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Enantioselective Hydrolysis of Glycidyl Methylphenyl Ethers by *Botryosphaeria Dothidea* ZJUZQ007: Effect of Substitution Pattern on Enantioselectivity

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Abstract Resolution of racemic glycidyl (*o, m, p*)-methylphenyl ethers by using a newly isolated *Botryosphaeria dothidea* ZJUZQ007 with epoxide hydrolase activity affords enantiopure epoxides with enantiomeric excesses (e.e.$_s$) of 91–99% and enantiomeric ratios (*E*) of 18.1 to 48.6. The *(R)*-enantiomer was obtained from *rac*-glycidyl (*o* or *m*)-methylphenyl ethers whereas the *(S)*-epoxides was obtained from glycidyl *p*-methylphenyl ether. Substitution pattern of the methyl group exerted effects both on configurations of the remaining epoxides and enantioselectivities of epoxide hydrolase. The observations were explained by enzyme-substrate docking studies. It is the first example showing that for kinetic resolution of glycidyl methylphenyl ethers, fungal species of *B. dothidea* was applied.

Keywords *Botryosphaeria dothidea* · Docking study · Enantioselectivity · Epoxide hydrolase · Glycidyl methylphenyl ethers

Introduction

Due to the increasing demands for modern enantiopure pharmaceuticals, agrochemicals, there is a growing interest for enantiopure epoxides and the corresponding vicinal diols [1–3]. For example, optically pure glycidyl methylphenyl ethers (GMPE) are key chiral building blocks for analog of Zatebradine, an active cardiovascular hybrid drug [4], and Toliprolol, a β-adrenergic blocker [5] (Scheme 1). Therefore, the explorations of efficient and environmentally friendly methods for preparing these valuable synthons are of utmost importance. The direct epoxidation of an olefin or the kinetic resolution of racemic epoxides catalyzed by heavy-metal-based catalysts is one of the most promising methods [6–8]. However, these hazardous metals often lead to environmental problems and safety concerns in the operating process. Additionally, the remaining metals from the catalysts restrict the applications of these chemical methods in the pharmaceutical industry. During

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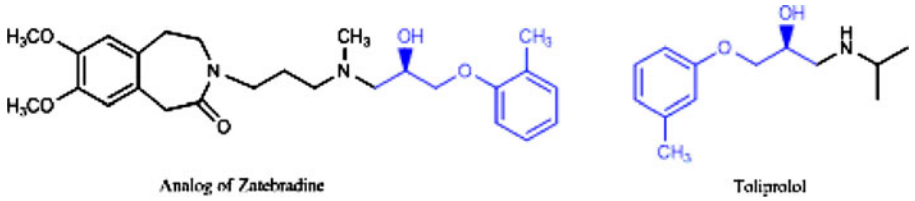


Scheme 1 Structures of analog of Zatebradine and Toliprolol

the past few years, microbial epoxide hydrolase (EH), as a new promising means to enantioselectively resolve racemic epoxides has gained much recent interest due to: (1) high yields; (2) excellent enantiomeric purity of the remaining epoxides and the corresponding vicinal diols; (3) no cofactors; (4) environmental friendliness [1, 3, 9, 10].

But to the best of our knowledge, only a few examples of biocatalysis resolution of GMPEs were reported due to the limited availability of EH produced by microorganisms especially with high enantioselectivity. For example, *Aspergillus niger* was utilized a decade ago in the kinetic resolutions of the three racemic GMPEs and (*R*)-enantiomers were obtained with e.e. of 100%, whereas the yields ranged from 17% to 29% [11]. Recently, yeast *Trichosporon loubierii* ECU1040 was reported to offer optically pure (*R*)-enantiomers of GMPEs with *E* value of 17–41[12].

In this study, a newly isolated filamentous fungus *B. dothidea* ZJUZQ007 for the resolution of the three GMPEs with good yields and excellent enantioselectivity was reported. Moreover, docking studies were successfully employed to discuss the effect of methyl substitution pattern on the phenyl ring on the configurations and enantioselectivities of epoxides.

