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Enzyme-catalyzed domino reaction: Efficient construction of spirocyclic oxindole skeleton using porcine pepsin

Yan-Hong He*, Tao He, Jun-Tao Guo, Rui Li, Yang Xiang, Da-Cheng Yang, Zhi Guan*

Key Laboratory of Applied Chemistry of Chongqing Municipality, School of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, PR China

Fax: +86-23-68254091; e-mails: heyh@swu.edu.cn (for Yan-Hong He); guanzhi@swu.edu.cn (for Zhi Guan)

Abstract: The pepsin from porcine gastric mucosa was used as a sustainable and environmentally friendly biocatalyst in the domino Knoevenagel/Michael/Michael reaction for the synthesis of spirooxindole derivatives in methanol. A wide range of isatins and α,β-unsaturated ketones reacting with malononitrile provided the corresponding products in yields of up to 99% with diastereoselectivity of up to >99:1 dr. This pepsin-catalyzed domino reaction provided a novel case of enzyme catalytic promiscuity.

Key words: porcine pepsin; domino reaction; spirocyclic oxindole; biocatalysis; enzyme promiscuity

1. Introduction

Enzyme catalysis is experiencing an unprecedented revolution since Klibalov's early 1980s discovery which showed that enzymatic activity can be stabilized in nearly anhydrous organic solvents[1-3] allowing for the active site of an enzyme in the appropriate solvent environment[4] to catalyze unnatural chemical reactions. This behavior is known as enzymatic promiscuity[5, 6].

Research into enzymatic promiscuity has brought enzymatic organic reactions significant attention and recognition from chemists and biochemists alike. Advantages including environmental friendliness, easy operation and mild reaction conditions demonstrate a great potential for future applications of enzyme catalysis in organic synthesis and significant advances have been made in the field of enzymatic promiscuity[7, 8]. For instance, some elegant work on enzymatic promiscuity have been reported including enzyme-catalyzed aldol[9-15], Michael[16-19], Mannich[20], Henry[21-23], Knoevenagel condensation[24], multi-component[25, 26] and domino reactions[27]. Among these examples, multi-step reactions catalyzed by a single enzyme are relatively rare.

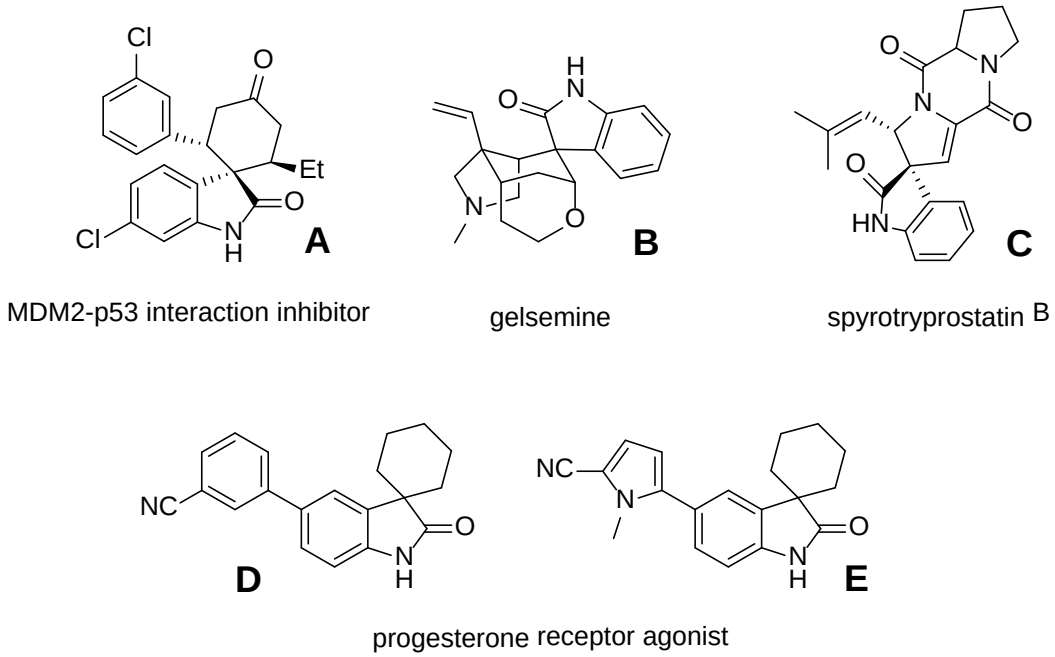


Fig. 1 Example of some natural products and pharmaceutically relevant compounds containing spriocyclic oxindoles

The spirocyclic oxindole skeletons are important structural motifs often found in natural products as well as biologically active compounds[28-35] (**Fig. 1**). Due to these compounds' unique

