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New view of acylase promiscuity: An extended study on the acylase-catalyzed Markovnikov addition

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ABSTRACT

Acylases have shown several promiscuities towards non-natural substrates. For example, acylases have been proved to be able to catalyze Markovnikov addition of *N*-heterocycles to vinyl esters recently. Some interesting and unexplainable observations drew our attention, and the acylase-catalyzed Markovnikov addition was investigated further in this paper. We detected an acylation product in the reaction and found that acetaldehyde was able to improve the reaction rate. Moreover, Markovnikov adduct could be formed using isopropenyl acetate or 2,2,2-trifluoro-ethyl acetate as substrates in the presence of acetaldehyde. Based on these results, it was proposed that the so-called acylase-catalyzed Markovnikov addition actually consisted of three steps identified as acylation, hemiaminal intermediate formation and transesterification. This discovery revealed that the promiscuity of acylase for Markovnikov addition was *pseudo*-promiscuity.

1. Introduction

Enzymatic promiscuities in organic media cause enormous interest, since the new catalytic activities of enzymes with non-natural substrates provide useful methods in organic synthesis [1,2]. The development of catalytic promiscuities expands the application of enzymes in organic chemistry largely [3–7]. Investigation of enzymatic promiscuities offers several novel avenues to understand enzymes. Hydrolases have been studied widely and shown diverse functions [8–11]. Berglund revealed the mechanism of CAL-B-catalyzed Michael addition and Aldol reaction by calculation. And the catalytic activities of CAL-B towards these two reactions were improved by mutation [12–15]. Acylases have also been recognized as very promising biocatalysts. They could promote Michael addition, which has been applied for the synthesis of a series of *N*-heterocycles derivatives successfully [16–18]. Some studies demonstrated that acylases could also catalyze the Markovnikov addition between *N*-heterocycles and vinyl esters [19–21]. In the preliminary work, several control experiments by using bovine serum albumin or deactivated acylases as catalysts were carried out to prove that the active site of acylases was responsible for the Markovnikov addition. Acylases could not catalyze Markovnikov addition between *N*-heterocycles and vinyl ether, although the reactivity of vinyl ether is higher than that of vinyl ester. This unexpected result was hardly explained, which attracted our interest. Thus, some work needs to be done to go deep into this reaction. Here, we explored the enzymatic process and reported the new discovery of the acylase-catalyzed Markovnikov addition.

2. Materials and methods

2.1. Materials

D-Aminoacylase from *Escherichia coli* and lipase PS Amano were purchased from Amano Enzyme Inc. Lipase immobilized on acrylic resin from *Candida antarctica* was purchased from Sigma. Solvents were dried over 3 Å molecular sieves for 24 h prior to use. All other chemicals were of the highest purity commercially available.

2.2. Analytical methods

^{1}H NMR spectra were recorded on a Bruker AMX-400 MHz spectrometer, using $CDCl_3$ as a solvent and TMS as internal reference. Analytical HPLC was performed using a SHIMADZU LC-10AVP HPLC with a UV detector. IR spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. Mass spectra were obtained by electrospray ionization (ESI) on a Bruker esquire 3000 plus mass spectrometer. GC–MS was obtained by Agilent 5973.

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2.3. General procedure

A mixture of dideuterated imidazole (1 mmol) and vinyl acetate (4 mmol) was dissolved in 2 mL DMSO. The reaction was incubated at 50 °C and 200 rpm and catalyzed by 25 mg D-amino acylase. After 96 h, the enzyme was filtered off to terminate the reaction. The crude residue was purified by flash column chromatography on silica gel using petroleum/ethyl acetate.

A mixture of 4-nitro-imidazole (1 mmol) and vinyl acetate (3 mmol) was dissolved in 2 mL DMSO. Different amount of acetaldehyde or water ranging from 0 to 100% (refer to the molar of 4-nitro-imidazole) was added to the reaction mixture. The reaction was catalyzed by 25 mg D-amino acylase and incubated at 50 °C and 200 rpm. Eight samples of 20 μL were taken from the reaction mixture after fixed intervals of time. Then samples were diluted to 1 mL with methanol and analyzed by GC–MS or HPLC. The initial reaction rate was calculated from the time curve plot of the concentration of product versus time. The apparent turnover numbers (k_{cat}^{app}) were calculated by dividing the number of millimoles of product by the reaction time and number of millimoles of enzyme at low conversion. The turnover numbers were apparent since they were only measured at one concentration (500 mM).