Materials and Methods

Materials

(R) and *(S)*-epichlorohydrin were purchased from Sigma–Aldrich Co. (*o, m, p*)-Methyl phenols and other analytical grade chemicals were purchased from Huadong Chemicals Co., Hangzhou, China. The screening medium for screening culture contained (per liter): 2.0 g NH_4NO_3, 1.5 g $Na_2HPO_4 \cdot 12H_2O$, 1.5 g $KH_2PO_4 \cdot 3H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg $ZnSO_4 \cdot 7H_2O$, 0.1 g yeast extract 0.1 g corn flour, pH 6. Fermentation medium contained (per liter): 20 g corn flour, 20 g sucrose, 10 g NaCl, pH 4.

Synthesis of Racemic and Optical GMPEs

rac-GMPEs were synthesized from corresponding phenols according to the method reported previously [13]. (*S*)- or (*R*)-GMPEs were synthesized in good yields from (*R*)-or (*S*)-epichlorohydrin respectively using the same method [13]. Enantiopurities of each sample determined by chiral HPLC were above 99% and thus they were used as standard reagents.

General Procedure for Enzymatic Kinetic Resolution of GMPEs

Racemic epoxides hydrolysis was conducted in 10 ml KH_2PO_4/K_2HPO_4 buffer (KPB, 100 mM, pH 7.6) containing 0.8-g wet mycelium of *B. dothidea* ZJUZQ007 and 12-mg

racemic epoxides dissolved in dimethyl sulfoxide (DMSO) (1:10 *v*/*v*). Reaction mixtures were incubated at 30 °C for 40 min on a rotary shaker at 220 rpm. Then the mixtures were extracted four times with 10 mL EtOAc. After extraction, the combined organic layers were dried and the $e.e._s$ and *E* of the remaining GMPEs were further determined by chiral HPLC.

Screening of GMPE-Utilizing Strains

Microorganisms derived from soil samples and rotten fruits were inoculated into agar plates of the screening medium containing GMPE as the sole carbon source. The strains which survived on agar plates were isolated to test their hydrolysis activities and enantioselectivities. These strains were transferred to 30-ml fermentation medium and cultivated at 30 °C for 72 h on a rotary shaker at 220 rpm. Then the culture was centrifuged to deposit the cells. After washed with 100 mM KPB (pH 8.0) twice, the cells was used to determine their hydrolysis activities and enantioselectivities as described above.

HPLC Conditions

The enantiomeric excess of epoxides ($e.e._s$) was determined by HPLC equipped with a Daicel CHIRALPAK AS-H column (0.46 cm×25 cm 4 μm). The mobile phase was hexane/isopropanol (95:5 for *o*-GMPE, 90:10 for *m*-GMPE, and 97:3 for *p*-GMPE, *v*/*v*) and the flow rate was set at 0.8 ml/min. The detection was carried out at a wavelength of 269 nm.

Results and Discussion

The value of e.e. is expressed as enantiomeric excess of the remaining epoxides, which was calculated using the following equation: $e.e._s = ([R] - [S])/([R] + [S])$.

E value was enantiomeric ratios of epoxides, which was calculated using the following equation [14]: $E = \ln[(1-c)(1-e.e._s)]/\ln[(1-c)(1+e.e._s)]$. (*c*, conversion; $e.e._s$, ee of the remaining expoide)

Isolation and Identification of GMPE-Utilizing Microorganisms

Epoxides could be degraded to vicinal diols by EHs. Therefore, the strains utilizing *o*-GMPE as a sole carbon source might have EH activity. All microorganisms which survived on agar plates were tested for hydrolysis activities and their enantioselectivities toward GMPEs. More than 500 microbes were isolated and 21 strains were found to possess EH activity, but, as shown in Table 1, only five of them had enantioselectivity. Among these strains, fungi ZJUZQ007 preferentially hydrolyzed *(S)-o*-GMPE, accumulating *(R)*-enantiomer with the highest e.e. of 75.8%. It was further identified as *Botryosphaeria dothidea* ZJUZQ007 based on its morphology and ITS sequence analysis. The sequence data has been submitted to the GenBank databases (http://www.ncbi.nlm.nih.gov) under accession NO.GU295945. The strain was deposited in China Center for Type Culture Collection (CCTCC AF 209016). Therefore, the study on enantioselective hydrolysis of (*o, m, p*)-GMPEs using this strain was performed in the following experiments.