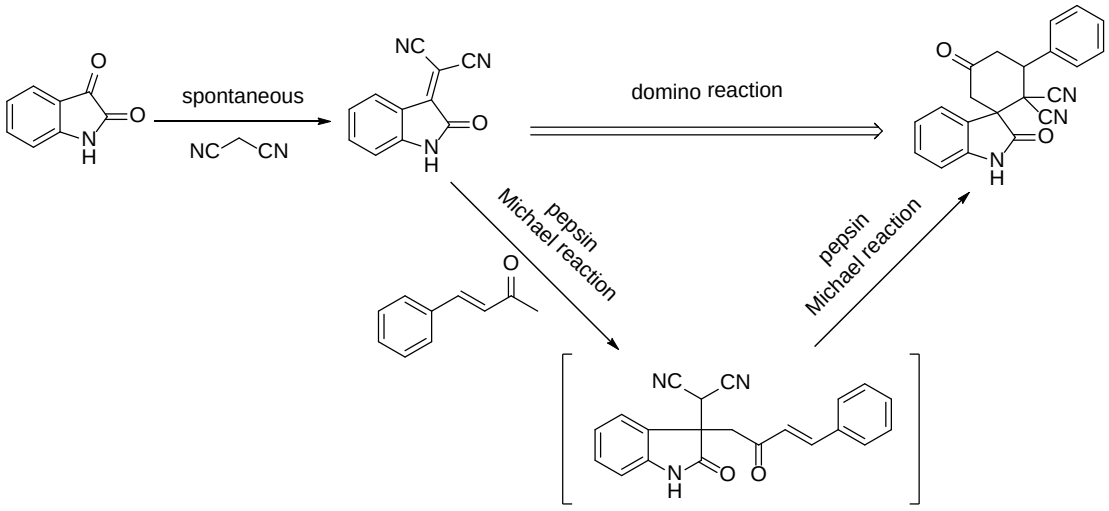
architecture and important biological activity, methods for the synthesis of spirocyclic oxindoles have aroused the attention and interest from chemists. Many excellent synthetic protocols from a variety of starting materials have been developed to access the spirocyclic oxindole skeletons, especially spiro[cyclohexanone-oxindole] backbones[29, 36-41]. Melchiorre and co-workers disclosed an asymmetric synthesis of spirocyclic oxindoles with multiple stereocenters via a tandem Michael/Michael addition in 2009[42]. Gong and co-workers reported an asymmetric formal [4+2] cycloaddition reaction between Nazarov reagents and methyleneindolinone catalyzed by bifunctional catalysts in 2010[34]. Wang and co-workers developed a cascade Michael-ketone aldol-dehydration domino reaction involving 2-hydroxy-3-acetyl indoles and enones catalyzed by Epi-Qui.-NH_2 (a Cinchona-based primary catalyst) in 2010[43]. Wang and coworkers also reported a method to access spirocyclic oxindoles via a tandem double Michael reaction starting from oxindoles and dienones catalyzed by chiral primary amine in 2011[44]. Lan and coworkers used the combination of a cinchona-based chiral primary amine and a BINOL-phosphoric acid as a synergistic catalyst system for the double Michael addition of isatylidene malononitriles (pre-generated from isatins and malononitrile) with α,β-unsaturated ketones to afford chiral spiro [cyclohexane-1,3'-indoline]-2',3-diones at 80 °C in 2011[45]. Hu and co-workers disclosed an asymmetric synthesis of multistereogenic spirocyclic oxindoles via a Morita–Baylis–Hillman reaction catalyzed by chiral phosphine in 2014[46]. Ramachary and co-workers developed a tandem Michael/Michael process to access spiro[cyclohex[3]ene-1,3'-indoline]-2',5-dione derivatives based on the reaction of unmodified ynones and isatylidene malononitriles[47]. Although many elegant synthetic protocols have been reported in this field in recent years, the development of novel catalysts for the synthesis of spirocyclic oxindoles that are environmentally friendly,

operationally simple and cheap are still in great demand.

Very recently, we reported a one-pot three-component reaction to construct 3,3-disubstituted oxindoles and spirooxindoles by using *α*-amylase from hog pancreas as a biocatalyst. Acetone, nitromethane or indole could participate in the reaction with isatin and malononitrile to form 3,3-disubstituted oxindoles via Knoevenagel and Michael reaction, and spirooxindole pyrans could be obtained via Knoevenagel, Michael and intramolecular cyclization reactions of acetylacetone, 4-hydroxylcoumarin or dimedone with isatin and malononitrile[48]. To continue exploring synthetic applications of enzymes, based on Lan and coworkers' research[45], herein, we report a novel discovery that pepsin from porcine gastric mucous could catalyze the domino Knoevenagel/Michael/Michael reaction of isatins, malononitrile and α,β-unsaturated ketones in methanol without any additives to afford spiro[cyclohexanone-oxindole] derivatives.

2. Results and discussion

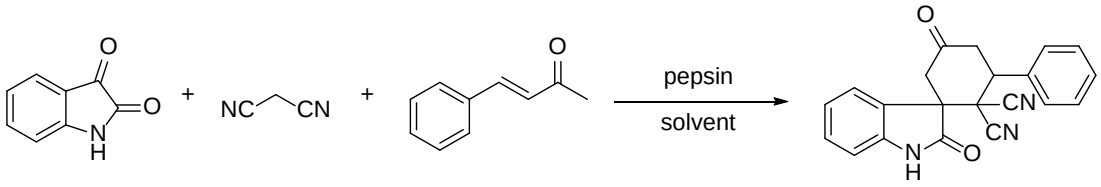
Isatin, malononitrile and an α,β-unsaturated ketone were used as the substrates in this pepsin-catalyzed domino reaction (**Scheme 1**). According to previous reports[48], isatin and malononitrile can spontaneously form a Knoevenagel adduct (isatylidene malononitrile). We speculated that the product spiro[cyclohexanone-oxindole] derivatives were formed via the domino Michael/Michael reaction (the Michael addition of isatylidene malononitrile and α,β-unsaturated ketone, followed by intramolecular Michael addition) (**Scheme 1**).



Scheme 1 The sequence of pepsin-catalyzed domino reaction.

The reaction between isatin, malononitrile and benzalacetone was used as a model reaction. Because reaction medium has obvious influence on enzyme activity, enzyme stability and the ability to catalyze unnatural reactions[5], the effect of different organic solvents on the pepsin-catalyzed model reaction was investigated (**Table 1**). As can be seen from the table, reaction medium played a crucial role in the pepsin-catalyzed domino reaction. The highest yield of 91% was obtained in methanol and a yield of 79% was achieved in ethanol after 5 days (**Table 1**, entries 1 and 2). The reaction in DMF, DMSO, THF and isopropanol provided the product in yields of 20-40% (**Table 1**, entries 3-6). In other tested solvents very poor yields were received or only trace amounts of product were observed (**Table 1**, entries 8-12). The observed results may be attributed to both the solubility of substrates or intermediates and specific interactions between the solvent and pepsin. Thus, methanol was chosen as the optimum solvent for the reaction in the following studies.

