Biosynthesis of α-substituted β-ketoesters via the tandem Knoevenagel condensation-reduction reaction using a single enzyme

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Abstract

Saturated α-substituted β ketoesters are important building blocks in synthesis of pharmaceuticals and agrochemicals. Herein, we report a one-pot biosynthesis of α-substituted β ketoesters via Knoevenagel condensation and reduction of received unsaturated alkenes in situ, catalyzed by single ene-reductase (NerA). A series of inexpensive and readily available aldehydes and 1,3-diketones were condensed and reduced by NerA in aqueous solution at room temperature. We also note that low loadings (3 mg/ml) of NerA are sufficient to facilitate the cascade process, both E and Z isomeric intermediates can be reduced effectively and improved the overall yield up to 95%. Meanwhile, the method can be applied in preparative-scale synthesis of pharmaceutical intermediate. This process conforms to the concepts of green chemistry and shows advantages for synthesis of high value saturated α-substituted β ketoesters.

1 Introduction

In the pharmaceutical and fine chemistry, α-substituted β ketoesters are widely used as intermediates. For example, ethyl-α-(2'-furfuryl) acetoacetate is used with iridium in organic light-emitting diode screens (Saha, Rozenberg, & Lemcoff, 2015). Ethyl-2-pentylacetoacetate is used to synthesize porphyrins (Mikhalitsyna et al., 2012) which are important components in supramolecular chemistry. Similarly, ethyl 2-[(4-chlorophenyl)methyl]-3-oxobutanoate and ethyl 2-benzylacetoacetate have pharmaceutical uses. Their downstream products, including pyridine benzimidazole derivatives, have been used to develop anti-tuberculosis and schistosomiasis drugs (Okombo et al., 2017; Pieroni et al., 2011). Currently, the main strategy to synthesize α-substituted β ketoesters is through alkylation of 1,3-dicarbonyl compounds, during which nucleophiles attack highly reactive carbons, leading to the formation of carbon-carbon bonds (Shirakawa & Kobayashi, 2007). For the nucleophilic substitution reactions, halogenated alkanes are traditional substrates. However, these substrates have several disadvantages, such as salt treatment and effluent disposal restrictions due to halogen removal. From the perspectives of atomic economic efficiency and product processing, alcohols and enols have been identified as ideal substrates. For example, Makoto Yasuda and co-workers (Yasuda, Somyo, & Baba, 2006) reported that indium can be used as a catalyst to generate carbon-carbon bonds between alcohols and active methylene in toluene. In this method the byproduct, water, requires comparatively easy post-treatment. The disadvantage of this strategy is that catalytic activation of alcohols is difficult due to the poor leaving ability of the hydroxyl group. Thus, significant amounts of a Brønsted acid or of Lewis acid must be employed to promote the reactions.

Enzymes are green catalysts widely used in organic synthesis due to their excellent chemoselectivity, stereoselectivity, and mild reaction conditions (Yang, Hechavarria Fonseca, & List, 2004; Yang, Hechavarria Fonseca, Vignola, & List, 2005; Ouellet, Tuttle, & MacMillan, 2005; Tuttle, Ouellet, & MacMillan, 2006; Shoda, Uyama, Kadokawa, Kimura, & Kobayashi, 2016). Some researchers promote the reduction of products of Knoevenagel condensation to synthesize α-substituted β ketoesters. For example, Jimenez et al.