A mixture of 4-nitro-imidazole (1 mmol), acetaldehyde (3 mmol) and isopropenyl acetate or 2,2,2-trifluoro-ethyl acetate (3 mmol) was dissolved in 2 mL DMSO. The reaction was catalyzed by 25 mg D-amino acylase, and incubated at 50 °C and 200 rpm. After 96 h, samples were taken for GC–MS or ESI or HPLC detection.

A mixture of imidazole derivatives (1 mmol), isopropenyl acetate (2 mmol) and aldehyde (2 mmol) was dissolved in 1 mL organic solvent. The reaction was initiated by adding 20 mg lipases and was incubated at 50 °C and 200 rpm. After 72 h, the enzyme was filtered off to terminate the reaction. The crude residue was purified by flash column chromatography on silica gel using petroleum/ethyl acetate mixtures.

2.4. Characterization

2.4.1. Acetic acid 1-(4-nitro-imidazol-1-yl)-ethyl ester (**1a**)

White solid; ^{1}H NMR (DMSO-d_6, 400 MHz, δ, ppm): 8.68 (s, 1H, N=CH–N), 8.11 (s, 1H, N–CH=C), 6.77 (q, 1H, J = 6.2 Hz, N–CH–O–C=O), 2.05 (s, 3H, O=C–CH_3), 1.78 (d, 3H, J = 6.20 Hz, –CH–CH_3). ^{13}C NMR (DMSO-d_6, 125 MHz, δ, ppm): 169.8, 147.8, 137.2, 120.1, 77.3, 21.1, 20.2; IR (KBr, cm^{-1}): 3445, 1748, 1511, 1213.

2.4.2. Acetic acid 4-nitro-imidazol-1-yl-methyl ester (**1b**)

White powder; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.47 (s, 1H), 8.01 (s, 1H), 5.99 (s, 2H), 2.07 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 170.2, 147.5, 138.6, 122.2, 69.0, 20.8; IR (neat, cm^{-1}): 3448, 3084, 1753, 1543, 1491, 1349, 1217, 823.

2.4.3. Acetic acid 1-(4-nitro-imidazol-1-yl)-propyl ester (**1c**)

Yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 1.92 (d, 1H, J = 1.2 Hz, N=CH–N), 7.68 (s, 1H, J = 1.2 Hz, N–CH=C), 6.42 (t, 1H, J = 7.6 Hz, N–CH–O–C=O), 2.12–2.04 (m, 5H, –CH_2–CH_3, O=C–CH_3), 0.99 (t, 3H, J = 7.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 169.4, 148.4, 135.4, 117.0, 80.3, 27.3, 20.6, 9.0; IR (KBr, cm^{-1}): 3442, 1745, 1510, 1209, 825.

2.4.4. Acetic acid (4-nitro-imidazol-1-yl)-phenyl-methyl ester (**1d**)

Yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.78 (s, 1H), 7.67 (s, 1H), 7.63 (s, 1H), 7.52 (m, 5H), 2.27 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 169.0, 135.9, 132.7, 130.6, 128.4, 126.2, 118.1, 78.8, 20.7; IR (neat, cm^{-1}): 3583, 3136, 1761, 1547, 1495, 1348, 1278, 1213, 1025, 827, 735.

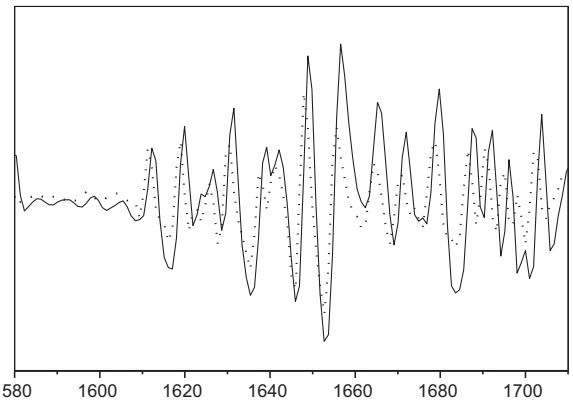


Fig. 1. Comparison of the second derivative spectra of D-amino acylase in DMSO (line) and in H_2O (dot).

