Microbiological transformations. Part 48: Enantioselective biohydrolysis of 2-, 3- and 4-pyridyloxirane at high substrate concentration using the *Agrobacterium radiobacter* AD1 epoxide hydrolase and its Tyr215Phe mutant

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Abstract—The epoxide hydrolase (EH) from *Agrobacterium radiobacter* AD1 wild type (ArWT) and its Tyr215Phe mutant were compared for the biocatalyzed hydrolytic kinetic resolution (BHKR) of 2-, 3- and 4-pyridyloxirane. The regioselectivity of the oxirane ring opening as well as the substrate concentration limit and the inhibitory effect of the diol were determined. A gram scale preparation of enantiopure 2-pyridyloxirane (ee>98%) at a concentration as high as 127 mM (15.5 g/L) could be achieved with each of these two enzymes. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

In recent years, epoxide hydrolases (EHs) (EC 3.3.2.3) have been shown to be ubiquitous in nature, to be cofactor independent and to catalyze the enantioselective addition of water to an epoxide, thus leading to the corresponding diol and the recovery of the less reactive substrate enantiomer via a biocatalysed hydrolytic kinetic resolution (BHKR).[1–7] One general advantage of this approach is the fact that it constitutes a typical 'green chemistry' process, which can be run using water as the only solvent and reactant, and only implies a natural and biodegradable catalyst, i.e. an enzyme. Using such a strategy, both the epoxide and the diol can be obtained in—ideally—enantiopure form. Moreover, since this process is a kinetic resolution, it is generally possible to tune the ee of the remaining epoxide up to enantiopurity, simply by choosing the appropriate extent of conversion. Furthermore, combination of enantio- and regiocomplementary enzymes can, in certain cases, enable to develop highly valuable enantioconvergent processes allowing to overcome the 50% yield limitation intrinsic to a common resolution process. We have for instance recently illustrated the possible use of a fungal epoxide hydrolase for the synthesis of enantiomerically pure bioactive compounds like Ibuprofen[8] or Eliprodil.[9]

This method may be even more attractive if it allows the preparation of epoxides which cannot be obtained by using the presently available conventional chemistry approaches. This is for instance the case for 2-, 3- and 4-pyridyloxirane **1–3**,[10] which are key-step building blocks for the synthesis of several biologically active compounds, such as β-adrenergic receptor antagonists or anti-obesity drugs.[11–13] We have recently shown that these substrates can be conveniently obtained in enantiopure form using partially purified EH from *Aspergillus niger*,[14] and were interested in testing other enzymes in this context. In particular, some of us have recently isolated, cloned and over-expressed the epoxide hydrolase from the bacterium *Agrobacterium radiobacter* AD1 and studied some of its enzyme/substrate properties.[15–18] Both the *A. niger* and the *A. radiobacter* EHs have recently been crystallized and their three-dimensional structure has been determined.[19,20]
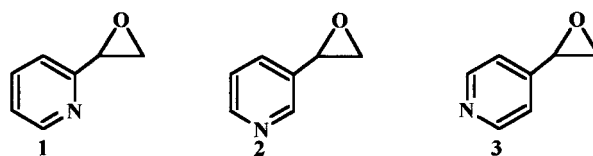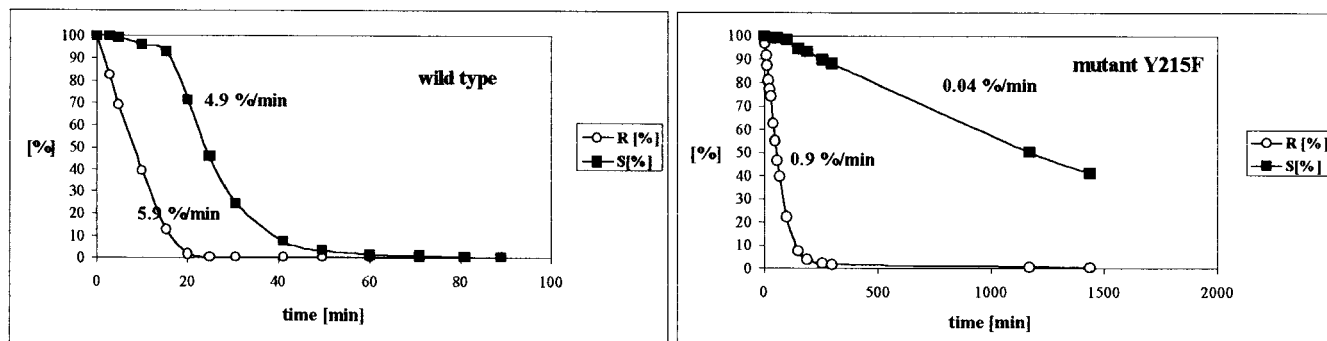

