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Promiscuous Acylases-Catalyzed Markovnikov Addition of N-Heterocycles to Vinyl Esters in Organic Media

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Abstract: Three acylases, including D-aminoacylase from *Escherichia coli*, acylase "Amano" from *Aspergillus oryzae* and immobilized penicillin G acylase from *Escherichia coli* have been found to possess novel activity to catalyze the Markovnikov addition reaction of N-heterocycles to vinyl esters. The aza-Markovnikov addition reactions of 4-nitroimidazle to vinyl acetate catalyzed by D-aminoacylase, acylase "Amano" and immobilized penicillin G acylase were up to 1260-fold, 720-fold and 320-fold faster than the respective non-enzymatic reaction. Some control experiments have been designed to demonstrate the catalytic specificity of acylases. Under the catalysis of these promiscuous acylases, a number of N-heterocycles, including some pentacyclic N-heterocycles, pyrimidines and purines, were successfully added to a series of vinyl esters in moderate to excellent yields to prepare N-heterocycle derivatives. The acylase-catalyzed Markovnikov addition reaction has provided a new strategy to perform the Markovnikov addition and expanded the application of biocatalysts.

Keywords: biotransformations; catalytic promiscuity; enzyme catalysis; Markovnikov addition; N-heterocycles

Introduction

With the discoveries of highly stereoselective enzymes with broad substrate specificity, the study of enzyme catalysts has expanded rapidly in the last decades.[1] During the exploration of new activities of enzymes, a growing number of them have been found to be capable of catalyzing not only their "natural" reaction but also one or more alternative reactions.[2] This "catalytic promiscuity" of enzymes has potential impact on the evolution of biocatalysts since duplication of the gene, followed by a series of mutations to amplify the desired activity, may generate a new enzyme now having the progenitor's secondary activity as the primary one.[3] The catalytic promiscuity also provides new tools for organic and bioorganic transformations, and thus expands the application of enzymes.[4] Research in this area has attracted much attention of chemists and biochemists in recent years.

The addition reaction is among the most fundamental types of reactions in organic synthesis. However, there were only scarce reports about enzymes which are able to catalyze general addition reactions. Among them, the Michael addition has been most extensively studied. Some hydrolytic enzymes were used to catalyze the Michael-type addition of trifluorinated α,β-unsaturated carbonyl compounds in buffer solution.[5] Our group reported that an alkaline protease from *Bacillus subtilis* showed a remarkable activity to catalyze the Michael addition of N-nucleophiles to acrylates in organic solvents.[6] Several elegant works have described designed combinations of experiments to investigate the possible catalytic mechanism of "promiscuous" lipase.[7] Besides Michael addition, aldol addition reactions were achieved under the catalysis of an engineered mutant of CAL B.[8] In order to enrich enzymatic addition reactions, exploration of enzymes with new activities becomes particularly fascinating and remains a great challenge.

The Markovnikov addition is one type of useful carbon-carbon, oxygen-carbon or nitrogen-carbon bond-forming reaction. It is especially important to synthesize bioactive N-heterocycle derivatives with a nitrogen-carbon linkage which could be achieved by an addition reaction. Such aza-Markovnikov additions can be traditionally performed under harsh chemical conditions in which bases, acids and strong heating were usually used to promote the reaction.[9] In many cases, the yields and selectivities are far from satisfactory due to the occurrence of several side reactions. Enzyme catalysts

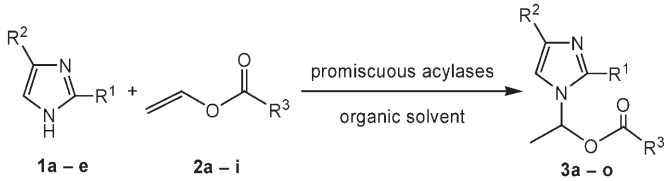
with high regioselectivity and stereoselectivity have gained recognition as favorable, environmentally benign alternatives.

