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Enantioselective hydrolysis of racemic naproxen nitrile and naproxen amide to *S*-naproxen by new bacterial isolates

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Abstract

Bacteria were enriched from soil samples with succinate as a carbon source and racemic naproxen nitrile [2-(6-methoxy-2-naphthyl)propionitrile] as sole source of nitrogen. Since naproxen nitrile was only poorly soluble in water media amended with different water-immiscible organic phases were used for the enrichments. With pristane (2,6,10,14-tetramethylpentadecane) as the organic phase two bacterial strains were isolated (strain C3II and strain MP50) which were identified as rhodococci. Cells of both strains converted naproxen nitrile via naproxen amide to naproxen. From racemic naproxen nitrile *Rhodococcus* sp. C3II formed *S*-naproxen amide and subsequently *S*-naproxen. Racemic naproxen amide was hydrolysed to *S*-naproxen. *Rhodococcus* sp. MP50 converted racemic naproxen nitrile predominantly to *R*-naproxen amide and racemic naproxen amide to *S*-naproxen. With both strains racemic naproxen amide was converted to *S*-naproxen with an enantiomeric excess > 99% at a conversion rate up to 80% of the theoretical value. In strain C3II the enzymes which hydrolysed naproxen nitrile and naproxen amide were present only at a low constitutive level. In contrast, in *Rhodococcus* sp. MP50 these activities were induced when grown in the presence of various nitriles.

Key words: Amidase; Enantioselective enzyme; Naproxen; Nitrile; Nitrile hydratase; *Rhodococcus*

1. Introduction

2-Arylpropionic acids such as ibuprofen and naproxen are important non-steroidal antiinflammatory drugs (Shen, 1972). They contain a chiral center and in vitro studies on inhibition of prostaglandin synthesis show that their activity resides almost exclusively in the *S*(+)-isomers (Caldwell et al., 1988). In the case of naproxen the *S*-form is 28-fold more active than the corresponding *R*-enantiomer (Harrison et al., 1970; Roszkowski et al., 1971).

Up to now the chiral synthesis of *S*-naproxen was achieved by chemical methods (Tsuchihashi et al., 1982; Franck and Rüchardt, 1984; Giordano et al., 1984; Castaldi et al., 1987; Parrinello and Stille, 1987; Wolber and Rüchardt, 1991; Sonawane et al., 1992; Brown, 1992) and by enantioselective hydrolysis of racemic esters of

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naproxen using microbial esterases (Gu et al., 1986; Battistel et al., 1991). In the present study the ability of two bacterial strains to produce pure *S*-naproxen (e.e. > 99%) from racemic naproxen nitrile or naproxen amide is described.

2. Materials and methods

Chemicals

Racemic naproxen nitrile (NapN) and naproxen amide (NapA) were prepared from 2-methoxynaphthalene and racemic ibuprofen nitrile from isobutylbenzene (Arsenijevic et al., 1973; Hall and Gisler, 1976; Hiyama et al., 1986). *S*-Naproxen was obtained from Sigma (Deisenhofen). *S*-NapA was synthesized from *S*-Nap via the corresponding acid chloride. Octane, nonane, pristane, heptamethylnonane and diethyl phosphoramidate were supplied from Aldrich (Steinheim, Germany). Naphthalene-2-carbonitrile and 2-naphthalenecarboxamide were purchased from Lancaster Synthesis (Eastgate, UK) and naphthalene-2-carboxylic acid from Fluka (Buchs, Switzerland). All other culture media, chemicals and apparatus were the same as described before (Layh et al., 1992).

Media

Bacterial cultures were grown in a nitrogen-free mineral medium according to Layh et al. (1992) with succinate (10 mM) as sole source of carbon and energy. Naproxen nitrile was added as sole source of nitrogen. Naproxen nitrile was dissolved in methanol (stock solution: 20 or 40 mM) or in a water-immiscible organic phase and added to the cultures to a final concentration of 0.5 mM. In certain experiments mineral media containing ammonia were used as described by Dorn et al. (1974).

