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Highly efficient and large-scalable glucoamylase-catalyzed Henry reactions†

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Eco-friendly, highly efficient and large-scalable Henry reactions of aromatic aldehydes and nitroalkanes catalyzed by glucoamylase from *Aspergillus niger* (*An*GA) are described. The reactions were carried out at 30 °C in the mixed solvents of ethanol and water, and the corresponding β-nitro alcohols were obtained in yields of up to 99%. Only 3 mg of enzyme was sufficient to catalyze the reaction of 1 mmol aldehydes. The natural activity and promiscuous activity of *An*GA were compared under different conditions. Experiments demonstrated that the product of the Henry reaction could inhibit *An*GA at 80 °C. This enzymatic Henry reaction showed a broad substrate scope, and could be facilely enlarged to gram scale. The possible mechanism was also discussed.

1. Introduction

The Henry or nitroaldol reaction is a powerful reaction and an important atom-economical methodology to furnish a β-nitro alcohol by a carbonyl compound and an alkyl nitro compound in organic synthesis.[1–4] The resulting β-nitro alcohols, very interesting functional compounds, have been used in various beneficial organic transformations.[5–8] Therefore, many catalytic Henry reactions have been reported including metal[9–11] and small-molecule catalysis.[12] However, high-efficiency and eco-friendly catalytic synthetic methods that satisfy increasingly stringent environmental constraints are still in great demand by the pharmaceutical and chemical industries. As a kind of important green catalyst, enzymes have shown immense advantages such as mild reaction conditions, high yields, and good selectivities. In recent years, enzymatic promiscuity has been paid much attention by chemists and biochemists.[13,14] Many enzyme promiscuous activities have been revealed, such as aldol reactions,[15–17] Michael additions,[18,19] Mannich reactions,[20,21] Baeyer–Villiger oxidations,[22,23] and so on. However, to date, only a few enzymatic Henry reactions have been reported. Griengl and co-workers reported the asymmetric enzymatic Henry reactions using hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) (EC 4.1.2.39).[24,25] Lin and co-workers reported the enzymatic Henry reactions to form β-nitro alcohols followed by enzymatic kinetic resolution of the stereoisomers,[26] and D-aminoacylase-catalyzed Henry reactions in DMSO.[27] Gotor and co-workers reported that protease from *Bacillus licheniformis* immobilized as cross-linked enzyme aggregates (Alcalase-CLEA) catalyzed the Henry reaction between 4-nitrobenzaldehyde and nitromethane.[28] Our group reported transglutaminase-catalyzed Henry reactions in a biphasic system composed of water and CH_2Cl_2.[29]

Glucoamylase, also known as amyloglucosidase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3), is an inverting *exo*-acting hydrolase that catalyzes the hydrolysis of α-1,4 and α-1,6 glucosidic linkages to release β-D-glucose from the non-reducing ends of starch and related saccharides.[30,31] Glucoamylase is widely used in the food industry to produce glucose syrup, high-fructose corn syrup and so on.[32] Herein we wish to report that glucoamylase from *Aspergillus niger* (*An*GA) is an efficient catalyst for Henry reactions of aromatic aldehydes and nitroalkanes.

2. Results and discussion

Initially, the reaction of 4-cyanobenzaldehyde and nitromethane was used as a model reaction. In view of the fact that the reaction medium is one of the most important factors influencing the enzymatic reactions,[33] different kinds of solvents were screened (Table 1). Generally, the *An*GA-catalyzed model Henry reaction in polar solvents (such as ethanol, acetonitrile, DMSO, water and polyethylene glycol) gave products in higher yields (85–99%) in relatively shorter reaction times (5–22 h) (Table 1, entries 1–3, 6 and 7) than in the non- or low-polar solvents (such as butyl acetate, toluene, cyclohexane and chloroform) (Table 1, entries 8, 9, 12 and 14). The reaction in DMSO achieved an excellent yield of 94% within only 5 h (Table 1, entry 3), while the reaction in water gave a good yield of 89% after 22 h (Table 1, entry 6). Moreover, under solvent-free conditions a good yield of 92% was

