Asymmetric Epoxidation and Sulfoxidation Catalyzed by a New Styrene Monooxygenase from *Bradyrhizobium*

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Abstract

Asymmetric epoxidation catalyzed with styrene monooxygenase (SMO) is a powerful enzymatic process producing enantiopure styrene epoxide derivatives. To establish a more diversified reservoir of SMOs, a new SMO from *Bradyrhizobium* sp. ORS 375, named *Br*SMO, was mined from the database and characterized. *Br*SMO was constituted of an epoxygenase component of 415 amino acid residues and an NADH-dependent flavin reductase component of 175 residues. *Br*SMO catalyzed the epoxidation of styrene and 7 more styrene derivatives, yielding the corresponding (*S*)-epoxides with excellent enantiomeric excesses (95– > 99% ee), with the highest activity achieved for styrene. *Br*SMO also catalyzed the asymmetric sulfoxidation of 7 sulfides, producing the corresponding (*R*)-sulfoxides (20–90% ee) with good yields.

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Introduction

Enantiopure epoxides are important synthons for the production of pharmaceuticals and fine chemicals [1–8]. The synthesis of chiral epoxides in high yield and with excellent enantiopurity is a challenge in organic synthesis. Until now, many chemical and enzymatic strategies have been developed to synthesize chiral epoxides, among which, the biocatalytic epoxidation of alkenes has been approved as a powerful route to enantiopure epoxides [9–12].

Styrene monooxygenase (SMO; EC 1.14.14.11), also known as styrene epoxygenase, is an excellent biocatalyst for the epoxidation of many aromatic conjugated and unconjugated alkenes [13–21], as well as several aliphatic alkenes with exceptional stereoselectivity, producing the corresponding epoxides with enantiomeric excesses of up to > 99% ee [22–24]. They also show good activity in the sulfoxidation of sulfides [25–27] and the oxidative coupling of indole to form indigo dyes, most probably through an unstable epoxide intermediate [28].

SMO is a two-component flavoprotein composed of a FAD-specific styrene epoxidase (StyA) and NADH-specific flavin reductase (StyB), which catalyzes the formation of reduced FAD [29, 30]. Up until now, 48 SMO genes have been identified from styrene-degrading strains or metagenomes, or through database mining, and functionally characterized, including a very recent work involving the assay of 31 monooxygenases from different microbes with 23 of which showed activity on the epoxidation of styrene with high enantioselectivity [31, 32]. Some of them are E1-type flavin-dependent monooxygenases, which are two-protein systems composed of a monooxygenase and a reductase, and the others belong to group E2-type, where the oxygenase and reductase components are fused to form a self-sufficient protein [26, 33].

The first one was identified from *Pseudomonas taiwanensis* VLB120 (StyAB) [34], heterologously expressed and developed to catalyze the pilot-scale production of (*S*)-styrene oxide from styrene, resulting in producing 388 g of styrene oxide in a two-liquid phase 30-L fed-batch system (170 U per liter) [35]. Further efforts have improved the volumetric productivities to 1800 U per liter [36], and the product concentration doubled to 36.3 g L_{tot}^{-1}, making it one of the most productive biocatalytic oxyfunctionalization processes [37, 38]. Then, more SMOs were identified from *Pseudomonas* species, such as *P. fluorescens* ST [21], *P. putida* SN1 [39], and *Pseudomonas* sp. LQ26 [22], with the protein sequences of StyA sharing a high similarity of > 90%. SMOs from microbes other than *Pseudomonas* share lower similarity at protein level, such as those from the genus of *Rhodococcus* (*R. opacus* 1CP, *Rhodococcus* sp. ST-5 and ST-10) [18], a metagenome library [40], and strains of *Variovorax paradoxus* EPS [26] and *Gordonia rubripertincta* CWB2 [41]. Nevertheless, all the abovementioned SMOs are of (*S*)-selectivity, producing (*S*)-epoxides from styrene and derivatives with good to excellent enantioselectivity.

In our continuous effort to exploit the potential of new SMOs, we have recently identified two new SMOs from marine microbes *Paraglaciecola agarilytica* NO2 and *Marinobacterium litorale* DSM 23545 [28], as well as a new epoxidase component from *Streptomyces* sp. NRRL S-31 [42]. While the former two have displayed the same (*S*)-selectivity as expected, the latter one has shown excellent (*R*)-selectivity, complementary to all the known SMOs, yielding the (*R*)-epoxides with excellent enantioselectivity (91–99% ee) [42].

The present work reports the epoxidation and sulfoxidation activity of a new SMO (*Br*SMO) from *Bradyrhizobium* sp. ORS 375 [43], which belongs to the class of α-proteobacteria. Very recently, another hypothetic monooxygenase (GenBank No.