Isolation of microorganisms

Bacterial cultures were isolated by enrichment from soil in Erlenmeyer flasks with baffles (300 ml with 15 ml of medium) on a rotary shaker (100 rpm). Enrichments were started with different soils taken from the area of Stuttgart (Germany) and carried out in a nitrogen free mineral salts medium containing succinate (10 mM) as carbon source, naproxen nitrile (0.5 mM) as a nitrogen source and different organic solvents (see Results). After 1 week incubation cultures were transferred (1 : 10 v/v) to fresh medium with the same composition. These cultures were transferred (after 1 week) to agar plates (mineral medium with succinate and NapN) and single colonies purified by standard methods on solid media. After two transfers single colonies were used to inoculate aqueous organic media as described above. After 9 d incubation the aqueous phases were analyzed by HPLC for the formation of naproxen (Nap). The Nap forming cultures were purified on Nutrient Broth-agar plates, transferred to succinate/NapN-agar and finally grown in liquid culture with NapN as sole nitrogen source.

Initial characterization and taxonomic classification of the isolates

Strain C3II and MP50 formed on Nutrient Broth agar plates white mucoid colonies, which reached a diameter of about 5 mm after 3 d incubation at 30°C. Strain C3II and MP50 were Gram-positive non motile rods. Strain C3II formed in aqueous cultures structures resembling the 'snapping' of coryneform bacteria (Stanier et al., 1976). The isolates were characterized by using the API Coryne test (API-System S.A., La Balme les Grottes, France). Thus strain C3II and MP50 were found to belong to *Rhodococcus equi* (probability for strain C3II = 93% and for strain MP50 = 99.8%). According to Dr. R. Kroppenstedt (Deutsche Sammlung von Mikroorganismen (DSM); Braunschweig, Germany) strain C3II belongs most probably to a yet undescribed species in the genus *Rhodococcus*.

Analytical methods

The formation of naproxen was analyzed by HPLC. If no separation of enantiomers was necessary a reverse-phase column (Grom-Sil C8; Grom, Herrenberg, Germany) was used (solvent system: 45% (v/v) acetonitrile, 54.7% (v/v) water, 0.3% (v/v) H_3PO_4). For separation of the enantiomers of naproxen (Nap) an AGP-column (with guard column) (column size: 100 × 4 mm; 5

μm silicagel particles with α_1-acid glycoprotein; ChromTech, Norsborg, Sweden) with 10 mM Na-phosphate buffer pH 7.0 as solvent was used (flow rate 0.9 ml min^{-1}; R_t (*S*-Nap) = 5.7 min, R_t (*R*-Nap) = 3.5 min). For the separation of the enantiomers of naproxen amide (NapA) a HSA-column (with guard column) (column size: 100 × 4 mm; 5 μm silicagel particles with human serum albumin; ChromTech), with 100 mM Na-phosphate buffer, pH 7.0 and 5% (v/v) isopropanol as solvent, was used (flow rate 0.9 ml min^{-1}; R_t (*S*-NapA) = 17 min, R_t (*R*-NapA) = 15 min). The enantiomeric excess (e.e.) was defined as the ratio $((R - S)/(R + S)) \times 100\%$ (or vice versa), where *R* and *S* are, respectively, the concentrations of the *R*- and *S*-enantiomers. The e.e. values were calculated by comparison of the peak areas obtained by HPLC analysis.

The identity of *S*-Nap was determined by cochromatography with an enantiopure standard (Sigma, Deisenhofen). All other chromatographic conditions have been described before (Layh et al., 1992).

For the determination of enzyme activities resting cells (A_{546nm} = 0.05–20 in 50 mM Na/K-phosphate buffer, pH 7.4) were incubated on a rotary shaker at 30°C with naproxen nitrile or naproxen amide. Every 3 min aliquots (0.15 ml) were taken, cells removed by centrifugation (3 min, 14000 rpm) and the supernatants analysed by HPLC for the formation of naproxen.

Growth measurement

Growth of bacterial cultures was monitored spectrophotometrically by measuring the optical density at 546 nm with a Kontron Uvikon 820 Spectrophotometer (Kontron, Eching, Germany). For both strains an optical density (A_{546nm}) of 1 corresponded to 220 mg of bacterial dry weight per liter of culture.