Table 1 Screening of microorganisms with enantioselective EH activity toward o-GMPE

Microorganisms	Number	e.e.s(%)/abs conf[a]	E[b]
Fungi	ZJUZQ007	75.8/(*R*)	12.7
Fungi	ZJUZQ023	63.5/(*R*)	9.6
Fungi	ZJUZQ051	37.9/(*R*)	4.8
Yeast	ZJUZQ079	40.4/(*S*)	7.3
Bacterium	ZJUZQ113	51.2/(*S*)	8.1

[a] Absolute configuration of most abundant epoxide after resolution

[b] The *E* value was enantiomeric ratios of epoxides and it was calculated using the following equation [14]: $E = \ln[(1-c)(1-\text{e.e.}_s)]/\ln[(1-c)(1+\text{e.e.}_s)]$

Optimization of Biotransformation Conditions

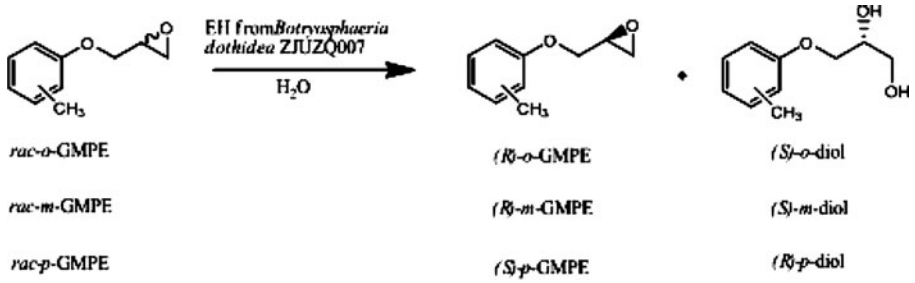
In order to fully investigate the enantioselectivity of the strain, (*o*, *m* and *p*)-GMPE were employed in the following experiments (Scheme 2). Spontaneous hydrolysis of GMPEs was not detected under the following conditions.

To check the stability of the biocatalyst, the whole wet mycelium was stored under the reaction condition without substrates, and it's EH activity remained 90% after 24 h. In addition, the same results were obtained with the recycled mycelium under the same condition. Therefore the whole wet mycelium biocatalyst is quite stable in the reaction system, and is suitable for the further study.

Temperature effects on the activity and apparent enantioselectivity of EH in KPB were investigated from 25 to 45 °C. The results are shown in Fig. 1. The *E* values and the enantioselectivities of the three GMPEs are at their maximum when temperature is 30 °C. The e.e._s and the *E* value reach 88.4% and 26.3 for *(R)-o*-GMPE, 66.07% and 9.3 for *(R)-m*-GMPE, and 77.8% and 15 for *(S)-p*-GMPE, respectively.

The pH of the buffer has an obvious effect on the EH activity and enantioselectivity. The microorgansim exhibits high activity over a broad range of pH varied from 7.2 to 8.0. The fact is probably due to the self-regulation in the microenvironment to resist the pH change in the external environment [15]. As shown in Fig. 2, maximum EH enantioselectivities and the apparent *E* values were obtained at pH 7.6. The e.e._s and the *E* value reached 90.3%, 41.3 for *(R)-o*-GMPE, 75.6%, 12.7 for *(R)-m*-GMPE, and 83.8%, 23.8 for *(S)-p*-GMPE, respectively.

