Molecular redesign of Baeyer-Villiger Monooxygenases. Understanding and improvement of their biocatalytic properties
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Chapter 3

Baeyer-Villiger Monooxygenases as Biocatalysts in the Synthesis of Optically Pure Sulfoxides

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3.1 Abstract

4-hydroxyacetophenone monooxygenase (HAPMO) from *Pseudomonas fluorescense* ACB and phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* are Baeyer-Villiger monooxygenases that oxidize aromatic organic compounds such as ketones and sulfides. The products of these compounds are known to have various interesting properties for pharmaceutical applications. In this chapter we describe the biocatalytic properties of the newly identified PAMO, revealing its reactivity with a large range of sulfides and ketones. In addition, we studied the enantioselective oxidation of several prochiral compounds using PAMO and HAPMO. These enzymes were found to oxidize a number of phenyl sulfides with high enantioselectivity. In addition, HAPMO showed to be an excellent biocatalyst for the synthesis of enantiopure benzyl sulfoxides and an aromatic ester.

3.2 Introduction

Baeyer-Villiger monooxygenases (BVMOs) are flavin-containing enzymes that use NAD(P)H and molecular oxygen in order to catalyze the nucleophilic oxygenation of ketones and boron as well as the electrophilic oxygenation of various heteroatoms. In the last years, the use of BVMOs has been shown to be an excellent methodology in Baeyer-Villiger reactions, sulfoxidations, amine oxidations and epoxidations.\[1-5\] In many of these reactions, conversions occur with high enantio- and/or regioselectivity, while using environmentally friendly conditions. In general, the observed selectivities are difficult to achieve by chemical means.

Products that can be obtained using BVMOs are of great value in organic chemistry. Recently, mono- and polycyclic lactones have received considerable attention as therapeutic agents and as intermediates in pharmaceutical synthesis.\[6,7\] These products are easily obtained by the Baeyer-Villiger oxidation of the corresponding cyclic ketones. Additionally, optically active sulfoxides have become a well-established interest in organic synthesis. The configurational stability of the sulfinyl group as well as their synthetic versatility has increased
their application in the synthesis of enantiomerically enriched materials. Nowadays, sulfoxides are applied as chiral auxiliaries in numerous asymmetric reactions such as Michael addition, C-C bond formation, Diels-Alder reaction and radical addition.\[8,9\] Many organic sulfoxides also possess biological activity. They play an important role as therapeutic agents displaying anti-ulcer (proton pump inhibition), antibacterial and antifungal properties. Furthermore, they can be used as psychotonic and vasodilators.\[8-10\] The oxidation of sulfides is the most straightforward method for the synthesis of these sulfoxides and a great number of (in)organic reagents are available for this reaction.\[11,12\] However, these are environmentally unfriendly approaches compared to the use of BVMOs or peroxidases for this type of oxygenations.\[13-16\]

Only a limited number of BVMOs are available in recombinant form. In fact, until a few years ago, only cyclohexanone monooxygenase (CHMO, EC 1.14.13.22) from Acinetobacter sp. NCIMB 9871 had been extensively studied and applied in Baeyer-Villiger reactions and other selective oxidation processes.\[17-22\] In 2001 the first BVMO primarily active on aromatic compounds was isolated from Pseudomonas fluorescens ACB. Purification, characterization and overexpression of this enzyme showed that it oxidizes 4-hydroxyacetophenone to 4-hydroxyphenylacetate, hence its name 4-hydroxyacetophenone monooxygenase (HAPMO, EC 1.14.13.84).\[23,24\] This enzyme is a homodimer of 145 kDa with each subunit containing a tightly non-covalently bound FAD cofactor. Previous studies on substrate specificity have demonstrated that HAPMO prefers acetophenones and benzaldehydes bearing an electron-donating substituent at the para-position, although heteroaromatic and aliphatic substrates are also accepted.\[25-28\] A recent study has revealed that HAPMO can be used as a biocatalyst in aqueous and aqueous-organic media.\[29\]

The recent recognition of a protein sequence motif that can be used to identify BVMOs has enabled mining of the genome database. Using this sequence motif, a large number of putative BVMO genes can be annotated including the previously mentioned CHMO and HAPMO.\[30\] Through this approach of enzyme discovery, a novel BVMO has been obtained from the therophilic actinomycete Thermobifida fusca. The initial characterization of this biocatalyst has shown that it represents a thermostable and monomeric enzyme, containing FAD as a cofactor and being
NADPH dependent. Previous studies reported that the best substrate was phenylacetone and therefore the enzyme was named phenylacetone monooxygenase (PAMO, EC 1.14.13.92).[31] It was also demonstrated that the enzyme is able to oxidize other aromatic and aliphatic ketones and organic sulfides. PAMO represents the first BVMO of which the X-ray structure has been solved and this enables mechanistic and enzyme redesign studies (see Chapters 4 and 5).[32]

The main goal of this work was to explore the synthetic repertoire of HAPMO and PAMO by exploring the biocatalytic asymmetric oxidation of aromatic sulfides and a prochiral diketone. Furthermore, to improve our knowledge on the biocatalytic potential of the newly identified thermostable PAMO, the kinetic parameters of a set of ketones, sulfides, sulfoxides and tertiary amines in enzyme-catalyzed oxidations were also determined.

3.3 Experimental

3.3.1 General
Recombinant HAPMO and histidine-tagged PAMO were overexpressed and purified according to previously described methods.[23,31] Oxidation reactions were performed using the purified enzymes. One unit of enzyme oxidizes 1.0 µmol of thioanisole 3.1 to methyl phenyl sulfoxide 3.1a per minute at pH 9 and 25 °C in the presence of NADPH. Glucose-6-phosphate dehydrogenase (G6PDH) from Leuconostoc mesenteroides was obtained from Fluka-BioChemika. Glucose-6-phosphate and NADP(H) were purchased from Sigma-Aldrich-Fluka. Sulfides 3.1, 3.2, 3.4, 3.7, 3.8, 3.10, 3.13, 3.20, 3.21, 3.22, 3.23, thiantrone, p-tolyl disulfide, 1,3-dithiao; racemic sulfoxides (+)-3.1a, (+)-3.7a, (+)-3.13a, (–)-nicotine 3.42, phenylboronic acid (3.36), phenol (3.37), ketones 3.43-3.47, 3.49 and 3.50 were purchased from Sigma-Aldrich-Fluka. Phenyl sulfides 3.12, 3.18, benzyl sulfides 3.24-3.25 and amine 3.41 were products from Lancaster. Compounds 3.6, 3.9, 3.11, 3.17, 3.19 and ketone 3.50 were from Acros-Organics. Diketone 3.38 was purchased by TCI Europe. (R)-1-Hydroxy-1-phenylacetone 3.40 was a kind gift from Dr. M. Breuer (BASF). Sulfides 3.3[33] 3.5[33] 3.14[44] 3.15[33] 3.16[36] 3.24-3.28[37] 3.29[33] 3.30[38] 3.31[39] 3.32[37] 3.33[33] 3.34[40] 3.35[33],
Chapter 3

(±)-3.39\textsuperscript{[41]} and compound (±)-3.40\textsuperscript{[41]} were synthesized according to the literature. Sulfoxides were prepared by chemical oxidation from the corresponding sulfides and exhibited physical and spectral properties in agreement with those reported.\textsuperscript{[33,35-39,42-53]} N,N-dimethyl benzylamine N-oxide 3.41a and cis-(S)-(−)-nicotine N-1'-oxide 3.42a were obtained by chemical oxidation with 30 % H\textsubscript{2}O\textsubscript{2}. All other reactants and solvents were of the highest quality grade available, commercialized by Sigma-Aldrich-Fluka.

