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α-Chymotrypsin-Catalyzed Povarov Reaction: One-Pot Synthesis of Tetrahydroquinoline Derivatives

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Abstract: The three-component one-pot Povarov reaction for the synthesis of tetrahydroquinoline derivatives was catalyzed by α-chymotrypsin from bovine pancreas (BPC) for the first time. The products were obtained in moderate to good yields with a wide range of substrates. Based on the control and comparison experiments of natural and promiscuous activities, a tentative mechanism was discussed. Molecular docking and energy calculation of quantum chemistry were used to explain the experimental results in theory. As a novel case of enzyme catalytic promiscuity, this work expands the application of BPC. Exploring the untapped catalytic promiscuity of natural enzymes may also provide useful information about enzyme evolution.

Key words: Povarov reaction; tetrahydroquinoline derivatives; *α*-chymotrypsin; enzyme catalytic promiscuity; molecular docking

Introduction

The tetrahydroquinoline skeleton is an important structural unit found in many biologically active natural products and synthetic pharmaceutical preparations.[1] Tetrahydroquinoline derivatives display a broad range of biological, medicinal, and pharmacological properties and are constituents of bradykinin antagonist,[2] antiallergic agent,[3] antitumor agent,[4] and NMDA receptor antagonist.[5] Among the currently available synthetic methodologies, the Povarov reaction,[6] an inverse electron-demand aza-Diels-Alder reaction between N-arylimines and electron-rich olefins, is one of the most facile and synthetically practical approach to the synthesis of tetrahydroquinolines in high structural diversity. A series of Lewis acids such as $GdCl_3$,[7] SmI_2,[8] lanthanide complexes,[9] chiral phosphoric acid,[10] and selectfluor[11] have been used as efficient catalysts for this reaction. However, many of these catalysts are not fully satisfied with operational simplicity and mild reaction conditions, and some of the catalysts suffer disadvantages such as expensiveness, moisture-sensitivity, multistep synthesis, and toxicity to the environment and humans. Therefore, the development of sustainable, environmentally-benign, and cost-efficient catalysts for the synthesis of tetrahydroquinoline derivatives still remains a significant challenge.

Enzymes as sustainable, eco-friendly and biodegradable catalysts have attracted increasing attention from organic chemists. Enzyme catalytic promiscuity, which means the ability of active site of enzyme to catalyze distinctly different chemical transformations no matter natural or non-natural substrates, contributes to the natural evolution of new enzymes and brings new

catalysts for organic synthesis.[12] Some enzymes have exhibited their promiscuity through catalyzing the formation of C-C and C-heteroatom bonds,[13] such as the aldol reactions,[14] Markovnikov additions,[15] Michael additions,[16] Mannich reactions,[17] the asymmetric synthesis of α-aminonitrile amides,[18] and multi-component cascade or domino reactions[19] etc. The study on enzyme catalytic promiscuity has expands rapidly in recent years. However, at the present stage, no general methods are available to profile enzyme catalytic promiscuity. Thus, it is necessary to explore the enzyme catalytic promiscuity for existing enzymes case by case. It is especially important to investigate the new activities for those enzymes which are well-known and widely used in industry.

Herein we report a novel discovery that α-chymotrypsin could promote the one-pot, three-component Povarov reaction for the synthesis of tetrahydroquinoline derivatives. α-Chymotrypsin from bovine pancreas (BPC), a serine protease that hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu) on the carboxyl end of the peptide bond, is one of the most thoroughly studied enzymes. Moreover, BPC has been cheaply available for many years because of the simple procedure for purification, good storage stability, and the abundant supply of the starting materials from slaughter houses.[20]

Results and discussion

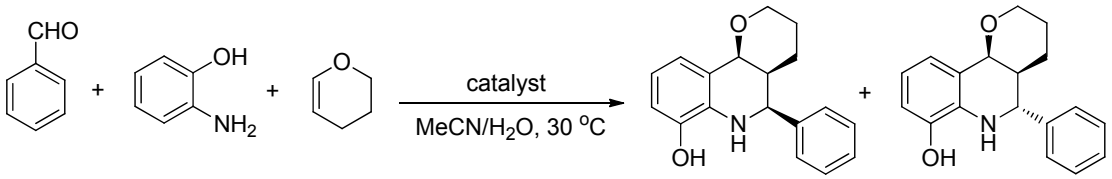
Povarov reaction requests N-arylimines and electron-rich olefins to take place an inverse electron-demand aza-Diels-Alder reaction. In consideration of the fact that aromatic aldehydes can form stable imines with N-arylamines, benzaldehyde (**1a**) was selected as an aldehyde substrate. With a view to getting better interaction between the substrate and the enzyme via the hydroxyl

group, 2-aminophenol (**2**) was chosen as a N-arylamine substrate. Moreover, 2,3-dihydropyran (**3**) as one of readily available electron-rich olefins was used as an olefin substrate. Thus, the one-pot Povarov reaction of benzaldehyde (**1a**), 2-aminophenol (**2**) and 2,3-dihydropyran (**3**) was used as a model reaction. Based on our previous experience in the enzyme catalytic promiscuity, the mixed solvents of MeCN/water were temporarily chosen as the medium. Several commercially available enzymes were screened, and the results were summarized in **Table 1**. Among the tested enzymes, only BPC displayed catalytic effect on the model reaction, giving the isolated products (**4a** + **5a**) in a yield of 31% with 82:18 dr (*trans:cis*) (**Table 1**, entry 8). For the reaction with other enzymes only a trace amount of product was detected on TLC (**Table 1**, entries 1-7).