catalyzed Knoevenagel condensation between aryl aldehydes and malononitrile in methanol and then reduced the unsaturated alkenes using whole cells of Penicillium citrinum CBMAI 1186, with excellent yields (˜98%) (Jimenez, Ferreira, Birolli, Fonseca, & Porto, 2016). However, the reaction requires separation and purification between two sub-steps. Generally, cascade reactions contain several reactions in a single pot, lowers consumption of energy, eliminates intermediate separation and purification steps when compared with traditional stepwise synthesis (Kroutil & Rueping, 2014; Schrittwieser et al., 2013; Pellissier, 2013; Oroz-Guinea & García-Junceda, 2013). Remarkable developments have recently occurred in the use of heterogeneous, homogeneous, organic, and biological catalysts in cascade systems (Pellissier, 2012; Wende & Schreiner, 2012; Albrecht, Jiang, & Jørgensen, 2011; Ricca, Brucher, & Schrittwieser, 2011; Grondal, Jeanty, & Enders, 2010; Climent, Corma, & Iborra, 2009). Recently, researchers found that metal nanoparticles such as Ni and Pd could promote Knoevenagel condensation (Javad Kalbasi, Mesgarsaravi, & Gharibi, 2019; H. Wang et al., 2019). Sequentially, H2 was used to reduce condensation compounds in one pot to produce saturated α-substituted β ketoesters. But the condensation and reduction must be operated in sequence, and difficult removing of the metal catalyst also restricted the application in pharmaceutical synthesis. In most of one-pot systems, several catalysts work together to accomplish the desired reaction. Multi-step reactions using a single catalyst have rarely been studied. Bifunctional catalyst, Pd-ZIF-8/rGO, was used as an efficient catalyst for the Knoevenagel condensation-reduction tandem reaction (Scheme 1). However, both conversion and selectivity were lower towards less active methylene compounds, such as diethyl malonate and ethyl cyanoacetate. Meanwhile, condensation and reduction are still required to operate one by one (Cuetos, Bisogno, Lavandera, & Gotor, 2013; Willemsen, van Hest, & Rutjes, 2013; Foulkes, Malone, Coker, Turner, & Lloyd, 2011; Kraußer et al., 2011; Tenbrink, Seßler, Schatz, & Gröger, 2011).

In this work, we show that ene-reductases (ERs) are effective biocatalysts, which directly catalyzed the synthesis of saturated α-substituted β ketoesters 3a-k from readily available aldehydes 1a-k and 1,3-diketones. This one-pot two sub-steps reaction which underwent the steps as Knoevenagel condensation to form α, β-unsaturated intermediates 2a-k, which were immediately reduced to the saturated products by the same single enzyme, NerA. Without the use of precious metals, heating and hazardous H2, the tandem reaction was simply performed in an aqueous phase at room temperature, yield and selectivity as high as 95% and 100%, respectively. As shown in Scheme 1, this cascade reaction fulfills the requirements of green chemistry and provides an environmentally friendly method for synthesizing saturated α-substituted β ketoesters.

2 Material and Experimental Section

2.1 General Information

Unless otherwise noted, reagents and organic solvents were obtained from chemical suppliers in reagent grade quality and used without further purification. All reactions were monitored by thin-layer chromatography (TLC) with Haiyang GF254 silica gel plates. Flash column chromatography was carried out using 100-200 mesh silica gel at increased pressure. For visualization, TLC plates were either placed under ultraviolet light, or stained with iodine vapor, or acidic vanillin. NMR spectra were recorded on a 400 MHz spectrometer.

2.2 Gene cloning and protein generation.

A synthetic gene in *E. coli* codon usage, encoding for the GTN reductase (CAA74280.1), OYE2.6 (XP_001384055), IPR (Q6WAU1) and GDH (WP_003246720), were ordered from Beijing Genomics Institute (BGI, Beijing, China). The synthetic genes were flanked with *Nco* I and*Xho* I and cloned into the *Nco* I/*Xho* I restriction sites of the pET28a vector (NerA and IPR), pET41a vector (OYE2.6), and pET32a vector (GDH) respectively, to generate the expression plasmid pET28a-NerA, pET28a-IPR, pET32a-GDH and pET41a-OYE2.6. The sequence of the resulting expression plasmid was verified by DNA sequencing (Sangon Biotech, Shanghai, China).

Chemically competent *E. coli* BL 21 (DE3) cells were transformed with pET28a-NerA, pET28a-IPR, pET32a-GDH and pET41a-OYE2.6 and protein expression was performed by standard protocols. Precultures were grown in lysogeny broth (LB) supplemented with kanamycin (50 μg/mL) for NerA, IPR, OYE2.6 and ampicillin (100 μg/mL) for GDH at 37 °C for 12 h and were used to inoculate main cultures (800 mL

LB medium containing 50 μg/mL kanamycin or 100 μg/mL ampicillin) to an initial OD600 of 0.05. Cells were grown at 37 °C to an OD600 of 0.6, and protein expression was induced by adding isopropyl-β-d-thiogalactopyranoside (IPTG) with a final concentration of 0.1 mM. The cultures were incubated at 35 °C for an additional 10 h, and cells were harvested by centrifugation. Cell pellets were washed with a NaCl solution (0.9%, w/v) and frozen at -20 °C for later purification.