IR spectra were recorded on a Jasco FTIR 610. Optical rotations were determined on a Perkin Elmer 141 polarimeter. Chemical reactions were monitored by analytical TLC, performed on Merck silica gel 60 F\textsubscript{254} plates and visualized by UV irradiation. Flash chromatography was carried out with silica gel 60 (70-230 mesh, Merck). \textsuperscript{1}H- and \textsuperscript{13}C-NMR spectra at 300 MHz and 72.5 MHz were recorded on a Bruker AC-300. Mass spectra were performed on a GCMS-EI (Finnigan-Thermo). Chiral HPLC analyses were performed on a Jasco HPLC instrument (model 880-PU pump, model 870-UV/Vis detector) equipped with a Chiralcel OD (Daicel), a Chiralcel OJ (Daicel) or a Chiralcel OB (Daicel) chiral column. Acetanilide was used as internal standard. Retention times of the chiral samples were in agreement with the purified racemic ones. Chiral and achiral GC analyses were performed on a Shimadzu GC17 instrument equipped with a FID-detector and a Chiraldex G-TA column (Agilent, 30 m × 0.25 mm × 0.125 mm) or a HP1 column (Agilent, 30 m × 0.25 mm × 0.25 mm), respectively. The kinetic measurements were carried out with a Perkin Elmer Lambda Bio40 spectrophotometer.

Unless otherwise stated, absolute configurations of chiral sulfoxides were established by comparison of the HPLC chromatograms with the patterns described in previous experiments for the known configurations. For sulfoxides 3.3a\textsuperscript{[33]}, 3.4a\textsuperscript{[50]}, 3.5a\textsuperscript{[54,55]}, 3.7a\textsuperscript{[42]}, 3.10a\textsuperscript{[48]}, 3.18a\textsuperscript{[47]}, 3.19a\textsuperscript{[46]} and 3.27a\textsuperscript{[37]} absolute configuration was established by comparison of the specific rotation measured with the ones reported. Configuration of sulfoxide (\textit{R})-3.8a was established by comparison with a sample prepared from the chemical chlorination of (S)-methyl phenyl sulfoxide with \textit{N}-chlorosuccinimide.\textsuperscript{[56]} For sulfoxide (\textit{R})-3.9a the configuration was assigned by comparison with a sample prepared from treatment of (S)-phenyl vinyl sulfoxide with trimethylsilyl chloride.\textsuperscript{[57]} Configuration of (\textit{R})-
3.39 was established by comparison with an authentic sample prepared from chemical acetylation of (R)-1-hydroxy-1-phenylacetone (R)-3.40.

3.3.2 Typical procedure for the enzymatic oxidation of substrates
Substrates (15-20 mM, except for 3.38, 2.5 mM) were dissolved in a buffer Tris/HCl (50 mM, pH 9.0, 1.0 mL, except for substrate 3.38, pH 7.5) buffer, containing glucose-6-phosphate (1.5 equiv.), glucose-6-phosphate dehydrogenase (10.0 units), NADP⁺ (0.02 mM), acetanilide (0.02 mg) as internal standard and 1.0 unit of HAPMO or PAMO. The mixture was shaken at 250 rpm and 25 °C in a rotatory shaker for the times established. The reactions were then stopped, extracted with dichloromethane (3 × 0.5 mL), dried over Na₂SO₄ and analyzed by chiral HPLC to determine the conversion and the enantiomeric excesses of the sulfoxides obtained. Conversion and optical purity of (R)-3.39 were established by means of GC. Control experiments in absence of enzyme were performed for all substrates tested, and no reaction was observed.

3.3.3 General procedure for the enzymatic oxidation at multimilligram scale by HAPMO
The sulfides (50.0 mg, 0.30-0.36 mmol) were dissolved in a Tris/HCl buffer (50 mM, pH 9.0, 25 mL) containing glucose-6-phosphate (1.2 equiv.), glucose-6-phosphate dehydrogenase (125 units), NADP⁺ (0.02 mM), acetanilide (0.1 mg) and 4-hydroxyacetophenone monooxygenase (12.5 units). Reactions were stirred at 25 °C and 250 rpm in a rotatory shaker for 30 h (sulfides 3.5 and 3.10) or 40 h (sulfides 3.7 and 3.19). The reactions were then extracted with dichloromethane (4 × 15 mL) and the organic layers were dried over Na₂SO₄, filtered and evaporated under reduced pressure. The crude residues were purified by flash chromatography on silica gel (petroleum ether / ethyl acetate 9:1, except for substrate 3.19, petroleum ether / ethyl acetate 8:2) to afford the chiral sulfoxides: (S)-3.5a (colorless oil, 26.7 mg, 49 % yield), (S)-3.7a (colorless oil, 21.6 mg, 39 % yield), (R)-3.10a (colorless oil, 20.8 mg, 38 % yield) and (S)-3.19a (yellow pale oil, 17.6 mg, 31 %).
(S)-n-Butyl phenyl sulfoxide, (S)-3.5a
Determination of e.e. by HPLC analysis: Chiralcel OB, petroleum ether / i-propanol (88:12), 1.0 mL.min⁻¹, 254 nm. \( t_R \) 11.9 (R) and 19.7 (S) min. \([\alpha]_D^{25} = -131.5\) (c 0.98, acetone) e.e. 71 %.

(S)-Allyl phenyl sulfoxide, (S)-3.7a
Determination of e.e. by HPLC analysis: Chiralcel OB, petroleum ether / i-propanol (85:15), 1.0 mL.min⁻¹, 254 nm. \( t_R \) 13.6 (S) and 17.9 (R) min. \([\alpha]_D^{25} = -164.8\) (c 1.08, EtOH) e.e. 98 %.