Previously, we reported the unprecedented Markovnikov-type addition of allopurinol to vinyl ester by penicillin G acylase from *Escherichia coli* (PGA).[10] In the present work, we surprisingly found that another two zinc-binding acylases, D-aminoacylase from *Escherichia coli* and acylase "Amano" from *Aspergillus oryzae*, which naturally catalyze the hydrolysis of *N*-acyl-D-amino acids and *N*-acyl-L-amino acids, respectively, also possess the promiscuous activity to catalyze the Markovnikov addition of a broad range of N-heterocycles to vinyl esters and exhibit even higher activities. Under the catalysis of these promiscuous acylases, a number of pentacyclic N-heterocycles, pyrimidines and purines were successfully added to a series of vinyl esters in moderate to high yields. The N-heterocycle derivatives obtained are usually pharmacologically active and may be applied as potential therapeutic alternatives.[11] The catalytic specificity of acylases was demonstrated by the combination of different control experiments. The influence of the structure of the N-heterocycles or vinyl esters to the enzymatic reactions has been systematically evaluated. This novel Markovnikov addition activity of acylases is of practical significance in expanding the application of enzymes and in the evolution of new biocatalysts.

Results and Discussion

In the light of the above observations, we designed some control experiments to demonstrate the catalytic specificity of acylases. From the data in Table 1 and Figure 1, reaction yields and initial rates were calculated and compared.

The reaction of 4-nitroimidazle (**1a**) with vinyl acetate (**2a**) in the absence of enzyme led to the Markovnikov adduct in very low yield ($<0.4\%$) even after 5 days. In contrast, the reactions in the presence of D-aminoacylase, acylase "Amano" and immobilized penicillin G acylase were up to 1260-fold, 720-fold and 320-fold faster, respectively (entries 2, 6 and 7, Table 1). Besides, the initial reaction rate is practically proportional to the enzyme amount, also suggesting the catalytic effect of the enzyme (entries 2 and 3). When the reactants were incubated with denatured D-aminoacylase (pre-treated with urea at 100 °C for 6 hours) or bovine serum albumin (BSA), both the initial rates were almost equal to the background reaction (entries 4 and 8), ruling out the possibility that the similar amino acid distribution on the protein surface has promoted the process. Catalysis of the reactions by two widely used hydrolases (lipase from *Candida cylindracea* and lipozyme) could only accelerate the process by 11- and 4-fold, respectively (entries 9 and 10). To further establish that the reaction takes place in the enzyme's active site, control reactions were run with inhibited D-aminoacylase by adding 50 mM non-competitive inhibitor $ZnCl_2$. Ligation of the inhibitory zinc ion by the highly conserved residues Asp^{366}, His^{67} and His^{69} in the active site would lower their pK_a values and/or hold the nucleophile to perturb the proton shuttle and intermediate stabilization.[12] The in-



Scheme 1. Markovnikov addition of imidazoles to vinyl esters.

Table 1. Markovnikov addition between 4-nitroimidazole (**1a**) and vinyl acetate (**2a**) in the presence of different catalysts.

Entry	Catalyst	Time [h]	Yield [%][a]	V_0 ($\mu M \cdot min^{-1}$)	V_r[b]
1	No catalyst	120	0.4	0.17	1.0
2	D-Aminoacylase	72	97.3	213.96	1258.6
3	D-Aminoacylase[c]	72	70.8	114.03	670.8
4	Denatured[d]	96	1.9	1.03	6.1
5	Inhibited[e]	96	0.7	0.32	1.9
6	Acylase "Amano"	72	80.4	122.57	721.0
7	Immobilized PGA	72	55.2	54.89	322.9
8	BSA	96	2.2	1.18	6.9
9	CCL	96	3.5	1.85	10.9
10	Lipozyme	96	1.3	0.71	4.2

Conditions: 4-nitroimidazole (0.6 mmol), vinyl acetate (4 equivs.), D-aminoacylase (100 mg), DMSO (2 mL), 50 °C.
[a] Values in entries 2, 3, 6 and 7 are isolated yields and others were detected by HPLC.
[b] Relative initial reaction rate to the reaction in absence of enzyme.
[c] 25 mg/mL D-aminoacylase was used.
[d] D-Aminoacylase predenatured with urea at 100 °C for 6 h.
[e] D-Aminoacylase inhibited by 50 mM $ZnCl_2$.