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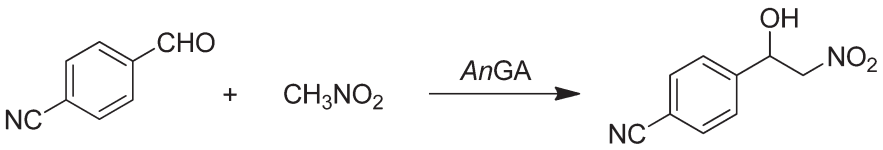
Table 1 Solvent screening and control experiments[a]



Entry	Solvent	Time [h]	Yield [%][b]
1	Ethanol	19	99
2	Acetonitrile	22	97
3	DMSO	5	94
4	Solvent-free	95	92
5	Isopropyl ether	95	89
6	Water	22	89
7	Polyethylene glycol	9	85
8	Butyl acetate	95	79
9	Toluene	95	76
10	2-Propanol	6.5	75
11	THF	22	73
12	Cyclohexane	105	58
13	1,4-Dioxane	25	50
14	Chloroform	105	39
15	Ethanol (no enzyme)	20	8
16	Ethanol (bovine serum albumin)	20	23
17	Ethanol (egg white albumin)	20	5
18	Ethanol (purified *An*GA)[c]	20	52

[a] Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (10 mmol), *An*GA (20 mg), solvent (0.90 mL), and deionized water (0.10 mL) at 30 °C. [b] Yield of the isolated product after silica gel chromatography. [c] Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (5 mmol), purified *An*GA (1 mg), ethanol (0.85 mL), and deionized water (0.15 mL) at 30 °C.

obtained after a long reaction time of 95 h (Table 1, entry 4). Based on the results of solvent screening, in view of both environmental protection and a good ability to dissolve a wide scope of substrates, we chose ethanol as the optimum solvent for further investigation.

Several control experiments were designed to verify the specific catalytic effect of *An*GA on the model Henry reaction in ethanol. In the absence of *An*GA, the reaction only gave the product in a yield of 8% after 20 h (Table 1, entry 15). As a comparison, some albumins instead of *An*GA were used to catalyze the reaction. The reaction with egg white albumin only gave the product in a yield of 5%, similar to the blank (Table 1, entry 17). However, the reaction with bovine serum albumin gave the product in a yield of 23% (Table 1, entry 16), suggesting that a protein without enzyme function was able to catalyze the Henry reaction in a very unspecific way. On the other hand, *An*GA showed much higher activity than the albumins indicating that some special group/s is/are responsible for this high yield observed.

To rule out the possibility that another protein or inorganic impurities catalyzed the reaction, the commercial preparation of *An*GA was checked with SDS-PAGE, and purified by centrifugal ultrafiltration. (For the procedures of enzyme purification and the SDS-PAGE of the commercial preparation, please see the ESI†.) The purified protein showed a glucoamylase activity of 27 U mg^{-1} (the glucoamylase activity of the commercial preparation was 79 U mg^{-1}). The decrease of activity may be due to the process of purification (in order to rule out that buffer catalyzed the reaction, we did not use any buffer during the whole process). The purified protein was then used to catalyze the model Henry reaction, and 1 mg of this protein as a catalyst gave the product in a yield of 52% after 20 h (Table 1, entry 18). These experiments confirmed that *An*GA indeed catalyzed the Henry reaction.

Moreover, in order to understand the relation between natural and promiscuous activities of *An*GA, we investigated the catalytic activity of *An*GA on the Henry reaction and natural substrate (starch) under different conditions (Table 2). The natural activity of the enzyme under normal conditions was 79 U mg^{-1}, but it was only 17 U mg^{-1} when tested in mixed solvents (water/(water + ethanol) = 0.15, v/v) (Table 2, entry 1). Since metal ions can inactivate the enzyme by reacting with some groups *e.g.* sulfhydryl groups and/or by disrupting the bonds that hold the enzyme together to cause the enzyme to undergo a conformational change, *An*GA was pretreated with Ag^+ and Cu^{2+}, respectively. It can be seen that the metal-pretreated *An*GA almost competely lost its catalytic activity on the model Henry reaction (Table 2, entries 5 and 6). The blank reactions were performed using the same amounts of Ag^+ and Cu^{2+} to catalyze the reaction, which only gave trace amounts of the product proving that Ag^+ or Cu^{2+} alone did not catalyze this transformation (Table 2, entries 3 and 4). Another blank reaction in which *An*GA was pretreated in water at 30 °C (Table 2, entry 2) showed that Ag^+ and Cu^{2+} indeed inactivated

