Microbiological Transformations. 28. Enantiocomplementary Epoxide Hydrolyses as a Preparative Access to Both Enantiomers of Styrene Oxide

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The value of epoxides as synthetic intermediates in the total synthesis of optically active natural products or drugs emphasizes the need to obtain these compounds in a high state of enantiomeric purity. One appealing way to solve this problem could be the enantioselective enzymatic hydrolysis of racemic epoxides. In fact, a tremendous amount of work has been carried out in order to elucidate the characteristics of such reactions using subcellular fractions of liver cells.[1] The epoxide hydrolases (EH) in the microsomal and cytosolic fractions have been purified and characterized,[2] and it has been shown that epoxide hydrolysis, leading to the chemically and toxicologically less active vicinal diol, constitutes a major mechanism for detoxication. A second mechanism involves the action of glutathione S-transferases which catalyse the nucleophilic attack of oxirans by glutathione, leading to water-soluble conjugates.[3] However, because of obvious practical reasons, these techniques, which have been shown to be highly enantioselective in certain cases, are of low practical value for organic synthesis. In the course of our work related to the use of microorganisms for organic synthesis, we have been interested in trying to devise processes which would use easily accessible whole-cell microbial cultures in order to achieve enantioselective epoxide hydrolysis. Our previous work has shown that an epoxide hydrolase (EH) of the fungus *Aspergillus niger* is very efficient as a hydrolytic kinetic resolving agent for racemic 6,7-epoxygeraniol *N*-phenylcarbamate, giving access to the 6*S* epoxide in a high state of optical purity.[4] We have also shown that this fungus similarly allows the separation of diastereoisomeric mixtures of 8,9-epoxylimonene.[4] In order to explore the scope of this very interesting new type of preparative microbiological transformations, it was of interest to investigate the hydrolysis of other racemic epoxides with this fungus, but also to find some other microorganisms able to achieve the same type of biohydrolysis. Moreover, another very challenging goal was to find microorganisms showing epoxide hydrolase activity of *opposite enantioselectivity* for the same substrate. This would allow very desirable approaches to the preparation of either enantiomer of a given starting substrate or to those of their hydrolysis products. To start this study, we chose styrene oxide as a substrate because it has often been used as a standard model for mammalian microsomal and cytosolic epoxide hydrases[5] thus allowing eventually comparison of the observed regio- and enantioselectivities of the microbiologically-mediated hydrolyses *versus* these mammalian enzymes mechanisms. We here describe that two microorganisms, *Aspergillus niger* (LCP 521) and *Beauveria sulfurescens* (ATCC 7159), are able to perform highly enantioselective hydrolysis of racemic styrene epoxide. Moreover, these strains were revealed to be enantiocomplementary since they achieve the hydrolysis of the styrene oxide enantiomers of *opposite configuration*.

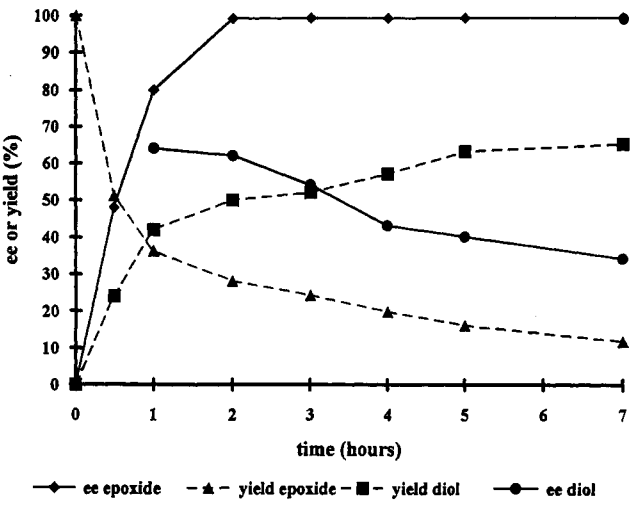
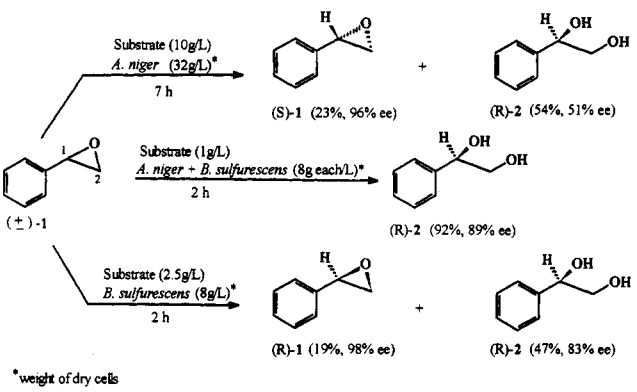


Figure 1. Yield and ee of 1 and 2 during the biohydrolysis of *rac*-1 (1 g/L) by *A. niger* (8 g/L, weight of dry cells).

