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Enzyme-Catalyzed Asymmetric Domino Thia-Michael/Aldol Condensation Using Pepsin

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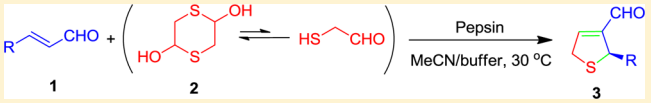
Supporting Information

ABSTRACT: The novel catalytic promiscuity of pepsin from porcine gastric mucosa for the asymmetric catalysis of the domino thia-Michael/aldol condensation reaction in MeCN and buffer was discovered for the first time. Broad substrate specificity was tested, and a series of corresponding products were obtained with enantioselectivities of up to 84% ee. This specific catalysis was demonstrated by using recombinant pepsin and control experiments with denatured and inhibited pepsin. The reaction was also shown to occur in the active site by site-directed mutagenesis (the Asp32Ala mutant of pepsin), and a possible mechanism was proposed.



INTRODUCTION

Enzymes, as green and efficient biocatalysts, are greatly superior in many important respects such as mild reaction conditions, good stereoselectivity, easy processing, economic and ecological advantages, etc.[1] Enzymes exhibit specific substrate recognition in metabolism and have been optimized through the evolution of a specific chemical transformation. Despite this, more and more enzymes have been found to have the ability to catalyze reactions, or act on substrates, other than those for which they evolved; this phenomenon is called enzyme promiscuity.[2] Until recently, the wider implications of the "darker" side of enzyme promiscuity were largely ignored. Actually, promiscuous activities are not rare exceptions but are rather widespread inherent features of enzymes and proteins in general.[3] It is believed that promiscuous activities serve as starting points for the divergence of new enzymes in natural evolution. Broad-specificity enzymes acted as progenitors for today's specialized enzymes.[4] Thus, enzyme catalytic promiscuity is a key factor in the evolution of new enzyme functions. Many examples of enzyme catalytic promiscuity have been reported,[2c,5] such as the aldol reactions,[6] Henry reactions,[7] Markovnikov additions,[8] Michael additions,[9] Mannich reactions,[10] asymmetric synthesis of α-aminonitrile amides,[11] multicomponent cascade or domino reactions,[1a−c,12] etc. However, only very few mechanisms of promiscuous functions have been proven by site-directed mutation of enzymes.[6a,13] Valuable insights regarding the catalytic mechanisms can be provided by a systematic research of the hidden skills of enzymes.[14] Thus, exploring more reaction types and verifying the catalytic mechanism are still greatly in demand.

Dihydrothiophenes as valuable S-heterocycles have attracted a great deal of attention because of their special value in biological and medical properties,[15] versatile synthetic intermediates,[16] and material science.[17] Thus, many efficient synthetic approaches have been devoted to the development of these compounds.[16c,18] However, only few successful procedures achieved the enantioselective formation of dihydrothiophenes. In 2009, the De Risi[19] group reported the synthesis of chiral 4,5-dihydrothiophene; in 2010, the Xu[20] group developed the enantioselective formation of 2,5-dihydrothiophenes by domino thia-Michael/aldol condensation between 1,4-dithiane-2,5-diol and α,β-unsaturated aldehydes using chiral diphenylprolinol TMS ether as an organocatalyst. On the basis of the work of the Xu[20] group, in 2015, the De Risi[21] group developed a one-pot, four-step organocatalytic process catalyzed by (*S*)-diphenylprolinol TMS ether. This process consists of the domino thia-Michael/aldol condensation between 1,4-dithiane-2,5-diol and α,β-unsaturated aldehydes, and the derived chiral dihydrothiophene adducts subsequently react with bromonitromethane via the domino Michael/α-alkylation reaction to yield chiral nitrocyclopropanes. Because the biological activities and pharmacological activities are often related to the configurations of most natural products and pharmaceuticals, the enantioselective syntheses of dihydrothiophenes are greatly important. Therefore, the development of novel catalysts that are environmentally friendly, sustainable, and inexpensive with respect to chiral synthesis of dihydrothiophenes is still strongly desired.

Pepsin is an aspartic protease that participates in food digestion in the mammal stomach. In recent years, some promiscuous activities of pepsin from porcine gastric mucosa have been disclosed gradually. In 2010, the Yu group reported the pepsin-catalyzed aldol reaction between acetone and substituted benzaldehydes in which an enantioselectivity of up to 44% ee was observed.[22] Our group also has investigated the catalytic promiscuity of pepsin. In 2015, we found that pepsin can catalyze direct asymmetric aldol reactions for the synthesis of vicinal diol compounds, and products were obtained with an enantioselectivity of up to 75% ee.[23] In

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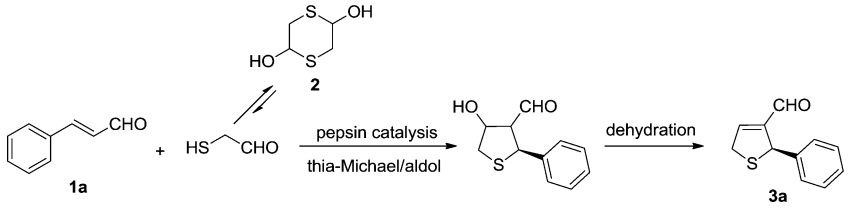
Scheme 1. Model Asymmetric Domino Thia-Michael/Aldol Condensation Reaction