Reaction time is one of the most important parameters affecting the enantioselectivity in kinetic resolution. Therefore the e.e._s of epoxides is investigated for different time. Racemic



Scheme 2 The enantioselective hydrolysis of *rac*-GMPEs using whole cells of *B. dothidea* ZJUZQ007

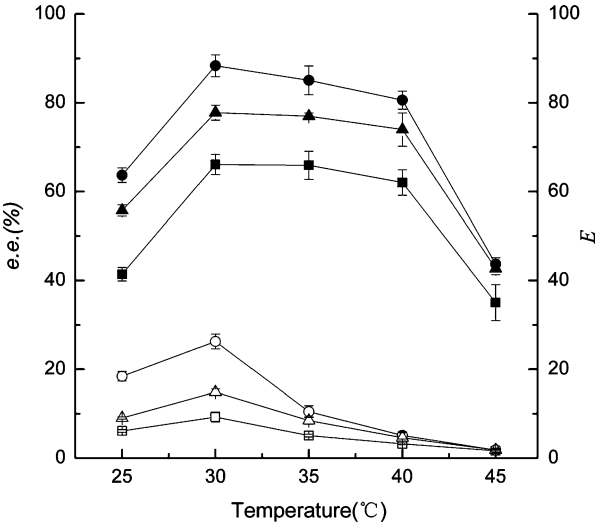


Fig. 1 Effect of reaction temperature on the enantioselectivity using wet mycelium of *B. dothidea* ZJUZQ007. Biotransformation condition: time 30 min, pH 8.0. *Black circle* (●), ee of *(R)-o*-GMPE; *white circle* (○), *E* of *(R)-o*-GMPE; *black up-pointing triangle* (▲), ee of *(S)-p*-GMPE; *white up-pointing triangle* (△), *E* of *(S)-p*-GMPE; *black square* (■), ee of *(R)-m*-GMPE; *white square* (□), *E* of *(R)-m*-GMPE

epoxide (12 mg) in DMSO (1:10 *v*/*v*) was hydrolyzed at 30 °C in 10 ml KPB (100 mM, pH 7.6) using the wet mycelium of ZJUZQ007 (0.8 g) by shaking the mixture (220 rpm) at six different reaction times from 10 to 70 min. As shown in Fig. 3, the highest *E* of these epoxides is obtained at 40 min. *(R)-o*-GMPE could be obtained in $e.e._s$ of >99%, *E* of 48.6,

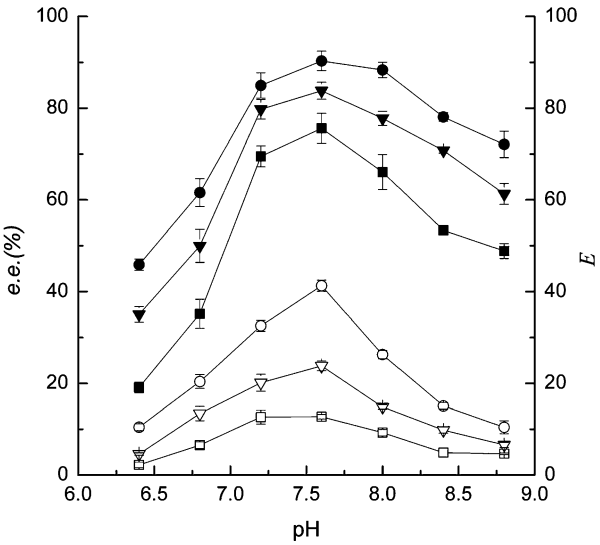


Fig. 2 Effect of pH of buffer on the enantioselectivity using wet mycelium of *B. dothidea* ZJUZQ007. Biotransformation condition: time 30 min, temperature 30 °C. *Black circle* (●), ee of *(R)-o*-GMPE; *white circle* (○), *E* of *(R)-o*-GMPE; *black down-pointing triangle* (▼), ee of *(S)-p*-GMPE; *white down-pointing triangle* (▽), *E* of *(S)-p*-GMPE; *black square* (■), ee of *(R)-m*-GMPE; *white square* (□), *E* of *(R)-m*-GMPE

