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PAPER

Biocatalytic domino reaction: synthesis of 2*H*-1-benzopyran-2-one derivatives using alkaline protease from *Bacillus licheniformis*†

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A novel BLAP (alkaline protease from *Bacillus licheniformis*) catalyzed synthesis of 2*H*-1-benzopyran-2-one derivatives was achieved by domino Knoevenagel/intramolecular transesterification reaction. The control of enzymatic chemoselectivity between Knoevenagel/intramolecular transesterification and Knoevenagel/intramolecular hemiketalization could be realized by adjusting parameters including solvent, water content and temperature. The products were obtained in acceptable yields. This BLAP catalyzed selective domino reaction provided an alternative synthetic method for 2*H*-1-benzopyran-2-one derivatives.

1. Introduction

The synthesis of 2*H*-1-benzopyran-2-one and its derivatives has engrossed substantial attention from organic and medicinal chemists for many years as they belong to a class of compounds with proven utility in medicinal chemistry.[1] The nucleus of 2*H*-1-benzopyran-2-one were called privileged structures in biological chemistry, and numerous pharmaceuticals were based on development of this basic scaffold (Fig. 1).[2] Of these, warfarin was the most well-known of a class of 2*H*-1-benzopyran-2-one derivatives used as anticoagulants. In principle, 2*H*-1-benzopyran-2-one derivatives might provide access to compounds like warfarin and also allowed the synthesis of a wide variety of 3-alkylcoumarins for biological screening. However the conventional methods for the preparation of 2*H*-1-benzopyran-2-one derivatives suffered from major drawbacks[3] (drastic conditions, stoichiometric amounts of Lewis or mineral acids, multistep protocols, troublesome work-up procedures). Some attempts have been reported to expand the synthetic approach to functionalized 2*H*-1-benzopyran-2-one derivatives by using heteropolyacid-catalyzed procedures,[4] ionic liquids,[5] microwave irradiation,[6] and the calcined Mg–Al hydrotalcite as solid base.[7] Yet, to the best of our knowledge, enzyme-catalyzed synthesis of 2*H*-1-benzopyran-2-one and its derivatives, which could be performed under facile and mild reaction conditions, has never been reported.

Since Klibanov (re)discovered that enzymes could maintain their activities in organic solvents,[8] enzymes as biocatalysts have been paid more and more attention. Not only do enzymes work in anhydrous organic media, but they acquire remarkable properties such as enhanced stability, altered substrate and enantiomeric specificities, and the ability to catalyze unusual reactions which are impossible in aqueous media.[9] Some elegant works on enzyme catalyzed reactions in organic solvents have been reported.[10] While a multitude of single enzyme-catalyzed single-step transformations was known, few examples of the single enzyme catalyzed multistep conversions have been described.[11] Therefore, the development of new enzymatic catalysts in domino reaction to prepare 2*H*-1-benzopyran-2-one derivatives is not only in great demand in enzymology and synthetic methodologies, but also of practical importance. Herein, we wish to report a novel discovery that the readily available alkaline protease BLAP (alkaline protease from *Bacillus licheniformis* 2709, EC 3.4.21.14) promotes the domino Knoevenagel/intramolecular transesterification reaction to form 2*H*-1-benzopyran-2-one derivatives in organic solvents.

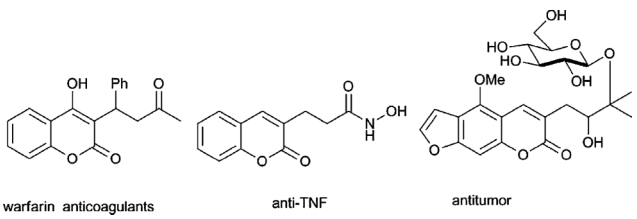

Fig. 1 Biologically active 2*H*-1-benzopyran-2-one derivatives.

