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Chiral Resolution of Racemic *p*-Methylsulfonylphenyl Serine Ethyl Ester with Lipases: The Mechanism of Side Reaction and Its Suppression

Rui Guo, Yong-Xian Fan, Xiao-Long Chen,* and Yin-Chu Shen

Institute of Fermentation Engineering, College of Biological and Environmental Engineering, Zhejiang University of Technology, 18 Chaowang Road, Hangzhou 310014, People's Republic of China

Supporting Information

ABSTRACT: The D-*threo* form of *p*-methylsulfonylphenyl serine ethyl ester (MPSE) is a key intermediate for the synthesis of florfenicol. In this study, chiral resolution of DL-*threo*-*p*-MPSE with lipases was investigated. Among a series of lipases, Novzyme 435 was the best to resolve DL-*threo*-*p*-MPSE with the conversion rate of 36.83% and ee value of 35.13%. To improve the conversion rate and ee value, a number of byproducts were identified and characterized using reverse-phase HPLC, normal-phase HPLC, ^{1}H NMR, and LC-MS when *threo*-*p*-MPSE was hydrolyzed by lipases in organic medium. Mechanisms of generating main byproducts are proposed, and a suppressing method is provided. The results showed that byproduct *p*-methylsulfonyl benzaldehyde serves as the key intermediate during the whole side reaction process. It was also observed that *threo*-*p*-MPSE with a proper hydrolytic velocity served as a driving force to generate *p*-methylsulfonyl benzaldehyde and accelerated the side reactions. Finally, a feasible approach to suppress side reactions in enzymatic catalysis is offered. The conversion rate and ee value were greatly improved by 69.29 and 46.26%, respectively, using Zn^{2+} compared to those without Zn^{2+}.

KEYWORDS: *p-methylsulfonylphenyl serine ethyl ester, enzymatic hydrolysis, lipase, side reaction, suppression*

INTRODUCTION

Because different enantiomers of a drug can exert different pharmacological and toxic effects to an organism, chirality is a crucial factor for the efficacy and safety of many drug products. Thus, production of single enantiomers of drug intermediates has become increasingly important in the pharmaceutical industry.[1−3] Single enantiomers can be produced through both chemical and biocatalytic approaches. Compared to chemical methods, enzymatic methods are preferable because they are usually highly enantio- and regioselective. They can also be carried out at ambient temperature and under atmospheric pressure, avoiding the use of extreme conditions and various chemical reagents. Therefore, enzymatic approaches are the trend in chiral separation of drugs and their intermediates.[4−6]

The veterinary antibiotic florfenicol is the derivative of chloramphenicol and of great therapeutic value for infectious diseases of animals.[7] The D-*threo* form of *p*-methylsulfonylphenyl serine ethyl ester (*p*-MPSE) (Figure 1) is one of the key intermediates in the synthesis of florfenicol, whereas the other isomers possess extremely weak antimicrobial activity. The two main chemical routes to prepare D-*threo*-*p*-MPSE are the "*p*-methylsulfonylphenyl serine" routine and the "*p*-methylsulfonylphenyl serine copper routine".[8,9] Enzymatic approaches to gain enantiomerically pure D-*threo*-*p*-MPSE have rarely been reported. However, there are papers about the enzymatic ways to obtain enantiomerically pure *p*-methylthiophenyl serine ethyl ester, which is also an intermediate to synthesize florfenicol and is an analogue of *p*-MPSE.[10−12] There are also papers about the isolation of single enantiomers having structures similar to that of *p*-MPSE, such as enzymatic regioselective production of chloramphenicol ester through acylation,[13] and preparing D-*threo*-*p*-hydroxyphenyl glycine through enzymatic asymmetric hydrolysis of racemic *p*-hydroxyphenylglycine methyl ester.[14]

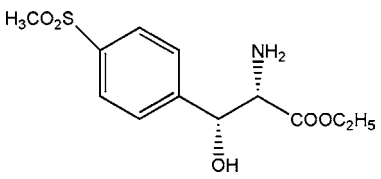

Figure 1. Structure of D-*threo*-*p*-MPSE.

In this paper, chiral resolution of racemic *p*-MPSE with lipases was investigated. It was found that DL-*threo*-*p*-MPSE could easily undergo side reactions either in water at ambient conditions or in organic solvent when enzymatic hydrolysis was carried out. This resulted in the generation of a series of byproducts, which had seriously decreased the production yield and optical purity of the target product. Thus, it would affect the feasibility of the enzymatic way to isolate D-*threo*-*p*-MPSE. Therefore, it was necessary to determine the mechanism of the side reactions and to come up with a feasible way to suppress them. Existing chromatographic methods, such as reverse-HPLC, normal-HPLC, preparative HPLC, and LC-MS, were employed to purify and identify the products of the side reactions. A reasonable mechanism was established and a suppressing method was tested. Overall, this paper provides important

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information for an enzymatic resolution technique of DL-*threo*-*p*-MPSE and its derivatives.

EXPERIMENTAL PROCEDURES

Chemicals. DL-*threo*-*p*-MPSE and D-*threo*-*p*-methylsulfonyl phenyl serine ethyl ester were provided as gifts by Kangmu Pharmaceutical Co., Ltd., Zhejiang, China, with a purity of 99% and were used without further purification. Novzyme 435 lipase (lipase from *Candida antarctica*), pig pancreatic lipase, and *Penicillium expansum* lipase were purchased from Sigma. *Candida lipalytica* lipase was bought from Wuxi Enzyme Factory, Jiangshu, China. *Aspergillus niger* lipase was purchased from Shenzhen Leveking Bio-Engineering Co., Ltd., Guangdong, China. *tert*-Butyl alcohol (TBA) was employed with a water content of <0.3%. All other chemicals used in this work were of analytical grade and commercially available in the local market. Buffer solutions, Tris-HCl buffer (0.02 M) and disodium hydrogen phosphate−citric acid buffer (0.02 M) with required pH, were employed, too.

Enzymatic Reaction Procedure. Chiral hydrolysis of DL-*threo*-*p*-MPSE by lipases was performed in water or in organic solvent. If the organic solvent was used, 5% (v/v) 20 mM Tris-HCl buffer (pH 7.5) was added. In the reaction flask, 25 mg of DL-*threo*-*p*-MPSE, 25 mg of lipase, and 10 mL of water or the organic solvent were added. Experiments were carried out in 50 mL batch conical flasks with lids in a rotating shaker. The rotating rate and temperature were set at 150 rpm and 30 °C, respectively. The reaction time was 72 h.

The conversion rate and ee value were calculated by applying the following equations, which are valid for reactions:

$$\text{conversion rate} = [D_e]/[I] \times 100\%$$

$$\text{ee} = [([D] - [L])/([D] + [L])] \times 100\%$$

$[D_e]$ and $[I]$ are the decreasing and initial concentrations of DL-*threo*-*p*-MPSE; $[D]$ and $[L]$ are the concentrations for D-*threo*-*p*-MPSE and L-*threo*-*p*-MPSE, respectively.