Table 2 Catalytic activities of *An*GA on the Henry reaction[a] and natural substrate[b]

Entry	Catalyst	Yield [%][c]	Natural activity [U mg^{-1}][d]
1	*An*GA	99	79 (17, in mixed solvents)[e]
2	*An*GA (pretreated at 30 °C)[f]	95	—
3	$AgNO_3$ (42 mg)	Trace	—
4	$CuSO_4$ (39.9 mg)	Trace	—
5	*An*GA (pretreated with 250 mM Ag^+)[g]	3	6
6	*An*GA (pretreated with 250 mM Cu^{2+})[h]	8	28
7	*An*GA (pretreated at 100 °C)[i]	95	—
8	urea (100 mg)	5	—
9	guanidine hydrochloride (100 mg)	6	—
10	*An*GA (pretreated with urea)[j]	63	0
11	*An*GA (pretreated with guanidine hydrochloride)[k]	70	0
12	miglitol (100 mg)	51	—
13	*An*GA (pretreated with miglitol)[l]	70	30

[a] Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (10 mmol), *An*GA or pretreated-*An*GA (20 mg), ethanol (0.90 mL), and deionized water (0.10 mL) at 30 °C for 20 h. [b] Reaction conditions: *An*GA or pretreated-*An*GA dissolved in deionized water (0.01 mg mL^{-1}), starch (1% W V^{-1}), and NaAc–HCl (50 mM, pH = 4.5), at 60 °C. [c] Yield of the isolated product after silica gel chromatography. [d] Unit definition (U mg^{-1}): one unit corresponds to the amount of enzyme that liberates 1 μmol of glucose per minute at pH 4.5 and 60 °C. [e] *An*GA dissolved in mixed solvents (water/(water + ethanol) = 0.15, v/v) to get a solution (0.01 mg mL^{-1}) used for the enzyme assay. [f] *An*GA (20 mg) in deionized water (1 mL) was stirred at 30 °C for 24 h, and then water was removed under reduced pressure before use. [g] *An*GA (20 mg) in Ag^+ solution (250 mM) [$AgNO_3$ (42 mg) in deionized water (1 mL)] was stirred at 30 °C for 24 h, and then water was removed under reduced pressure before use. [h] *An*GA (20 mg) in Cu^{2+} solution (250 mM) [$CuSO_4$ (39.9 mg) in deionized water (1 mL)] was stirred at 30 °C for 24 h, and then water was removed under reduced pressure before use. [i] *An*GA (20 mg) in deionized water (1 mL) was stirred at 100 °C for 24 h, and then water was removed under reduced pressure before use. [j] *An*GA (20 mg) in urea solution (1.67 M) [urea (100 mg) in deionized water (1 mL)] was stirred at 100 °C for 24 h, and then water was removed under reduced pressure before use. [k] *An*GA (20 mg) in guanidine hydrochloride solution (1.04 M) [guanidine hydrochloride (100 mg) in deionized water (1 mL)] was stirred at 100 °C for 24 h, and then water was removed under reduced pressure before use. [l] *An*GA (20 mg) in miglitol (0.97 M) [miglitol (100 mg) in deionized water (0.5 mL)] was stirred at 30 °C for 24 h, and then water was removed under reduced pressure before use.

the enzyme. At the same time, the metal-pretreated *An*GA displayed a much lower activty towards natural function (Table 2, entries 5 and 6). Next, urea and guanidine hydrochloride were used to denature *An*GA, which made the enzyme lose its natural activity completely (Table 2, entries 10 and 11). In the meantime, urea and guanidine hydrochloride decreased the enzyme activity towards the model Henry reaction from 99% to 63% and 70%, respectively (Table 2, entries 10 and 11). Finally, miglitol, as a competitive inhibitor of amyloglucosidase, was used to pretreat the enzyme. The blank reaction with miglitol gave the Henry product in a yield of 51% (Table 2, entry 12), while the Henry reaction with miglitol-pretreated *An*GA only gave the product in a yield of 70% (Table 2, entry 13), indicating that miglitol strongly inhibited the enzyme activity in the Henry reaction. Miglitol-pretreated *An*GA also showed a lower natural activity (Table 2, entry 13). These data demonstrated that both the natural and promiscuous activities of the enzyme were inhibited by miglitol. Based on the above experiments, it can be inferred that the natural active center of *An*GA was responsible for its activity in the Henry reaction.

