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Bio-Catalytic Bis-Michael Reaction for Generating Cyclohexanones with a Quaternary Carbon Center Using Glucoamylase

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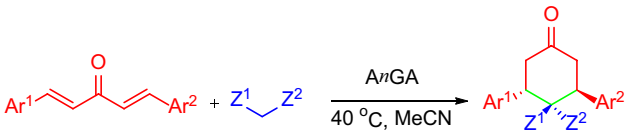
Abstract Bio-catalytic bis-Michael reaction for the construction of quaternary carbon centers is reported. Glucoamylase from *Aspergillus niger* (*An*GA) was used as a sustainable and eco-friendly catalyst. Various highly substituted *trans*-cyclohexanones with a quaternary carbon center were obtained with yields of up to 92%. As a novel case of enzyme promiscuity, this work provides a bio-catalytic alternative for construction of quaternary carbon centers.

Graphical Abstract Glucoamylase from *Aspergillus niger* (*An*GA) catalyzed the bis-Michael addition of (1E,4E)-1,5-diarylpenta-1,4-dien-3-ones with active methylene compounds to form various highly substituted *trans*-cyclohexanones with a quaternary carbon center



12 examples, yields up to 92%, Dr >99:1

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Keywords Bis-Michael reaction · Glucoamylase · Enzyme catalysis · Quaternary carbon center

1 Introduction

Enzymes as sustainable, practical, eco-friendly, economic and biodegradable catalysts have attracted increasing attention from organic chemists [1, 2]. Enzymatic methods as part of green technologies for organic synthesis were considered to possess great potential and were exploited increasingly in organic synthesis [3–5]. Primitively, enzyme catalytic reactions had a narrow scope of application since reactions were restricted to natural substrates and aqueous reaction media [5, 6]. Since Klibanov discovered that enzymes can maintain their activities in organic solvents in the early 1980s [7–9], enzymatic reactions in organic media have drawn significant attraction. Nowadays, more and more enzymes have been found to have the ability to catalyze chemical transformations that vary from their natural reactions [4, 10–12]. This phenomenon is called enzymatic promiscuity which brings novel enzyme activities for organic synthesis [13]. Many reactions were reported with enzyme catalytic promiscuity by catalyzing the formation of C–C and C–heteroatom bonds [4, 14–16]. These reactions included the Markovnikov additions [17], aldol reactions [18, 19], Mannich reactions [20, 21], Michael additions [22–25], multi-component cascade or domino reactions [26, 27], and the asymmetric synthesis of α-aminonitrile amides [28, 29] The study of enzyme promiscuity not only provided novel research methods and tools for organic synthesis, but also formed a bond that led to developments on interdisciplinary sciences between biology and organic chemistry [5, 30–38]. Thus, it is necessary

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to study enzyme catalytic promiscuity, especially for those enzymes that are well-known and widely used in industries.

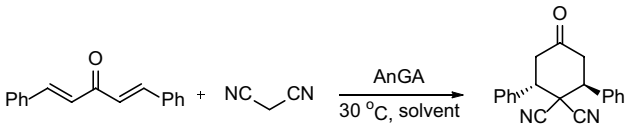
Synthesis of carbon atoms with four nonhydrogen substituents has become a hot topic because of their properties of stereochemistry and biological activities [5, 39, 40]. The synthesis of quaternary carbons is a challenge because of the extreme steric congestion. Conjugate addition of nucleophilic reagents to Michael acceptors is one of the most powerful methods for constructing the quaternary carbon centers. A few catalysts have been reported for the bis-Michael additions to construct the quaternary carbon centers, such as ruthenium(II) complexes [41, 42], basic ionic liquid [bmIm]OH [43, 44], silica nanoparticles (NPs) [45], KF/basic alumina under ultrasound irradiation [46]. Asymmetric double-conjugate additions have also been reported to form the quaternary carbon centers using 9-amino-9-deoxyepiquinine [47], cinchona-based primary amines/(S)-BINOL-phosphoric acid [48] and quinine [49] as chiral organocatalysts. Although some elegant synthetic methods have been reported in this field, the exploration of biocatalysts for the bis-Michael additions to construct quaternary carbon centers that are safe, environmentally benign and operationally simple is still desirable. We found that glucoamylase from *Aspergillus niger* (*An*GA) as a green catalyst could catalyze the bis-Michael addition of (1E,4E)-1,5-diarylpenta-1,4-dien-3-ones (**1**) with active methylene compounds (**2**) to form *trans*-cyclohexanones with a quaternary carbon center (Scheme 1). Herein, we wish to report this work as a complement for existing methods, and a novel case of enzymatic promiscuity.

