D-Aminoacylase-Catalyzed Markovnikov Addition of Azoles to Vinyl Esters in Organic Solvents

Wei-Bo Wu, Jian-Ming Xu, Qi Wu, De-Shui Lv, Xian-Fu Lin*

Department of Chemistry, Zhejiang University, Hangzhou 310027, P. R. of China
Fax +86(571)87952618; E-mail: llc123@css.zju.edu.cn

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Abstract: A novel strategy to effect Markovnikov addition between azoles and vinyl esters was developed, in which a 'promiscuous' enzyme, D-aminoacylase from *Escherichia coli*, was utilized as the catalyst. The enzymatic Markovnikov addition is dependent on the organic solvent chosen. By this strategy, a series of pharmaceutically active azole derivatives were synthesized in moderate to excellent yields.

Key words: enzymes, addition reactions, vinyl esters, D-aminoacylase, catalysis

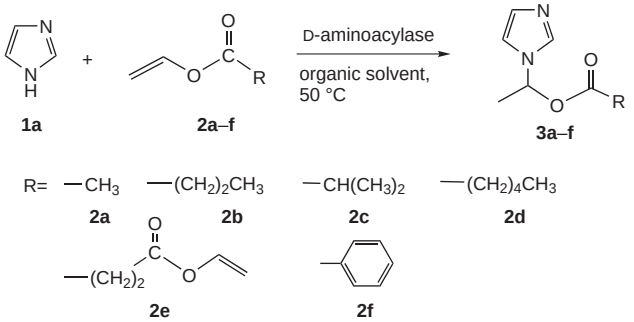
Enzyme catalysts are efficient tools for biotransformation in both organic and bioorganic synthesis. Many of them have displayed activity with unnatural substrates in organic solvents, which has been investigated in the past decade.[1] During the exploration of new synthetic applications of enzymes, a growing number of them have been found to be able to catalyze more than one chemical transformation in their active sites.[2] This 'catalytic promiscuity' contributes to the natural evolution of new enzymes[3] and also provides new catalysts for synthesis.[4]

The addition reaction is one of the most fundamental types of reactions in organic synthesis. However, relevant reports about enzymes that are able to catalyze general addition reactions are very scarce. Among them, Michael addition has been studied most extensively; various hydrolases, including lipases and proteases, have been found to catalyze this type of addition.[5] There are several elegantly designed combinations of experiments to investigate the possible catalytic mechanism of these 'promiscuous' enzymes.[5f,g] Besides Michael addition, aldol addition reactions were achieved by the catalysis of an engineered mutant of CAL B.[6] In order to enrich enzymatic addition reactions, the search for new enzymes with greater activity has become particularly fascinating and remains a great challenge.

Markovnikov-type addition is among the most useful carbon–carbon, oxygen–carbon or nitrogen–carbon bond-forming reactions. It is especially important to synthesize bioactive *N*-azole derivatives with a nitrogen–carbon linkage, which may be achieved by an addition reaction. Such aza-Markovnikov addition is traditionally performed in harsh chemical conditions in which bases, acids, or strong heating were usually used to promote the reaction.[7] 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 esters catalyzed by Penicillin G Acylase from *Escherichia coli* (PGA) and designed a group of experiments to demonstrate the 'catalytic promiscuity' of acylase.[8] Surprisingly, in the present work, we found that D-aminoacylase from *Escherichia coli*, a zinc binding metalloenzyme, which naturally catalyzes the hydrolysis of *N*-acetyl-D-amino acids, possesses even higher Markovnikov addition activity and was applicable to a broad spectrum of addition substrates. Herein, we would like to report a novel strategy to effect Markovnikov addition of azoles (imidazole, 1,2,4-tiazole, and pyrazole) to vinyl esters under 'promiscuous' D-aminoacylase catalysis. The *N*-azole derivatives obtained are usually pharmacologically active and may be applied as potential therapeutic alternatives.[9]

In view of the observation, we began our study with imidazole (**1a**). When the reaction was carried out with eight equivalents of vinyl acetate (**2a**) at 50 °C, a single product formed; the Markovnikov adduct **3a**, formed by addition to the N-1 position, was isolated after flash chromatography (Scheme 1). The structure of this compound was confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy, as well as ESI-MS. The progress of the reaction was monitored by TLC and HPLC; it is worth mentioning that the enzymatic Markovnikov addition reaction catalyzed by D-aminoacylase afforded no byproducts resulting from anti-Markovnikov addition, acylation, or other reactions.