(R)-Chloromethyl phenyl sulfoxide, (R)-3.8a
Determination of e.e. by HPLC analysis: Chiralcel OD, petroleum ether / i-propanol (95:5), 1.0 mL.min⁻¹, 254 nm. \( t_R \) 14.8 (S) and 17.6 (R) min. \([\alpha]_D^{25} = -83.3\) (c 0.73, acetone) e.e. 89 %.

(R)-Chloroethyl phenyl sulfoxide, (R)-3.9a
Determination of e.e. by HPLC analysis: Chiralcel OD, petroleum ether / i-propanol (97:3), 1.0 mL.min⁻¹, 254 nm. \( t_R \) 27.9 (S) and 30.8 (R) min. \([\alpha]_D^{25} = -101.8\) (c 1.39, acetone) e.e. 98 %.

(R)-Methoxymethyl phenyl sulfoxide, (R)-3.10a
Determination of e.e. by HPLC analysis: Chiralcel OD, petroleum ether / i-propanol (9:1), 1.0 mL.min⁻¹, 254 nm. \( t_R \) 11.8 (S) and 14.6 (R) min. \([\alpha]_D^{25} = -207.1\) (c 0.84, CHCl₃) e.e. 97 %.

(S)-4-Aminophenyl methyl sulfoxide, (S)-3.19a
Determination of e.e. by HPLC analysis: Chiralcel OD, petroleum ether / i-propanol (75:25), 1.0 mL.min⁻¹, 254 nm. \( t_R \) 20.3 (R) and 28.8 (S) min. \([\alpha]_D^{25} = -85.1\) (c 0.82, EtOH) e.e. 95 %.
3.3.4 General procedure for the enzymatic oxidations at multimilligram scale by PAMO

The sulfides (0.33 mmol for 3.3 and 3.4; 0.29 mmol for 3.18 and 0.30 mmol for 3.27) were dissolved in a Tris/HCl buffer (50 mM, pH 9.0, 25 mL) containing glucose-6-phosphate (1.5 equiv.), glucose-6-phosphate dehydrogenase (2.5 units), NADPH (0.02 mM), acetanilide (0.5 mg) and phenylacetone monooxygenase (0.25 units). The mixtures were shaken at 25 °C and 250 rpm during 48 hours for substrates 3.4 and 3.27, 60 hours for sulfide 3.3 and 72 hours for compound 3.18. Once finished, the reactions were extracted with dichloromethane (3 × 25 mL) and the combined organic layers were dried over Na$_2$SO$_4$, filtered and evaporated under reduced pressure. The residues were purified by flash chromatography on silica gel with petroleum ether/ethyl acetate 9:1 to afford the corresponding sulfoxides: (S)-3.3a (colorless oil, 23.2 mg, 42 % yield), (S)-3.4a (colorless oil, 17.6 mg, 32 % yield), (R)-3.18a (yellow pale solid, 26.2 mg, 49 % yield) and (S)-3.16a (yellow pale oil, 25.8 mg, 48 % yield).

(S)-Phenyl propyl sulfoxide, (S)-3.3a

Determination of e.e. by HPLC analysis: Chiralcel OB, petroleum ether / i-propanol (85:15), 1.0 mL.min$^{-1}$, 254 nm. $t_R$ 13.4 (S) and 29.3 (R) min. $[\alpha]_D^{25}$ -51.8 (c 0.88, CHCl$_3$), e.e. 21 %.

(S)-Cyclopropyl phenyl sulfoxide, (S)-3.4a

Determination of e.e. by HPLC analysis: Chiralcel OD, petroleum ether / i-propanol (95:5), 1.0 mL.min$^{-1}$, 254 nm. $t_R$ 13.1 (R) and 17.3 (S) min. $[\alpha]_D^{25}$ +56.6 (c 0.71, acetone), e.e. 48 %.

(R)-Methyl p-nitrophenyl sulfoxide, (R)-3.18a

Determination of e.e. by HPLC analysis: Chiralcel OB petroleum ether / i-propanol (75:25), 1.0 mL.min$^{-1}$, 254 nm. $t_R$ 37.9 (S) and 51.0 (R) min. $[\alpha]_D^{25}$ +84.1 (c 1.30, CHCl$_3$), e.e. 76 %.
(R)-Benzyl isopropyl sulfoxide, (R)-3.27a

Determination of e.e. by HPLC analysis: Chiralcel OD, petroleum ether / i-propanol (95:5), 1.0 mL.min\(^{-1}\), 254 nm. \( t_R \) 26.8 (S) and 28.9 (R) min. \([\alpha]_D^{25}\) +53.7 (c 1.29, EtOH), e.e. 41 %.

3.3.5 Procedure for the determination of the kinetic parameters

For determination of the steady-state kinetic parameters of PAMO with various ketones, sulfides and amines, the enzyme activity was determined by monitoring the decrease in NADPH concentration at 340 nm (\( \varepsilon_{340} = 6.22 \text{ mM}^{-1}\text{.cm}^{-1} \)) or 370 nm (\( \varepsilon_{370} = 3.7 \text{ mM}^{-1}\text{.cm}^{-1} \)). A reaction mixture of 1.0 mL usually contained 50 mM Tris/HCl, pH 7.5, 100 µM NADPH, 1 % (v/v) DMSO and 0.5 µM PAMO. The presence of 1 % DMSO resulted in only a slight decrease in PAMO activity (< 1 %), while a higher solubility of certain compounds could be obtained. The steady-state kinetic parameters were determined at 30 °C using air-saturated buffers.

\[
\text{D-glucurate-6P} \xrightarrow{\text{G6PDH}} \text{NADPH} + H^+ \xrightarrow{\text{HAPMO or PAMO}} \text{NADP}^+ \xrightarrow{\text{D-glucurate-6P}} \]

\( n: 0, 1, 2, 3. \)

Scheme 3.1 General procedure for the enzymatic oxidation of organic sulfides.

3.4 Results and Discussion

The oxidation of a set of organic sulfides (3.1-3.35) to their corresponding sulfoxides by recombinant 4-hydroxyacetophenone monooxygenase\(^{[23]}\) and phenylacetone monooxygenase\(^{[31]}\) was coupled to an ancillary enzymatic reaction in order to regenerate NADPH (Scheme 3.1). As a NADPH regeneration system,
glucose-6-phosphate and glucose-6-phosphate dehydrogenase (G6PDH) were employed.\[4,5\] All oxidations were carried out in a Tris/HCl buffer at pH 9. The results obtained in the enzymatic catalyzed oxidation of different aromatic sulfides (Figure 3.1) are summarized in Table 3.1.