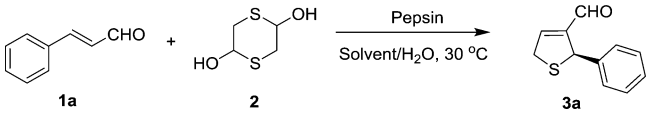


2016, we reported the pepsin-catalyzed Morita–Baylis–Hillman (MBH) reaction between aromatic aldehydes and 2-cyclohexen-1-one or 2-cyclopenten-1-one, and an enantioselectivity of up to 38% ee was achieved.[24] Very recently, we found that pepsin can catalyze the domino Knoevenagel/Michael/Michael reaction for the synthesis of spirooxindole derivatives with diastereoselectivities of up to >99:1 dr without enantioselectivity.[12b] In view of the amazing catalytic versatility of pepsin and its stereoselectivity toward some synthetically useful molecules, it is necessary to gain further insight into its catalytic promiscuity. Herein, we report a novel activity of pepsin for the asymmetric domino thia-Michael/aldol condensation reaction of 1,4-dithiane-2,5-diol with α,β-unsaturated aldehydes. Broad substrate specificity was tested, and a series of corresponding products were obtained with enantioselectivities of up to 84% ee. The specific catalytic effect of pepsin was clearly shown to occur in the active site by site-directed mutagenesis (the Asp32Ala mutant of pepsin). This is the first study utilizing site-directed mutagenesis to confirm the promiscuous activity of pepsin, and a possible mechanism was proposed.

RESULTS AND DISCUSSION

The asymmetric domino thia-Michael/aldol condensation reaction of cinnamaldehyde (**1a**) and 1,4-dithiane-2,5-diol (**2**) was chosen as a model reaction, and pepsin from porcine gastric mucosa was used as a catalyst. In this reaction, 1,4-dithiane-2,5-diol (**2**), the mercaptoacetaldehyde dimer, was used as a convenient and efficient synthon to provide an *in situ*-generated mercaptoacetaldehyde (Scheme 1).[25] It is known that the reaction medium plays an important role in enzymatic reactions as the reaction medium has a strong effect on the stability and catalytic activity of an enzyme, particularly on enantioselectivity and regioselectivity.[26] To optimize experimental conditions, we first examined the effect of solvent on the model reaction (Table 1). It could be seen that the catalytic effects of pepsin, especially the enantioselectivity, were obviously influenced by different media. The enzyme showed the best enantioselectivity of 41% ee in MeCN with a yield of 17% (Table 1, entry 1). Better yields of 20–21% were obtained in $CHCl_3$, PhMe, and $ClCH_2CH_2Cl$, but with very low enantioselectivity (Table 1, entries 6–8, respectively). The reactions in the other tested solvents gave either lower yields or lower ee's, and only trace amounts of the product were observed in several solvents, including water (Table 1, entries 2–5 and 9–16). These results may be attributed to the solubility of substrates and specific interactions between the solvent and pepsin. To obtain the best enantioselectivity, MeCN was chosen as a suitable solvent for the reaction. The absolute configuration of product **3a** was determined to be the *R* configuration by comparison with the results of chiral HPLC analysis.[20]

Table 1. Solvent Screening for the Model Reaction[a]



entry	solvent	yield (%)[b]	ee (%)[b]
1	MeCN	17[c]	41
2	MeOH	10	35
3	THF	11	15
4	EtOAc	11	11
5	CH_2Cl_2	17	6
6	$CHCl_3$	21	5
7	PhMe	20	4
8	$ClCH_2CH_2Cl$	21	3
9	Et_2O	11	3
10	PhOMe	trace	
11	1,4-dioxane	trace	
12	EtOH	trace	
13	DMF	trace	
14	DMSO	trace	
15	xylene	trace	
16	H_2O	trace	

[a]For the reaction, a mixture of cinnamaldehyde **1a** (0.5 mmol), 1,4-dithiane-2,5-diol **2** (0.35 mmol), solvent (0.90 mL), deionized water (0.10 mL), and pepsin (6.5 kU) was stirred at 30 °C for 96 h. [b]Determined by chiral HPLC using a Chiralpak AS-H column. [c]Yield of the isolated product after silica gel chromatography.

Because the pH value of the reaction medium significantly affects the stability and catalytic activity of enzymes,[27] phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 4.7–8.7) was used to replace the water in the reaction system [1/9 (v/v) buffer/MeCN] to obtain the optimal reaction conditions (Table 2). It could be seen that addition of buffer obviously enhanced the enantioselectivity of the reaction. Pepsin showed the best enantioselectivity in the presence of phosphate buffer (pH 6.5), giving the product with a yield of 16% and 57% ee (Table 2, entry 4). However, the addition of phosphate buffer could not increase the yield. Thus, the phosphate buffer [pH 6.5, 1/9 (v/v) buffer/MeCN] was selected as the optimal condition for the reaction.