WP_012044467) from the same genus (*Bradyrhizobium* sp. BTAi1) has been analyzed by Tischler et al., but it did not show epoxidation activity toward styrene [32]. Here, *Br*SMO was found to catalyze the asymmetric (*S*)-epoxidation of styrene and derivatives with excellent stereoselectivity, and the sulfoxidation of thioanisole with moderate stereoselectivity.

Materials and methods

Chemicals

Substrates of **1a-15a** were purchased from Alfa-Aesar (Tianjin, China) or Acros Organics (Geel, Belgium) and used without further purification. Racemic styrene epoxide (**1b**) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Racemic epoxides were prepared from the corresponding substrates using *m*-chloroperoxybenzoic acid (**2b-8b**) [22] or H_2O_2 (**9b-15b**) as the oxidant [44].

Plasmids construction and gene manipulation

StyA from *Pseudomonas* sp. LQ26 (*Ps*StyA) [22] was used as the query template to perform BLASTP [45] search in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The hypothetic monooxygenase (*Br*SMO) from *Bradyrhizobium* sp. ORS 375 (GenBank no: NZ_CAFI01000479.1) having a 48% identity with *Ps*StyA was selected. The accession numbers are WP_009031806.1 and WP_009031805.1 for *Br*StyA and *Br*StyB, respectively. The DNA sequence encoding *Br*SMO synthesized by GenScript Biotech Co. (Nanjing, China). The synthesized DNA fragment and pET-28a (+) (Novagen, Madison, WI) were digested with *Bam*H I and *Sac* I (New England Biolabs, Beverly, MA, USA), and ligated to achieve the expression plasmid pET-*Br*SMO. The DNA sequence encoding *Br*SMO was verified by sequencing the plasmid at Invitrogen Biotechnologies (Shanghai, China).

Protein expression of recombinant *Br*SMO

The plasmid pET-*Br*SMO was introduced into *E. coli* BL21 (DE3). A single colony was picked up and grown in LB medium containing kanamycin (50 mg/L) at 37 °C and 180 rpm. Then 200 mL Terrific Broth containing kanamycin (50 mg/l) was inoculated with the overnight culture and incubated at 37 °C and 220 rpm. After OD_{600} reached 0.8, IPTG (0.05 mM) was added, and the incubation was continued at 20 °C for 20 h. Cells were harvested by centrifugation ($8 \times 10^3 g$ and 4 °C) for 10 min, washed twice. The collected cells were then used for SDS-PAGE analysis (stained with Coomassie blue) and as a catalyst for biotransformation, as described in the next section.

Whole-cell bioconversion and product analysis

Specific epoxidation activity was measured using whole cells following the literature [23, 46, 47]. Briefly, the biotransformation was carried out in 4 mL potassium phosphate buffer (100 mM, pH 7.0) containing 0.5 g CDW/L of the recombinant cells, 0.5% (wt/vol) glucose, and incubated at 30 °C on a rotary shaker at 200 rpm. After 10 min, styrene was added to a final concentration of 1.5 mM (30 mM stock solution in ethanol). The reaction was continued for 5

min. The mixtures were extracted by ethyl acetate and analyzed with gas chromatography (GC). Activities were calculated from the amount of product formed and were expressed in units per g CDW, with one unit (U) being defined as the activity that produces 1 μmol of oxide per min.

For optimal reaction pH and temperature determination, the biotransformation was carried out in 5 mL potassium phosphate buffer or Tris-HCl buffer (100 mM) containing 0.05 g CDW of the cells expressed *Br*SMO, 0.5% (wt/vol) glucose, 2% (v/v) cyclohexane, and 8 mM styrene. The reaction was carried out at a varied temperature for 15 min with shaking at 200 rpm, terminated by extraction with ethyl acetate, and analyzed with GC.

The relative activities of the whole cells toward various substrates were determined in 5 mL potassium phosphate buffer (100 mM, pH 7.5) containing 0.05 g CDW of the cells expressed *Br*SMO, 0.5% (wt/vol) glucose, 2% (v/v) cyclohexane, and 8 mM substrate. The reaction was carried out at 30 °C for 30 min with shaking at 200 rpm, terminated by extraction with ethyl acetate, and analyzed with GC.

The yield and optical purity of the whole cells toward various substrates were determined in 5 mL potassium phosphate buffer (100 mM, pH 7.0) containing 0.2 g CDW of the resting cells, 10% (v/v) cyclohexane, and 5 mM substrate. The reactions were performed at 30 °C and 200 rpm for 4 h, and then the mixtures were extracted by ethyl acetate. The organic phases were combined, dried with anhydrous sodium sulfate, and concentrated under vacuum for analysis.

GC analysis was performed on an Agilent Technologies 7890B GC system connected to an FID detector using a Cyclodex-B column (30 m × 0.25 mm × 0.25 μm, USA). The temperatures of the injector and detector were set at 260 °C and 280 °C, respectively. The column temperatures were set at 100 °C for **1b**, 140 °C for **2b-4b** and **6b-7b**, 90 °C for **5b**, and 160 °C for **8b-15b**.