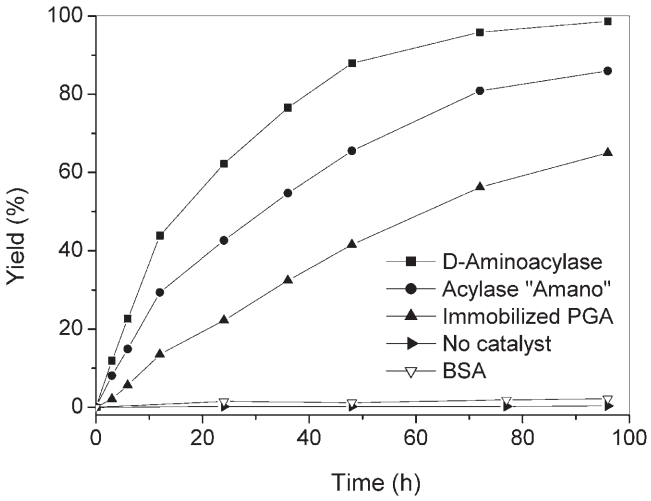


Figure 1. Progress curves of the Markovnikov addition of 4-nitroimidazole (**1a**) to vinyl acetate (**2a**) catalyzed by different catalysts.

hibited enzyme did not show any acylase activity to catalyze the hydrolysis of *N*-acetyl-D-methionine, and the specific activity for the Markovnikov addition was the same as that of non-enzymatic reaction (entry 5). All these results suggest that the tertiary structure and the specific active site of acylases are responsible for the Markovnikov addition reaction.

We then examined the generality of the enzymatic conditions to imidazoles, and the results are listed in Table 2. We focused on D-aminoacylase as biocatalyst since this enzyme has demonstrated high activity toward the Markovnikov addition reaction. In order to prevent the hydrolytic reactions and favor the addition reaction and to reach higher conversions, organic solvents and ratios of vinyl esters have also been established. For imidazole or alkylimidazoles, an organic solvent with a higher log P value was found to better facilitate the enzymatic reactions (entries 10–12, Table 2). Consequently, the hydrophobic solvent hexane was employed as the reaction media. For nitroimidazoles, which could only dissolve in a strongly polar solvent, the reactions were carried out in DMSO. When the formation of products was followed by TLC and HPLC, it is noteworthy to mention that the enzymatic addition reaction catalyzed by all three acylases afforded no by-products resulting from anti-Markovnikov addition, acylation reaction, hydrolytic reactions or other reactions.

Generally, the Markovnikov addition of imidazoles **1a**–**e** to a variety of vinyl esters proceeded favorably, furnishing the corresponding products in moderate to excellent isolated yields. The influence of the structure of the vinyl ester was evaluated using 4-nitroimidazole (**1a**) as the substrate. The enzymatic addition reactivity was found to decrease as the chain length of the vinyl ester increased (entries 1–6), and the Markovnikov addition of more sterically hindered vinyl esters provided lower yields (entries 2 and 3). When the chain lengths are comparable, divinyl dicarboxylates reacted faster than monoacidic vinyl esters (entries 7 and 8). The Markovnikov addition of 4-nitroimidazole to vinyl benzoate (**2i**) is rather slow and provided only a 36% yield of product even with more equivalents of vinyl benzoate after a prolonged reaction time of192 h (entry 9). This may be due to the relatively weaker nucleophilicity of aromatic acid vinyl esters in comparison to fatty acid vinyl esters.

