Enzymatic Baeyer–Villiger Oxidation of Benzaldehydes

Mariëlle J. H. Moonen,[a] Adrie H. Westphal,[a] Ivonne M. C. M. Rietjens,[b]
Willem J. H. van Berkel[a,]*

[a] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
Phone: (+31)-317-482861, fax: (+31)-317-484801, e-mail: willem.vanberkel@wur.nl
[b] Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, The Netherlands

Received: October 8, 2004; Revised: March 18, 2005; Accepted: March 19, 2005

Abstract: The selectivity of the chemical Baeyer–Villiger oxidation of benzaldehydes depends on steric and electronic factors, the type of oxidizing agent and the reaction conditions. Here we report on the enzymatic Baeyer–Villiger oxidation of fluorobenzaldehydes as catalyzed by the flavoprotein 4-hydroxyacetophenone monooxygenase (HAPMO). HAPMO was most active with 4-amino- and 4-hydroxybenzaldehydes. With these compounds significant substrate inhibition occurred. Monofluoro- and difluorobenzaldehydes were readily oxidized by HAPMO without substrate inhibition. ^{19}F NMR analysis revealed that 4-fluoro-, 2,6-difluoro-, 3,4-difluoro-, 2-fluoro-4-hydroxy- and 3-fluoro-4-hydroxybenzaldehyde were quantitatively converted by HAPMO to the corresponding fluorophenyl formates. These products spontaneously hydrolyzed to fluorophenols. The HAPMO-mediated conversion of 2-fluoro-, 3-fluoro-, 2,3-difluoro- and 2,4-difluorobenzaldehyde yielded, besides fluorophenols, also minor amounts of fluorobenzoic acids. The high preference of HAPMO for the production of fluorophenols is in disagreement with the rule derived from chemical studies that electron-poor benzaldehydes form mainly benzoic acids. This suggests that interactions of the benzaldehyde substrates with amino acids and/or the flavin cofactor in the enzyme active site influence the selection of the migratory group in favor of the phenyl ring.

Keywords: Baeyer–Villiger oxidation; benzaldehyde; enzymatic synthesis; flavoprotein; ^{19}F NMR; 4-hydroxyacetophenone monooxygenase; substrate inhibition

Introduction

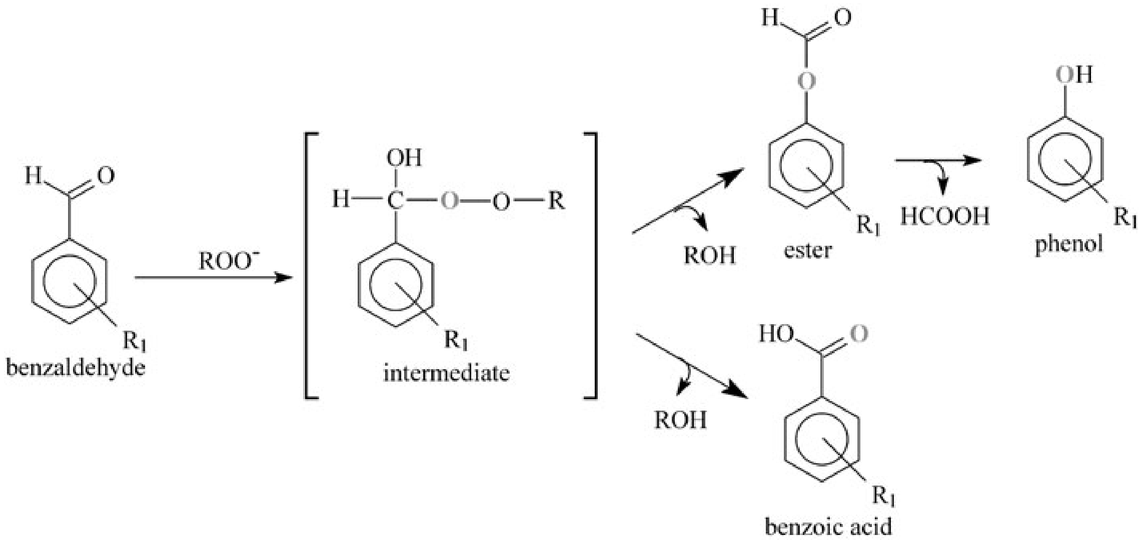
Fluorinated chemicals are gaining in industrial importance, with applications in pharmaceuticals, agrochemicals and material products.[1] Among these fluorinated chemicals, fluorophenols have attracted much attention due to their ability to modify the biological activity of molecules. Fluorophenols can serve as synthons for the production of drugs, including enzyme inhibitors[2,3] and receptor antagonists.[4] Catecholamines and amino acids with a radiolabelled [^{18}F]-fluorophenol moiety have found applications for the *in vivo* imaging of amino acid metabolism[5] and protein synthesis[6] using positron emission tomography (PET).[7]

Chemical methods to produce fluorophenols include diazotization/Sandmeyer decomposition with fluoroanilines, diazotization/thermal decomposition with aminophenols, alkaline hydrolysis with substituted fluorobenzene and F_2/N_2 gas/liquid phase conversion with phenol. All these methods display both economic or technological benefits and disadvantages, and none of them seems to be entirely satisfactory for the industrial production of fluorophenols.[8]

Newer methods for the chemical synthesis of fluorophenols make use of the Baeyer–Villiger oxidation of aromatic ketones and benzaldehydes.[8–11] In these reactions a nucleophilic peroxy moiety is incorporated into the starting compound yielding a tetrahedral "Criegee" intermediate.[12,13] This unstable species undergoes a rearrangement involving migration of one of the substituents of the carbonyl carbon and subsequent heterolytic cleavage of the peroxidic bond, yielding either the ester or benzoic acid (Scheme 1). By using an aqueous base, the ester is hydrolyzed to give the desired phenol.

