Highly Efficient Access to (S)-Sulfoxides Utilizing a Promiscuous Flavoprotein Monooxygenase in a Whole-Cell Biocatalyst Format

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Chiral sulfoxides have gained attention as synthons and precursors for API synthesis. Flavoproteins such as Baeyer-Villiger or styrene monooxygenases mainly provide access to (R)-sulfoxides and often suffer from low selectivity, activity, and/or limited substrate scope. The flavoprotein monooxygenase AbIMO from Acinetobacter baylyi ADP1 initiates indole degradation. Here, AbIMO was expressed recombinantly in E. coli and characterized for its sulfoxidation activity and substrate spectrum. Next to indole and styrene, AbIMO was found to accept numerous alkyl aryl sulfoxides as substrates, transforming them to (S)-sulfoxides with high enantioselectivity (95% to >99% for most sulfoxides). The formulation as a whole-cell biocatalyst allowed specific production rates of up to 370 U g<sub>cdw</sub>⁻¹ – the highest specific oxygenase activity achieved in whole cells so far – and the preparative synthesis of enantiopure (S)-aryl alkyl sulfoxides. With its extraordinarily high specific activity, high specificity, ease of handling, and high stability (catalyst is stable for >16 days at 4°C), the designed whole-cell biocatalyst adds enormous value to the portfolio of chemical and biological catalysts for asymmetric sulfoxide synthesis.

Introduction

Chiral sulfoxides have gained huge attention as synthons and precursors for the synthesis of APIs,[1,2] flavors and fragrances,[3] or as chiral auxiliaries in chemical syntheses.[4,5] As the potency of one enantiomer often outperforms the one of the antipode (e.g., shown for esomeprazole),[6–8] enantiopure sulfoxide preparation is required. This can be achieved by costly and labor intensive resolution of racemates[9] or direct asymmetric synthesis.[5] The latter can be accomplished by the application of chemical[4,5] or biological catalysts, with the biocatalysts typically having a selectivity advantage.[11]

A number of enzymes such as cytochrome P450 monooxygenases,[12] Baeyer-Villiger monooxygenases (BVMOs)[13,14] peroxidases,[15] and styrene monooxygenases (SMOs)[16] among others catalyze regio- and enantioselective sulfoxidation, which has been pioneered by work on peroxidases and BVMOs.[11] However, (enantio)selectivity, versatility with respect to the structural diversity of accepted prochiral sulfoxides, as well as activity and stability of respective biocatalysts are properties, which often hinder industrial application. Thus, improving described systems or identifying novel ones is still necessary. P450pyrI83H from Sphingomonas sp. HXN-200 expressed in E. coli BL21 (DE3) is one of the most promising biocatalysts described so far and converted sulfoxides 1, 3, and 9

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to respective (R)-sulfoxides with remarkable enantioselectivities (e.e. 99.3, 98.2, and 96.0%, respectively) and specific activities up to 16 \( U_{\text{gcdw}}^{-1} \). For sulfides 2 and 6, however, this biocatalyst afforded an e.e. of only 61.8 and 66.9% for the (R)-enantiomers, respectively.\(^{[17]}\) A similarly limited substrate scope was observed with whole cells of \textit{Rhodococcus} sp. strain ECU066 primarily producing (S)-sulfoxides at rather low rates (< 1 \( U_{\text{gcdw}}^{-1} \)).\(^{[18]}\) For \textit{Rhodococcus} sp. CCZU10-1, a high (S)-selectivity for the sulfoxidation of sulfides 1–4 (e.e. > 99.9%) was reported, but specific activities remained low (< 1 \( U_{\text{gcdw}}^{-1} \)), increased up to 6.4 \( U_{\text{gcdw}}^{-1} \) upon application of \( n \)-octane as second liquid organic phase.\(^{[19]}\) Recently, an impressive study described asymmetric sulfoxidation employing an isolated BVMO for the production of an anti-cancer drug candidate precursor at the kilogram scale.\(^{[14]}\) The applied BVMO was chosen from a Codexis enzyme library obtained by directed evolution. This approach is appropriate when it comes to the synthesis of high-value drug candidates, but is highly costly as it requires the purification of two enzymes, one of them for cofactor regeneration. From an economic and ecological point of view, an universally applicable biocatalyst would be desirable featuring high specific activity and operational stability, cost-efficient catalyst formulation, and high enantioselectivity (> 99%) for a broad set of prochiral sulfides.