Scheme I



Results

Biohydrolysis of Styrene Oxide 1 with *A. niger*. Preliminary studies were performed on an analytical scale by incubating *rac*-1 (1 g/L) with a buffered resting-cell suspension of the fungus *A. niger*. The disappearance of 1, after different incubation periods, is shown in Figure 1 and Scheme I. These results show, first, that rapid hydrolysis of 1 occurs under these conditions[6] and, second,

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(3) (a) Seidegard, J.; Depierre, J. W. *Biochim. Biophys. Acta* **1983**, *695*, 251. (b) Meijer, J.; Depierre, J. W. *Chem.-Biol. Interact.* **1988**, *64*, 207.

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(5) See, for instance: Splinter, K.; Bauer, D.; Seidel, W. *Exp. Pathol.* **1990**, *39*, 195.

(6) We have tested that only a slight chemical hydrolysis (about 2% per h) occurs in these experimental conditions using phosphate buffer (pH 8) without and with the same quantity of heat-inactivated microbial cells.

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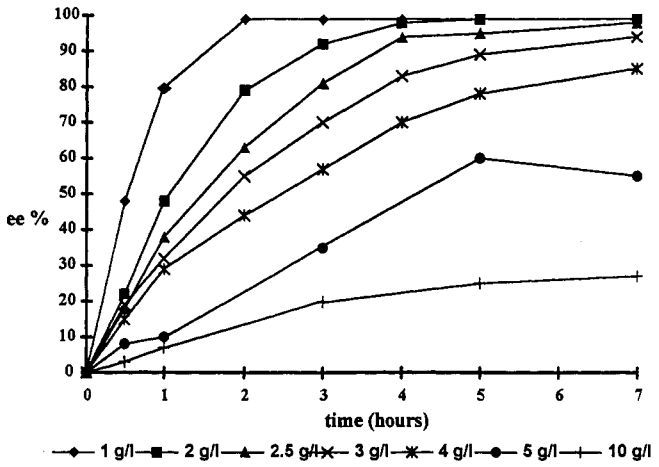

Figure 2. Effect of the substrate concentration (1–10 g/L) on the ee of 1 during the biohydrolysis of *rac*-1 by *A. niger* (8 g/L, weight of dry cells).

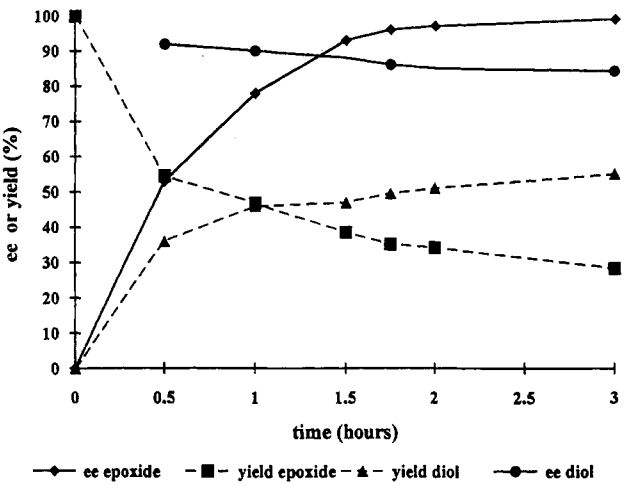

Figure 3. Yield and ee of 1 and 2 during the biohydrolysis of *rac*-1 (1 g/L) by *B. sulfurescens* (8 g/L, weight of dry cells).

that (*R*)-1 reacted much more rapidly than its (*S*) enantiomer, so that (*S*)-1 can be recovered with a very high enantiomeric excess (ee > 99%, 28% analytical yield) if the reaction is interrupted after 2 h. During the first 2 h, the formed (*R*)-diol 2 showed an ee in the range of 60–70%. After 24 h, 1 was totally transformed, leading to a 76% yield of 2 (which still showed a slight ee (10%) in favor of its (*R*) enantiomer). The ees of both 1 and 2 (measured *via* its acetonide derivative) have been determined using chiral GC. Their absolute configurations have been assigned on the basis of their optical rotation and confirmed by comparison of their elution order with those of known samples.

