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Enzymatic transformations. Part 55: Highly productive epoxide hydrolase catalysed resolution of an azole antifungal key synthon

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Abstract—A highly productive bioprocess for the preparation of enantiopure azole antifungal chirons is described. These are key building blocks for the synthesis of new triazole drug derivatives known to display valuable activity against such infections as for instance fluconazole-resistant oro-oesophageal candidiasis. Using commercially available recombinant *Aspergillus niger* epoxide hydrolase under optimised experimental conditions, the hydrolytic kinetic resolution of 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane was performed in plain water, at room temperature, using a two-phase reactor. This methodology allowed the process to be run at a substrate concentration as high as 500 g/L (i.e., 2.5 M) and afforded the (unreacted) epoxide and the corresponding vicinal diol, both in nearly enantiopure form and quantitative yield.

1. Introduction

The preparation of enantio-enriched epoxides or of their corresponding vicinal diols, two types of chiral intermediates which offer high chemical versatility, has been considered for years as one of the most significant goals for asymmetric synthesis. Identification of new methodologies for access to these targets has been an area of active effort for several decades. Diverse conventional chemistry approaches have been described, the best ones based on the use of different types of transition-metal based catalysts.[1,2] We (and others) have also explored, over the last decades, the possibility offered by the use of appropriate enzymes. In particular, we have described that microbial epoxide hydrolases allowed efficient biocatalysed hydrolytic kinetic resolution (BHKR) of various epoxides.[3] Although this approach surprisingly seems to be sometimes ignored, for some unclear cultural reasons, by organic chemists, there is no doubt that this methodology could provide interesting complementarity[4] as well as valuable practical advantages to the conventional chemistry approach, particularly for industrial application. For example, the 'green chemistry' aspect, the very high turnover numbers generally observed, and the fact that some of these enzymes offer complementary regioselectivities (thus allowing to set up an enantioconvergent process, i.e., affording 100% ee, 100% yield starting from a racemic substrate) are important advantages which are surely worth considering.[5]

With the exception of lipases and baker's yeast, nowadays commonly accepted as 'classical reagents', the use of biocatalyts is very often considered to be hampered by several practical drawbacks. These are for instance: (a) the fact that the appropriate enzyme is rarely commercially available (b) the (possible) limited substrate specificity of a given enzyme (c) the low substrate concentration usable owing to the (generally) poor solubility of organic substrates in water (thus leading to low volumetric productivity of the bioreactor) or, else (d) the reaction limitation linked to substrate and/or product inhibition. Although, depending on the substrate, these limitations should indeed not be neglected, they may in many cases be circumvented by using appropriate methodologies. We describe herein our results, focused on the BHKR of epoxide **1**, where these drawbacks could be efficiently overcome. Thus, we show that using the commercially available recombinant *Aspergillus niger* epoxide hydrolase† it is possible to set up a highly productive bioprocess for the preparation of enantiopure epoxide **1** as well as of the corresponding chloro-diol **2**. Both these chirons are key building blocks for the synthesis of new triazole drug derivatives, like for instance D0870, known to display valuable activity against such infections—as for instance fluconazole-resistant oro-oesophageal candidiasis—often encountered in HIV-infected (and other immunocompromised) patients[6,7] (Scheme 1).

Keywords: Biocatalysis; Enzyme resolution; Epoxide; Kinetic resolution; Enantioselective; Hydrolysis.
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† This enzyme is presently commercially available from FLUKA (ref. 71832).

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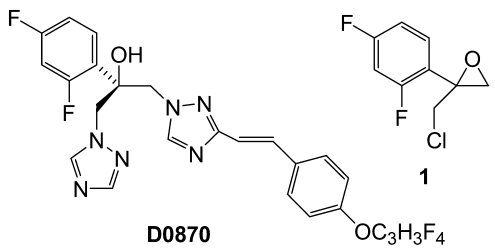


Scheme 1.