The optical purities were determined by chiral HPLC using a Daicel Chiralpak IC column (**1b**, **2b**, **6b**, and **8b**) or Chiralpak OJ-H column (**3b**) or Chiralpak OB-H column (**9b-15b**). (See Table S1 and Table S2 of the Supporting information for separation conditions and retention times). The absolute configuration of each product was determined by comparing the elution orders on chiral HPLC or GC (**1b**, **3b**, **6b**, **8b** [42], **2b** [48], **4b** [49], **5b** [50], **7b** [22], and **9b-15b** [51]) with published data.

Results and Discussion

Sequence Analysis of *Br*SMO

StyA from *Pseudomonas* sp. LQ26 was used as the reference to mine new SMOs from the NCBI nr protein database. *Pseudomonas* belongs to the class of γ-proteobacteria in the phylum of proteobacteria, which is a major phylum of Gram-negative bacteria [52]. The search returned a hypothesized monooxygenase (*Br*StyA) from *Bradyrhizobium* sp. ORS 375, which is a kind of plant symbiotic bacteria and belongs to the class of α-proteobacteria. Previously two SMOs were identified from α-proteobacteria, either from *Sphingopyxis* sp. Kp5.2 [53] or *Gemmobacter nectariphilus* DSM 15620 [32]. Both of them could catalyze styrene epoxidation to produce enantiopure (*S*)-oxide.

*Br*StyA contains 415 amino acid residues, similar to known SMOs (about 406-427 residues). Its amino acid sequence shares 48% identity to the reference sequence from

Pseudomonas sp. LQ26 [22], and 47% identity to StyA$_{ST-10}$ from *Rhodococcus* sp. ST-10 [18]. Further analysis showed that the DNA coding *Br*StyA is followed by a 528 bp sequence coding a putative flavin reductase (*Br*StyB). However, other genes in a typical styrene degradation operon, such as styrene oxide isomerase (*styC*) gene or phenylacetaldehyde dehydrogenase (*styD*) gene, were not found in the neighboring region of *styAB*. *Br*StyB contains 175 amino acid residues and has 41% and 37% identities to that from *Pseudomonas* sp. LQ26 and *Rhodococcus* sp. ST-10, respectively. Based on known mechanisms of SMO, together *Br*StyA and *Br*StyB would form a two-component flavoprotein, named *Br*SMO.

Multiple sequence alignment (Fig. 1) showed that *Br*SMO contained several common sequence motifs compare to other SMOs of known function. In the reductase component, the signature motif of TANxxxSx$_{10}$S, important for interacting with isoalloxazine and NADH [54, 55], is conserved in the N-terminus (residues 37–54) (Fig. 1b). In the epoxygenase component, the elements include the FAD-binding fingerprints GxGxxG, GG, and Dx6G (residues 9–14, 117–118, and 138–145, respectively) and the putative dual function motif GDx6P (residues 298–306) related to FAD and/or NAD(P)H interaction (Fig. 1a). The residue Thr47, which is considered functionally important to catalytic activity in StyA was also conserved in *Br*SMO (Fig. 1a). Notably, a recently identified fingerprint motif is present in *Br*SMO as N46-V48-H50-Y73-H76-S97 (Fig. 1a), which corresponds to the N46-V48-H50-Y73-H76-S96 motif in *Pp*StyA S12 from *P. putida* S12 (PDB ID: 3IHM) [56]. This motif is shared by many SMOs and found to lead to (*R*)-enantioselectivity in sulfoxidation, while the motif S46-Q48-M50-V/I73-I76-A96 in indole monooxygenase often leads to the (*S*)-enantiomer [32].

Heterologous expression and functional screening

E. coli BL21 (DE3) cells harboring the pET-*Br*SMO construct were grown at 20 °C to expressing soluble proteins of *Br*StyA and *Br*StyB. SDS-PAGE analysis showed a prominent band at 45 kDa, as expected from the size of *Br*StyA (Fig. 2), and only vague bands appeared at around 19 kDa, indicating low expression of *Br*StyB (Fig. 2). In previous works, several SMOs have also been reported of much higher expression of StyA than StyB [18, 28, 29]. The overexpression of *Br*StyB is often achieved by using the plasmid containing only the coding DNA of StyB but not of StyA [18, 29], which is also the case for *Br*StyB (Fig. 2).

During the cultivation of *E. coli* BL21 (DE3)/pET-*Br*SMO, the formation of blue dye (indigo) was distinctly observed, which was in accordance with the known indigo-forming capacity of SMOs [42, 57], indicating the functional expression of *Br*SMO. After harvesting, the whole cells expressing *Br*SMO were used as the catalyst for the epoxidation of styrene, yielding styrene epoxide with > 99% ee, which indicated the *Br*SMO did have the function as a styrene monooxygenase.

