Pergamon

Tetrahedron: Asymmetry, Vol. 8, No. 16, pp. 2749–2755, 1997

Printed in Great Britain
PII: S0957-4166(97)00335-2
0957-4166/97 $17.00 + 0.00

Preparation of (*S*)-naproxen by enantioselective hydrolysis of racemic naproxen amide with resting cells of *Rhodococcus erythropolis* MP50 in organic solvents[1]

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Abstract: Racemic naproxen amide (*RS*)-**1** was hydrolyzed to enantiomerically pure naproxen (*S*)-**2** in water-saturated organic solvents with log *P* values between 2.0 and 0.17 using immobilized resting cells of the bacterial strain *Rhodococcus erythropolis* MP50. Alkyl acetates are the most suitable solvents concerning solubility of (*RS*)-**1** and enzyme activity. In butyl acetate with 3 vol% DMSO as cosolvent and an increased water content of the immobilized cells the rate of hydrolysis was an optimum. Under these reaction conditions a preparative scale application gave after 45 h, at a conversion rate of 48%, (*S*)-**2** with >99% *ee* and an isolated yield of 84%. © 1997 Elsevier Science Ltd

2-Arylpropanoic acids are an important class of nonsteroidal, anti-inflammatory compounds.[2] Several representatives of this class of compounds, such as naproxen, ibuprofen, ketoprofen or flurbiprofen, have been successfully introduced as pharmaceuticals. In all cases, the (*S*)-enantiomers are more effective drugs than the racemates. In case of naproxen, for example, the (*S*)-enantiomer is 28 times more active than the (*R*)-enantiomer.[3] For this reason, many investigations have been carried out with the objective of preparing 2-arylpropanoic acids in the enantiomerically pure (*S*)-configuration.

In the last few years, many stereoselective syntheses of optically active 2-arylpropanoic acids have been described.[4] The major chemical procedures for the preparation of optically active 2-arylpropionic acids are summarized in a recent review.[4f] A resolution of racemic 2-arylpropanoic acids can be achieved by enzymatic methods. With esterases or lipases, for example, enantioselective hydrolysis of the corresponding racemic esters and enantioselective esterification of the racemic carboxylic acids, respectively, have been described.[3,5]

The first patents concerning the kinetic resolution of racemic 2-arylpropionitriles with resting cells of *Brevibacterium* or *Corynebacterium* were published in 1989.[6] In the years after, several papers have been published on the enantioselective hydrolysis of racemic 2-arylpropionitriles with resting cells.[7]

For the preparation of the pharmaceutically most important (*S*)-naproxen, we have developed an enantioselective hydrolysis of racemic naproxen nitrile,[8] using new bacterial isolates enriched from soil samples.[9] Although the optical and the chemical yields by this method are excellent,[8] the procedure is unsatisfactory for the preparation of (*S*)-naproxen in larger quantities, because of the low space–time yield, which is mainly due to the very poor solubility of naproxen nitrile in water.[8] For this reason, we have now investigated the enantioselective hydrolysis of racemic naproxen amide with resting cells in organic solvents.

Solvent dependence of the activity of resting cells of *Rhodococcus erythropolis* MP50

By enrichment in a two-liquid-phase system with pristane (2,6,10,14-tetramethylpentadecane) as the organic phase, two interesting nitrile hydrolyzing bacterial strains were isolated — strain C3II and

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† Part of dissertation, Universität Stuttgart, 1996.
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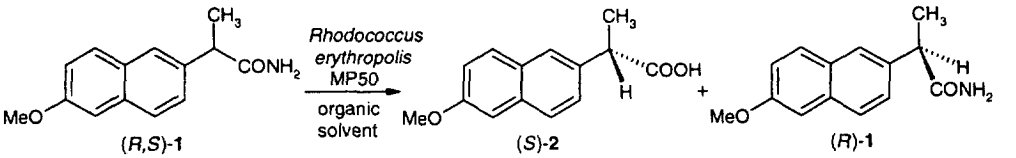
strain MP50 — which were identified as rhodococci.[9] Cells of both strains convert racemic naproxen nitrile via naproxen amide to (*S*)-naproxen.[9] The strain C3II yields (*S*)-naproxen amide first and subsequently (*S*)-naproxen. The strain MP50 gives at first predominantly (*R*)-naproxen amide and then (*S*)-naproxen. With both strains, racemic naproxen amide can be converted to (*S*)-naproxen with an enantiomeric excess ≥99% at a conversion rate up to 100% of the theoretical value.[8,9]