Next, to verify the specific catalytic effect of BPC on the Povarov reaction, some control experiments were performed (**Table 1**, entries 9-14). In the absence of BPC, only a trace amount of product was detected on TLC (**Table 1**, entry 9), indicating that BPC preparation had a catalytic effect on the Povarov reaction. Then, the albumins from chicken egg white and bovine serum were used as non-enzyme proteins to catalyze the model reaction separately, and only trace amounts of products were observed (**Table 1**, entries 10 and 11), which excluded the possibility of protein catalysis, meaning that catalysis was not simply a result of the amino acid residues on the surface of the protein. Furthermore, urea as a denaturing agent was used to pretreat the BPC, and it was found that the urea-denatured BPC lost its activity in the reaction (**Table 1**, entry 12). Meanwhile, it was proved that urea alone could not catalyze this transformation (**Table 1**, entry 13). These results indicated that the specific natural fold of BPC was responsible for its activity in the Povarov reaction. Next, since BPC is a serine protease, phenylmethylsulfonyl fluoride (PMSF), an irreversible inhibitor of serine proteases, was used to pretreat it. The data showed that the

PMSF-inhibited BPC lost its activity in the model reaction (**Table 1**, entry 14), suggesting that the Povarov reaction may take place on the catalytic site of BPC, and the active site residues, especially the serine, may be responsible for this promiscuous reaction.

Table 1. Enzyme screening and control experiments for the BPC-catalyzed Povarov reaction[a]



1a **2** **3** ***cis*** **4a** ***trans*** **5a**

Entry	Catalyst	Yield (%)[b]	dr (*trans/cis*)[c]
1	*α*-amylase from hog pancreas	trace	--
2	*β*-glucanase from *trichoderma longibriatum*	trace	--
3	lipase from *Candida rugosa*	trace	--
4	lipase, immobilized on immobead 150, from *Psedomnas cepacia*	trace	--
5	trypsin, from porcine pancreas	trace	--
6	proteinase, from *Aspergillus melleus*	trace	--
7	papain, from *Carica Papaya*	trace	--
8	BPC	31	82/18
9	none	trace	--
10	albumin from chicken egg white	trace	--
11	albumin from bovine serum	trace	--
12	BPC denatured with urea[d]	trace	--
13	urea[e]	trace	--
14	BPC inhibited with PMSF[f]	trace	--
15	another BPC preparation as comparison (TLCK-chymotrypsin)[g]	99[h]	89/11[i]
16	BPC[g]	80[h]	89/11[i]

[a] Unless otherwise noted, reaction conditions: **1a** (0.5 mmol), **2** (0.5 mmol), **3** (2.0 mmol), and catalyst (25 mg) in MeCN (0.90 mL) and deionized water (0.10 mL) at 30 °C for 120 h. [b] Yield of the isolated products (**4a** + **5a**) after silica gel chromatography. [c] Calculated according to the isolated weights of **4a** and **5a**. [d] BPC (25 mg) in 0.89 M urea solution [urea (80 mg) in deionized water (1.5 mL)] was stirred at 30 °C for 24 h, and then the water was removed under reduced pressure. The residue was used as a catalyst in the reaction. [e] Urea (80 mg) was used instead of BPC. [f] BPC (25 mg) in 0.019 M PMSF solution (in THF) was stirred at 30 °C for 24 h, and then THF

was removed under reduced pressure. The residue was used as a catalyst in the reaction. [g] **1a** (0.125 mmol), **2** (0.250 mmol), **3** (1.125 mmol), and BPC (10 mg) in MeCN (0.225 mL) and deionized water (0.025 mL) at 30 °C for 96 h. [h] Yield of the products (**4a** + **5a**) determined by HPLC. [i] Determined by HPLC.

The BPC we used is a commercial enzyme preparation, a lyophilized powder [Type II, molecular weight 25 kDa, 94.1% protein (UV); 45.2 units/mg protein; purchased from Sigma-Aldrich, product number: C4129; lot number: 060M7007V]. It was produced from 3×crystallized chymotrypsinogen. The quality level was premium. To rule out the possibility of the catalysis from some impure proteins, we checked the purity of BPC by SDS-PAGE (for the SDS-PAGE image and experiment details, please see the Supplementary Information). It showed a very clear BPC band that had a molecular weight of 25 kDa. In addition to this major band, a 15 kDa band could be seen, which was one of peptide chains of BPC decomposed in the course of SDS-PAGE (BPC consists of 241 amino acid residues. The molecule has three peptide chains: an A chain of 13 residues, a B chain of 131 residues, and a C chain of 97 residues). Moreover, to further confirm the catalytic effect of BPC on the Povarov reaction, another BPC preparation (alternative name: TLCK-chymotrypsin) [TLCK treated to inactivate residual trypsin activity (preparation note: TLCK treatment inactivates trypsin which may be present in chymotrypsin, without affecting the chymotrypsin activity), Type VII, molecular weight 25 kDa, essentially salt-free, lyophilized powder, 94% protein (UV); 64 units/mg protein; purchased from Sigma-Aldrich, product number: C3142; lot number: SLBK5967V] was used as a comparison. The model Povarov reaction with this TLCK-chymotrypsin gave the products (**4a** + **5a**) in a yield of 99% with 89:11 dr (*trans:cis*) determined by HPLC, under the optimized molar ratio of

substrates with increased enzyme concentration (**Table 1**, entry 15). Under the same reaction conditions, the BPC preparation gave the products (**4a** + **5a**) in a yield of 80% with 89:11 dr (*trans:cis*) (**Table 1**, entry 16). These comparison experiments further confirmed that BPC indeed catalyzed the Povarov reaction.