The effects of the substrate molar ratios on the model reaction were investigated (Table 3). The results showed that the reaction was clearly influenced by changes in the molar ratio of substrates. As the amount of cinnamaldehyde (**1a**) was increased, enhanced yields were obtained but with reduced ee's (Table 3, entries 1–6). When the amount of 1,4-dithiane-2,5-diol (**2**) was increased, a slight improvement in both yield and ee was observed (Table 3, entries 1 and 7–10). The best ee of 60% and yield of 19% were obtained at a 1/3 **1a**/**2** molar ratio (Table 3, entry 8), which was chosen as the optimal ratio for further studies.

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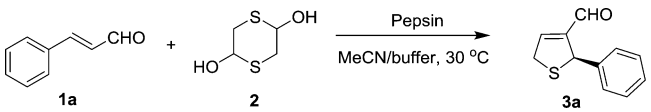
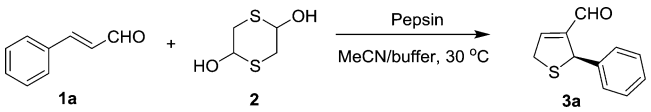
Table 2. Influence of pH Conditions (phosphate buffer) on the Model Reaction[a]



entry	pH of phosphate buffer	yield (%)[b]	ee (%)[b]
1	4.7	17	50
2	5.6	19	54
3	6.3	16	56
4	6.5	16	57
5	7.0	16	55
6	7.4	16	56
7	8.0	14	56
8	8.7	14	53

[a]For the reaction, a mixture of cinnamaldehyde **1a** (0.5 mmol), 1,4-dithiane-2,5-diol **2** (0.35 mmol), MeCN (0.90 mL), phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 4.7−8.7, 0.10 mL), and pepsin (6.5 kU) was stirred at 30 °C for 96 h. [b]Determined by chiral HPLC using a Chiralpak AS-H column.

Table 3. Effect of the Molar Ratio of Substrates on the Model Reaction[a]



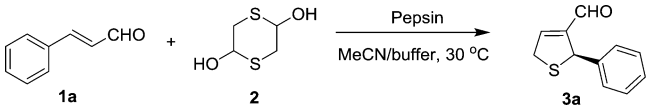
entry	molar ratio (**1a**/**2**)	yield (%)[b]	ee (%)[b]
1	1/1	15	56
2	1.4/1	16	57
3	2/1	23	46
4	3/1	29	36
5	4/1	29	33
6	5/1	28	28
7	1/2	18	56
8	1/3	19	60
9	1/4	19	59
10	1/5	19	60

[a]For the reaction, a mixture of cinnamaldehyde **1a** (0.35−1.75 mmol), 1,4-dithiane-2,5-diol **2** (0.35−1.75 mmol), MeCN (0.90 mL), phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 6.5, 0.10 mL), and pepsin (6.5 kU) was stirred at 30 °C for 96 h. [b]Determined by chiral HPLC using a Chiralpak AS-H column.

Next, to further optimize the reaction conditions, the influence of the phosphate buffer content in the reaction system on the model reaction was investigated. The best yield of 24% with 59% ee was realized in the mixed solvent of phosphate buffer (pH 6.5) and MeCN [1/4 (v/v) buffer/MeCN], which was chosen as the optimal condition for the reaction (for details, see the Supporting Information). The influence of the mixed solvent volume on the model reaction was also examined. To our delight, as the volume increased from 1.00 to 1.50 mL, the ee was increased from 59 to 70% with a slight decrease in yield from 24 to 18%. Thus, to obtain better enantioselectivity, a mixed solvent volume of 1.50 mL [1/4 (v/v) buffer/MeCN] was chosen as the optimal reaction condition (for details, see the Supporting Information).

The influence of enzyme loading on the model reaction of cinnamaldehyde (0.35 mmol) and 1,4-dithiane-2,5-diol (1.05 mmol) was examined. The yield was visibly affected by enzyme loading (Table 4). A great enhancement in yield was obtained

Table 4. Effect of Enzyme Loading on the Model Reaction[a]



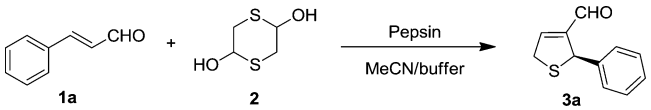
entry	enzyme loading (kU)	yield (%)[b]	ee (%)[b]
1	3.9	11	70
2	6.5	18	70
3	9.1	28	70
4	11.7	30	72
5	14.3	37	71
6	16.9	46	70
7	19.5	46	69
8	22.1	53	65
9	26.0	55	63

[a]For the reaction, a mixture of cinnamaldehyde **1a** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL), and pepsin (3.9−26.0 kU) was stirred at 30 °C for 96 h. [b]Determined by chiral HPLC using a Chiralpak AS-H column.

when the enzyme loading was increased from 3.9 to 16.9 kilounits (kU) (Table 4, entries 1−6) with the ee remaining approximately the same. Though there was a slight enhancement in yield with further increases in the amount of enzyme from 16.9 to 26.0 kU, the ee began to decline (Table 4, entries 6−9). Thus, we chose an enzyme loading of 16.9 kU (Table 4, entry 6) as the optimal condition for further studies.