We have previously observed that the bioconversion of geraniol epoxide under anaerobic conditions (N_2 or Ar) prevented the remaining epoxide from hydrolysis and/or other metabolic degradation.[4] In the case of 1, we did not observe any change in the outcome of the reaction (identical yield, same ee) during the first hours of incubation when the reaction was conducted under nitrogen. After 24 h only 6% of 1 (instead of 40% in the case of geraniol epoxide) remained unchanged in the medium. This result is very probably due to the fact that the rate of enzymatic hydrolysis of *rac*-1 is higher (about eight times) than the rate of geraniol epoxide hydrolysis. Thus, nearly the total amount of 1 is probably hydrolyzed before denaturation of the fungus which is presumed to occur after a few hours of anaerobic conditions.

These very promising analytical results led us to pursue this study by exploring the range of authorized substrate concentrations. Thus, it appeared, as shown in Figure 2, that epoxide hydrolysis indeed occurs in the range of 1–10 g of substrate per L using 8 g/L of dry cell weight. In these conditions the best choice is obtained for a substrate concentration of 2.5 g/L which allows us to reach an ee higher than 95% for the remaining epoxide 1. Using higher concentrations lowers the hydrolysis rate considerably, and indicates an important inhibition phenomenon. This is probably due to a product inhibition effect. Indeed, we have shown that the same fungal cells can be reused, and are still active for hydrolysis, if the culture is filtered off in order to eliminate the water-soluble formed diol 2. We have also observed that the important factor is not the concentration of substrate itself but rather the ratio of substrate to cells. Thus, it is possible to use higher substrate concentration (up to 10 g/L) by using higher cell concentration (going from 8 to 32 g of dry cell weight per L). This led us to the conclusion that a very satisfactory preparative-scale experiment can be conducted by using 32 g (dry cell weight) of fungus—obtained from a 4-L culture—as resting cells suspended in a 1-L buffer solution; thus, 10 g of racemic 1 were resolved over a 7 h period. After normal workup, this led to a 23% isolated yield (2.3 g) of (*S*)-1 showing an ee of about 96% and to a 54% yield of (*R*)-2 (ee 51%).

Biohydrolysis with *B. sulfurescens*. In order to find different microorganisms able to carry out the biohydrolysis of styrene oxide we have tested another fungus, *B. sulfurescens*, well known to carry out biohydroxylation of nonactivated carbon atoms[7] as well as olefin epoxidation.[8] Thus, it could be presumed that this fungus might also possess the enzymatic capability to effect epoxide detoxication. The results obtained using 1 and a resting cell suspension of this fungus over different incubation times are given in Figure 3 and Scheme I. It appears that, like *A. niger*, this fungus is able to perform the hydrolysis of *rac*-1. Moreover, this biohydrolysis also appeared to be enantioselective, the remaining epoxide showing a high enantiomeric excess (ee 98%, 34% yield) after only 2 h. Surprisingly, the absolute configuration of this remaining epoxide enantiomer was opposite to the one obtained with *A. niger*, since it proved to be the (*R*) instead of the (*S*) enantiomer. Even more astonishing was the fact that the formed diol 2 was again of (*R*) absolute configuration and showed an ee of about 84% after 2 h incubation. After complete hydrolysis (24 h) 2 was not racemic, but still showed an ee of about 68% for its (*R*) enantiomer.