2.4.5. Acetic acid (4-phenyl-phenyl)-(4-nitro-imidazol-1-yl)-methyl ester (**1e**)

Yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.82 (s, 1H), 7.72–7.39 (m, 11H), 2.27 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 169.1, 148.2, 143.6, 139.5, 135.9, 131.5, 128.9, 128.1, 127.1, 126.7, 118.2, 78.8, 20.7; IR (neat, cm^{-1}): 3575, 1758, 1547, 1485, 1350, 1213, 1012, 824, 731.

2.4.6. 1-(1-Imidazole)-propyl acetate (**1f**)

Yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.73 (s, 1H, N–CH=N), 7.08 (s, 2H, N–CH=CH–N), 6.43 (t, 1H, J = 7.6 Hz, N–CH–O), 2.16–2.03 (m, 5H, CH_3CO, CH_2), 0.92 (t, 3H, J = 7.6 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 169.5, 136.7, 129.5, 116.7, 79.7, 27.2, 20.7, 9.0; IR (neat, cm^{-1}): 2977, 1749, 1495, 1216, 1046, 828, 740.

2.4.7. 1-(1-(2-Methyl-imidazole))-propyl acetate (**1g**)

Yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 6.94 (s, 2H, N–CH=N, N–CH=C), 6.37 (t, 1H, J = 7.6 Hz, N–CH–O), 2.47 (s, 3H, CH_3), 2.08–1.93 (m, 5H, CH_3CO, CH_2), 0.85 (t, 3H, J = 7.6 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 169.4, 145.2, 128.0, 152.3, 78.8, 28.0, 20.7, 13.1, 8.9; IR (neat, cm^{-1}): 2969, 1755, 1490, 1218, 1044, 831, 736.

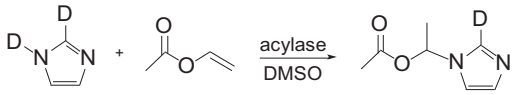
2.4.8. 1-(1-(4-Methyl-imidazole))-propyl acetate (**1h**)

Yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.58 (s, 1H, N–CH=N), 6.73 (s, 1H, N–CH=C), 6.29 (t, 1H, J = 7.6 Hz, N–CH–O), 2.15 (s, 3H, CH_3), 2.08–1.93 (m, 5H, CH_3CO, CH_2), 0.86 (t, 3H, J = 7.6 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 169.5, 138.4, 136.1, 113.0, 79.7, 27.1, 20.7, 13.3, 9.0; IR (neat, cm^{-1}): 2975, 1753, 1492, 1212, 1039, 822, 743.

3. Results and discussion

3.1. Characterization of D-amino acylase

Second derivative IR spectra were used as a particularly sensitive probe of protein conformation [22,23]. The IR spectrum of D-amino acylase in DMSO was almost the same as that in water (Fig. 1), which indicated that the conformation of D-amino acylase was preserved in DMSO. Thus, it can be concluded that D-amino acylase may keep its natural activity in DMSO.



Scheme 1. Acylase-catalyzed Markovnikov addition of dideuterated imidazole with vinyl acetate.

3.2. Isotope experiment

An isotope labeling experiment in the process of enzymatic Markovnikov addition was carried out by using dideuterated imidazole (Fig. S1) as nucleophile in the reaction (Scheme 1). According to the Markovnikov addition rule, a methyl group containing one deuterium atom should be formed. However, a methyl group that did not contain deuterium atoms appeared in the final product (Fig. S2). This result indicated that the apparent Markovnikov adduct was not formed from Markovnikov addition through one step.

