Penicillin G acylase catalyzed Markovnikov addition of allopurinol to vinyl ester†

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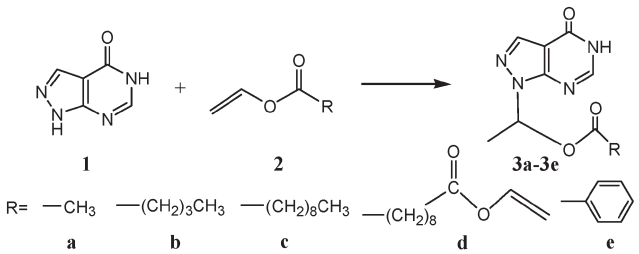
A new enzymatic process is reported, in which penicillin G acylase from *Escherichia coli* displays a promiscuous activity in catalyzing the Markovnikov addition of allopurinol to vinyl ester.

Enzymes are efficient catalysts in organic and bioorganic chemistry and many of them have displayed activity with unnatural substrates in organic media, thus they have been widely used to carry out organic synthesis.[1] During the exploration of new synthetic applications of enzymes, it was found that some of them have catalytic promiscuity, which indicates they have one or more secondary activities in addition to their primary, physiological activity.[2] For example, some lipases can be used as carboxylic acid esterases, thioesterases, peptidases, dehalogenases, epoxide hydrolases and halo peroxidases, *etc*.[3] Since the catalytic promiscuity may lead to the evolution of new biocatalysts and expand the application of biotransformation, this has become an area attracting much attention.[4]

Addition reaction is one of the most basic types of reactions in organic synthesis, however, relevant reports about enzymes which are able to catalyze general addition reactions are very scarce. Recently, an engineered mutant of CAL B was developed to catalyze aldol addition reactions.[5] T. Kitazume and coworkers used hydrolytic enzymes to catalyze the Michael addition of fluorine-containing compounds such as 2-(trifluoromethyl)-propenoate with nucleophiles to synthesize asymmetric fluorinated molecules.[6] Our group reported that alkaline protease from *Bacillus subtilis* showed a remarkable activity in catalyzing Michael addition of *N*-nucleophiles to acrylates.[7] A more recent work by V. Gotor and coworkers demonstrated that lipase was also capable of catalyzing Michael type addition.[8] Considering these elegant discoveries of addition activity of common enzymes, we prefigured that other types of addition could also be catalyzed through an enzymatic process. Here we report the unprecedented observation of the novel Markovnikov addition activity of penicillin G acylase from *Escherichia coli* (PGA), a commercially available and widely used biocatalyst in the enzymatic synthesis of β-lactam antibiotics. In this preliminary communication, addition of allopurinol to vinyl ester is reported and a mechanism is tentatively proposed.

In view of the observation, we examined the addition activity of PGA. After incubation of 0.37 M allopurinol and 0.74 M vinyl acetate with immobilized PGA (50 mg/mL) in DMSO for 144 h, Markovnikov adduct at the N-9 position was prepared in ~ 60%



Scheme 1

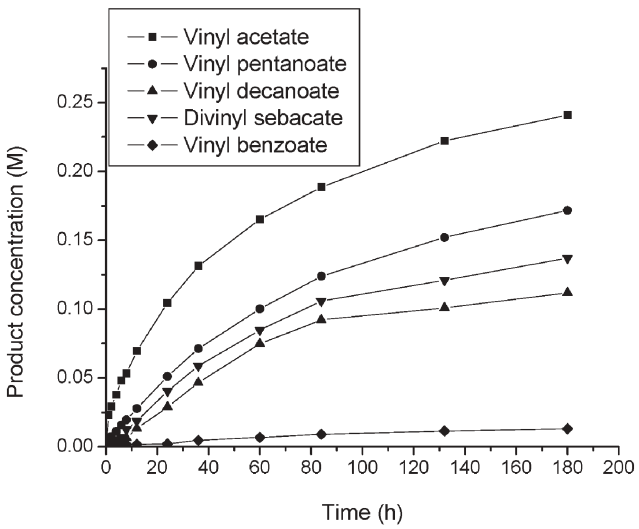


Fig. 1 Progress curve of Markovnikov addition of allopurinol to different vinyl esters catalyzed by immobilized PGA in DMSO.