2. Results and discussion

It has been reported recently that protease could catalyze the transesterification reaction for the synthesis of N-substituted imidazole derivatives containing a glucose branch in organic solvents.[11] Furthermore, we found that alkaline protease could

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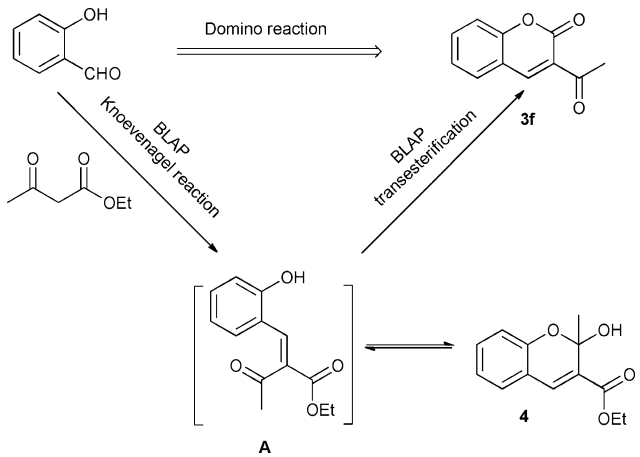
catalyze Knoevenagel reaction in organic solvents which was currently in progress in our laboratory. Based on this, we speculated that the Knoevenagel reaction between salicylaldehyde and ethyl acetoacetate catalyzed by BLAP might take place to generate intermediate A (Scheme 1), and 2*H*-1-benzopyran-2-one might be fulfilled in succession under the same conditions. Therefore, we decided to investigate the possibility of using BLAP as a catalyst for the synthesis of 2*H*-1-benzopyran-2-one derivatives by domino Knoevenagel/intramolecular transesterification reaction. Initially, the condensation of salicylaldehyde and ethyl acetoacetate catalyzed by BLAP in DMSO at r.t. was tested, and the desired product 2*H*-1-benzopyran-2-one **3f** was obtained successfully.



Scheme 1 BLAP-catalyzed reaction of salicylaldehyde with ethyl acetoacetate.

In consideration of the various advantages of using organic solvents in enzymatic reactions,[9] a solvent screen was performed using salicylaldehyde and ethyl acetoacetate as a model reaction. Interestingly, when BLAP was used as a catalyst in different organic solvents, the desired product 2*H*-1-benzopyran-2-one **3f** was obtained, but a by-product hemiketal **4** was also observed which was formed by domino Knoevenagel/intramolecular hemiketalization (Scheme 1). The results of various organic solvents were given in Fig. 2. As can be seen from Fig. 2, 2*H*-1-benzopyran-2-one **3f** could be obtained in the highest yield with the best selectivity in DMSO, while hemiketal **4** could be received with the highest yield in DMF. It was notable that the chemoselectivity of BLAP could be adjusted, even reversed, by changing solvents. In DMSO and ethanol, **3f** was received as the major product, while **4** was obtained as the major product in such solvents as DMF, acetone, THF and so on. Thus, we chose DMSO as the optimum solvent for the preparation of **3f** in the following studies.

It was known that all enzymes need essentially bound water, and the enzymatic activity in organic solvent depends on water content.[12] Moreover, the water content required to reach the maximal activity is different in different organic solvents. Thus, the experiments were performed to ascertain the catalytic activity of BLAP with different amounts of water in the model reaction system. From Fig. 3, it was found that the yield of the enzymatic reaction could be enhanced by increasing the concentration of water, and reached the highest yield. The

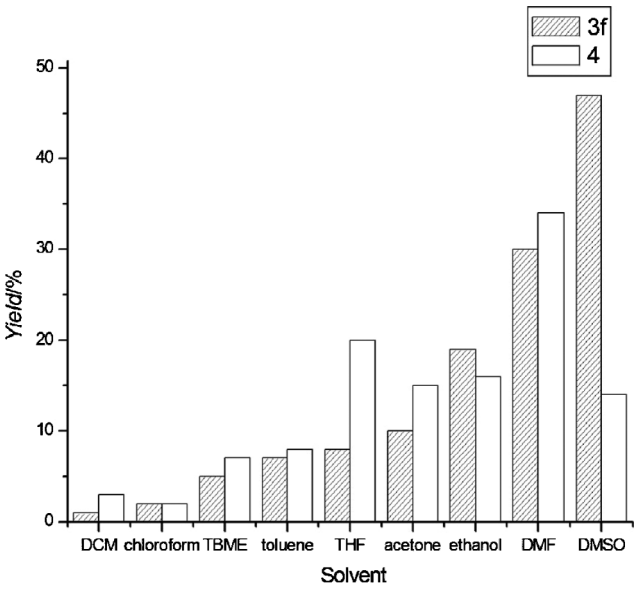


Fig. 2 The influence of organic solvents on chemoselectivity and yield of BLAP-catalyzed domino reaction. Conditions: salicylaldehyde (200 mg, 1.64 mmol), BLAP (40 mg ml^{-1}), ethyl acetoacetate (4.90 mmol), deionized water (0.5 ml) and organic solvent (5 ml) at 25 °C.