2.3 Cell disruption and purification:

The cell pellets were thawed on ice and resuspended in lysis buffer (50 mM phosphate buffer containing 300 mM NaCl and 10 mM imidazole, pH 8.0), with use of 10 mL buffer per 5 g of wet cells. Then the cells were disrupted by sonication (2 x 4 min, 50% duty cycle; Sonicator QSonica Q500 Ultra Sonicator) with cooling on ice. Cell debris was removed by centrifugation at 18000g for 30 min at 4 °C. The supernatant was filtered through a 0.25 μm PVDF filter and was loaded onto a Ni-NTA column (Thermo scientific) equilibrated with lysis buffer. After loading of the filtered lysate, the column was washed with six column volumes of wash buffer (50 mM phosphate buffer containing 300 mM NaCl and 20 mM imidazole, pH 8.0). Finally, the protein was eluted with elution buffer (50 mM phosphate buffer containing 300 mM NaCl and 250 mM imidazole), and fractions containing target enzyme (determined by SDS-PAGE) were pooled and dialyzed at 4 °C against Tris-HCl buffer (20 mm, pH 7.5). Aliquots of concentrated enzymes with approximately 10-20 μM, flash-frozen on powdered dry ice, and stored at -80 °C until later use. Protein expression and purity was assessed by SDS-PAGE (Figure S3).

2.4 General experimental procedure for the Knoevenagel reaction (1a-k-2a–k)

A 5 mL microcentrifuge tube, was charged with solvent (2 mL), to which the 1a-k (50 mM) and 1,3-dicarbonyl compound (100 mM) were introduced. The resulting mixture was stirred for the specified amount of time at 25 oC. After completion, the product was extracted into EtOAc (4x5 mL), The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under a vacuum. The residue was purified by flash column chromatography with petroleum ether and ethyl acetate to afford the desired products.

2.5 General experimental procedure for ERs catalytic reduction of intermediates (2a-k-3a–k)

A 2 mL microcentrifuge tube, was charged with solvent (1 mL), 10 mM (2a-k), NAD(P)H (0.1 mM), ERs (0.8 mg mL-1), sodium phosphate buffer (50 mM, pH 7), Incubate at 25 degC for 5 minutes, then The reducing activity was determined by measuring the decrease of NAD(P)H at 340 nm.

2.6 General experimental procedure for the domino Knoevenagel reaction (1a-k-3a–k)

A 25 mL round-bottomed flask was charged with NerA (3 mg mL-1), GDH (1.5 mg mL-1), NAD+ (1 mM), deionized water (10 mL), to which the aldehydes (50 mM) and 1, 3-dicarbonyl compound (100 mM) were introduced. The resulting mixture was stirred for the specified amount of time at 25 oC. After completion, the product was extracted into EtOAc (4x10 mL), The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under a vacuum. The residue was purified by flash column chromatography with petroleum ether/ethyl acetate to afford the desired products.

2.7 Molecular docking calculations with Surflex-Dock

NerA was docked on each one of the target ligands (ethyl (E)/(Z)-2-acetylhept-2-enoate) using the Surflex-Dock module of Sybyl-X 2.0 program and visualized with PyMOL. All docking calculations were set to equal parameters (10 poses each), only using the highest ranked pose by Surflex-Dock.

2.8 General Experimental Procedure for the NerA Catalyzed Tandem Reaction

A 25 mL round-bottomed flask was charged with NerA (3 mg mL^{-1}), GDH (1.5 mg mL^{-1}), NAD^+ (1 mM), deionized water (10 mL), to which the aldehydes (50 mM) and 1, 3-dicarbonyl compound (100 mM) were introduced. The resulting mixture was stirred for the specified amount of time at 25 degC. After completion, the product was extracted into EtOAc (4 x 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under a vacuum. The residue was purified by flash column chromatography with

petroleum ether/ethyl acetate (4:1) to afford the desired products ethyl 2-(4-chlorobenzyl)-3-oxobutanoate and ethyl 2-benzyl-3-oxobutanoate.