### 3.4.1 Enantioselective sulfoxidations by HAPMO

First, a set of phenyl sulfides (3.1-3.10) were analyzed as possible HAPMO substrates by determining their conversion and the enantiomeric excess of the products obtained. With some exceptions, high enantiomeric excesses (e.e. > 95 %) were achieved for the compounds tested, indicating an enzyme preference for this structure in terms of enantioselectivity. In all cases, oxygenation resulted in the same absolute configuration (Table 3.1). As previously described,\[25\] thioanisole oxidation was almost complete after 24 hours, resulting in the formation of enantiopure (S)-methyl phenyl sulfoxide 3.1a. Similar enantioselectivities were measured for sulfides with alkyl chains shorter than the cyclopropyl group 3.2-3.4. Butyl phenyl sulfide 3.5 led to the formation of (S)-3.5a with only moderate enantiomeric excess (e.e. = 71 %). Conversions were slightly decreased by increasing the size of the alkyl moiety of the sulfides. HAPMO is able to catalyze the enzymatic sulfoxidation of phenyl alkenyl sulfides into the corresponding (S)-sulfoxides with excellent enantioselectivities. Both vinyl and allyl sulfides 3.6 and 3.7 were oxidized into (S)-3.6a and (S)-3.7a with e.e. = 98 % and conversions close to 70 %. Phenyl alkyl sulfides containing a heteroatom in the alkyl chain were also tested. The presence of a chloride atom at two bond lengths from the sulfur atom affected negatively the enantioselectivity of the enzyme with respect to the corresponding non-chlorinated sulfide 3.1. (R)-Methylchloride phenyl sulfoxide (R)-3.8a was obtained with moderate enantiomeric excess (e.e. = 76 %) and conversion (56 %). When the chloride atom was placed further away from the sulfur atom (sulfide 3.9), both the conversion and the enantioselectivity measured for (R)-3.9a were slightly improved. In contrast, the oxidation of a sulfide containing an electron-donating atom in the alkyl chain (3.10), led to the formation of almost enantiomerically pure sulfoxide (R)-3.10a.

The bicyclic aromatic compound 2-naphthyl methyl sulfide 3.12 was oxidized to (S)-3.12a with high enantiomeric excess but low conversion. The low conversion
was probably due to the low solubility of the sulfide in the aqueous medium. The presence of a bulky aromatic system did not affect the enantioselectivity of the biocatalyst to a great extent.

Figure 3.1 Aromatic sulfides used as substrates for enantioselective oxidation by 4-hydroxyacetophenone and phenylacetone monooxygenase.

When HAPMO was employed in the sulfoxidation of a set of benzyl alkyl sulfides, it was found that the absolute configuration of the products was strongly dependent on the size of the alkyl group. The (S)-enantiomer predominated in the case of small alkyl substituents \(3.24\) and \(3.25\). However, when the alkyl chain was relatively bulky, the corresponding (R)-sulfoxides \(3.26a-3.28a\) were formed. The enantiomeric excesses of the benzyl alkyl sulfoxides obtained were in all cases close to 80 %, with exception of \(3.26a\) (e.e. = 65 %). Furthermore, it was found that the measured conversions were between 44 % and 59 %, indicating that the catalytic efficiency of HAPMO is not very sensitive to the alkyl group variation of these substrates. Upon extending the alkyl chain between the sulfur atom and the phenyl moiety, there was a clear trend towards lower conversions and decreased enantioselectivity. For both 2-phenylethyl and 3-phenylpropyl methyl sulfides \(3.33\) and \(3.35\), the corresponding (R)-sulfoxides were obtained with moderate enantiomeric excesses (e.e. around 50 %) and conversions, especially in the case of compound \(3.35\) (e = 29%).
### Table 3.1 Oxidation of aromatic sulfides catalyzed by HAPMO and PAMO\[a\]

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>time (h)</th>
<th>HAPMO conv. (%)</th>
<th>e.e. (%) [b,c]</th>
<th>time (h)</th>
<th>PAMO conv. (%)</th>
<th>e.e. (%) [b,c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>CH₃</td>
<td>24</td>
<td>96</td>
<td>99 (S)</td>
<td>24</td>
<td>94</td>
<td>44 (R)</td>
</tr>
<tr>
<td>3.2</td>
<td>CH₂CH₃</td>
<td>24</td>
<td>86</td>
<td>99 (S)</td>
<td>24</td>
<td>79</td>
<td>33 (S)</td>
</tr>
<tr>
<td>3.3</td>
<td>Propyl</td>
<td>24</td>
<td>85</td>
<td>97 (S)</td>
<td>24</td>
<td>56</td>
<td>21 (S)</td>
</tr>
<tr>
<td>3.4</td>
<td>Cyclopropyl</td>
<td>24</td>
<td>74</td>
<td>97 (S)</td>
<td>24</td>
<td>67</td>
<td>48 (R)</td>
</tr>
<tr>
<td>3.5</td>
<td>Butyl</td>
<td>24</td>
<td>61</td>
<td>71 (S)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.6</td>
<td>CH=CH₂</td>
<td>24</td>
<td>70</td>
<td>98 (S)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.7</td>
<td>CH₂CH=CH₂</td>
<td>24</td>
<td>69</td>
<td>98 (S)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.8</td>
<td>CH₂Cl</td>
<td>24</td>
<td>56</td>
<td>76 (R)[d]</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.9</td>
<td>CH₂CH₂Cl</td>
<td>24</td>
<td>69</td>
<td>81 (R)[d]</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.10</td>
<td>CH₂OCH₃</td>
<td>24</td>
<td>63</td>
<td>98 (R)[d]</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.11</td>
<td>CH₂COOH</td>
<td>n.d.</td>
<td>n.d.</td>
<td>48</td>
<td>≤3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.12</td>
<td>-</td>
<td>24</td>
<td>31</td>
<td>95 (S)</td>
<td>24</td>
<td>22</td>
<td>41 (S)</td>
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<tr>
<td>3.13</td>
<td>CH₃</td>
<td>24</td>
<td>77</td>
<td>99 (S)</td>
<td>24</td>
<td>68</td>
<td>10 (R)</td>
</tr>
<tr>
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<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>24</td>
<td>31</td>
<td>17 (S)</td>
</tr>
<tr>
<td>3.15</td>
<td>Octyl</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>48</td>
<td>25</td>
<td>≤3 (-)</td>
</tr>
<tr>
<td>3.16</td>
<td>CH₂-COOCH₂CH₃</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>24</td>
<td>12</td>
<td>≤3 (-)</td>
</tr>
<tr>
<td>3.17</td>
<td>p-CHO₃</td>
<td>24</td>
<td>78</td>
<td>99 (S)</td>
<td>24</td>
<td>47</td>
<td>25 (R)</td>
</tr>
<tr>
<td>3.18</td>
<td>p-NO₂</td>
<td>24</td>
<td>32</td>
<td>87 (S)</td>
<td>24</td>
<td>27</td>
<td>76 (R)</td>
</tr>
<tr>
<td>3.24</td>
<td>CH₃</td>
<td>20</td>
<td>55</td>
<td>85 (S)</td>
<td>6</td>
<td>29</td>
<td>94 (S)</td>
</tr>
<tr>
<td>3.25</td>
<td>CH₂CH₃</td>
<td>20</td>
<td>52</td>
<td>81 (S)</td>
<td>6</td>
<td>36</td>
<td>98 (S)</td>
</tr>
<tr>
<td>3.26</td>
<td>Propyl</td>
<td>20</td>
<td>59</td>
<td>65 (R)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.27</td>
<td>Isopropyl</td>
<td>20</td>
<td>44</td>
<td>82 (R)</td>
<td>8</td>
<td>36</td>
<td>41 (R)</td>
</tr>
<tr>
<td>3.28</td>
<td>Butyl</td>
<td>20</td>
<td>57</td>
<td>77 (R)</td>
<td>18</td>
<td>32</td>
<td>28 (R)</td>
</tr>
<tr>
<td>3.29</td>
<td>Isopentyl</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>18</td>
<td>21</td>
<td>17 (R)</td>
</tr>
<tr>
<td>3.30</td>
<td>CH₂-COOCH₃</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6</td>
<td>30</td>
<td>48 (n.d.)</td>
</tr>
<tr>
<td>3.31</td>
<td>CH₂CH₂OAc</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6</td>
<td>21</td>
<td>66 (n.d.)</td>
</tr>
<tr>
<td>3.32</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>30</td>
<td>18</td>
<td>≤3 (-)</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.33</td>
<td>CH₃</td>
<td>24</td>
<td>44</td>
<td>51 (R)</td>
<td>8</td>
<td>27</td>
<td>80 (R)</td>
</tr>
<tr>
<td>3.34</td>
<td>CH₂CH₃</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6</td>
<td>23</td>
<td>83 (n.d.)</td>
</tr>
<tr>
<td>3.35</td>
<td>-</td>
<td>24</td>
<td>29</td>
<td>57 (R)</td>
<td>8</td>
<td>21</td>
<td>70 (R)</td>
</tr>
</tbody>
</table>