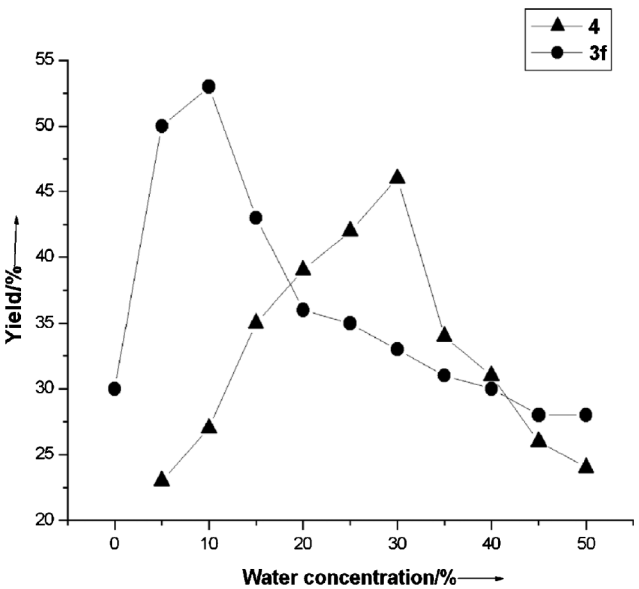


Fig. 3 The influence of water content on BLAP-catalysed domino reaction. Conditions: BLAP (40 mg ml^{-1}), salicylaldehyde (200 mg, 1.64 mmol), ethyl acetoacetate (4.90 mmol), water+DMSO (5 ml), deionized water from 0 to 50% (water/[water+DMSO], v/v) at r.t. (25–30 °C) for 86 h.

desired product 2*H*-1-benzopyran-2-one **3f** could be obtained in the highest yield with the best selectivity in DMSO at 10% (water/[water+DMSO], v/v) water content. However, the yield of **3f** decreased evidently once the water content surpassed 10%. When the water content reached 30%, the by-product hemiketal **4** could be formed dominately. All the results indicated that water was obviously essential in the biocatalytic domino reaction. The chemoselectivity could be achieved by adjusting the water

content in organic solvent. Thus, the optimum water content for the synthesis of 2*H*-1-benzopyran-2-one **3f** was 10%.

Temperature also plays an important role in enzyme catalyzed reaction, due to their effects on the enzyme stability and reaction rate. Thus, a temperature screen was performed from 30 to 80 °C using the model reaction of salicylaldehyde with ethyl acetoacetate (Fig. 4). It was found that the yield of the by-product hemiketal **4** was slightly higher than the desired product 2*H*-1-benzopyran-2-one **3f** at 35 °C. However, once the temperature reached 55 °C, **3f** could be obtained in the highest yield with the best selectivity. The results indicated that the chemoselectivity also could be achieved by adjusting the temperature. Thus, the optimum temperature for the synthesis of **3f** was 55 °C.