2. Results

We have recently described our preliminary results aimed at offering an alternative pathway to the synthesis of the enantiopure epoxide (*S*)-**1** and of the corresponding diol (*R*)-**2**.[8] This involved the BHKR of *rac*-**1**, performed using the commercially available (recombinant) *A. niger* epoxide hydrolase. We have shown that, when conducted at a 2 mM (0.4 g/L) concentration of *rac*-**1** in a 100 mM phosphate buffer solution (pH 7) containing 10% DMSO, this could efficiently lead to enantiopure (*S*)-**1** and to the corresponding chloro-diol (*R*)-**2**, also obtained in nearly enantiopure form (ees>98%) (Scheme 2). The observed *E* value of this resolution was higher than 200, and the α(*S*)/β(*S*) and α(*R*)/β(*R*) regioselectivity factors, determined for each one of the two enantiomers of **1**, were calculated to be 5/95 and 1/99, respectively, indicating that both enantiomers were essentially attacked at the less substituted carbon atom.

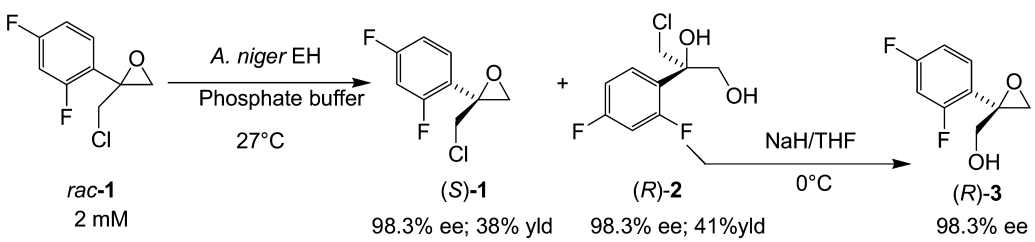
These interesting results encouraged us to pursue this study in order to set up an improved preparative scale process. In particular, we wanted to address some of the drawbacks cited above, in order to demonstrate the practical applicability of such a biocatalytic procedure. To reach this goal we explored the possibility (a) to simplify the experimental protocol (b) to increase the substrate concentration (c) to optimise the enzyme/substrate ratio (d) to perform this BHKR at a several-gram scale using the thus defined optimal experimental conditions.

2.1. Simplification of the experimental protocol

2.1.1. Use of plain water instead of phosphate buffer. As a general trend, one of the important issues for industrial application is, whatever the type of reactant or catalyst (chemical or enzymatic) involved, the set up of so-called 'salt free' processes. This obviously allows to simplify, and therefore to lower the cost, of the subsequent downstream processing. Our previously described procedure—which involved in the use of a 100 mM (pH 7) phosphate buffer—in fact implied an amount of 15 g/L of phosphate salts, which would translate into 15 kg/m^3 in case of an industrial application. Therefore, we have explored the possibility to perform this resolution using plain water instead of phosphate buffer. Indeed, we had shown previously—using rather water soluble pyridine oxirans—that to the contrary of many enzymes, the *A. niger* epoxide hydrolase retained nearly its total activity in plain water. It remained to be checked whether this was also applicable to the rather water insoluble epoxide **1**. The results of a comparative study between the BHKR of *rac*-**1**, using a phosphate buffer solution on one hand or plain water on the other hand, are reported on Figure 1. Interestingly no significant difference was observed between these two experiments, indicating that a salt free process could also be used in this case.

2.1.2. Optimisation of the substrate concentration. As far as the practical/industrial applicability of such a process is concerned, one major bottleneck obviously could be the usable substrate concentration. We have determined that the aqueous solubility of **1** is about 0.7 g/L, precluding any high concentration application using a monophasic aqueous medium. Addition of a water miscible solvent, i.e., DMSO as described in our preliminary results, obviously would allow to slightly increase the aqueous concentration, but only to a very limited extend keeping in mind that this solvent is only tolerated by the enzyme at less than about 10% concentration (at this concentration the residual activity is about 70%). Therefore, we decided to explore the possibility to set up a 'high concentration' two-phase reactor, the substrate playing itself the role of the organic phase. We have already described the possibility to efficiently operate such a two-phase BHKR reaction in the case of *para*-bromo-α-methylstyrene oxide[9] or *para*-chlorostyrene oxide,[5] for which a substrate concentration as high as 80 and 300 g/L, respectively, could be reached. It should be noted that these two reactions were carried out at low temperature (4 and 0 °C respectively) in order to limit the spontaneous chemical hydrolysis of these substrates. In the present case, epoxide **1** appeared to be very stable towards chemical hydrolysis (less than 0.1% per hour), thus allowing the preparative experiments to be carried out at nearly room temperature (27 °C).