Table 2. Markovnikov addition of imidazoles with vinyl esters catalyzed by D-aminoacylase.

Entry	Substrate			Vinyl ester			Solvent	Product	Time [h]	Yield[a] [%]
	No.	R^1	R^2		R^3	[equivs.]				
1	**1a**	H	NO_2	**2a**	CH_3	4	DMSO	**3a**	72	97
2	**1a**	H	NO_2	**2b**	$(CH_2)_2CH_3$	8	DMSO	**3b**	84	94
3	**1a**	H	NO_2	**2c**	$CH(CH_3)_2$	8	DMSO	**3c**	84	88
4	**1a**	H	NO_2	**2d**	$(CH_2)_3CH_3$	8	DMSO	**3d**	95	95
5	**1a**	H	NO_2	**2e**	$(CH_2)_4CH_3$	8	DMSO	**3e**	96	93
6	**1a**	H	NO_2	**2f**	$(CH_2)_6CH_3$	8	DMSO	**3f**	95	74
7	**1a**	H	NO_2	**2g**	$(CH_2)_2COOCH{=}CH_2$	8	DMSO	**3g**	84	92
8	**1a**	H	NO_2	**2h**	$(CH_2)_4COOCH{=}CH_2$	8	DMSO	**3h**	96	95
9	**1a**	H	NO_2	**2i**	Ph	16	DMSO	**3i**	192	36
10	**1b**	H	H	**2a**	CH_3	8	DMSO	**3j**	84	48
11	**1b**	H	H	**2a**	CH_3	8	toluene	**3j**	84	55
12	**1b**	H	H	**2a**	CH_3	8	hexane	**3j**	84	85
13	**1b**	H	H	**2b**	$(CH_2)_2CH_3$	8	hexane	**3k**	96	82
14	**1b**	H	H	**2g**	$(CH_2)_2COOCH{=}CH_2$	8	hexane	**3l**	96	88
15	**1c**	H	CH_3	**2a**	CH_3	8	hexane	**3m**	96	80
16	**1d**	CH_3	NO_2	**2a**	CH_3	8	DMSO	**3n**	96	55
17	**1e**	CH_3	H	**2a**	CH_3	8	hexane	**3o**	96	63

Conditions: substrate (0.6 mmol), vinyl ester (relevant equivs.), D-aminoacylase (100 mg), solvent (2 mL), 50 °C.
[a] Isolated yields.

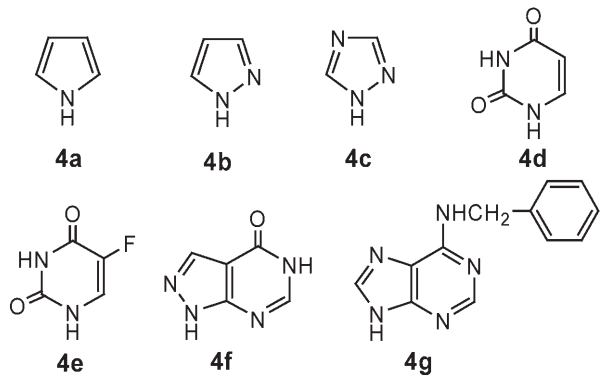


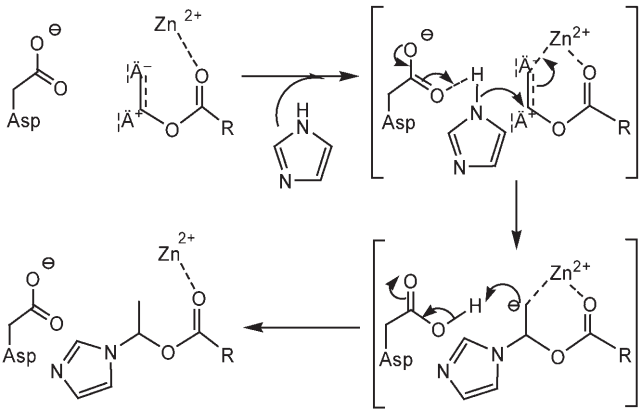
Figure 2. Other N-heterocyclic substrates used for the enzymatic Markovnikov addition.