The specific epoxidation activity of *Br*SMO was determined to be 86 ± 2 U/g CDW, which was comparable to that of the other SMOs measured under similar conditions, such as that from *P. taiwanensis* VLB120 (79 ± 5 U/g CDW) [23], *P. putida* SN1 (55 ± 5 U/g CDW) [46], and *Pseudomonas* sp. LQ26 (66.5 U/g CDW) [47].

Effects of reaction temperature and pH

The effect of reaction temperature on the epoxidation of styrene was measured at temperatures ranging from 20 to 40 °C in potassium phosphate buffer at pH 7.0 using whole cells (Fig. 3).

a

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                 GXGXXG                          N V H                        Y
BrStyA     : MEKSIGIVGAGIGGLHLALYLQKHGIQATVLTDREPEQYAATRLMNTVAHHGITVARENELGVNHWDDPNVVY : 73
SN1StyA    : MKKRIGIVGAGTAGLHLGLFLRQHDVDVTVYTDRKPDEYSGQRLLNTVAHNAVTVQREVALDVNEWPSEEFGY : 73
VLB120StyA : MKKRIGIVGAGTAGLHLGLFLRQHDVDVTVYTDRKPDEYSGLRLLNTVAHNAVTVQREVALDVNEWPSEEFGY : 73
LQ26StyA   : MKKRIGIVGAGTAGLHLGLFLRQHDHDVTVYTDRQPDEYAGLRLLNTVAHHAVTVQREVDLGVNEWPSDEFGY : 73
RhStyA     : MSKSIAIVGAGTAGLHLALYLQQNGVESTIFTDRRPEEYRDVRLLNTVAHHAVTVEREVGLDVDHWPG--AGY : 71
STStyA     : MKKRIGIVGAGTAGLHLGLFLRQHDVDVTVYTDRKPDEYSGLRLLNTVAHHAVTVQREVALDVNEWPSEEFGY : 73
                                                      + + +                         +

             H                   S                     GG             DXXXXXXG
BrStyA     : HHHDHFFNFPGSPILLFRGAFKQ-PSRAVDYRIYLPALMKDFEDRGGTIEYASIQDDDIAALVARFDLLVVSTG : 145
SN1StyA    : FGHYYYVGGP-QPMRFYGDLKA-PSRAVDYRLYLPMLMRALEARGGKFCYDAVSAEDLGGLSEQYDLLVVCTG : 144
VLB120StyA : FGHYYYVGGP-QPMRFYGDLKA-PSRAVDYRLYQPMLMRALEARGGKFCYDAVSAEDLEGLSEQYDLLVVCTG : 144
LQ26StyA   : FGHYYYVGGP-QPMRFYGDLKA-PSRAVDYRLYQPMLMRALEARGGKFCYDAVSVEDLESLSDQYDLLVVCTG : 144
RhStyA     : YGHYYYIGVPGMPIEFYGDLSAGPSRAIDYRLYLPTLMNDFLQRGGKIEYRNIELSDVEDLSDAYDLVLIGTG : 144
STStyA     : FGHYYYVGGS-QPMRFYGDLKA-PSRAVDYRLYQPMLMRALEARGGKSCYDDVSTEDLEGLSEQYDLLVVCTG : 144
                          +                    +

BrStyA     : KGALGRMFNHRPELSPYNQPQRLLCVGLYDGVDHGSPDGDDPRGVTLSVSPGHGEMIVIPTLTFGGMKTALLM : 218
SN1StyA    : KYALGKVFVKQSENSPFEKPQRALCVGLFKGIKEAP-----IRAVTMSFSPGHGELIEIPTLSFNGMSTALVL : 212
VLB120StyA : KYALGKVFEKQSENSPFEKPQRALCVGLFKGIKEAP-----IRAVTMSFSPGHGELIEIPTLSFNGMSTALVL : 212
LQ26StyA   : KNSLGQLFEKQPENSPFEKPQRALCVGLFKGIKEAP-----IRAVTMSFSPGHGELIEIPTLSFNGMTTALVL : 212
RhStyA     : KGGLGTIFARDEDSSPYSEPQRQLCVGMFKGIADQE-----TRAVTFGIAPGNGEMIEIPSVSFNGNATALVF : 212
STStyA     : KNALGKVFEKQSENSPFEKPQRALCVGLFKGIKEAP-----IRAVTMSFSPGHGELIEIPTLSFNGMSTALVL : 212

BrStyA     : ENIPGGDMAELVSLNYDADPAGFRQTMLDKLEKHHPHTYNKIDTHRFDLQ--PLDLLQGAVVPTVRRSSVSFD : 289
SN1StyA    : ENHIGSDLEVLAHTKYDDDPRAFLDLMLEKLRKHHPSVAERIDPAEFDLANSSLDILQGGVVPVFRDGHATLN : 285
VLB120StyA : ENHIGGDLEVLAHTKYDDDPRAFLDLMLEKLGKHHPSVAERIDPAEFDLANSSLDILQGGVVPAFRDGHATLN : 285
LQ26StyA   : ENHIGGDLEVLAHTKYEENPRAFLDLLFQKLHAHHPSIAERIDPEEFDLANSSLDILQGSVTPVFRNGHATLK : 285
RhStyA     : ENHIGGDLEVLAKTRYDDDPRAFLDLVLDKLRTYYPLTADRVNEDDFDLANSSLDILQGAVTPGVRHGHVKLG : 285
STStyA     : ENHIGSDLEVLAHTKYDDDPRAFLDLMLEKLRKHHPSVAERIDPAEFDLANSSLDILQGGVVPAFRDGHATLS : 285