In general, the migration preference of the Baeyer–Villiger rearrangement depends on the steric and electronic properties of the groups attached to the carbonyl carbon atom. The migration ability is also influenced by the type of oxidizing agent and the reaction conditions.[8,10,11,13–16] Since all groups attached to the carbonyl carbon of acetophenones migrate in preference to the methyl group, substituted acetophenones show a strong preference for ester formation.[17,18] For other aromatic alkyl ketones, migration of the alkyl group depends on its structure, while the migration of the aryl group depends on the electron-donating or electron-withdrawing properties of the substituents.[8,13] For benzophenones it

DOI: 10.1002/adsc.200404307



Scheme 1. Baeyer–Villiger oxidation of benzaldehydes.

was established that the product selectivity is strongly dependent on the type and position of the substituents in the phenyl rings.[8,10,19] Benzaldehydes with electron-donating substituent(s) in *ortho* and *para* positions stimulated migration of the phenyl ring and formation of phenyl formates (upper pathway in Scheme 1). On the other hand, benzaldehydes lacking electron-donating substituents and/or possessing electron-withdrawing groups yielded benzoic acids (lower pathway in Scheme 1).[9,10]

Ekaeva et al. reported the Baeyer–Villiger oxidation of [^{18}F]-fluorobenzaldehydes.[9] Using *m*-chloroperbenzoic acid in the presence of trifluoroacetic acid, it was shown that, in contrast to the above rules, fluorobenzaldehydes can be converted to fluorophenols to a significant extent.

The chemical Baeyer–Villiger reaction requires the use of potentially hazardous oxidants. Therefore, efforts have been made to replace the traditionally used peracids with safer oxidants. Most of these strategies rely on the use of transition metal catalysts or organocatalytic compounds in combination with hydrogen peroxide, peroxy acids or molecular oxygen.[11,16,17,20–27] However, the most challenging "green chemistry" approach for the selective Baeyer–Villiger oxidation of ketones and aldehydes is offered by enzyme-mediated reactions.[28–30]

Fluorobenzaldehydes are the most attractive starting compounds for the synthesis of fluorophenols because they are available *via* sustainable pathways. Several white-rot fungi have a great potential for the biocatalytic production of haloaromatic aldehydes.[31] Bioconversion experiments with whole cells of *Bjerkandera adusta* showed that 2-fluoro-, 3-fluoro- and 4-fluorobenzoic acid and 2-fluoro- and 4-fluorocinnamic acid were reduced to the corresponding fluorobenzaldehydes.[32] Fluorobenzaldehydes can also be obtained from the corresponding alcohols through the biooxidative activity of alcohol dehydrogenases[33] and aryl-alcohol oxidases.[34–36]

Recently, we described the purification, characterization and heterologous expression of 4-hydroxyacetophenone monooxygenase (HAPMO) from *Pseudomonas fluorescens* ACB.[37,38] This homodimeric flavoenzyme is a type I Baeyer–Villiger monooxygenase[30] that is active with a wide range of aromatic ketones.[38,39] HAPMO also catalyzes the conversion of aliphatic ketones to lactones[28,29,39] and the oxidation of aryl sulfides to chiral sulfoxides.[39] Being highly active with aromatic compounds, HAPMO can be discriminated from the extensively studied cyclohexanone monooxygenase from *Acinetobacter* sp. NICMB 9871.[28,40–43]

Relatively little is known about the reactivity of HAPMO with benzaldehydes. Preliminary experiments revealed that, in line with the chemical Baeyer–Villiger reaction, 4-hydroxybenzaldehyde is converted to 4-hydroxyphenyl formate, which after hydrolysis yields hydroquinone.[39] To study in more detail the selectivity of the enzyme-mediated Baeyer–Villiger oxidation of aromatic compounds we addressed in this paper the reactivity and product specificity of HAPMO with fluorinated benzaldehydes. Unlike most chemical procedures, the enzymatic approach gives high yield and selectivity towards the sustainable production of fluorophenols.

Results

Substrate Specificity

Kinetic studies revealed that HAPMO is active with a wide range of substituted benzaldehydes. 4-Aminobenzaldehyde appeared to be the best HAPMO aldehyde substrate (Table 1). However, with this compound significant substrate inhibition occurred (Fig. 1A). 4-Hydroxybenzaldehydes were also good HAPMO substrates. With these compounds, weak substrate inhibition was observed (Table 1). The kinetic parameters estimated with 4-hydroxybenzaldehyde slightly deviate from previous data where substrate inhibition was not taken into account.[38]

The HAPMO-mediated oxidation of fluorinated benzaldehydes was not sensitive to substrate inhibition (Table 1, Fig. 1B). As found for the corresponding acetophenones (Table 1), introduction of fluoro substituents in the benzaldehyde ring slightly decreased the reaction rate and increased the Michaelis constant to some extent. 3-Bromo-4-fluorobenzaldehyde was not a substrate for HAPMO.

Product Analysis of the Conversion of Fluorinated Benzaldehydes by HAPMO

The aromatic products formed in the reactions of HAPMO with fluorinated benzaldehydes were analyzed by ^{19}F NMR. Fig. 2A presents, as an example, the ^{19}F NMR spectrum recorded after incubation of 2-fluorobenzaldehyde (– 124.7 ppm) with a limiting amount of NADPH. From this spectrum it is clear that two products are formed. On the basis of reference compounds, the main product (95%) was assigned to 2-fluorophenol (– 142.0 ppm), whereas the minor product (5%) was assigned to 2-fluorobenzoic acid (– 120.3 ppm). Under the conditions applied, no ester formation was observed.