Group E flavoproteins are promising candidates for efficient and selective sulfoxidations (Scheme 1).\(^{[16–20]}\) Well-known representatives of this group have been shown to initiate the degradation of styrene by highly stereo- and enantioselective epoxidation and also to catalyze sulfoxidation reactions, but with lower activities and enantioselectivities.\(^{[20–22]}\) However, highly enantioselective sulfoxidation was found for StyA1-like enzymes from \textit{Rhodococcus opacus} 1CP and \textit{Variovorax paradoxus} EPS\(^{[21,23,24]}\) belonging to subgroup E2 of these flavoproteins.\(^{[25]}\) It was assumed that they play a role in the degradation of heterocyclic compounds.\(^{[26]}\) Just recently, indole was discovered as a native substrate for two subgroup E2 oxygenases from \textit{Acinetobacter}, which thus can be referred to as indole monoxygenases (IMOs).\(^{[27,28]}\)

In this study, we investigated the sulfoxidation potential of a closely related monoxygenase system from \textit{Acinetobacter baylyi} ADP1 (AbIMO, earlier designated as SMOA-ADP1), for which epoxidation activity has been reported before.\(^{[29,30]}\) First, we explored the sulfoxidation capability of AbIMO utilizing the purified enzyme. Styrene and thioanisole (1) were employed as standard substrates to allow for a comparison with related catalysts. With promising results in hand, we set out to develop a robust whole-cell biocatalyst for enantioselective sulfoxidation.

**Results and Discussion**

In \textit{vitro} characterization of AbIMO. In order to investigate the sulfoxidation capability of AbIMO, its monoxygenase component containing an N-terminal His\(_{6}\)-tag was produced recombinantly in \textit{E. coli} BL21 (DE3), purified, and assayed with different sulfide substrates. During cultivation on LB medium, the recombinant cells developed a dark blue color, which indicated the presence of an active monoxygenase transforming indole (derived from tryptophan) to indoxyl with subsequent spontaneous formation of indigo as reported before for similar oxygenases.\(^{[30–32]}\) This fits to the recent classification of the enzyme as indole monoxygenase catalyzing the initial step of indole degradation.\(^{[23,24,35]}\)

The versatility of AbIMO for sulfoxidation was tested via \textit{in vitro} biotransformations of sulfides 1–11 (assays comprised 2 mM substrate; Figure 1) providing FADH\(_2\) in excess by adding a flavin reductase component (PStyB; 28700 \( \text{U g}^{-1} \)), which utilized NADH to reduce FAD.\(^{[33]}\) The product amounts formed within 2 h of biotransformation were used to calculate the yields and rates given in Table 1. AbIMO converted most sulfides with

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**Scheme 1.** Reactions catalysed by group E flavoprotein monoxygenases.\(^{[20–30]}\) These are two component enzymes composed of a NADH-dependent FAD reductase (B) and FADH\(_2\)-utilizing monoxygenase (A). The flavin cofactor can be transferred directly or via diffusion and allows to form a hydroperoxy-FAD (FADOH) intermediate in the monoxygenase for selective oxygenation of substrates to corresponding epoxides or sulfoxides. Chiral centres might be generated in dependence of the substrate and enzyme employed. Here, the standard substrates styrene, indole and thioanisole are shown.