The possibility to increase the substrate concentration was explored in the range of 2 mM–3.6 M concentration. The reaction was performed using plain water at a constant enzyme/substrate ratio of 93 U/mmol of *rac*-**1**, and was followed by monitoring the ee of the remaining epoxide at 20, 40 and 60 min reaction time periods (Fig. 2). Interestingly, it thus appeared that (a) the reaction did very nicely proceed in such a two-phase system (b) no mass transfer limit was apparent (c) the BHKR reaction was very efficient



Scheme 2.

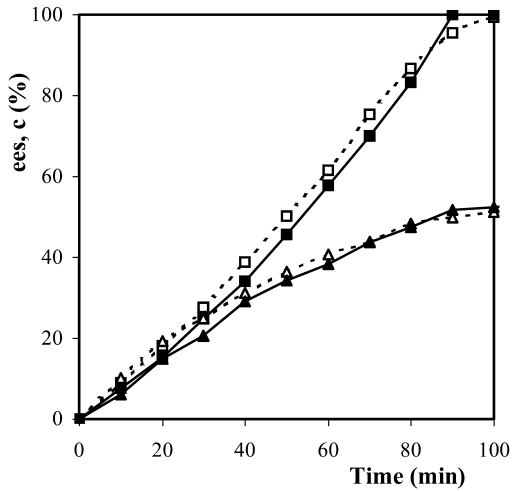


Figure 1. Comparative study between the BHKR of *rac*-**1** using a phosphate buffer solution or plain water. Full line: phosphate buffer; dashed line: plain water; squares: ee of **1**; triangles: conversion ratio c.

up to a substrate concentration as high as 2.5 M (500 g/L) (d) above this value, the reaction rate slowed down noticeably (e) surprisingly—at an identical reaction time—the ee of the remaining epoxide appeared to reach a higher value for experiments conducted at higher substrate concentration. Thus, a comparative study, carried out in a homogeneous system (at 2 mM, i.e., 0.4 g/L) on one hand, and using a two-phase system (2.5 M, i.e., 500 g/L) at the other hand, clearly showed that at high substrate concentration the rate of reaction was faster by a factor of about 1.5 than at low concentration. In these cases, the ee of the remaining epoxide reached nearly 100% after only 40 min in the biphasic system, whereas 60 min were necessary to reach the same value for the monophasic system (Fig. 3). A more detailed study of this interesting effector effect, which will be published elsewhere, showed that this activation was due to the presence of diol **2** formed during biohydrolysis.

2.1.3. Optimisation of the enzyme over substrate ratio. One other important aspect of the cost effectiveness of such a bioprocess is obviously the amount of enzyme necessary to run the reaction, and therefore, the enzyme/substrate ratio is one of the important factors to be optimised. This is particularly true in the case of epoxide resolution, racemic epoxides being generally relatively cheap compounds. We

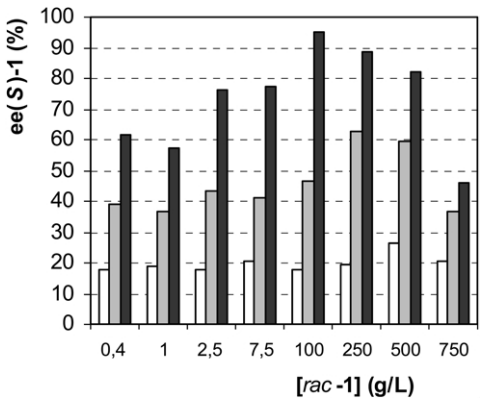


Figure 2. Substrate concentration increase. The enzyme/substrate ratio was kept constant at 93 U/mmol of *rac*-**1** throughout the scale up. Reaction time: white box: 20 min; grey box: 40 min; black box: 60 min.

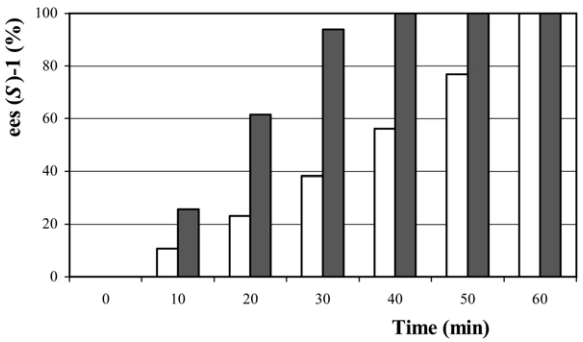


Figure 3. Influence of the substrate concentration on the ee of the residual epoxide-**1** as a function of time. □ %ee of (*S*)-**1** at 0.4 g/L (2 mM); ■ %ee of (*S*)-**1** at 500 g/L (2.5 M).

therefore have explored the possibility to decrease the enzyme/substrate ratio, and the results we have obtained are shown in Figure 4. It appeared that the resolution process could be carried out even when the enzyme/substrate ratio was decreased from 100 to 25 U/mmol of *rac*-**1**, the reaction time being then increased from 60 to 300 min for reaching the 50% conversion ratio (ee>99%).