From a preparative point of view, a study of the acceptable substrate concentration showed (Figure 4) that, as in the case of *A. niger*, an excellent resolution (ee > 98%) of *rac*-1 can be obtained using a concentration of 2.5 g/L and 8 g/L of cells (dry weight). At higher concentration a drastic limitation is in operation. However, contrary to the previous result, this limitation was proven to be independent of the cell concentration and was presumably due to the toxicity of the substrate toward the fungus. Therefore, these conditions were selected as being the best preparative-scale choice. In a typical experiment, the

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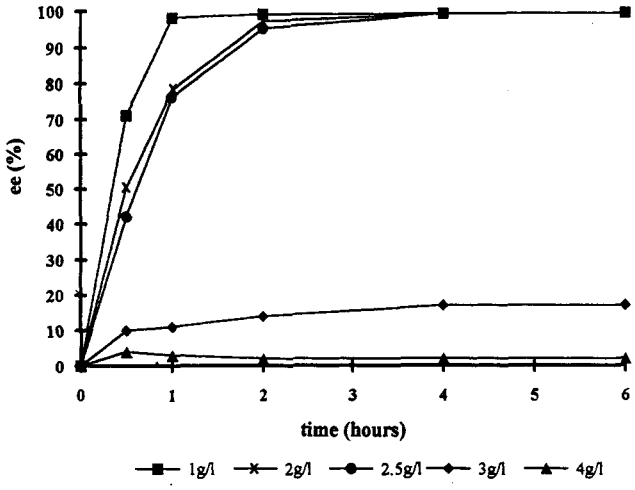

Figure 4. Effect of the substrate concentration (1–4 g/L) on the ee of 1 during the biohydrolysis of *rac*-1 by *B. sulfurescens* (8 g/L, weight of dry cells).

reaction was stopped after 2 h giving 0.47 g (19% yield) of (*R*)-1 (ee 98%) and 1.36 g (47% yield) of (*R*)-2 (ee 83%).

Biohydrolysis of 1 Using a Mixture of *A. niger* and *B. sulfurescens* Cells. From the results described above it appears that the biohydrolyses carried out with *A. niger* and *B. sulfurescens* each lead preferentially to (*R*)-2 during the early hours of reaction. Since this (*R*) diol 2 results either from hydrolysis of the (*R*) epoxide with *A. niger* or of the (*S*) enantiomer with *B. sulfurescens*, it was tempting to try to obtain (*R*)-2 in a high state of enantiomeric purity using a mixture of the two fungi. This proved to be nicely operative using 1 g of *rac*-1 and a 1/1 mixture of *A. niger* and *B. sulfurescens* cells (8 g of dry weight of each), suspended in a 1-L phosphate buffer at pH 8. As expected, complete hydrolysis of the substrate occurred within 2 h leading to a 92% yield of (*R*)-2 which showed an ee as high as 89% (Scheme I). Since both epoxide enantiomers are processed to the same diol enantiomer it is noteworthy that this reaction allows the synthesis of (*R*)-2[9] without appreciable loss of substrate.

Discussion

As far as the mechanism of these enzymatic hydrolyses is concerned, it clearly appears that both fungi operate by using an EH enzyme, since 2 is formed without important loss of starting material. As stated before, it was also possible that a glutathione S-transferase enzyme would be implied which would account for the small portion of unrecovered substrate.[9,10] In addition, it has been shown that this last type of enzymes can act enantioselectively toward racemic epoxides, a phenomenon which could have complicated interpretation of our results.[9,11] This hypothesis was, however, ruled out since we have checked by HPLC analysis[9] that no accumulation of phenyloxirane–(*S*)-glutathione conjugate was observed during the course of these hydrolyses.

Coming back to the mechanism of these hydrolyses, a common feature is the fact that both fungi are enantioselective. In regard to the stereochemical outcome of both reactions, the most obvious scheme seems to involve a *trans* opening process implying nucleophilic attack of a water molecule on the oxirane ring. However, it is obvious that the two fungal EH operate with a different regioselectivity. Thus, *A. niger* seems to behave similarly to the mammalian microsomal and cytosolic epoxide hydrases. Indeed, in order to account for retention of the C(1) absolute configuration, one should consider that both enantiomers of 1 are opened *via* attack of a water molecule at the less substituted oxirane carbon atom C(2), the (*R*) enantiomer reacting more rapidly than its (*S*) antipode.[12] Interestingly, both these stereochemical features occur as a general rule for the mammalian enzymes. On the contrary, the *B. sulfurescens* EH seems to involve a more complicated mechanism and leads to very peculiar results as far as stereochemical features are concerned. In this case, the most obvious explanation would be to consider, in order to explain the high ee of the obtained (*R*)-2 diol, first, that opening of the faster reacting (*S*) enantiomer must involve nucleophilic attack at the more substituted carbon atom C(1), thus leading to inversion of configuration to afford the (*R*)-2 diol and, second, that, as in the case of *A. niger*, the slower reacting (*R*) enantiomer is still opened *via* nucleophilic attack at the less substituted C(2) carbon atom.