Preparation of Reaction Mixtures for the Detection of Byproducts. Reaction mixture of the spontaneous hydrolysis: 25 mg of DL-*threo*-*p*-MPSE was dissolved in 10 mL of water with an initial pH of 7.0. Then, the solution was rotated in a water bath shaker at 130 rpm and 30 °C for 24 or 48 h. Alkaline hydrolysis was carried out under pH of 11.0−12.0 in 0.5 M sodium hydroxide solution. Reaction mixture of enzymatic hydrolysis: for a solution of 25 mg of DL-*threo*-*p*-MPSE in 10 mL of TBA, 12.5 mg of Novzyme 435 lipase or *P. expansum* lipase was added. The reaction was then carried out in the water bath shaker at 130 rpm and 30 °C for 48 h.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis. RP-HPLC analysis was performed on an LC-20AT system (Shimadzu) combined with an SPD-20A UV detector (Shimadzu) (λ = 226 nm) and a Thermo ODS-2 HYPERSIL (C18) column (4.6 mm × 250 mm, particle size = 5 μm) using water/acetonitrile (90:10 v/v, 0.1% trifluoroacetic acid) as the mobile phase, 1 mL min^{-1} as the flow rate, and 20 μL as the injection volume.

Normal-Phase High-Performance Liquid Chromatography (NP-HPLC) Analysis. NP-HPLC analysis was performed on an LC-20AT system (Shimadzu) combined with an SPD-20A UV detector (Shimadzu) (λ = 226 nm) and a chiral column of Daicel ChiralcEL OJ-3 (4.6 mm × 250 mm, particle size = 3 μm) using *n*-hexane/ethanol (60:40 v/v, 0.5% diethylamine) as the mobile phase, 1 mL min^{-1} as the flow rate, and 20 μL as the injection volume.

^{1}H NMR Analysis. D_2O was used as a magnetic field frequency lock signal. ^{1}H NMR spectra were recorded for each sample on a Bruker AVANCE III 500 spectrometer at 500 MHz using tetramethylsilane (TMS) as an internal standard.

Mass Spectrometry (MS) and Liquid Chromatography−Mass Spectrometry (LC-MS) Analysis. The analytical methods of MS and LC-MS were employed for the determination of molecular weight of each byproduct. LC-MS analyses were conducted with an Agilent 6210 LC-TOF combined with a Thermo ODS-2 Hypersil(C18) column (4.6 mm × 250 mm, particle size = 5 μm) or a Thermo Finnigan LCQ-Advantage combined with an Eclipse Plus (C18) column (2.1 mm × 100 mm, particle size = 3.5 μm), both with ESI source operated in positive mode. Two elution modes were applied in the experiments. In mode I, the method of isocratic elution was employed, which employed a mobile phase of water/acetonitrile (95:5 v/v, 0.1% formic acid). In mode II, the method of gradient elution was applied: mobile phase A was water containing 0.1% ammonium acetate, and mobile phase B was acetonitrile; the condition of gradient elution was B% from 10 to 90% in 10 min, which was then kept for 15 min. MS data were acquired over scan ranges of m/z 50−2000 and 100−800, respectively.

RESULTS AND DISCUSSION

Effect of Different Lipases on the Enzymatic Resolution. Five commercial lipases from different sources were employed to investigate the effect on the enzymatic resolution of DL-*threo*-*p*-MPSE. The results are shown in Table 1. From Table 1, Novezyme 435 was much better than the other lipases in resolving DL-*threo*-*p*-MPSE, with a conversion rate of 36.83% and ee value of 35.13%. Therefore, in the next study, Novezyme 435 was selected and used in the reaction.

Table 1. Effect of Different Lipases on the Enzymatic Resolution

enzyme	conversion rate (%)	ee value
Novezyme 435	36.83	35.13
Penicillium expansum	80.30	6.17
Candida lipalytica	1.77	0
Aspergillus niger	1.52	0
pig pancreatic lipase	1.05	0

However, the yield of D-*threo*-*p*-MPSE and the conversion rate of *p*-MPSE were very low, which increases the cost of the reaction. Therefore, how to raise the yield of D-*threo*-*p*-MPSE and the conversion rate of *p*-MPSE became the questions in the study. In the HPLC chromatograph, there were new unidentified peaks, indicating the generation of byproducts. How did the side reactions take place, and how could the side reactions be stopped? To answer questions such as these, the byproducts should be identified and the reaction mechanism should be proposed. Then reasonable measures could be taken to stop the side reactions, which could improve the yield of D-*threo*-*p*-MPSE and *p*-MPSE's conversion to *p*-methylsulfinophenyl serine rather than byproducts.

Detection of Byproducts Employing RP-HPLC. *Detection of Byproducts in the Spontaneous Hydrolysis.* When DL-*threo*-*p*-MPSE was spontaneously hydrolyzed in water for 24 h, the byproducts of spontaneous hydrolysis were observed, especially the hydrolyzed product, DL-*threo*-*p*-methylsulfinophenyl serine (DL-*threo*-*p*-MPS). Besides the peaks of *p*-MPS and *p*-MPSE, two unknown peaks were also observed (Figure 2a). After hydrolysis for another 24 h, four more unknown peaks appeared, altogether with the two peaks mentioned above. Therefore, there were six byproducts, BP1, BP2, BP3, BP4, BP5, and BP6, generated in the spontaneous hydrolysis of *p*-MPSE (Figure 2b). The peaks of BP3 and BP6 were relatively bigger than the others. Moreover, from Figure 2b, there was a shoulder peak in front of the BP6 peak, indicating there was possibly one more byproduct, BPX.

Detection of Byproducts in the Enzymatic Hydrolysis. To confirm whether there were the same byproducts in the enzymatic hydrolysis, asymmetric hydrolysis of *p*-MPSE with Novzyme 435 was designed in 100% TBA for 48 h. The result is shown in Figure 3. In the reaction, a series of byproducts had also been detected except for BP1 and BP2 (Figure 3b). Thus, the byproducts in the enzymatic hydrolysis in TBA were two peaks