[a] For reaction details see the Experimental section.

[b] Conversion and enantiomeric excess determined by HPLC.

[c] Sign of specific rotation in parentheses.

[d] Absolute configuration is reversed due to a change in the priority according to the sequence rules.

n.d.: not determined.
Table 3.2 HAPMO catalyzed oxidation of thioanisole derivatives R-Ph-S-CH₃ to the corresponding (S)-sulfoxides.[a]

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>σ[b]</th>
<th>Time (h)</th>
<th>conv. (%)</th>
<th>e.e. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.19</td>
<td>p-NH₂</td>
<td>-0.66</td>
<td>24</td>
<td>41</td>
<td>96 (S)</td>
</tr>
<tr>
<td>3.17</td>
<td>p-OCH₃</td>
<td>-0.27</td>
<td>24</td>
<td>78</td>
<td>99 (S)</td>
</tr>
<tr>
<td>3.13</td>
<td>p-CH₃</td>
<td>-0.17</td>
<td>24</td>
<td>77</td>
<td>99 (S)</td>
</tr>
<tr>
<td>3.1</td>
<td>H</td>
<td>0</td>
<td>24</td>
<td>96</td>
<td>99 (S)</td>
</tr>
<tr>
<td>3.21</td>
<td>p-Cl</td>
<td>0.23</td>
<td>24</td>
<td>37</td>
<td>44 (S)</td>
</tr>
<tr>
<td>3.23</td>
<td>m-Cl</td>
<td>0.37</td>
<td>24</td>
<td>42</td>
<td>93 (S)</td>
</tr>
<tr>
<td>3.20</td>
<td>p-CN</td>
<td>0.66</td>
<td>24</td>
<td>64</td>
<td>96 (S)</td>
</tr>
<tr>
<td>3.18</td>
<td>p-NO₂</td>
<td>0.78</td>
<td>24</td>
<td>32</td>
<td>87 (S)</td>
</tr>
<tr>
<td>3.22</td>
<td>o-Cl</td>
<td>n.a.</td>
<td>24</td>
<td>76</td>
<td>96 (S)</td>
</tr>
</tbody>
</table>

[a] For reaction details see Experimental section.
[b] Values taken from Hansch et al.[58]
[c] Conversion and enantiomeric excess determined by HPLC.
[d] Sign of specific rotation in parentheses.

In a previous report, it was established that the affinity of HAPMO for a set of para-substituted acetophenones depended on the electronic properties of the substituents.[25] The enzyme showed better conversions on ketones possessing an electron-donating group than on those with electron-withdrawing ones. Here, the effect of para-substitution has been studied on several derivatives of thioanisole 3.1, which is the best sulfide substrate found for HAPMO so far (Scheme 3.1). The results obtained in the HAPMO catalyzed oxidation of different p-substituted-phenyl methyl sulfides are summarized in Table 3.2. We examined the correlation between the conversion and the enantiomeric excess of the sulfoxide products and the parameter σ for the substituents, which represents a contribution of factors such as resonance and both field and inductive effects. For all the substrates tested, the (S)-enantiomer was mainly obtained. It was observed that on average, the sulfides with an electron-donating group (σ < 0; NH₂, OCH₃ and CH₃) were oxidized with higher conversions and enantioselectivities that those bearing an electron-withdrawing group (σ > 0; Cl, CN and NO₂). Additionally, HAPMO shows the highest conversion with 3.1, indicating that the enzyme prefers no substituent on the phenyl ring. The position of the chloride substituent in the phenyl ring had only a marginal effect on the conversion and enantioselectivity, ortho being the best position (Table 3.2).
HAPMO has also been studied in kinetic resolution processes with a number of racemic sulfoxides. The biocatalyst was able to oxidize (±)-phenyl methyl sulfoxide 3.1a, (±)-benzyl isopropyl sulfoxide 3.27a and (±)-methyl phenylethyl sulfoxide 3.33a to the corresponding sulfones (30 %, 23 % and 14 % conversion after 20 hours, respectively), but showed no enantioselectivity ($E \approx 1$ for the three compounds).[59] From this, it can be concluded that the enantiomeric excess of the sulfoxides obtained with HAPMO are only due to the asymmetric oxidations of the starting material, with no contribution from a kinetic resolution of the sulfoxides formed.

As previously found for cyclohexanone monooxygenase,[54,55] HAPMO was also able to catalyze the nucleophilic oxidation of the boron atom, specifically of phenylboronic acid 3.36. This substrate was converted into phenol 3.37 ($c = 24 \%$ after 48 hours), in a process analogous to a Baeyer-Villiger oxidation. This is the first example of boron oxidation for this biocatalyst.