Table 1 Solvent screening [a]



1a **2** **3a** **4a**

Entry	Solvent	Yield (%) [b]
1	methanol	91
2	ethanol	79
3	DMF	40
4	DMSO	30
5	isopropanol	20
6	THF	20
7	1,4-dioxane	16
8	chloroform	6
9	water	4
10	butyl acetate	trace
11	acetonitrile	trace
12	hexane	trace

[a] Reaction conditions: isatin (0.25 mmol), malononitrile (0.25 mmol), benzalacetone (0.50 mmol), pepsin (9.5 kU), solvent (0.90 mL), and deionized water (0.10 mL) at 30 °C for 5 days.

[b] Yield of the isolated product after purification by silica gel chromatography.

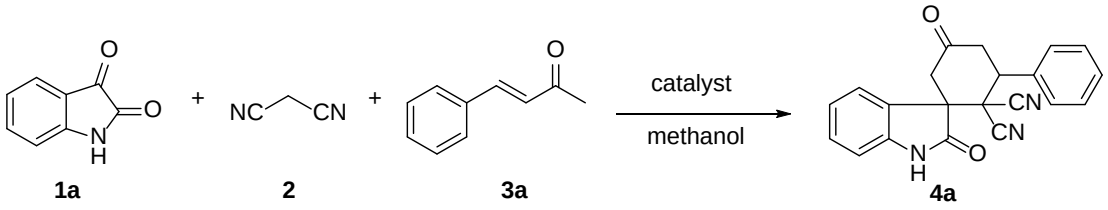
To demonstrate the specific catalytic effect of pepsin on the domino Michael/intramolecular Michael sequence in methanol, a series of control experiments were carried out (**Table 2**). We performed the model domino reaction in the absence of pepsin, and only a trace amount of desired product was observed after 5 days (**Table 2**, entry 1). The pepsin-catalyzed model reaction gave the product in a good yield of 91% (**Table 2**, entry 2), indicating that the pepsin preparation catalyzed the reaction. Urea as a denaturation agent was employed to pretreat the pepsin, and the urea-pretreated pepsin was then used to catalyze the model reaction which only gave the product with a very low yield of 13% (**Table 2**, entry 4). At the same time, to compare the natural activity with the non-natural activity of the both pretreated and untreated pepsin, an enzymatic assay of pretreated and untreated pepsin in a natural reaction (hydrolyzing hemoglobin) was conducted.

Results showed that the urea-pretreated pepsin lost most of its natural activity, with a reduction from 106 U mg^{-1} to 42 U mg^{-1} (**Table 2**, entries 2 and 4). The urea treatment caused a great degree of denaturation of the pepsin, which accordingly decreased its catalytic ability in the unnatural reaction. In order to ascertain the effects of urea on the model reaction, urea alone was used to catalyze the model reaction, and no catalytic effect was observed from urea (**Table 2**, entry 3). Heavy metal ions can also be used to inactivate enzymes since they can react with some structural groups (*e.g.* sulfhydryl groups) resulting in irreversible damage, or they can interact with some amino acid residues causing changes in tertiary structure. Thus, Cu^{2+} and Ag^{+} were used to pretreat the pepsin, separately. The metal ion-pretreated pepsin almost lost its ability to catalyze the model reaction, and only 8% and 15% yields were obtained, respectively (**Table 2**, entries 6 and 8). The metal ion treatment also caused a serious decrease on the natural activity of pepsin reducing activity from 106 U mg^{-1} to 25 U mg^{-1} (**Table 2**, entries 2, 6 and 8). Blank reactions using Cu^{2+} or Ag^{+} alone as the catalyst failed to give the product (**Table 2**, entries 5 and 7). The above control experiments indicated that pepsin catalyzed the model domino reaction, and its specific three-dimensional structure was crucial for this catalytic activity.

Moreover, we previously reported the addition of other nucleophiles such as active methyl or active methylene compounds (acetone, nitromethane, indole, acetylacetone, 4-hydroxylcoumarin and dimedone) to *in situ* generated isatylidenemalononitriles to form 3,3'-disubstituted oxindoles and spirooxindole pyrans using α-amylase from hog pancreasas as a biocatalyst[48]. To verify if α-amylase can also catalyze the present reaction, the α-amylase from hog pancreas was used as an enzyme catalyst for the model reaction which only gave a low yield of 22% (**Table 2**, entry 9).

The result showed that *α*-amylase has a certain degree of catalytic ability to this reaction, but the catalytic activity is very low in comparison with the pepsin.

Table 2 Control experiments [a]



1a **2** **3a** **4a**

Entry	Catalyst	Yield (%) [b]	Natural activity (U mg^{-1} solid) [c]
1	none	trace	-
2	pepsin	91	106
3	urea (200 mg)	trace	-
4	pepsin (pretreated with urea) [d]	13	42
5	$CuSO_4$ (39.9 mg)	trace	-
6	pepsin (pretreated with 250 mM Cu^{2+}) [e]	8	25
7	$AgNO_3$ (42 mg)	trace	-
8	pepsin (pretreated with 250 mM Ag^{+}) [f]	15	25
9	*α*-amylase from hog pancreas [g]	22	--

[a] Reaction conditions: isatin (0.25 mmol), malononitrile (0.25 mmol), benzalacetone (0.50 mmol), pepsin (9.5 kU), methanol (0.90 mL), deionized water (0.10 mL) at 30 ºC for 5 days.

[b] Yield of the isolated product after purification by silica gel chromatography.

[c] Unit definition (U mg^{-1} solid), one unit will produce a change in A280 of 0.001 per minute at pH 2.0 at 37 ºC, measured as TCA-soluble products using hemoglobin as substrate.

[d] Pepsin (9.5 kU) in urea solution (6.7 M) [urea (400 mg) in deionized water (1 mL)] was stirred at 25 ºC for 24 h, and then water was removed by lyophilization before use.