Scheme 1.

Figure 1. BHKR of **1** (7 mM, 28°C, 500 rpm) using the *A. radiobacter* wild type enzyme (ArWT) (0.02 g/L) or the Tyr215Phe mutant EHs (0.34 g/L).

Moreover, a Tyr215Phe mutant of the *A. radiobacter* enzyme has been constructed and was shown to exhibit a substantial improvement of the enantioselectivity (*E* value) for (substituted) styrene oxides.[17] The experiments described here were aimed at obtaining kinetic resolution of **1–3** (Scheme 1) using the wild type *A. radiobacter* epoxide hydrolase (ArWT) and its Tyr215Phe mutant.

2. Results and discussion

2.1. Resolution of pyridyloxiranes by wild-type and mutant *A. radiobacter* epoxide hydrolase

Previous work has shown that the *A. radiobacter* epoxide hydrolase was able to hydrolyze styrene oxide and various derivatives thereof.[15] One of the most intriguing facts is the existence of a so-called 'sequential' mechanism. Thus, owing to the fact that the K_M ratio of the two enantiomers is very high ($K_M(S)/K_M(R)=46$), hydrolysis of the (*S*)-enantiomer is almost completely inhibited by the (*R*)-enantiomer as long as the latter has not totally disappeared. After consumption of the (*R*)-enantiomer, the (*S*)-enantiomer is consumed much faster, due to the fact that its k_{cat} value is higher than that of the (*R*) enantiomer ($k_{cat}(S)/k_{cat}(R)=2.5$). This is evidently an important drawback for the use of such a catalyst at a preparative scale, since it necessitates to very accurately tune the extent of conversion of such a BHKR in order to obtain a reasonable ee/yield compromise. Surprisingly enough, this is no more the case for the mutated enzyme, where a classical kinetic profile implying a consistent rate difference between the two enantiomers was observed.[17,21] A similar reaction profile was similarly observed for compounds **1–3** (Fig. 1). Thus, the ArWT EH again showed a sequential mechanism for all three substrates, while the Tyr215Phe EH clearly hydrolyzed

one enantiomer much slower than the other one, even after consumption of the preferred enantiomer.[22]

This difference in behavior is illustrated by Fig. 1 in the case of epoxide **1**. For both enzymes, the remaining antipode was of (*S*)-configuration. At 7 mM substrate concentration, we observed as expected a significant increase in enantioselectivity for the mutated EH (Table 1).

2.2. Enantio- and regioselectivity determination

Due to the possible combination of enantio- and regioselectivity inherent to epoxide hydrolase catalyzed resolutions,[23] the *E*-values corresponding to the various experiments were calculated on the basis of the ee of the remaining substrate and the degree of conversion (Table 1). As expected, the mutated enzyme exhibited a higher enantioselectivity then the wild type enzyme towards substrates **1–3**. This clearly confirms that replacement of the active site Tyr215 residue by a phenylalanine constitutes an important factor for enhancing the overall enantioselectivity of this epoxide hydrolase for structurally different substrates.