In conclusion, these results show clearly that the two fungi *A. niger* and *B. sulfurescens* are able to achieve an enantioselective hydrolysis of *rac*-1. In addition, these two microorganisms present an opposite enantioselectivity for the styrene oxide enantiomers. This allows both the (*S*) enantiomer (with *A. niger*) and the (*R*) antipode (with *B. sulfurescens*) of styrene epoxide 1 to be obtained in a high state of enantiomeric purity. It should be noted that these two microbiological transformations can be easily carried out on large-scale quantities, thus allowing production of several grams of either enantiomer. Surprisingly enough, in spite of this opposite enantioselectivity toward the epoxide, the absolute configuration of the product diol is not opposite, since the (*R*) enantiomer is obtained in either case. An application of this interesting observation was, by carrying out the biohydrolysis with a mixture of the two fungi, to devise a process allowing the high yield production of (*R*)-2 which was obtained with an ee as high as 89%. Work is in progress in our laboratory in order to explore the scope and limitations of this very interesting biohydrolysis of epoxides, as well as to gain some more insight into the nature of these enzymes as well as of the mechanism involved in these bioconversions.

Experimental Section

General. The strain of *A. niger* used in this work is registered at the Museum d'Histoire Naturelle (Paris) under no. LCP 521 (Lab. de Cryptogamie, 12 rue Buffon, 75005 Paris, France). Cornsteep Liquor (CSL) is from Roquette S.A. Vapor-phase chromatography analyses were performed by using a classic 25-m capillary column (OV 1701) at 130 °C, a chiral 25-m capillary column[13] Lipodex E [octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-

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cyclodextrin in OV 1701 (1/1)] at 90 °C for the determination of enantiomeric excess of styrene oxide (elution order: (*S*)-1 t_R = 6 min; (*R*)-1 t_R = 6.7 min; α = 1.13) or a heptakis(6-*O*-methyl-2,3-di-*O*-pentyl)-β-cyclodextrin at 80 °C for the determination of enantiomeric excess of diol *via* its acetonide derivative (elution order: (1*R*)-2 t_R = 21.5 min; (1*S*)-2 t_R = 22.6 min; α = 1.05). Separation and purification of the products were achieved by flash chromatography (silica gel 60 H from Merck and solvent mixtures consisting of pentane and ether in the range of 100% pentane to 100% ether) or by bulb-to-bulb distillation. Melting points are uncorrected.

Biohydrolysis of Styrene Oxide with *A. niger*. Analytical Biohydrolysis Experiments. Hydrolysis of racemic styrene oxide were carried out in Erlenmeyer flasks (0.5 L) containing phosphate buffer (0.1 L, 0.1 M, pH 8) and 10% by weight of a fungal cake obtained as described previously[4] from a 2-L fermentor. A solution of styrene oxide (range of 0.1–1 g) in EtOH (1 mL) was poured into the medium, and the flasks were stirred at 27 °C. The course of the bioconversion was followed by withdrawing samples (2 mL) at time intervals. After saturation with NaCl the samples were extracted each with a solution of ethyl acetate (2 mL) containing tetradecane as an internal standard for subsequent direct GC analysis of epoxide and diol. At the same time, samples (2 mL) were extracted with pentane aliquots and 1 was purified through silica gel chromatography before chiral GC analysis. The ee's of the diols were determined by chiral GC analysis of their acetonide derivatives after ether extraction of diol, purification through silica gel, and derivatization.