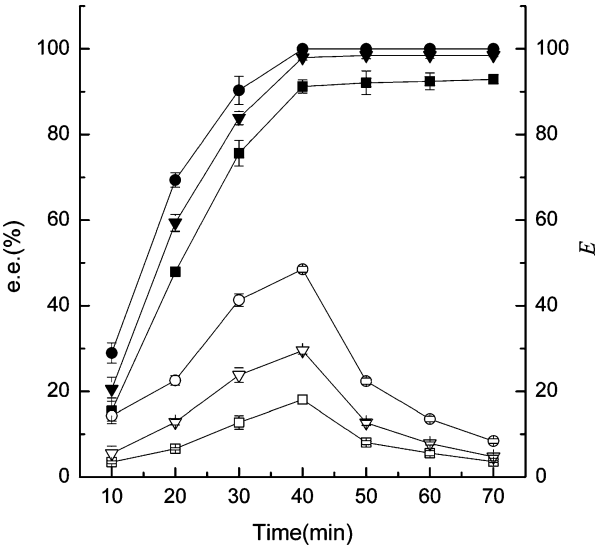


Fig. 3 Effect of reaction time on the enantioselectivity using wet mycelium of *B. dothidea* ZJUZQ007. Biotransformation condition: pH 7.6, temperature 30 °C. *Black circle* (●), ee of *(R)-o*-GMPE; *white circle* (○), *E* of *(R)-o*-GMPE; *black down-pointing triangle* (▼), ee of *(S)-p*-GMPE; *white down-pointing triangle* (▽), *E* of *(S)-p*-GMPE; *black square* (■), ee of *(R)-m*-GMPE; *white square* (□), *E* of *(R)-m*-GMPE)

whereas *(R)-m*-GMPE was offered in $e.e._s$ of 91.2%, *E* of 18.1, and *(S)-p*-GMPE in $e.e._s$ of 98%, *E* of 29.5, respectively.

Effects of Substitution Patterns of Methyl Group on Enantioselectivities of *B. Dothidea* ZJUZQ007

As can be seen from Table 2, *B. dothidea* ZJUZQ007 shows high enantioselectivities towards GMPEs under the optimized condition (temperature 30 °C, pH 7.6 100 mM KPB, reaction time 40 min). It also indicates that, by utilizing *B. dothidea* ZJUZQ007, substitution pattern of the methyl group exerts effects both on configurations of the remaining epoxides and enantioselectivities of EH. For *o*-GMPE and *m*-GMPE, *(S)*-enantiomers were hydrolyzed and afford *(R)*-configurations epoxides, whereas *p*-GMPE

Table 2 Preparation of optical GMPE by *B. dothidea* ZJUZQ007[a]

Substrate	Configuration	Yield (%)	e.e. (%)	E[b]
o-GMPE	*R*(*R*[c])	41.1	>99	48.6(41[d])
m-GMPE	*R*(*R*[c])	40.7	91.2	18.1(21[d])
p-GMPE	*S*(*R*[c])	42.5	98.0	29.5(17[d])

[a] Racemic epoxide (12 mg) was hydrolyzed using wet mycelium of *B. dothidea* ZJUZQ007 (0.8 g) at 30 °C in 10 ml KPB (100 mM, pH 7.6) for 40 min.

[b] The *E* value was enantiomeric ratios of epoxides and it was calculated using the following equation (14): $E = \ln[(1-c)(1-e.e._s)]/\ln[(1-c)(1+e.e._s)]$.

[c] The reserved configuration reported by Choi et al. in 1998 and Xu et al. in 2004.

[d] The *E* values of glycidyl methylphenyl ethers reported by Xu et al. in 2004

affords *(S)*-enantiomer. These unexpected results indicate that the substitution pattern of the methyl group on the phenyl ring plays an important role in the resolution of GMPE. To study the mechanism, *o*-GMPE and *p*-GMPE are selected for enzyme-substrate docking studies by using Discovery Studio 2.1 software. The reference epoxide hydrolase (PDB ID:1VJ5) is obtained from X-ray structure deposited in Protein Data Bank (http://www.rcsb.org/pdb) [16].