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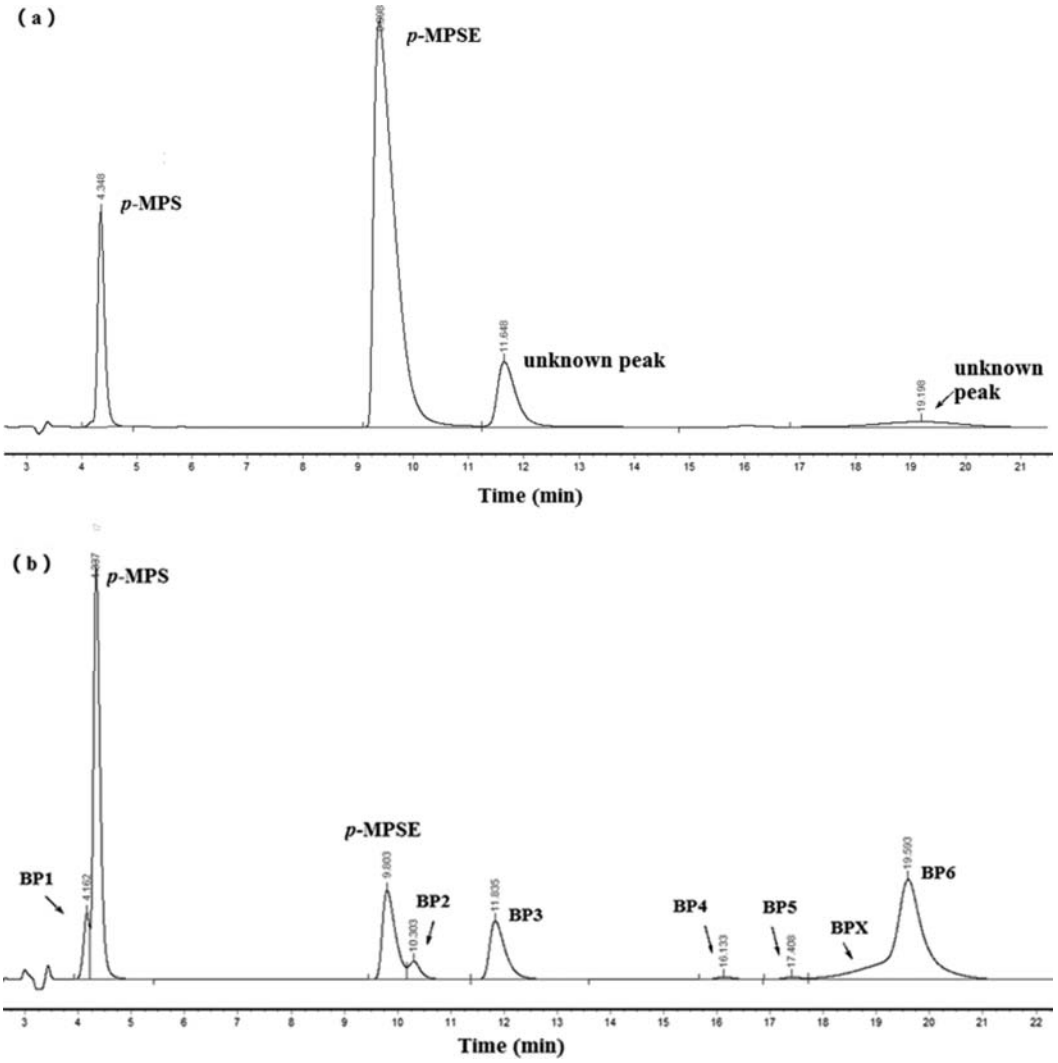

Figure 2. RP-HPLC analysis of *p*-MPSE spontaneous hydrolysis reaction mixture, with *p*-MPSE initial concentration of 2.5 mg mL^{-1}: (a) reaction time of 24 h; (b) reaction time of 48 h.

less than those in the spontaneous hydrolysis, only four peaks in the HPLC spectrum.

Identification of Byproducts. *Alkaline Hydrolysis.* The pH was adjusted to 11.0−12.0 in the spontaneous hydrolysis to afford the complete hydrolysis. After the complete hydrolysis, it was observed from the HPLC spectrum that peaks of *p*-MPSE and BP3 disappeared while other peaks remained. At the same time, the peaks of *p*-MPS, BP1, and BP6 became much bigger. BP3 was then separated and hydrolyzed in alkali. The result showed that BP3 could be hydrolyzed by alkali. The product of the hydrolysis was BP1. Therefore, BP3 was supposed to be an ester. There was no considerable change in the peaks of other byproducts after alkaline hydrolysis. Moreover, the amount of *p*-MPS was much lower than the theoretical amount. Probably the generation of BP6 might be the main cause.

NP-HPLC Analysis of BP3. NP-HPLC with a chiral column was employed to analyze BP3. The sample of BP3 was prepared through preparative RP-HPLC and was mixed with DL-*threo*-*p*-MPSE in a proper proportion. The mixture was then separated by the chiral column. Four peaks (1, 2, 3, and 4) presented on the spectrum (Figure 4). Peaks 1 and 4 were L-*p*-MPSE and D-*p*-MPSE, respectively, as already known. Thus, peaks 2 and 3 were BP3, which clearly demonstrated that BP3 was also a chiral compound, comprising two enantiomers.

After D-*threo*-*p*-MPSE was spontaneously hydrolyzed in water at 40 °C for 12 h, the reaction mixture was detected. As shown in Figure 5, there were also four peaks on the spectrum, two enantiomers of BP3 as well as the L-*threo*-*p*-MPSE, which indicated that D-*threo*-*p*-MPSE had undergone the process of racemization. The racemization was supposed to occur after the process of epimerization when there were two chiral carbons in the compound. The molecular weight of BP3 was proved to be 287 (see section 3.2.3), indicating that BP3 was an isomer of *p*-MPSE. As mentioned above, BP3 might have the ester structure and could be hydrolyzed to BP1, having a molecular weight of 259, the same as that of *p*-MPS, as proved later in section 3.2.4.1. All of these strongly indicated that BP3 was the *erythro*-form of *p*-MPSE. In addition, it could be concluded that two chiral carbons had been engaged in the configuration change when epimerization of *threo*-*p*-MPSE happened.

^{1}H NMR and MS Analysis of the Main Byproducts BP3 and BP6. To identify the structures of BP3 and BP6, ^{1}H NMR and MS analyses were performed. ^{1}H NMR analysis of *p*-MPSE was also carried out. From the MS analysis, the molecular weights of BP3 and BP6 were 287 and 184, respectively. When the MS

