring for 1 h. After being quenched with 0.1 M HCl solution (5 ml) and then 0.1 M NaOH solution (5 ml) for three times in succession, the organic phase was separated, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure, giving the desired and stable liquid mixture of 3(5)-methylpyrazole-*N*-carboxylate or 3(5)-methyl-4-bromopyrazole-*N*-carboxylate regioisomers. Similar procedures were carried out for preparing other alkyl 3(5)-methylpyrazole-*N*-carboxylates, except that 5 ml benzene containing 0.5 mmol alcohol and 0.5 mmol diphosgene was added dropwise 1 mmol triethylamine at the room temperature with stirring for 1 h. Then, the resultant mixture was further added 1 mmol triethylamine and 1 mmol 3(5)-methylpyrazole with stirring for 1 h. All the synthesized substrates were confirmed from the retention time in HPLC analysis and NMR spectra.

The hydrolysis of 3(5)-methylpyrazole-*N*-carboxylate or 3(5)-methyl-4-bromopyrazole-*N*-carboxylate regioisomers was monitored by HPLC at 220 nm by using a chiral column (Chiralcel OD-H, Daicel Chemical Industries, Japan) that was capable of separating the internal standard of 2-nitrotoluene and the substrate. The mobile phase was a HEX/IPA mixture (v/v) at a flow rate of 2.0 ml/min. As shown in Scheme 1, the resultant 3-methylpyrazole will rapidly tautomerize and alkyloxy acid product is not stable to further decompose to $CO_2$ and alcohol, e.g. (*R*)-1-phenylethanol that can be confirmed from the retention time in HPLC spectra. Detailed HPLC analytical conditions, $^1$H NMR, and $^{13}$C NMR spectra recorded on Brucker Avance DRX 500 spectrometer in $CDCl_3$ solution with TMS as an internal standard are given as follows.

(*R*)-1-Phenylethyl 3-methylpyrazole-*N*-carboxylate (**1a**); HPLC analysis: mobile phase (HEX:IPA = 99.3:0.7, v/v), retention time (2-nitrotoluene:**1a** = 3.3:9.4, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.75 (3H, t, α-$CH_3$), 2.34 (3H, s, 3-$CH_3$), 4.89 (1H, s, α-H), 6.19 (H, d, 4-H), 7.25–7.38 (5H, m, aromatic H), 8.01 (1H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 14.02 (3-$CH_3$), 22.03 (α-$CH_3$), 70.38 (α-C), 109.73 (4-C), 125.38–128.69 (2′-C–6′-C, aromatic C), 140.24 (5-C), 143.03 (1′-C), 148.74 (3-C), 149.85 (CO).

(*R*)-1-Phenylethyl 5-methylpyrazole-*N*-carboxylate (**1b**); HPLC analysis: mobile phase (HEX:IPA = 99.3:0.7, v/v), retention time (2-nitrotoluene:**1b** = 3.3:6.0, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.75 (3H, t, α-$CH_3$), 2.53 (3H, s, 5-$CH_3$), 4.89 (1H, s, α-H), 6.11 (H, d, 4-H), 7.25–7.38 (5H, m, aromatic H), 7.48 (1H, d, 3-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 14.25 (5-$CH_3$), 22.22 (α-$CH_3$), 70.38 (α-C), 109.82 (4-C), 125.39–128.69 (2′-C–6′-C, aromatic C), 143.03 (1′-C), 144.18 (3-C), 145.88 (5-C), 149.85 (CO).

Methyl 3-methylpyrazole-*N*-carboxylate (**2a**); HPLC analysis: mobile phase (HEX:IPA = 98:2, v/v), retention time (2-nitrotoluene:**2a** = 2.8:7.1, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 2.34 (3H, s, 3-$CH_3$), 4.05 (3H, s, $CH_3$), 6.23 (H, d, 4-H), 8.04 (H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 13.91 (3-$CH_3$), 54.72 (α-C), 109.83 (4-C), 143.05 (3-C), 144.27 (5-C), 149.93 (CO).

Methyl 5-methylpyrazole-*N*-carboxylate (**2b**); HPLC analysis: mobile phase (HEX:IPA = 98:2, v/v), retention time (2-nitrotoluene:**2b** = 2.8:6.2, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 2.57 (3H, s, 5-$CH_3$), 4.04 (3H, s, $CH_3$), 6.15 (H, d, 4-H), 7.57 (H, d, 3-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 14.02 (5-$CH_3$), 54.48 (α-C), 109.88 (4-C), 143.05 (3-C), 144.27 (5-C), 149.93 (CO).

Ethyl 3-methylpyrazole-*N*-carboxylate (**3a**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**3a** = 3.2:6.8, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.44–1.48 (3H, t, β-$CH_3$), 2.36 (3H, s, 3-$CH_3$), 4.47–4.53 (2H, m, α-$CH_2$), 6.23 (H, d, 4-H), 8.04 (H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 13.78 (β-C), 14.10 (3-$CH_3$), 64.15 (α-C), 109.52 (4-C), 148.32 (3-C), 149.19 (5-C), 150.23 (CO).