**Figure 1.** Chemical structures of the (mostly) prochiral sulfides applied in this study.
a high enantiomeric excess to respective (S)-sulfoxides except for the relatively bulky sulfides 7 and 8. The rates calculated for the 2 h of biotransformation, however, were much lower than initial rates (within the range of the first 15 min) determined in the same setup for styrene epoxidation ($139 \pm 16 \text{ U g}^{-1}$) or benzyl methyl sulfide (10) oxidation ($539 \pm 102 \text{ U g}^{-1}$) with a sampling time of only about 5 min for these substrates. This activity drop most likely was due to enzyme deterioration caused by the presence of substrate and/or product as it has been found for related monooxygenases.[24,29,34]

Overall, AbiMO was found to be rather slow and unselective enzyme with respect to styrene epoxidation ($v_{\text{max}}$ 139 U g$^{-1}$, biotransformation rate 13.2 U g$^{-1}$, (S)-87.1 % ee). In contrast, most sulfides were converted at substantially higher rates and enantioselectivities. Based on these promising sulfoxidation properties, we set out to develop a powerful AbiMO-based whole-cell system.

**Sulfoxidation by means of AbiMO-based whole-cell biocatalysts.** As shown in Table 1, purified AbiMO catalyzes the bioconversion of prochiral sulfides with a remarkably high enantioselectivity, but suffers from a decreasing activity limiting achievable product yields. Furthermore, utilization of isolated AbiMO not only requires the laborious purification of the respective two enzyme components, but also costly cofactor addition or regeneration. Thus, a whole-cell approach was chosen as a means to develop a stable, highly active, and easily producible and applicable biocatalyst. A possible complication of the downstream processing may be overcome via in situ product removal, e.g., a second liquid organic phase, as shown before for oxygenase-based whole-cell biocatalysis.[35]

A whole-cell biocatalyst expressing the two AbiMO genes under control of the pLacUV5 promoter was constructed. In resting cell biotransformations, the resulting heterologous biocatalyst *E. coli* JM101 (pCom10-L-AbiMO) efficiently oxidized most of the tested thioanisole derivatives to respective sulfones with excellent enantioselectivity. Extraordinarily high specific activities up to 370 U g$_{\text{cdw}}$^{-1} (cdw, cell dry weight) were obtained, and the activity pattern among the different substrates (Figure 2) correlated quite well with the observations made in *in vitro* bioconversions. This underlines that AbiMO qualifies very well for the sulfoxidation of a broad range of substituted thioanisole derivatives. The very high biocatalyst-specific sulfoxidation activities obtained via the whole-cell approach deserve special emphasis. The active enzyme portion of the biomass was ≤10% of the cell dry weight (g$_{\text{cdw}}$). Thus, the specific enzyme activities obtained in *in vivo* ($\geq 3080 \text{ U g}^{-1}$ for thioanisole oxidation; Figure 2) are approximately one order of magnitude higher than initial activities measured in *in vitro* (539 U g$^{-1}$ for thioanisole, see also Table 1). Sulfides 1 (thioanisole), 6 (4-methoxy-phenyl-methyl-sulfide), and 10 (benzyl-methyl-sulfide) were converted at biocatalyst-specific rates exceeding 300 U g$_{\text{cdw}}$^{-1}. These rates substantially exceed the highest specific production rates reported so far for oxygenase-containing whole cells, that is, 180 U g$_{\text{cdw}}$^{-1} for the oxygenation of styrene to (S)-styrene oxide.[36]

Moreover, no overoxidation to sulfones was observed, which poses a common drawback among asymmetric sulfoxidations employing chemical[23] or biological catalysts such as Baeyer-Villiger monooxygenases,[13,37] P450 monooxygenases, peroxidases, and peroxidases.[12,18,28,39] The specific sulfoxidation catalysts described so far mainly give access to (R)-enantiomers at lower rates emphasizing the attractiveness of the whole-cell biocatalyst reported here.[40] Interestingly, the BVMO designated 4-hydroxyacetophenone monooxygenase (HAPMO) has been reported to produce highly pure (S)- and (R)-enantiomers in dependence of the nature of the sulfide

![Figure 2. Specific sulfoxide production rates of resting-cell biocatalysts.](image)

Product formation was assessed 5 min after addition of the prochiral sulfide. One U corresponds to 1 μmol sulfoxide formed per min.