First investigations of naproxen nitrile hydrolysis in organic solvents have shown, that by both bacterial strains naproxen nitrile was not hydrolyzed, i.e. both strains have no nitrile hydratase activity in organic solvents.

Since the amidase activity of *Rhodococcus erythropolis* MP50 was found to be markedly higher than that of *Rhodococcus* sp. C3II,[9] we have focused our further investigations on the enantioselective hydrolysis of racemic naproxen amide (*RS*)-**1** to (*S*)-naproxen (*S*)-**2** in organic solvents exclusively on the catalysis with resting cells of strain MP50 (Scheme 1).



Scheme 1.

It is well known, that polar organic solvents can deactivate enzymes.[10a] This effect is also known for resting cells. Despite various attempts to clarify the correlation between solvent properties, especially its polarity, and deactivation of enzymes in resting cells,[10b,c] a prediction of these effects is still difficult.[10a] Since cell immobilization can prevent deactivation and clumping of cells in organic solvents, we have immobilized the resting cells of *Rhodococcus erythropolis* MP50 in polyurethane PU3 (see experimental part).[8,11]

The organic solvents applied should therefore combine high solubility for (*RS*)-**1** with lowest possible solvent polarity. As a measure for the deactivating effect of solvent polarity on enzymes,[10a] the log *P* values of the corresponding solvents can be used.

Table 1 summarizes the solubility of naproxen amide (*RS*)-**1** in water-saturated organic solvents and the initial activity of immobilized resting cells of strain MP50 in the hydrolysis of (*RS*)-**1**.

While the immobilized resting cells of strain MP50 were inactivated in ethyl methyl ketone and in dichloromethane immediately (Table 1), a complete inactivation in *tert*-butyl methyl ether and in benzene was observed within 24 hours. Although the initial activities in methyl acetate and

Table 1. Solubility of naproxen amide **1**[a] in various water-saturated organic solvents and the initial activities of immobilized resting cells of *Rhodococcus erythropolis* MP50 in these solvents

Solvent	log *P*	Solubility of **1** (mM)	Activity (U/mg)[b]
benzene	2.0	5.5	$5.5 \cdot 10^{-4}$
butyl acetate	1.7	23.6	$9.7 \cdot 10^{-4}$
tert-butyl methyl ether	1.4	6.4[c]	$6.4 \cdot 10^{-4}$
cyclohexanone	0.96	96.3	$2.5 \cdot 10^{-4}$
dichloromethane	0.8	64.1	0
ethyl acetate	0.68	64.9	$5.5 \cdot 10^{-4}$
ethyl methyl ketone	0.29	203.3	0
methyl acetate	0.17	69.1	$2.7 \cdot 10^{-4}$

[a]Solubility of (*RS*)-**1** in phosphate buffer 0.3 mM. [b]Activity referred to the dry weight of resting cells (57.7 mg) used for immobilization, concentration of (*RS*)-**1** fixed at 6.4 mM. [c]See Gu *et al.*[3]