Ethyl 5-methylpyrazole-*N*-carboxylate (**3b**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**3b** = 3.2:5.7, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.38–1.40 (3H, t, β-$CH_3$), 2.63 (3H, s, 5-$CH_3$), 4.30–4.38 (2H, m, α-$CH_2$), 6.15 (H, d, 4-H), 7.57 (H, d, 3-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 13.69 (β-C), 14.04 (5-$CH_3$), 63.91 (α-C), 109.58 (4-C), 142.70 (5-C), 143.95 (3-C), 150.23 (CO).

Propyl 3-methylpyrazole-*N*-carboxylate (**4a**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**4a** = 3.2:5.8, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 0.97–1.05 (3H, m, γ-$CH_3$), 1.81–1.88 (2H, m, β-$CH_2$), 2.34 (3H, s, 3-$CH_3$), 4.31–4.37 (2H, t, α-$CH_2$), 6.22 (H, d, 4-H), 8.03 (H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 9.90 (γ-C), 13.71 (3-$CH_3$), 21.78 (β-C), 69.47 (α-C), 109.48 (4-C), 148.44 (3-C), 149.21 (5-C), 151.1(CO).

Propyl 5-methylpyrazole-*N*-carboxylate (**4b**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**4b** = 3.2:5.1, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 0.97–1.05 (3H, m, γ-$CH_3$), 1.71–1.78 (2H, m, β-$CH_2$), 2.57 (3H, s, 5-$CH_3$), 4.21–4.24 (2H, t, α-$CH_2$), 6.15 (H, d, 4-H), 7.57 (H, d, 3-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 9.78 (γ-C), 13.91 (5-$CH_3$), 21.74 (β-C), 69.34 (α-C), 109.52 (4-C), 142.68 (5-C), 143.82 (3-C), 151.1 (CO).

Isopropyl 3-methylpyrazole-*N*-carboxylate (**5a**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**5a** = 3.1:4.8, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.43–1.46 (6H, q, 2$CH_3$), 2.34 (3H, s, 3-$CH_3$), 5.24–5.29 (1H, m, α-H), 6.14 (H, d, 4-H), 8.02 (H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 14.03 (3-$CH_3$), 21.44 (α-$CH_3$), 72.82 (α-C), 109.58 (4-C), 148.01 (5-C), 148.94 (3-C), 150.02 (CO).

Isopropyl 5-methylpyrazole-*N*-carboxylate (**5b**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**5b** = 3.1:4.2, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.43–1.46 (6H, q, 2$CH_3$), 2.56 (3H, s, 5-$CH_3$), 5.24–5.29 (1H, m, α-H), 6.20 (H, d, 4-H), 7.57 (H, d, 3-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 14.27 (5-$CH_3$), 21.78 (α-$CH_3$), 72.56 (α-C), 109.74 (4-C), 142.83 (3-C), 144.06 (5-C), 150.02 (CO).

Butyl 3-methylpyrazole-*N*-carboxylate (**6a**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**6a** = 3.2:5.7, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 0.93–0.98 (3H, m, δ-$CH_3$), 1.37–1.50 (2H, m, γ-$CH_2$), 1.78–1.85 (2H, m, β-$CH_2$), 2.34 (3H, s, 3-$CH_3$), 4.42–4.45 (2H, t, α-$CH_2$), 6.22 (H, d, 4-H), 8.03 (H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 13.29 (δ-C), 13.71 (3-$CH_3$), 13.71 (γ-C), 30.37 (β-C), 67.87 (α-C), 109.46 (4-C), 148.44 (5-C), 149.22 (3-C), 150.33 (CO).

Butyl 5-methylpyrazole-*N*-carboxylate (**6b**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**6b** = 3.2:4.7, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 0.93–0.98 (3H, m, δ-$CH_3$), 1.10–1.13 (2H, m, γ-$CH_2$), 1.67–1.73 (2H, m, β-$CH_2$), 2.57 (3H, s, 5-$CH_3$), 4.25–4.28 (2H, t, α-$CH_2$), 6.14 (H, d, 4-H), 7.34 (H, d, 3-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 13.13 (δ-C), 13.91 (5-$CH_3$), 13.91 (γ-C), 30.06 (β-C), 67.69 (α-C), 109.54 (4-C), 142.68 (5-C), 143.83 (3-C), 150.33 (CO).

Hextyl 3-methylpyrazole-*N*-carboxylate (**7a**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**7a** = 3.2:5.5, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 0.88–0.91 (3H, m, ζ-$CH_3$), 1.29–1.34 (6H, m, ε,δ,γ-$CH_2$), 1.80–1.84 (2H, m, β-$CH_2$), 2.34 (3H, s, 3-$CH_3$), 4.41–4.44 (2H, t, α-$CH_2$), 6.22 (H, d, 4-H), 8.03 (H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 13.77 (ζ-C and 3-$CH_3$), 22.28 (ε-C), 25.16 (γ-C), 28.41 (β-C), 31.09 (δ-C), 68.22 (α-C), 109.51 (4-C), 148.48 (5-C), 149.27 (3-C), 150.27 (CO).

Hextyl 5-methylpyrazole-*N*-carboxylate (**7b**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**7b** = 3.2:4.7, min); $^1$H NMR (500 MHz, $CDCl_3$) δ = 0.88–0.91 (3H, m, ζ-$CH_3$), 1.29–1.34 (6H, m, ε,δ,γ-$CH_2$), 1.80–1.84 (2H, m, β-$CH_2$), 2.57 (3H, s, 5-$CH_3$), 4.26–4.27 (2H, t, α-$CH_2$), 6.21 (H, d, 4-H), 7.57 (H, d, 3-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 13.77 (ζ-C and 3-$CH_3$), 22.30 (ε-C), 25.01 (γ-C), 28.38 (β-C), 31.19 (δ-C), 68.05 (α-C), 109.59 (4-C), 142.73 (5-C), 143.87 (3-C), 150.27 (CO).