Enzymatic incubations with other fluorinated benzaldehydes mainly resulted in the production of fluorinated phenols (Table 2). With 2-fluoro-, 3-fluoro-, 2,3-difluoro- and 2,4-difluorobenzaldehyde, some fluorinated benzoic acid was also formed. The enzymatic conversions of 2-

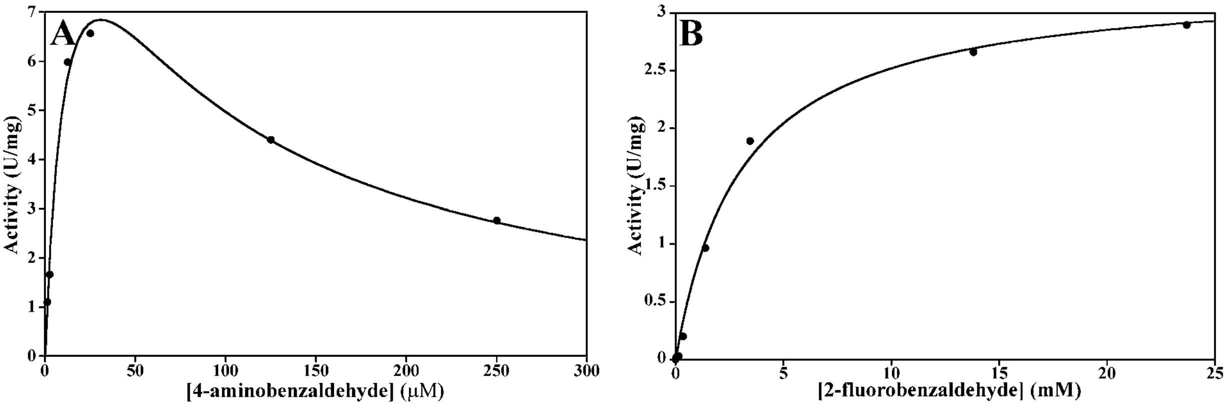


Figure 1. Michaelis–Menten kinetics of the 4-hydroxyacetophenone monooxygenase-mediated conversion of substituted benzaldehydes, (**A**) 4-aminobenzaldehyde, (**B**) 2-fluorobenzaldehyde.

Table 1. Kinetic parameters of the conversion of substituted benzaldehydes and acetophenones by 4-hydroxyacetophenone monooxygenase from *P. fluorescens* ACB.

Substitution	Benzaldehyde			Acetophenone		
	K'_m [mM]	k'_{cat} [s^{-1}]	K'_s [mM]	K'_m [mM]	k'_{cat} [s^{-1}]	K'_s [mM]
parent compound	3.4 ± 0.5	5.2 ± 0.3	–	2.0 ± 0.8	4.6 ± 1.2	–
2-fluoro-	3.0 ± 0.4	3.9 ± 0.1	–	1.6 ± 0.2	1.0 ± 0.1[a]	–
3-fluoro-	4.8 ± 0.4	4.0 ± 0.1	–	0.7 ± 0.1	1.0 ± 0.1[a]	–
4-fluoro-	4.0 ± 0.1	2.6 ± 0.0	–	1.0 ± 0.3[b]	0.7 ± 0.1[a]	–
2-fluoro-4-hydroxy-	0.8 ± 0.6	3.2 ± 1.6	1.5 ± 1.2	2.0 ± 0.2	12.4 ± 0.8	3.7 ± 0.4
3-fluoro-4-hydroxy-	3.4 ± 0.3	7.0 ± 0.3	–	1.8 ± 2.0	14.2 ± 10.2	4.8 ± 5.6
2,3-difluoro-	14.7 ± 1.4	0.7 ± 0.0	–	ND	ND	ND
2,4-difluoro-	5.7 ± 0.7	2.3 ± 0.1	–	1.7 ± 0.5	0.5 ± 0.1[a]	–
2,6-difluoro-	4.7 ± 0.8	2.4 ± 0.1	–	ND	ND	ND
3,4-difluoro-	7.9 ± 1.0	0.7 ± 0.0	–	ND	ND	ND
4-hydroxy-	0.27 ± 0.02	12.5 ± 0.4	4.8 ± 0.4	0.006 ± 0.001	8.3 ± 0.3	0.9 ± 0.1
4-amino-	0.014 ± 0.004	15.9 ± 2.4	0.07 ± 0.02	0.006 ± 0.003	20.4 ± 3.4	0.3 ± 0.2

[a] From ref.[44]
[b] From ref.[38]
ND = not determined.