### Table 1. Biotransformation of styrene and sulfides 1–11 with purified AbiMO.

| Substrate | Rate$^{(a)}$ [U g$^{-1}$] | Conversion$^{(b)}$ [%] | Enantiomeric excess [%]$^{(c)}$ |
|-----------|--------------------------|----------------------|-------------------------------|
| styrene   | 13 ± 1                   | 9.4                  | (5) 87                        |
| 1         | 67 ± 2                   | 47.8                 | (5) > 99                      |
| 2         | 58 ± 7                   | 41.5                 | (5) 98                        |
| 3         | 77 ± 1                   | 54.8                 | (5) > 99                      |
| 4         | 48 ± 1                   | 34.1                 | (5) 96                        |
| 5         | 82 ± 13                  | 58.1                 | (5) 98                        |
| 6         | 176 ± 7                  | 100.0                | (5) > 99$^{(d)}$              |
| 7         | 17 ± 2                   | 12.4                 | n.d.                          |
| 8         | < 1                      | 0.4                  | n.d.                          |
| 9         | 76 ± 5                   | 53.8                 | (5) 99                        |
| 10        | 127 ± 7                  | 90.1                 | (5) 99                        |
| 11        | 58 ± 7                   | 41.0                 | (5) > 99                      |

Assays were performed in triplicates with a reconstituted enzyme system comprised of the monooxygenase component of AbiMO and P5StyB. Reaction mixtures were supplemented with surplus amounts of NADH and FAD. (a) One unit U corresponds to 1 μmol product formed per min. (b) The conversion was determined by quantifying product concentrations by HPLC after 2 h of incubation at 30°C under constant shaking. (c) Mean values are presented. Enantioselectivity was measured multiple times for each product and the standard deviation was always less than 2 %. (d) Only the (S)-enantiomer was detected.

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supplied as substrate. A whole-cell approach with HAPMO would be interesting.

The high specific production rates achieved with E. coli JM101 (pCom10 L:AbIMO) constitute a milestone for the synthesis of chiral sulfoxides, but at the same time pose a major challenge for the microbial metabolism that supplies reduction equivalents in the form of NADH. NADH is produced by glucose oxidation via glycolysis and the TCA cycle. By employing flux balance analysis, Blank et al. estimated a maximum NADH regeneration rate of ∼370 μmol min⁻¹ gcdw⁻¹ for resting E. coli cells metabolizing glucose at a typical rate of 2.4 mmol gcdw⁻¹ h⁻¹. The sulfoxidation rate achieved for benzyl-methyl sulfide (10) of 370 μmol min⁻¹ gcdw⁻¹ thus fully exploits this theoretical metabolic capacity of resting E. coli cells, given an equimolar stoichiometry of NADH consumption and sulfoxide production. Higher rates of glucose catabolism, as a target for metabolic engineering and as achieved with solvent-tolerant Pseudomonads as host strains, may enable even higher NADH regeneration rates, further advancing the presented whole-cell sulfoxidation approach.

The relatively bulky sulfoxides 7 and 8 were oxidized at substantially lower specific rates of 10.6 and 0.3 μmol gcdw⁻¹ h⁻¹, respectively. According to the general similarity of in vitro and in vivo activity patterns, a higher activity for those bulky sulfoxides was expected. Hence, these low activities may, beside enzyme specificity, have been caused by substrate uptake limitation over the cell membranes. Cell permeabilization with polymyxin B, however, did not lead to increased rates (Figure S2). Thus, a sulfide uptake limitation can be excluded. Together with the consistent in vitro data, this finding indicates that AbIMO hardly converts bulky sulfoxides with two phenyl substituents, but efficiently converts a broad set of thioanisole and benzyl phenyl sulfide derivatives.