Preparative Biohydrolysis Experiment. The large-scale bioconversion was carried out in a 7-L fermentor (SETRIC) jar containing 4 L of culture medium (20 g of CSL, 10 g of glucose in 1 L of tap water). Before sterilization (30 min at 115 °C), 0.5 g of pluronic PE 8100 (BASF) and 0.2 mL of antifoam silicone 426 R (PROLABO) were added to prevent overflowing during the growth. The medium was maintained at 27 °C, stirred at 500 rpm, and aerated with sterilized air (60 L/h). The broth was inoculated by transferring a piece of gelose (about 5 cm^2 of area) supporting the mycelium and the black spores from a 3-day-old agar slant of *A. niger* (LCP 521) to the medium. After incubation for 43 h, the mycelium was filtered off, washed with water, and then transferred to a 2-L fermentor containing 1 L of a pH 8 (0.1 M) phosphate buffer. The medium was stirred at 1100 rpm and maintained at 27 °C. Racemic 1 (10 g) as a solution in ethanol (20 mL) was added to the medium. After incubation for 7 h, the reaction was stopped by addition of ether. The medium was filtered off, and the fungal cake was separately extracted two times with ether. After decantation, the aqueous phase was saturated with NaCl and then continuously extracted with dichloromethane (20 h). The combined organic layers were dried ($MgSO_4$) and evaporated in vacuum. 1 and 2 were separated and purified from the crude product by bulb-to-bulb distillation (110 °C, 5 mm for 1; 150 °C, 0.1 mm for 2). (*S*)-1 (2.3 g, 23%) (ee 96%) was obtained as a colorless oil $[\alpha]^{25}_D$ −14.3 (*c* 2.26, acetone) (lit.[14] $[\alpha]^{20}_D$ −6.8 (*c* 4.9, acetone)) and the (*R*)-phenylethanediol 2 (6.2 g, 54%) (ee 51%) as a white solid: mp 55 °C; $[\alpha]^{25}_D$ = −20.9 (*c* 2.36, EtOH) (lit.[15] $[\alpha]^{8}_D$ −39.6 (*c* 2.44, EtOH)).

Biohydrolysis of Styrene Oxide with *B. sulfurescens*. Analytical Biohydrolysis Experiments. The experimental conditions used were the same as described above for biohydrolysis of styrene oxide with *A. niger* except that the fungal cake was obtained as described in the following Preparative Biohydrolysis experiment.

Preparative Biohyrolysis Experiment. The fermentation was carried out in a 2-L fermentor as previously described except the broth was inoculated by transferring 10 mL of a 3-day-old preculture of *B. sulfurescens* (ATCC 7159) to the medium. After 48 h of growth, the mycelium was filtered off, washed with water, and then replaced in the same fermentor containing 1 L of a pH 8 (0.1 M) phosphate buffer before addition of a solution of 2.5 g of 1 in 5 mL of ethanol. The experimental conditions used were identical to those described above for the fungus *A. niger*. The course of the bioconversion was followed by the same procedure as described for the biohydrolysis by *A. niger*. After 2 h of incubation, the bioconversion was stopped by addition of ether (500 mL) in the fermentor when epoxide exhibited ee as high as 98%. Workup of the reaction was identical to that described for the preparative biohydrolysis with *A. niger*. (*R*)-1 (0.47 g, 19%) (ee 98%) was obtained as a colorless oil $[\alpha]^{25}_D$ +5.6 (*c* 2.47, acetone) (lit.[14] $[\alpha]^{20}_D$ +6.7 (*c* 4.9, acetone)) and (*R*)-2 (1.36 g, 47%) as a white solid: mp 55–56 °C; $[\alpha]^{25}_D$ −29.4 (*c* 2.46, EtOH) (lit.[15] $[\alpha]^{8}_D$ −39.6 (*c* 2.44, EtOH)).

Biohydrolysis of Styrene Oxide with a Mixture of *A. niger* and *B. sulfurescens* Cells. The fermentation of each fungus was carried out in a 2-L fermentor jar as described previously. After 43 h of growth for *A. niger* and 48 h of growth for *B. sulfurescens*, the cultures were filtered off and the two fungal cakes were washed with water and transferred to a 2-L fermentor containing 1 L of a pH 8 (0.1 M) phosphate buffer before addition of a solution of 1 (1 g) in EtOH (5 mL). After 2.5 h the reaction was stopped by adding ether (500 mL). The mycelium was filtered off and the fungal cake washed with ether. After decantation the aqueous phase was continuously extracted with dichloromethane (20 h). The combined organic layers were dried ($MgSO_4$) and evaporated in vacuum. The crude product was purified by flash chromatography to afford 1.06 g (92%) of (*R*)-2 (ee 89%): mp 62 °C; $[\alpha]^{25}_D$ −32.5 (*c* 2.5, EtOH).

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