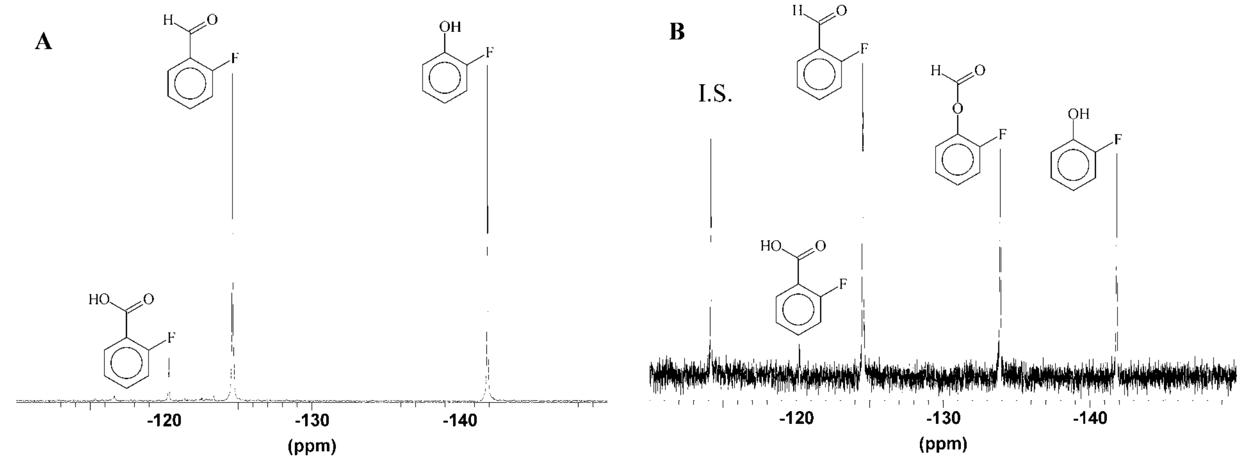


Figure 2. ^{19}F NMR analysis of the enzymatic conversion of 2-fluorobenzaldehyde by 4-hydroxyacetophenone monooxygenase. (**A**) Reaction at pH 8.0 with a limiting amount of NADPH to have about 50% substrate left at the end of the incubation (50,000 scans). (**B**) Reaction at pH 6.0 with excess NADPH (5,000 scans). I. S. is the internal standard (4-fluorobenzoic acid, -114.2 ppm). See Experimental Section for further details.

Table 2. Product ratio and yield of the conversion of fluorinated benzaldehydes by 4-hydroxyacetophenone monooxygenase from *P. fluorescens* ACB as determined by ^{19}F NMR. The mean standard error in the concentrations of fluorinated compounds was about 3%.

Substrate	Yield [%][a]	Product ratio	
		Phenol [%]	Benzoic acid [%]
2-fluorobenzaldehyde	88	95	5
3-fluorobenzaldehyde	83	82	18
4-fluorobenzaldehyde	75	100	0
2-fluoro-4-hydroxybenzaldehyde	86	100	0
3-fluoro-4-hydroxybenzaldehyde	91	100	0
2,3-difluorobenzaldehyde	25	73	27
2,4-difluorobenzaldehyde	64	95	5
2,6-difluorobenzaldehyde	70	100	0
3,4-difluorobenzaldehyde	31	100	0

[a] Total yield of phenol and benzoic acid in incubations with stoichiometric amounts of benzaldehyde and NADPH (see Experimental Section for further details).

fluoro-4-hydroxybenzaldehyde and 3-fluoro-4-hydroxybenzaldehyde both resulted in the exclusive formation of 2-fluorohydroquinone (Table 2). The chemical shift values of fluorinated benzaldehydes and their products obtained after conversion by HAPMO are listed in Table 3.

Factors Governing Product Yield

The product yield of the HAPMO-mediated conversion of fluorinated benzaldehydes was dependent on the type of substrate, the reaction conditions and the amount of enzyme used. With all substrates tested, no product inhibition was observed. Monofluorinated benzaldehydes were completely converted, providing that excess NADPH and oxygen were supplied. Using an NADPH generating system consisting of NADP$^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and air-saturating conditions, it was possible to convert mg amounts of 2-fluorobenzaldehyde into 2-fluorophenol (95%) and 2-fluorobenzoic acid (5%).

2,3-Difluorobenzaldehyde and 3,4-difluorobenzaldehyde were rather poor substrates for HAPMO (*cf.* Table 1). Substantial conversion of these compounds was only obtained at relatively high enzyme concentrations. Moreover, for high yields large excesses of NADPH and oxygen were needed, because of the competing non-productive NADPH oxidase activity ($k'_{cat} = 0.11\ s^{-1}$).[38]

Additional studies on factors governing product yield were performed using 2-fluorobenzaldehyde as the model substrate. Absorption spectral analysis of the enzymatic conversion of 2-fluorobenzaldehyde pointed at the initial formation of a compound with a low absorp-

Table 3. ^{19}F NMR chemical shifts [ppm] of fluorinated benzaldehydes and their products after conversion by 4-hydroxyacetophenone monooxygenase from *P. fluorescens* ACB.

Substitution	Benzaldehyde	Benzoic acid	Phenol
2-fluoro-	−124.7	−120.3	−142.0[a]
3-fluoro-	−116.7	−117.9	−116.6[a]
4-fluoro-	−106.3	−114.2	−129.2[a]
2-fluoro-4-hydroxy-	−121.8	−116.4	−138.8
3-fluoro-4-hydroxy-	−141.3	ND	−138.6
2,3-difluoro-	−142.2 (F3) and −150.1 (F2)	−142.8 (F3) and −46.4 (F2)	−143.1 (F3) and −167.1 (F2)
2,4-difluoro-	−101.8 (F4) and −119.8 (F2)	−111.5 (F4) and −15.1 (F2)	−126.3 (F4) and −137.3 (F2)
2,6-difluoro-	−119.6	−120.1	−139.3
3,4-difluoro-	−131.2 (F4) and −140.7 (F3)	−139.0 (F4) and −42.6 (F3)	−140.9 (F3) and −154.3 (F4) [a]