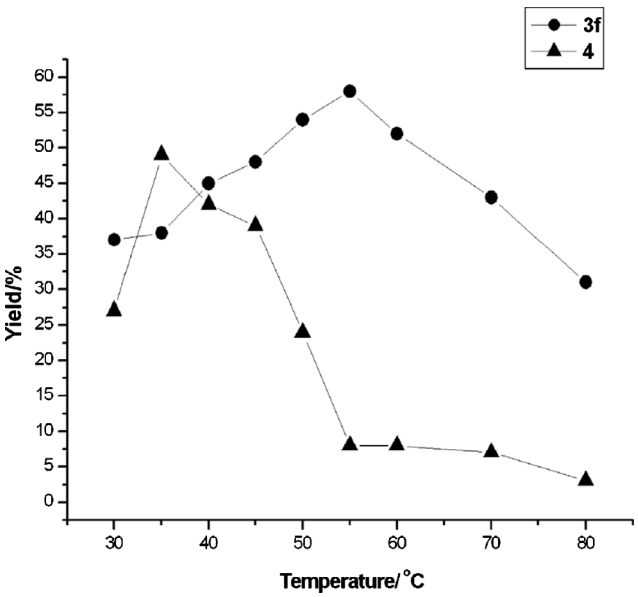


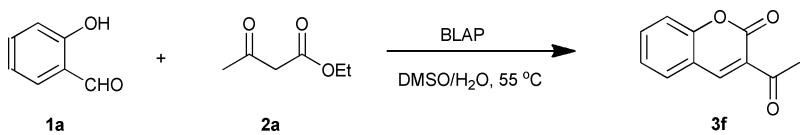
Fig. 4 The influence of temperature on BLAP-catalyzed domino reaction. Conditions: BLAP (40 mg ml^{-1}), salicylaldehyde (200 mg, 1.64 mmol), ethyl acetoacetate (4.90 mmol), deionized water (0.5 ml) and DMSO (4.5 ml) at specified temperature for 45 h.

Next, we investigated the effects of BLAP loading and molar ratio of substrates on the enzymatic domino reaction (Table 1). The results showed that 20 mg ml^{-1} of BLAP loading and the molar ratio 1 : 3 of salicylaldehyde to ethyl acetoacetate were the optimum conditions.

To demonstrate the specific catalytic effect of BLAP on the domino Knoevenagel/intramolecular transesterification sequence under optimized conditions, we performed some control experiments (Table 2). The reaction between salicylaldehyde and ethyl benzoylacetate catalyzed by BLAP under the optimized conditions gave product **3d** in satisfied yield of 73% after 48 h (Table 2, entry 1), but no product was detected in the absence of enzyme after 48 h (Table 2, entry 2). This clearly indicated that BLAP had specific catalytic effect on the reaction. Furthermore, since BLAP is a Ca^{2+}-dependent enzyme, EDTA (ethylene diamine tetraacetic acid) was used to denature the enzyme. We found that the EDTA-denatured BLAP completely lost its catalytic activity for the domino reaction (Table 2, entry 3), which demonstrated that the catalytic activity of BLAP was not simply caused by the amino acids of the enzyme, and the native tertiary structure of the enzyme might also be necessary for this catalysis. In addition, to further prove that the catalytic activity of BLAP for the domino reaction did not arise from unspecific protein-derived activation of the reagents by, *e.g.*, the surface of the enzyme. The experiment catalyzed by inhibited enzyme was conducted, and a complete inhibition of the catalytic activity of BLAP was observed by using serine protease inhibitor PMSF (phenylmethanesulfonyl fluoride) (Table 2, entry 4). This indicated that the enzyme catalyzed domino reaction occurred in the active site of BLAP or in close proximity similar to other reported enzymes.[13] On the other hand it could be assumed that the protein surface of BLAP was predominantly catalytically inactive in the process. Above experiments also excluded the possibility that the catalysis was caused by the impurities of the enzyme.

Furthermore, in order to verify that the second step (the intramolecular transesterification) was also catalyzed by BLAP, we attempted to synthesize the intermediate A (Scheme 1). Unexpectedly, we could not obtain intermediate A although

Table 1 Effects of BLAP loading and molar ratio of salicylaldehyde to ethyl acetoacetate on the yield of **3f** in the domino reaction[a]

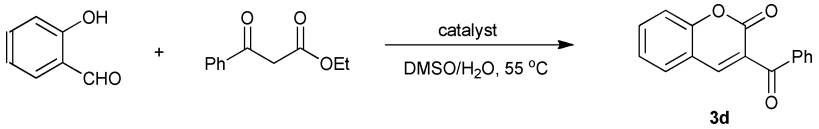


1a **2a** **3f**

Entry	**1a** : **2a**	BLAP (mg ml^{-1})	Time [h]	Yield **3f** [%][b]
1	1 : 3	10	112	51
2	1 : 3	20	112	58
3	1 : 3	30	112	56
4	1 : 3	40	112	55
5	1 : 3	50	112	43
6	1 : 1	20	80	47
7	1 : 2	20	80	50
8	1 : 3	20	80	58
9	1 : 4	20	80	48

[a] All reactions were carried out using salicylaldehyde (200 mg, 1.64 mmol), deionized water (0.5 ml) and DMSO (4.5 ml) at 55 °C. [b] Yield of the isolated product after chromatography on silica gel.