(*R*)-1-Phenylethyl 3-methyl-4-bromo-pyrazole-*N*-carboxylate (**8a**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**8a** = 3.1:5.5, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.75 (3H, d, α-$CH_3$), 2.31 (3H, s, 3-$CH_3$), 6.08 (1H, s, α-H), 7.22–7.41 (5H, m, aromatic H), 8.06 (1H, d, 5-H); $^{13}$C NMR (125 MHz, 27 °C, $CDCl_3$) $\delta$ = 12.34 (3-$CH_3$), 21.84 (α-$CH_3$), 99.69 (4-C), 125.34–128.69 (2′-C to 6′-C, aromatic C), 143.27 (5-C), 148.98 (3-C).

(*R*)-1-Phenylethyl 5-methyl-4-bromo-pyrazole-*N*-carboxylate (**8b**); HPLC analysis: mobile phase (HEX:IPA = 99:1, v/v), retention time (2-nitrotoluene:**8b** = 3.1:4.4, min); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ = 1.75 (3H, t, α-$CH_3$), 2.52 (3H, s, 5-$CH_3$), 6.08 (1H, s, α-H), 7.22–7.41 (5H, m, aromatic H), 7.61 (1H, d, 3-H); $^{13}$C NMR

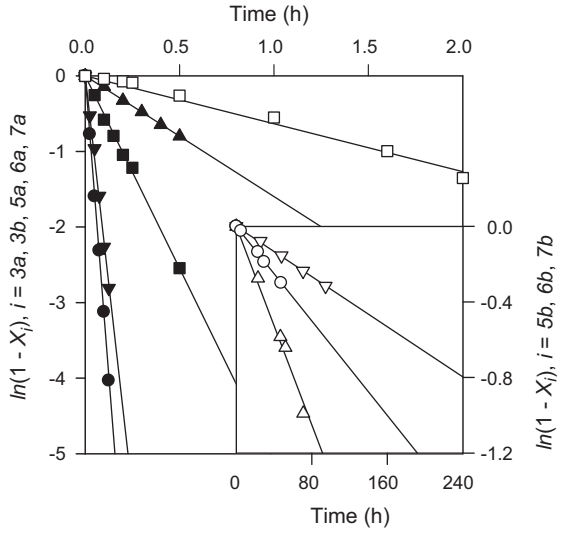


**Fig. 1.** Time-course variations of $\ln(1-X_i)$ for Novozym 435-catalyzed hydrolysis of **3a** (■), **3b** (□), **5a** (▲), **5b** (△), **6a** (●), **6b** (○), **7a** (▼), and **7b** (▽) in water-saturated MTBE at 45 °C; reaction conditions given in Table 1; (––) best-fit results by using first-order irreversible kinetics for each regioisomer.

(125 MHz, 27 °C, $CDCl_3$) $\delta$ = 12.66 (5-$CH_3$), 22.03 (α-$CH_3$), 99.69 (4-C), 125.34–128.69 (2′-C–6′-C, aromatic C), 139.76 (3-C), 140.01 (5-C).

### 2.3. Hydrolysis in organic solvents

To 10 ml water-saturated MTBE containing 3 mM regioisomer mixture at 45 °C was added a specific amount of Novozym 435, Lipase MY-30, or Lipase PS-D in water-saturated CYC. The resultant solution was stirred with a magnetic stirrer, and samples were removed at different time intervals for the HPLC analysis. From the time-course conversions $X_i$ ($i$ = 1*a*–8*b*), specific initial rates $V_i/(E_t)$ based on several conversion determinations were determined. Similar experiments were performed for Novozym 435 at other temperature and water-saturated organic solvents. Fig. 1 illustrated typical time-course variations of $\ln(1-X_i)$ ($i$ = 3*a*, 3*b*, and 5*a*–7*b*) for Novozym 435 in water-saturated MTBE, with which the specific initial rate for each regioisomer was estimated from the slope of each line. More experiments were performed in MTBE containing 0%, 20%, 40%, and 60% of saturated water, 3 mM of **1a** and **1b** (or **5a** and **5b**) mixture, and 40 mg/ml of Novozym 435 at 45 °C for studying effects of water content on the activity and regioselectivity.

In order to determine the lipase regioselectivity, the hydrolysis using 15 mM of **1a** and **1b** (21 mM of **5a** and **5b**, or 32 mM of **8a** and **8b**) mixture and 40 mg/ml Novozym 435 was repeated for 2 h. After filtrating the lipase, the resultant solution was extracted by using 0.1 M HCl solution (5 ml) and 0.1 M NaOH solution (5 ml) for three times in succession. The organic phase was then separated, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure, giving the remaining pure 5-methyl regioisomer of **1b**, **5b**, or **8b** as confirmed from $^1$H NMR and $^{13}$C NMR spectra.