An epoxide can a priori be attacked at either carbon atom of the oxirane ring. We therefore determined the $\alpha(S)/\beta(S)$ and $\alpha(R)/\beta(R)$ ratio for **1–3**, using the ee of the diol obtained upon biohydrolysis of the enantiopure (*S*)-epoxide and the ee of the diol formed after total conversion of the racemic epoxide.[23] The results obtained for the EH of ArWT and the Tyr215Phe mutant are summarized in Table 2. No difference of regioselectivity could be observed between the wild type and the mutant enzyme, both enzymes showing a highly preferential attack at the β-carbon atom in all cases. Since for all three substrates the $\alpha(S)$ and $\alpha(R)$ values were identical for both enantiomers, racemic diol was formed upon completion of the reaction (ee$_f$=0%). However, as also observed for the *A. niger* EH,[14] the regioselectivity was found to be a function of the position of the nitrogen atom and showed a slight increase of the α/β ratio **1** to **3**. Thus, the best regioselectivity was observed for **1**, with only 3% of the attack occurring at the α position.

2.3. Inhibition by the diol

Product inhibition can be a serious problem for scaling up kinetic resolutions. If the product formed is a strong competitive inhibitor, the conversion rate will slow down during

Table 1. *E*-values for hydrolysis of **1–3** (5–10 mM) with the ArWT and Tyr215Phe *A. radiobacter* EHs

EH-source	**1**	**2**	**3**
A. radiobacter ArWT	28	11	7
A. radiobacter Tyr215Phe mutant	55	33	31

The spontaneous hydrolysis of **1** at 28°C was estimated to be about 0.005%/min. Therefore, the *E*-values given in this table are apparent values, and the actual enzyme enantioselectivity must be somewhat higher.

Table 2. Regioselectivity for the hydrolysis of **1–3** using the ArWT epoxide hydrolase and its Tyr215Phe mutant

EH-source	1			2			3		
	$\alpha(S)/\beta(S)^a$	$\alpha(R)/\beta(R)^b$	ee_f^c	$\alpha(S)/\beta(S)$	$\alpha(R)/\beta(R)$	ee_f	$\alpha(S)/\beta(S)$	$\alpha(R)/\beta(R)$	ee_f
ArWT	3/97	3/97	~0	8/92	6/94	~0	13/87	13/87	~0
Tyr215Phe	2/98	2/98	~0	8/92	8/92	~0	12/88	13/87	~0

[a] $\alpha(S)/\beta(S)$: regioselectivity coefficient, α-carbon atom attack/β-carbon-atom attack of the (S)-enantiomer, given as percentages.[23]
[b] $\alpha(R)/\beta(R)$: regioselectivity coefficient, α-carbon atom attack/β-carbon-atom attack of the (R)-enantiomer, given as percentages.[23]
[c] ee_f=enantiomeric excess of the diol at total conversion of the racemic epoxide. These values were calculated from the equation $\alpha(R)=\alpha(S)-ee_f$ as described previously.[23]

progress of the reaction and thus limit a large-scale conversion. Inhibition of the activity of Tyr215Phe mutant EH by diol **1d** was determined by investigating its effect on the conversion of (R)-para-nitrostyrene oxide. The obtained K_i was 122 mM. One should note that these experiments were performed using racemic **1d**, which implies that the strongest inhibiting enantiomer could have an up to two-fold lower K_i value. This implies that, for a kinetic resolution with, for example, a starting concentration of 122 mM, the apparent k_{cat}/K_M is still at 50% or higher when the preferred enantiomer is completely converted.