3 Results and Discussion

3.1 Preparation for appropriate catalysts

In 1909, Dakin found that primary amino acids can catalyze Knoevenagel condensation (DAKIN, 2009). The fact that bovine serum albumin (BSA), which slightly catalyzed the condensation between benzaldehyde and ethyl cyanoacetate in water phase (W. Li et al., 2015) encouraged us to investigate other enzymes which may also possess the ability to promote Knoevenagel condensation. To test the idea, the enzymes NerA (CAA74280), OYE2.6 (XP_001384055), IPR (Q6WAU1), GDH (WP_003246720), and ADH_4 (XP_-001387122) were chosen to compare with glycine (Chaudhry et al., 2016), proline, and lysine (Y. Wang, Shang, Wu, Fan, & Chen, 2006; Y. Zhang, Sun, Liang, & Shang, 2010), as catalysts in the model reaction (Figure S3). To date, it hasn't been recorded in the literature that these enzymes have a direct role in Knoevenagel condensation and natural amino acids can promote the reaction obviously. For the pretest, ethyl acetoacetate (EAA) and valeraldehyde **1b** were used as substrates. As shown in Table S1, both enzymes and amino acids were able to efficiently promote Knoevenagel condensation in neat aqueous solvent. Interestingly, the yield of the reaction was not significantly affected by the species of enzymes (Table S1, list 4-8), but were positively correlated with the amount of catalyst (Table S1, list 4-8 and 9-13).

According to the literature (W. Li et al., 2015), lipases like PPL, MJL, YILip2 can also promote Knoevenagel condensation in a water-ethanol solvent system. 10 mg mL^{-1} enzymes and 96.7% ethanol by volume were employed to catalyze the reaction efficiently. Interestingly, unspecific reaction in lipase-catalyzed Knoevenagel condensation was also observed in addition to specific reaction. Our work demonstrated that several enzymes could catalyze the condensation, with the exception of lipases. 2.4 mg mL^{-1} enzymes in neat aqueous solvent maintained the ability to catalyze Knoevenagel condensation. On the basis of the mechanism as reported by Knoevenagel, we thought that the hydrophilic amino acids on the surface of enzymes were associated with the formation of a Schiff base with aldehyde substrates, before the obtained imine intermediates condensed with carbanions (Zheng, Li, Tao, & Zhang, 2019).

The Knoevenagel condensation catalyzed by a certain concentration of enzymes is comparable to that of the amino acids, which inspired us to consider that a low catalytic turnover would be further promoted if the condensation products could be converted by the same enzyme. Ene-reductases, which catalyze the reduction of α, β-unsaturated alkene, have highlighted the potential reduction of Knoevenagel condensation products. Subsequently, we tried to construct the tandem reaction using single ER which catalyzes both Knoevenagel condensation and*in situ* reduction of the condensation products to obtain more stable and valuable products. Several ERs were investigated to ascertain which one can reduce intermediates **2a -k** . Following a review of the literature, three of ERs (NerA, OYE2.6 and IPR) were cloned and expressed (Oberdorfer et al., 2013; B. Zhang, Zheng, Lin, & Wei, 2016). The reduction activity of each catalyst towards intermediates **2a-k** was then tested, shown in Figure 1, except that IPR had no obvious catalytic activity for any of the products, the other two reductases had wide substrate ranges. In particular, NerA presented a broader range of substrate species and higher activity on nearly all the products we were interested in. It's worth noting that the products derived from the condensation of benzaldehyde (**2f**) and furan aldehyde (**2h** , **2i**) with EAA were reduced effectively. These bulky substrates are often difficult to reduce using other ERs (Peters, Frasson, Sievers, & Buller, 2019). Phenyl group bound with the electron withdrawing groups (**2g**) was reduced faster than **2f** , which may facilitate hydrogen transfer between olefin and reduced flavin cofactor (de Paula, Zampieri, Nasário, Rodrigues, & Moran, 2017). OYE2.6 also had a wide reduction capacity, but the relative activity on all substrates was at least 40% lower than that of NerA. OYE2.6 had relatively low reduction activities, especially towards the large size compounds (**2g-2k**), which may attribute to the smaller activity centre (Oberdorfer et al., 2013; B. Zhang, Zheng, Lin, & Wei, 2016). In addition, NerA takes NADH as a co-factor while the co-factor of OYE2.6 is NADPH. Considering economy and usability, NerA was chosen for the one-pot reaction presented here.