2.2. Gram scale reactor

Using these optimised experimental conditions, a gram scale preparative experiment was conducted at a 2.5 M (500 g/L) concentration in a two-liquid-phase reactor. This was performed using 2 g of *rac*-**1** in the presence of 275 U of crude recombinant *A. niger* epoxide hydrolase (corresponding to 25 mg of crude powder at 11 U/mg) i.e., an enzyme/substrate ratio of 28, dissolved in 1.8 mL plain water and 0.2 mL DMSO. This was stirred for 2 h at 27 °C, and the reaction was quenched by addition of 1 mL of acetonitrile to the reaction medium. Unreacted **1** was extracted with pentane (3×15 mL), whereas diol **2** was further extracted with ethyl acetate (3×15 mL). Both products were purified by flash chromatography, then by bulb-to-bulb distillation, which led to 0.83 g of (*S*)-**1** [99.9% ee; 41.5% yield; $[\alpha]_D^{25}=+46.5$ (*c* 1; THF)], and to 0.95 g of diol (*R*)-**2** [94.5% ee; 43.5% yield; $[\alpha]_D^{25}=+3.4$ (*c* 1; THF)]. This experiment allowed us to determine (a) the turnover frequency (TOF) of the enzyme against **1**, which was

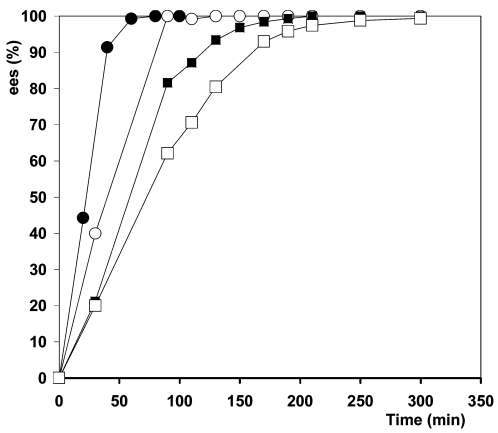


Figure 4. Influence of the enzyme/substrate ratio on the ee of the residual epoxide-**1** as a function of time at an initial concentration of 500 g/L. The enzymatic powder used throughout this study showed an activity of 9.6 U/mg. Enzyme/substrate ratio (U/mmol): ●, 100; ○, 50; ■, 37.5; □, 25.

estimated to be about 1000 h^{-1}; (b) the total turn over number (TON) which was about 21,500 and (c) the space time yield of the reactor which was about 2850 g/L/day. As can be seen, these values are very satisfactory and indicate that the industrial implementation of such a process could be interestingly envisaged (Scheme 3).

As a complement to this process it is to be stressed that, as we have described previously,[8] it is possible to chemically cyclise the obtained chloro-diol (*R*)-**2** into epoxy-alcohol (*R*)-**3**, which can itself be transformed back into epoxide (*S*)-**1** via treatment with triphenylphosphine in carbon tetrachloride (Scheme 3). Both these reactions were shown to occur without loss of stereochemical integrity. Thus, by pooling the 41.5% of the unreacted and the thus obtained (*S*)-**1** epoxide, it is possible to render this BHKR strategy enantioconvergent (i.e., to overcome the 50% yield limitation linked to a classical resolution process), the epoxide (*S*)-**1** of 100% ee being easily obtained by performing a second-round BHKR of the thus obtained highly enantiomerically enriched substrate.