3. Results

3.1. Isolation of bacteria

Bacteria were enriched from soil in a nitrogen-free mineral medium containing succinate (10 mM) as carbon source and naproxen nitrile (0.5 mM; Fig. 1) as sole source of nitrogen. Naproxen nitrile (NapN) was only slightly soluble in aqueous media. Therefore NapN was dissolved in methanol or different water-immiscible organic solvents and the solutions added to the different enrichment cultures. Octane, nonane, pristane or heptamethylnonane (10–50% (v/v)) were used as the substrate carrying organic phases. The strains C3II and MP50 were obtained from different enrichment cultures with pristane as the organic phase. NapN-dependent growth of strain C3II and MP50 on agar plates with succinate (10 mM) and NapN (1 mM) suspended as fine particles resulted in the disintegration of crystalline NapN forming a clear halo around the colonies.

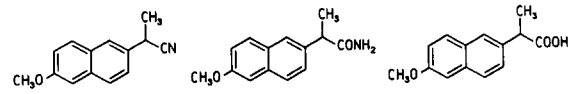

Fig. 1. Structural formulas of naproxen nitrile (left), naproxen amide (middle) and naproxen (right).

The two isolates were designated tentatively as *Rhodococcus* sp. C3II and *Rhodococcus* sp. MP50 (see Material and methods).

3.2. Formation of naproxen from naproxen nitrile by resting cells of Rhodococcus sp. C3II and Rhodococcus sp. MP50

The isolates were grown with succinate (10 mM) and NapN (0.5 mM) as nitrogen source. Cells were harvested by centrifugation and resting cells (A_{546nm} = 4–5) were incubated with NapN (0.3 mM, dissolved in methanol). By HPLC the formation of naproxen (Nap) (R_t = 4.1 min) and naproxen amide (NapA) (R_t = 2.9 min) from NapN (R_t = 7.8 min) was demonstrated. Due to the low solubility of NapN in water during almost the entire experiment the apparent concentration of NapN in the aqueous phase was constant and low (about 0.04 mM). Only at the end of the biotransformation it dropped to zero.

With both strains NapA accumulated during the first minutes and its concentration slowly decreased towards the end of the experiment. The maximal concentrations of NapA were 30–

50% of the starting concentration of NapN. Nap was not further metabolized indicating that the isolates could not use it as a carbon source.

3.3. Formation of S-naproxen from racemic naproxen nitrile or naproxen amide by Rhodococcus sp. C3II

Resting cells of strain C3II (A_{546nm} = 4.4) were incubated in two separate flasks with NapN or NapA (0.2 mM each). Within the initial 5 min turn-over of racemic NapN resulted in the predominant formation of *S*-NapA (enantiomeric excess (e.e.) = 43%). Subsequently the concentration of *S*-NapA decreased. Thereby, the e.e. of *R*-NapA increased to an e.e. value > 99%. After 15 min 40% of the racemic NapN was transformed to *S*-Nap with an e.e. > 99%. Later on small amounts of *R*-Nap appeared in the supernatant. The specific activity for the formation of *S*-Nap from NapN was only 0.0034 μmol min^{-1} and mg of dry weight. For the formation of NapA from NapN an activity of 0.15 μmol min^{-1} mg^{-1} was observed. In a parallel experiment with racemic NapA as a substrate it was found that about one half of the substrate was rapidly converted to *S*-Nap. After conversion of 39% an e.e. of 98.5% was found (Fig. 2). In both experiments the formation of *S*-Nap proceeded with almost the same velocity. It was thus concluded that the nitrile hydratase activity was significantly higher than the amidase activity and that hydrolysis of NapA to *S*-Nap was the enantioselective and rate-limiting step.

3.4. Formation of S-naproxen from racemic naproxen nitrile or naproxen amide by Rhodococcus sp. MP50

The Nap formed from NapN (0.3 mM) by resting cells of *Rhodococcus* sp. MP50 (A_{546nm} = 4.6) showed only a low enantiomeric excess for *S*-Nap (e.e. ≤ 47%). In contrast, enantiomeric pure *R*-NapA was found (e.e. > 99%). In a parallel experiment racemic NapA (0.3 mM) was incubated with resting cells of *Rhodococcus* sp. MP50 (A_{546nm} = 0.05). This resulted in the formation of pure *S*-Nap (e.e. > 99%) until 46% of the racemic