             GDXXXXXXP
BrStyA     : DGKLAIALGDVHSVVDPMMGQGANMASYAAFELGKAIVDAVAFDDRFVETVDRARENRVIAAARWTNLMLQPP : 362
SN1StyA    : NGKTIIGLGDIQATVDPVLDQGANMASYAAWILGEEILAHSVYDLRFSEHLERRQDRVLCATRWTNFTLSAF : 358
VLB120StyA : NGKTIIGLGDIQATVDPVLGQGANMASYAAWILGEEILAHSVYDLRFSEHLERRQDRVLCATRWTNFTLSAL : 358
LQ26StyA   : NGKTIIGLGDVQATVDPVLGQGANMASHAAWILGEEILSHSVYDHRFCEHLERRQDRVLCATRWTNFTMGAL : 358
RhStyA     : NGKIVGLLGDAHATVDPVLGQGQGANMASYAAHVLGEEIIKTDVYDERFFEVVNARRAVRVLGATRWTNYMLKNL : 358
STStyA     : NGKTIIGLGDVQATVDPVLGQGANMASHAAWILGEEILAHSVYDLRFSEHLERRRQDRVLCATRWTNFILSAL : 358

BrStyA     : SE---AMGRLIVTMAQNRALCDEFTDNFNYPERQWDRLASDRRIHAWIDERTPLAA------------ : 415
SN1StyA    : TELPPEFLTFLQILSQSREMADEFTDNFNYPERQWDRFSSPERIGQWCSQYAPTIAA----------- : 415
VLB120StyA : SALPPEFLAFLQILSQSREMADEFTDNFNYPERQWDRFSSPERIGQWCSQFAPTIAA---------- : 415
LQ26StyA   : QALPPEFIAFLQVLSQSREMADEFTDNFNYPERQWDRFSSPERIGHWCSQYAPTIAA---------- : 415
RhStyA     : RELPNELVEFLGAVSLDRNLADKFTTNFNYPEKQWDIFSSPESMRSWVELNAGSAEVTDARPFAGTGV : 426
STStyA     : SALPPEFLAFLQILSQSREMADEFTDNFNYPERQWDRFSSPERIGQWCNQYAPTIAA---------- : 415
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b

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                                                 TANXXXSXXXXXXX
BrStyB     : ------------------MSHHPDPAS------FRAAASRFSTGVTVVTSSDAEGAPVGMTANSFTTVSMQPPT : 50
SN1StyB    : ------------------MTLKKDVVVDIDSTSFRQAVALFATGIAVLSAETDEGVHGMTVNSFTSISLDPPT : 56
VLB120StyB : ------------------MTLKKDMAVDIDSTNFRQAVALFATGIAVLSAETEEGVHGMTVNSFTSISLDPPT : 56
LQ26StyB   : ------------------MTLKTDAAVEIDAASFRQAVALFATGIAVLSAETADGVHGMTVNSFTSISLDPPT : 56
RhStyB     : ------MSPQASTTSPTLTPTPEEVSAIAQMNFRTSVALFATGIAVVTMDDGDGGVHGVTVNSFTSISLDPPT : 67
STStyB     : ------------------MTLKKDVAVDIDSTCFRQAVALFATGIAVLSAETEEGVHGMTVNSFTSISLDPPT : 56

             XXXS
BrStyB     : VLVSLKRGRTWHAVTATRRYAVNVLAADDVAIGRHFAGAPLAQG-APALEARDGFFLLPQAIAQFGCEVVSSV : 122
SN1StyB    : VMVSLKSGRMHELLTQGGRFGVSLLGESQKMLSAFFSKRVIDGTPPPAFTAQAGLPTLRDAMAWFECEVESTV : 129
VLB120StyB : VMVSLKSGRMHELLTQGGRFGVSLLGESQKVFSAFFSKRAMDDTPPPAFTIQAGLPTLQGAMAWFECEVESTV : 129
LQ26StyB   : VMVSLKTGRMHELLTQGRRFGVSLLGEQKVLSAFFSKRMLDDSPPPAFTVQNSLPTLQDAMAWFECEVESTV : 129
RhStyB     : VMVSLRPGRAHDLISSAGVYGVSVLTQAQQNHSRYFTG-AREDNWSPEFLTGATVPTLAGSLARFECRVTDTI : 139
STStyB     : VMVSLKSGRMHELLTRGGRFGVSLLGESQKVFSAFFSKRVMDDTPSPAFTIQAGLPTLRDAMAWFECEVESTV : 129

