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4-Hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* ACB as an oxidative biocatalyst in the synthesis of optically active sulfoxides

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Abstract—Recombinant 4-hydroxyacetophenone monooxygenase (HAPMO) from *Pseudomonas fluorescens* ACB has been tested as a catalyst in sulfoxidation reactions on a set of aromatic sulfides. With a few exceptions, excellent enantioselectivities in the synthesis of chiral phenyl and benzyl sulfoxides were achieved. The bacterial Baeyer–Villiger monooxygenase was also shown to accept racemic sulfoxides, a prochiral diketone and an organoboron compound as substrates. This study demonstrates the great biocatalytic potential of this novel oxidative enzyme.

1. Introduction

Optically active sulfoxides have become a well-established interest in organic synthesis. The high configurational stability of the sulfinyl group as well as their synthetic versatility have 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.[1]

Many organic sulfoxides also possess high 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 psychotonics and vasodilators.[1,2]

The oxidation of sulfides is the most straightforward method for the synthesis of sulfoxides and a great number of reagents are available for this reaction.[1–3] Also the enzymes are able to catalyze the enantioselective oxidation of sulfides, in processes carried out in water under mild reaction conditions. This makes a biocatalytic approach environmentally friendly and most appealing for industrial applications. There are two main types of enzymes used for sulfoxidations reactions: peroxidases[4] and Baeyer–Villiger monooxygenases (BVMOs).[4c,5]

BVMOs are NAD(P)H dependent flavoenzymes, which are able to catalyze both the nucleophilic oxygenation of ketones and boron as well as the electrophilic oxygenation of various heteroatoms. They often display high regio- and/or enantioselectivities.[6]

4-Hydroxyacetophenone monooxygenase (HAPMO), a novel BVMO from *Pseudomonas fluorescens* ACB that catalyzes the NADPH-dependent oxidation of 4-hydroxyacetophenone to 4-hydroxyphenylacetate, has recently been purified, characterized and overexpressed.[7] This enzyme is a homodimer of 145 kDa with each subunit containing a tightly non-covalently bound FAD cofactor. It represents the first BVMO being primarily active on aromatic compounds. Previous studies on substrate specificity have demonstrated that HAPMO prefers acetophenones and benzaldehydes bearing an electron-donating substituent at the *para*-position.[8] The enzyme is also able to catalyze the Baeyer–Villiger

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oxidation of a wide variety of other ketones and aldehydes, including heteroaromatic and aliphatic compounds.[9] A recent study has revealed that HAPMO can be used as a biocatalyst in aqueous or in aqueous-organic media.[10]

The main aim of the present paper was to improve our knowledge on the catalytic properties of HAPMO by exploring the biocatalytic asymmetric sulfoxidation of aromatic sulfides. Other organic systems have been also studied in HAPMO catalyzed oxidations.

2. Results and discussion

The oxidation of aromatic sulfides by recombinant 4-hydroxyacetophenone monooxygenase (Tables 1 and 2), was coupled to a second enzymatic reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH), in order to regenerate the NADPH consumed.[6c,d] All oxidations were carried out in a Tris/HCl buffer at pH 9.0.

First, a set of phenyl sulfides **1**–**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 (ee > 95%) were achieved for the compounds tested, indicating an enzyme preference for this structure in terms of enantioselectivity. In all cases, (*S*)-sulfoxides were formed.

As previously described,[8] thioanisole oxidation was almost complete after 24 h, resulting in the formation of enantiopure (*S*)-methyl phenyl sulfoxide **1a**. Similar enantioselectivities were measured for sulfides with alkyl chains no longer than the cyclopropyl group **2**–**4**. Butyl phenyl sulfide **5** led to the formation of (*S*)-**5a** with only moderate enantiomeric excess (ee = 71%). Conversions were slightly decreased by increasing the size of the alkyl moiety of the sulfides.

Table 2. HAPMO catalyzed oxidation of thioanisole derivatives X–Ph–S–CH_3 to the corresponding (*S*)-sulfoxides[a]

Compound	X	σ[b]	Conv. (%)[c]	ee (%)[c]
1	H	0	96	99
12	*p*-NH_2	−0.30	41	96
13	*p*-OCH_3	−0.27	78	99
14	*p*-CH_3	−0.17	77	99
15	*p*-CN	0.23	64	96
16	*p*-NO_2	0.66	32	87
17	*p*-Cl	0.78	37	44
18	*o*-Cl	—	76	96
19	*m*-Cl	0.37	42	93

[a] Reaction time 24 h. For other details, see Section 4.
[b] Values taken from Ref. 11.
[c] Conversion and enantiomeric excess determined by HPLC.