We were very curious about the relation between "natural" and "promiscuous" activities of BPC. As one of proteolytic enzymes, BPC facilitates the cleavage of peptide bonds by a hydrolysis reaction. On the other hand, like many other proteases, BPC will also hydrolyze ester bonds, which is the most convenient reaction for the investigation of BPC catalysis by virtue of that enabled the use of substrate analogs such as N-benzoyl-L-tyrosine ethyl ester (BTEE) for BPC assay.[21] Therefore, catalyzing the hydrolysis of BTEE could reflect the "natural" activity of BPC. With UV spectrophotometer, enzymatic assay of BPC on the hydrolysis of BTEE was performed, which showed an activity of 45.2 units/mg protein (**Table 2**, entry 1). Since the urea-denatured BPC lost its catalytic ability for the Povarov reaction (**Table 1**, entry 12), as a comparison we checked its hydrolysis activity on BTEE, and only 3.8 units/mg protein of activity was detected (**Table 2**, entry 2). Furthermore, because PMSF inhibited the catalytic activity of BPC towards Povarov reaction (**Table 1**, entry 14), we tested the hydrolysis activity of the PMSF-inhibited BPC on BTEE, and only 3.5 units/mg protein of activity was observed (**Table 2**, entry 3). The above results indicated that both urea and PMSF caused BPC lost its activity not only on hydrolysis reaction but also on Povarov reaction. These comparison experiments further suggested that like the "natural" activity, the "promiscuous" activity may also take place in the active site of BPC.

Next, to verify the influence of organic solvent/water system on the BPC activity, the

enzymatic assay for the hydrolysis of BTEE was investigated using the BPC pretreated in MeCN/water [$H_2O/(H_2O+MeCN)$ = 0.2, v/v] at 38 °C (Our optimized conditions for the BPC-catalyzed Povarov reaction. For details, please see the Supplementary Information) for different times. As can be seen from **Table 2**, after pretreated in MeCN/water for 24 h and 60 h, the BPC still maintained most activity (26.0 and 22.6 units/mg protein, respectively) (**Table 2**, entries 4 and 5), while only a very low activity (6.6 units/mg protein) was observed after pretreated in MeCN/water for 120 h (**Table 2**, entry 6). These data demonstrated that BPC can tolerate the MeCN/water system for a reasonable time. (For the UV spectrophotometric determination of the kinetics of BPC catalyzed-hydrolysis of BTEE, please see the Supplementary Information).

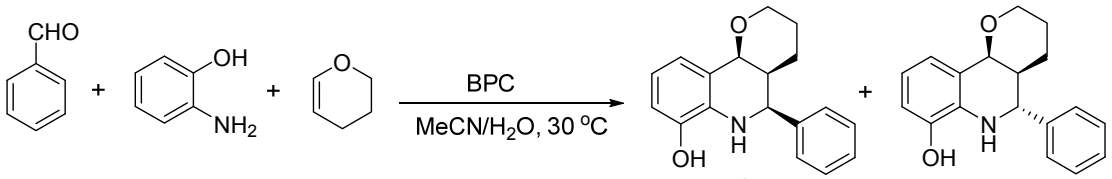
Table 2. The enzyme assay of BPC on the hydrolysis of BTEE [a]

Entry	Enzyme	Activity (units/mg protein)
1	BPC	45.2
2	urea-denatured BPC[b]	3.8
3	PMSF-inhibited BPC[c]	3.5
4	BPC pretreated in MeCN/water for 24 h[d]	26.0
5	BPC pretreated in MeCN/water for 60 h[d]	22.6
6	BPC pretreated in MeCN/water for 120 h[d]	6.6

[a] Enzyme assay: a solution of Tris-HCl buffer (80 mM, 1.42 mL, pH 7.8), BTEE (1.18 mM, 1.40 mL), calcium chloride (2 M, 0.08 mL) and BPC (0.1 mg/mL, 0.10 mL) was added to the cuvette and measured at 256 nm for 360 s by UV spectrophotometric (for details, please see the Supplementary Information). [b] Urea-denatured BPC (see **Table 1**, entry 12). [c] PMSF-inhibited BPC (see **Table 1**, entry 14). [d] The mixture of BPC (50 mg) in MeCN (0.80 mL) and deionized water (0.20 mL) was stirred at 38 °C for 24 h, 60 h or 120 h, and then BPC was collected through filtration, which was used for the enzyme assay.

In order to further improve the yield of BPC-catalyzed Povarov reaction, the effect of molar ratio of substrates on the model reaction was investigated (**Table 3**). It can be seen that the molar ratio of substrates had a remarkable influence on the yield and selectivity of the reaction. When the ratio of **1a** / **2** / **3** was 1:2:9 (**Table 3**, entry 13), the product was obtained in the best yield of 50% with 82/18 dr (*trans:cis*). Thus, the molar ratio (**1a** / **2** / **3** = 1:2:9) was chosen as the optimal ratio for further studies.