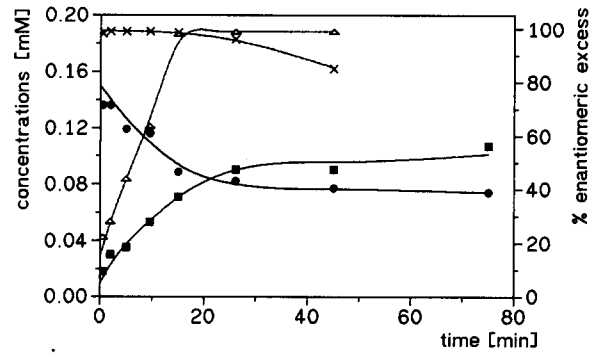

Fig. 2. Conversion of naproxen amide to naproxen by resting cells of *Rhodococcus* sp. C3II. Strain C3II was grown in a mineral medium (NH_4-containing) with succinate (10 mM) and Nutrient Broth (0.45 g l^{-1}). Cells were harvested by centrifugation and resuspended in Na/K-phosphate buffer (pH 7.4, 50 mM) to an optical density A_{546nm} = 4.4. Naproxen amide was added to a starting concentration of 0.2 mM (stock solution: 20 mM in methanol). At the indicated time intervals aliquots (0.15 ml) were taken and bacterial cells removed by centrifugation (3 min, 14000 rpm). The supernatants were analyzed using a reverse-phase column (naproxen (■); naproxen amide (●)). The e.e. values of *S*-naproxen (×) and *R*-naproxen amide (△) were calculated by comparison of the peak areas of the respective *S*- and *R*-enantiomers obtained by chiral HPLC-analysis.

NapA was converted. The remaining NapA was pure *R*-NapA (Fig. 3). The specific activity for the formation of *S*-Nap from racemic NapA was 0.74 μmol min^{-1} mg^{-1}.

3.5. Enantioselectivity of the nitrile hydratases from Rhodococcus sp. C3II and Rhodococcus sp. MP50

A dilute suspension of resting cells of *Rhodococcus* sp. C3II (A_{546nm} = 0.73) was incubated with NapN. Turnover of the amide was the rate-limiting step during biotransformation of NapN (see above). Therefore at a low cell concentration the nitrile was mainly converted to the amide and only low amounts of Nap were formed (< 2% compared to the amide). Thus it was found that preferentially *S*-NapA was formed from racemic NapN. The highest e.e. of *S*-NapA was 94% after 30% turnover.

During the turnover of NapN by *Rhodococcus* sp. MP50 the formation of enantiopure *R*-NapA had been observed (see above). This could be

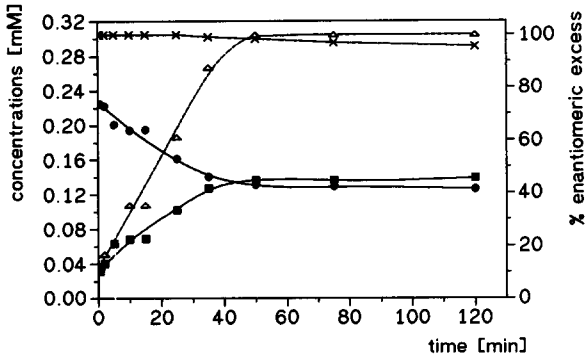

Fig. 3. Formation of *S*-naproxen from racemic naproxen amide by resting cells of *Rhodococcus* sp. MP50. Strain MP50 was grown in a mineral medium with succinate (10 mM) and naproxen amide (0.5 mM). Cells were harvested by centrifugation and resuspended in Na/K-phosphate buffer (pH 7.4, 50 mM) to an optical density $A_{546nm} = 0.05$. Naproxen amide was added to a starting concentration of 0.3 mM (stock solution: 20 mM in methanol). At the indicated time intervals aliquots (0.15 ml) were taken and bacterial cells removed by centrifugation (3 min, 14000 rpm). The supernatants were analyzed using a reverse-phase column (naproxen (■); naproxen amide (●)). The e.e. values of *S*-naproxen (×) and *R*-naproxen amide (△) were calculated by comparison of the peak areas of the respective *S*- and *R*-enantiomers obtained by chiral HPLC-analysis.

either explained by a highly enantioselective nitrile hydratase forming *R*-NapA or by the highly active enantioselective amidase which converted all *S*-NapA formed by the nitrile hydratase. To answer this question resting cells were incubated with NapN and diethyl phosphoramidate to inhibit the amidase (Dr. Piel, personal communication). Actually the nitrile hydratase from *Rhodococcus* sp. MP50 formed *R*-NapA from NapN with a low enantiomeric excess (Fig. 4).