**Scheme 3.2** Biooxidation of 3-phenyl-penta-2,4-dione 3.38

HAPMO was also tested as a biocatalyst in the oxidation of 3-phenyl-penta-2,4-dione 3.38. Conversion of this diketone can lead to the formation of an interesting pharmaceutical intermediate, $(R)$-phenylacetylcarbinol $(R)$-3.40 (Scheme 3.2), a well-known precursor in the synthesis of ephedrine and pseudoephedrine.[60] Currently, this compound is produced by an enzymatic process on industrial scale, using pyruvate and benzaldehyde as starting compounds.[61,62] As shown in Scheme 3.2, diketone 3.38 is indeed converted by HAPMO with almost complete selectivity (e.e. $> 99 \%$) into $(R)$-1-acetoxy-phenylacetone $(R)$-3.39. Hydrolysis of this ester would yield $(R)$-3.40. The production of $(R)$-3.39 by an enzymatic Baeyer-Villiger oxidation shows the potential of HAPMO to convert prochiral phenyldiketones.
3.4.2 Enantioselective oxidations by PAMO

A similar set of sulfides were analyzed as possible substrates for enantioselective oxidation by phenylacetone monoxygenase (Table 3.1). With alkyl sulfides 3.1-3.4, low to moderate enantiomeric excesses were obtained. Oxidation of thioanisole led to the formation of (R)-3.1a with almost complete conversion after 24 hours, but with moderate enantiomeric excess (e.e. = 44 %). Sulfides with a longer alkyl chain 3.2-3.3 yielded the (S)-sulfoxides instead of the (R) ones, with lower conversions and enantiomeric excess compared to substrate 3.1. Conversion of cyclopropyl phenyl sulfide 3.4 led to the preferential formation of the (R)-enantiomer, with an enantiomeric excess of 48 %, close to that obtained with a methyl group. No oxidation was observed when a carboxylic acid group was present in the sulfide structure 3.11. As described before,[31] the oxidation of 3.13 was not selective, leading to (R)-3.13a with low enantiomeric excess (e.e. = 10 %) and 68 % conversion after 24 hours. The enzyme also showed very low selectivity with the other p-tolyl sulfides tested (compounds 3.14 - 3.16; e.e. of the products ≤ 17 %). The introduction a methoxy group as a para-substituted (compound 3.17) slightly decreased the (R)-selectivity (e.e. = 25 %) compared to 44 % for thioanisole 3.1. Surprisingly, when a strong electron-withdrawing group was located at the para-position 3.18, the enantiomeric excess of the obtained sulfoxide (R)-3.18a was higher (e.e. = 76 %) than the rest of the phenyl sulfides previously examined. Despite its bulkiness, 2-naphthyl methyl sulfide 3.12 was also converted by PAMO to yield (S)-3.12a with a similar selectivity compared to 3.1.

The alkyl benzyl sulfides possessing a small alkyl chain appeared to be very good PAMO substrates in terms of selectivity. The oxidations carried out with compounds 3.24 and 3.25 led to the formation of the (S)-sulfoxides in high enantiomeric excesses (e.e. = 94 - 98 %) and good conversions (nearly 35 % after 6 hours). It is interesting to note that the structure of these sulfides is very similar to phenylacetone, the best PAMO substrate reported so far. When the size of the alkyl chain for this kind of sulfide was larger than the ethyl group (isopropyl, butyl and isopentyl), the conversion and the enantiomeric excess of the sulfoxides decreased, as shown in Table 3.1 (3.27 - 3.29). For chain lengths longer than ethyl, a change from (S)- to the (R)-configuration of the products was observed. Introduction of an ester group in the alkyl moiety of benzyl sulfides 3.30 and 3.31 gave sulfoxides
3.30a and 3.31a with moderate enantiomeric excesses. Conversions for these reactions were very similar to those obtained with methyl or ethyl groups.

Sulfides possessing the sulfur moiety further away from the aromatic ring were also converted with good selectivities. (R)-Methyl 2-phenylethyl sulfoxide (R)-3.33a was obtained with 80 % e.e. and 27 % conversion after 8 hours using 3.33 as substrate. Furthermore, similar behavior was observed for the ethyl derivative 3.34 as shown in Table 3.1 (e.e. = 83 %). When methyl 3-phenylpropyl sulfide 3.35 was used as PAMO substrate, moderate enantiomeric excess was achieved (e.e. = 70 %) with 21 % conversion after 8 hours. Besides the compounds listed in the Table 3.1, some disulfides with bulky chains such as p-tolyl disulfide or thiantrene were found not to be oxidized by PAMO, presumably because of steric hindrance. A nonaromatic disulfide such as 1,3-dithiane was also examined as enzyme substrate. However, after 48 hours only the racemic monosulfoxide was obtained with low conversion (c = 18 %).

Based on the obtained results, we can deduce in terms of PAMO enantioselectivity for different sulfur positions in aromatic methyl sulfides, that the benzyl structure 3.24 is preferred by PAMO. The 2-phenylethyl group 3.33 also led to good enantiomeric excesses. The enantiomeric excess of the sulfoxide obtained decreased when the sulfur atom was further away from the aromatic ring, as shown for compound 3.35. Finally, the presence of the sulfur atom next to the aromatic ring 3.1 seemed to have a marked negative effect on PAMO enantioselectivity. HAPMO displays opposite properties, as the phenyl structure is preferred by this enzyme in terms of enantioselectivity. These observations are in agreement with the physiological substrates of both enzymes: HAPMO is involved in the degradation of acetophenones, while PAMO is primarily active with phenylacetones.

Previous studies carried out with CHMO revealed that the oxidation of the sulfoxide products to the corresponding sulfones was very slow and could not be exploited for kinetic resolution purposes.\[18\] Instead, an increase in the enantiomeric excess of the sulfoxide products as a function of time was observed in some of the PAMO catalyzed oxidations. This indicated that the enzyme was not only able to catalyze the asymmetric oxidation from sulfide to sulfoxide, but also the kinetic resolution of the sulfoxide to the sulfone. Table 3.3 summarizes the
results obtained in the kinetic resolution of a set of sulfoxides. Oxidation of both
(±)-3.1a and (±)-3.18a occurred, but did not show any selectivity. More interesting
results were obtained with the benzyl sulfoxides (±)-3.24a and (±)-3.25a, where the
enantioselectivities \( E \)[59], especially in the case of the ethyl derivate \( E = 110 \),
were very high. This allowed the recovery of (S)-3.25a with high optical purity at
conversions near 50 % in short reaction times. Instead, when the isopropyl or
methylcarboxymethyl benzyl derivates (3.27a and 3.30a) were used, the reactions
were slower and the selectivities were very low. Finally, the (S)-enantiomer of
sulfoxide (±)-3.33a was selectively oxidized \( E = 57 \) to sulfone, leaving the (R)-
enantiomer behind (e.e. = 95 %). The high enantiomeric excess of compounds (S)-
3.24a, (S)-3.25a and (R)-3.33a (Table 3.1) were the result of a combination of
asymmetric oxidation of the starting sulfides and, in part, of the resolution process
of the sulfoxides formed. The kinetic resolution of racemic sulfoxides by bacterial
dimethyl sulfoxide reductases has been recently described and moderate to good
enantioselectivities were found depending on the sulfoxide.[63] The resolution for
these biocatalysts was based on the selective reduction of the starting sulfoxide to
sulfides, not through their oxidation, as we described in this chapter by using
PAMO.