### 2.4. Kinetic analysis

In order to study effects of changing the *N*-carboxylate moiety on the lipase performance, the hydrolysis of **2a** and **2b** (**4a** and **4b**, or **5a** and **5b**) mixture of various concentrations in water-saturated MTBE at 45 °C was carried out. The specific initial rates $V_{ia}/(E_t)$ and $V_{ib}/(E_t)$ varied with the regioisomer concentrations can be employed for estimating the kinetic parameters $k_{2,ia}/K_{m,ia}$, $k_{2,ib}/K_{m,ib}$, $[K_{m,ia}{}^{-1} + (S_{ib})(S_{ia})^{-1}K_{m,ib}{}^{-1}]$, and hence regiomeric ratio $R_i$ defined as $k_{2,ia}K_{m,ib}/[k_{2,ib}K_{m,ia}]$ from the following equations [30].

$$\frac{V_{ia}}{(E_t)} = \frac{k_{2,ia}(S_{ia})/K_{m,ia}}{1 + (S_{ia})/K_{m,ia} + (S_{ib})/K_{m,ib}} \quad (1)$$

$$\frac{V_{ib}}{(E_t)} = \frac{k_{2,ib}(S_{ib})/K_{m,ib}}{1 + (S_{ia})/K_{m,ia} + (S_{ib})/K_{m,ib}} \quad (2)$$

Notations $(E_t)$, $(S_{ia})$, and $(S_{ib})$ represent the initial concentrations of enzyme, fast-reacting and slow-reacting regioisomers, respectively.

## 3. Results and discussion

### 3.1. Substrate analysis

Depending on the nature of solvent, temperature, substituent, and concentration, the 3(5)-substituted-pyrazole can exist in two tautomeric forms having different tautomer ratio [10,15,31–34]. This implies that the hydrogen atom may be bound to $N_1$ or $N_2$ atom, in which the electron-withdrawing substituent such as $BH_2$ can stabilize the $N_1$-H tautomer (or 3-substituted pyrazole), while the electron-donating substituent such as OH stabilizes the $N_2$-H tautomer (or 5-substituted pyrazole). The two 3(5)-methylpyrazole tautomers can be distinguished from the different chemical shifts of methyl substituent by $^1$H NMR, or the chemical shift appearing at 146–152 ppm for the substituted C3 and 137–141 ppm for the substituted C5 by $^{13}$C NMR [10]. Therefore from the chemical shift of 2.34 ppm at C3 or 2.53–2.56 ppm at C5 for the methyl substituent by $^1$H NMR and $^{13}$C NMR spectra for the remaining substrate after extraction, the lipase regioselectivity for 3-methylpyrazole-*N*-carboxylate and 3-methyl-4-bromopyrazole-*N*-carboxylate can be determined.

The tautomer ratio of 46:54 for 3- and 5-methylpyrazoles has been estimated from $^{13}$C NMR spectra in hexamethylphosphoramide at −17 °C or methanol at −43 °C [32]. In the present report, the HPLC spectra were employed for determining the regioisomer ratio as 45:55 for **1a**:**1b**, 39:61 for **5a**:**5b**, 7:3 for **8a**:**8b**, or more than 2:1 for **2a**:**2b**, **3a**:**3b**, **4a**:**4b**, **6a**:**6b**, and **7a**:**7b** without containing 1-methyl side-chain in the *N*-carboxylate group (Table 1). Moreover, a variation of the *N*-protected group to trimethylsilyl or 2-phenylpropionyl moiety has resulted in the presence of only the 3-methylpyrazole regioisomer [31,35]. All the results imply a probable role of steric hindrances between the protected group and 3(5)-methyl or 3(5)-methyl-4-bromo substituent on varying the regioisomer ratio.

### 3.2. Effects of solvent, temperature, lipase sources, N-protected groups, and water content

Table 1 also demonstrates effects of water-saturated solvent, temperature, lipase sources, and *N*-protected groups on the specific initial rates and regioselectivity in terms of the ratio of $V_{ia}/V_{ib}$. In general, the specific initial rate for **1a** or **1b** increases with the solvent hydrophobicity in terms of *log P* (*P* as the partitioning coefficient of solvent between *n*-octanol and water), except that a minimum $V_{1a}/(E_t)$ exists for IPE. The highest regioselectivity for MTBE (i.e. $V_{1a}/V_{1b}$ = 8035) is obtained, especially when deleting the substrate concentration effect (i.e. $R_1 = V_{1a}(S_{1b})/[V_{1b}(S_{1a})] = k_{2,1a}K_{m,1b}/[k_{2,1b}K_{m,1a}]$ = 12,567 for $(S_{1a})$:$(S_{1b})$ = 39:61). This implies that Novozym 435 can hardly accept **1b** containing a leaving 5-methylpyrazole as the substrate. Similar arguments have been reported for the hydrolysis of (*R*,*S*)-2-phenylpropionyl azolides containing a leaving benzotriazole or 3,5-dimethylpyrazole moiety [35].