[a] See ref.[62]

tion in the 300–400 nm region (not shown). This absorption disappeared in time, indicative for the non-enzymatic hydrolysis of the intermediate ester product. Similar transient absorption changes were observed in the enzymatic conversion of other fluorobenzaldehydes. To get additional insight into ester formation, the HAPMO-mediated conversion of 2-fluorobenzaldehyde was studied as a function of pH and at relatively high enzyme concentration (Table 4). At pH 6.0, the enzymatic reaction was significantly slower than at pH 8.0, but ^{19}F NMR product analysis clearly showed the initial formation of 2-fluorophenyl formate (−133.9 ppm, Fig. 2B). After 10 min incubation, 80% of the total amount of products was the ester which slowly decayed to yield 2-fluorophenol. After measuring for 1 h at 7 °C, nearly half the amount of ester was hydrolyzed to the phenol (Table 4), whereas after 2 h less than 25% of the ester remained. At pH 7.0, 2-fluorobenzaldehyde was more readily converted, but almost no formate ester was detected by ^{19}F NMR. Table 4 summarizes the product yields and product ratios of the reactions of HAPMO with 2-fluorobenzaldehyde at different pH values. At all pH values, formation of about 5% of 2-fluorobenzoate was observed.

Discussion

Fluorophenols are attractive building blocks for the synthesis of high-value fluorinated chemicals. Fluorophenols can be produced by the chemical Baeyer–Villiger oxidation of fluorobenzophenones, fluoroacetophenones and fluorobenzaldehydes. With all these compounds, the amount of fluorophenol produced is dependent on the substituents in the aromatic ring(s), the type of oxidizing agent and the reaction conditions.[8–10,14] The chemical Baeyer–Villiger oxidation of 3-bromo-4-fluorobenzaldehyde led to the corresponding fluorobenzoic acid as opposed to the desired phenol.[26] With monofluorobenzaldehydes, the chemical conversion resulted in nearly equal amounts of fluorophenols and fluorobenzoic acids with a final yield of fluorophenols of about 25%.[9]

In a previous study we reported that 4-fluorophenols can be produced from 4-fluoroacetophenones using HAPMO as biocatalyst.[44] Oxyfunctionalization by HAPMO at pH 6 resulted in the exclusive production of 4-fluorophenyl acetates. At pH 8.0, the reaction rate increased and the fluorinated phenyl acetate products readily hydrolyzed to the corresponding fluorophenols.[44] Here we show that HAPMO is active with a wide range of fluorinated benzaldehydes. Under the conditions applied (pH 8.0), these reactions mainly resulted in the production of fluorophenols. At pH 6.0, transient formation of the initial ester product could be observed.

The enzymatic conversion of 4-amino-, 4-hydroxy- and 3-fluoro-4-hydroxybenzaldehyde was sensitive to substrate inhibition. Interestingly, there was a clear correlation between the Michaelis constants of the aromat-

Table 4. Product ratio and yield of the conversion of 2-fluorobenzaldehyde by 4-hydroxyacetophenone monooxygenase from *P. fluorescens* ACB at different pH values. ^{19}F NMR spectra were recorded for 1 h at 7 °C. The mean standard error in the concentrations of fluorinated compounds was about 3%.

pH	Product yield [%]	Product ratio [%]		
		2-fluorophenyl formate	2-fluorophenol	2-fluorobenzoate
8.0	100	0	95	5
7.0	95	2	93	5
6.0	57	53	42	5

ic substrates (K'_m) and the degree of substrate inhibition (K'_s). This suggests that the inhibition is directly related to catalysis and is not caused by binding of the substrate at a secondary site. For the related cyclohexanone monooxygenase, it was reported that NADP$^+$ remains bound during the entire reaction cycle and that the interaction between the reduced enzyme and pyridine nucleotide is needed for the stabilization of the flavin C4a peroxide oxygenation species.[45] Thus, it is unlikely that the inhibitory substrates are binding at the NADP(H) binding site. A more plausible explanation for the observed substrate inhibition is that the aromatic substrate competes with the aromatic product for binding to (one of) the enzyme intermediates which are formed after substrate oxygenation. A similar competition between substrate and product binding has been observed with flavoprotein aromatic hydroxylases.[46–48] The HAPMO-mediated Baeyer–Villiger oxidation of other fluorobenzaldehydes was not sensitive to substrate inhibition. In line with the above notion, this might be related to a relatively weak binding of these compounds in the active site.

The HAPMO-mediated conversion of fluorobenzaldehydes mainly resulted in the production of fluorophenols. The high preference for the formation of phenols is in disagreement with the rule derived from chemical studies that aromatic substrates with electron-withdrawing substituents mainly form benzoic acids.[8,10,16] Because the rearrangement of the tetrahedral Criegee intermediate (Scheme 1) of the enzymatic reaction is believed to be governed by the same stereoelectronic effects as in the non-enzymatic Baeyer–Villiger oxidation,[28,49,50] this suggests that interactions of the benzaldehyde substrates with amino acids or the flavin cofactor in the active site influence the selection of the migratory group in favor of the phenyl ring. The first insight into the active site topology of type I Baeyer–Villiger monooxygenases has recently come from the crystal structure determination of the uncomplexed form of phenylacetone monooxygenase.[51] From this structure and sequence comparison it was deduced that an arginine residue (Arg440) plays a critical role in HAPMO catalysis. However, a deeper insight into the enzyme-substrate interaction must await the crystal structure determination of a protein complex.