Since a specific amount of water is essential to the enzymes to enhance activity in non-aqueous solvents,[21,34] the water content [$V_{water}/V_{(water + ethanol)}$] from 0 to 0.5 was screened (Fig. 1). It can be seen that the yield was raised from 87% to 99% as the water content increased from 0 to 0.15. Continuing to increase the water content to 0.40 did not cause any obvious effects on the yield. Once the water content passed 0.40, the yield of the Henry product began to decrease. Thus, the water content of 0.15 was chosen as the optimal water content for the reaction.

Then, the effect of the molar ratio of substrates on the *An*GA-catalyzed Henry reaction was investigated (Fig. 2). It can be seen that an excellent yield of 99% was obtained when the molar ratio of 4-cyanobenzaldehyde to nitromethane was 1 : 5.

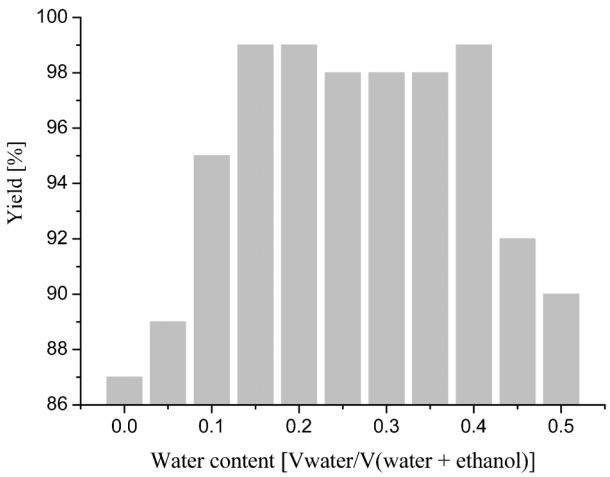


Fig. 1 Influence of water content on the *An*GA-catalyzed Henry Reaction. Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (10 mmol), *An*GA (20 mg), water content [0–0.5, water/(water + ethanol), v/v], water + ethanol = 1 mL, at 30 °C for 20 h. Yield of the isolated product after silica gel chromatography.

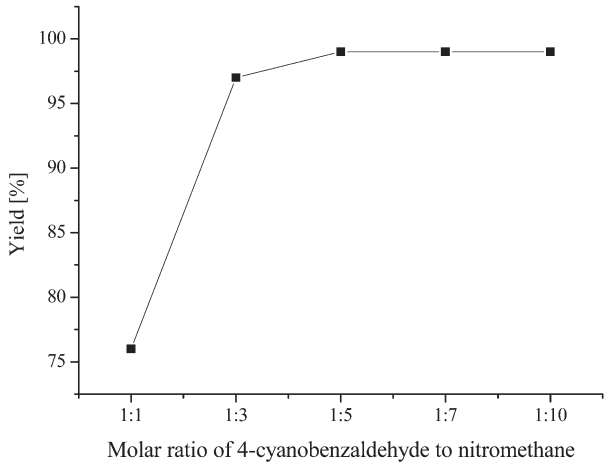


Fig. 2 Influence of molar ratio of substrates on the *An*GA-catalyzed Henry reaction. Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (1–10 mmol), *An*GA (20 mg), ethanol (0.85 mL), and deionized water (0.15 mL) at 30 °C for 19 h. Yield of the isolated product after silica gel chromatography.