**Table 1**
Effects of lipase sources, water-saturated solvent, temperature, and alcohol moiety on hydrolysis of 3(5)-methylpyrazole-*N*-carboxylates.

| Lipase | Solvent | $(E_t)$ (mg/ml) | $V_{ia}/(E_t)$ (mmol/h g) | $V_{ib}/(E_t)$ (mmol/h g) | $V_{ia}/V_{ib}$ | Time (h) | $X_{ia}$ (%) | Time (h) | $X_{ib}$ (%) |
|---|---|---|---|---|---|---|---|---|---|
| (*R*)-1-Phenylethanol 3(5)-methylpyrazole-*N*-carboxylates (regioisomer ratio **1a**:**1b** = 39:61) | | | | | | | | | |
| Novozym 435 | ISO | 4 | 1.00 | 2.05E−4 | 4878 | 0.5 | 83.5 | 121.0 | 5.1 |
| Novozym 435 | CYC | 15 | 5.41E−1 | 8.73E−5 | 6197 | 0.25 | 82.2 | 163.6 | 11.6 |
| Novozym 435 | IPE | 40 | 6.77E−2 | 1.57E−5 | 4312 | 0.5 | 67.2 | 190.0 | 6.1 |
| Novozym 435 | MTBE | 40 | 7.36E−2 | 9.16E−6 | 8035 | 0.5 | 69.2 | 213.8 | 4.3 |
| Novozym 435 (35 °C) | MTBE | 40 | 5.20E−2 | ND | ND | 0.5 | 57.2 | 180.0 | ND |
| Novozym 435 (55 °C) | MTBE | 40 | 1.23E−1 | 1.59E−5 | 7735 | 0.4 | 82.3 | 166.3 | 5.4 |
| Lipase MY-30 | CYC | 80 | 7.75E−4 | 5.11E−5 | 15 | 5.5 | 25.1 | 143.3 | 27.7 |
| Methanol 3(5)-methylpyrazole-*N*-carboxylates (regioisomer ratio **2a**:**2b** = 68:32) | | | | | | | | | |
| Novozym 435 | MTBE | 1 | 4.81 | 1.79E−1 | 27 | 1.5 | 97.4 | 2.6 | 48.3 |
| Novozym 435 | ISO | 0.5 | 7.70 | 1.40 | 5 | 0.5 | 89.3 | 1.5 | 84.7 |
| Lipase MY-30 | CYC | 50 | 1.67E−1 | 9.99E−3 | 16 | 0.5 | 86.3 | 1.0 | 39.2 |
| Ethanol 3(5)-methylpyrazole-*N*-carboxylates (regioisomer ratio **3a**:**3b** = 82:18) | | | | | | | | | |
| Novozym 435 | MTBE | 2 | 6.50 | 1.63E−1 | 40 | 0.5 | 92.2 | 2.0 | 74.1 |
| Lipase MY-30 | CYC | 50 | 1.95E−1 | 5.42E−3 | 36 | 1.0 | 95.7 | 2.5 | 60.3 |
| Propanol 3(5)-methylpyrazole-*N*-carboxylates (regioisomer ratio **4a**:**4b** = 67:33) | | | | | | | | | |
| Novozym 435 | MTBE | 8 | 5.86 | 5.12E−2 | 114 | 0.125 | 94.6 | 2.5 | 66.4 |
| Lipase MY-30 | CYC | 50 | 8.07E−2 | 4.09E−3 | 20 | 0.5 | 61.1 | 2.5 | 39.5 |
| Isopropanol 3(5)-methylpyrazole-*N*-carboxylates (regioisomer ratio **5a**:**5b** = 45:55) | | | | | | | | | |
| Novozym 435 | MTBE | 40 | 5.35E−2 | 5.45E−4 | 98 | 0.5 | 55.1 | 71.0 | 63.0 |
| Lipase MY-30 | CYC | 40 | 7.10E−3 | 8.02E−5 | 88 | 22.4 | 90.3 | 172.3 | 33.9 |
| Butanol 3(5)-methylpyrazole-*N*-carboxylates (regioisomer ratio **6a**:**6b** = 78:22) | | | | | | | | | |
| Novozym 435 | MTBE | 8 | 9.32 | 5.05E−4 | 18,467 | 0.125 | 98.2 | 47.5 | 25.9 |
| Lipase MY-30 | CYC | 50 | 3.69E−2 | 3.79E−3 | 10 | 0.5 | 31.9 | 0.5 | 13.1 |
| Hexanol 3(5)-methylpyrazole-*N*-carboxylates (regioisomer ratio **7a**:**7b** = 77:23) | | | | | | | | | |
| Novozym 435 | MTBE | 8 | 6.89 | 2.83E−4 | 24,331 | 0.13 | 94.4 | 94.5 | 27.1 |
| Lipase MY-30 | CYC | 50 | 1.37E−2 | 1.01E−3 | 13 | 2.0 | 44.4 | 2.0 | 14.0 |

Conditions: unless specified, 10 ml solvent containing 3 mM regioisomer mixture at 45 °C and 400 rpm. Symbol of E−1 as $10^{-1}$. ND as not detected at 180 h.

The temperature effects on varying the enzyme activity and regioselectivity in MTBE are represented in Table 1. A liner relationship of $\ln[V_{1a}/(E_t)]$ with the inverse of absolute temperature can be found, implying that the lipase is thermally stable at 55 °C. In general, increasing of temperature may result in enhancements of the enzyme activity but not regioselectivity. This is similar to the temperature effect on varying the enantioselectivity in lipase-catalyzed kinetic resolutions for the racemates. In comparison with the performance of Novozym 435, very low specific activity and regioselectivity for Lipase MY-30 ($V_{1a}/V_{1b} = 15$) and Lipase PS-D (data not shown) is shown in water-saturated CYC.