Table 3. Effect of molar ratio of substrates on the BPC-catalyzed Povarov reaction[a]



1a **2** **3** *cis* **4a** *trans* **5a**

Entry	**1a** / **2** / **3**	**1a** (mmol)	**2** (mmol)	**3** (mmol)	Yield (%)[b]	dr (*trans/cis*)[c]
1	1:1:4	0.5	0.5	2.0	31	82/18
2	2:1:4	1.0	0.5	2.0	30	67/33
3	3:1:4	1.5	0.5	2.0	23	57/43
4	1:2:4	0.5	1.0	2.0	38	82/18
5	1:3:4	0.5	1.5	2.0	22	74/26
6	1:2:1	0.5	1.0	0.5	11	82/18
7	1:2:2	0.5	1.0	1.0	17	91/9
8	1:2:3	0.5	1.0	1.5	30	87/13
9	1:2:5	0.5	1.0	2.5	35	87/13
10	1:2:6	0.5	1.0	3.0	38	87/13
11	1:2:7	0.5	1.0	3.5	48	83/17
12	1:2:8	0.5	1.0	4.0	48	90/10
13	1:2:9	0.5	1.0	4.5	50	82/18
14	1:2:10	0.5	1.0	5.0	49	82/18
15	1:2:12	0.5	1.0	6.0	48	82/18

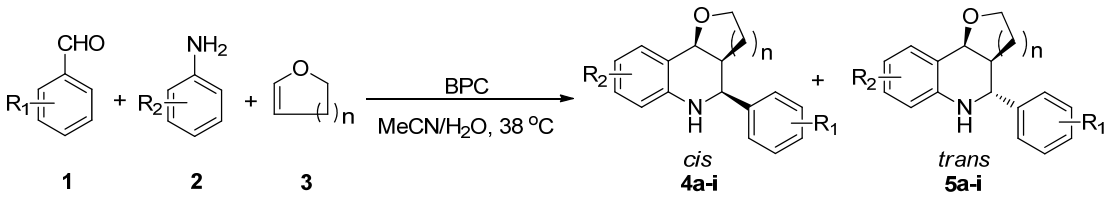
[a] Reaction conditions: **1a**, **2**, **3**, and BPC (25 mg) in MeCN (0.90 mL) and deionized water (0.10 mL) at 30 °C for 120 h. [b] Yield of the isolated products (**4a** + **5a**) after silica gel chromatography. [c] Calculated according to the

isolated weights of **4a** and **5a**.

The effect of other parameters (solvents, water contents, BPC concentration, temperature, and phosphate buffer solution) on the model Povarov reaction was also investigated (For details, please see the Supplementary Information). The experiments showed that using phosphate buffer solution to replace the water content in the reaction medium did not improve the reaction results. The optimized reaction conditions were found to consist of the following: MeCN as a solvent, water content of 0.2 [H_2O/(H_2O+MeCN), v/v], BPC concentration of 50 mg/mL, and temperature of 38 °C.

To check the generality and scope of the BPC-catalyzed three-component Povarov reaction, some other substrates were tested under the optimized conditions. As shown in **Table 4**, aromatic aldehydes bearing either electron-withdrawing or electron-donating substituents could participate in the reaction smoothly. 2-Aminophenol, aniline and 4-toluidine could be used as arylamines. When 2,3-dihydropyran was used, the *trans*-isomers were obtained as the major products (**Table 4**, entries 1-7). However, using 2, 3-dihydrofuran gave the *cis*-isomers as the major products (**Table 4**, entries 8 and 9). Among the tested reactions, the highest yield of 83% and the best diastereoselectivity of 92:8 (*trans:cis*) were received. All the *trans* and *cis* products could be easily separated by silica gel column chromatography. Unfortunately, no obvious enantiomeric excess of the products were observed by chiral HPLC analysis.

Table 4. Substrate scope of the BPC-catalyzed Povarov reaction[a]



1 2 3 **4a-i** **5a-i**

Entry	R_1	R_2	n	Product	Time (h)	Yield (%)[b]	dr (*trans/cis*)[c]
1	H	2-OH	2	**4a**, **5a**	60	80	89/11
2	H	H	2	**4b**, **5b**	84	80	85/15
3	4-NO_2	2-OH	2	**4c**, **5c**	96	83	89/11
4	4-Cl	2-OH	2	**4d**, **5d**	96	81	92/8
5	4-F	2-OH	2	**4e**, **5e**	108	83	91/9
6	4-Me	2-OH	2	**4f**, **5f**	96	78	88/12
7	H	4-Me	2	**4g**, **5g**	96	74	88/12
8	H	H	1	**4h**, **5h**	30	70	29/71
9	H	4-Me	1	**4i**, **5i**	12	66	38/62

[a] Reaction conditions: **1** (0.5 mmol), **2** (1.0 mmol), **3** (4.5 mmol), and BPC (50 mg) in MeCN (0.80 mL) and deionized water (0.20 mL) at 38 °C. [b] Yield of the isolated products [**4(a-i)** + **5(a-i)**] after silica gel chromatography. [c] Calculated according to the isolated weights of **4 (a-i)** and **5 (a-i)**.