Scheme 1 D-Aminoacylase-catalyzed Markovnikov addition of imidazole (**1a**) to vinyl esters **2a–f**

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In order to achieve high conversions, conditions for the enzymatic Markovnikov addition were optimized using the addition of imidazole (**1a**) and vinyl acetate (**2a**) as the model reaction. Since the reaction media has been recognized to be one of the most important factors influencing the enzymatic reaction, we conducted the reaction of **1a** and **2a** in various organic solvents, whose log P value ranged from –1.3 to 4.9, to find a favorable solvent. As shown in Figure 1, the solvent plays a crucial role on the enzymatic Markovnikov addition. With the exception of DMSO, very little Markovnikov adduct was formed in organic solvents with a strong polarity such as dioxane, acetone, and THF after 84 hours. As the log P value of the solvent increases, the yield of Markovnikov addition also increases. When the reaction was performed in non-polar solvents such as hexane or octane, Markovnikov addition occurred efficiently and provided the desired product **3a** in good isolated yield (85%, Table 1, entry 1). The dramatic rate acceleration in non-polar solvents may be attributed to the fact that these solvents could preserve the catalytic activity without disturbing the micro-aqueous layer of the enzyme. The discrepancy in the results obtained in the protein-dissolving solvent DMSO may be explained by its special property; it has been widely reported that DMSO might cause some conformational changes of enzymes and thus stimulate special catalytic activity or induce high enantioselectivity.[10] Besides the solvent effect, other influencing factors, such as temperature, concentration of catalyst and ratios of vinyl esters, have also been established.

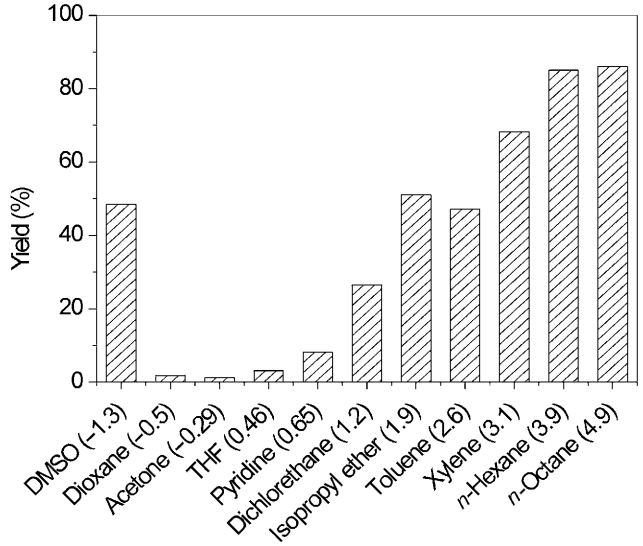

Figure 1 D-Aminoacylase-catalyzed Markovnikov addition of imidazole (**1a**) to vinyl acetate (**2a**) in different organic solvent (log P)

With optimal conditions in hand, we next examined the generality of these conditions to the reactions between imidazole and other vinyl esters, and the results are listed in Table 1. Generally, the Markovnikov addition of imidazole to a series of vinyl esters **2a**–**e** proceeded favorably, furnishing the corresponding products in moderate to high isolated yields. The addition reactivity decreases as the chain length of the vinyl ester increased (Figure 2, Table 1, entries 1–4), the more sterically hindered vinyl ester provided a lower yield (Table 1, entry 2 and 3). When vinyl esters with a comparable chain length were utilized, divinyl dicarboxylate reacted faster than monoacid vinyl ester (Table 1, entry 5). Markovnikov addition of imidazole to vinyl benzoate (**2f**) is rather slow and provided only 26% yield of the product after a prolonged reaction time of 192 hours (Table 1, entry 6). This may be due to the relatively weaker nucleophilicity of aromatic acid vinyl esters in comparison to fatty acid vinyl esters.

To confirm the versatility to this enzymatic reaction, other azoles were introduced. When similar processes were carried out with 1,2,4-triazole (**1b**) and vinyl acetate (**2a**), or divinyl succinate (**2e**) comparable behaviors were observed. D-Aminoacylase showed high Markovnikov activity toward this substrate, compounds **3g** and **3h** were isolated exclusively with excellent yields (Table 1, entries 7 and 8). The enzymatic addition of pyrazole (**1c**) and vinyl acetate (**2a**) also proceeded smoothly to give the corresponding Markovnikov adducts in moderately high yield (Table 1, entry 9). The addition reactivity of the three pentacyclic N-heterocycles examined in our research decreases in the order of 1,2,4-triazole, imidazole, and pyrazole. These results were in accordance with their nucleophilicity. No enantioselectivity could be achieved for any of the tested substrates with D-aminoacylase. The reason for this will be further investigated.