BrStyB     : EIADHTLFIGEVRWCRHRDG---LPLAFYASRFRNGLGAEISPGDALAYPAEGWSI : 175
SN1StyB    : EVHDHTLFIARVSACGVPEANAPQPLLFFASRYHG---------NPLPLN------ : 170
VLB120StyB : QVHDHTLFIARVSACGTPEANTPQPLLFFASRYHG---------NPLPLN------ : 170
LQ26StyB   : QIHDHTLFFARVSACGRPEATAPQPLLFFASRYHG---------NPLPLN------ : 170
RhStyB     : SVHDHTLFIARVEHCDSDQG---SPLMFFASKYHQ---------PALTISDKAT-- : 181
STStyB     : QVHDHTLFIARVSACGTPEANAPQPLLFFASRYHG---------KPLPLN------ : 170
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Fig. 1 Multiple sequence alignment of the two subunits of SMOs, StyA (**a**) and StyB (**b**). Several sequence motifs conserved in the flavoprotein hydroxylase family are indicated. A (+) indicates a residue that is part of the new fingerprint motif (N-V-H-Y-H-S) that defines the enantioselectivity of SMOs in sulfoxidation. Shown are *Br*StyA, *Br*StyB (*Bradyrhizobium* sp. ORS 375, WP_009031806, and WP_009031805), SN1StyA, SN1StyB (*Pseudomonas putida* SN1, ABB03727, and ABB03728), VLB120StyA, VLB120StyB (*Pseudomonas taiwanensis* VLB120, AAC23718, and AAC23719), LQ26StyA, LQ26StyB (*Pseudomonas* sp. LQ26, ADE62390, and ADE62391), *Rh*StyA, *Rh*StyB (*Rhodococcus* sp. ST-10, BAL04129, and BAL04130), STStyA, and STStyB (*Pseudomonas fluorescens* ST, CAB06823, and CAB06824)

The epoxidation appeared to be sensitive to the reaction temperature. The highest conversion of 47% was achieved at 30 °C after 15-min incubation. The conversion dropped to < 35% when the reaction temperature was maintained at 20 °C or 40 °C.

The effect of reaction pH was measured in potassium phosphate buffer (pH 6.0 to 8.0) or Tris-HCl buffer (pH 7.5 to 9.0). *Br*SMO yielded the highest conversion at pH 7.5 in potassium phosphate buffer and a slightly lower conversion in Tris-HCl buffer of the same pH (Fig. 4). In all cases, the epoxidation yielded (*S*)-styrene oxide with excellent enantioselectivity (> 99% ee).
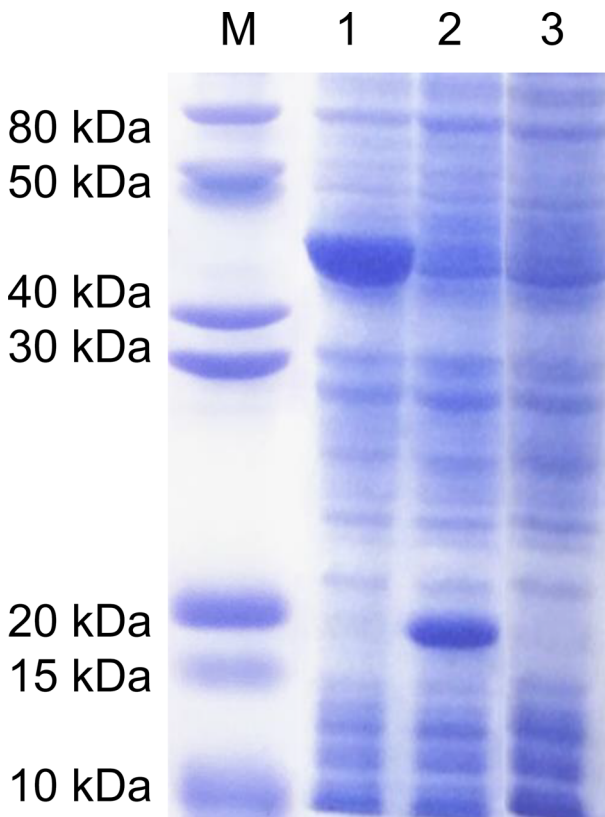
Fig. 2 SDS-PAGE analysis of the *Br*SMO expression. Lane M represents molecular marker. Lanes 1, 2, and 3 represent cell-free extracts of *E. coli* harboring the plasmids of pET-*Br*SMO, pET-*Br*StyB, and pET-28a (+), respectively