2 Results and Discussion

In initial research, (1E,4E)-1,5-diphenylpenta-1,4-dien-3-one (**1a**) and malononitrile (**2a**) was used as a model reaction. The solvent screening was performed to find the favorable solvent for this bio-transformation (Table 1). It can be seen that only trace amounts of product were observed for the reactions in non- or low-polar solvents (such as cyclohexane, hexane, methyl *tert*-butyl ether and toluene) (Table 1, entries 1–4). The model reaction gave a moderate yield of 42% in MeCN (Table 1, entry 16). Many other solvents were also screened, which gave yields of 6–30% (Table 1, entries 5–15). Only *trans*-cyclohexanone (determined by ^{1}H NMR) was obtained for the model reaction in all the tested solvents. Unfortunately, there was no enantiomeric excess of the products observed by the chiral HPLC analysis. Therefore, MeCN was chosen as the optimal solvent for the following studies.

In order to further improve the yield of the *An*GA-catalyzed bis-Michael reaction, effects of molar ratio of substrates on the model reaction were investigated (Table 2). When increasing the ratio of (1E, 4E)-1,5-diphenylpenta-1,4-dien-3-one (**1a**) to malononitrile (**2a**) from 1:1 to 5:1, a good yield of 71% was obtained at a ratio of 4:1 (**1a:2a**) (Table 2, entry 4), while other ratios only got moderate

Table 1 Effects of solvents on the *An*GA-catalyzed bis-Michael reaction



1a 2a 3a (+)

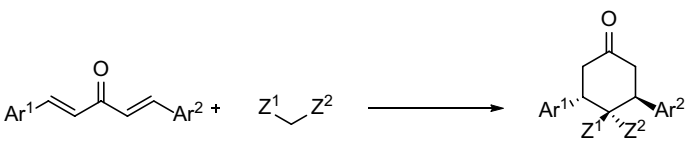
Entry	Solvent	Yield[a] (%)	Dr[b]
1	Cyclohexane	trace	–
2	Hexane	trace	–
3	Methyl *tert*-butyl ether	trace	–
4	Toluene	trace	–
5	DMSO	6	>99:1
6	Anisole	14	>99:1
7	*N*-methylpyrrolidone	15	>99:1
8	Isopropanol	17	>99:1
9	EtOH	18	>99:1
10	MeOH	20	>99:1
11	DMF	22	>99:1
12	1,2-Dichloroethane	22	>99:1
13	Butylacetate	26	>99:1
14	1,4-dioxane	30	>99:1
15	MeCN	42	>99:1

Reaction conditions: **1a** (0.25 mmol), **2a** (0.25 mmol) and *An*GA (3.6 kU) in solvent (1.0 mL) at 30 °C stirring for 60 h

[a]Isolated yield

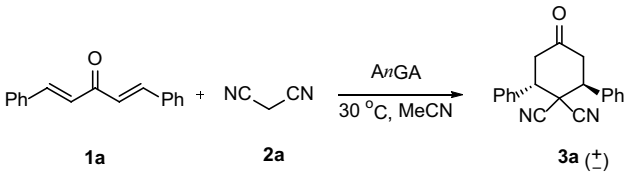
[b]Diastereomeric ratio (dr): determined by ^{1}H NMR analysis

Scheme 1 Bis-Michael addition



1 2 3

Table 2 Effects of molar ratio of substrates on the *An*GA-catalyzed bis-Michael reaction



1a **2a** **3a** (±)

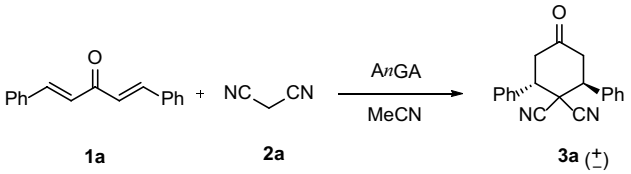
Entry	Molar ratio (**1a:2a**)	Yield[a] (%)	Dr[b]
1	1:1	42	>99:1
2	2:1	59	>99:1
3	3:1	66	>99:1
4	4:1	71	>99:1
5	5:1	61	>99:1
6	1:2	5	>99:1
7	1:3	3	>99:1