Table 2 Control experiments for the reaction of salicylaldehyde and ethyl benzoylacetate[a]

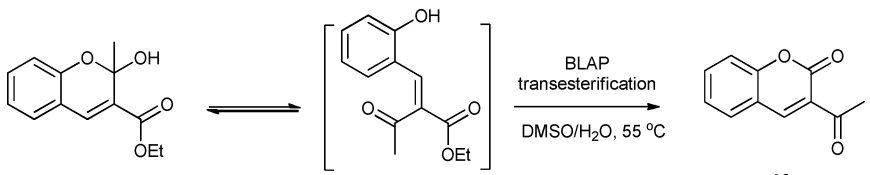


3d

Entry	Catalyst	Time [h]	Yield [%][b]
1	BLAP	48	73
2	no enzyme	48	ND
3	BLAP denatured with EDTA[c]	48	ND
4	BLAP inhibited with PMSF[d]	48	ND

[a] All reactions were carried out using salicylaldehyde (200 mg, 1.64 mmol), ethyl benzoylacetate (4.90 mmol), deionized water (0.5 ml), DMSO (4.5 ml) and catalyst (20 mg ml^{-1}) at 55 °C. [b] Yield of the isolated product after chromatography on silica gel. [c] Pre-treated with EDTA at 100 °C for 24 h. [d] Pre-treated with PMSF at 25 °C for 24 h.

Table 3 Control experiments of intramolecular transesterification[a]



4 A 3f

Entry	Catalyst	Time [h]	Yield [%][b]
1	BLAP	48	75
2	no enzyme	48	52
3	BLAP denatured with EDTA[c]	48	53
4	BLAP inhibited with PMSF[d]	48	49

[a] All reactions were carried out using hemiketal **4** (38 mg), deionized water (0.05 ml), DMSO (0.45 ml) and catalyst (20 mg ml^{-1}) at 55 °C. [b] Yield of the isolated product after chromatography on silica gel. [c] Pre-treated with EDTA at 100 °C for 24 h. [d] Pre-treated with PMSF at 25 °C for 24 h.

we have tried many different methods, but instead hemiketal **4** was received. It was apparent that the intermediate A is unstable and so hemiketal **4** is easily formed. Because there is a chemical equilibrium between the intermediate A and hemiketal **4**, we used **4** to conduct the same set of control experiments presented in Table 2 to demonstrate the catalytic effect of BLAP on the intramolecular transesterification (Table 3). The intramolecular transesterification catalyzed by BLAP under the optimized conditions gave product **3f** in satisfied yield of 75% after 48 h (Table 3, entry 1). In the absence of BLAP, **3f** was only obtained in a low yield of 52% (Table 3, entry 2). The experiments using denatured and inhibited BLAP gave the results similar to the blank experiment (Table 3, entries 3 and 4). It is obvious that BLAP promoted the intramolecular transesterification. Based on the results of control experiments, the BLAP catalyzed domino reaction was validated.

We then detected the reaction process, and the time courses were shown in Fig. 5. A quick increase of product **3f** and by-product **4** was observed during 0–10 h. After 10 h, the concentration of **3f** increased continually, but the concentration of **4** decreased slightly. After 50 h, the product **3f** reached the equilibrium, and the concentration of **4** and salicylaldehyde did not continue to decrease. The results showed that the catalytic activity of the BLAP for Knoevenagel/intramolecular transesterification is higher than the activity for Knoevenagel/hemiketalization under the optimal conditions. It was also confirmed that the synthesis of 2*H*-1-benzopyran-2-one

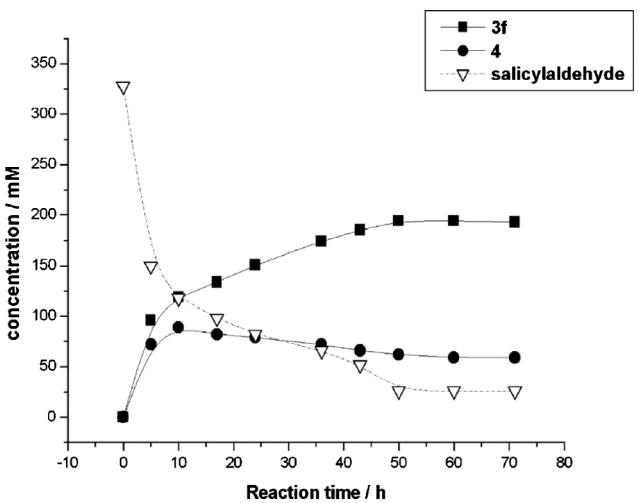
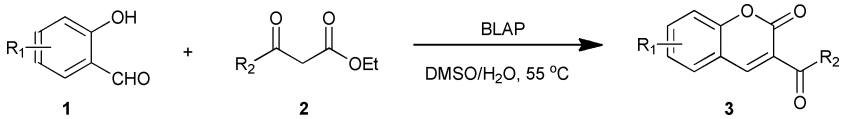