Table 1 Initial rates of Markovnikov addition between allopurinol (0.37 M) and vinyl acetate (0.74 M) in the presence of different catalysts

Entry	Catalyst	Concentration (mg/mL)	V_0 (mM·h^{-1})	V_R[a]
1	No enzyme	—	0.036	1.0
2	Immobilized PGA[b]	50	4.273	118.0
3	Immobilized PGA	25	2.006	55.4
4	Free PGA[c]	50	1.264	35.1
5	Denatured	50	0.047	1.3
6	BSA	50	0.042	1.1
7	PPL	50	0.085	2.4
8	Lipozyme	50	0.063	1.8

[a] Relative initial rate to the reaction in absence of enzyme. [b] PGA immobilized on acrylic beads. [c] PGA aqueous solution.

† Electronic supplementary information (ESI) available: experimental section. See http://www.rsc.org/suppdata/cc/b5/b501338k/

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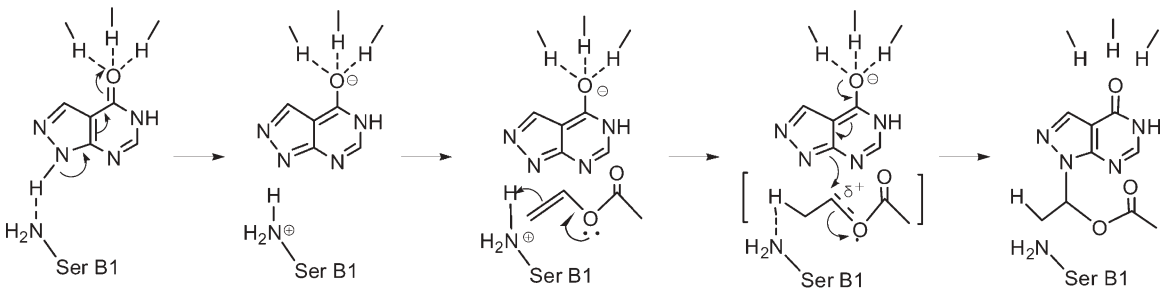

Scheme 2 Proposed mechanism of PGA catalyzed Markovnikov addition.

yield (Scheme 1) and found to have optical activity.‡ The nonenzymatic reaction of this type of addition requires much harsher alkaline conditions.[9] When reaction was catalyzed by NaOH (20 mg/mL) at 50 °C for 24 h, 90% allopurinol converted to more than 5 products, only 25% of which was the same adduct as the enzymatic process.

In addition to vinyl acetate, the reaction also takes place with other fatty acid vinyl esters and aromatic acid vinyl esters (Fig. 1); the reaction rates depend on the structure. We have followed the formation of the products by HPLC in order to quantify their concentration and compare reaction rates. As the chain of the fatty acid vinyl ester increases, the enzymatic Markovnikov addition activity decreases (vinyl acetate **1** > vinyl pentanoate **2** > vinyl decanoate **3**). When the chain length is comparable, divinyl dicarboxylate reacts faster than mono-acid vinyl ester (divinyl sebacate **4** > vinyl decanoate **3**). The reactivity of vinyl benzoate **5** is rather low in comparison to the fatty acid vinyl esters.

A control experiment was designed to demonstrate that the Markovnikov addition of allopurinol to vinyl esters is an enzyme-catalyzed process. The reaction of allopurinol with vinyl acetate in the absence of enzyme led to very low yield adduct (< 0.3%) in 3 days. In contrast, the reaction in the presence of immobilized PGA is up to 120-fold faster (entry 2 in Table 1). Although the free PGA catalyzed process is less efficient, the enzyme itself can increase 35-fold the initial reaction rate. Besides, the rate is practically proportional to the enzyme amount (entries 2 and 3), suggesting the catalytic effect of the enzyme.