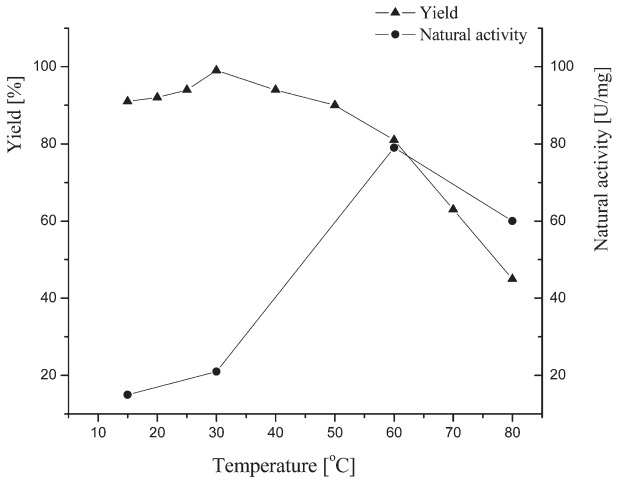


Fig. 3 Influence of temperature on the *An*GA-catalyzed Henry reaction[a] and natural activity[b]. [a] Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (5 mmol), *An*GA (3 mg), ethanol (0.85 mL), and deionized water (0.15 mL) at 15–80 °C for 19 h. Yield of the isolated product after silica gel chromatography. [b] Reaction conditions: *An*GA (0.01 mg mL^{-1}) dissolved with deionized water, starch (1% W V^{-1}), and NaAc–HCl (50 mM, pH = 4.5), at 15–60 °C.

Thus, the molar ratio of aromatic aldehyde to nitroalkane 1 : 5 was selected as the optimal ratio for further studies.

The effect of enzyme concentration on the model Henry Reaction of 4-cyanobenzaldehyde (1 mmol) and nitromethane (5 mmol) was examined (Table 3). *An*GA displayed a prominent catalytic activity on the model Henry reaction. The Henry product could be furnished in a high yield of 95% with the enzyme concentration of 1 mg mL^{-1} (Table 3, entry 1). The best yield of 99% was obtained with the enzyme concentration of 3 mg mL^{-1} (Table 3, entry 2). Thus, we chose 3 mg mL^{-1} as the optimal enzyme concentration for the *An*GA-catalyzed Henry reaction.

Temperature is another important factor for enzyme-catalyzed reactions, due to its effect on the rate of the reaction and the stability of the enzyme. Thus, a temperature screening was performed (Fig. 3). Yields of $\geqslant$ 90% were obtained at a temperature range from 15 to 50 °C. However, once the temperature surpassed 50 °C, the yield decreased evidently, and only 43% yield was gained at 80 °C. The best yield of 99% was obtained at 30 °C. Thus, we chose 30 °C as the optimal temperature for the *An*GA-catalyzed Henry reaction.

Table 3 Effect of enzyme concentration on the *An*GA-catalyzed Henry Reaction[a]

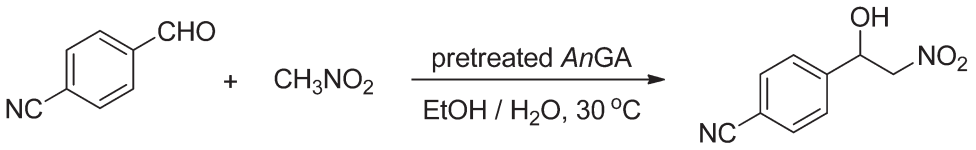
Entry	Enzyme concentration [mg mL^{-1}]	Yield [%][b]
1	1	95
2	3	99
3	5	97
4	10	96
5	20	99

[a] Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (5 mmol), *An*GA (1–20 mg), ethanol (0.85 mL), and deionized water (0.15 mL) at 30 °C for 19 h. [b] Yield of the isolated product after silica gel chromatography.

The influence of temperature on the natural activity of *An*GA was also investigated (Fig. 3). The optimum temperature for the promiscuous activity was 30 °C, while the optimum temperature for the natural activity was 60 °C. However, both promiscuous and natural activities decreased at 80 °C.

To explore the reasons for a low yield at a temperature of 80 °C, we designed and performed several experiments (Table 4). Firstly, to verify whether *An*GA could be inactivated in the mixed solvents at 80 °C, *An*GA was pretreated in the mixed solvents of ethanol and deionized water at 80 °C for 19 h, and then used to catalyze the model reaction, which gave the product in a yield of 96%, demonstrating that the mixed solvents at 80 °C caused no loss of the *An*GA's catalytic activity (Table 4, entry 1). Secondly, we examined whether the substrates could inactivate *An*GA at 80 °C. *An*GA, pretreated with the substrates 4-cyanobenzaldehyde and nitromethane at 80 °C, respectively, was used to catalyze the model reaction, and good yields of 99% and 92% were obtained in turn (Table 4, entries 2 and 3). The results indicated that the substrates did not cause the loss of *An*GA's catalytic activity. The experiment (Table 4, entry 3) also demonstrated that the possible volatilization of nitromethane was not the reason for low yield at 80 °C. Thirdly, we determined whether the Henry product 4-(1-hydroxy-2-nitroethyl)benzonitrile could inactivate *An*GA. *An*GA, pretreated with Henry product at 30 °C and 80 °C separately, was used to catalyze the model reaction, and the yields of 90% and 27% were obtained, respectively (Table 4, entries 4 and 5). The results clearly indicated that the Henry product inactivated *An*GA at a high temperature (80 °C); however, it almost did not affect the activity of *An*GA at low temperature (30 °C). Therefore, we could conclude that the low yield at a temperature of 80 °C is mainly due to the inactivation