The influence of different substituted groups on the imidazole ring was also examined. Addition of 4-nitroimidazole (**1a**) to vinyl acetate (**2a**) afforded a much higher yield (97%, entry 1) than imidazole (85%, entry 9) in a shorter time with less equivalents of vinyl ester, indicating that an electron-withdrawing group improves the addition reactivity of the N-heterocycle. Oppositely, the additions of alkylimidazoles proceeded more slowly (entries 15–17). A substituted group adjacent the N-1 position sterically hindered the addition reaction dramatically, giving a much lower isolated yield (entries 16 and 17).

Having obtained favorable results with imidazoles, we then examined the addition of other pentacyclic N-heterocycles to vinyl acetate (**2a**). The results are summarized in Table 3. When similar processes were carried out with pyrrole (**4a**), pyrazole (**4b**), and 1,2,4-triazole (**4c**), comparable behaviors were observed. D-Aminoacylase showed good Markovnikov activity toward these substrates, providing exclusively compounds **5 g** and **5b** and **5c** with moderate to high yields (entries 1–3, Table 3). The addition reactivity of the four pentacyclic N-heterocycles examined in our research decreases in the order of 1,2,4-triazole (**4c**), imidazole (**1b**), pyrazole (**4b**), and pyrrole (**4a**). These results were in accordance with their nucleophilicity.

To confer versatility to this enzymatic Markovnikov reaction, more complicated N-heterocycles, such as pyrimidines and purines, were introduced. The analogous reaction of uracil (**4d**) with vinyl acetate (**2a**) provided a very low yield of the Markovnikov adduct (5%) after 8 days, since the N–H of the amide is much less nucleophilic than that of the amine (entry 4, Table 3). With an electron-withdrawing group on the heterocycle, 5-fluorouracil reacted faster (entry 5). Although the acylases showed low activity toward pyrimidines, excellent regioselective was achieved and single N-1 adducts were prepared (also monitored by TLC and HPLC). The enzymatic addition of allopurinol (**4f**) and 6-benzylaminopurine (**4g**), two purine-type heterocycles, proceeded smoothly to give Markovnikov adducts at the N-9 position in moderately high yields (entry 6 and 7).

We also have examined the acylase-catalyzed reactions between N-heterocycles and vinyl ethers, but no products were observed. This strongly indicated that the carboxyl group in the vinyl ester plays a significant role in the enzymatic process. With all these results in hand, we propose a tentative mechanism for the enzyme-catalytic Markovnikov addition reaction (Scheme 2). The generally accepted acylase mechanism usually involves the polarization of a carbonyl group by the



Scheme 2. Proposed mechanism of D-aminoacylase-catalyzed Markovnikov addition.

Table 3. Markovnikov addition of other N-heterocycles with vinyl acetate catalyzed by D-aminoacylase.[a]

Entry	Substrate	Product	Solvent	Time [h]	Yield [%][a]	Alkylated position
1	**4a**	**5a**	*n*-hexane	96	64	N-1
2	**4b**	**5b**	*n*-hexane	96	76	N-1
3	**4c**	**5c**	*n*-hexane	84	89	N-1
4[b]	**4d**	**5d**	DMSO	192	5	N-1
5	**4e**	**5e**	DMSO	120	24	N-1
6	**4f**	**5f**	DMSO	96	71	N-9
7	**4g**	**5g**	DMSO	96	77	N-9

Conditions: substrate (0.6 mmol), vinyl acetate (8 equivs.), D-aminoacylase (100 mg), solvent (2 mL), 50 °C.
[a] Isolated yields.
[b] This was carried out on a 5-mL scale, with same concentration of reactants and enzyme.