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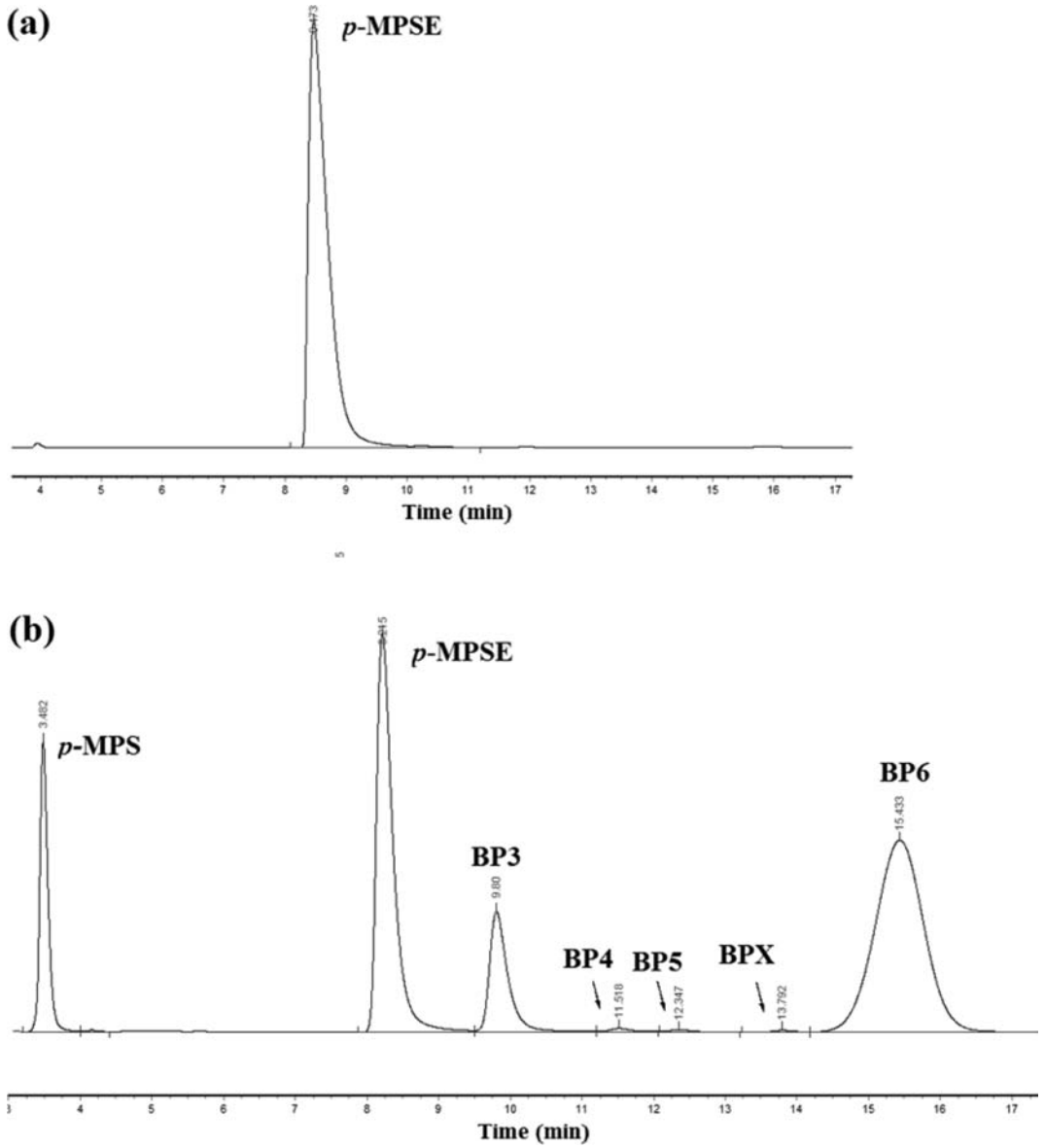

Figure 3. RP-HPLC analysis of reaction mixture of *p*-MPSE enzymatic hydrolysis: (a) control system without lipase; (b) lipase hydrolysis (reaction time of 48 h).

results were combined with ^{1}H NMR results, BP6 could be identified as *p*-methylsulfonyl benzaldehyde (*p*-MBA), and it could be observed that there was no difference between the structures of BP3 and *p*-MPSE, which could confirm that BP3 was the *erythro*-form of *p*-MPSE. The slight difference between BP3 and *p*-MPSE mainly existed at the α- and β-positions.

Identification of Other Byproducts Employing LC-MS. *LC-MS Analysis Employing Isocratic Elution.* As for the isocratic elution (mode I), the ideal mobile phase for LC-MS analysis was water/acetonitrile (95:5 v/v, 0.05% formic acid). There were totally seven peaks of byproducts as shown in the TIC spectrum. The molecular weights of unidentified byproducts were M_{BP1} = 259, M_{BP2} = 186, M_{BP4} = 369, M_{BP5} = 369, M_{BP7}= 103, and M_{BP8} = 200. BP7 and BP8 were new byproducts, which had not been detected by RP-HPLC.

Structures of most of the byproducts could easily be deduced. BP1 had the same molecular weight as *p*-MPS, giving evidence to the assumption that BP3 was the *erythro*-form of *p*-MPSE. Thus, BP1 should be the *erythro*-form of *p*-MPS; BP2 was *p*-methylsulfonyl benzenemethanol (*p*-MBM); BP7 was glycine ethyl ester. The resolution between glycine ethyl ester and *p*-MPS was improved when LC-MS with higher sensitivity was employed; BP8 was *p*-methylsulfonylbenzoic acid (*p*-MBAC). MBAC might just be the BPX mentioned above. When LC-MS was employed, it could be seen that the ionizing MBAC had been "separated" from MBA, which was difficult to ionize, in the TIC spectrum. The structures of BP4 and BP5 could still not be determined only by their molecular weight result, but it was probable that they were also a pair of diastereoisomers with either *threo*- or *erythro*-form.

LC-MS Analysis Employing Gradient Elution. When mode II was employed for LC-MS analysis of a reaction mixture of the spontaneous hydrolysis, two more new byproducts had been detected with the same molecular weight of 453, suggesting that they were isomers and, probably, were diastereoisomers. These two new products could easily be determined to be Schiff base compounds; each of them had *threo*- or *erythro*-form (Figure 6).

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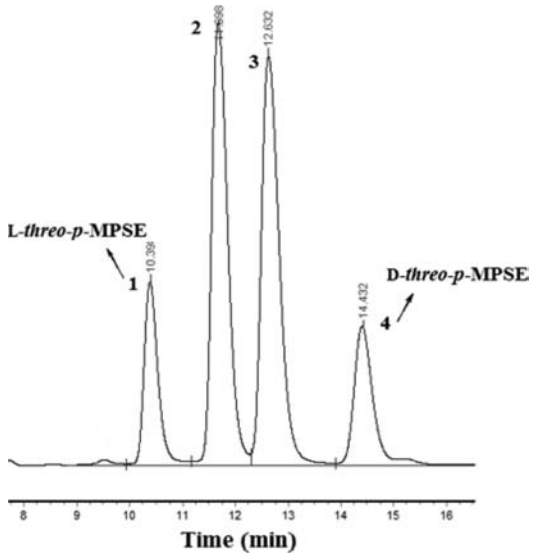


Figure 4. NP-HPLC analysis of a mixture of D-*threo*-*p*-MPSE and BP3.

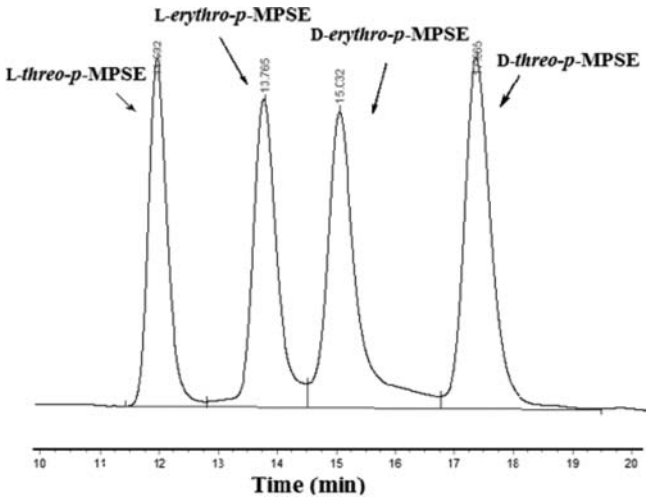


Figure 5. NP-HPLC analysis of the reaction mixture of D-*threo*-*p*-MPSE been spontaneously hydrolyzed in water for 12 h at 40 °C.