[e] Pepsin (9.5 kU) in Cu^{2+} solution (250 mM) [$CuSO_4$ (39.9 mg) in deionized water (1 mL)] was stirred at 25 ºC for 24 h, and then water was removed by lyophilization before use.

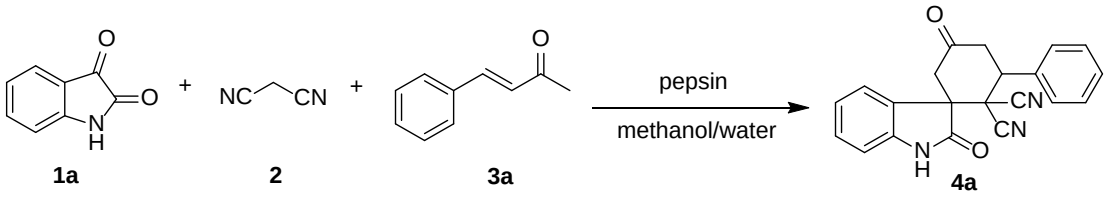
[f] Pepsin (9.5 kU) in Ag^{+} solution (250 mM) [$AgNO_3$ (42 mg) in deionized water (1 mL)] was stirred at 25 ºC for 24 h, and then water was removed by lyophilization before use.

[g] *α*-Amylase from hog pancreas (30 mg, lyophilized powder) was used instead of pepsin.

Water content usually affects enzymatic reactions in organic solvents because water can affect the conformational flexibility of an enzyme. To confirm the effect of water on this pepsin-catalyzed domino reaction in methanol, the addition of water from 0 to 50% [$V_{water}/V_{(water+methanol)}$%] was investigated (**Table 3**). The reaction yield significantly decreased with the addition of water into

the reaction system, likely due to decreases in solubility of both substrates and intermediates as water content increased. Thus, methanol without addition of water was chosen as the optimum reaction medium. The methanol employed was A.R. grade, and it was used directly without drying treatment.

Table 3 Effect of water addition on the pepsin-catalyzed domino reaction [a]



1a **2** **3a** **4a**

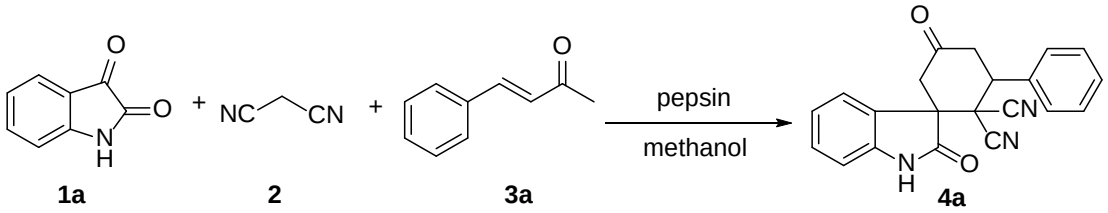
Entry	Addition of water [$V_{water}/V_{(water+methanol)}$%]	Yield (%) [b]
1	0	96
2	10	91
3	20	52
4	30	38
5	40	18
6	50	10

[a] Reaction conditions: isatin (0.25 mmol), malononitrile (0.25 mmol), benzalacetone (0.50 mmol), pepsin (9.5 kU), $V_{(methanol+water)}$ = 1.0 mL at 30 ºC for 5 days.

[b] Yield of the isolated product after purification by silica gel chromatography.

The effects of catalyst loading on the pepsin-catalyzed model domino reaction were examined (**Table 4**). From the results we obtained, there was a linear relationship between enzyme loading and the yield of the domino reaction when 1-9.5 kU of pepsin was used (**Table 4**). However, the reaction with higher enzyme loading (11.7 kU) gave product in a slightly decreased yield (**Table 4**, entry 6). Therefore, 9.5 kU of pepsin was chosen as the optimum enzyme loading for the reaction of isatin (0.25 mmol), malononitrile (0.25 mmol) and benzalacetone (0.50 mmol) in methanol (1.0 mL).

Table 4 Effect of enzyme loading on the pepsin-catalyzed domino reaction [a]



1a **2** **3a** **4a**

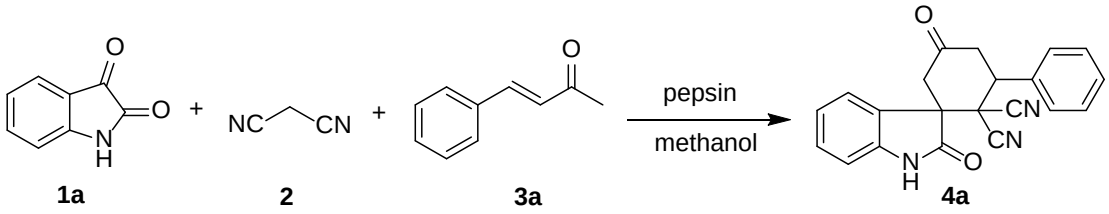
Entry	Pepsin (kU)	Yield (%) [b]
1	1	10
2	3.2	30
3	5.3	56
4	7.4	74
5	9.5	96
6	11.7	92

[a] Reaction conditions: isatin (0.25 mmol), malononitrile (0.25 mmol), benzalacetone (0.50 mmol), pepsin (1-11.7 kU) and methanol (1.0 mL) at 30 ºC for 5 days.

[b] Yield of the isolated product after purification by silica gel chromatography.