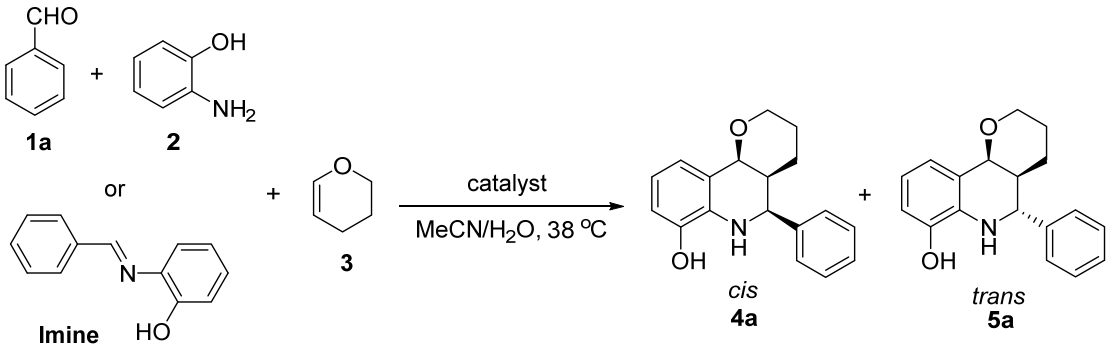
The mechanism of the Povarov reaction between N-arylimines and electron-rich olefins has been a topic of controversy for decades. However, more evidence supports stepwise process rather than a concerted manner.[22] The stepwise process can be explained as a Mannich reaction of *in situ* generated imine attacked by the electron rich nucleophile, and followed by an intramolecular cyclization. To verify if there is any difference between utilizing preformed imine and *in situ* generated imine for the BPC-catalyzed Povarov reaction, some comparison experiments were performed using the model reaction. Firstly, aldehyde and amine were replaced by imine, and the reaction of imine (0.5 mmol) and 2,3-dihydropyran **3** (4.5 mmol) gave the product in a yield of 43% with 76:24 dr (*trans:cis*) (**Table 5**, entry 1). As comparison, the same amount of benzaldehyde **1a** (0.5 mmol) and 2-aminophenol **2** (0.5 mmol) were used in the Povarov reaction,

and an extremely similar result was obtained (**Table 5**, entry 2). In addition, to further confirm this observation, we performed the comparison experiments with the optimized molar ratio of substrates. The reaction of imine (0.5 mmol), 2-aminophenol **2** (0.5 mmol), and 2,3-dihydropyran **3** (4.5 mmol) provided the product in a yield of 78% with 89:11 dr (*trans:cis*) (**Table 5**, entry 3), which was almost the same to the results from the reaction of benzaldehyde **1a** (0.5 mmol), 2-aminophenol **2** (1.0 mmol), and 2,3-dihydropyran **3** (4.5 mmol) (**Table 5**, entry 4). The above comparison experiments indicated that there was no difference between using preformed imine and *in situ* generated imine for the BPC-catalyzed Povarov reaction. In other words, it could be inferred that the critical role of BPC was to catalyze the reaction between imine and 2,3-dihydropyran.

Aspartate-102, histidine-57, and serine-195 have been identified as the residues of active centre, forming the Asp-His-Ser catalytic triad in α-chymotrypsin.[23] We were curious about whether the free amino acid Asp, His or Ser could catalyze the Povarov reaction. Thus, some comparison experiments were conducted using these amino acids (10 mol %, about 25 times of BPC in mol) to replace BPC under the optimized conditions. The reaction with Asp gave the product in a yield of 45% with 79:21 dr (*trans:cis*) (**Table 5**, entry 5), and a lower yield of 20% with 69:31 dr (*trans:cis*) was obtained with Ser (**Table 5**, entry 7). His did not promote the reaction (**Table 5**, entry 6). The above experiments showed that Asp, and Ser could catalyze the Povarov reaction, but catalytic efficiency and selectivity were much lower than BPC, and His as a basic amino acid could not catalyze the reaction. Moreover, Asp (10 mol %) + His (10 mol %) + Ser (10 mol %) were used together to catalyze the Povarov reaction, and the product was only received in a low yield of 24% with 74:26 dr (*trans:cis*) (**Table 5**, entry 8). It seemed like that

acidic Asp formed a salt with basic His, and the reaction was mainly promoted by Ser. From the results of the above experiments, it could be inferred that the specific fold of BPC and the location of the Asp, His, and Ser residues in the active centre are crucial for this enzymatic Povarov reaction.

Table 5. Comparison experiments [a]



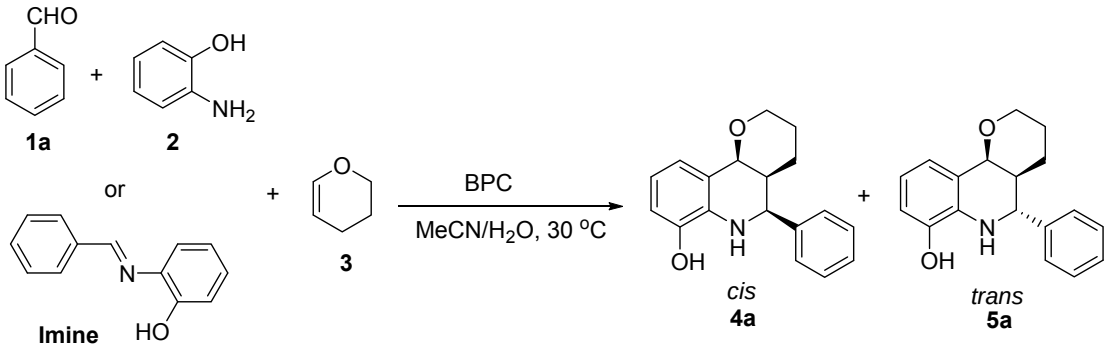
Entry	Imine (mmol)	**1a** (mmol)	**2** (mmol)	**3** (mmol)	Catalyst[b]	Yield[c] (%)	dr (*trans/cis*)[d]
1	0.5	--	--	4.5	BPC	43	76/24
2	--	0.5	0.5	4.5	BPC	44	76/24
3	0.5	--	0.5	4.5	BPC	78	89/11
4	--	0.5	1.0	4.5	BPC	80	89/11
5	--	0.5	1.0	4.5	Asp	45	79/21
6	--	0.5	1.0	4.5	His	trace	--
7	--	0.5	1.0	4.5	Ser	20	69/31
8	--	0.5	1.0	4.5	Asp+His+Ser	24	74/26