Reaction conditions: **1a, 2a** and *An*GA (3.6 kU) in MeCN (1.0 mL) at 30 °C stirring for 60 h

[a]Isolated yield

[b]Determined by ^{1}H NMR analysis

Table 3 Effects of temperature on the *An*GA-catalyzed bis-Michael reaction



1a **2a** **3a** (±)

Entry	Temperature (°C)	Yield[a] (%)	Dr[b]
1	25	67	>99:1
2	30	71	>99:1
3	35	74	>99:1
4	40	92	>99:1
5	45	85	>99:1
6	50	84	>99:1
7	55	79	>99:1
8	60	68	>99:1

Reaction conditions: **1a** (1.00 mmol), **2a** (0.25 mmol) and *An*GA (4.8 kU) in MeCN (2.0 mL) stirring for 60 h

[a]Isolated yield

[b]Determined by ^{1}H NMR analysis

yields (Table 2, entries 1–3 and 5). But, when changing the ratio of **1a** to **2a** into 1:2 and 1:3, the yields decreased rapidly (Table 2, entries 6 and 7). Thus, molar ratio of 4:1 (**1a:2a**) was selected as the optimal condition for the further investigation. Excess **1a** can be easily removed during the purification of product **3a** by flash column chromatography on silica gel.

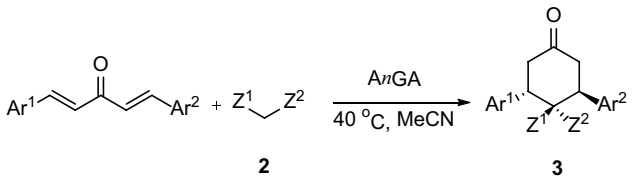
Considering about that the enzyme loading and concentration of the reactants and catalysts in the reaction also play key roles, their effects on this *An*GA-catalyzed model bis-Michael reaction were tested next. The optimized reaction conditions consist of the following: 4.8 kU of enzyme loading (One unit corresponds to the amount of enzyme which liberates 1 μmole of glucose per minute at pH 4.8 and 60 °C with starch as substrate.) and 2.0 mL of MeCN (for details, please see Supplementary Information Tables S1 and S2).

Since enzymes come from organisms, their work temperature is mild. Therefore, the environmental temperature is very important for their stability and activity. To find a suitable temperature for the *An*GA-catalyzed bis-Michael reaction, a temperature screening was conducted from 25 to 60 °C at 5 °C intervals (Table 3). Among the results, 92% yield was exhibited at 40 °C which was also proven to be the best choice accordingly (Table 3, entry 4). Temperature higher than 40 °C caused a decrease in the yield, probably due to the partial denaturation of the enzyme at higher temperature.

With the optimized conditions in hand, several substrates were tested to expand upon this novel enzymatic bis-Michael reaction (Table 4). (1E, 4E)-1,5-Diarylpenta-1,4-dien-3-one (**1**) with different substituents on the benzene ring were applied to react with malononitrile, ethyl cyanoacetate and ethyl nitroacetate. When there are Cl- or Br- on the *ortho* or *para* position of benzene ring substituents (as Ar^1 and Ar^2), the yields are not as good as expected (Table 4, entries 3, 4, 9 and 10). But, when Cl- is on the *meta* position of benzene ring, there were good yields from 74 to 76% (Table 4, entries 2, 8 and 12). The highest yield of 92% was obtained with the reaction of **1a** and **2a** (Table 4, entry 1). However, the lowest yield of 24% was achieved when both benzene rings (Ar^1 and Ar^2) were substituted by CH_3- on the *para* position (Table 4, entry 5). In general, the reactivity of malononitrile is higher than ethyl cyanoacetate and ethyl nitroacetate (Table 4, entries 1, 7 and 11). All the products obtained are *trans*-cyclohexanones (determined by ^{1}H NMR). Unfortunately, there was no enantiomeric excess of the products observed by the chiral HPLC analysis. To further confirm the configuration of the products, product **3h** as a representative example was determined by single-crystal X-ray analysis (Fig. 1).