Fig. 3 Effect of reaction temperature on the whole-cell-catalyzed epoxidation. Reaction conditions: a mixture of the resting cells (0.05 g CDW), glucose (0.5%), cyclohexane (2%), and styrene (8 mM) in 5 mL potassium phosphate buffer (0.1 M, pH 7.0) was incubated at varied temperature with shaking at 200 rpm for 15 min
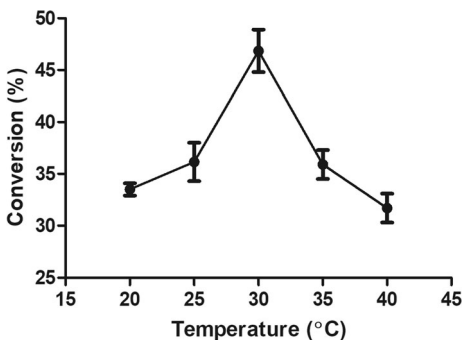
*Br*SMO-catalyzed asymmetric epoxidation and sulfoxidation

Fourteen more substrates besides styrene were tested using the whole-cell biotransformation system to probe the substrate spectrum and stereoselectivity of *Br*SMO. The results showed that *Br*SMO could catalyze the asymmetric epoxidation of 8 aromatic alkenes (Table 1), as well as the sulfoxidation of 7 sulfides (Table 2).

The epoxidation of styrene derivatives afforded the corresponding (*S*)-epoxides with excellent enantiomeric excesses of > 99% ee (for **1a**, **3a**-**4a**, **6a**-**8a**), 97% ee (for **2a**), and 95% ee (for **5a**) (Table 1). The enantioselectivity of *Br*SMO is similar to that of SMOs from *P. taiwanensis* VLB120 [29], *Pseudomonas* sp. LQ26[22], *Rhodococcus* sp. ST-5 and ST-10 [18], metagenome [33, 40], and *Marinobacterium litorale* DSM 23545 and *Paraglaciecola agarilytica* NO2 [28], which produce the (*S*)-epoxides with enantiomeric excesses ranging from 95 to > 99% ee.

*Br*SMO showed the highest relative activity toward styrene (**1a**), and the activity decreased when a substituent was present on the benzene ring (Table 1). Compared to the *meta*-substituted styrene (**3a** and **6a**), the relative activities of *ortho*- and *para*-substituents (**2a**, **4a**, **5a**, and **7a**) were low. Consequently, their product yields after 4-h reaction were also lower than *meta*-substituted styrenes. In addition, each of the chloro-substituted substrates showed higher relative activity than their bromo-substituted counterparts (**2a** vs **5a**, **3a** vs **6a**, **4a** vs **7a**) (Table 1). The results indicated that the position and type of substituents affected the enantioselectivity and conversion rate. Besides, aliphatic alkenes, such as 1-heptene, were also tested, but no product was detected.

Fig. 4 Effect of reaction pH on the whole-cell-catalyzed epoxidation. Reaction conditions: a mixture of the resting cells (0.05 g CDW), glucose (0.5%), cyclohexane (2%), and styrene (8 mM) in 5 mL (0.1 M) potassium phosphate buffer (black circle) or Tris-HCl buffer (black square) was incubated at 30 °C with shaking at 200 rpm for 15 min
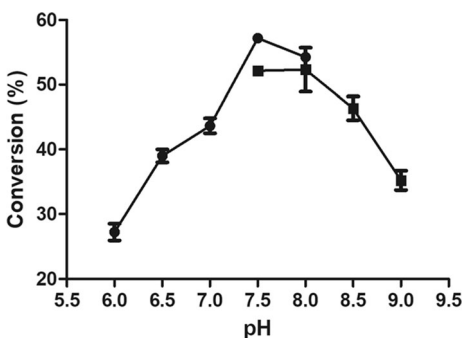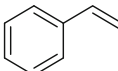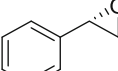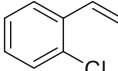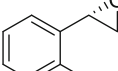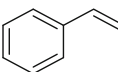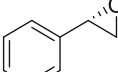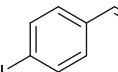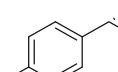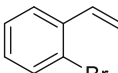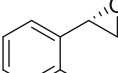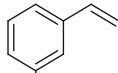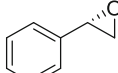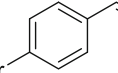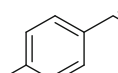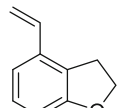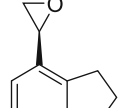
Table 1 Biotransformation of alkenes with *E. coli* whole cells expressing *Br*SMO

Substrate		Product		Relative Activity (%)[a]	Yield (%)[b]	ee (%)[b]
1a		**1b**		100 ± 1.1	100	>99, *S*
2a		**2b**		4.8 ± 0.1	51	97, *S*
3a		**3b**		45.4 ± 0.4	90	>99, *S*
4a		**4b**		11.6 ± 1.4	50	>99, *S*
5a		**5b**		3.5 ± 0.1	34	95, *S*
6a		**6b**		37.9 ± 2.5	85	>99, *S*
7a		**7b**		4.5 ± 1.1	29	>99, *S*
8a		**8b**		3.4 ± 0.2	69	>99, *S*