[a] Unless otherwise noted, reaction conditions: substrates and catalyst in MeCN (0.80 mL) and deionized water (0.20 mL) at 38 °C for 60 h. [b] For entries 1-4: BPC (50 mg, about 0.002 mmol). For entries 5-7: Asp, His or Ser (0.05 mmol). For entry 8: Asp (0.05 mmol) + His (0.05 mmol) + Ser (0.05 mmol). [c] Yield of the isolated products (**4a** + **5a**) after silica gel chromatography. [d] Calculated according to the isolated weights of **4a** and **5a**.

Furthermore, the kinetic parameters of the BPC-catalyzed Povarov reaction, Michaelis constant Km and catalytic constant Kcat, for each of the substrates and imine were determined

(**Table 6**). It can be seen that the Km for each substrate was quite high, showing that the affinity of BPC with non-native substrates was very poor. Meanwhile, the Kcat was quite slow, indicating that the activity of BPC for the promiscuous substrates in the Povarov reaction was very low. However, even though the affinity between enzyme and imine was not as good as the affinity of the enzyme with benzaldehyde (**1a**) or 2-aminophenol (**2**), the reaction of the BPC catalyzed Povarov reaction with imine was still much faster than the reaction with aldehyde and amine directly (**Table 6**, entries 1, 2 and 4). These results suggested that the BPC-catalyzed one-pot three-component Povarov reaction of benzaldehyde, 2-aminophenol and 2,3-dihydropyran possibly took place through the intermediate imine.

Table 6. Kinetic Parameters for the BPC-catalyzed Povarov reaction [a]

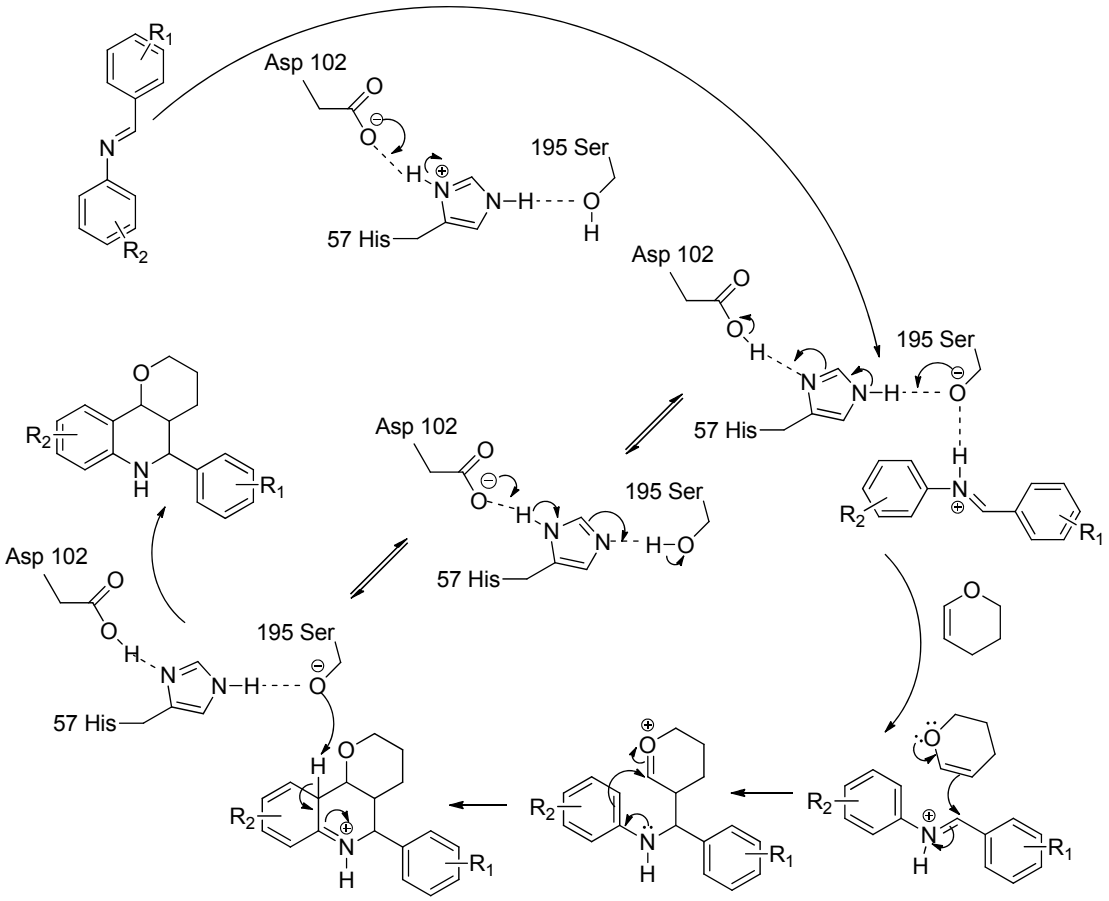


Entry	Substrate	Km (M)	Kcat (h^{-1})
1[b]	benzaldehyde (**1a**)	0.46	9.62
2[c]	2-aminophenol (**2**)	0.06	5.60
3[d]	2,3-dihydropyran (**3**) (reacting with **1a** and **2**)	0.60	9.08
4[e]	imine	7.75	24.02