2.4. Activity at high substrate levels

Another limiting factor we have previously observed for scaling up some EH-mediated resolutions was the solubility of the substrate in the reaction medium (aqueous buffer). If the substrate concentration exceeds its solubility, an emulsion is formed which can quickly inactivate the enzyme.[21] Interestingly, substrates **1–3** exhibit much higher solubilities in water (i.e. **1**: 454 mM (55 g/L) at 28°C) than styrene oxide derivatives, which should avoid this limitation. As **1** showed the best enantioselectivity with both EHs, it was used to explore the possibility to achieve conversion at high substrate concentration. Using the ArWT EH, a substrate concentration as high as 200 mM (24.3 g/L) still led to an excellent ee (ee >99%, c=58%, 1 h reaction) of the remaining epoxide **1** after a reaction time of about 70 min (28°C) indicating that product inhibition was still not predominant. At higher concentration however, enzyme inhibition clearly occurred, thus slowing down the reaction and affording the remaining substrate in only very low ee (ee=7%, c≈12%, 6 h reaction, 250 mM). Using the Tyr215Phe EH, the substrate concentration limit was even slightly higher, a 95% ee being obtained at 300 mM (36.3 g/L) (c=56%, 7 h reaction). Higher concentration again led to enzyme inhibition. It is interesting to emphasize the fact that, in contrast to what we observed with the A. niger EH,[14] the E-value remained constant for both enzymes upon increase of substrate concentration.
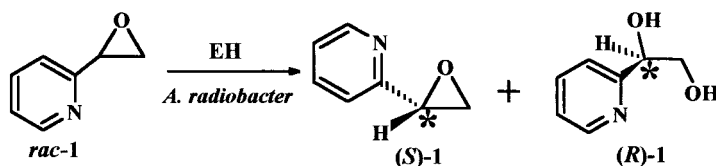
2.5. Preparative scale conversion

Based on these results, a preparative scale conversion of **1** using both A. radiobacter EHs was carried out at a concentration of 127 mM (Scheme 2). The reaction was stopped when the ee of the residual epoxide reached a value of about 98%, as a good compromise between ee and overall yield. Enantiomerically pure **1** could of course be obtained by leaving the reaction proceed for some additional time.

In a typical experiment, 2 g (16.5 mmol) of rac-**1** was submitted to a BHKR procedure using 9 mg of a partially purified enzyme extract of ArWT (specific activity for **1**: 13 U/mg protein). The corresponding concentrations were, respectively, 15.4 g/L (127 mM) of substrate and 0.07 g/L of enzyme extract. The reaction was carried out at 28°C in 130 mL phosphate buffer. After 160 min, usual work-up led to 0.69 g (34.5% purified yield) of (S)-**1**—which showed an ee of >99% and 1.3 g (R)-**1d** (56.6% purified yield) with an ee of 71%. The calculated turnover number (TON) was estimated to be about 13,200 mol substrate/mol enzyme/h and the space time yield was about 48 g/L/day.

Similarly, a preparative scale BHKR was carried out using the Tyr215Phe EH. Thus, 2 g (16.5 mmol) of rac-**1** was hydrolyzed using a solution of 128 mg (15.4 mL) of a partially purified enzyme extract (specific activity for **1**: 0.11 U/mg protein) at 28°C in 130 mL phosphate buffer. The corresponding concentrations were respectively 15.4 g/L (127 mM) of substrate and 0.98 g/L enzyme extract. After 7 h and usual work-up, this led to 0.72 g (36% purified yield) of (S)-**1** which showed an ee of 98% and 0.925 g of (R)-**1d** (40% purified yield) with an ee of 81%. These ee values were determined using chiral GC analysis as described in the experimental part. The calculated turnover number (TON) was estimated to be about 360 mol substrate/mol enzyme/h and the space time yield was about 19 g/L/day.

It is to be emphasized that, in our hands, neither of the conventional chemistry approaches known to date (i.e. the

Scheme 2. Preparative scale conversion of **1**.

heavy metal-based Jacobsen epoxidation or HKR strategy) were effective under standard experimental conditions for the preparation of enantiopure **1–3**.[14] This is probably due to the low reactivity of the corresponding olefin and/or to a complexation (or reaction) of the pyridine substrate with the metal catalyst (or reagents).