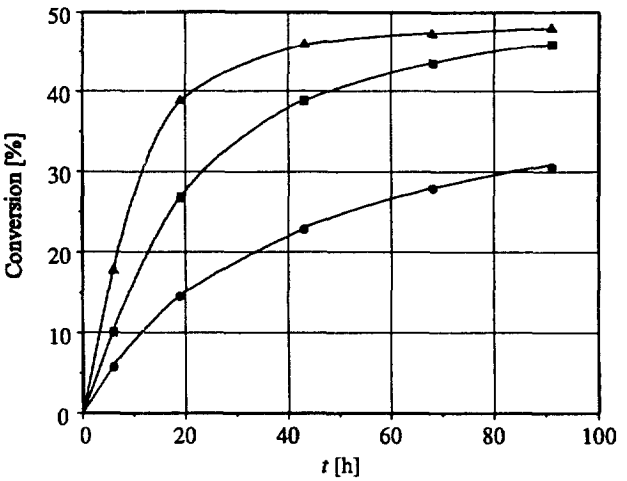


Figure 1. Hydrolysis of racemic naproxen amide (*RS*)-**1** (21.8 mM) to (*S*)-naproxen (*S*)-**2** with resting cells of strain MP50 (33.1 mg dry weight) in 10 ml of methyl acetate (●), ethyl acetate (■), and butyl acetate (▲) at 30°C.

cyclohexanone are similar, only low conversions were obtained in cyclohexanone indicating partial deactivation in this solvent. In alkyl acetates, however, conversion rates >40% were obtained. With, in comparison to phosphate buffer (0.3 mM), significantly increased solubility of (*RS*)-**1** (64.9 mM), combined with high enzyme activity, ethyl acetate appeared especially attractive for a large scale preparation of (*S*)-naproxen (*S*)-**2**. Indeed, the hydrolysis of racemic naproxen amide in ethyl acetate provided the enantiomerically pure (*S*)-naproxen (*S*)-**2** (>99% *ee*) at nearly quantitative conversion rate with an isolated yield of 68% of the theoretical value.

Table 1 shows some discrepancies between the log *P* values and the observed biocatalytic activities of the immobilized resting cells. Whereas resting cells of MP50 were immediately inactivated in ethyl methyl ketone (log *P*=0.29), only a diminished activity was observed over several days in the even more polar methyl acetate (log *P*=0.17). The interaction of the solvents and the polyurethane, used for immobilization, must be decisive for this result. With exception of alkyl acetates, in all other solvents the polyurethane polymer is swelling.

In many cases isolated enzymes are more active and easier to handle than resting cells. Therefore we have tried to isolate the amidase (EC 3.5.1.4) from *Rhodococcus erythropolis* MP50. The enzyme could be isolated in pure form by anion exchange chromatography.[12] Despite immobilization on Avicel cellulose the cell-free enzyme was nearly completely inactivated in ethyl acetate in 2 hours. In this solvent immobilized resting cells are stable for several days. Therefore, the cell-free amidase cannot be used for the preparation of (*S*)-naproxen in organic solvents.

Optimization of the enantioselective hydrolysis of racemic naproxen amide (*RS*)-1 in alkyl acetates

Due to the results described before we concentrated the optimization of the process to alkyl acetates as solvents. Comparative hydrolyses of naproxen amide (*RS*)-**1** in water-saturated butyl, ethyl and methyl acetate were carried out with immobilized resting cells of strain MP50 (Figure 1).

The initial activity of the biocatalyst decreases in the order butyl>ethyl>methyl acetate corresponding to the solvent polarity (see Table 1). This difference of activities of the cells in the various alkyl acetates is observed during the whole course of the reactions and results in decreasing conversion rates from butyl to methyl acetate. The enantiomeric excess, however, was not influenced by the enzyme activity. (*S*)-Naproxen was obtained in all cases with >99% *ee*.

It has been reported that dimethyl sulfoxide (DMSO) as a cosolvent accelerates markedly enzyme-