3. Conclusion

The aim of this work was to optimise the experimental conditions of the BHKR of epoxide *rac*-**1**. This epoxide is a key building block for the synthesis of D0870, a triazole drug derivatives known to display efficient activity against infections—as for instance fluconazole-resistant oro-oesophageal candidiasis—often encountered in HIV-infected (and other immunocompromised) patients. By using our commercially available (recombinant) *A. niger* epoxide hydrolase as biocatalyst, we have shown that (a) this enzyme was very active against this particular substrate, (b) this BHKR could be performed at nearly room temperature, in plain water (and 5% DMSO) i.e., using a salt free methodology (c) a two-phase reactor could be conducted at a concentration as high as 2.5 M (i.e., 500 g/L) without apparent substrate and/or product inhibition (d) both the unreacted epoxide (*S*)-**1** and the formed chloro-diol (*R*)-**2** could be obtained in nearly enantiopure form and nearly quantitative yield. Interestingly, both the TOF and the TON, as well as the space time yield of this reaction, were excellent.

Moreover we have previously shown that (*R*)-**2** could be easily transformed chemically into (*S*)-**1** without loss of stereochemical integrity. Thus, in this particular case, it is possible to set up an enantioconvergent process, i.e., to overcome the theoretical 50% yield limitation theoretically linked to a resolution process. This is an additional illustration to the fact that this novel green chemistry type of process should offer very interesting opportunities for industrial preparation of both enantio-enriched epoxides and of their corresponding vicinal diols. Further work is going on in our laboratory in order to scale up this type of process to a multigram scale and to explore other possible applications of this two phase methodology.

4. Experimental

4.1. General

Gas chromatography (GC) analysis were performed using the chiral column Lipodex G (25 m×0.25 mm, Macherey–Nagel, 1 kg/cm^2 helium). NMR spectra were recorded in $CDCl_3$ using a Bruker 250 MHz instrument. The crude powders of the recombinant *A. niger* epoxide used throughout this study respectively showed an activity of 7, 8 and 11 U per mg against styrene oxide (Units are expressed in μmol/min/mg of powder), as measured by our new (sodium periodate based) spectrophotometric assay.[10]

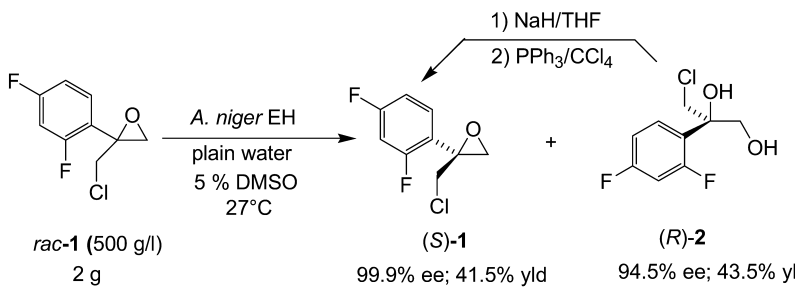
4.1.1. Synthesis of 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane 1. This was synthesised following the procedure described by Murakami and Mochizuki.[11] This epoxide was obtained as a low-melting solid. Mp=42 °C. ^{1}H NMR δ: 2.9 (d, *J*=5 Hz, 1H, OCH_2), 3.2 (d, *J*=5 Hz, 1H, OCH_2), 3.7 (d, *J*=12 Hz, 1H, CH_2Cl), 4.1 (d, *J*=12 Hz, 1H, CH_2Cl), 6.9 (m, 2H, ArH), 7.4 (m, 1H, ArH); GC-condition: 100 °C; (*R*)-**1**=28.7 min; (*S*)-**1**=29.7 min.

4.2. Comparative study between the BHKR of *rac*-1 conducted in phosphate buffer solution or in plain water

8 mg of chloro-epoxide **1** dissolved in 2 mL of DMSO were mixed to 18 mL of plain water (or pH 7 phosphate buffer, 0.1 M). To this solution, 0.5 mg of *A. niger* EH powder (7 U/mg solid) dissolved in 100 μL of water were added and the medium was stirred at 27 °C. Aliquots of 400 μL were withdrawn at different time intervals; 600 μL of acetonitrile were added to stop the reaction. The epoxide was extracted with 600 μL of isooctane containing 4-bromo-acetophenone (2 mM) as internal standard and analysed by chiral GC for conversion ratio and ee.