To verify the catalytic effect of *An*GA on the bis-Michael addition, some control experiments were performed with the model reaction of **1a** and **2a** (Table 5). In the absence of the enzyme, only trace amount of product was observed (Table 5, entry 1), while in the presence of

Table 4 Substrate scope of the *An*GA-catalyzed bis-Michael addition



2 3

Entry	Ar^1	Ar^2	Z^1	Z^2	Product	Time (h)	Yield[a] (%)	Dr[b]
1	Ph	Ph	CN	CN	**3a**	48	92	> 99:1
2	*m*-ClC_6H_4	*m*-ClC_6H_4	CN	CN	**3b**	36	76	> 99:1
3	*o*-ClC_6H_4	*o*-ClC_6H_4	CN	CN	**3c**	48	44	> 99:1
4	*p*-ClC_6H_4	*p*-ClC_6H_4	CN	CN	**3d**	40	50	> 99:1
5	*p*-$CH_3C_6H_4$	*p*-$CH_3C_6H_4$	CN	CN	**3e**	36	24	> 99:1
6	*p*-$CH_3C_6H_4$	Ph	CN	CN	**3f**	36	90	> 99:1
7	Ph	Ph	CO_2Et	CN	**3g**	48	66	> 99:1
8	*m*-ClC_6H_4	*m*-ClC_6H_4	CO_2Et	CN	**3h**	40	76	> 99:1
9	*p*-ClC_6H_4	*p*-ClC_6H_4	CO_2Et	CN	**3i**	48	30	> 99:1
10	*p*-BrC_6H_4	*p*-BrC_6H_4	CO_2Et	CN	**3j**	40	40	> 99:1
11	Ph	Ph	CO_2Et	NO_2	**3k**	48	74	> 99:1
12	*m*-ClC_6H_4	*m*-ClC_6H_4	CO_2Et	NO_2	**3l**	48	74	> 99:1

Reaction conditions: **1a** (1.00 mmol), **2a** (0.25 mmol) and *An*GA (4.8 kU) in MeCN (2.0 mL) at 40 °C

[a]Isolated yield

[b]Determined by ^{1}H NMR analysis

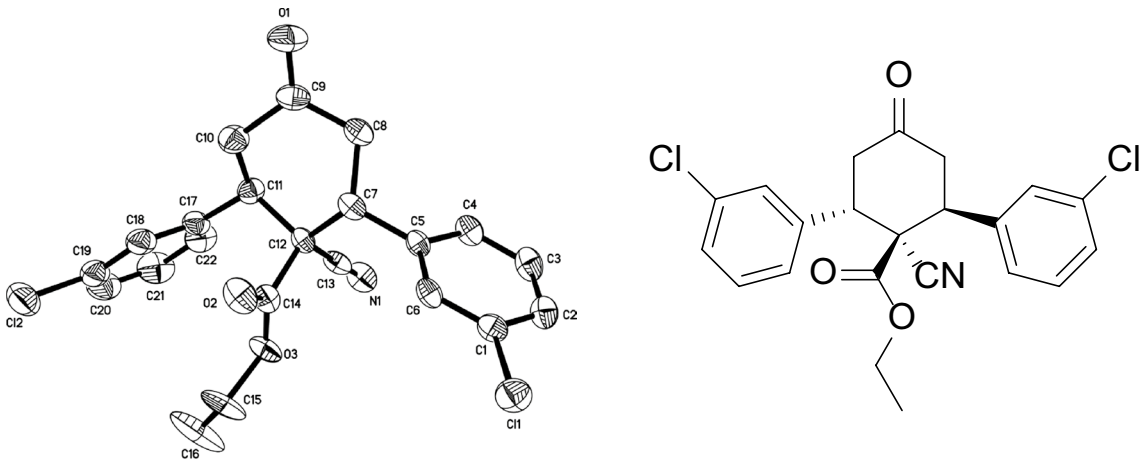
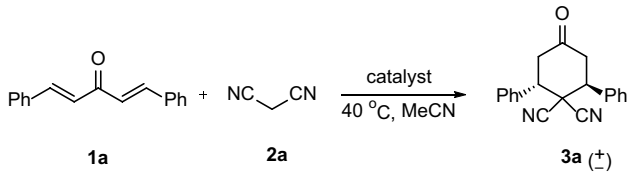


Fig. 1 X-ray crystal structure of **3h**

*An*GA, product **3a** was obtained in an excellent yield of 92% (Table 5, entry 2), indicating that *An*GA indeed had a catalytic effect on the bis-Michael addition. Since metal ions can change the conformation of enzymes, disrupt the active site and ultimately denature enzymes, *An*GA was pretreated with Ag^+ and Cu^{2+}. Metal-denatured *An*GA was then used to catalyze the model reaction, and only trace amounts of product was observed (Table 5, entries 3 and 4). The fact that metal ions completely destroyed the activity of *An*GA on the model reaction indicated that the specific natural fold of *An*GA is responsible for its catalytic activity in the bis-Michael addition. Moreover, miglitol (Fig. 2) is structurally similar to glucose, it has been frequently used as a competitive inhibitor of glucoamylase [50–52]. Thus,