In conclusion, we have shown in this paper that HAPMO catalyzes the conversion of a wide range of benzaldehydes to the corresponding esters. The latter compounds spontaneously hydrolyze to the corresponding phenols. The enzymatic reactions with fluorobenzaldehydes show high selectivity and product yield and are not sensitive to substrate inhibition. A main advantage of the enzymatic Baeyer–Villiger reaction is the use of a mild oxidant (i.e., the flavin C4a peroxide). Recent studies have shown that recombinant whole cells expressing HAPMO can be used for the biooxidation of prochiral cyclobutanones.[52] Such a whole-cell system is of particular interest from a preparative point of view because it avoids expensive cofactor recycling.[53,54] Therefore, with proper upscaling and downstream processing,[41,55–58] HAPMO may develop as an environmentally benign alternative for the synthesis of fluorophenols.

Experimental Section

Materials

4-Fluorobenzaldehyde, 3-fluorobenzoic acid and 4-hydroxyacetophenone were purchased from Aldrich. 2-Fluorophenol, 3-fluorophenol and 4-fluorophenol were from Acros. 2-Fluoro-4-hydroxybenzaldehyde was synthesized as described earlier.[59] All other fluorinated aromatic compounds were obtained from Fluorochem. Dimethylformamide (DMF) and potassium phosphate were from Merck. Glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase (grade I), NADPH and NADP$^+$ were from Roche.

Enzyme Purification

HAPMO was purified from *Pseudomonas fluorescens* ACB as described earlier.[38]

Analytical Methods

The activity of 4-hydroxyacetophenone monooxygenase (HAPMO) was routinely determined spectrophotometrically by monitoring the aromatic substrate-stimulated oxidation of NADPH at 370 nm ($\varepsilon_{370} = 2.7\ \mathrm{mM^{-1}\ cm^{-1}}$). The standard enzyme activity was measured at 25 °C in 1.0 mL air-saturated 50 mM potassium phosphate, pH 8.0, containing 250 μM NADPH, and 200 μM 4-hydroxyacetophenone.[38] The reaction was started by the addition of 10 μg HAPMO. Specific activities were calculated from initial rate determinations and were corrected for endogenous NADPH oxidase activity. One unit of HAPMO activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per minute under the standard assay conditions. Steady-state kinetics were performed under the above conditions with 250 μM NADPH as the fixed substrate and varying concentrations (1 μM–20 mM) of aromatic substrate. With slow substrates, the enzyme concentration was increased ten-fold. The kinetic parameters K'_m and k'_{cat} were determined from non-linear regression analysis using the Michaelis–Menten equation. In case of substrate inhibition, the kinetic data were treated according to Eq. (1):

$$k'_{obs} = \frac{k'_{cat} * [S]}{K'_m + [S] + ([S]^2 / K'_s)} \tag{1}$$

where k'_{obs} is the apparent initial rate observed, k'_{cat} is the apparent maximal rate at saturating substrate concentration, K'_m is the apparent Michaelis constant for the aromatic substrate at fixed values of the other substrates (NADPH and O_2) and K'_s

is the apparent secondary-binding inhibition constant for the aromatic substrate.[60]

Reactions with Fluorinated Benzaldehydes

Stock solutions of aromatic substrates (100 mM) were prepared in DMF. Enzymatic incubation mixtures (2.0 mL) contained 0.6 mM fluorinated benzaldehyde, 6 mM sodium ascorbate, and 0.4–1.0 mM NADPH in 50 mM potassium phosphate pH 8.0. Ascorbate was added to prevent autooxidation of aromatic substrates and products. Reactions were performed for 1 h at 30 °C. At 10 min intervals, the reaction mixtures were saturated with air. Reactions were started by the addition of 10–100 μg HAPMO and stopped by freezing the samples in liquid nitrogen.

The enzymatic conversion of 2-fluorobenzaldehyde was studied at pH 6.0, 7.0 and 8.0 at 30 °C. The incubation mixture (2.0 mL) contained 0.5 mM 2-fluorobenzaldehyde, 0.6 mM NADPH in 50 mM potassium phosphate. Reactions were started by the addition of 0.7 mg HAPMO. Incubation mixtures were exposed to air to have maximal conversion. Recording of ^{19}F NMR spectra at 7 °C was initiated after 10 min of incubation.

2-Fluorobenzaldehyde was also converted by HAPMO using an NADPH generating system. To that end, 5 mM 2-fluorobenzaldehyde was incubated for 2 h at 30 °C in 50 mM potassium phosphate (pH 8.0) with 0.9 mg HAPMO, 0.25 mM NADP$^+$, 10 mM glucose 6-phosphate, and 7.5 mM ascorbate. The reaction (2.0 mL) was started by the addition of 0.5 mg glucose 6-phosphate dehydrogenase. At 10 min intervals, the reaction mixture was saturated with air.

^{19}F NMR Product Analysis

^{19}F NMR experiments were performed on a Bruker DPX 400 NMR spectrometer, essentially as described elsewhere.[61] The temperature was 7 °C. A dedicated 10 mm ^{19}F NMR probehead was used. The spectral width for the ^{19}F NMR measurements was 50,000 Hz. The number of data points used for data acquisition was 32768. Pulse angles of 30° were used. Between 1000 and 66,000 scans were recorded, depending on the concentrations of the fluorine-containing compounds and the signal-to-noise ratio required. The detection limit for an overnight run (60,000 scans) is 1 μM. ^{1}H decoupling was achieved with a Waltz16 decoupling sequence.