Temperature plays an important role in enzymatic reactions due to its effect on the reaction rate and the stability of enzymes. Thus, the effect of temperature on the pepsin-catalyzed model domino reaction was examined (**Table 5**). Rising temperature from 20 to 50 ºC led to a remarkable increase of yield from 9 to 91% after reacting for 20 h (**Table 5**, entries 1-4). The reaction at 60 ºC gave the product in an excellent yield of 96% after only 12 h (**Table 5**, entry 5). To exclude the possibility that high temperature promoted the reaction instead of pepsin itself, a blank reaction was conducted at 60 ºC and only 6% yield was obtained after 12 h (**Table 5**, entry 6). It was proved that raising temperatures to 60 ºC improved the catalytic activity of pepsin in the model domino reaction. However, further increasing the temperature to reflux temperature of the solvent did not give better yield (**Table 5**, entry 7). Thus, 60 ºC was selected as the optimum temperature for the reaction.

Table 5 Effect of temperature on the pepsin-catalyzed domino reaction [a]



1a **2** **3a** **4a**

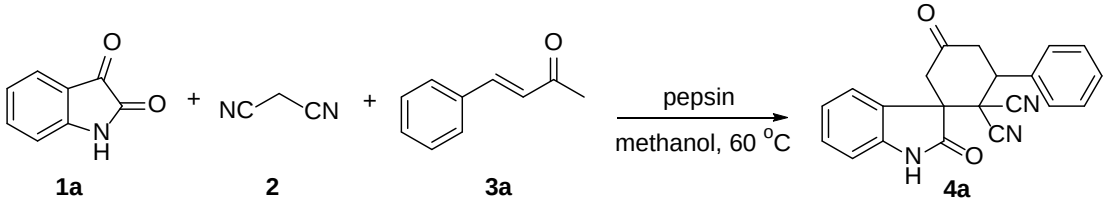
Entry	Temperature (ºC)	Time (h)	Yield (%) [b]
1	20	20	9
2	30	20	30
3	40	20	73
4	50	20	91
5	60	12	96
6	60 (without pepsin)	12	6
7	reflux	12	91

[a] Reaction conditions: isatin (0.25 mmol), malononitrile (0.25 mmol), benzalacetone (0.50 mmol), pepsin (9.5 kU), methanol (1.0 mL).

[b] Yield of the isolated product after purification by silica gel chromatography.

The influence of the substrate molar ratio was also tested and the best yield could be achieved with a molar ratio of 1:1:2 (isatin to malononitrile to benzalacetone).

Table 6 Influence of molar ratio of substrates on the pepsin-catalyzed domino reaction [a]



1a **2** **3a** **4a**

Entry	Molar ratio of **1a**/**2**/**3a**	Yield (%) [b]
1	1:1:1	48
2	1:1:1.5	58
3	1:1:2	96

[a] Reaction conditions: isatin (0.25 mmol), malononitrile (0.25 mmol), benzalacetone (0.25-0.50 mmol), pepsin (9.5 kU), methanol (1.0 mL) at 60 °C for 12 h.

[b] Yield of the isolated product after purification by silica gel chromatography.

Reaction time of the pepsin-catalyzed model domino reaction was also investigated (**Fig. 2**).

Product yield clearly increased in the first 4 h. The yield reached its limit of 95% after 12 h.

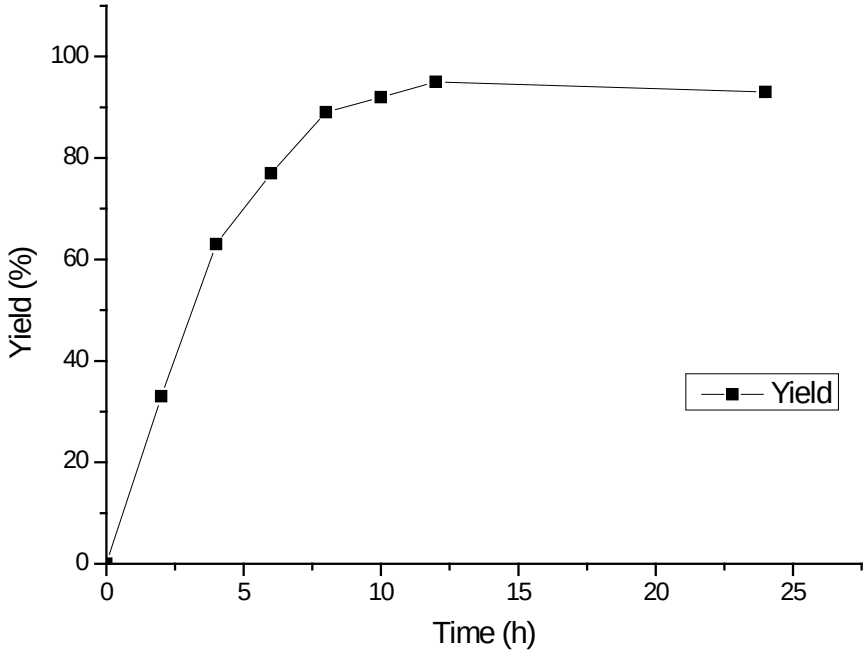


Fig. 2 Reaction time of the pepsin-catalyzed domino reaction.

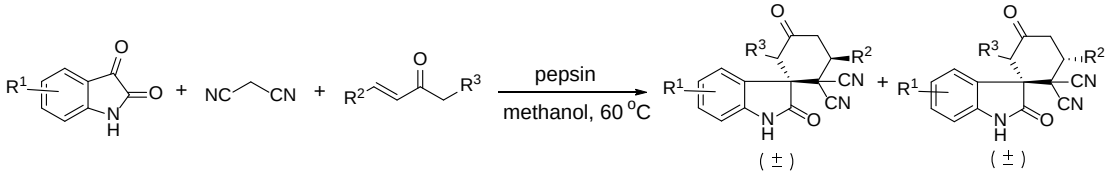
The reactions were carried out with isatin (0.25 mmol), malononitrile (0.25 mmol) and benzalacetone (0.50 mmol) in the presence of pepsin (9.5 kU) in methanol (1.0 mL) at 60 ºC for 4-24 h. Yield of the isolated product after purification by silica gel chromatography.