Temperature also plays an important role in enzyme-catalyzed reactions because of its effects on enzyme stability as well as the rate and selectivity of the reaction.[28] To further characterize the activity and selectivity of pepsin in the model reaction, the influence of temperature was investigated (Table 5). When the temperature was increased from 20 to 35 °C, the

Table 5. Influence of Temperature on the Model Reaction[a]



entry	T (°C)	yield (%)[b]	ee (%)[b]
1	20	33	69
2	25	44	69
3	30	46	70
4	35	47	69
5	38	45	65
6	40	38	60
7	50	7	29
8	60	7	19

[a]For the reaction, a mixture of cinnamaldehyde **1a** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL), and pepsin (16.9 kU) was stirred at 20−60 °C for 96 h. [b]Determined by chiral HPLC using a Chiralpak AS-H column.

yield improved from 33 to 47% with the ee remaining at approximately the same level (Table 5, entries 1−4). However, once the temperature surpassed 35 °C, the yield and ee began to decrease (Table 5, entries 5−8). When the temperature was higher than 40 °C, the yield and ee sharply declined (Table 5, entries 7 and 8), which we believe to be due to the high temperature causing denaturation of pepsin. Overall, a relatively

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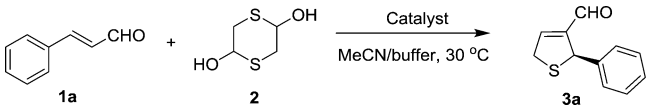
good result of 46% yield with 70% ee was achieved at 30 °C (Table 5, entry 3). Thus, performing the reaction at 30 °C was proven to be the optimal reaction condition.

The time course of the model reaction was investigated under the aforementioned optimal conditions. Generally, the yield increased with reaction time in the early stages and finally remained at an almost constant level. No significant change in ee was detected over the whole time course (for details, see the Supporting Information).

To verify the specific catalytic effect of pepsin on the domino reaction, some control experiments were performed (Table 6).

Table 6. Control Experiments for the Model Reaction[a]



1a 2 3a

entry	catalyst	yield (%)[b]	ee (%)[b]
1	pepsin	46	70
2	none	trace	-
3	pepsin (pretreated with CDI)[c]	4	5
4	pepsin (pretreated with urea)[d]	1	2
5	CDI (200 mg)	trace	–
6	urea (200 mg)	trace	–
7	recombinant pepsin[e]	60	43
8	mutant pepsin (Asp32Ala)[e]	trace	–

[a]Unless otherwise noted, for the reaction, a mixture of cinnamaldehyde **1a** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL), and pepsin (16.9 kU) was stirred at 30 °C for 96 h. [b]Determined by chiral HPLC using a Chiralpak AS-H column. [c]Pepsin (16.9 kU) in a CDI solution (1.2 M in THF) (200 mg of CDI in 1.0 mL of THF) was stirred at rt for 4 h, and THF was removed under reduced pressure before use. [d]Pepsin (16.9 kU) in a urea solution (3.3 M) (200 mg of urea in 1.0 mL of deionized water) was stirred at rt for 24 h, and water was removed by lyophilization before use. [e]For the reaction, a mixture of cinnamaldehyde **1a** (0.035 mmol), 1,4-dithiane-2,5-diol **2** (0.105 mmol), MeCN (0.24 mL), phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 6.5, 0.06 mL), and recombinant pepsin (2.34 mg, 0.7 kU) (for entry 7) or mutant pepsin (Asp32Ala) (2.34 mg) (for entry 8) was stirred at 30 °C for 96 h.

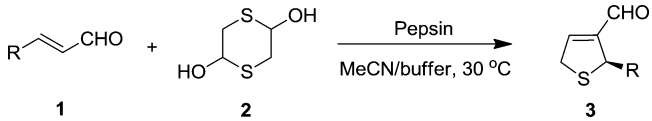
In the absence of pepsin, the reaction gave only a trace amount of the product (Table 6, entry 2), which indicated that the pepsin preparation had a catalytic effect on the domino reaction. The catalytic site of pepsin consists of two aspartate residues, Asp32 and Asp215. *N,N′*-Carbonyldiimidazole (CDI) can be irreversibly covalently bound to carboxyl. Thus, CDI was used to pretreat the pepsin, and the reaction with CDI-pretreated pepsin gave the product in a low yield of 4% with 5% ee (Table 6, entry 3), which indicated that CDI strongly inhibited enzyme activity in the domino reaction. At the same time, CDI alone was verified to have no effect on the domino reaction (Table 6, entry 5). The results suggested that the enzymatic process may proceed in the active site. Moreover, urea as a denaturing agent can change the conformational structure of enzymes and ultimately denature the enzyme. Hence, urea was used to denature pepsin, which nearly led to the complete loss of the catalytic activity of pepsin (Table 6, entry 4). Meanwhile, the blank experiment showed that urea itself did not catalyze the domino reaction (Table 6, entry 6). The experiments described above indicated that the specific natural fold of pepsin was responsible for its activity in the domino reaction.

To further verify that the pepsin is truly the catalyst of the investigated domino reaction, we have conducted a series of studies involving the cloning, expression, purification, and activation of recombinant porcine pepsinogen A,[29] and the obtained recombinant pepsin was used to catalyze the model domino reaction. A good yield of 60% with 43% ee was obtained from the recombinant pepsin-catalyzed reaction (Table 6, entry 7). The result clearly confirmed that pepsin indeed catalyzed the domino reaction in an asymmetric manner.