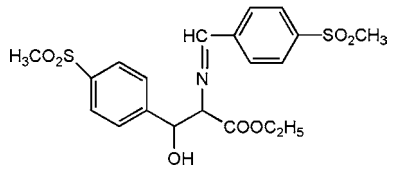
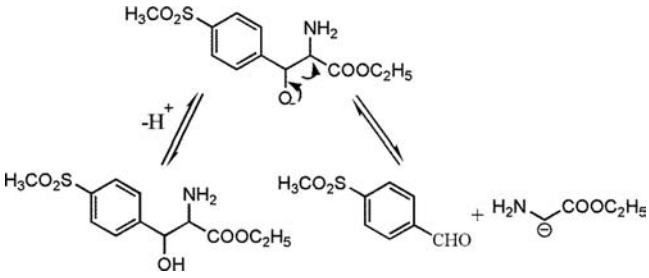


Figure 6. Schiff base compound.

Schiff base compounds are unstable in acid environments and will decompose before being eluted from the column if TFA or formic acid is used in the mobile phase. Therefore, the two new byproducts, Schiff base compounds, were not detected in mode I.

Chemical Mechanisms of Side Reactions. *Mechanism of Generating p-MBA.* Yan[15] reported that, during the process of racemization of L-*threo*-*p*-methylsulfonylphenyl serine copper under alkaline condition with a pH ranging from 8 to 11, *p*-MBA had been detected, with the hypothesis that a reverse reaction of condensation had happened between *p*-MBA and glycine copper. In the current study, it was found that, as the spontaneous hydrolysis or enzymatic hydrolysis of *p*-MPSE proceeded in its water solution with an initial pH ranging from 7 to 7.5 or in organic solvent of TBA at room temperature, a similar cleavage, the retro-aldol reaction, could also occur with the generation of a considerable amount of *p*-MBA and its corresponding byproduct glycine ethyl ester. When *p*-MPSE was under the mentioned conditions, the hydroxyl proton went off and then formed the negative oxygen ion. Next the electron transfer reaction occurred, which would result in carbon−carbon bond rupture between α-C and β-C (Scheme 1). Therefore, the

Scheme 1



bond between α-C and β-C of *p*-MPSE was rather unstable when in water or organic solvent of TBA. Bishwajit's work[15] had showed that ethyl *threo*-2-(1-adamantyl)-3-hydroxybutyrate, an ester of β-hydroxy acid, would mainly experience the retro-aldol reaction rather than hydrolysis in alkaline solution. However, its parent, ethyl 3-hydroxybutyrate, with no substituent on α-C would merely undergo the hydrolysis process under similar conditions. This was mainly because the different substituents on the substrate had different activated barriers and then would have different reaction pathways. Similarly, as for *p*-MPSE, the types of substituents on α-C and β-C and their internal interaction, as well as the entire molecular configuration, might be favorable for both hydrolysis and retro-aldol reaction to occur under the above-mentioned conditions. When *p*-MPS was under the identical reaction condition, retro-aldol reaction did not take place. Therefore, the molecular structure of *p*-MPS was stable under the same conditions, indicating that no MBA generated from the cleavage of *p*-MPS.

Mechanism of Generating Schiff Base Compound. There are two possible mechanisms for generating Schiff base compounds under the related conditions. In mechanism I, aldol reaction of two molecules of MBA with glycine ethyl ester occurs. In this aldol reaction, the Schiff base of glycine ethyl ester is initially formed and then reacts with a second molecule of MBA due to the deprotonated α-position (Scheme 2). In mechanism II, one molecule of MBA just formed the Schiff base compound with *p*-MPSE directly (Scheme 3).

From the LC-MS results, the *threo*- and *erythro*-forms of the Schiff base compound were equal in quantity, and the *threo*-form of *p*-MPSE predominated over its *erythro*-form in amount. Therefore, if *erythro*-*p*-MPSE was generated through a certain mechanism before the generation of the Schiff base compound, it was impossible for the Schiff base compound to form through mechanism II. If the Schiff base compound was generated through mechanism I, it was clear that the *threo*- and *erythro*-forms of the Schiffbase compound were equal in quantity from the equation, which was in accordance with the result of the spectrum. Therefore, mechanism I was suitable and reasonable to explain the generated byproducts.

Epimerization and Racemization of p-MPSE. The earliest racemic technique of the ineffective enantiomer L-*threo*-*p*-MPSE was that a 50% ethanol solution of L-*threo*-*p*-MPSE was heated to

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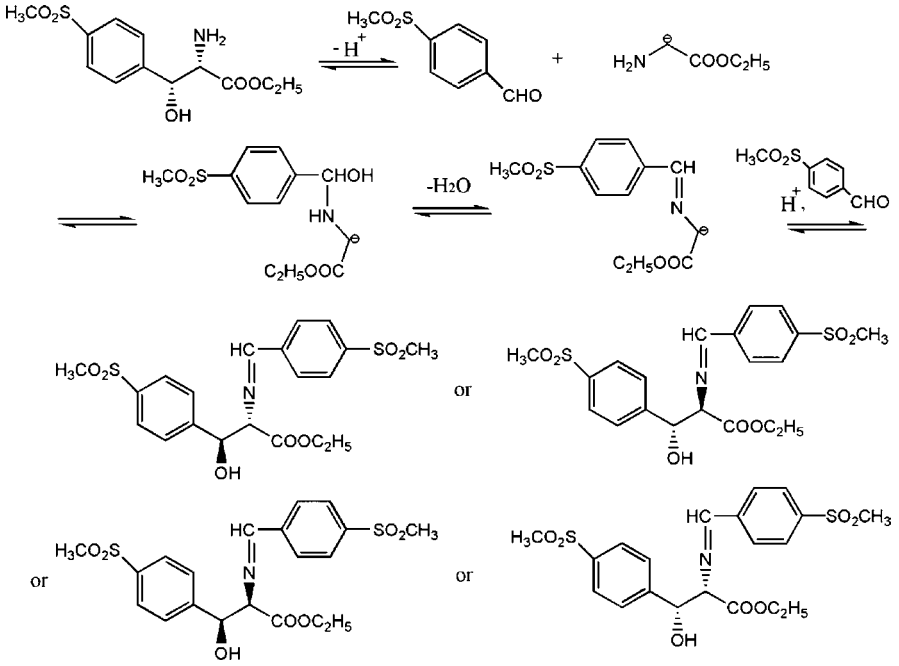
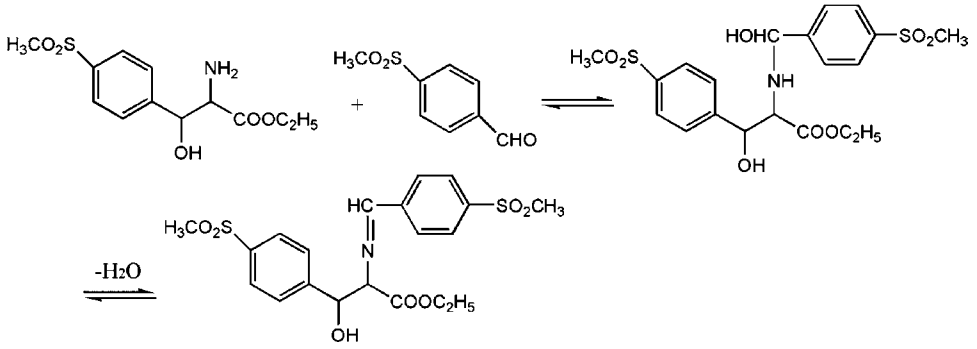
Scheme 2