Some further experiments were performed to focus on the catalytic specificity of PGA. When the reactants are incubated with denatured immobilized PGA (pre-treated with urea at 100 °C for 6 hours) or bovine serum albumin (BSA), both the initial rates are practically equal to the background reaction (entries 5 and 6), ruling out the possibility that the polymeric support or the similar amino acid distribution on the protein surface has promoted the process. The reactions catalyzed by two widely used hydrolases (Lipase from *porcine pancreas* and Lipozyme) can only accelerate the process by 2.4 and 1.8 fold respectively (entries 7 and 8). All these results suggest that the tertiary structure and the specific catalytic site of PGA are necessary to promote the Markovnikov addition.

A mechanism might explain the observed new addition activity (Scheme 2). The generally accepted acylase mechanism of PGA involves the stabilization of a carbonyl group by main-chain amides of B23 and B69 and the Nδ of Asn B241 (namely the oxyanion hole). The α-amino group of the N-terminal serine B1 residue (Ser B1) plays a key role in the proton transference from the nucleophile to the leaving group.[10] The proposed mechanism would start with the accommodation of the substrate (allopurinol) in the active site. The oxyanion hole interacts with the carbonyl group of allopurinol and draws electron density away from the conjugated heterocycle, meanwhile, the α-amino group of Ser B1 functions as a general base and deprives the proton of the N-9 position. Then, the α-amino group of Ser B1, now functioning as a general acid, would deliver the proton to the unsaturated C-β position of the vinyl ester to form a transition state; the resulting positive charge at the C-α position could be stabilized by the lone-pair electrons of the adjacent oxygen. Subsequently, allopurinol is released from the oxyanion hole and added to the transition state to complete the reaction.

The proposed binding of allopurinol to the active site is based on experimental facts. We confirmed that PGA could not catalyze the addition reaction of common heterocyclic compounds (*e.g.* imidazole) with vinyl ester. However, conjugated heterocyclic compounds with a carbonyl group similar to allopurinol (*e.g.* uracil) could be specified as addition substrates, indicating the catalysis may be initiated from the interaction of these specific substrates with enzyme. Study toward this direction is in progress in our laboratory.

In conclusion, we herein report an unprecedented penicillin G acylase catalyzed Markovnikov addition reaction. The effect of the vinyl ester structure is preliminarily measured. The catalytic effect of the enzyme is demonstrated by the combination of different experiments. This new addition activity of penicillin G acylase provides a clear example of catalytic promiscuity. Moreover, it provides a potential synthetic route for bioactive *N*-derivatives of heterocyclic compounds.

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Notes and references

‡ Spectral data for **3a**: $[\alpha]_D^{20}$/MeOH: −24.43°. ESI-MS: *m*/*z* = 222.7 (M + 1).^{1}H NMR (DMSO-d_6, 500 MHz, δ, ppm): 11.76 (s, 1H, O=C–NH), 8.70 (s, 1H, N=CH–N), 7.97 (s, 1H, N=CH–C), 6.91 (q, 1H, *J* = 6.00 Hz, N–CH–O–C=O), 2.05 (s, 3H, O=C–CH_3), 1.79 (d, 3H, *J* = 6.05 Hz , –CH–CH_3). ^{13}C NMR (DMSO-d_6, 125 MHz, δ, ppm): 169.71 (–O–C=O), 159.30 (–NH–C=O), 159.14 (C7), 148.13 (C9), 129.88 (C4), 107.62 (C3), 80.81 (–N–CH–O–), 21.14 (O=C–CH_3), 19.83 (CH_3 of –CH–CH_3). IR (cm^{-1}): 3435 (ν_{N-H}), 3179, 3068 ($\nu_{N=C-H}$), 1746 ($\nu_{-O-C=O}$), 1677 ($\nu_{-N-C=O}$), 1277, 1231, 1181,1140 (ν_{C-O} and ν_{C-N}).

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