Table 5 Control experiments for the *An*GA-catalyzed bis-Michael addition



1a **2a** **3a** (±)

Entry	Catalyst	Yield[a] (%)	Dr[b]
1	None	trace	–
2	*An*GA (4.8 kU)	92	> 99:1
3	*An*GA (4.8 kU) pretreated with Ag^{+}[c]	trace	–
4	*An*GA (4.8 kU) pretreated with Cu^{2+}[d]	trace	–
5	*An*GA (4.8 kU) pretreated with miglitol[e]	11	> 99:1
6	miglitol[f]	85	> 99:1
7	*An*GA (4.8 kU) pretreated with CDI[g]	trace	–
8	CDI[h]	trace	–

Reaction conditions: **1a** (1.00 mmol), **2a** (0.25 mmol) and catalyst in MeCN (2.0 mL) at 40 °C stirring for 60 h

[a]Isolated yield

[b]Determined by ^{1}H NMR analysis

[c]The mixture of *An*GA (4.8 kU), deionized water (1 mL) and silver nitrate (0.25 mmol) was stirred at 40 °C for 24 h, then water was removed by lyophilization

[d]The mixture of *An*GA (4.8 kU), deionized water (1 mL) and copper sulfate (0.25 mmol) was stirred at 40 °C for 24 h, then water was removed by lyophilization

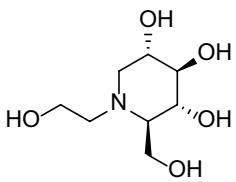
[e]The mixture of *An*GA (4.8 kU), deionized water (1 mL) and miglitol (1.0 mmol) was stirred at 40 °C for 12 h, then water was removed by lyophilization

[f]Miglitol (1.0 mmol) was used instead of *An*GA

[g]The mixture of *An*GA (4.8 kU), dichloromethane (1 mL) and CDI (1.85 mmol) was stirred at 40 °C for 4 h, and dichloromethane was removed by vacuum rotary evaporation. The excess CDI was removed by dialysis against water and the enzyme solution was lyophilized

[h]CDI (1.85 mmol) was used instead of *An*GA

Fig. 2 Structure of miglitol



miglitol was used to pretreat *An*GA. Miglitol alone can catalyze the model bis-Michael addition very well, giving a good yield of 85% (Table 5, entry 6), while the model reaction with miglitol-pretreated *An*GA only gave the product with a low yield of 11% (Table 5, entry 5), suggesting that miglitol strongly inhibited the enzyme activity in the bis-Michael addition. In addition, it is known that catalytic site of *An*GA includes Glu179 and Glu400 [53, 54], 1,1-Carbonyldiimidazole (CDI) can irreversibly react with carboxylic acids [55]. Thus, CDI was used as an inhibitor to pretreat *An*GA, and the model reaction with the CDI-inhibited *An*GA only gave a trace amount of product (Table 5, entry 7). At the same time, CDI alone had no detectable effect on the model reaction (Table 5, entry 8). Based on the control experiments with miglitol and CDI, it can be inferred that the natural active center of *An*GA is responsible for its activity in the bis-Michael addition.

Finally, based on the above control experiments and the previous literature [53, 54], we attempted to propose the possible mechanism of the *An*GA-catalyzed bis-Michael reaction (Scheme 2). The catalytic site of *An*GA consists of Glu179 and Glu400 located at the bottom of a pocket. Based on the widely accepted mechanism of hydrolysis of *An*GA, we hypothesized the possible mechanism with the model reaction of **1a** and **2a** as an example. Glu400, as a base, deprotonates malononitrile (**2a**), and the formed nucleophile attacks one of the double bonds of **1a**. And at the same time, Glu179, as an acid, donates a proton, forming the first Michael adduct **I**. Then in the same way Glu179 deprotonates adduct **I** and an intramolecular Michael addition occurs in which Glu400 as an acid, donates a proton, to get the final product **3a**.