[a] The reaction was carried out in MeCN (0.90 mL) and deionized water (0.10 mL) at 30 °C. The kinetic parameters were obtained as final enzyme concentration as 0.998 mM (for entries 1 and 2) and 0.941 mM (for entries 3 and 4). The experiments were based on HPLC determination of the products. [b] Concentration of **1a** varied from 0.25 M to 1.5 M, with **2** (0.5 M) and **3** (4.5 M). [c] Concentration of **2** varied from 0.25 M to 1.5 M, with **1a** (0.5 M) and **3** (4.5

M). [d] Concentration of **3** varied from 0.5 M to 4.5 M, with **1a** (0.5 M) and **2** (1.0 M). [e] Concentration of imine varied from 0.25 M to 1.5 M, with **3** (4.5 M).

Based on the above control and comparison experiments as well as the mechanism proposed by Dagousset[24] and Kumar,[25] we hypothesized a tentative mechanism of BPC catalyzed three-component Povarov reaction of aromatic aldehyde, aromatic amine, and 2,3-dihydropyran shown in **Scheme 1**. Firstly, *in situ* generated imine is activated by Ser-195 from the Asp-His-Ser catalytic triad in the active centre of BPC. Secondly, the protonated imine is attacked by the electron-rich nucleophile 2,3-dihydropyran through a Mannich process. Eventually, an intramolecular Friedel-Crafts reaction furnishes the final product tetrahydroquinoline skeleton with the aid of BPC.

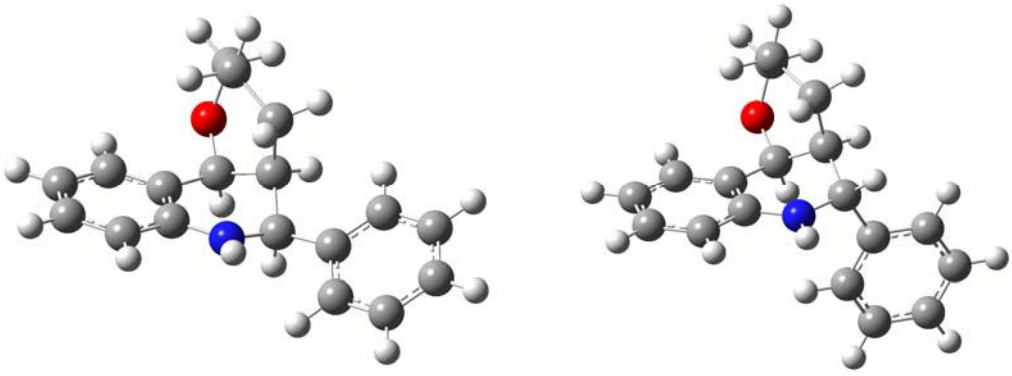


Scheme 1. A proposed tentative mechanism for the BPC-catalyzed Povarov reaction.

Finally, molecular docking and energy calculation of quantum chemistry were used to explain the experimental results in theory. Firstly, the geometry optimization and the single point energy calculations were conducted on products **4b** and **5b**, respectively, using semi-empirical theory with the PM6 method and density functional theory (DFT) at the B3LYP level of theory and the 6-31G* basis set. The results showed that the energy of **5b** (*trans*) is lower than **4b** (*cis*) (**Table 7** and **Fig. 1**), indicating that **5b** is more stable than **4b**, which coincides with the experimental results (**5b**:**4b** = 85:15, **Table 4**, entry 2). Secondly, the docking of both **4b** and **5b** into the active site pocket of BPC was carried out using AutoDock (V4.2). The results showed that both **4b** and **5b** could combine with the active site very well (**Fig. 2**), and the binding free energy of them are almost the same, meaning the affinity is undifferentiated (**Table 8**). Thus, it is possible to say that the active site of the enzyme did not have selectivity on the *cis* (**4b**) and *trans* (**5b**). To sum up, the lower energy of **5b** may be the key point to make it as a major product, and the active site of BPC did not have the selectivity during the course of the formation of **4b** and **5b**.

Table 7. Energies of **4b** (*cis*) and **5b** (*trans*) calculated by PM6 and DFT (B3LYP/6-31G*)

Molecule	PM6 (Kcal/mol)	DFT (B3LYP/6-31G*) (Kcal/mol)
5b (*trans*)	-0.609	-519024.732
4b (*cis*)	1.906	-519021.139
∆E [**4b** (*cis*) - **5b** (*trans*)]	2.515	3.593



4b (*cis*) **5b** (*trans*)

Fig 1 Conformations of **4b** (*cis*) and **5b** (*trans*) after geometry optimization.