By replacing the (*R*)-1-phenylethyl moiety with a methyl for **2a** and **2b**, more than 100-fold enhancements of specific initial rate for each regioisomer are obtained, but lead to moderate regioselectivity of $V_{2a}/V_{2b} = 27$ in MTBE or 5 in ISO for Novozym 435, and 16 in CYC for Lipase MY-30. This indicates that a minute change of the *N*-protected group may cause profound influences on varying the enzyme activity and regioselectivity. By employing **3a** and **3b** mixture containing an ethyl in the *N*-carboxylate moiety as the substrate, only minor changes of the specific activity and regioselectivity for both lipases are shown. However for **4a** and **4b** mixture containing a propyl moiety, more than 3-fold decreases of $V_{4b}/(E_t)$ but not $V_{4a}/(E_t)$, leading to good regioselectivity (i.e. $V_{4a}/V_{4b} = 114$ and $R_4 = V_{4a}(S_{4b})/[V_{4b}(S_{4a})] = k_{2,4a}K_{m,4b}/[k_{2,4b}K_{m,4a}] = 56$) for Novozym 435 but not Lipase MY-30, are demonstrated.

On the contrary for **5a** and **5b** mixture containing an isopropyl in the *N*-carboxylate moiety, more than two order-of-magnitudes lower specific activity for each regioisomer are shown, yet leading to excellent regioselectivity (i.e. $V_{5a}/V_{5b} = 98$ and hence $R_5 = V_{5a}(S_{5b})/[V_{5b}(S_{5a})] = k_{2,5a}K_{m,5b}/[k_{2,5b}K_{m,5a}] = 120$) for Novozym 435, as well as those of 88 and 107 for Lipase MY-30). However, no activity was detected for Lipase PS-D at 180 h, implying that the enzyme active site can hardly accommodate the substrate. When further changing the isopropyl group to a butyl for **6a** and **6b**, the specific activity for the fast-reacting but not slow-reacting regioisomer increases to yield the highest $V_{6a}/(E_t)$ and excellent regioselectivity (i.e. $V_{6a}/V_{6b} = 18{,}467$ and $R_6 = V_{6a}(S_{6b})/[V_{6b}(S_{6a})] = k_{2,6a}K_{m,6b}/[k_{2,6b}K_{m,6a}] = 5208$) for Novozym 435. Similarly by using **7a** and **7b** mixture containing a hexyl moiety as the substrate, Novozym 435 but not Lipase MY-30 gives high specific activity $V_{7a}/(E_t)$ and excellent regiomeric ratio (i.e. $R_7 = V_{7a}(S_{7b})/[V_7/(S_{7a})] = k_{2,7a}K_{m,7b}/[k_{2,7b}K_{m,7a}] = 7267$).

All the above results indicate that a straight alkyl chain longer than propyl in the *N*-carboxylate group is critical for obtaining the excellent regioselectivity. A possible explanation is that the moiety has induced fine-tuning of the 5-methyl regioisomer in the active site such that the nucleophilic attack and proton transfer *via* catalytic triads may greatly decrease. Similarly in comparison with **3a** and **3b**, introducing of the 1-phenyl moiety for **1a** and **1b** (or 1-methyl group for **5a** and **5b**) also yields excellent regioselectivity for Novozym 435.

Typical examples of using **1a** and **1b** (or **5a** and **5b**) mixture as the substrate in MTBE for studying effects of water content on Novozym 435 performances are demonstrated in Table 2, in which $k_{2,ia}/K_{m,ia}$ and $k_{2,ib}K_{m,ib}$ are estimated as $V_{ia}/[(E_t)(S_{ia})]$ and $V_{ib}/[(E_t)(S_{ib})]$, respectively, owing to the low substrate concentration employed. More than 9 folds lower $k_{2,ia}/K_{m,ia}$ and $k_{2,ib}K_{m,ib}$, but with an enhancement of regiomeric ratio $R_i$, are shown when replacing the anhydrous MTBE with water-saturated MTBE

**Table 2**
Effects of water content on Novozym 435 performances by using **1a** and **1b** (or **5a** and **5b**) mixture as the substrate.

| | Water content (%) | | | | |
|---|---|---|---|---|---|
| | 0 | 20 | 40 | 60 | 100 |
| $k_{2,1a}/K_{m,1a}$ (l/h g) | 5.78E−1 | 3.33E−1 | 1.54E−1 | 8.94E−2 | 6.29E−2 |
| $k_{2,1b}/K_{m,1b}$ (l/h g) | 1.61E−4 | 4.00E−5 | 1.50E−5 | 6.00E−6 | 5.00E−6 |
| $R_1$ | 3592 | 8346 | 10,243 | 14,896 | 12,576 |
| $k_{2,5a}/K_{m,5a}$ (l/h g) | 7.17E−1 | 2.67E−1 | 1.12E−1 | 6.24E−2 | 4.02E−2 |
| $k_{2,5b}/K_{m,5b}$ (l/h g) | 1.06E−2 | 2.55E−3 | 7.80E−4 | 4.21E−4 | 3.11E−4 |
| $R_5$ | 67 | 105 | 152 | 148 | 129 |

Conditions: 10 ml MTBE containing 40 mg/ml Novozym 435 and 3 mM mixture at 45 °C and 400 rpm. Symbol of E−1 as $10^{-1}$.