It was found that HAPMO was able to catalyze the enzymatic sulfoxidation of phenyl alkenyl sulfides into the corresponding (*S*)-sulfoxides with excellent enantioselectivities. Both vinyl and allyl sulfides **6** and **7** were oxidized into (*S*)-**6a** and (*S*)-**7a** with ee = 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 **1**. (*R*)-Methylchloride phenyl sulfoxide (*R*)-**8a** was obtained with moderate enantiomeric excess (ee = 76%) and conversion (56%). When the chloride atom was placed further away from the sulfur atom (sulfide **9**), both the conversion and the enantioselectivity measured for (*R*)-**9a** were slightly improved.

In contrast, the oxidation of a sulfide containing an electron-donating atom in the alkyl chain (**10**), led to the formation of the almost enantiomerically pure sulfoxide (*R*)-**10a**.

Table 1. Oxidation of aromatic sulfides catalyzed by 4-hydroxyacetophenone monooxygenase[a]

Compound	Structure	Time (h)	Conv. (%)[b]	ee (%)[b]	Configuration
1	C_6H_5–S–CH_3	24	96	99	*S*
2	C_6H_5–S–CH_2CH_3	24	86	99	*S*
3	C_6H_5–S-propyl	24	85	97	*S*
4	C_6H_5–S-cyclopropyl	24	74	97	*S*
5	C_6H_5–S-butyl	24	61	71	*S*
6	C_6H_5–S–CH=CH_2	24	70	98	*S*
7	C_6H_5–S–CH_2CH=CH_2	24	69	98	*S*
8	C_6H_5–S–CH_2Cl	24	56	76	*R*[c]
9	C_6H_5–S–CH_2CH_2Cl	24	69	81	*R*[c]
10	C_6H_5–S–CH_2OCH_3	24	63	98	*R*[c]
11	2-Naphthyl-S–CH_3	24	31	95	*S*
20	C_6H_5–CH_2–S–CH_3	20	55	85	*S*
21	C_6H_5–CH_2–S–CH_2–CH_3	20	52	81	*S*
22	C_6H_5–CH_2–S-propyl	20	59	65	*R*
23	C_6H_5–CH_2–S-isopropyl	20	44	82	*R*
24	C_6H_5–CH_2–S-butyl	20	57	77	*R*
25	C_6H_5–$(CH_2)_2$–S–CH_3	24	44	51	*R*
26	C_6H_5–$(CH_2)_3$–S–CH_3	24	29	57	*R*

[a] For reaction details, see Section 4.
[b] Conversion and enantiomeric excess determined by HPLC.
[c] Absolute configuration is reversed due to a change in the substituent priority according to the sequence rules.

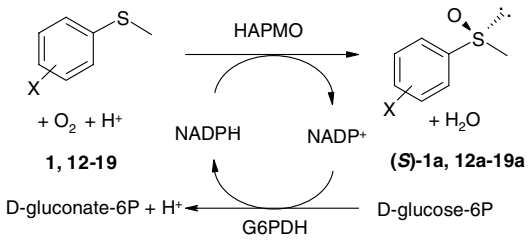
The bicyclic aromatic compound 2-naphthyl methyl sulfide **11** was oxidized to (*S*)-**11a** with high enantiomeric excess but low conversion, mainly because of the low solubility of the sulfide in the aqueous medium. In contrast to phenylacetone monooxygenase (PAMO),[5c] the presence of a bulky aromatic system did not affect the enantioselectivity of the biocatalyst to a great extent.

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 **20** and **21**. However, when the alkyl chain was relatively bulky, the corresponding (*R*)-sulfoxides **22**–**24** were formed. The enantiomeric excesses of the benzyl alkyl sulfoxides obtained were in all cases close to 80%, with exception of **22a** (ee = 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.

By 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 **25** and **26**, the corresponding (*R*)-sulfoxides were obtained with moderate enantiomeric excesses (ee around 50%) and conversions, especially in the case of compound **26** ($c = 29\%$).

The results obtained indicate that in sulfide oxidation reactions, HAPMO displays opposite properties with respect to the recently studied PAMO, where the benzyl structure was preferred by the enzyme in terms of enantioselectivity.[5c] This is in agreement with the physiological substrates for both enzymes: HAPMO is involved in the degradation of acetophenones, while PAMO is primarily active with phenylacetones.