PAMO was also tested in the enzymatic oxidation of organic compounds
possessing heteroatoms different from sulfur. Tertiary amine N-oxides play an
important role in chiral catalysis and in biological processes.[64,65] These
compounds are prepared by oxidation of the corresponding amines and have been
synthesized by biocatalytic methods using CHMO.[66] Herein, \( N,N \)-
dimethylbenzylamine 3.41 was oxidized by PAMO to the corresponding N-oxide
3.41a with a 73 % conversion after 48 hours. When a bulky tertiary amine such as
(S)-(−)-nicotine (S)-3.42 was subjected to PAMO oxidation, the unaltered starting
material was fully recovered after 2 days incubation. As described for HAPMO, we
also tested phenylboronic acid 3.36 as PAMO substrate, which led to the formation
of phenol 3.37 (\( e = 11 \) % after 24 hours). The same product can be formed by
chemical oxidation.[67] This adds another type of oxidative reactivity to the broad
catalytic repertoire of PAMO.

Finally, the ability of PAMO to convert the aromatic prochiral diketone, 3-phenyl-
penta-2,4-dione 3.38, was investigated. Oxidation of this compound resulted in the
**Table 3.3 Kinetic resolution of racemic sulfoxides catalyzed by PAMO at 25 °C.**[^a]

<table>
<thead>
<tr>
<th>Compound structure</th>
<th>time (h)</th>
<th>conv. (%)[^b]</th>
<th>e.e. (%)[^b]</th>
<th>config.</th>
<th>E[^c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(±)-3.1a</td>
<td>24</td>
<td>52</td>
<td>≤ 3</td>
<td>~ 1</td>
<td>-</td>
</tr>
<tr>
<td>(±)-3.18a</td>
<td>24</td>
<td>21</td>
<td>≤ 3</td>
<td>~ 1</td>
<td>-</td>
</tr>
<tr>
<td>(±)-3.24a</td>
<td>4</td>
<td>39</td>
<td>60</td>
<td>53</td>
<td>S</td>
</tr>
<tr>
<td>(±)-3.25a</td>
<td>4</td>
<td>49</td>
<td>93</td>
<td>110</td>
<td>S</td>
</tr>
<tr>
<td>(±)-3.27a</td>
<td>6</td>
<td>25</td>
<td>30</td>
<td>17</td>
<td>R</td>
</tr>
<tr>
<td>(±)-3.30a</td>
<td>4</td>
<td>30</td>
<td>24</td>
<td>4.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>(±)-3.33a</td>
<td>8</td>
<td>51</td>
<td>95</td>
<td>57</td>
<td>R</td>
</tr>
</tbody>
</table>

[^a]: For reaction details see the Experimental section.
[^b]: Conversion and optical purity determined by HPLC.
[^c]: Enantiomeric ratio, $E = \ln[(1-c)(1+e.e.)/\ln(1-c)(1+e.e.)]$.[^59]

n.d.: not determined.

formation of (R)-acetoxy-phenylacetone (R)-3.39 with a good enantioselectivity (e.e. = 82 %) in a relatively fast process (c = 88 % after 90 minutes). However, the oxidation of this prochiral diketone by PAMO was not as enantioselective as the oxidation performed by HAPMO (see previous paragraph). Still, it shows the potential of PAMO to convert prochiral phenyl diketones in an enantioselective way.
3.4.3 Determination of the kinetic parameter of PAMO

To obtain a better understanding on the catalytic efficiency of PAMO, the steady-state kinetic parameters for various ketones (3.38, 3.43-3.50), sulfides (3.1, 3.24, 3.25, 3.33) and their corresponding sulfoxides (3.1a, 3.24a, 3.25a, 3.33a) were determined using isolated enzyme (Table 3.4). The maximal catalytic rate ($k_{cat}$) was found to be remarkably similar for all substrates (1.2-3.6 s$^{-1}$). Only for some substrates, the exact value for $k_{cat}$ could not be determined due to solubility problems of those substrates. More variation was found for the $K_M$ values suggesting differences in substrate affinity, while the rate of catalysis is probably restricted by a common substrate-independent kinetic step. The oxidation of the sulfur atom at the $\alpha$-position from the phenyl ring 3.1 occurred with a low catalytic efficiency ($k_{cat}/K_M$) due to a high $K_M$. A shift of the sulfur atom to the $\beta$- or $\gamma$-position (sulfides 3.24, 3.25, 3.33) resulted in a 30- to 50-fold increase in catalytic efficiency. This increase coincided with an increased enantioselectivity. The racemic sulfoxides turned out to be rather poor substrates due to relatively high $K_M$ values. It is interesting to note that sulfoxide 3.25a not only displayed a relatively high catalytic efficiency but also showed the highest enantioselectivity. Surprisingly, the sulfoxides showed a higher conversion in shorter times than the corresponding sulfides, while the catalytic efficiency of the sulfoxides was significant lower. A reason for this observation might be that the produced sulfoxides inhibited the conversion of the sulfides to a greater extent than the sulfones inhibit the oxidation of the sulfoxides. The latter statement is however not true for thioanisole 3.1, which showed a higher conversion than 3.1a with a lower catalytic efficiency.