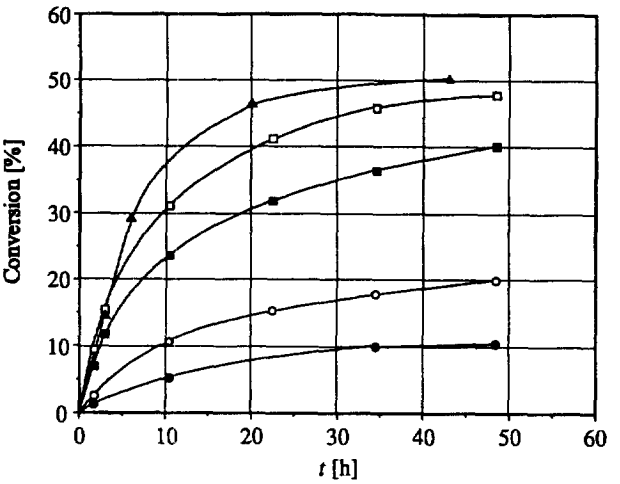


Figure 2. Hydrolysis of (*RS*)-**1** (23.9–24.2 mM) to (*S*)-**2** at 30°C with immobilized resting cells (23.6 mg dry weight) in 10 ml alkyl acetate with DMSO as cosolvent: ethyl acetate +1 vol% DMSO (○), ethyl acetate +3 vol% DMSO (●), butyl acetate +1 vol% DMSO (□), butyl acetate +3 vol% DMSO (■) and butyl acetate +3 vol% DMSO +0.54 ml water (▲). The water uptake (0.54 ml) results from resuspension of immobilized resting cells (23.1 dry weight) in phosphate buffer prior to use.

catalyzed transesterification reactions in organic solvents.[13] We have therefore investigated the activating effect of DMSO as cosolvent on the rate of hydrolysis of (*RS*)-**1** in water-saturated ethyl and butyl acetate. The results are summarized in Figure 2.

As can be seen from Figure 2, the cosolvent DMSO has a favourable effect only in butyl acetate as solvent by increasing the enzymatic activity compared to that in pure butyl acetate. The hydrolysis proceeds faster with 1 vol% DMSO than with 3 vol% DMSO. Under water uptake of the immobilized cells (Figure 2), however, a further enhancement of enzymatic activity is observed in the case of butyl acetate with 3 vol% DMSO. The enantiomeric excess was not influenced by the cosolvent DMSO. In all cases, (*S*)-**2** was obtained with >99% *ee*.

The activating effect of DMSO is not based on enhancement of the solubility of naproxen amide (*RS*)-**1**. The solubility of (*RS*)-**1** was only slightly increased from 23.6 mM in pure butyl acetate (Table 1) to 27.3 mM in butyl acetate and 3 vol% DMSO as cosolvent.

Using the optimized reaction conditions, we have carried out the hydrolysis of (*RS*)-**1** on a preparative scale. After 45 hours, at 48% conversion, the reaction was terminated by filtration of the immobilized biocatalyst. In order to separate (*S*)-**2** from (*R*)-**1** the evaporated filtrate was treated with NaOH solution and filtered. By acidifying the filtrate to pH 4 (*S*)-**2** precipitated. After recrystallization, enantiomerically pure (*S*)-naproxen (*S*)-**2** (>99% *ee*) was isolated with a yield of 84% of the theoretical value.

Experimental

Materials and methods

Optical density (OD): Perkin–Elmer Lambda 7 spectrometer. HPLC: Pharmacia LKB system, Chiral AGP-column (100×4 mm) (ChromTech, Norsborg, Sweden), and RP-C_{18}, Vertex column (120×4 mm), Nucleosil 100, 5 μm (Knaur). PU3 prepolymer was provided from Toyo Rubber Ind. Co., Osaka, Japan, and (*S*)-naproxen was purchased from Sigma. Racemic naproxen amide (*RS*)-**1** was prepared according to Effenberger and Böhme;[8] for media and isolation of *Rhodococcus erythropolis* MP50 see Layh *et al.*[9] In all cases immobilized cells are derived from the same bacterial culture and immobilization for one test series. All solvents were distilled prior to use.

Immobilization of resting cells

According to Effenberger and Böhme,[8] the PU3 prepolymer was heated to ca. 50°C and after cooling to room temperature, a cell suspension (OD_{546} between 100 and 290) was added. The mixture was vigorously stirred, the viscous foam erased on a glass plate and cooled to 4°C for 2–3 h. Then the polymer was cut into pieces of 3–5 mm^3.