3. Conclusions

The results we have obtained throughout this work indicate that substrates **1–3** were efficiently and enantioselectively hydrolyzed using the wild-type epoxide hydrolase from the bacteria *A. radiobacter* as well as by its Tyr215Phe mutant. The wild type enzyme clearly showed a so-called sequential mechanism for all three epoxides, as already observed with other substrates, while in contrast the Tyr215Phe mutant exhibited a classical kinetic profile. These results show that the active-site mutation introduced into the *Agrobacterium* enzyme on basis of the X-ray structure has yielded a more suitable catalyst for various kinetic resolutions, making the enzyme more versatile. Since Tyr215 is conserved in other epoxide hydrolase sequences, it is well conceivable that the same mutation can also be used to improve the applicability of other epoxide hydrolases.

The enantio- and regioselectivity of these BHKR were determined. As expected from previous observations, the mutant enzyme exhibited a slight increase in enantio-selectivity and both enzymes showed the same regioselec-tivity. Interestingly, in contrast to the previously observed results using the same enzymes but different substrates, 2-pyridyloxirane **1** could be operated at concentrations as high as 200 mM for the wild-type epoxide hydrolase and up to 300 mM for the mutant enzyme. These were used to achieve a preparative scale synthesis of enantiopure (*S*)-**1** (ee>99%) at a 127 mM (15.4 g/L) concentration. Due to the fact that conventional chemistry approaches, including the Jacobsen HKR cobalt-based catalyst, were not successful in our hands, these results, together with those obtained using the *Aspergillus niger* epoxide hydrolase, are particularly interesting from the 'chirotechnology' point of view. Work is in progress in our laboratories in order to widen the scope of this epoxide hydrolase based green chemistry technology.

4. Experimental

4.1. General

For gas chromatographic analysis, the chiral columns Chiralsil Dex CB (Chrompack) and Lipodex E (Macherey and Nagel) were used. This allowed to determine the ee of **1–3**. The enzyme solutions were prepared as described by Rink et al.[16] The synthesis of **1–3** and **1d–3d** have been described.[14]

4.1.1. Determination of the ee of 2-pyridyldiol 1d by derivatisation to the dimethylether. The ee was deter-mined by adding 100 μL DMSO, KOH and 12 μL iodo-methane to the dry diol. This was stirred for 30 min at RT. The formed dimethylether was extracted with diethyl-ether and analyzed by GC (col. Lipodex E, 90°C; 23 and 25 min).

4.1.2. Determination of inhibition constants. The com-petitive inhibition constant K_i of diol **1d** to the Ty215Phe mutant was obtained from depletion curves of (*R*)-*para*-nitrostyrene oxide with varying concentrations of racemic diol. The apparent k_{cat}/K_m values, $(k_{cat}/K_m)^{app}$ were deter-mined by fitting a single exponential to the depletion curves. The inhibition constant K_i was obtained by fitting the formula $(k_{cat}/K_M)^{app}=(k_{cat}/K_M)/(1+[\mathbf{1d}]/K_i)$ to the $(k_{cat}/K_M)^{app}$ values, using several concentrations of **1d**.[24]

4.2. General procedure for biohydrolysis of 1. Preparative scale conversion

An amount of 2 g (16.5 mmoles) of rac-epoxide **1** was dissolved in 0.1 M Na-phosphate buffer pH8. The total volume together with the added enzyme solution (1.29 mL; 0.07 g/L in Tris-buffer pH9 for ArWT and 15.4 mL; 8.3 g/L in Tris-buffer pH9 for the Tyr215Phe mutant) was 130 mL (28°C; 250 rpm). The reaction was stopped at ee>98% by extracting the epoxide with CHCl₃. The aqueous phase was then lyophilized, and **1d** was directly extracted with acetone from the resulting dry diol-salt and purified by flash chromatography. From the CHCl₃ phase the epoxide and the diol were purified by flash chromatography: epoxide: pentane/ethylacetate 4/6; diol: ethylacetate/methanol: 9/1. The fractions containing the epoxide and respectively the diol were pooled, dried over Na₂SO₄, and the solvent was removed by stripping. The fractions containing the diol were treated similarly. The epoxide was further purified by bulb-to-bulb distillation (4×10^{-2} mbar, 40°C).

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