With the optimized conditions in hand, we explored the scope of substrates and generality of the pepsin catalyzed domino reaction (**Table 7**). A series of isatins containing various substituents with different electronic properties were investigated (**Table 7**, entries 1-10). Reactions with isatins bearing an electron-donating group in the 5-position gave better yields (**Table 7**, entries 3 and 4) than those bearing a strong electron-withdrawing group in the 5-position (**Table 7**, entries 5 and 6). The position of substituents also had effects on the reaction yield. The isatins with a substituent in the 5-position gave higher yields than those with a substituent in the 1- or 7-position (**Table 7**, entries 2, 4, 7 and 10). A series of α,β-unsaturated ketones were also used to examine the

tolerance of pepsin (**Table 7**, entries 11-27). A wide range of substituted benzalacetones containing either electron-donating or electron-withdrawing groups in the *o*, *m*, or *p*-position of the benzene ring performed very well, affording the products in good to excellent yields (**Table 7**, entries 11-21). The α,β-unsaturated ketones containing naphthyl, thienyl or furyl were also tested and the products were obtained in satisfactory yields with excellent diastereoselectivity (**Table 7**, entries 22-24). Besides aromatic α,β-unsaturated ketones, an aliphatic α,β-unsaturated ketone was tested and gave a low yield of 32% with 99:1 dr (**Table 7**, entry 25). Not only methyl styryl ketones reacted satisfactorily in this reaction, but also ethyl and propyl styryl ketones participated in the reaction smoothly giving corresponding products in good yields with lower diastereoselectivity after longer reaction time (**Table 7**, entries 26 and 27). In all circumstances, the major products are **4** the minor products are **5** and the relative configuration are confirmed by comparing with NMR recorded by literature[45].

Unfortunately, there was no enantiomeric excess of the products observed by the chiral HPLC analysis. The products obtained were all racemic. Griengl and coworkers previously reported lipase-catalyzed Michael-type carbon–carbon bond formations. The reactions proceeded without enantioselectivity[49]. Moreover, Brinck and Berglund *et al.* explored the active-site of a rationally redesigned lipase for catalysis of Michael-type additions, and the products were produced also without enantioselectivity[50]. Enzyme induced enantioselectivity was not found in the present work as well as these literatures indicating that the catalytic mechanism of enzyme promiscuity is not as highly specific as its natural reaction.

Table 7 Substrate scope of the pepsin-catalyzed domino reaction [a]



Entry	R^1	R^2	R^3	Product	Time (h)	Yield (%) [b]	Dr (**4**:**5**) [c]
1	H	Ph	H	**4a**	12	96	97:3
2	1-Me	Ph	H	**4b**	15	84	95:5
3	5-MeO	Ph	H	**4c**	17	99	>99:1
4	5-Me	Ph	H	**4d**	16	99	90:10
5	5-NO_2	Ph	H	**4e**	16	75	90:10
6	5-F	Ph	H	**4f**	16	72	>99:1
7	5-Cl	Ph	H	**4g**	17	95	88:12
8	5-Br	Ph	H	**4h**	19	92	95:5
9	5-I	Ph	H	**4i**	16	99	87:13
10	7-Cl	Ph	H	**4j**	12	73	88:12
11	H	*o*-$MeOC_6H_4$	H	**4k**	15	85	82:18
12	H	*o*-ClC_6H_4	H	**4l**	12	99	>99:1
13	H	*m*-$MeOC_6H_4$	H	**4m**	19	95	95:5
14	H	*m*-ClC_6H_4	H	**4n**	15	86	>99:1
15	H	*m*-BrC_6H_4	H	**4o**	12	85	99:1
16	H	*p*-MeC_6H_4	H	**4p**	19	97	87:13
17	H	*p*-$CF_3C_6H_4$	H	**4q**	15	95	87:13
18	H	*p*-$NO_2C_6H_4$	H	**4r**	17	98	87:13
19	H	*p*-FC_6H_4	H	**4s**	12	95	94:6
20	H	*p*-ClC_6H_4	H	**4t**	18	81	94:6
21	H	*p*-BrC_6H_4	H	**4u**	18	81	92:8
22	H	1-naphthyl	H	**4v**	15	82	>99:1
23	H	2-thienyl	H	**4w**	17	72	>99:1
24	H	2-furyl	H	**4x**	17	78	99:1
25	H	Me	H	**4y**	17	32	99:1
26	H	Ph	Me	**4z**	48	89	58:42
27	H	Ph	Et	**4aa**	60	71	73:27

[a] Reaction conditions: isatin (0.25 mmol), malononitrile (0.25 mmol), α,β-unsaturated ketones (0.50 mmol), pepsin (9.5 kU), methanol (1.0 mL) at 60 °C.

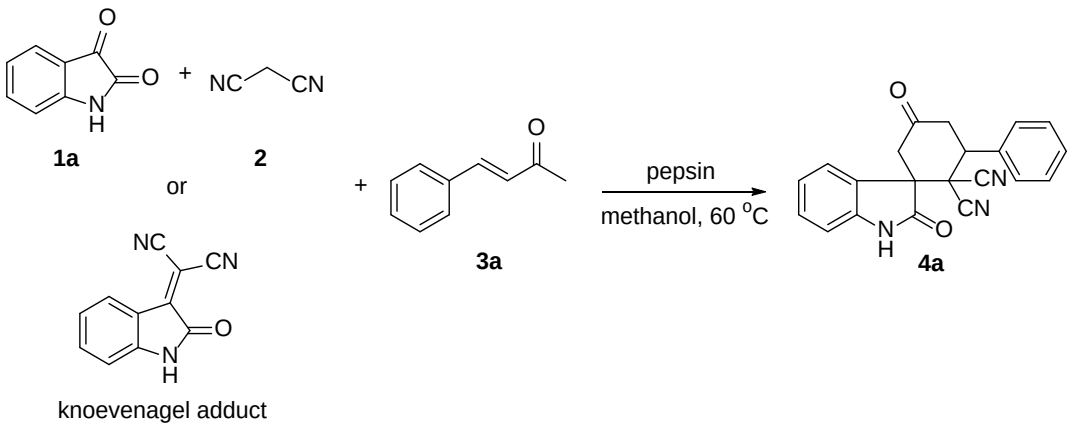
[b] Yield of the isolated product after purification by silica gel chromatography.