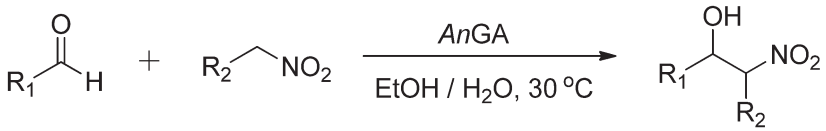
Table 4 Exploring the reasons for low yield at 80 °C[a]



Entry	Pretreatment of *An*GA	Yield [%][b]
1	*An*GA pretreated in the mixed solvents of ethanol and deionized water at 80 °C[c]	96
2	*An*GA pretreated with substrate 4-cyanobenzaldehyde at 80 °C[d]	99
3	*An*GA pretreated with substrate nitromethane at 80 °C[e]	92
4	*An*GA pretreated with Henry product 4-(1-hydroxy-2-nitroethyl)benzonitrile at 30 °C[f]	90
5	*An*GA pretreated with Henry product 4-(1-hydroxy-2-nitroethyl)benzonitrile at 80 °C[f]	27

[a] Reaction conditions: 4-cyanobenzaldehyde (1 mmol), nitromethane (5 mmol), *An*GA (3 mg), ethanol (0.85 mL), and deionized water (0.15 mL) at 30 °C for 19 h. [b] Yield of the isolated product after silica gel chromatography. [c] *An*GA (3 mg) was pretreated in the mixed solvents of ethanol (0.85 mL) and deionized water (0.15 mL) at 80 °C for 19 h. [d] *An*GA (3 mg) was pretreated with 4-cyanobenzaldehyde (1 mmol) in mixed solvents of ethanol (0.85 mL) and deionized water (0.15 mL) at 80 °C for 19 h, and then nitromethane (5 mmol) was added and the mixture was stirred at 30 °C for another 19 h. [e] *An*GA (3 mg) was pretreated with nitromethane (5 mmol) in mixed solvents of ethanol (0.85 mL) and deionized water (0.15 mL) at 80 °C for 19 h, and then 4-cyanobenzaldehyde (1 mmol) was added and the mixture was stirred at 30 °C for another 19 h. [f] *An*GA (3 mg) was pretreated with Henry product 4-(1-hydroxy-2-nitroethyl)benzonitrile (85 mg) in mixed solvents of ethanol (0.85 mL) and deionized water (0.15 mL) at 30 °C (entry 4) or 80 °C (entry 5) for 19 h, and then 4-cyanobenzaldehyde (1 mmol) and nitromethane (5 mmol) were added and the mixture was stirred at 30 °C for another 19 h.