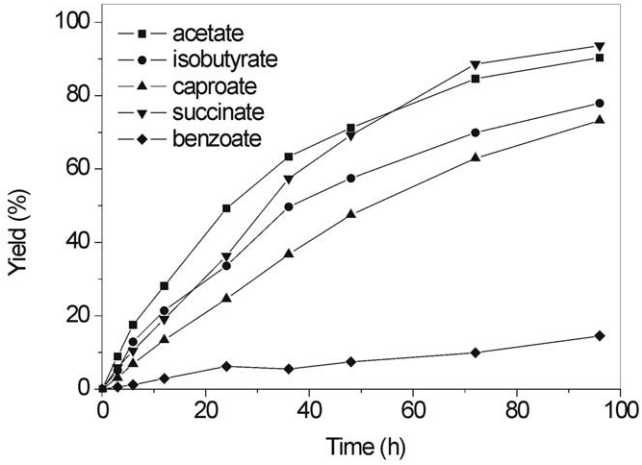

Figure 2 Progress curve of enzymatic Markovnikov addition of imidazole (**1a**) to different vinyl esters

Some control experiments were performed to demonstrate the catalytic effect of D-aminoacylase. The reaction of 1,2,4-triazole (**1b**) with vinyl acetate (**2a**) in the absence of enzyme led to the Markovnikov adduct in very low yield (< 0.3%) even after five days. The bovine serum albumin or denatured D-aminoacylase-catalyzed reactions were almost equal to the background reaction, ruling out the possibility that amino acid distribution on the protein surface has promoted the process. When control reactions

Table 1 D-Aminoacylase-Catalyzed Markovnikov Addition of Azoles to Vinyl Esters[a]

1a

1b

1c

Entry	Azole	Vinyl Ester	Product	Time (h)	Yield[b] (%)
1	**1a**	**2a**	**3a**	84	85
2	**1a**	**2b**	**3b**	96	82
3	**1a**	**2c**	**3c**	96	75
4	**1a**	**2d**	**3d**	96	72
5	**1a**	**2e**	**3e**	96	91
6	**1a**	**2f**	**3f**	192	26
7	**1b**	**2a**	**3g**	84	93
8	**1b**	**2e**	**3h**	96	90
9	**1c**	**2a**	**3i**	94	76

[a] Substrate (0.6 mmol), vinyl ester (8 equiv), D-aminoacylase (100 mg), hexane (2 mL), 50 °C.
[b] Isolated yields.

were run with covalently inhibited D-aminoacylase by adding 50 mM of the noncompetitive inhibitor $ZnCl_2$,[11] the inhibited 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 that of the non-enzymatic reaction. These results suggest that the tertiary structure and the specific active site of D-aminoacylase are responsible for the Markovnikov addition reaction.

The generally accepted acylase mechanism of D-aminoacylase usually involves the polarization of a carbonyl group by the binding of a zinc ion followed by proton transfer from water to a leaving group mediated by Asp.[12] Herein, we propose a tentative mechanism for the enzymatic Markovnikov addition. The tightly bound zinc ion first interacts with the carbonyl group of the vinyl ester and draws electron density away from the carbon; 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, removing the *N*-proton, while the nucleophile simultaneously adds to the C-β position. The resulting negative charge at C-β could be stabilized by the zinc ion. Finally, the Asp, now functioning as a general acid, would deliver the proton to C-β to complete the reaction. Preliminary experiments confirmed that D-aminoacylase was not able to catalyze any reaction between azoles and vinyl ethers. This indicated that the carboxyl group of vinyl ester played an extremely significant role. Further study of the catalytic mechanism is in progress in our laboratory.

In conclusion, we have developed a facile biotransformation path to perform Markovnikov additions between azoles and vinyl esters by D-aminoacylase catalysis. Solvents play an essential role in the enzymatic addition reaction. The result of addition was affected by the structure of substrate and vinyl ester. By this novel strategy, a number of pharmacologically active azole derivatives were successfully synthesized in moderate to excellent yields.

D-Aminoacylase-Catalyzed Markovnikov Addition of Azoles to Vinyl Esters; General Procedure

A suspension of substrate (0.6 mmol) and D-aminoacylase (100 mg) from *Escherichia coli* (EC 3.5.1.81, purchased from Amano Enzyme Inc.) in hexane (2 mL) was incubated at 50 °C at 200 r.p.m. (orbitally shaken) for 5 min. Then, vinyl ester (8 equiv) was added in order to initiate the reaction. After the time indicated (Table 1), the reaction was terminated by filtering off the enzyme. The crude product was purified by chromatography on silica gel (petroleum ether–EtOAc).

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