[c] Determined by ^{1}HNMR.

Furthermore, the kinetic parameters (Michaelis constant K_m, catalytic constant K_{cat} and K_{cat} / K_m) of the pepsin-catalyzed domino reaction for each of the substrates in the model reaction were

determined (**Table 8**). According to our previous research, isatylidene malononitrile can be formed via a spontaneous Knoevenagel reaction of isatin and malononitrile[48]. Thus based on Lan and coworkers' report[45], we speculated that the pepsin-catalyzed domino reaction actually is the double Michael addition of Knoevenagel adduct with α,β-unsaturated ketone. Therefore, to understand the reaction mechanism, the Knoevenagel adduct was also considered as a substrate (**Table 8**). It can be seen that the K_m for each substrate was generally quite high, showing that the affinity of pepsin with non-native substrates was very poor. But the K_m of the Knoevenagel adduct was still lower than other substrates (**Table 8**, entry 4). Meanwhile, the K_{cat} of each substrate was quite slow, indicating that the catalytic activity of pepsin for the promiscuous reaction was very low. However, K_{cat} / K_m of the Knoevenagel adduct was still higher than the other substrates, suggesting that the efficiency of pepsin catalysis with the Knoevenagel adduct was higher than with the other substrates. These results suggested that the pepsin-catalyzed domino reaction of isatin, malononitrile and benzalacetone possibly takes place through the intermediate Knoevenagel adduct.

Table 8 Kinetic parameters for the pepsin-catalyzed domino reaction [a]

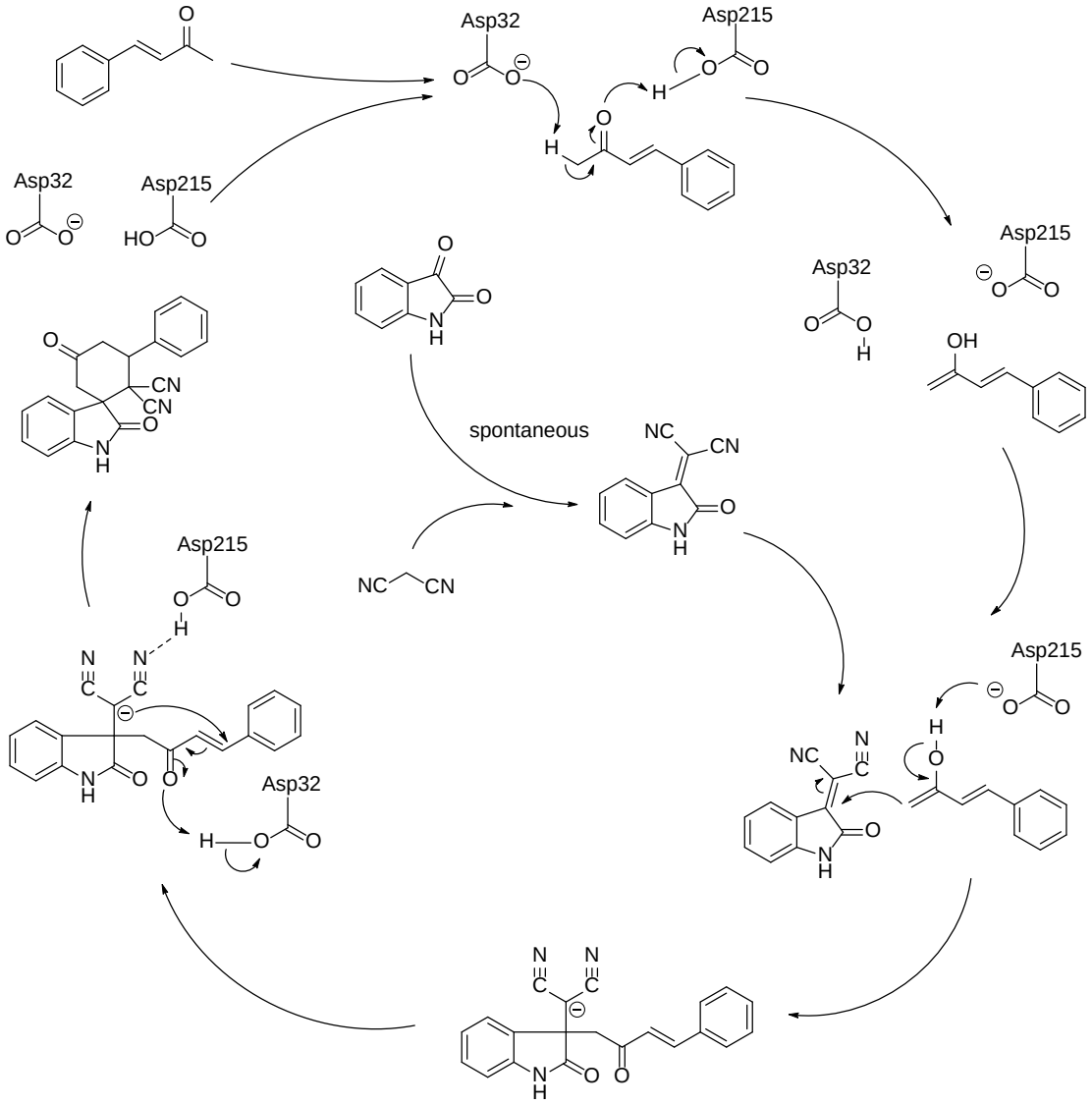


Entry	Substrate	K_m (M)	K_{cat} (h^{-1})	K_{cat} / K_m ($M^{-1}h^{-1}$)
1[b]	Isatin (**1a**)	0.0398	122.9	3088