General procedure for the enzymatic hydrolysis of (RS)-***1*** *to* (S)-***2*** *in organic solvents*

The immobilized resting cells were added to a solution of (*RS*)-**1** in the respective water-saturated solvent or solvent/DMSO mixture (10 ml) and incubated with stirring at 30°C. In order to determine the concentration of (*S*)-**2**, samples of 100 µl each were taken from the mixture, and filled up to 1 ml with ethyl acetate. 5–100 µl of them were taken, and the solvent was removed in a nitrogen stream. The residue was taken up in the respective eluent and analyzed by HPLC.

Enzymatic hydrolysis of naproxen amide (RS)-***1*** *to naproxen* (S)-***2*** *on a preparative scale in ethyl acetate*

10 ml of a cell suspension (OD 244) were immobilized in PU3 prepolymer (4 g). 4 g of immobilized resting cells (273 mg dry weight) were added at 30°C to a suspension of (*RS*)-**1** (1.15 g, 5.0 mmol) in water-saturated ethyl acetate (30 ml), and the mixture was mechanically stirred. Samples of 100 µl each were taken from the mixture. The solvent was removed, the residue taken up in 10 ml of phosphate buffer (50 mM, pH 7.4), and analyzed by HPLC. At a conversion rate of 48% the immobilized cells were filtered off and washed with 100 ml of ethyl acetate. The combined filtrates were concentrated, and the crude (*S*)-**2** chromatographed on silica gel with petroleum ether/ethyl acetate (3:7) to give 0.39 g (68%) (*S*)-**2** as white crystals. The physical data correspond to those in the literature.[14]

Enzymatic hydrolysis of (RS)-***1*** *on a preparative scale in the system butyl acetate/DMSO/water*

Immobilized resting cells (176.5 mg dry weight) were resuspended in phosphate buffer for 1 h prior to use and filtered by vacuum, resulting in an additional water uptake of ca. 4 ml. These immobilized cells were added to a stirred suspension of (*RS*)-**1** (1.15 g, 5.0 mmol) in 50 ml of butyl acetate/3% DMSO (v/v) and incubated at 30°C. As described above, samples of 100 µl were taken and prepared for HPLC analysis. For termination of the reaction the immobilized biocatalyst was filtered by vacuum, carefully pressed and washed with ethyl acetate. The combined filtrates were concentrated, and the residue dried under high vacuum. (*S*)-**2** was separated from (*R*)-**1** by extracting with 250 ml of a 1 M NaOH solution and filtration. By acidifying the filtrate with HCl to pH 4 (*S*)-**2** was precipitated, filtered off and dried. Recrystallization from ca. 500 ml of water/ethanol (8:2) gave 0.48 g (84%) (*S*)-**2** with >99% *ee*.

Analytical methods[8]

The formation of naproxen (*S*)-**2** was analyzed by HPLC. The enantiomers of naproxen were separated on a Chiral AGP-column (with guard column) with phosphate buffer (10 mM, pH 7.0) as eluent [flow rate 0.6 ml/min, R_t (*R*)-**2**=3.5 min, R_t (*S*)-**2**=5.7 min, detection wavelength λ=230 nm]. The enantiomeric excess of (*S*)-**2** was calculated by comparison of the peak areas obtained by HPLC analyses, and its identity confirmed by co-injection of an enantiopure standard. The conversion was determined on a RP-C_{18} column with phosphate buffer (50 mM, pH 2.3)/acetonitrile (40% (v/v)) as eluent [flow rate 1.0 ml/min, R_t=4.3–4.7 min, detection wavelength λ=230 nm].

Growth measurement

Growth of bacterial cultures was monitored by measuring the optical density at λ=546 nm. For the measurements, the cell suspensions were diluted with phosphate buffer in order to keep the extinction below 0.3. The optical density of 1.0 corresponded to 224 mg bacterial dry weight per liter culture.[9]

Acknowledgements

This work was generously supported by the Bundesministerium für Bildung und Forschung (Zentrales Schwerpunktprogramm Bioverfahrenstechnik, Stuttgart). We would like to thank Prof. H.-J. Knackmuss and his co-workers for providing the bacterial strain used.

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(*Received in UK 16 June 1997; accepted 28 July 1997)*