3.6. Induction of the naproxen nitrile and naproxen amide hydrolysing activities in Rhodococcus sp. C3II and Rhodococcus sp. MP50

In *Rhodococcus* sp. C3II the activity that converted NapN to Nap was constitutively expressed and also found in cells grown with ammonia as nitrogen source.

Strain MP50 was grown with succinate (10 mM) and NapN or NapA (0.5 mM each) in mineral media with or without added ammonia. The cells were harvested and resting cells incubated with NapN or NapA (0.5 mM each). After growth with NapN without ammonia the turnover rate of NapN was 0.003 μmol min^{-1} mg^{-1} of dry weight and of NapA was 0.71 μmol min^{-1} mg^{-1}. Surprisingly, after growth with NapN or NapA in the presence of ammonia only 2–4% of the respective enzyme activities were found. After growth with NapA without added ammonia only the amide hydrolysing activity (0.73 μmol min^{-1} mg^{-1} of dry weight), but no nitrile converting enzyme was induced. Induction of these activities (= 90–120% of the value found with NapN) was also observed after growth with benzylcyanide, α-methylbenzylcyanide, isobutyronitrile, isovaleronitrile or urea.

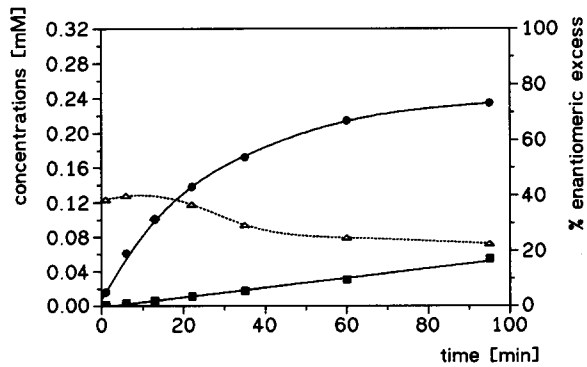

Fig. 4. Conversion of naproxen nitrile to naproxen amide by resting cells of *Rhodococcus* sp. MP50 in the presence of diethyl phosphoramidate. Strain MP50 was grown overnight in a mineral medium with succinate (10 mM) and naproxen nitrile (0.5 mM). Cells were harvested by centrifugation and resuspended in Na/K-phosphate buffer (pH 7.0, 50 mM) and diethyl phosphoramidate (20 mM) to an optical density $A_{546nm} = 36$. Naproxen nitrile was added to a starting concentration of 0.3 mM (stock solution: 20 mM in methanol). At the indicated time intervals aliquots (0.15 ml) were taken and bacterial cells removed by centrifugation (3 min, 14000 rpm). The supernatants were analyzed using a reverse-phase column (naproxen (■); naproxen amide (●). The e.e. of *R*-naproxen amide (△) was calculated by comparison of the peak areas of the *S*- and *R*-enantiomers obtained by chiral HPLC-analysis.

Table 1
Conversion of different nitriles and amides by resting cells of *Rhodococcus* sp. C3II and MP50

Compound	Relative activity (%) of strain	
	C3II	MP50
Naproxen nitrile	100	100
Benzylcyanide	650	1400
α-Methylbenzylcyanide	30	400
α-Ethylbenzylcyanide	10	40
α-Methoxybenzylcyanide	<1	90
Benzonitrile	500	nd
Naphthylcarbonitrile	nd	2700
Ibuprofen nitrile	<1	<1
Naproxen amide	100	100
Benzylamide	400	20
α-Methylbenzylamide	40	80
Naphthylcarbamid	nd	130