The sample volume was 1.6 mL. For calibration, an insert containing D_2O and a calibrated amount of 4-fluorobenzoate was used which also served as deuterium lock for locking the magnetic field. For the enzymatic conversion of 4-fluorobenzaldehyde, a 1625 μL sample with addition of 100 μL D_2O was used without the insert. Concentrations of the various fluorinated compounds were calculated by comparison of the integrals of their ^{19}F NMR resonances with the integral of the 4-fluorobenzoate resonance. Chemical shifts are reported relative to $CFCl_3$. ^{19}F NMR chemical shift values of the various fluorine-containing compounds were identified using authentic fluorinated benzaldehydes, benzoic acids and phenols or previously reported resonances. The ^{19}F NMR spectral data were corrected for impurities ($<5\%$) of fluorobenzoic acids and fluorophenols present in some substrate solutions.

Acknowledgements

We thank Barend van Lagen for assistance in NMR experiments. This work was supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO), division "Procesvernieuwing voor een Schoner Milieu".

References

[1] C. Dong, F. Huang, H. Deng, C. Schaffrath, J. B. Spencer, D. O'Hagan, J. H. Naismith, *Nature* **2004**, *427*, 561–565.
[2] R. P. Robinson, E. R. Laird, K. M. Donahue, L. L. Lopresti-Morrow, P. G. Mitchell, M. R. Reese, L. M. Reeves, A. I. Rouch, E. J. Stam, S. A. Yocum, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1211–1213.
[3] A. Tanaka, T. Terasawa, H. Hagihara, N. Ishibe, M. Sawada, Y. Sakuma, M. Hashimoto, H. Takasugi, H. Tanaka, *J. Med. Chem.* **1998**, *41*, 4408–4420.
[4] A. M. Ismaiel, J. De Los Angeles, M. Teitler, S. Ingher, R. A. Glennon, *J. Med. Chem.* **1993**, *36*, 2519–2525.
[5] J. T. Konkel, J. Fan, B. Jayachandran, K. L. Kirk, *J. Fluorine Chem.* **2002**, *115*, 27–32.
[6] W. Vaalburg, H. H. Coenen, C. Crouzel, P. H. Elsinga, B. Langstrom, C. Lemaire, G. J. Meyer, *Int. J. Rad. Appl. Instrum. [B]* **1992**, *19*, 227–237.
[7] H. H. Coenen, in: *PET studies on amino acid metabolism and protein synthesis*, (Eds.: B. Mazoyer, W. D. Weiss, D. Comar), Kluwer Academic Publisher, Dordercht, Netherlands, **1993**, pp. 109–129.
[8] L. Conte, M. Napoli, G. P. Gambaretto, A. Guerrato, F. M. Carlini, *J. Fluorine Chem.* **1994**, *67*, 41–45.
[9] I. Ekaeva, L. Barre, M.-C. Lasne, F. Gourand, *Appl. Radiat. Isot.* **1995**, *46*, 777–782.
[10] T. Ludwig, J. Ermert, H. H. Coenen, *Nucl. Med. Biol.* **2002**, *29*, 255–262.
[11] A. Corma, V. Fornes, S. Iborra, M. Mifsud, M. Renz, *J. Catal.* **2004**, *221*, 67–76.
[12] R. Criegee, *Justus Liebigs Ann. Chem.* **1948**, *560*, 127–135.
[13] M. Renz, B. Meunier, *Eur. J. Org. Chem.* **1999**, *4*, 737–750.
[14] Y. Ogata, Y. Sawaki, *J. Org. Chem.* **1969**, *34*, 3985–3991.
[15] A. Corma, L. T. Nemeth, M. Renz, S. Valencia, *Nature* **2001**, *412*, 423–425.
[16] G. J. ten Brink, I. W. Arends, R. A. Sheldon, *Chem. Rev.* **2004**, *104*, 4105–4124.
[17] G. R. Krow, in: *Organic Reactions*, (Ed.: L. A. Paquette), John Wiley & Sons, New York, **1993**, pp. 251–798.
[18] S. L. Friess, A. H. Soloway, *J. Am. Chem. Soc.* **1951**, *73*, 3968–3972.
[19] W. v. E. Doering, L. Speers, *J. Am. Chem. Soc.* **1950**, *72*, 5515–5518.
[20] G. Strukul, *Angew. Chem. Int. Ed.* **1998**, *37*, 1198–1209.
[21] S. Murahashi, S. Ono, Y. Imada, *Angew. Chem. Int. Ed.* **2002**, *41*, 2366–2368.
[22] C. Mazzini, J. Lebreton, R. Furstoss, *J. Org. Chem.* **1996**, *61*, 8–9.

[23] O. Fukuda, S. Sakaguchi, Y. Ishii, *Tetrahedron Lett.* **2001**, *42*, 3479–3481.

[24] C. Bolm, C. Palazzi, G. Francio, W. Leitner, *Chem. Commun.* **2002**, 1588–1589.

[25] M. Hudlicky, in: *Oxidations in Organic Chemistry,* ACS Monograph 186, American Chemical Society, Washington, DC, **1990**, p. 164.

[26] A. Adejare, J. Shen, A. M. Ogunbadeniyi, *J. Fluorine Chem.* **2000**, *105*, 107–109.

[27] G. J. ten Brink, J. M. Vis, I. W. Arends, R. A. Sheldon, *J. Org. Chem.* **2001**, *66*, 2429–2433.

[28] M. D. Mihovilovic, B. Müller, P. Stanetty, *Eur. J. Org. Chem.* **2002**, *2002*, 3711–3730.

[29] M. W. Fraaije, J. Wu, D. P. Heuts, E. W. van Hellemond, J. H. Spelberg, D. B. Janssen, *Appl. Microbiol. Biotechnol.* **2005**, *66*, 393–400.

[30] N. M. Kamerbeek, D. B. Janssen, W. J. H. van Berkel, M. W. Fraaije, *Adv. Synth. Catal.* **2003**, *345*, 667–678.