3.2 Investigation of the key reaction parameters

The compatibility of all involved catalysts and their reaction conditions, especially the solvent, is a critical factor that requires a compatibility window that is often difficult to meet in one pot (Gómez Baraibar et al., 2016). To construct the tandem reaction, each sub-step was pre-tested. The kinetics of Knoevenagel condensation in H_2O was investigated. The highest concentration of**1b** was set as 50 mM due to the tolerance of the enzymes. As shown in Figure 2 and Table S2, the condensation efficiency markedly accelerated with increasing the concentration of **1b** in first 1 hours, and significantly slowed down and reached equilibrium in 2 hours, due to a decrease in substrate concentration and reversibility of the Knoevenagel condensation. In addition, another possible reason may be because 1,3-diketones tend to form stable six membered cyclic enols resulting in lower reactivity in water (Hu, Guan, Deng, & He, 2012). To solve this problem, we considered the solvent in this system. Traditionally, Knoevenagel condensation is always conducted in DMF and DMSO because aprotic solvents may lead to the deprotonation of the active hydrogen substrate, which is why water is seemed as negative factor (Siebenhaar, Casagrande, Studer, & Blaser, 2001). It was understood that all enzymes need essential bound water, and that enzymatic activity in organic solvents depends on water content (C.-H. Wang, Guan, & He, 2011). Thus, we selected an organic solvent optimized by mixing with water to promote Knoevenagel condensation. As expected, the species of the solvent affected the yield obviously (Figure S1 and S2). 30% DMSO promoted the yield up to 35%, in contrast to the yield of only 20% in water. This shows that deprotonation is indeed vital in a reaction that is more facile in non-protonic solvents, as expected according to the mechanism of Knoevenagel condensation. Meanwhile, compared to classical Knoevenagel condensation in DMF combined with a molecular sieve, this sub-step presented a very low yield due to aqueous environment (C.-J. Li & Chen, 2006).

Although the condensation process is significantly different in presence and absence of DMSO, the gap may be narrowed when the condensation and reduction are synergistic. Firstly, the high cost of NADH requires*in situ* regeneration, as a common cofactor cycling system, GDH was selected. **1b** and EAA were chosen as substrates to conduct tandem reactions. As shown in Figure 3A, the addition of GDH greatly increases the yield. But **3b** was not synthesized in GDH alone, while GDH promotes the Knoevenagel condensation. Meanwhile, all available NADH were used and 8% yield was obtained in NerA alone, indicating that NerA may accomplish the condensation of **1b** and EAA and reduction of **2b**independently. To verify this conclusion, the reaction with NerA or GDH on their own were conducted, shown in Table S3. It was found that NerA on its own was able to facilitate the reaction, achieving a yield of 84%.

Then, the yield was determined in both water and 30% DMSO. As shown in Figure 3B, the yield in 30% DMSO achieved 53% in 1 hour, whereas only reached 36% in aqueous solvent. However, we noticed that the product**3b** grows linearly in both systems (Figure 3B), prolonging the reaction time and improving the loading of enzymes may compensate for the loss of yield due to the removal of organic solvent. To test the idea, the same amount of NerA (2.4 mg mL^{-1}) was added in the aqueous phase, along with 30% DMSO and the reaction time was extended to reach equilibrium. As shown in Figure 3C, the yield in water reached 89% in 4 hours, still lower than that after 3 hours in 30% DMSO (90%). There was almost no residue of **2b** in water, and the yield was the same as in 30% DMSO at 5 hours, so that condensation is a limiting step in water. In fact, some ERs, such as YersER, tend to reduce the E -isomer and the isomerization is required to reduce the isomeric mixture (Litman, Wang, Zhao, & Hartwig, 2018). Both the$E$ and Z isomers formed by condensation of aldehydes with 1, 3-dicarbonyl compounds in this reaction can be effectively reduced by NerA. Molecular docking experiments showed that carbonyl oxygen atom in either of the acetyl or carboxylic acid group of **2b** can form hydrogen bonds with H178 and N181 in catalytic pocket, and the side chain extends into the hydrophobic cavity formed by Y65 and Y356 (Figure 4). Therefore, the unsaturated double bond is correctly fixed and efficiently reduced. Next, 3 mg mL^{-1} NerA was added with **1b** and EAA in aqueous solvent, as shown in Figure 3D, equilibrium was achieved in 3 hours in both systems. The yield in water was comparable with that of 30% DMSO (90% vs. 91%, respectively) while 30% DMSO encumbered the separation and purification of the products. In addition, using water as the medium has received considerable attention in organic synthesis due to its economic, environmental, safety, and other advantages (Patel, Sharma, & Jasra, 2008). Based on these results, a neat aqueous solvent was chosen.