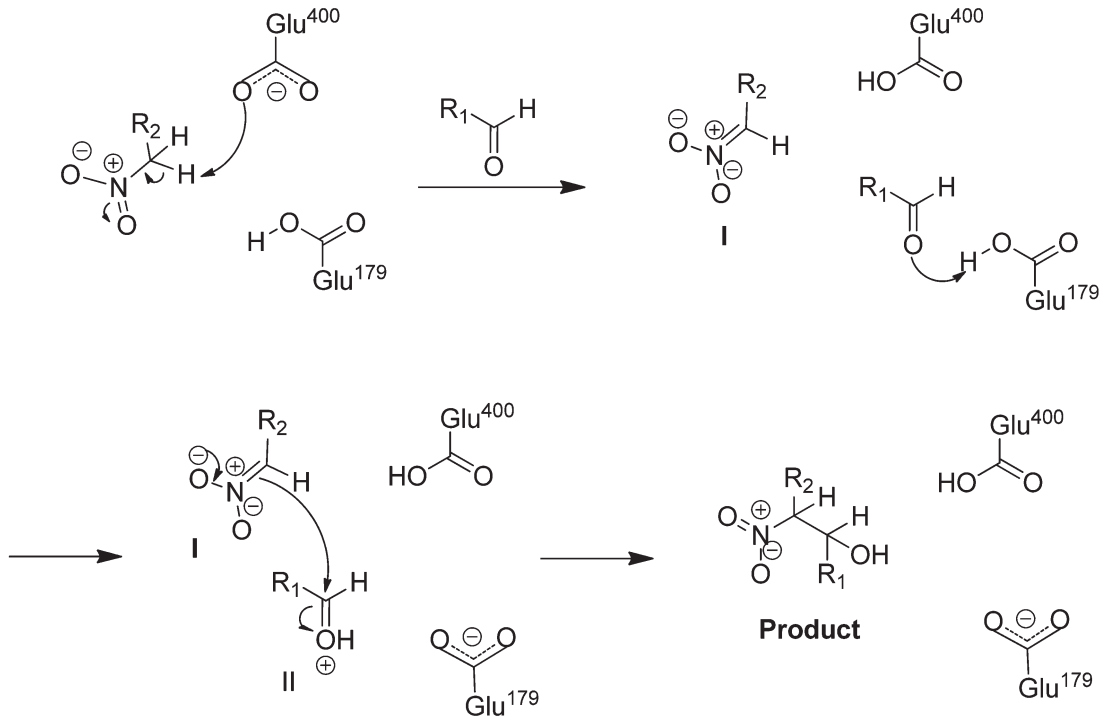
Table 5 Substrate scope of the *An*GA-catalyzed Henry reactions[a]



1 **2** **3a-v**

Entry	R_1	R_2	Product	Time [h]	Yield [%][b]	dr [*syn*/*anti*][c]
1	2-$MeOC_6H_4$	H	**3a**	72	86	—
2	3-$MeOC_6H_4$	H	**3b**	72	77	—
3	2-$NO_2C_6H_4$	H	**3c**	10	95	—
4	3-$NO_2C_6H_4$	H	**3d**	21	96	—
5	4-$NO_2C_6H_4$	H	**3e**	21	98	—
6	3-ClC_6H_4	H	**3f**	96	52	—
7	4-ClC_6H_4	H	**3g**	48	82	—
8	3-CNC_6H_4	H	**3h**	11	97	—
9	4-CNC_6H_4	H	**3i**	19	99	—
10	2,6-$Cl_2C_6H_3$	H	**3j**	10	93	—
11	2,4-$Cl_2C_6H_3$	H	**3k**	10	98	—
12	4-BrC_6H_4	H	**3l**	48	89	—
13	2-Furyl	H	**3m**	96	6	—
14	2-Thienyl	H	**3n**	96	8	—
15	2-$MeOC_6H_4$	Me	**3o**	71	72	57 : 43
16	4-$NO_2C_6H_4$	Me	**3p**	40	87	58 : 42
17	4-CNC_6H_4	Me	**3q**	46	89	50 : 50
18	4-BrC_6H_4	Me	**3r**	88	65	64 : 36
19	2-$MeOC_6H_4$	Et	**3s**	71	61	50 : 50
20	4-$NO_2C_6H_4$	Et	**3t**	40	87	61 : 39
21	4-CNC_6H_4	Et	**3u**	46	87	70 : 30
22	4-BrC_6H_4	Et	**3v**	88	74	77 : 23
23 (large-scale)[d]	4-CNC_6H_4	H	**3i**	19	95	—

[a] Reaction conditions: aldehyde (1 mmol), nitroalkane (5 mmol), *An*GA (3 mg), ethanol (0.85 mL), and deionized water (0.15 mL) at 30 °C. [b] Yield of the isolated product after silica gel chromatography. [c] dr was determined by ^{1}H NMR, and *syn* and *anti* isomers were assigned by chemical shifts according to the literature. [d] Reaction conditions: 4-cyanobenzaldehyde (20 mmol, 2.62 g), nitromethane (100 mmol, 6.10 g), *An*GA (60 mg), ethanol (17 mL), and deionized water (3 mL) at 30 °C.



Scheme 1 Possible mechanism of the *An*GA-catalyzed Henry reaction.

of *An*GA caused by the Henry product that is formed during the course of the reaction.