[31] A. Hage, H. E. Schoemaker, J. A. Field, *Appl. Microbiol. Biotechnol.* **1999**, *52*, 834–838.

[32] F. R. Lauritsen, A. Lunding, *Enz. Microb. Technol.* **1998**, *22*, 459–465.

[33] W. Kroutil, H. Mang, K. Edegger, K. Faber, *Adv. Synth. Catal.* **2004**, *346*, 125–142.

[34] F. Guillen, A. T. Martinez, M. J. Martinez, *Eur. J. Biochem.* **1992**, *209*, 603–611.

[35] R. H. H. van den Heuvel, C. Laane, W. J. H. van Berkel, *Adv. Synth. Catal.* **2001**, *343*, 515–520.

[36] P. Ferreira, M. Medina, F. Guillén, M. J. Martínez, W. J. H. van Berkel, A. T. Martínez, *Biochem. J.* **2005**, in press.

[37] M. J. H. Moonen, I. M. C. M. Rietjens, W. J. H. van Berkel, in: *13th International Congress on Flavins and Flavoproteins*, (Eds.: S. Ghisla, P. Kroneck, P. Macheroux, H. Sund), Agency for Scientific Publication, Berlin, **1999**, pp. 375–378.

[38] N. M. Kamerbeek, M. J. H. Moonen, J. G. M. van der Ven, W. J. H. van Berkel, M. W. Fraaije, D. B. Janssen, *Eur. J. Biochem.* **2001**, *268*, 2547–2557.

[39] N. M. Kamerbeek, A. J. Olsthoorn, M. W. Fraaije, D. B. Janssen, *Appl. Environ. Microbiol.* **2003**, *69*, 419–426.

[40] C. T. Walsh, Y.-C. J. Chen, *Angew. Chem.* **1988**, *100*, 342–352.

[41] V. Alphand, G. Carrea, R. Wohlgemuth, R. Furstoss, J. M. Woodley, *Trends Biotechnol.* **2003**, *21*, 318–323.

[42] M. T. Reetz, F. Daligault, B. Brunner, H. Hinrichs, A. Deege, *Angew. Chem. Int. Ed.* **2004**, *43*, 4078–4081.

[43] M. T. Reetz, B. Brunner, T. Schneider, F. Schulz, C. M. Clouthier, M. M. Kayser, *Angew. Chem. Int. Ed.* **2004**, *43*, 4075–4078.

[44] M. J. H. Moonen, I. M. C. M. Rietjens, W. J. H. van Berkel, *J. Ind. Microbiol. Biotechnol.* **2001**, *26*, 35–42.

[45] D. Sheng, D. P. Ballou, V. Massey, *Biochemistry* **2001**, *40*, 11156–11167.

[46] M. Husain, B. Entsch, D. P. Ballou, V. Massey, P. J. Chapman, *J. Biol. Chem.* **1980**, *255*, 4189–4197.

[47] A. P. Jadan, M. J. H. Moonen, S. A. Boeren, L. A. Golovleva, I. M. C. M. Rietjens, W. J. H. van Berkel, *Adv. Synth. Catal.* **2004**, *346*, 367–375.

[48] M. G. Taylor, V. Massey, *J. Biol. Chem.* **1990**, *265*, 13687–13694.

[49] J. M. Schwab, *J. Am. Chem. Soc.* **1981**, *103*, 1876–1878.

[50] J. M. Schwab, W. Li, L. P. Thomas, *J. Am. Chem. Soc.* **1983**, *105*, 4800–4804.

[51] E. Malito, A. Alfieri, M. W. Fraaije, A. Mattevi, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13157–13162.

[52] M. D. Mihovilovic, P. Kapitan, J. Rydz, F. Rudroff, F. H. Ogink, M. W. Fraaije, *J. Mol. Catal. B: Enzym.* **2005**, *32*, 135–140.

[53] V. Alphand, R. Furstoss, *J. Org. Chem.* **1992**, *57*, 1306–1309.

[54] J. D. Stewart, K. W. Reed, C. A. Martinez, J. Zhu, G. Chen, M. M. Kayser, *J. Am. Chem. Soc.* **1998**, *120*, 3541–3548.

[55] N. M. Kamerbeek, M. W. Fraaije, D. B. Janssen, *Eur. J. Biochem.* **2004**, *271*, 2107–2116.

[56] A. Z. Walton, J. D. Stewart, *Biotechnol. Prog.* **2002**, *18*, 262–268.

[57] S. D. Doig, P. J. Avenell, P. A. Bird, P. Gallati, K. S. Lander, G. J. Lye, R. Wohlgemuth, J. M. Woodley, *Biotechnol. Prog.* **2002**, *18*, 1039–1046.

[58] I. Hilker, V. Alphand, R. Wohlgemuth, R. Furstoss, *Adv. Synth. Catal.* **2004**, *346*, 203–214.

[59] W. J. H. van Berkel, M. H. M. Eppink, W. J. Middelhoven, J. Vervoort, I. M. C. M. Rietjens, *FEMS Microbiol. Lett.* **1994**, *121*, 207–215.

[60] W. van Berkel, A. Westphal, K. Eschrich, M. Eppink, A. de Kok, *Eur. J. Biochem.* **1992**, *210*, 411–419.

[61] J. Vervoort, P. A. de Jager, J. Steenbergen, I. M. C. M. Rietjens, *Xenobiotica* **1990**, *20*, 657–670.

[62] S. Peelen, I. M. C. M. Rietjens, M. G. Boersma, J. Vervoort, *Eur. J. Biochem.* **1995**, *227*, 284–291.