Moreover, to validate if this observed promiscuous activity arose from the active site of the pepsin, a site-directed mutagenesis was conducted. The catalytic site of pepsin is formed by two aspartate residues, Asp32 and Asp215. We changed, by site-directed mutagenesis, pepsin active site Asp32 to an Ala.[29] Only a trace amount of product was observed for the model domino reaction with the mutant pepsin (Asp32Ala) (Table 6, entry 8). It can be inferred that the enzymatic process indeed proceeds in the active site, and the Asp residues located in the active center are also crucial for this enzymatic domino reaction.

With the optimal reaction conditions in hand, to investigate the generality and scope of the pepsin-catalyzed domino reaction, various *α,β*-unsaturated aldehydes were investigated. As shown in Table 7, aromatic *α,β*-unsaturated aldehydes

Table 7. Investigation of the Substrate Scope for the Pepsin-Catalyzed Domino Reaction[a]



1 2 3

entry	R	product	time (h)	yield (%)[b]	ee (%)[c]
1	Ph	**3a**	96	46	70
2	4-MeC_6H_4	**3b**	132	36	72
3	2-OMeC_6H_4	**3c**	132	44	71
4	4-OMeC_6H_4	**3d**	132	50	70
5	4-ClC_6H_4	**3e**	144	42	66
6	3-ClC_6H_4	**3f**	120	41	56
7	2-ClC_6H_4	**3g**	120	40	50
8	4-BrC_6H_4	**3h**	144	39	65
9	2-BrC_6H_4	**3i**	120	36	55
10	4-$NO_2C_6H_4$	**3j**	144	40	60
11	2-furanyl	**3k**	144	53	70
12	propyl	**3l**	168	35	40
13	isopropyl	**3m**	168	38	84

[a]For the reaction, a mixture of *α,β*-unsaturated aldehyde **1** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL), and pepsin (16.9 kU) was stirred at 30 °C. [b]Yield of the isolated product after silica gel chromatography. [c]Determined by chiral HPLC using a Chiralpak AS-H or Chiralcel OD-H column.

bearing either electron-withdrawing or electron-donating substituents could participate in this domino reaction smoothly. Substituents on the aromatic ring had a significant influence on the enantioselectivities. Generally, aromatic *α,β*-unsaturated aldehydes with electron-donating groups provided enantioselectivities higher than those with electron-withdrawing substituents (Table 7, entries 2−10). When the substituents

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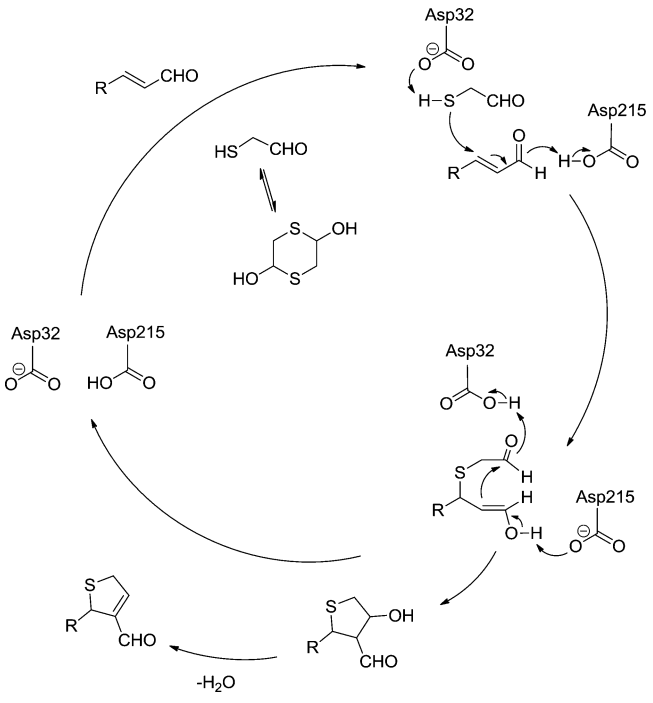
on the aromatic ring were halogen atoms, the position of the substituents had effects on the enantioselectivity. Substituents at the *para* position gave ee values higher than those at *ortho* or *meta* positions (Table 7, entries 5−9). The heteroaromatic α,β-unsaturated aldehyde, 3-(2-furyl)acrylaldehyde, could participate in the reaction, as well, giving the best yield of 53% with 70% ee (Table 7, entry 11). Besides aromatic α,β-unsaturated aldehydes, aliphatic α,β-unsaturated aldehydes could also be applied to the reaction. The straight chain aliphatic α,β-unsaturated aldehyde gave a less efficient result of 35% yield and 40% ee (Table 7, entry 12), while the branched aliphatic α,β-unsaturated aldehyde exhibited the best ee of 84% with a 38% yield (Table 7, entry 13). In general, the desired products were obtained with moderate to good enantioselectivities, but yields were not so high. In all cases, no obvious byproducts were observed. A long reaction time was required in this investigated promiscuous activity of pepsin, which indicated that the reaction rates with substrates that are not natural to an enzyme are usually inefficient and slower than those observed for native substrates.