3.3. Influence of the amount of vinyl ester on the enzymatic Markovnikov addition

To identify the intermediate, the reaction between 4-nitro-imidazole and vinyl acetate was monitored by GC–MS. To our surprise, a small amount of **2a** was detected in the reaction system besides the corresponding adduct (Scheme 2 and Fig. S3). **2a** was also isolated from the reaction in large scale and the structure of **2a** was characterized (Fig. S4). It demonstrated that the acylation catalyzed by D-amino acylase took place in DMSO. The concentration of **2a** increased slightly at the beginning and then decreased, but the amount of **2a** still kept at a very low level in the whole procedure. Therefore, the yield may decrease as the amount of vinyl acetate decreasing, because vinyl acetate could be consumed during the acylation reaction. When the molar ratio of vinyl acetate to 4-nitro-imidazole was 1, the maximum yield was 64%. It was consistent with our hypothesis.

3.4. Influence of the amount of acetaldehyde on the enzymatic Markovnikov addition

In the enzymatic Markovnikov addition reaction, the acylation of 4-nitro-imidazole catalyzed by D-amino acylase would produce acetaldehyde. The question whether the amount of acetaldehyde could affect the reaction arose. Thus, the influence of the molar ratio of acetaldehyde to 4-nitro-imidazole on the reaction was examined. The data from a plot of reaction rate versus concentration of acetaldehyde implied that the reaction was dependent on the concentration of acetaldehyde. All the reaction rates were improved. The rate was increased nearly six times in the presence of 0.1 equiv acetaldehyde (Entries 1 and 2, Table 1). However, the effect of acetaldehyde on the reaction became weaker and weaker as its concentration increasing (Entries 2–5, Table 1). It may be attributed to the deactivation of enzyme caused by a large number of acetaldehyde [24]. Another control reaction between 4-nitro-imidazole and vinyl acetate with adding 3 equiv acetaldehyde in the absence of acylase was carried out. The reaction could not take place, even prolonging reaction time to 120 h. This result ruled out the possibility that the transesterification could be spontaneous in the presence of acetaldehyde without D-amino acylase. Moreover, it indicated that the role of the acylase is not only to generate acetaldehyde. It should be taken into account that the acetaldehyde was an aqueous solution. Thus, an experiment by adding water was carried out. As seen from Fig. 2, there was no evident difference between the yields in the presence of water or without water. This result excluded the influence of water on the reaction. Thus, it can be concluded that acetaldehyde is very important in enzymatic Markovnikov addition.

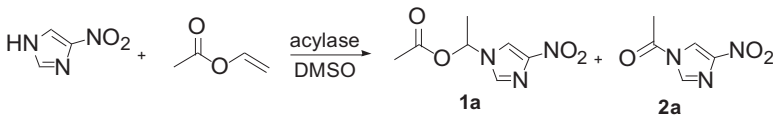
Table 1
The influence of acetaldehyde on the Markovnikov addition catalyzed by D-amino acylase in DMSO at 50 °C.[a]

Entry	Aldehyde	Yield[b] (%)	Vo (mM h^{-1})	Vr[c]	k_{cat}^{app} (h^{-1})
1	0	86	31.1	1	69.7
2	10%	95	183	5.87	410
3	20%	94	92.2	2.96	206
4	50%	91	76.2	2.45	171
5	100%	90	64.8	2.08	145

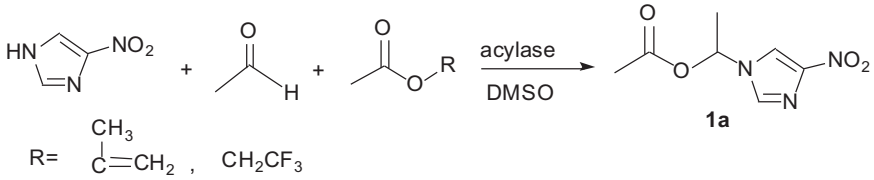
[a] Reaction condition: 1 mmol 4-nitro-imidazole, 3 mmol vinyl acetate, 25 mg D-amino acylase, 2 mL DMSO, 50 °C.
[b] Determined by HPLC.
[c] Relative initial reaction rate to the reaction in the absence of acetaldehyde.