Comparison of the steady-state kinetic parameters of ketones 3.43 and 3.45 indicated that the presence of an electron-withdrawing fluorine group at the para-position of phenylacetone 3.45 enhanced the catalytic efficiency of the enzyme by increasing the rate of catalysis. When fluorine atoms were positioned close to the carbonylic function (3.46), a 50-fold lower catalytic efficiency was observed when compared with phenylacetone, resulting from an increase in $K_M$. As mentioned above, PAMO also converted the prochiral diketone 3.38. Steady-state analysis showed that this substrate was oxidized with a reasonable catalytic rate while displaying a relatively large $K_M$, suggesting a low affinity. The apparent affinity
Table 3.4 Kinetic parameters of PAMO for sulfides, sulfoxides and ketones.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td></td>
<td>≥ 2.5 $^a$</td>
<td>≥ 0.12</td>
<td>47</td>
</tr>
<tr>
<td>3.24</td>
<td></td>
<td>1.6</td>
<td>1.8</td>
<td>1,100</td>
</tr>
<tr>
<td>3.25</td>
<td></td>
<td>1.3</td>
<td>2.3</td>
<td>1,800</td>
</tr>
<tr>
<td>3.33</td>
<td></td>
<td>0.75</td>
<td>1.5</td>
<td>2,000</td>
</tr>
<tr>
<td>(±)-3.1a</td>
<td></td>
<td>19.5</td>
<td>1.9</td>
<td>96</td>
</tr>
<tr>
<td>(±)-3.24a</td>
<td></td>
<td>23.7</td>
<td>1.2</td>
<td>51</td>
</tr>
<tr>
<td>(±)-3.25a</td>
<td></td>
<td>8.7</td>
<td>2.7</td>
<td>310</td>
</tr>
<tr>
<td>(±)-3.33a</td>
<td></td>
<td>19</td>
<td>1.3</td>
<td>68</td>
</tr>
<tr>
<td>3.38</td>
<td></td>
<td>6.9</td>
<td>1.4</td>
<td>200</td>
</tr>
<tr>
<td>3.43 $^b$</td>
<td></td>
<td>0.059</td>
<td>1.9</td>
<td>32,000</td>
</tr>
<tr>
<td>3.44 $^b$</td>
<td></td>
<td>0.36</td>
<td>1.8</td>
<td>5,000</td>
</tr>
<tr>
<td>3.45</td>
<td></td>
<td>0.056</td>
<td>3.6</td>
<td>65,000</td>
</tr>
<tr>
<td>3.46</td>
<td></td>
<td>4.0</td>
<td>2.3</td>
<td>580</td>
</tr>
<tr>
<td>3.47</td>
<td></td>
<td>≥ 2.5 $^a$</td>
<td>≥ 0.03</td>
<td>10</td>
</tr>
<tr>
<td>3.48</td>
<td></td>
<td>≥ 5.0 $^a$</td>
<td>≥ 0.06</td>
<td>10</td>
</tr>
<tr>
<td>3.49</td>
<td></td>
<td>≥ 10.0 $^a$</td>
<td>≥ 0.08</td>
<td>8</td>
</tr>
<tr>
<td>3.50</td>
<td></td>
<td>8.9</td>
<td>3.6</td>
<td>410</td>
</tr>
</tbody>
</table>

$^a$ Due to limited solubilities of the compounds, the substrate concentration could not be increased upon the indicated values.

$^b$ Values taken from Fraaije et al.$^{[31]}$
Chapter 3

decreased even further when larger and bulkier groups (3.47 - 3.49) were introduced, resulting in a very poor catalytic efficiency. Other substrates such as 2-phenylcycloheptanone and 2-indanone also showed a low catalytic efficiency (28 and 18 M$^{-1}$s$^{-1}$, respectively) indicating that PAMO has difficulties accepting bulky aromatic ketones. Besides phenylacetone derivatives, $p$-hydroxybenzylacetone 3.50 was also included in this study in order to compare with benzylacetone 3.44. The electron donating hydroxyl group at the para-position resulted in a ~ 10-fold lower catalytic efficiency. When also taking into account the above mentioned effect of a fluorine substituent, this indicates that the enzyme prefers electron-withdrawing para-substituents. However, the effect of the hydroxyl group could also reflect steric hindrance. Finally, the steady-state kinetic parameters of $N,N$-benzylidimethylamine 3.41 were determined. However, by monitoring the consumption of NADPH in time, a catalytic rate of only 0.02 s$^{-1}$ was measured.

3.5 Conclusions

4-Hydroxyacetophenone monooxygenase is able to catalyze the sulfoxidation reaction of a large number of aromatic sulfides. In general, phenyl sulfides seem to be the best substrates for the enzyme, yielding (S)-sulfoxides with high enantioselectivities. Low enantiomeric excesses were obtained with benzyl sulfides, and inversion of enantipreference from (S) to (R) was observed for alkyl chains longer than ethyl. Reversal in enantiopreference, with moderate selectivities, was also found when the sulfur atom was located further away from the aromatic ring. With para-substituted phenyl methyl sulfides, the enzyme showed high selectivity for electron-donating groups, while strong withdrawing ones had a negative effect on selectivity and efficiency. The biocatalyst was not significantly affected in terms of selectivity by changing the location of the aromatic substituents. It was also found that HAPMO can oxidize aromatic sulfoxides but with no enantioselectivity. The enzyme is also able to convert 3-phenyl-penta-2,4-dione with high enantiopreference and to catalyze the boron atom oxidation. This study and previous reports have shown that HAPMO can accept a number of substituents on the phenyl moiety as substrate. This indicates that this monooxygenase can be applied for a wide variety of selective oxidation reactions
resulting in formation of, for example, optically active sulfoxides or aromatic esters.

Additionally, we described in this chapter the substrate acceptance and enantioselectivity of phenylacetone monooxygenase. This revealed that the enzyme is able to oxidize a wide range of sulfides and sulfoxides with varying degrees of selectivity, depending on the substrate structure. Interestingly, the enzyme complements HAPMO in terms of enantioselective oxidation of the sulfides tested; PAMO shows good enantioselectivity towards the benzyl sulfides, whereas HAPMO shows to be an excellent biocatalyst for the enantioselective oxidation of phenyl sulfides. In terms of enantioselectivity the enzymes seem to prefer substrates that resemble the physiological substrate of the corresponding enzyme, i.e. 4-hydroxyacetophenone for HAPMO and phenylacetone for PAMO. However, an exception has to be made for diketone 3.38, which is oxidized more enantioselectively by HAPMO. The broad substrate specificity and reactivity makes PAMO a valuable tool for performing selective oxidation of either the sulfur atom at the β- or γ-position from the phenyl ring, whereas oxidation at the α-position was either rather poor or did not occur at all.\[31\] The low apparent affinity of PAMO towards the tested sulfoxides in comparison with their corresponding sulfides indicates that an oxygen atom adjacent to the sulfur is poorly accepted by the enzyme. Apart from this, the introduction of bulky groups in the phenylacetone derivatives 3.47-3.49 resulted in a large decrease of catalytic efficiency, indicating that PAMO has difficulties accepting bulky aromatic ketones. It was also established that PAMO prefers electron withdrawing para-substituents in the aromatic moiety in order to obtain high catalytic efficiencies. As described in the next chapter and by Bocola and coworkers, the substrate acceptance of this enzyme can be increased even further by the creation of various enzyme mutants.\[32,68\]

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3.7 References

BVMOs as Biocatalysts in the Synthesis of Optically Pure Sulfoxides