zinc ion bound in the active sites of D-aminoacylase and acylase "Amano" (or by the oxyanion hole of PGA). A highly conserved Asp (or α-amino group of N-terminal Ser B1 of PGA) plays a key role in the proton transfer from the nucleophile water to the leaving group.[13] The proposed mechanism would start with the accommodation of an addition acceptor (vinyl ester) in the active site. For D-aminoacylase and acylase "Amano", the tightly bound zinc ion interacts with the carbonyl group of the vinyl ester and draws electron density away. Owing to the electron-withdrawing effect of the carboxyl group, the α-carbon of the vinyl group carries a partial positive charge. When the substrate enters the active site, the Asp functions as a general base and extracts the N-proton and the nucleophile simultaneously adds to the partially positive charged C-α position. The resultant negative charge at the C-β carbon could be stabilized by the zinc ion. Finally, the Asp, now functioning as a general acid, would deliver the proton to complete the reaction. For penicillin G acylase, the oxyanion hole might play a similar role as the zinc ion, and the α-amino group of N-terminal Ser B1 facilitates the proton transfer.

To our surprise, none or little optical activity was observed for any tested Markovnikov adducts. A similar result was also found in the exploration of the active-site of CAL-B for catalysis of Michael-type additions. No enantioselectivity could be achieved in that study.[7b] These findings reveal that the active sites might perform the promiscuous activity in some specific way. The reasons for this will be the subject of further investigations.

Conclusion

A facile biotransformation path to perform Markovnikov additions between N-heterocycles and vinyl esters has been developed by utilizing three promiscuous acylases as biocatalysts. The catalytic promiscuity of the acylases was demonstrated by the combination of different control experiments. These acylases could specify a broad range of N-heterocycles as addition substrates, including pentacyclic N-heterocycles, pyrimidines and purines. The influence of the structure of the N-heterocycles and vinyl ester to the enzymatic addition has been systematically examined. The ease and mildness of this method provides an attractive route to the synthesis of natural and unnatural N-heterocycle derivatives which may used as pharmacological alternatives. The complete study of the biological activity of these new derivatives will be reported in due course.

Experimental Section

Materials and General Methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE DMX-500 spectrometer at 500 MHz and 125 MHz in DMSO-d_6, respectively. Chemical shifts are reported in ppm (δ), relative to the internal standard of tetramethylsilane (TMS). HR-MS were obtained on a Bruker 7-tesla FT-ICR MS equipped with an electrospray source (Billelica, MA, USA). Melting points were determined using an XT-4 apparatus and were not corrected. All chemicals were obtained from commercial suppliers and used without further purification. For all reactions dry (molecular sieve), analytical grade solvents were used. Solvents for column chromatography were distilled before use. D-Aminoacylase from *Escherichia coli* (EC 3.5.1.81, lyophilized powder) and acylase "Amano" from *Aspergillus oryzae* (EC 3.5.1.14, lyophilized powder) were purchased from Amano Enzyme Inc. (Japan). Immobilized penicillin G acylase from *Escherichia coli* (EC 3.5.1.11, immobilized on acrylic beads) was purchased from Hunan Flag Biotech Co. (P. R. China).

General Procedure for the Enzymatic Markovnikov Addition of N-Heterocycles to Vinyl Esters

A suspension of **1a** (0.6 mmol) and 100 mg D-aminoacylase in 2 mL DMSO was incubated at 50 °C and 200 rpm. (orbitally shaken) for 5 minutes. Then, the corresponding equivalents of vinyl ester (**2a**–**i**) were added in order to initiate the reaction. After the indicated time (Table 1), the enzyme was filtered off to terminate the reaction and washed with MeOH (3–5 mL). Solvent was evaporated under vacuum to dryness. The crude residue was purified by flash chromatography on silica gel using petroleum/ethyl acetate mixtures. Product-containing fractions were combined, concentrated, and dried to give **3a**–**i**.

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