Table 8. Binding free energies of **4b (***cis***)** and **5b (***trans***)** calculated by Autodock 4.2

Molecule	Binding free energy (Kcal/mol)
4b (*cis*)	-7.56
5b (*trans*)	-7.65
∆ E [**4b** (*cis*) - **5b** (*trans*)]	0.09

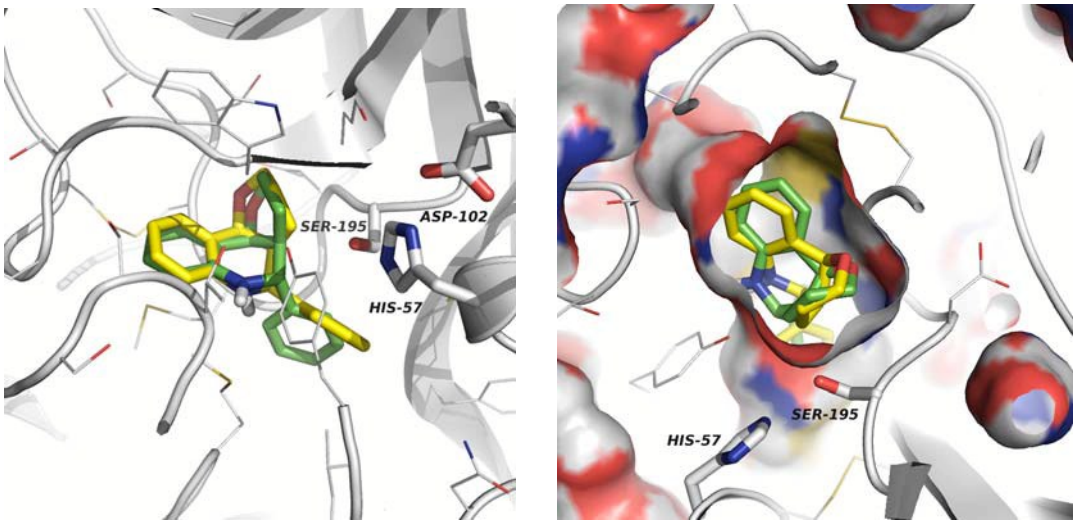


Fig. 2 The binding site pocket of **4b** and **5b** on BPC. Yellow is **4b**, and green is **5b**.

Conclusion

We described a novel methodology for the synthesis of tetrahydroquinoline derivatives via a three-component one-pot Povarov reaction using BPC as a safe, economical, and eco-friendly

catalyst. The products were obtained in moderate to good yields with a wide range of substrates. To understand this enzyme catalytic promiscuity, some control experiments with denatured and inhibited BPC were conducted, and the relation between natural and promiscuous activities of BPC was also investigated. The results suggested that the specific natural fold and the active site of BPC were responsible for its catalytic activity towards Pavonov reaction. The effect of organic solvent/water system on the BPC activity was also observed, which demonstrated that BPC can tolerate the MeCN/water system for a reasonable time. Based on the control and comparison experiments, a tentative mechanism was discussed. Molecular docking and energy calculation of quantum chemistry were used to explain the experimental results in theory. As a novel case of enzyme catalytic promiscuity, this work expands the application of BPC. Exploring the untapped catalytic promiscuity of natural enzymes may also provide useful information about enzyme evolution.

General procedure for the BPC-catalyzed Povarov reaction

BPC (50 mg) was added to a round bottom flask containing aromatic aldehyde (0.5 mmol), arylamine (1.0 mmol), 2, 3-dihydropyran or 2, 3-dihydrofuran (4.5 mmol), MeCN (0.80 mL) and deionized water (0.20 mL). The resultant mixture was stirred at 38 °C for the specified reaction time and monitored by TLC. The reaction was terminated by filtering off the enzyme. The filter cake was washed with ethyl acetate. Then, the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography (ethyl acetate/petroleum ether) to give the product.

Acknowledgements

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Supporting Information Available:

Materials, General methods, Molecular Docking, Energy calculation of quantum chemistry, Optimization of reaction conditions for the BPC-catalyzed Povarov reaction, Time course of the reaction, The kinetics of BPC catalyzed-hydrolysis of BTEE, Kcat for the BPC- or Asp-catalyzed Povarov reaction, SDS-PAGE analysis of BPC, Enzymatic assay of BPC, Characterization of Povarov products, NMR spectra, and HRMS spectra of new products. This material is available free of charge via the Internet at

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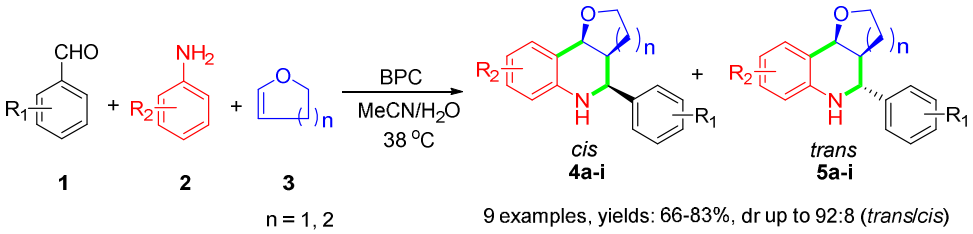

The α-chymotrypsin from bovine pancreas (BPC) catalyzed three-component one-pot Povarov reaction for the synthesis of tetrahydroquinoline derivatives was reported.