The mechanism of hydrolysis of epoxides catalyzed by EH has been validated [17]. As shown in Fig. 4, it was proposed that four amino acid residues (Tyr383, Tyr466, Asp335, and His524) constituted the catalytic center of the enzyme. The configuration of the substrate in the active center is directed by a hydrogen bond between the oxygen from the oxirane ring and the hydroxyl groups of the two Tyr residues. The formation of hydrogen bond determines the enantioselectivity towards GMPEs. The preferential conformations of *o*-GMPE and *p*-GMPE in the enzyme active center are presented in Fig. 4. For *o*-GMPE, only the *(S)*-enantiomer forms hydrogen bond with Tyr383 and Tyr466, and *(R)*-enantiomer cannot. Additionally, compared with the *(R)*-enantiomer, the van der Waals force helps the hydrophobic methyl group of the *(S)*-enantiomer approaches the hydrophobic amino acid residues (Val and Leu) in the EH-binding pocket, which makes the *(S)*-enantiomer binds more strongly to the enzyme. This observation reveals that, for *o*-GMPE, the *(S)*-enantiomer is a preferential substrate for the EH (Fig. 4a). For *p*-GMPE, the *(R)*-enantiomer binds to Tyr via a hydrogen bond and thus lead to hydrolysis and *(S)-p*-GMPE is retained (Fig. 4b).

In summary, a newly isolated *B. dothidea* ZJUZQ007 with epoxide hydrolase activity was utilized for the resolution of racemic glycidyl (*o, m, p*)-methylphenyl ethers, which affords *(R)-o*-GMPE in e.e.$_s$ of >99%, *E* of 48.6, *(R)-m*-GMPE in e.e.$_s$ of 91.2%, *E* of 18.1, and *(S)-p*-GMPE in e.e.$_s$ of 98%, *E* of 29.5, respectively. It is the first example that fungi species of *B. dothidea* could be applied to kinetic resolution of glycidyl methylphenyl ethers. The results also show that for this epoxide hydrolase producing strain, the methyl substitution pattern on the phenyl ring has different effects on the enantioselectivity. The observations are in agreement with our enzyme-substrate docking studies.

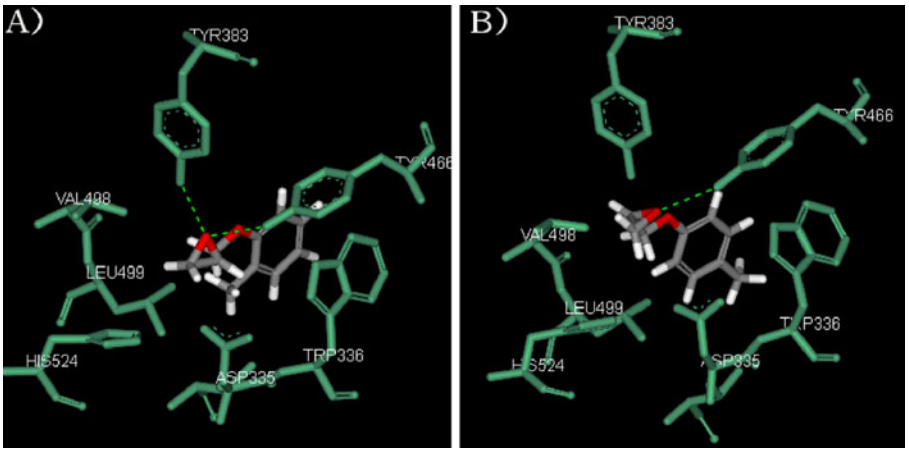


Fig. 4 Different configurations of epoxides binding to the epoxide hydrolase's active center. Different configurations of epoxides binding to the epoxide hydrolase's active center. Hydrogen bonds were displayed as *green dashes*. **a**, *(S)-o*-GMPE; **b**, *(R)-p*-GMPE

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