Fig. 5 Concentration curves of **3f**, **4** and salicylaldehyde in the process of BLAP catalyzed two-step domino reaction. Conditions: BLAP (20 mg ml^{-1}), salicylaldehyde (200 mg, 1.64 mmol), ethyl acetoacetate (4.90 mmol), deionized water (0.5 ml) and DMSO (4.5 ml) at 55 °C. 9 reactions were set up at the same time, and each reaction was terminated at a specified time. The concentrations were calculated by isolated weights of **3f**, **4** and salicylaldehyde.

Table 4 Substrate scope of the domino Knoevenagel/intramolecular transesterification reaction[a]



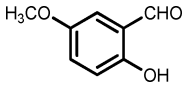
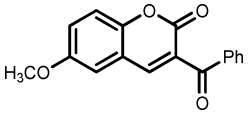
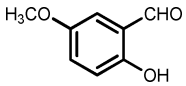
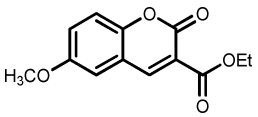
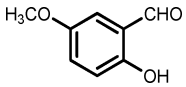
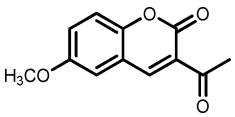
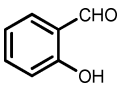
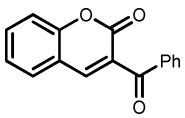
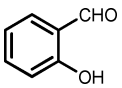
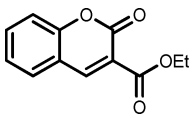
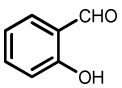
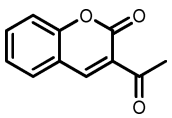
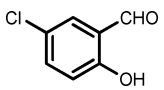
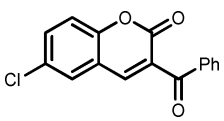
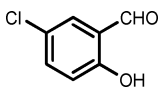
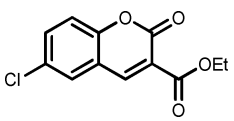
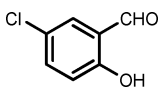
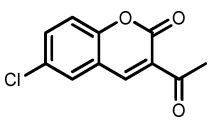
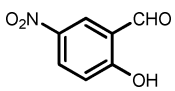
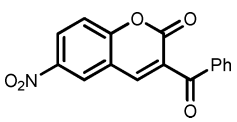
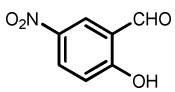
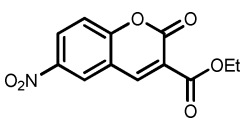
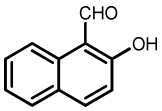
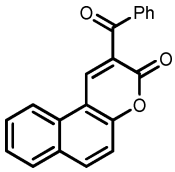
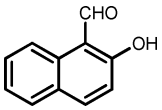
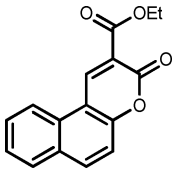
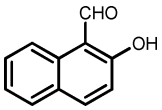
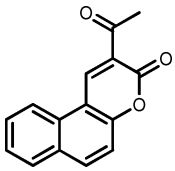
Entry	1	R_2	Product	Prod. No.	Time [h]	Yield [%][b]
1		Ph		**3a**	48	75
2		OEt		**3b**	163	68
3		CH_3		**3c**	117	60
4		Ph		**3d**	48	73
5		OEt		**3e**	163	69
6		CH_3		**3f**	48	58
7		Ph		**3g**	48	45
8		OEt		**3h**	117	56
9		CH_3		**3i**	117	30
10		Ph		**3j**	48	48
11		OEt		**3k**	117	25

Table 4 *(Contd.)*

Entry	1	R_2	Product	Prod. No.	Time [h]	Yield [%][b]
12		Ph		**3l**	192	18
13		OEt		**3m**	192	10
14		CH_3		**3n**	288	48

[a] All reactions were carried out using salicylaldehyde derivative (1.64 mmol), β-keto ester (4.90 mmol), deionized water (0.5 ml), DMSO (4.5 ml) and BLAP (20 mg ml^{-1}) at 55 °C. [b] Yield of the isolated product after chromatography on silica gel.

derivatives by domino Knoevenagel/intramolecular transesterification could be catalyzed by BLAP.