[a] The biotransformation was carried out at 30 °C for 30 min in 5 mL potassium phosphate buffer (100 mM, pH 7.5) containing 0.05 g CDW of the resting cells, 0.5% glucose, 2% (v/v) cyclohexane, and 8 mM substrate. Activities were normalized as percentages of the activity toward styrene
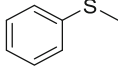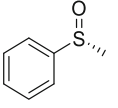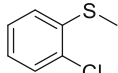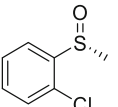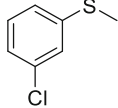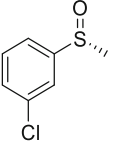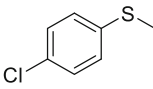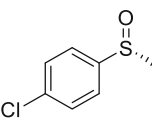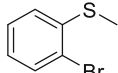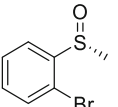
[b] The biotransformation was carried out at 30 °C for 4 h in 5 mL potassium phosphate buffer (100 mM, pH 7.0) containing 0.2 g CDW of the resting cells, 10% (v/v) cyclohexane, and 5 mM substrate

*Br*SMO also catalyzed the sulfoxidation of sulfides, affording (*R*)-sulfoxide with medium stereoselectivity (59–90% ee), except 2-chlorothioanisole (**10a**) (20% ee). Among them, 4-chlorothioanisole (**12a**) and 4-bromothioanisole (**15a**) yielded products with the highest enantiomeric excess of 89% ee and 90% ee, respectively (Table 2). The relative activity of *Br*SMO was the highest toward thioanisole (**9a**), followed by 3-chlorothioanisole (**11a**) and 4-

bromothioanisole (**15a**). The product yields of **9a**, **11a**, **12a**, and **15a** reached 100% after 4-h reaction, while others ranged from 67 to 87% (Table 2).

The (*R*)-selectivity of *Br*SMO is complementary to some (*S*)-selective P450s [51, 58], and is in accordance with the fingerprint motif of N46-V48-H50-Y73-H76-S97 that defines the enantioselectivity of SMOs in sulfoxidation [32]. Other SMOs with varied stereoselectivity have also been reported. For example, SMO from *P. taiwanensis* VLB120 catalyzed sulfoxidation reactions of thioanisole, but with poor enantioselectivity (26% ee) [25]. SMOs from *Gordonia polyisoprenivorans* catalyzed sulfoxidation reactions and produced the (*R*)-

Table 2 Biotransformation of sulfides with *E. coli* whole cells expressing *Br*SMO

Substrate		Product		Relative Activity (%)[a]	Yield (%)[b]	ee (%)[b]
9a		**9b**		100 ± 2	100	84, *R*
10a		**10b**		58.0 ± 1.1	87	20, *R*
11a		**11b**		98.1 ± 0.8	100	68, *R*
12a		**12b**		77.9 ± 1.9	100	90, *R*
13a		**13b**		62.1 ± 0.6	67	61, *R*
14a		**14b**		90.0 ± 0.1	80	59, *R*
15a		**15b**		93.8 ± 6.6	100	89, *R*

[a] The biotransformation was carried out at 30 °C for 30 min in 5 mL potassium phosphate buffer (100 mM, pH 7.5) containing 0.05 g CDW of the resting cells, 0.5% glucose, 2% (v/v) cyclohexane, and 8 mM substrate. Activities were normalized as percentages of the activity toward styrene
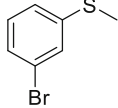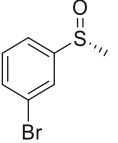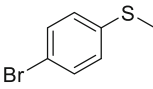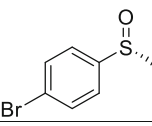
[b] The biotransformation was carried out at 30 °C for 4 h in 5 mL potassium phosphate buffer (100 mM, pH 7.0) containing 0.2 g CDW of the resting cells, 10% (v/v) cyclohexane, and 5 mM substrate

enantiomer with excellent enantioselectivity (98.6% ee) [59]. At the same time, several StyAs were able to catalyze the sulfoxidation and produced the (*S*)-enantiomer with excellent enantioselectivities (98–99% ee) [26, 32, 55, 59, 60].

Conclusion

In summary, we have identified a new two-component styrene monooxygenase, *Br*SMO, from the genome of *Bradyrhizobium* sp. ORS 375 and functionally expressed it in *E. coli*. The resulting whole-cell biocatalyst can catalyze the asymmetric epoxidation of styrene derivatives, yielding (*S*)-epoxides with excellent enantiomeric excesses; it can also catalyze the oxidation of sulfides, producing (*R*)-sulfoxide. The results presented here would enrich the diversity of styrene monooxygenase and implicate great potential for bio-epoxidation reactions.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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