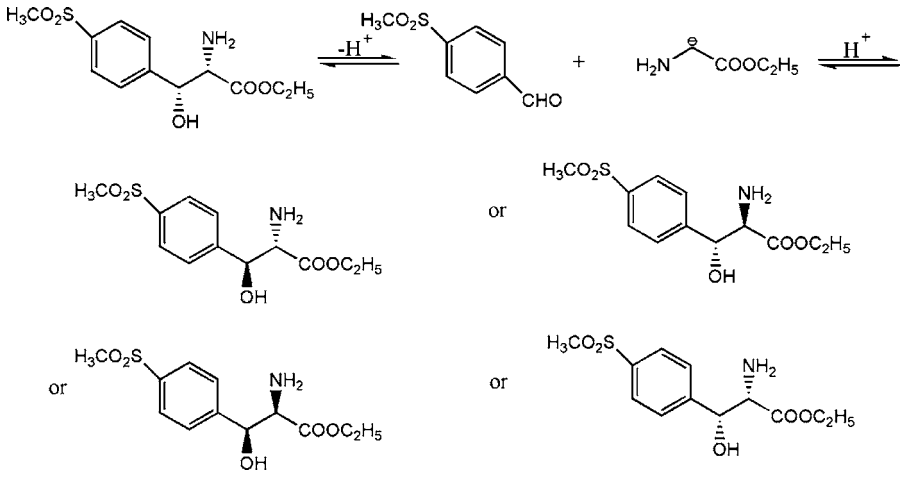


Scheme 3



threo or erytho form

Scheme 4



80 °C and kept for a few hours, and then L-*threo-p*-MPSE would be racemized into DL-*threo-p*-MPSE with the generation of the *erythro*-form at the same time. Later, the more mature technique was to hydrolyze L-*threo-p*-MPSE into L-*threo-p*-MPS with alkali first, and then the L-*threo-p*-MPS was racemized in metal salt solutions of Ca^{2+} and Cu^{2+} with a pH ranging from 8.0 to 10.0 at a temperature ranging from 30 to 100 °C, which would give a relative pure DL-*threo-p*-MPSE without generation of the *erythro*-

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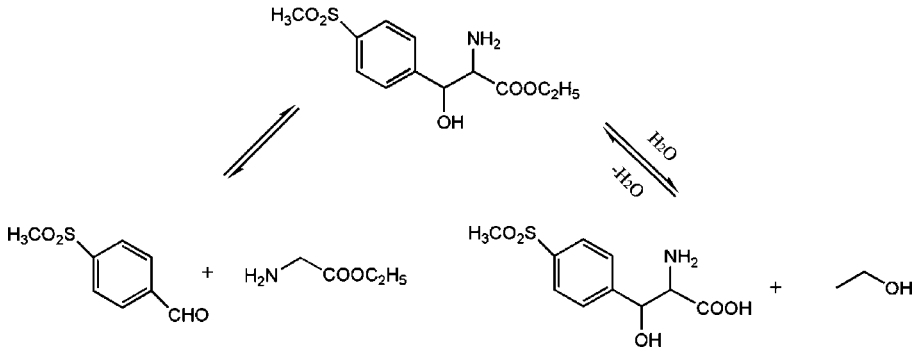
Scheme 5



form.[15,16] As to the racemic mechanism, Yan had supposed that MBA generated from the cleavage of *p*-MPS copper salt would condense again with glycine under alkali condition in the presence of Cu^{2+}.[15] Similarly, a probable mechanism of epimerization and racemization of *p*-MPSE in water or in enzymatic hydrolysis organic solvent system could be supposed, that MBA and glycine ethyl ester, generated from the cleavage of *p*-MPSE, could serve as the starting materials of aldol reaction and form *p*-MPSE with a different configuration (Scheme 4).

From the above discussions of mechanisms, a conclusion could be drawn that MBA served as the key byproduct during the whole process of side reactions, as it was the basis of the generation of other byproducts.

Effects of pH and Temperature on Side Reactions. The Schiff base compound was not stable in the acid surrounding. Solving the issue of how the surrounding pH influenced other side reactions was helpful in confirming the mechanism of the side reaction. Experiments were carried out to investigate the effect of pH and temperature on the side reactions. Buffers with pH ranging from 3.0 to 9.0 were employed for the spontaneous hydrolysis of *p*-MPSE. The following buffers were used to obtain the required pH: disodium hydrogen phosphate−citric acid buffer for pH 3.0−8.0 and Tris-HCl buffer for pH 7.5−9.0. After 24 h of reaction, each reaction mixture was sampled for RP-HPLC analysis. The results were similar when two different buffers with the same pH were employed, indicating the types of buffer employed had no influence on the hydrolysis as well as the side reactions. When *p*-MPSE was in a pH ranging from 3.0 to 5.0, the spontaneous hydrolysis rarely happened, and no byproduct was detected; when the pH was 7.5, side reactions occurred significantly, with a sharp increase in the generation of *erythro-p*-MPSE and MBA; when the pH was 8.0, the generation of MBA reached the highest. However, as the pH continued to increase, side reactions decreased abruptly. Therefore, pH has a significant influence on side reactions, which would achieve a maximum degree when the pH was between 7.5 and 8.0. A phenomenon could also be observed that when *p*-MPSE was under acidic or weakly acidic condition, hydrolysis could hardly happen, and no byproduct was detected at all. However, as long as MBA and *erythro-p*-MPSE could be detected, the hydrolysis was significantly increased. The situation of spontaneous hydrolysis and side reactions was also investigated in TBA with different contents of buffer solution (from 2.5 to 10%), and it was found that there was a significant positive correlation between side reactions and buffer content. As could be observed in section 3.2.2, in 100% TBA, the control with no hydrolysis had no generation of byproducts (Figure 3a), but as soon as enzymatic hydrolysis happened, a large amount of MBA, as well as *erythro-p*-MPSE, appeared in such an microwater environment, which rarely had the influence of pH (Figure 3b). Therefore, *p*-MPSE hydrolysis could serve as the driving force of side reactions. However, when *p*-MPSE was under stronger alkaline condition, *p*-MPSE could be rapidly hydrolyzed into MPS without any byproduct being generated. Therefore, hydrolysis with proper velocity, not too fast, could be a driving force of side reactions. When *p*-MPSE was exposed in 100% TBA for >10 days, a large amount of MBA and *erythro-p*-MPSE could still be generated, however, without hydrolysis of *p*-MPSE. Therefore, the spontaneous side reactions were not a unique phenomenon in water or in solution containing water, in which the hydrolysis occurred. Thus, the hydrolysis of *p*-MPSE with proper velocity was not the exclusive cause for side reactions, but a stimulating factor. Furthermore, when in a same hydrolysis level, the generating amount of MBA was higher in the enzymatic catalysis−TBA system than in water.