Preparative scale in vivo bioconversions provide access to enantiopure sulfoxides. In order to determine the usefulness of the whole-cell biocatalyst for preparative sulfoxide synthesis, a 50 mL suspension (100 mM potassium phosphate buffer, pH 7.2, 1.5% (w/v) glucose) of resting AbIMO containing cells were incubated (30°C, 200 rpm, 2.5 cm amplitude) in a baffled flask and supplied with 1 mM of the sulfoxides 1–6 and 9–11. Another 1 mM sulfide portion was added every 30 min to prevent biocatalyst toxification by the sulfide as well as its excessive evaporation. After 11 successive cycles of substrate supplementation and conversion (6 h), no remaining prochiral substrate was detected in either case, indicating high biocatalyst stability. Whereas the sulfoxides 1, 6, 9, and 10 were completely transformed into corresponding sulfoxides (Table 2), only 36 to 79% of the other substrates were converted. The apparently reduced conversion of sulfoxides 2–5 and especially 11 can be ascribed to their high volatility and consequent loss via evaporation. More than 70% of the initially available sulfide 3 evaporated from the reaction medium within 2 h in the absence of cells or in the presence of E. coli JM101 carrying an empty control plasmid (Figure S1). Substrate conversion or absorption could be excluded as control experiments with and without cells gave the same results. Thus, sulfide conversion efficiency depended on the rate, at which the sulfide was oxidized, and its volatility.

Prochiral sulfide was added every 30 min in 12 portions of 50 μmol each to the reaction mixture (total volume: 50 mL). Conversion is calculated based on the product amount formed (Figures S3, S4, and S5). The isolation yield relates the amount of product isolated to the amount product formed.

Resting, AbIMO containing E. coli JM101 converted all tested sulfoxides (1–6 and 9–11) to respective sulfoxides with enantiomeric purities exceeding 99% (Table 2), thus being even higher than those obtained in vitro with purified AbIMO. Often, no second enantiomer was detected (Figure S4). These differences typically fall into the range of limits of detection and standard deviations, which, depending on the enantiomer separation efficiency accounted for maximally 2%. However, as in case of sulfoxide 4, a significant difference among in vivo and in vitro sulfoxidations may indeed exist. Flavoprotein monoxygenases are well known for the uncoupling of NADH oxidation from oxygenation leading to the formation of H₂O₂ (excellently reviewed by Hollman and coworkers), which is known to oxidize sulfoxides to racemic sulfoxides. In addition, superoxide might be formed and destabilize the enzymes in the in vitro assays. On the one hand, uncoupling may be less pronounced in the native enzyme environment in vivo (lower O₂ concentration, high protein concentration etc.) and, on the other hand, the efficiency of H₂O₂ disproportionation by catalase, which was present both in in vivo and in vitro biotransformations, may vary depending on the enzyme’s environment and its abundance. The high specific activity, stability, and enantiospecificity of the whole-cell sulfoxidation biocatalyst developed in this study augur well for a straightforward scale-up in high cell density bioreactor setups giving rise to high product titers and space-time-yields as it has been reported for a related group E flavoprotein monoxygenase catalyzing enantioselective epoxidation.

A typical challenge connected to the application of whole-cell catalysts is their limited storability and thus complex handling. This means that cultivation to the desired cell density has to precede every biotransformation in a timely manner. In order to evaluate, if a temporal decoupling of biocatalyst production and biotransformation would be feasible with E. coli JM101 (pCom10 L:AbIMO), we investigated its storability by assessing the sulfoxidation activity of resting cells that were...
stored under similar conditions as during biotransformations (2 g<sub>cdw</sub>L<sup>-1</sup>, 30°C, 200 rpm, KPi buffer pH 7.4, but no glucose) or at 4°C (~100 g<sub>cdw</sub>L<sup>-1</sup> in the same buffer, no orbital shaking) for different time periods. Whereas the cells stored under biotransformation-like conditions lost their sulfoxidation activity within 3 days, the cells stored at 4°C retained more than 50% of their initial sulfoxidation activity after 16 days of storage (Figure 3). The high stability at 4°C substantially simplifies biocatalyst handling and thus facilitates the application of this whole-cell biocatalyst in the daily routine of chemical synthesis.