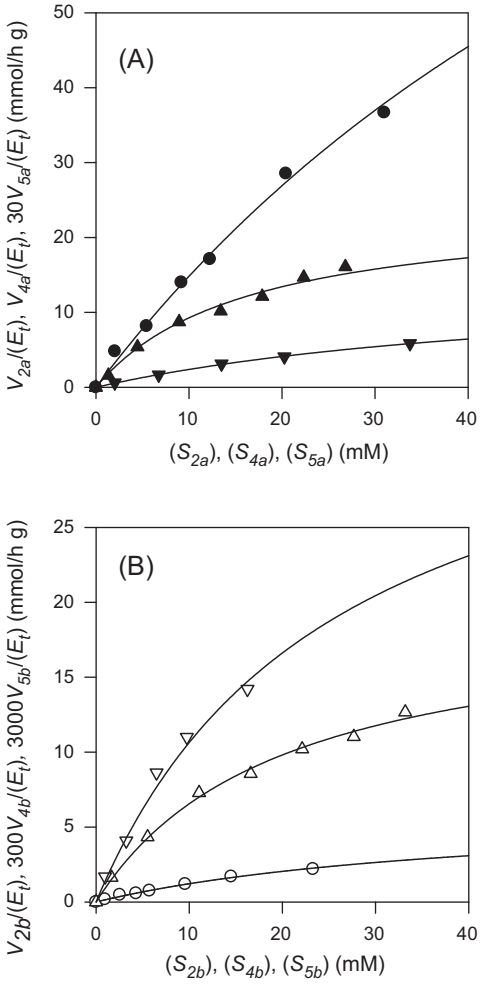


**Fig. 2.** Variation of specific initial rates with regioisomer concentrations at 45 °C in water-saturated MTBE; 1 mg/ml of Novozym 435 for **2a** (•) and **2b** (○), 8 mg/ml for **4a** (▼) and **4b** (▽), and 40 mg/ml for **5a** (▲) and **5b** (△), respectively.

**Table 3**
Kinetic constants and regiomeric ratio for Novozym 435-catalyzed hydrolysis of 3(5)-methylpyrazole-*N*-carboxylates at 45 °C in water-saturated MTBE.

| Entry | **2a** and **2b** | **4a** and **4b** | **5a** and **5b** |
|---|---|---|---|
| $k_{2,ia}/K_{m,ia}$ (l/h g) | 1.65 | 2.85E−1 | 4.99E−2 |
| $k_{2,ib}/K_{m,ib}$ (l/h g) | 1.51E−1 | 4.93E−3 | 3.28E−4 |
| $1/K_{m,ia} + (S_{ib})/[(S_{ia})K_{m,ib}]$ ($mM^{-1}$) | 1.13E−2 | 1.92E−2 | 6.17E−2 |
| $R_i$ | 11 | 58 | 152 |
| $k_{2,ia}$ (mmol/h g) | 2.15E+2 | 2.21E+1 | 1.79 |
| $k_{2,ib}$ (mmol/h g) | 1.97E+1 | 3.82E−1 | 1.18E−2 |
| $K_{m,ia}$ or $K_{m,ib}$ (mM) | 1.30E+2 | 7.76E+1 | 3.60E+1 |

The kinetic constants $k_{2,ia}$ and $k_{2,ib}$ were estimated by assuming $K_{m,ia}$ equal to $K_{m,ib}$.

containing 472.7 mM of water [36]. Similar kinetic behaviors have been reported for the influence of water content on the lipase activity and enantioselectivity for the hydrolytic resolution of (*R*,*S*)-pyrazolides [30,35]. Therefore, a compromise between the lipase activity and regioselectivity should been made for determining the best water content. In order to shed insights into how the substrate structure affects the lipase performance, a kinetic analysis based on Michaelis–Menten mechanism for **2a** and **2b** (**4a** and **4b**, or **5a** and **5b**) mixture in water-saturated MTBE at 45 °C was carried out.

### 3.3. Kinetic analysis

Fig. 2 illustrates the specific initial rates varied with the substrate concentrations of **2a**, **2b**, **4a**, **4b**, **5a**, and **5b**, with which the kinetic constants $k_{2,ia}/K_{m,ia}$, $k_{2,ib}/K_{m,ib}$, $(K_{m,ia}{}^{-1} + (S_{ib})(S_{ia})^{-1}K_{m,ib}{}^{-1})$ and hence regiomeric ratio can be estimated. As an approximation by further assuming the same substrate affinity to the active site, i.e. $K_{m,ia}$ equal to $K_{m,ib}$, the kinetic constants are then calculated and represented in Table 3. A change of the isopropyl to propyl moiety has resulted in slight decreasing of substrate affinity to the active site (i.e. $K_{m,4a}/K_{m,5a} = 2.15$), but great increasing of nuclephilic attack of catalytic serine and proton transfer from catalytic imidazolium to the leaving 3- or 5-methylpyrazole (i.e. $k_{2,4a}/k_{2,5a} = 12.3$ and $k_{2,4b}/k_{2,5b} = 33.4$). The decrease of specificity constants (i.e. $k_{2,5a}K_{m,4a}/[k_{2,4a}K_{m,5a}] = 0.175$ and $k_{2,5b}K_{m,4b}/[k_{2,4b}K_{m,5b}] = 0.0645$) indicates that an isopropyl moiety in **5a** and **5b** is not advantageous for increasing the enzyme activity, but still leads to an excellent regiomeric ratio of 152 (Table 4).