Finally, to investigate the generality and scope of this new enzyme activity, some other salicylaldehyde derivatives and β-keto esters were used to expand upon this BLAP catalyzed domino reaction under the optimized conditions (Table 4). It could be seen that a wide range of substrates could be accepted by the enzyme. Both electron-donating and electron-withdrawing functionalities were compatible (Table 4, entries 1–11). It was clear that the electronic factors of the substituents on the benzene ring appeared to have great effects on the yields of the reaction. When salicylaldehydes containing electron-donating substituents were condensed with different β-keto esters by this procedure, the products were obtained in good yields of 60–75% (Table 4, entries 1–3). In contrast, the substituted salicylaldehydes bearing electron-withdrawing substituents gave products in lower yields of 25–56% (Table 4, entries 7–11). Moreover, compared with other aromatic aldehydes, the yields of the reaction of 2-hydroxy-1-naphthaldehyde with β-keto esters were quite low probably due to the steric hindrance effect of naphthyl (Table 4, entries 12–14). In addition, ethyl benzoylacetate generally offered higher yields in shorter reaction times than ethyl acetoacetate and diethyl malonate when reacting with salicylaldehyde and substituted salicylaldehyde (Table 4, entries 1, 4, 7, 10). This was probably because ethyl benzoylacetate more easily produces stable carbanion with strong electron withdrawing character than ethyl acetoacetate and diethyl malonate. However, when reacting with 2-hydroxy-1-naphthaldehyde, ethyl benzoylacetate gave much lower yield than ethyl acetoacetate (Table 4, entries 12 and 14) due to the steric hindrance.

3. Conclusion

In conclusion, the synthesis of 2*H*-1-benzopyran-2-one derivatives has been achieved using a BLAP catalyzed domino Knoevenagel/intramolecular transesterification reaction between various salicylaldehydes and β-keto esters. Notably, we have demonstrated an example of the combination of a domino reaction with biocatalysis, in which the single enzyme displayed an activity to catalyze two step conversions. Furthermore, the control of enzymatic chemoselectivity between the Knoevenagel/intramolecular transesterification and the Knoevenagel/hemiketalization could be realized by adjusting parameters including solvent, water content and temperature. This BLAP catalyzed selective domino reaction provided an alternative synthetic method for 2*H*-1-benzopyran-2-one derivatives. It is also a novel case of unnatural activities of existing enzymes.

Experimental

General information

Alkaline protease from *Bacillus licheniformis* 2709 (200 U/mg. One unit of activity is the amount of enzyme that liberates 1.0 μg of tyrosine from casein per minute at 40 °C and pH 10.5) was purchased from Wuxi Xuemei Enzyme Co. Ltd.

(WuXi, China). Unless otherwise noted, all reagents were obtained from commercial suppliers and were 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 using100–200 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 300 MHz spectrometer. Melting points were determined on X-4 digital display micro melting point apparatus and were uncorrected.

General experimental procedure for the domino Knoevenagel/intramolecular transesterification reaction (products 3a–n)

A 25 ml round-bottomed flask was charged with BLAP (20 mg ml^{-1}), DMSO (4.5 ml) and deionized water (0.5 ml), to which the salicylaldehyde derivative (1.64 mmol) and β-keto ester (4.90 mmol) were introduced. The resulting mixture was stirred for the specified amount of time at 55 °C. The reaction was terminated by filtering the enzyme. CH_2Cl_2 was used to wash the filter paper to assure that products obtained were all dissolved in the filtrate. 20 ml of water was then added to the filtrate, and the filtrate was extracted three times with 20 ml of CH_2Cl_2. The combined extracts were dried over anhydrous Na_2SO_4 and the solvents were then removed under reduced pressure. The crude products were purified by column chromatography with petroleum ether/ethyl acetate as eluent.

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