nd, not determined; C3II *Rhodococcus* sp. C3II; MP50 *Rhodococcus* sp. MP50.
The strains were grown with succinate (10 mM) and naproxen nitrile (0.5 mM), harvested by centrifugation and resuspended in Na/K-phosphate buffer (50 mM, pH 7.4) to an optical density A_{546nm} of 13. The respective substrates were added (0.5 mM each) and the formation of the corresponding acids determined by HPLC analysis. For the nitriles the relative activities were compared with the value found with NapN taken as 100%. The relative activities for the amides were related to the value determined with NapA taken as 100%. The specific activities were: for strain C3II with NapN 0.011 μmol min^{-1} mg^{-1} of dry weight and with NapA 0.012 μmol min^{-1} per mg of dry weight; for strain MP50 with NapN 0.003 μmol min^{-1} per mg of dry weight and with NapA 0.5 μmol min^{-1} mg^{-1} of dry weight.

3.7. Substrate specificity of the naproxen nitrile and naproxen amide converting enzyme system

Rhodococcus sp. C3II and MP50 were grown with succinate (10 mM) and NapN (0.5 mM) and resting cells incubated with different nitriles and amides. Obviously both strains converted various subtituted nitriles and amides (Table 1).

4. Discussion

The microbial metabolism of nitriles proceeds via two different pathways. Nitrilases participate in the direct conversion of nitriles to their carboxylic acids and ammonia. The alternative pathway involves nitrile hydratases which mediate the conversion of nitriles to the corresponding amides. In a subsequent step amidases convert the amides to the corresponding carboxylic acids and ammonia (Harper, 1977a,b; Nagasawa et al., 1987, 1988a,b, 1990; Watanabe et al., 1987). The enzymatic hydrolysis of nitriles represents a very convenient synthetic method for amides and/or carboxylic acids due to the mild reaction conditions. Furthermore, these enzymatic reactions also allow the enantioselective synthesis of optical active amides and carboxylic acids from racemic precursors (Mayaux et al., 1990, 1991; Yamamoto et al., 1990; Kakeya et al., 1991; Bianchi et al., 1991; Bhalla et al., 1992; Cohen et al., 1992; Layh et al., 1992).

Rhodococcus sp. C3II and MP50 probably possess a nitrile hydratase and an amidase which hydrolyse naproxen nitrile or naproxen amide, respectively. Several other nitriles and amides were also converted. It was shown for both strains that the nitrile hydratase and the amidase are capable of hydrolysing their substrates with moderate to high enantiomeric excesses. Obviously the nitrile hydratase and amidase of *Rhodococcus* sp. C3II produced predominantly the *S*-enantiomers of naproxen amide or naproxen. It should be possible to use the purified nitrile hydratase from this strain to produce various *S*-amides from the corresponding racemic nitriles. In contrast, the nitrile hydratase from *Rhodococcus* sp. MP50 hydrolysed racemic NapN predominantly to *R*-NapA. Although both nitrile hydratases showed some enantioselectivity the high optical purity of *S*-naproxen formed was mainly due to the amidase reaction. For both isolates the amidase reaction occurred with high specificity allowing the recovery of both amide and acid with high optical activities.

Highly enantioselective amidases have also been observed by other investigators with various α-substituted amides (Kieny L'Homme et al., 1981; Asano et al., 1989; Mayaux et al., 1990, 1991; Kakeya et al., 1991; Cohen et al., 1992). In general, these activities were found after screening of strains from culture collections or in bacteria which were initially enriched with different often aliphatic nitriles or amides. Later on these microorganisms were analyzed for the enantiose-

lective hydrolysis of the substrate of interest. In the present study a different strategy was chosen and enrichments were carried out with the substrates to be biotransformed by the bacteria. Thus it was shown that bacteria could be obtained with the ability to hydrolyse enantioselectively naproxen nitrile to the industrially important compound *S*-naproxen.

Naproxen nitrile is only slightly soluble in water (about 0.04 mM). Therefore, bioavailability of NapN in water would be growth limiting. Similar problems are encountered in the bacterial degradation of polyaromatic hydrocarbons. Therefore we used a two-liquid-phase system for the enrichments. The observation that in two independent enrichments using NapN as sole nitrogen source bacteria were isolated only when pristane was present indicated a positive effect of this enrichment procedure.

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