The control experiments with denatured and inhibited pepsin as well as the experiments with recombinant and mutant enzyme clearly demonstrated that the domino reaction occurs in the active site of pepsin, and the Asp residues located in the active center are responsible for this promiscuous activity. According to the literature,[30] the catalytic site of pepsin is formed by two aspartate residues, Asp32 and Asp215, one of which (Asp215) has to be protonated, and the other (Asp32) deprotonated, for the protein to be active. Thus, on the basis of the control experiments and literature, we attempted to propose a possible mechanism for the pepsin-catalyzed domino reaction of α,β-unsaturated aldehydes and 1,4-dithiane-2,5-diol (Scheme 2). First, Asp32 acts as a base to remove a proton from the mercaptoacetaldehyde that is generated *in situ* from 1,4-dithiane-2,5-diol. Second, the activated mercaptoacetaldehyde and α,β-unsaturated aldehyde undergo intermolecular thia-Michael addition, and the derived adduct accepts the proton from Asp215 forming the enol. Third, the tetrahydrothiophene scaffold is provided through a subsequent intramolecular aldol reaction. Finally, the dehydration process occurs forming the dihydrothiophene skeleton.

Scheme 2. Proposed Mechanism for the Pepsin-Catalyzed Domino Reaction



CONCLUSION

In summary, we have shown a novel promiscuous pepsin-catalyzed asymmetric domino thia-Michael/aldol condensation reaction of aromatic, heteroaromatic, and aliphatic α,β-unsaturated aldehydes with 1,4-dithiane-2,5-diol in MeCN and buffer for the first time. The corresponding functionalized chiral dihydrothiophenes were obtained with 40−84% ee without an additive. The specific catalytic effect of pepsin was demonstrated by combining some control experiments and site-directed mutagenesis. This is the first study utilizing site-directed mutagenesis to confirm the promiscuous activity of pepsin. This work broadens the scope of enzyme-catalyzed transformations. The performance of pepsin in this work as well as its other promiscuous activities reported previously demonstrates that pepsin has considerable potential for broad catalytic promiscuity, which may allow organic chemists to rapidly develop new synthetic applications of pepsin to expand the repertoire of synthetic organic methodologies.

EXPERIMENTAL SECTION

Materials. Pepsin from porcine gastric mucosa [EC 3.4.23.1, CAS Registry Number 9001-75-6, product P7125-100G, lot SLBD7698V, 721 units/mg of protein, 18% protein (UV); 1 unit will produce a change in ΔA_{280} of 0.001 per minute at pH 2.0 and 37 °C measured as TCA-soluble products using hemoglobin as a substrate] was purchased from Sigma-Aldrich. Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification.

General Procedure for the Pepsin-Catalyzed Domino Thia-Michael/Aldol Reaction. Pepsin (16.9 kU) was added to a 10 mL round-bottom flask containing α,β-unsaturated aldehyde (0.35 mmol), 1,4-dithiane-2,5-diol (1.05 mmol), MeCN (1.20 mL), and phosphate buffer (NaH_2PO_4/Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL). The resultant mixture was stirred at 30 °C for the specified reaction time and monitored by thin-layer chromatography (TLC). The reaction was terminated by filtering the enzyme. The filter cake was washed with ethyl acetate (10 mL). Then, the filtrate was concentrated *in vacuo*. The crude product was purified by flash column chromatography with a petroleum ether/ethyl acetate (4/1 to 8/1) eluent.

(R)-2-Phenyl-2,5-dihydrothiophene-3-carbaldehyde (***3a***) (Table 7, entry 1).[20] Brown solid: yield 30.6 mg, 46%; mp 68−70 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.71 (s, 1H), 7.28−7.27 (m, 4H), 7.22−7.19 (m, 1H), 7.01−7.00 (m, 1H), 5.50 (d, J = 5.6 Hz, 1H), 4.23−4.19 (m, 1H), 4.02−3.98 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 187.0, 148.8, 148.7, 142.3, 128.6, 127.5, 54.9, 38.8; HPLC analysis via Chiralpak AS-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 240 nm, t_{major} = 15.4 min, t_{minor} = 19.3 min.

(R)-2-(p-Tolyl)-2,5-dihydrothiophene-3-carbaldehyde (***3b***) (Table 7, entry 2).[20] Brown solid: yield 25.7 mg, 36%; mp 158−160 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.73 (s, 1H), 7.17 (d, J = 8.1 Hz, 2H), 7.09 (d, J = 7.9 Hz, 2H), 7.02−7.01 (m, 1H), 5.49 (d, J = 5.5 Hz, 1H), 4.24−4.20 (m, 1H), 4.03−3.99 (m, 1H), 2.30 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 187.1, 148.9, 148.5, 139.3, 137.2, 129.3, 127.3, 54.7, 38.6, 21.1; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (95/5), flow rate 0.5 mL/min, λ = 244 nm, t_{major} = 23.0 min, t_{minor} = 25.8 min.