3.5. Influence of the leaving group in substrate on the enzymatic Markovnikov addition

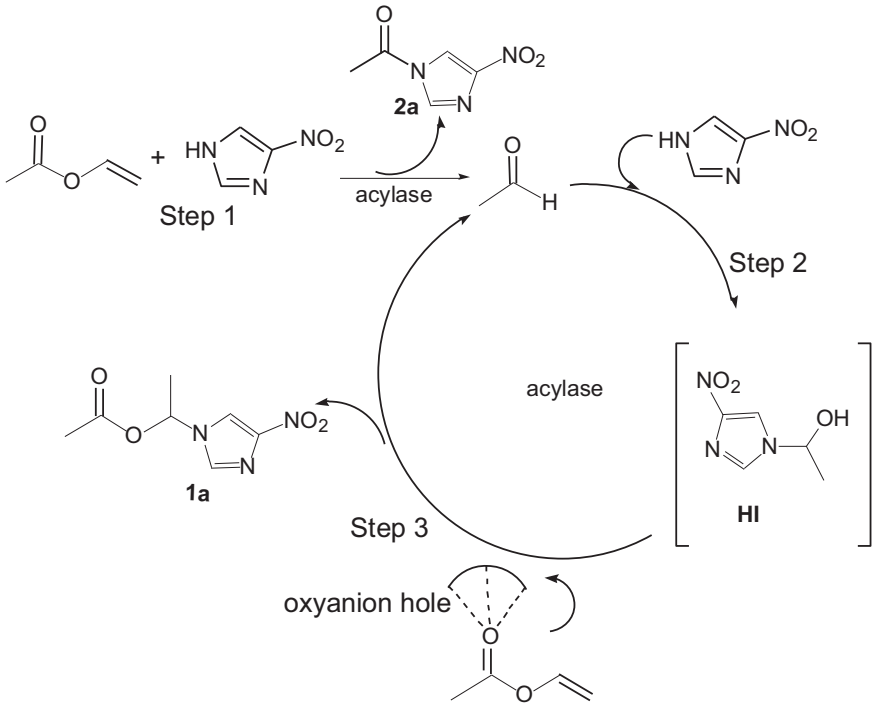
In order to distinguish from vinyl acetate, isopropenyl acetate was employed as the substrate in the presence of acetaldehyde (Scheme 3). The product **1a** was obtained in 70% yield confirmed by mass spectrum (Fig. S5) and HPLC analysis. This observation indicated that the ester bond was cleaved and a new one was formed during the process. However, 4-nitro-imidazole could not react with isopropenyl acetate in the absence of acetaldehyde. It may be ascribed to the low reactivity of acetone which generated from isopropenyl acetate. In addition, when isopropenyl acetate was replaced by 2,2,2-trifluoro-ethyl acetate, **1a** could also be synthesized (Fig. S6).



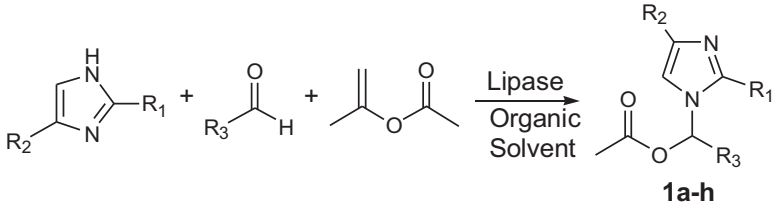
Scheme 2. Acylase-catalyzed Markovnikov addition of 4-nitro-imidazole with vinyl acetate.



Scheme 3. The reaction of 4-nitro-imidazole with ester in the presence of acetaldehyde catalyzed by D-amino acylase in DMSO at 50 °C.



Scheme 4. Proposed mechanism for enzymatic Markovnikov addition.



Scheme 5. Lipase-catalyzed "apparent" Markovnikov addition.

3.6. The process of enzymatic Markovnikov addition

Based on the above results, a hypothesis for the process of enzymatic Markovnikov addition was proposed as Scheme 4. Acylation of 4-nitro-imidazole with vinyl acetate was first catalyzed by acylase and acetaldehyde generated simultaneously. The *N*-heterocycle as nucleophile attacked the acetaldehyde and formed the hemiaminal intermediate (HI). HI was supposed to be stabilized by hydrogen bond in the catalytic site. Then, HI attacked the carbonyl group of vinyl ester which bonded with oxyanion hole in the catalytic site. Next, transesterification was accomplished by acylase and the final product was obtained. This hypothesis may provide an explanation for no reaction between *N*-heterocycles and vinyl ether.