Next, the dosage of NerA and the molar ratio of substrates were tested to maximize the final yield. Shown in Table 1, the yield was promoted by the addition of NerA. Furthermore, Figures 3C and 3D also showed that when the amount of NerA was increased to 3 mg mL^{-1}, the formation of **3b** was significantly accelerated compared with 2.4 mg mL^{-1}, and more **2b** accumulated in the first hour. Indicating that the increase of enzymes obviously promotes the condensation and reduction. However, 3 mg mL^{-1} of NerA is sufficient, since the yield of **3b** is similar to that of 3.6 mg mL^{-1}. To further optimize the reaction, we observed that the yield was maximized when the substrate molar ratio was 1:2. Finally, 3 mg mL^{-1} NerA and a substrate molar ratio of 1:2 were used.

4 Scope of the biocatalytic one-pot reaction

Having obtained a system capable of high productivity, we chose a variety of aldehydes **1a -k** , which were derived easily from biomass (e.g., primary, aromatic, and furan aldehydes) (Wu, Moteki, Gokhale, Flaherty, & Toste, 2016; Mahyari, Shaabani, & Bide, 2013; Boruah & Das, 2018; Lee et al., 2018) and mated with α, β-unsaturated carbonyl tandem reaction under optimized conditions. As shown in Table 2, the enzyme accepted a wide range of substrates. A high reaction rate was obtained using primary aldehydes whose corresponding intermediates may possess suitable sizes for adoption in the active center. The appropriate length branch-chain was vital in fastening the hydrophobic area in the active center of NerA(Peters, Frasson, Sievers, & Buller, 2019). Benzene (**3f**) and furan (**3h** , **3i**) derivatives also achieved high yields (80%, 75%, and 70% respectively), other ERs like DBVPG and OPRI had only 3% and 4% yields after 24 hours (Reß, Hummel, Hanlon, Iding, & Gröger, 2015). Compared to **3f** , the substrate with an electron-withdrawing group (**3g**) showed a higher yield (85%). These results are probably because benzene has electron donors with conjugating effects, which is not conducive to hydrogen transfer. This can be further proven as furan, a 5-center, 6-electron conjugate system with a higher electron cloud density, caused a lower yield. We also noticed that although enantioselectivity is a general property of most reductases including NerA. However, e.e% of **3a -k** are only around 10% (data not shown), since the intermediates can be flipped at the active center, and the keto-enol tautomerism of the ketone carbonyl in the products may be the other reason for racemization under an aqueous environment. Metal nanoparticles such as Ni and Pd were found to possess the ability to drive the cascade reactions containing Knoevenagel condensation and ene reduction as bifunctional catalysts similar to NerA (Javad Kalbasi, Mesgarsaravi, & Gharibi, 2019; H. Wang et al., 2019). Compared to our work, the reaction catalyzed by these metal nanoparticles must be separated into two sub-steps. Meanwhile, as an alternative to using metal catalysts, enzymes are coincident with the concept of green chemistry.

5 One pot synthesis towards pharmaceutical precursor

Ethyl 2-[(4-chlorophenyl)methyl]-3-oxobutanoate (**3g**) is the precursor of antituberculosis drugs[3] and can be produced by tandem reaction optimized above. Motivated by the pharmaceutical application, we tried to scale up the synthesis of**3g** . To avoid enzyme deactivation at high concentrations of*p* -chlorobenzaldehyde, fed-batch was selected. 50 mM **1g** , 50 mM EAA and 50 mM glucose were added at 2 h, 4 h, 7 h, 10 h, 13 h, 17 h and 20 h, respectively. The reaction progress was monitored by GC and the result was shown in Figure 5, the product was continuously synthesized within 13 hours. Then, 1.5 mg mL^{-1} NerA and 1 mg mL^{-1} GDH were compensated for the loss of enzyme activity. At 20 hours, the rate of reaction slowed down again and stopped feeding. The synthesis of **3g** was completed at 28 hours, and the yield of **1g** was 91%. Isolation of most products was simply extracted by ethyl acetate, resulting in 88% yield (880 mg). The transformation could be applied in preparative-scale synthesis without sacrificing yield.