By further replacing the propyl moiety to a methyl for **2a** and **2b**, similar results of $K_{m,2a}/K_{m,4a} = 1.68$, $k_{2,2a}/k_{2,4a} = 9.75$, $k_{2,2b}/k_{2,4b} = 51.2$, $k_{2,2a}K_{m,4a}/[k_{2,4a}K_{m,2a}] = 5.78$, and $k_{2,2b}K_{m,4b}/[k_{2,4b}K_{m,2b}] = 30.4$ were obtained, and led to decreasing of the regiomeric ratio from 58 to 11. From the linear relationships in Fig. 1 at the low substrate concentration of 3 mM, one may estimate the specificity constant $k_{2,i}/K_{m,i}$ from $V_i/[(E_t)(S_i)]$, i.e. $k_{2,1a}/K_{m,1a} = 6.27 \times 10^{-2}$ l/h g, $k_{2,1b}/K_{m,1b} = 5.00 \times 10^{-6}$ l/h g, $k_{2,6a}/K_{m,6a} = 3.98$ l/h g, $k_{2,6b}/K_{m,6b} = 7.63 \times 10^{-4}$ l/h g, and $k_{2,7a}/K_{m,7a} = 1.27$ l/h g, and $k_{2,7b}/K_{m,7b} = 4.10 \times 10^{-4}$ l/h g. These values imply that an introduction of a 1-methyl side-chain for **1a** and **1b**, or a straight alkyl chain longer than propyl, mainly decreases the specificity constant of slow-reacting regioisomer and gives excellent regioselectivity. Apparently, more studies are needed in order to determine which mixture (i.e. **1a** and **1b**, **4a** and **4b**, **5a** and **5b**, **6a** and **6b**, or **7a** and **7b**) is the best substrate, after considering effects of regioisomer ratio, specific activity for the fast-reacting regioisomer, regiomeric ratio, and recovery of alcohol product and remaining substrate on the production cost.

### 3.4. Hydrolysis of *8a* and *8b* mixture

The analysis has been extended to the hydrolysis of 3(5)-methyl-4-substituted-pyrazole-*N*-carboxylate from which the remaining pure regioisomer can be separated as the starting material for performing the *N*-alkylation or *C*-substitution for the leaving pyrazole [37,38]. With 3(5)-methyl-4-bromopyrazole-*N*-carboxylate mixture (**8a** and **8b**) as the model substrate, excellent regioselectivity of $V_{8a}/V_{8b} = 4836$ (or $R_8 = V_{8a}(S_{8b})/[V_{8b}/(S_{8a})] = k_{2,8a}K_{m,8b}/[k_{2,8b}K_{m,8a}] = 1881$) for Novozym 435 in water-saturated MTBE is shown in Table 3. However in comparison with the results for **1a** and **1b** (Table 1), an order-of-magnitude lower enzyme activity for each regioisomer was found and might be attributed to the difficult affinity of

**Table 4**
Effects of lipase sources and temperature on hydrolysis of **8a** and **8b** mixture.

| Lipase | Solvent | $(E_t)$ (mg/ml) | $V_{8a}/(E_t)$ (mmol/h g) | $V_{8b}/(E_t)$ (mmol/h g) | $V_{8a}/V_{8b}$ | Time (h) | $X_{8a}$ (%) | Time (h) | $X_{8b}$ (%) |
|---|---|---|---|---|---|---|---|---|---|
| Lipase PS-D | CYC | 40 | 3.85E−4 | 6.42E−5 | 6 | 167.5 | 69.4 | 191.7 | 39.7 |
| Novozym 435 | MTBE | 40 | 3.41E−3 | 7.05E−7 | 4836 | 5.5 | 31.2 | 311.4 | 1.0 |
| Lipase MY-30 | CYC | 80 | 2.22E−4 | 2.04E−6 | 108 | 23.0 | 17.8 | 215.0 | 3.9 |

Conditions: 10 ml solvent containing 3 mM substrate (regioisomer ratio **8a**:**8b** = 70:30) at 45 °C and 400 rpm. Symbol of E−1 as $10^{-1}$.

bulky 3(5)-methyl-4-bromopyrazole moiety to the active site. A similar change of the enzyme activity with slight increasing of regioselectivity (i.e. $V_{8a}/V_{8b}$ = 108 or $R_8$ = 38) for Lipase MY-30 but not Lipase PS-D is also demonstrated.

## 4. Conclusions

With lipase-catalyzed hydrolysis of **1a** and **1b** mixture as the model system, Novozym 435 gives excellent regioselectivity for the 3-methylpyrazole regioisomer in water-saturated MTBE. In general, increasing of the solvent hydrophobicity and temperature, or decreasing of water content, may enhance the enzyme activity but not regioselectivity. Aside from the solvent, temperature, lipase sources, and water content, a change of the *N*-protected group has resulted in profound influences on the regioisomer ratio, lipase activity and regioselectivity that are mainly attributed to the change of nucleophilic attack *via* catalytic serine and proton transfer from catalytic imidazolium to the leaving 3- or 5-methylpyrazole. In general, introducing of a 1-methyl side-chain for **1a** and **1b** (or **5a** and **5b**) or a straight alkyl chain longer than propyl moiety may yield excellent lipase regioselectivity for the 3-methyl regioisomer. The hydrolysis has been successfully extended to **8a** and **8b** mixture for preparing pure 5-methyl-4-bromopyrazole-*N*-carboxylate.

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