Conclusions

In summary, we have developed a promiscuous, selective, stable, and highly active whole-cell biocatalyst that provides access to chiral sulfoxides with an excellent enantiomeric purity. The synthetic applicability of the biocatalyst was demonstrated by preparative biotransformations affording the sulfoxides at the 100 mg scale. Further reaction engineering efforts are required to minimize sulfide loss by evaporation. This can be achieved by an increased biocatalyst concentration and/or a continuous substrate feeding strategy. The very high specific activity achieved by an increased biocatalyst concentration and/or a continuous substrate feeding strategy. The very high specific activity achieved by an increased biocatalyst concentration and/or a continuous substrate feeding strategy. The very high specific activity achieved by an increased biocatalyst concentration and/or a continuous substrate feeding strategy.

Experimental Section

Oligonucleotides and chemicals. Custom synthesized oligonucleotides were purchased from Eurofins (Ebersberg, Germany). All other chemicals were obtained from AppliChem (Darmstadt, Germany), Sigma Aldrich (Steinheim, Germany), or Carl Roth (Karlsruhe, Germany).

Substrates and Products. All substrates and some products (methyl phenyl sulfoxide, diphenyl sulfoxide, phenyl vinyl sulfoxide) were obtained from commercial sources (TCI Europe; Sigma Aldrich) at highest available purity. The other sulfoxides were synthesized by a protocol described earlier.<sup>[46]</sup> with batches of 7 mmol sulfide in 100 mL methanol serving as the starting material. Products obtained were dried with anhydrous magnesium sulfate. Success of chemical sulfoxidation was controlled by GC-MS as described elsewhere.<sup>[47]</sup>

Generation of AbIMO expression vectors. Cloning of genes was performed by restriction/ligation or in vitro assembly.<sup>[46]</sup> Reagents were obtained from Thermo Fisher Scientific (Waltham, MA). Phusion high fidelity or DreamTaq polymerases were used according to the manufacturer’s instructions. The indole monoxygenase gene was amplified from the chromosome of <i>A. baylyi</i> ADP1 using 50 pmol of the primers ADP1-MO-fw (CATATGCGTCGTATAGCAATTGTTG) and ADP1-MO-rev (GGCGGCGTTAACGCCATTCTCTGAC) containing Ndel and NotI restriction sites (underlined) to be introduced adjacent to the amplified gene. <i>A. baylyi</i> ADP1 freshly cultivated at 30°C on an LB agar plate served as the source of template DNA. The amplicon was ligated into the vector pET11.2 (Thermo Fisher Scientific), and the resulting plasmid was amplified in <i>E. coli</i> DH5α. The gene was then excised by restriction digestion with Ndel and NotI and inserted into the expressed plasmid vector pET11bP cut with the same enzymes. The resulting plasmid pETADP1MO was transformed into <i>E. coli</i> BL21 (DE3) pLysS and used for expression purposes.