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.[8] 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 **1**, which is the best sulfide substrate found for HAPMO so far (Scheme 1). The results obtained in the HAPMO catalyzed oxidation of different *p*-substituted-phenyl methyl sulfides are summarized in Table 2. We have looked for a correlation between the conversion and the enantiomeric excess of the sulfoxide products and the parameter σ for the substituents (described by the Hammet relationship), 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 ($\sigma < 0$; NH_2, OCH_3 and CH_3) were oxidized with higher conversions and enantioselectivities than those bearing an electron-withdrawing group ($\sigma > 0$; Cl, CN and NO_2).



Scheme 1. HAPMO catalyzed oxidation of thioanisole derivatives **12**–**19**.

The effect on conversion and enantioselectivity of the position of the chloride substituent in the phenyl ring was only marginal, as shown in Table 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 **1a**, (±)-benzyl isopropyl sulfoxide **23a** and (±)-methyl phenyethyl sulfoxide **25a** to the corresponding sulfones (30%, 23% and 14% conversion after 20 h, respectively), but showed no enantioselectivity ($E^{12} \approx 1$ for the three compounds). From this, it can be concluded that, differently from PAMO,[5c] the enantiomeric excesses of the sulfoxides obtained with HAPMO are only due to the asymmetric oxidation of the starting material, with no contribution from a kinetic resolution of the sulfoxides formed.

As previously found for cyclohexanone monooxygenase[13] and phenylacetone monooxygenases,[5c] HAPMO was also able to catalyze the nucleophilic oxidation of the boron atom, specifically of phenylboronic acid **27**. This substrate was converted into phenol **28** ($c = 24\%$ after 48 h), in a process analogous to a Baeyer–Villiger oxidation. This is the first example of boron oxidation reported for this biocatalyst.

The ability of HAPMO to convert the aromatic prochiral diketone, 3-phenyl-penta-2,4-dione **29** was also investigated. Oxidation of this compound in a Tris/HCl buffer pH 7.5 resulted in the formation of (*R*)-1-acetoxy-phenylacetone **30** with almost complete selectivity (ee > 99%). Hydrolysis of (*R*)-**30** led to enantiopure (*R*)-1-hydroxy-1-phenylacetone, a well-known precursor in the synthesis of ephedrine and pseudoephedrine.[14]

3. Conclusion

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 the (*S*)-sulfoxides with high enantioselectivities. Low enantio-

meric excesses were obtained with benzyl sulfides, and inversion of enantiopreference 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 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. Finally, the enzyme is also able to convert 3-phenylpenta-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 of the 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.

4. Experimental

4.1. General

Recombinant HAPMO was overexpressed and purified according to previously described methods.[7a] Oxidation reactions were performed using the purified enzyme. One unit of HAPMO will oxidize 1.0 μmol of thioanisole to methyl phenyl sulfoxide per minute at pH 9 and 25 °C in the presence of NADPH. Glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was obtained from Fluka–BioChemika. Glucose-6-phosphate and NADP(H) were purchased from Sigma–Aldrich–Fluka.

Sulfides **1**, **2**, **4**, **7**, **8**, **10**, **14**, **15, 17**, **18**, **19**, racemic sulfoxides (±)-**1a**, (±)-**7a**, (±)-**14a**, phenylboronic acid **27** and phenol **28** were purchased from Sigma–Aldrich–Fluka. Phenyl sulfides **11**, **16** and benzyl sulfides **20**–**21** were products from Lancaster. Compounds **6**, **9**, **12** and **13** were from Acros-Organics. Diketone **29** was purchased by TCI Europe. Sulfides **3**,[15] **5**,[15] **20**–**24**,[16] **25**–**26**[15] and compound (±)-**30**[17] 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.[5c,15,16,18] 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_{254} plates and visualized by UV irradiation. Flash chromatography was carried out with silica gel 60 (70–230 mesh, Merck). ^{1}H and ^{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 the internal standard to determine the conversion of the oxidation processes. 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 (Alltech, 30 m × 0.25 mm × 0.125 mm) or a HP1 column (Agilent, 30 m × 0.25 mm × 0.25 mm), respectively.