With the optimized conditions in hand, *An*GA-catalyzed Henry reactions of nitroalkanes with various aromatic and hetero-aromatic aldehydes were investigated (Table 5). A wide range of aromatic aldehydes can effectively participate in the reactions with nitromethane, nitroethane and nitropropane. This reaction tolerates both electron-donating and electron-withdrawing substituents of aromatic aldehydes. In general, the reactions with nitromethane gave better yields than those with nitroethane and nitropropane. Electron-withdrawing substituted aldehydes gave better yields than electron-donating ones. The best yield of 99% was achieved for the reaction of 4-cyanobenzaldehyde with nitromethane (Table 5, entry 9). Besides, disubstituted aromatic aldehydes such as 2,6- and 2,4-dichlorobenzaldehyde could react with nitromethane efficiently giving products in good yields (Table 5, entries 10 and 11). However, furfural and 2-thenaldehyde gave poor yields of 6 and 8%, respectively (Table 5, entries 13 and 14). In addition, when reacting with nitroethane and nitropropane, *An*GA displayed different degrees of diastereoselectivity for *syn*-isomers. The best d.r. value of 77 : 23 (*syn* : *anti*) was obtained (Table 5, entry 22). Unfortunately, no enantioselectivity was observed.

We further performed a large-scale Henry reaction of 4-cyanobenzaldehyde (20 mmol, 2.62 g) and nitromethane (100 mmol, 6.10 g) in ethanol (17 mL) and deionized water (3 mL) at 30 °C. Only 60 mg of *An*GA was sufficient to catalyze this large-scale reaction facilely using the same procedure as the small scale reactions, and an excellent yield of 95% was achieved after 19 h (Table 5, entry 23).

The Gotor group suggested an unspecific protein may catalyze the Henry reaction.[35] Based on the experiments with denatured and inhibited *An*GA, and the comparison of natural activity and promiscuous activity, we determine that the natural active center of *An*GA is responsible for its activity in the Henry reaction. Thus, we attempted to propose the possible mechanism of the *An*GA-catalyzed Henry Reaction of aldehyde with nitroalkane (Scheme 1). According to previous literature,[36–38] the catalytic site of *An*GA includes the general acid and base catalysts Glu179 and Glu400 situated at the bottom of a pocket. Based on the widely accepted mechanism of hydrolysis of *An*GA, we hypothesized the possible mechanism. Glu400, as a base, deprotonates the α-carbon of the nitroalkane providing intermediate **I**. At the same time, Glu179, as an acid, donates a proton to the carbonyl oxygen of the aldehyde generating intermediate **II**. Then, the α-carbon of **I**, as a nucleophile, attacks the carbonyl of **II** forming a new carbon-carbon bond. Finally, the product (β-nitro alcohol) is released from the active site.

3. Conclusions

A facile, environmentally friendly and large-scalable synthetic methodology of an *An*GA-catalyzed Henry reaction for the preparation of α-nitro alcohol derivatives was established. This reaction can be carried out under mild conditions without the need for additional cofactors or special equipment. The

remarkable catalytic activity of *An*GA on the Henry reaction was demonstrated adequately. Only 3 mg of *An*GA is sufficient to catalyze 1 mmol of aldehyde to give good yields in most cases. A wide range of substrates were accepted by the enzyme and yields of up to 99% were achieved. The control experiments with the denatured and inhibited enzyme and non-enzyme proteins indicated that the specific natural fold of *An*GA was responsible for its catalytic activity in the Henry reaction. The influence of several factors, including solvent, water content, molar ratio of substrates and temperature, was investigated. It was found that the Henry product could inactivate *An*GA at high temperature (80 °C); however, it almost did not affect the activity of *An*GA at low temperature (30 °C). This work broadens the scope of *An*GA-catalyzed transformations.

4. General procedure for the AnGA-catalyzed Henry reactions (products 3a–v)

To a mixture of aldehyde (1.0 mmol), *An*GA (3 mg), nitroalkanes (5.0 mmol) and ethanol (0.85 mL) was added deionized water (0.15 mL). The resultant mixture was stirred for the specified time at 30 °C, and monitored by thin-layer chromatography. The reaction was terminated by filtering the enzyme. Ethyl acetate was employed to wash the residue on the filter paper to ensure that the products obtained were all dissolved in the filtrate. The filtrate was dried over anhydrous Na_2SO_4, and the organic solvents were then removed under reduced pressure. The crude products were purified by silica gel column chromatography with petroleum ether/ethyl acetate as the eluent.

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