(R)-2-(2-Methoxyphenyl)-2,5-dihydrothiophene-3-carbaldehyde (***3c***) (Table 7, entry 3).[20] Brown solid: yield 33.9 mg, 44%; mp 152−154 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.80 (s, 1H), 7.20 (ddd, J = 1.6, 1.1, 1.6 Hz, 1H), 7.12−7.10 (m, 1H), 6.97 (dd, J = 1.7, 1.5 Hz,

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J. Org. Chem. 2016, 81, 6042−6048

1H), 6.87−6.85 (m, 2H), 5.88 (d, J = 5.6 Hz, 1H), 4.13−4.09 (m, 1H), 3.98−3.94 (m, 1H), 3.86 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 187.2, 156.5, 150.1, 148.0, 130.5, 128.5, 127.0, 120.7, 111.0, 55.7, 47.9, 38.2; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 220 nm, t_{major} = 18.8 min, t_{minor} = 15.1 min.

(R)-2-(4-Methoxyphenyl)-2,5-dihydrothiophene-3-carbaldehyde ***(3d)*** *(Table 7, entry 4).*[20] Brown solid: yield 38.5 mg, 50%; mp 134−136 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.73 (s, J = 1.0 Hz), 7.22−7.20 (m, 2H), 7.01−7.00 (m, 1H), 6.83−6.81 (m, 2H), 5.49 (d, J = 5.5 Hz, 1H), 4.24−4.20 (m, 1H), 4.03−4.00 (m, 1H), 3.77 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 187.1, 159.0, 148.9, 148.3, 134.4, 128.6, 114.0, 55.3, 54.5, 38.6; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 244 nm, t_{major} = 14.9 min, t_{minor} = 19.8 min.

(R)-2-(4-Chlorophenyl)-2,5-dihydrothiophene-3-carbaldehyde ***(3e)*** *(Table 7, entry 5).*[20] Brown solid: yield 33.0 mg, 42%; mp 145−146 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.73 (s, 1H), 7.26−7.24 (m, 4H), 7.04−7.03 (m, 1H), 5.48−5.46 (m, 1H), 4.25−4.21 (m, 1H), 4.05−4.02 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.8, 148.9, 148.6, 140.8, 133.2, 128.9, 128.7, 54.3, 38.8; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 240 nm, t_{major} = 11.4 min, t_{minor} = 13.1 min.

(R)-2-(3-Chlorophenyl)-2,5-dihydrothiophene-3-carbaldehyde ***(3f)*** *(Table 7, entry 6).*[20] Brown solid: yield 32.2 mg, 41%; mp 140−142 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.74 (s, 1H), 7.26 (s, 1H), 7.22−7.17 (m, 3H), 7.07−7.05 (m, 1H), 5.45 (d, J = 5.4 Hz, 1H), 4.26−4.22 (m, 1H), 4.06−4.02 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.8, 149.2, 148.4, 144.3, 134.4, 129.8, 127.7, 127.6, 125.9, 54.4, 38.9; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 240 nm, t_{major} = 11.3 min, t_{minor} = 12.9 min.

(S)-2-(2-Chlorophenyl)-2,5-dihydrothiophene-3-carbaldehyde ***(3g)*** *(Table 7, entry 7).*[20] Brown solid: yield 31.5 mg, 40%; mp 179−182 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.74 (s, 1H), 7.26 (s, 1H), 7.22−7.17 (m, 3H), 7.07−7.05 (m, 1H), 5.45 (d, J = 5.3 Hz, 1H), 4.26−4.22 (m, 1H), 4.06−4.02 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.8, 149.2, 148.4, 144.3, 134.4, 129.8, 127.7, 127.6, 125.9, 54.4, 38.9; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 228 nm, t_{major} = 15.7 min, t_{minor} = 19.2 min.

(R)-2-(4-Bromophenyl)-2,5-dihydrothiophene-3-carbaldehyde ***(3h)*** *(Table 7, entry 8).*[20] Brown solid: yield 36.7 mg, 39%; mp 107−110 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.73 (s, 1H), 7.41−7.40 (m, 2H), 7.18−7.16 (m, 2H), 7.04−7.03 (m, 1H), 5.45 (d, J = 5.5 Hz, 1H), 4.25−4.20 (m, 1H), 4.05−4.01 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.8, 149.0, 148.5, 141.3, 131.7, 129.3, 121.3, 54.4, 38.8; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 236 nm, t_{major} = 12.5 min, t_{minor} = 13.9 min.

(S)-2-(2-Bromophenyl)-2,5-dihydrothiophene-3-carbaldehyde ***(3i)*** *(Table 7, entry 9).*[20] Brown solid: yield 33.9 mg, 36%; mp 152−154 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.81 (s, 1H), 7.55 (d, J = 10.0 Hz, 1H), 7.24−7.20 (m, 2H), 7.09−7.05 (m, 2H), 5.93 (d, J = 5.5 Hz, 1H), 4.17−4.12 (m, 1H), 4.04−4.00 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.8, 150.6, 147.6, 141.4, 133.1, 128.8, 127.8, 123.8, 53.9, 38.3; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 236 nm, t_{major} = 19.0 min, t_{minor} = 23.5 min.