Temperatures ranging from 30 to 70 °C were employed for spontaneous hydrolysis of *p*-MPSE both in water (with initial pH 7.0) and in buffer (pH 7.0); the initial concentration of *p*-MPSE was 2.5 mg mL^{-1}. After 24 h of reaction, each reaction mixture was sampled for RP-HPLC analysis. In both solutions, the level of side reactions increased as the temperature rose, but the reaction in buffers was lower than that in water. When the temperature was 30 °C, the HPLC peak area of generating MBA was 2.91% in buffer and 3.12% in water. However, when the temperature was 70 °C, these values were 18.57% in buffer and 40.75% in water. During the reaction, the only different factor between these two solutions was pH variation. In buffer, the pH was kept at 7.0 all of the time; however, the pH in water decreased from 7.0 to 6.8−5.0 depending on different reaction temperatures or hydrolysis levels. For instance, when the reaction temperature was 50 °C, in 24 h of reaction, the pH in water decreased from 7.0 to 6.0, and the HPLC peak area of MBA was 26.14%, whereas in buffer, this value was only 8.97%, but its hydrolysis rate was higher than that in water. In buffer, the rise of temperature could accelerate the side reactions to a slight degree. When buffer was compared to water, side reactions were more significant under the variable pH condition than in the constant pH condition at the same temperature. As to the mechanism, according to section 3.3, an equilibrium equation could be drawn out, as shown in Scheme 5. *p*-MPSE could react toward two routes, the *p*-MPS route and the MBA route, respectively. On the one hand, as the temperature increased, the reaction rate would be sped in both routes. This was why side reactions increased along with the rise of temperature. However, its increasing rate was lower than that of hydrolysis. On the other hand, hydrolysis velocity that was too fast due to a high temperature was not

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favorable to side reactions, as more *p*-MPSE would be hydrolyzed to *p*-MPS rather than generate MBA when the reaction finished. Thus, as the hydrolysis proceeded in water and in a temperature >40 °C, the decrease of pH had remarkably slowed the fast hydrolysis velocity. Therefore, more *p*-MPSE had been used to generate MBA compared to that in buffer.

Some favorable factors, such as a relatively high temperature (>40 °C), would increase the generating velocity of MBA for a short period (<24 h) in the beginning, but when the reaction was finished, the generating amount of MBA would not be as high as that brought by a reaction which occurred with proper hydrolysis velocity under mild condition for a long period (>48 h).

From the effect of pH and temperature on the side reaction, the MBA generating mechanism was further confirmed.

Suppression of Side Reactions. It had been reported that copper(II) acetate would suppress the spontaneous oscillatory for the chiral conversion of α-substituted propionic acids by forming the chelating coordinate covalent.[17] Similarly, it was found that some kinds of metal ions would effectively suppress the generation of MBA and the subsequent byproducts. The mole ratio of *p*-MPSE and metal ion employed for investigation was 1:1 and each concentration, 8.71×10^{-7} mol L^{-1}.

Effect of Metal Ions on Side Reactions in Water and Buffer. *p*-MPSE was hydrolyzed spontaneously in water in the presence of several metal ions. After 24 h, the precipitation had formed in water with transition metal ions Fe^{3+}, Cu^{2+}, and Zn^{2+} or other metal ions, Al^{3+}, Sn^{2+}, and Pb^{2+}. The amounts of the precipitation for different metal ions were different, with Pb^{2+} and Zn^{2+} generating the most, Cu^{2+} and Al^{3+} the second most, and Fe^{3+} and Sn^{2+} the least. There was no precipitation in water with other tested ions. All precipitates generated above could be dissolved by water. From RP-HPLC analysis, the precipitate was identified as being *p*-MPS.

From the results of RP-HPLC, there were no byproducts generated along with *p*-MPS in the reactions with different metal ions. However, in these reactions, the hydrolysis rate was almost higher than the control with no metal ion. Byproducts were still generated in other tested ion reaction systems in which no precipitation formed.

p-MPSE could hardly be hydrolyzed in water with a pH below 5.0 as mentioned under section 3.4, but here, in the weak acidic solution of copper sulfate and acidic solutions of aluminum nitrate, ferric chloride, and stannic chloride, *p*-MPSE could still be greatly hydrolyzed. Thus, metal ions with suppressive effect would promote the hydrolysis of *p*-MPSE.

When ions with suppressive effect in water solution were added to buffer solution (pH 7.5), all ions had formed their corresponding hydroxide precipitation in such a pH environment except Zn^{2+}. Zn^{2+} could still suppress the formation of byproducts in buffer solution with a higher hydrolysis level of *p*-MPSE when compared to that in water solution. Therefore, Zn^{2+} could be used to suppress the side reaction in buffer.

Potential Mechanism of Suppression. From the above results, the mechanism of suppression could be supposed as the ion with suppressive effect could form the chelate with *p*-MPS (Figure 7a). On the one hand, the carboxyl group coordinated with the metal ion, which would make the carboxyl group hard to esterify with alcohol to form *p*-MPSE again. On the other hand, when *p*-MPS formed the chelate with the metal ion, its solubility decreased as compared to free *p*-MPS. When the concentration of chelate increased, the precipitation would happen. From the equilibrium equation (Scheme 5), it could be seen that both reasons would result in a sharp decrease of free *p*-MPS.

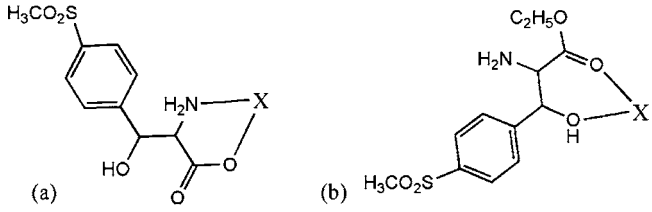

Figure 7. Chelate of (a) *p*-MPS with metal ions; (b) *p*-MPSE with metal ions. X = Pb^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+}, Fe^{3+} and Sn^{2+}.

Therefore, the reaction would tend to move toward the direction of *p*-MPSE hydrolysis rather than cleavage. Another mechanism might exist in the solution at the same time, the formation of *p*-MPSE chelate (Figure 7b). The lone pair electrons on the hydroxyl oxygen atom were stabilized when chelate formed. Thus, electron transfer would not happen and it was hard for the bond between α-C and β-C to rupture. In that case, no MBA would generate and the side reactions were suppressed.

Suppression of Side Reactions with Zn^{2+} in Enzymatic Reaction. Because Zn^{2+} could suppress side reactions effectively under the mildly alkaline condition (about pH 7.5), which was a favorable condition for alkaline lipase, it was practical for us to research the role of Zn^{2+} in suppressing side reactions in organic solvent.