6 Conclusion

Water, as an abundant, cost-efficient, environmentally compatible, nontoxic, and nonflammable substance, is widely recognized as an important solvent for organic synthesis. In this study, a variety of saturated α-substituted β ketoesters were efficiently prepared though the alkylation of EAA and aldehydes via a one-pot tandem reaction catalyzed by an only NerA in H_2O. In the first sub-step, the amino acid residues on the surface of NerA promoted the Knoevenagel condensation, and then, the enzyme reduced the received

intermediates immediately and shifted the equilibrium toward the final products, so that the substrates could be converted continuously. This method was conducted under mild conditions, and eliminated the use of organic solvents and toxic metals, consistent with the concept of green chemistry. The results also offer an efficient, practical, and environmentally friendly way to prepare value-added saturated α-substituted β ketoesters from readily available compounds. Moreover, the transformation was suitable for the high yield and preparative-scale synthesis of pharmaceutical precursor. All in all, our approach is facile and easy to scale up, has great potential for "greener" syntheses of pharmaceutical precursor, compared to the established methods.

Conflicts of interest

There are no conflicts to declare.

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Scheme 1. Design for the one-pot synthesis of saturated α-substituted β ketoesters catalyzed by single NerA in water.

Figure 1. The reduction activity of ERs toward **2a-2k** .[a]Conditions: **2a-k** (10 mM), NADH (0.1 mM) for NerA or NADPH (0.1 mM) for OYE 2.6 and IPR, enzyme (0.2-2.5 mg mL-1), sodium phosphate buffer (100 mM, pH 7), 25 °C,[b]The reducing activity was determined by measuring the decrease of NAD(P)H at 340 nm.[c]One unit of activity is as the amount of enzyme that convert 1 μmol NAD(P)H into $NAD(P)^+$ per minute at 25 °C.

Figure 2. The effect of different substrate concentration on the yield of intermediate. [a]Condition:**1b** (2.5 mM, 5 mM, 10 mM, 20 mM, 50 mM), EAA (5 mM, 10 mM, 20 mM, 40 mM, 100 mM), NerA (2.4 mg mL^{-1}), GDH (1.5 mg mL^{-1}), H_2O (1 mL) at 25 ºC.[b]The yield was determined by external standard method by GC.

Figure 3 . [a]Conditions: All reactions were carried out with **1b** (50 mM), EAA (100 mM), glucose (100 mM) and solvent (2 mL) at 25 ºC. [b]For 3A, NADH (5 mM), 30% DMSO (V/V), NerA (2.4 mg mL^{-1}) or GDH (1.5 mg mL^{-1}) [c]For 3B and 3C, NADH (1 mM), NerA (2.4 mg mL^{-1}), and GDH (1.5 mg mL^{-1}); [d]For 3D, NADH (1 mM), NerA (2.4 mg mL^{-1}) and GDH (1.5 mg mL^{-1}) in 30% DMSO, NerA (3 mg mL^{-1}) and

GDH (1.5 mg mL^{-1}) in water. All the yields were calculated by external standard method, and the external substance was 2-amyl acetoacetate.

Figure 4. Ethyl-2-acetylhept-2-enoates in the binding site. A stereo view of the region of the NerA molecule close to the ethyl (*E*)-2-acetylhept-2-enoate (A) and ethyl (*Z*)-2-acetylhept-2-enoate (B) molecule. Residues in close proximity to the substrate are represented as stick models. Oxygen and nitrogen atoms are colored in red and blue respectively. Ethyl-2-acetylhept-2-enoates are represented as cyan, and the distance (Å) between **2b** and amino acids and N5 of FMN were measured and shown as yellow dashed lines.

Figure 5. Preparative-scale synthesis of Ethyl 2-[(4-chlorophenyl)methyl]-3-oxobutanoate.[a]Conditions: **1g** (50 mM), EAA (100 mM), glucose (100 mM), NAD^+ (1 mM), NerA (3 mg mL^{-1}), GDH (1.5 mg mL^{-1}) and water (10 mL), 25 ºC, 28h.

Table 1. Optimize the dosage of NerA and the molar ratio of substrate. [a]Conditions: 1b (50 mM), GDH (1.5 mg mL-1), glucose (100 mM), water (1 mL), NADH (1 mM) at 25 ºC.[b]The yield was determined by external standard method using GC.

Table 2. Substrate scope of the domino Knoevenagel condensation. [a]Condition: **1a-k** (50 mM), β-keto esters (100 mM), glucose (100 mM), NAD^+ (1 mM), NerA (3 mg mL^{-1}), GDH (1.5 mg mL^{-1}) for coenzyme recycle, water (10 mL) 25 ºC.