Unless otherwise stated, the absolute configurations of the chiral sulfoxides were established by comparison of the HPLC chromatograms with the patterns described in previous experiments for the known configurations. For sulfoxides **5a**,[13] **7a**,[18a] **10a**[18g] and **12a**,[18e] the absolute configuration was established by comparison of the specific rotation measured with the ones reported. Configuration of the sulfoxide (*R*)-**8a** was established by comparison with a sample prepared from the chemical chlorination of (*S*)-methyl phenyl sulfoxide with *N*-chlorosuccinimide.[19] For sulfoxide (*R*)-**9a**, the configuration was assigned by comparison with a sample prepared from treatment of (*S*)-phenyl vinyl sulfoxide with trimethylsilyl chloride.[20] Configuration of (*R*)-**30** was established by comparison with an authentic sample prepared from chemical acetylation of (*R*)-1-hydroxy-1-phenylacetone.

4.2. Typical procedure for the enzymatic oxidation of substrates

Substrates (15–20 mM, except for **29**, 2.5 mM) were dissolved in a buffer Tris/HCl (50 mM, pH 9.0, 1.0 mL, except for substrate **29**, 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 4-hydroxyacetophenone monooxygenase. 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 Na_2SO_4 and analyzed by chiral HPLC to determine the conversion and enantiomeric excesses of the sulfoxides obtained. The conversion and enantiomeric excess of (*R*)-**30** were established by means of GC. Control experiments in absence of enzyme were performed for all substrates tested, and no reaction was observed.

4.3. General procedure for the enzymatic oxidation at multimilligram scale of sulfides 5, 7, 10 and 12

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 **5**

and **10**) or 40 h (sulfides **7** and **12**). The reactions were then extracted with dichloromethane (4×15 mL) and the organic layers dried over Na_2SO_4, 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 **12**, petroleum ether/ethyl acetate 8:2) to afford the chiral sulfoxides: (*S*)-**5a** (colourless oil, 26.7 mg, 49% yield), (*S*)-**7a** (colourless oil, 21.6 mg, 39% yield), (*R*)-**10a** (colourless oil, 20.8 mg, 38% yield) and (*S*)-**12a** (yellow pale oil, 17.6 mg, 31%).

4.3.1. (*S*)-*n*-Butyl phenyl sulfoxide, (*S*)-5a. Determination of ee by HPLC analysis: Chiralcel OB, petroleum ether-*i*-propanol (88:12), 1.0 mL/min, 254 nm, t_R 11.9 (*R*) and 19.7 (*S*) min. $[\alpha]_D^{25} = -131.5$ (*c* 0.98, acetone) ee 71%.

4.3.2. (*S*)-Allyl phenyl sulfoxide, (*S*)-7a. Determination of ee by HPLC analysis: Chiralcel OB, petroleum ether/*i*-propanol (85:15), 1.0 mL/min, 254 nm. t_R 13.6 (*S*) and 17.9 (*R*) min. $[\alpha]_D^{25} = -164.8$ (*c* 1.08, EtOH) ee 98%.

4.3.3. (*R*)-Chloromethyl phenyl sulfoxide, (*R*)-8a. Determination of ee by HPLC analysis: Chiralcel OD petroleum ether/*i*-propanol (95:5), 1.0 mL/min, 254 nm. t_R 14.8 (*S*) and 17.6 (*R*) min. $[\alpha]_D^{25} = -83.3$ (*c* 0.73, acetone) ee 89%. Enantiomeric excess obtained in the enzymatic reaction 76%.

4.3.4. (*R*)-Chloroethyl phenyl sulfoxide, (*R*)-9a. Determination of ee by HPLC analysis: Chiralcel OD petroleum ether/*i*-propanol (97:3), 1.0 mL/min, 254 nm. t_R 27.9 (*S*) and 30.8 (*R*) min. $[\alpha]_D^{25} = -101.8$ (*c* 1.39, acetone) ee 98%. Enantiomeric excess measured in the enzymatic oxidation 81%.

4.3.5. (*R*)-Methoxymethyl phenyl sulfoxide, (*R*)-10a. Determination of ee by HPLC analysis: Chiralcel OD petroleum ether/*i*-propanol (9:1), 1.0 mL/min, 254 nm. t_R 11.8 (*S*) and 14.6 (*R*) min. $[\alpha]_D^{25} = -207.1$ (*c* 0.84, $CHCl_3$) ee 97%.

4.3.6. (*S*)-4-Aminophenyl methyl sulfoxide, (*S*)-12a. Determination of ee by HPLC analysis: Chiralcel OD, petroleum ether/*i*-propanol (75:25), 1.0 mL/min, 254 nm. t_R 20.3 (*R*) and 28.8 (*S*) min. $[\alpha]_D^{25} = -85.1$ (*c* 0.82, EtOH) ee 95%.

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