Two controls and two enzymatic hydrolyses were employed according to Table 2. Ten milliliters of TBA−Tris-HCl buffer (pH 7.5) (19:1 v/v) was used as organic solvent in the reaction. The reaction had been carried out for 48 h, and then each reaction mixture was sampled for RP-HPLC analysis. From the spectra (Figure 8), it could be observed that there was no obvious influence of Zn^{2+} on *p*-MPSE spontaneous reaction, and in the presence of Zn^{2+}, the generation of MBA and *erythro*-*p*-MPSE had been suppressed remarkably during the enzymatic hydrolysis process. At the same time, its hydrolysis rate had been improved. Therefore, side reactions had been successfully suppressed by Zn^{2+} when lipase hydrolysis of *p*-MPSE was conducted in the organic solvent system. Meanwhile, Zn^{2+} could also improve the hydrolysis rate of lipase (Table 3). After 72 h of reaction, the conversion rate and ee value reached 62.35 and 51.38%, much higher than those without Zn^{2+} (36.83 and 35.13%), respectively. The longer the reaction time, the higher the conversion rate and ee value. This might be attributed to the formation of *p*-MPS chelate precipitation that had been observed in the enzymatic reaction system, but this promotion of hydrolysis rate would certainly exert some effects on the optical purity of the target product, and other issues, such as how Zn^{2+} would influence the enzyme activity and how to remove Zn^{2+} from chelate when in the preparation of the final product, still required further research.

Table 2. Solutions Designed for Ion Suppression Research in Enzymatic Hydrolysis

	Novzyme 435 (mg)	*p*-MPSE (mg)	$ZnSO_4 \cdot 7H_2O$ (mg)
control 1	0	25	0
lipase hydrolysis 1	25	25	0
control 2	0	25	25
lipase hydrolysis 2	25	25	25

Conclusions. Chiral resolution of DL-*threo*-*p*-MPSE with lipases was carried out successfully. Among the lipases, Novzyme 435 was the best to resolve DL-*threo*-*p*-MPSE with a conversion

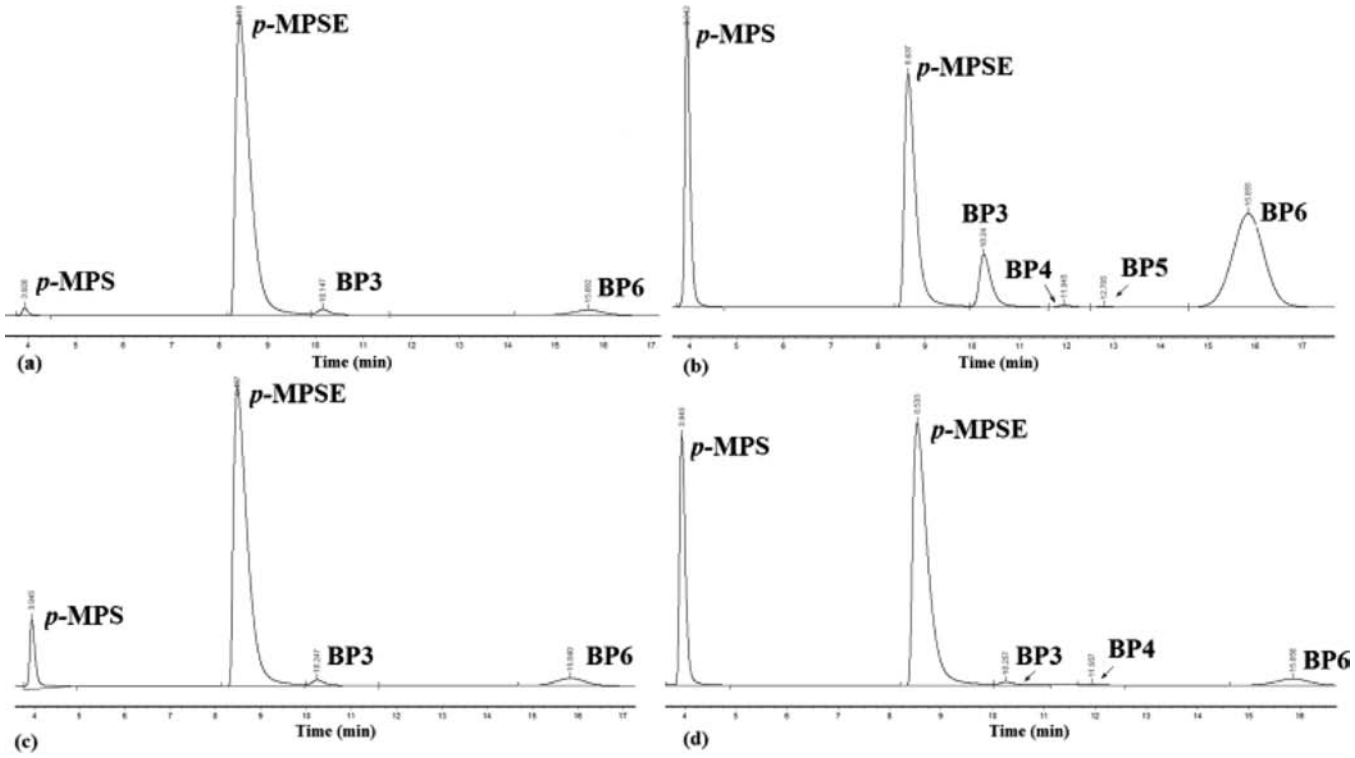

Figure 8. RP-HPLC analysis of reaction mixtures shown in Table 1, with a reaction time of 48 h: (a) control 1; (b) lipase hydrolysis 1; (c) control 2; (d) lipase hydrolysis 2.

Table 3. Effect of Zn^{2+} on the Enzymatic Resolution with Novzyme 435

reaction time (h)	conversion rate (%)	ee value
24	22.41	13.44
48	42.49	29.63
72	62.35	51.38
96	76.52	71.10

rate of 36.83% and an ee value of 35.13%. However, *p*-MPSE undergoes side reactions when it is spontaneously hydrolyzed in water or in organic solvent of TBA by lipase or exposed to 100% TBA for >10 days, which made the resolution inefficient. In the side reaction, there were 10 byproducts detected, and 8 of them were identified. Preliminarily, side reactions were successfully suppressed by adding Zn^{2+} during enzymatic hydrolysis of *p*-MPSE in organic solvent of TBA. Using Zn^{2+}, the conversion rate and ee value were significantly improved. Therefore, this ion-suppressing method was feasible in the organic solvent and had provided a potential approach for improving the productivity and efficiency as well as the optical purity of target product by further suppressing the generation of byproducts during the process of enzymatic asymmetric hydrolysis of *p*-MPSE.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86-571-88320571. Fax: +86-571-88320571. E-mail: Richard_chen@zjut.edu.cn.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

p-MPSE, *p*-methylsulfonylphenyl serine ethyl ester; *p*-MPS, *p*-methylsulfonylphenyl serine; MBA, *p*-methylsulfonyl benzaldehyde; MBM, *p*-methylsulfonyl benzenemethanol; MBAC, *p*-methylsulfonyl benzoic acid

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