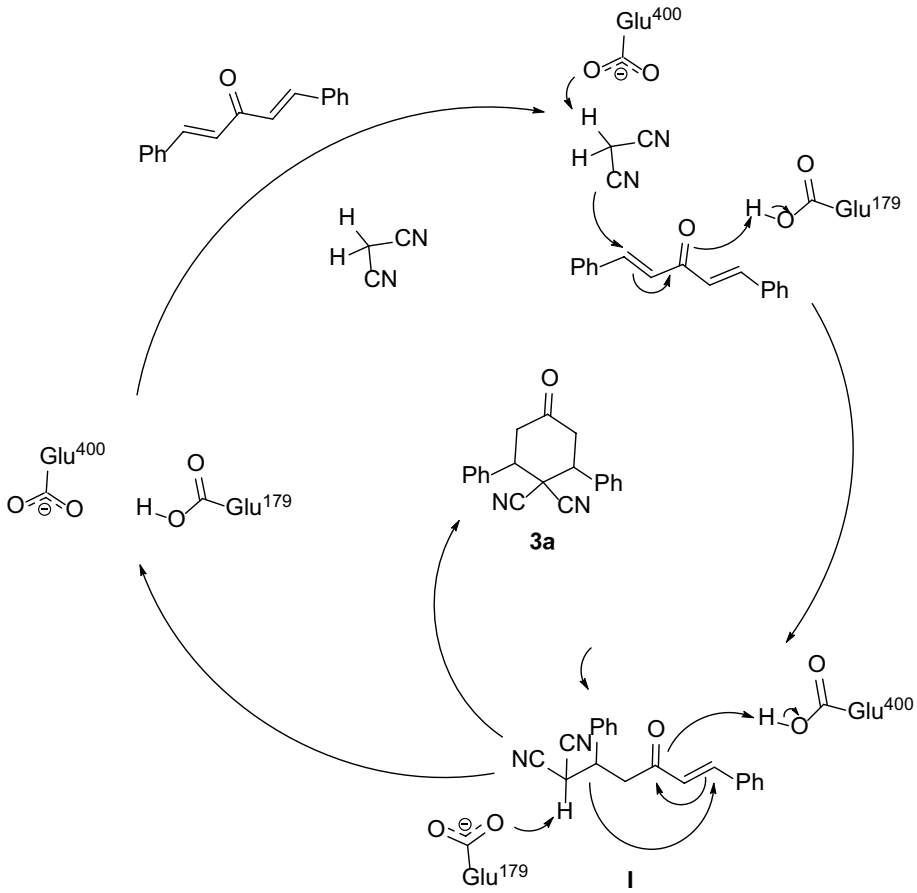
3 Materials

Amyloglucosidase from *Aspergillus niger* [glucoamylase, 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3, 120 U/mg (One unit corresponds to the amount of enzyme which liberates 1 μmole of glucose per minute at pH 4.8 and 60 °C with starch as substrate.)] was purchased from Sigma-Aldrich. (1E, 4E)-1,5-Diarylpenta-1,4-dien-3-ones were prepared according to literature [56, 57]. All reagents were purchased from commercial suppliers and used without further purification unless otherwise noted.

3.1 General Procedure for the *An*GA-Catalyzed Bis-Michael Addition

A mixture of (1E,4E)-1,5-diarylpenta-1,4-dien-3-one (**1**) (1.00 mmol), active methylene compound (**2**) (0.25 mmol) and *An*GA (40 mg, 4.8 kU) in MeCN (2.0 mL) was stirred at 40 °C for the specified reaction time and monitored by TLC. The reaction was terminated by filtering out the enzyme (with 40 mm Buchner funnel and qualitative filter paper), and the filter cake was washed with ethyl acetate (3 × 10 mL). The solvents were removed under reduced pressure. The crude products were purified by column chromatography on a silica gel (petroleum ether/EtOAc: 20/1–4/1) and gave the desired products.

Scheme 2 Proposed mechanism for the *An*GA-catalyzed bis-Michael reaction



4 Conclusion

In conclusion, we successfully demonstrated a sustainable and eco-friendly synthetic methodology for the construction of quaternary carbon centers in a single step by intermolecular and intramolecular bis-Michael addition. This reaction can be carried out under mild conditions without additional cofactors or special equipment. The remarkable catalytic activity of *An*GA on the bis-Michael addition was demonstrated adequately. Various highly substituted *trans*-cyclohexanones with a quaternary carbon center were obtained with yields of up to 92% from simple and readily available starting materials with a wide range of substrates. As a novel case of unnatural activities of existing enzymes in organic medium, this work provides a bio-catalytic alternative for construction of quaternary carbon centers.

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