To construct the expression vector for the whole-cell biocatalyst, the monoxygenase component was amplified from pETADP1MO using the primers PCW111 (<i>S’</i>-TAGAAGCAGTACTGAGGAGATTCCA-TATCGCGTCGTATAGCAATTGTTG-<i>S</i>’-ACCTCAGCGTGTAATTCGAAGT-3’) and PCW108 (<i>S’</i>-ATCCACCGGTAATTAAGTC-3’). After DpnI treatment of the PCR mix, the amplicon was purified from an agarose gel. The reductase gene was obtained by PCR amplification from pETADP1SMOB<sup>[30]</sup> using the primers PCW109 (<i>S’</i>-GGCGGCGTATATGTTACGGGATTAGAGA-GATATCATATGATATTTAATACATACATG-3’) and PCW110 (<i>S’</i>-ACGGATCTCCGGGCGCCGAAGATGTTGATCGGAGCTCTGGATG-3’) (65°C annealing temperature, 40 s elongation time). The two amplicons were fused using the primers PCW111 and PCW110. PCR settings were 65°C annealing temperature and 1 min elongation time, and the primers were added during the denaturation phase of the fourth PCR cycle. The fusion PCR construct was then recombined with Ndel digested pCom10ac leading to the expression plasmid pCom10 L:AbIMO.<sup>[45]</sup> Expression of the heterologous gene in this vector is driven by the IPTG inducible PlacUV5 promoter. Accuracy of all manipulations was assessed by sequence analysis.

Bacterial strains and cultivation conditions. <i>E. coli</i> DH5α was used for cloning purposes and <i>E. coli</i> BL21 (DE3) pLysS or <i>E. coli</i> JM101<sup>[50]</sup> were used for AbIMO expression/purification or whole-cell biotransformations, respectively. <i>E. coli</i> strains were cultivated on solid or liquid LB media with appropriate antibiotics. For expression/purification purposes, <i>E. coli</i> BL21 (DE3) pLysS was cultivated in 2-fold YPTG medium in order to reach a high cell density. Freshly cultivated <i>E. coli</i> BL21 (pETADP1MO) cells were inoculated in 15-mL LB medium with 100 μg mL<sup>-1</sup> ampicillin and 50 μg mL<sup>-1</sup> chloramphenicol and incubated overnight at 37°C on a rotary shaker.
The overnight culture was used to inoculate 1.5-L of 2xYPTG-medium with 100 μM IPTG at 30°C for 10 min. Prochiral sulfides were added from 100-fold stock solutions in isopropanol to a final concentration of 2 mM. Reactions were terminated after 5 min by addition of an equal volume of a methanol/acetonitrile mixture (1:1 v/v) for reverse-phase HPLC analysis or ethyl acetate for normal-phase HPLC analysis, followed by centrifugation (17,000 x g, 2 min) for biomass removal. Samples for normal-phase HPLC analysis were mixed vigorously for 5 min (2000 rpm, 3 mm amplitude) before centrifugation. The specific activities in Ugh⁻¹, with 1 U defined as 1 μmol of sulfide formed per min, were retrieved from accumulated product and applied biomass concentrations.

Preparative biocatalysis at the 100 mg scale was performed with resting cells obtained as described above with the following modification: after harvest, the cells were resuspended to approximately 100 gcdw⁻¹ in 0.1 M potassium phosphate buffer (pH 7.4) and stored overnight (16 h) at 4°C. Subsequently, the cells were diluted in the same buffer supplemented with 1.5% (w/v) glucose to a cell concentration of 2 gcdw⁻¹ in a total volume of 50 mL (screw-capped baffled Erlenmeyer flask, 1:10 aqueous:gas ratio) and revived by incubation in a rotary shaker for 30 min at 30°C. Then, sulfides were supplied as pure compounds to a concentration of 1 mM in the reaction mixture. This substrate addition was repeated 11 times every 30 min giving a total reaction time of 6 h. This procedure prevented toxification of the biocatalyst by high sulfide concentrations.

Isolation of enantiopure sulfoxides. Isolation of the biocatalytically produced sulfoxides was initiated by clarification of the reaction mixture via centrifugation (3,900 g, 15 min). Portions of 20 mL were extracted 4 times with 5 mL dichloromethane. After phase separation, the emulsion-like interphases were combined and extracted once again with 5 mL dichloromethane. The combined organic phases were dried over anhydrous Na₂SO₄ and the solvent was removed under reduced pressure (850 mbar, 60°C, 120 rpm). Solvent evaporation was considered complete when a constant weight was achieved.