2[c]	Malononitrile (**2**)	0.0619	33.2	536
3[d]	Benzalacetone (**3a**)	0.1580	82.9	525
4[e]	Knoevenagel adduct	0.0083	29.4	3555

[a] The reactions were carried out in methanol (1.0 mL) at 60 ºC for 1 h and the kinetic parameters were obtained at the enzyme concentration of 0.463 mM. The experiments were based on HPLC determination of the product. [b] The concentration of **1a** varied from 0.03 M to 0.19 M, with **2** (0.25 M) and **3a** (0.50 M). [c] The concentration of **2** varied from 0.03 M to 0.19 M, with **1a** (0.25 M) and **3a** (0.50 M). [d] The concentration of **3a** varied from 0.06 M to 0.38 M, with **1a** (0.25 M) and **2** (0.25 M). [e] The concentration of Knoevenagel adduct varied from 0.03 M to 0.13 M, with **3a** (0.50 M).

According to the literatures[51, 52], pepsin contains 385 residues. The active site of pepsin from porcine gastric mucosa contains Asp32 and Asp215 residues. The function of aspartic acid located in 32 has been confirmed to act as a base. Based on the literatures, the control experiments and the kinetic experiments, we speculated a tentative mechanism about the pepsin-catalyzed domino reaction of isatin, malononitrile and benzalacetone (**Scheme 2**). First, the carbonyl of benzalacetone is stabilized via a hydrogen bond with Asp215 in pepsin, and Asp32 acts as a base to take away a proton from the benzalacetone forming the enol. Second, an intermolecular Michael addition takes place through the enol attacking the isatylidene malononitrile which is formed via a spontaneous Knoevenagel reaction of isatin and malononitrile[48]. Finally, the intramolecular Michael addition occurs forming the spirocyclic oxindole skeleton.



Scheme 2 The speculated mechanism of pepsin-catalyzed domino reaction.

3. Conclusion

Pepsin from porcine gastric mucosa was used as a biocatalyst in the domino Knoevenagel/Michael/Michael reaction for the synthesis of spirooxindole derivatives in methanol. A wide range of isatins and α,β-unsaturated ketones reacting with malononitrile provided their corresponding products in yields of up to 99% with diastereoselectivity of up to >99:1 dr. The effects of a series of parameters such as solvent, water content, enzyme loading, temperature,

molar ratio of substrates, and reaction time on the pepsin-catalyzed domino reaction were investigated. The control experiments suggested that the specific three-dimensional structure of pepsin was responsible for the domino reaction, and a possible mechanism of the reaction was speculated. Using enzymes as a regenerable and environmentally friendly catalyst for organic synthesis meets the requirements of sustainable chemistry. This pepsin-catalyzed domino reaction provides a novel case of enzyme catalytic promiscuity.

Materials

Pepsin from porcine gastric mucosa (EC number 3.4.23.1, CAS number: 9001-75-6, product number: P7125-100G, ≥400 units/mg protein, 18% protein/mg) was purchased from Sigma-Aldrich. One unit will produce a ΔA280 of 0.001 per min at pH 2.0 at 37 °C, measured as TCA-soluble products using hemoglobin as substrate. Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification.

General procedure for the synthesis of substituted benzalacetones

A 50 mL round bottom flask was charged with aldehyde (20 mmol), acetone (5 mL) and H_2O (20 mL), and then 5% NaOH (aq) (2 mL) was slowly added to the mixture at 40 ^oC. After completion of the reaction (detected by TLC), acetone was removed under reduced pressure and ethyl acetate (20 mL) was added. The mixture was extracted with ethyl acetate (3 × 20 mL) and the aqueous phase was separated and discarded. The organic phase was concentrated in vacuum and the residue was purified by chromatography on silica gel with petroleum ether/ethyl acetate as the eluent to afford corresponding products.

General procedure for the pepsin-catalyzed domino reaction

Pepsin (9.5 kU) was added to a mixture of isatin (0.25 mmol), malononitrile (0.25 mmol), an α,β-unsaturated ketone (0.50 mmol), and methanol (1.0 mL). The resultant mixture was stirred for the specified time at 60 ºC, and monitored by TLC. The reaction was terminated by filtering the enzyme. Ethyl acetate was employed to wash the residue on filter paper to ensure that the products obtained were all dissolved in the filtrate. The organic solvents were then removed under reduced pressure. The crude products were purified by silica gel column chromatography with petroleum ether/ethyl acetate as the eluent (V_{PE}/V_{EA} = 2:1 to 5:1).

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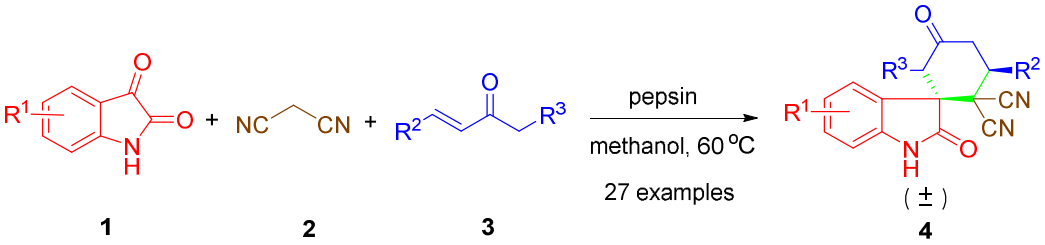
A table of contents entry



1 **2** **3** **4**

R^1 = H, Me, MeO, NO_2, F, Cl, Br, I
R^2 = phenyl, substituted phenyl, naphthyl, thienyl, furyl, Me
R^3 = H, Me, Et

yield up to 99%
dr up to >99:1

Pepsin from porcine gastric mucosa was used as a catalyst in the domino Knoevenagel/Michael/Michael reaction for the synthesis of spirooxindoles.