3.7. Lipase-catalyzed "apparent" Markovnikov addition

According to our proposed mechanism, it could be envisioned that lipases could catalyze this addition. The results in Table 2 showed that two lipases exhibited the ability to catalyze this reaction involving other aldehydes bearing the alkyl or aryl group and other imidazole derivatives (Scheme 5). The expected products were synthesized successfully. This reaction was favored by aliphatic aldehyde, while aromatic aldehydes exhibited lower reactivity (Table 2, Entries 1 and 5). Examination of substituted groups on imidazole revealed that electron-withdrawing group could decrease the reactivity of substrates (Table 2, Entries 3 and 6). In addition, steric hindrance showed negative effect on the reaction and slight decrease in yields was observed (Table 2, Entries 4–8). To our disappointment, no induction of stereoselectivity was

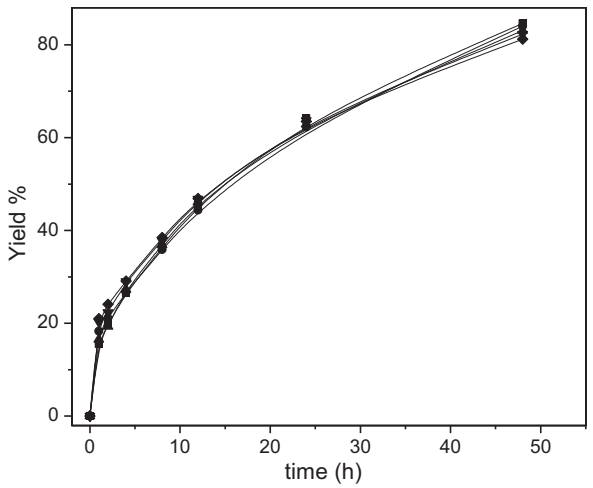
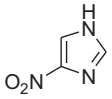
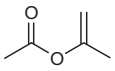
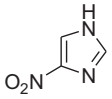
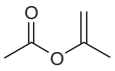
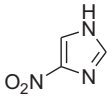
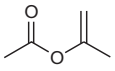
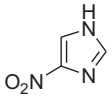
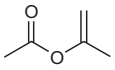
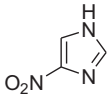
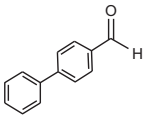
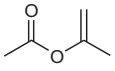
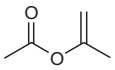
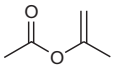
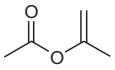


Fig. 2. The influence of water on the Markovnikov addition catalyzed by D-amino acylase in DMSO at 50 °C: (a) Blank (filled squares); (b) 10% (filled circles); (c) 20% (filled triangles); (d) 50% (filled inverted-triangles); (e) 100% (filled diamonds).

Table 2
Lipase-catalyzed "apparent" Markovnikov addition.[a]

Entry	Nucleophile	Aldehyde	Ester	Solvent	Enzyme	Yield[b] %
1				DMSO	PSL	**1a** (36)
2				DMSO	PSL	**1b** (48)
3				DMSO	PSL	**1c** (26)
4				DMSO	PSL	**1d** (13)
5				DMSO	PSL	**1e** (8)
6				MTBE	CAL-B	**1f** (53)
7				MTBE	CAL-B	**1g** (36)
8				MTBE	CAL-B	**1h** (40)

[a] All reactions were run on a 0.5 mmol scale of imidazole derivatives with 2 equiv of aldehydes and isopropenyl acetate in 1 mL of organic solvent catalyzed by lipases at 50 °C, 72 h.
[b] Detected by HPLC.

observed. Other study on the stereoselectivity of this reaction was still under way.

4. Conclusion

The enzymatic process of acylase-catalyzed Markovnikov addition has been clarified via several experiments. Strong evidence have shown that the reaction consisted of three processes identified as acylation, hemiaminal intermediate formation and transesterification. Acylases still exhibited their natural activity in the reaction. This work is important for enzymatic promiscuity because it reveals the possibility that some new activities of enzymes in non-natural reactions are *pseudo*-promiscuity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.08.002.

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