4.3. Optimisation of the substrate concentration

General procedure. These enzymatic reactions were carried



Scheme 3. Optimal biocatalysed hydrolytic kinetic resolution of *rac*-**1**, using a two-phase process at a substrate concentration of 500 g/L (i.e., 2.5 M).

out at 27 °C in microvials containing an adapted final volume of 1000–50 μL as a function of the substrate concentration (0.4–750 g/L). To different substrate emulsions in plain water containing 10% of DMSO a calculated enzyme extract quantity (7 U/mg solid) was added in order to keep constant the enzyme/substrate ratio at 93 U/mmol of *rac*-**1**. The determination of the ee of the residual epoxide after 20, 40 and 60 min of reaction time was carried out as described above using one microvial for each value.

4.4. Optimisation of the enzyme over substrate ratio

This study was carried out at 27 °C in microvials containing a final volume of 50 μL. In each vial 25 mg of chloro-epoxide **1** were mixed at 27 °C to 25 μL of plain water containing 10% of DMSO and different quantity of enzyme powder (7 U/mg solid; enzyme/substrate ratio: 25–100 U/mmol). Very small aliquots were withdrawn at different time intervals and analysed as described above.

4.4.1. Preparative scale resolution of *rac*-1 at 0.4 g/L. 400 mg of chloro-epoxide **1** dissolved in 100 mL of DMSO were mixed to 900 mL of phosphate buffer (0.1 M, pH 7). The biohydrolysis was started by addition of 13 mg of *A. niger* powder (8 U/mg). The medium was stirred and maintained at 27 °C. When the ee of **1** reached about 98% (4 h, 45 min) the reaction was stopped by adding 250 mL of acetonitrile. The medium was saturated with NaCl and then extracted with AcOEt (4×200 mL). After drying over $MgSO_4$ and concentration in vacuum (*S*)-**1** [163 mg, 41% yield, $[\alpha]_D^{26}$=+45 (*c* 1.2; THF)] and (*R*)-**2** [163 mg, 38% yield, $[\alpha]_D^{26}$=+3.6 (*c* 1; THF); ^{1}H NMR δ: 2.2 (dd, *J*=5.4, 7.7 Hz, 1H, OH), 3.3 (s, 1H, OH), 3.8–4.1 (m, 4H, CH_2Cl and CH_2O), 6.7–7.0 (m, 2H, ArH), 7.6–7.75 (m, 1H, ArH)] were separated and purified by flash chromatography then bulb-to-bulb distillation (130 °C, 5 mbar for **1** and 200 °C, 0.1 mbar for **2**). The diol **2** was obtained as a viscous oil.

4.4.2. Synthesis of (*R*)-1-hydroxy-2-(2,4-difluorophenyl)-2,3-epoxypropane 3 from (*R*)-2. To a stirred solution of 73 mg (0.33 mmol) of (*R*)-**2** in dry THF (4 mL) cooled on an ice bath, were added 20 mg (0.5 mmol) of sodium hydride (60% mineral oil dispersion). The resulting suspension was then agitated at 0 °C for 2 h. After addition of water (2 mL), the mixture was extracted twice with ether (5 mL). The combined extracts were washed twice with a saturated NH_4Cl solution (2 mL) then twice with brine (2 mL) and dried over magnesium sulfate. After concentration in vacuum and purification by bulb-to-bulb distillation (0.1 mbar, 200 °C) 59 mg of (*R*)-**3** (96% yield) were isolated. $[\alpha]_D^{26}$=+44.6 (*c* 1; THF); lit.[11] for (*S*)-**3**: $[\alpha]_D$=−42 (*c* 1; THF); GC-condition: 120 °C, (*S*)-**3**=19 min (small peak); (*R*)-**3**=20.2 min. ^{1}H NMR δ: 2.2 (s, 1H, OH), 2.85 (d, *J*=5 Hz, 1H, OCH_2), 3.3 (d, *J*=5 Hz, 1H, OCH_2), 4.0 (m, 2H, CH_2OH), 6.7–7.0 (m, 2H, ArH), 7.3–7.5 (m, 1H, ArH).

4.4.3. Synthesis of (*S*)-1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane 1 from (*R*)-3. 20 mg (0.107 mmol) of (*R*)-**3** obtained previously and 32 mg (0.122 mmol) of triphenylphosphine were dissolved in 1 mL carbon tetrachloride, and heated for 7 h under reflux.[11] After cooling and addition of water (2 mL), the mixture was extracted with methylene chloride (2 mL). The organic phase was washed with brine, dried over magnesium sulfate and evaporated. Injection on chiral GC at 100 °C led to identical retention time (29.7 min) as one recovered after biohydrolysis.

Acknowledgements

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