(R)-2-(4-Nitrophenyl)-2,5-dihydrothiophene-3-carbaldehyde ***(3j)*** *(Table 7, entry 10).*[20] Brown solid: yield 32.9 mg, 40%; mp 136−137 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.75 (s, 1H), 8.15 (d, J = 8.7 Hz, 2H), 7.46 (d, J = 8.6 Hz, 2H), 7.13 (s, 1H), 5.55 (d, J = 5.8 Hz, 1H), 4.31−4.26 (m, 1H), 4.13−4.09 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.7, 149.8, 149.5, 148.0, 128.5, 123.9, 54.2, 39.1; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 220 nm, t_{major} = 31.0 min, t_{minor} = 33.9 min.

(S)-2-(Furan-2-yl)-2,5-dihydrothiophene-3-carbaldehyde ***(3k)*** *(Table 7, entry 11).*[20] Brown oil: yield 33.4 mg, 53%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.79 (s, 1H), 7.31 (d, J = 1.1 Hz, 1H), 7.08−7.06 (m, 1H), 6.30−6.29 (m, 1H), 6.15 (d, J = 3.2 Hz, 1H), 5.60 (d, J = 5.3 Hz, 1H), 4.23−4.19 (m, 1H), 4.00−3.95 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 186.8, 153.4, 149.8, 146.0, 142.1, 110.7, 106.6, 47.3, 38.3; HPLC analysis via Chiralcel OD-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 232 nm, t_{major} = 16.2 min, t_{minor} = 14.1 min.

(R)-2-Propyl-2,5-dihydrothiophene-3-carbaldehyde ***(3l)*** *(Table 7, entry 12).*[20] Brown oil: yield 19.1 mg, 35%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.75 (s, 1H), 6.85 (dd, J = 4.1, 2.9 Hz, 1H), 3.97−3.93 (m, 1H), 3.85−3.81 (m, 1H), 2.01−1.95 (m, 1H), 1.57−1.51 (m, 2H), 1.43−1.35 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 187.9, 149.6, 149.4, 52.1, 38.3, 37.5, 20.5, 13.7; HPLC analysis via Chiralpak AS-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 236 nm, t_{major} = 6.6 min, t_{minor} = 7.4 min.

(R)-2-Isopropyl-2,5-dihydrothiophene-3-carbaldehyde ***(3m)*** *(Table 7, entry 13).*[20] Brown oil: yield 20.7 mg, 38%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.76 (s, 1H), 6.92−6.90 (m, 1H), 4.54−4.53 (m, 1H), 3.83−3.80 (m, 2H), 2.42−2.39 (m, 1H), 0.95 (d, J = 6.8 Hz, 3H), 0.77 (d, J = 6.7 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 187.8, 150.5, 148.6, 59.5, 37.7, 30.3, 21.8, 15.2; HPLC analysis via Chiralpak AS-H, *n*-hexane/*i*-PrOH (90/10), flow rate 1.0 mL/min, λ = 244 nm, t_{major} = 7.0 min, t_{minor} = 7.9 min.

Construction of the Expression Vector for the Production of Pepsinogen A and the Asp91Ala Mutant (see the Supporting Information for details). The cDNA for the swine pepsinogen A precursor (accession number GI:164603) was synthesized by GenScript Corp. (Nanjing, China). The fragment of pepsinogen A with a coding sequence that begins at amino acid position L16 (and thereby lacks the N-terminal signal peptide) was generated by PCR amplification, by using primers 5′-GGAATTCCATATGTTGGTAAAAGTTCCATTA-3′ and 5′-CCCAAGCTTAGCGACTGGGGCTAAA-3′ to introduce *Nde*I and *Hin*dIII restriction sites (underlined sequence) at the 5′ and 3′ ends, respectively. The PCR products were cloned into the pET28a (+) vector. The Asp91Ala point mutant construct was generated with a QuikChange Site-Directed Mutagenesis Kit (Stratagene). All constructs were confirmed by DNA sequencing.

Expression and Purification of Proteins (see the Supporting Information for details.). Wild-type pepsinogen A and the Asp91Ala mutant were produced in *Escherichia coli* BL21(DE3) by using the pET28a (+) expression system. Protein expression was induced overnight at 30 °C with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) after OD_{600} reached 0.6−0.8. A cell pellet was harvested by centrifugation, resuspended, and lysed by high-pressure homogenization, and soluble protein was fractionated from insoluble material by centrifugation. The targeted protein was purified by Ni-NTA affinity chromatography. Twenty-five milligrams of purified wild-type pepsinogen A and 20 mg of purified Asp91Ala mutant were obtained from 1 L of cell culture.

Activation of Pepsinogen A and the Asp91Ala Mutant. Pepsinogen A and the Asp91Ala mutant were activated as described in the literature.[29] After activation, amino acid residues 60−385 were retained to obtain the active pepsin and Asp32Ala mutant (numbering of porcine pepsin A). The enzyme assay was conducted as described in ref 31. The activity of the pepsin was 299 units/mg of protein (1 unit will produce a change in ΔA_{280} of 0.001 per minute at pH 2.0 and 37 °C measured as TCA-soluble products using hemoglobin as a substrate); no activity was detected for Asp32Ala mutant pepsin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01132.

General methods, extra information about the optimization of reaction conditions, detailed information about construction of the expression vector for the production of swine pepsinogen A and the Asp91Ala mutant, and expression and purification of proteins, ^{1}H NMR and ^{13}C NMR spectra of the products, and HPLC charts of the products (PDF)

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Notes

The authors declare no competing financial interest.

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