Product analysis. The conversion of styrene to styrene oxide as well as the enantiomeric configuration of the product were monitored by HPLC as described previously. For validation, commercial standards (Sigma Aldrich, Steinheim, Germany) were applied.

In vitro conversions of sulfides were analysed by means of a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with a DAD detector and a non-polar C18 reverse-phase column (Vertex Plus Eurospher 100–5, 4 x 125 mm, 5 μm particle size, Knauer, Berlin, Germany). Analytes were eluted at room temperature in a gradient of 40 to 90% methanol in water at a flow of 0.7 mL min⁻¹ and detected at 210 nm. Ten μL of samples (from stopped enzyme assays, diluted with methanol if necessary) were injected, and authentic standards were employed to calibrate the system. The enantiopurity of products was analyzed by means of chiral GC (see below).

In vivo conversions were analysed using the same HPLC system equipped with a C18 column (Accucore C18, 3 x 150 mm, 2 μm particle size, Thermo Fisher Scientific). Two μL of sample were injected, and analytes were eluted at 30°C with a mixture of 80% 10 mM ammonium phosphate buffer and 20% ACN at 6 min, followed by a gradient to 90% ACN (6-18 min) and detected at 210 nm. Sample preparation for analytical standards was performed as follows: 5 μL of 100-fold concentrated isopropanol stock solutions of sulfides or sulfoxides were added to 495 μL potassium phosphate buffer (pH 7.4, 0.1 M). Subsequently, 500 μL of a 1:1 (w/v)
The enantiomeric purity of sulfoxides obtained from whole-cell biotransformations was determined by normal-phase HPLC (6, 10, 11) or chiral gas chromatography (1, 2, 3, 4, 5, 9). Normal phase HPLC was performed using a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with a chiral Lux Cellulose-3 column (4.6 x 150 mm, 3 μm particle size, Phenomenex, Aschaffenburg, Germany) and a DAD detector. The oven was set to 25 °C and the analytes were eluted by an isocratic mixture of 80/20 % (v/v) n-hexane and isopropanol at 0.8 mL min⁻¹. n-Hexane half-saturated with water (by mixing equal volumes of water-saturated n-hexane with pure n-hexane) was applied, in order to minimize retention time shifts. Two μL of respective samples in ethyl acetate (see above) were injected. Organic compounds were detected at a wavelength of 210 nm. Chiral GC analysis was performed with a Trace 1310 gas chromatograph (Thermo Fisher Scientific) equipped with an Astec CHIRALDEX G-PN capillary GC column (30 m x 0.25 mm, 0.12 μm film thickness; Sigma-Aldrich). Prior to GC analysis, sulfoxide samples in ethyl acetate were appropriately diluted in the same solvent and dried over anhydrous Na₂SO₄. Oven and inlet temperatures were set to 170 and 90 °C, respectively. N₂ was used as carrier gas at a column flow rate of 1.5 mL min⁻¹ and a split ratio of 1:7. Separation was conducted for 25 °C and detection of the sulfoxides was achieved with a flame ionization detector (FID) at 250 °C.

The purified sulfoxides (synthesized from sulfides 1, 2, 3, 4, 5, 6, 9, 10, 11) were identified by GC-MS analysis on a Trace 1310 Gas chromatography system coupled to an ISQ LT Single Quadrupole Spectrometer equipped with a TG-5MS column (Thermo Fisher Scientific). Prior to analysis, sulfoxides samples in ethyl acetate were appropriately diluted in the same solvent and dried over anhydrous Na₂SO₄. GC-MS settings were: inlet temperature: 90 °C (2 min), 90–300 °C (15 °C min⁻¹). 300 °C (5 min), ionization mode: EI, ion source temperature: 180 °C.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Monooxygenase · Whole-cell biotransformation · Flavoprotein · Sulfoxidation · Biotransformation
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