Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron Letters 47 (2006) 8119–8123
Tetrahedron Letters

Biohydrolysis of (*S*)-3-(thiophen-2-ylthio)butanenitrile

M. Gelo-Pujic,* C. Marion, C. Mauger, M. Michalon, T. Schlama and J. Turconi

Rhodia Centre de Recherches et de Technologies, 85 rue de frères Perret, BP 62, F-69192 Saint Fons Cedex, France

Received 20 July 2006; revised 6 September 2006; accepted 8 September 2006
Available online 29 September 2006

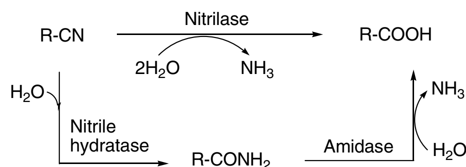
Abstract—Whole-cell enzymatic hydrolysis was shown to be the choice in the preparation of (*S*)-3-(thiophen-2-ylthio)butanoic acid. While all chemical methods of hydrolysis failed, 12 bacterial strains expressing nitrile hydratase and amidase activities have been identified to hydrolyze (*S*)-3-(thiophen-2-ylthio)butanenitrile **1** directly into the corresponding acid. The substrate was also shown to be an efficient inducer of the enzymatic activity. However, it inhibited microbial growth. Acid **3** was prepared on a gram scale with the recombinant *Rhodococcus erythropolis*, formerly *Brevibacterium* sp. pYG811b as shown by sequencing of its 16S rRNA.
© 2006 Elsevier Ltd. All rights reserved.

1. Introduction

Nitriles are readily transformed to corresponding amides and carboxylic acids by a variety of chemical processes that typically require strongly acidic or basic reaction conditions and high temperatures. These reactions often produce undesired side products and large amounts of inorganic salts as unwanted waste.

On the other hand, biocatalysis offers an option for a mild and selective hydrolysis. This includes nitrilase, nitrile hydratase and amidases enzymes. They represent one of the most important enzymes exploited industrially.[1,2] Biotechnological use and synthetic applications of nitrile-converting enzymes have been reviewed recently.[3,4]

Enzyme-catalyzed hydrolysis of the nitrile substrates into the corresponding carboxylic acids may be accomplished via a one-step or two-step reaction as represented in Scheme 1.



Scheme 1.

Nitrilase (E.C. 3.5.5.1) is a thiol type of enzyme, which converts a nitrile directly into the corresponding carboxylic acid in an aqueous solution without the intermediate formation of an amide. Nitrile hydratase (E.C. 4.2.1.84) has either a nonhaeme iron atom or noncorrinoid cobalt atom and catalyzes the hydration of nitriles to amides.[5]

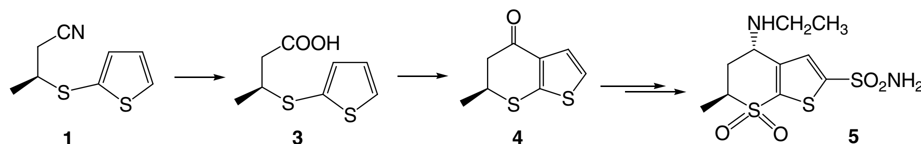
A wide variety of bacterial genera possess a diverse spectrum of nitrile hydratase, amidase or nitrilase activities, including *Rhodococcus*, *Pseudomonas*, *Alcaligenes*, *Bacillus*, *Corynebacterium*, *Micrococcus*, *Comamonas*, *Brevibacterium*, etc. Several nitrile hydratases from *Brevibacterium* sp. strain R312,[6] *Pseudomonas* strain B23,[7] *Rhodococcus* sp. strain YH3-3[8] and many others have been isolated, characterized and cloned. The microorganisms of the genera *Rhodococcus* are among the most common sources of nitrile hydratases and amidases.[9] It has been shown that all *Rhodococcus* strains recovered from distinct geographical regions have nitrile hydratase and amidases hydrolyzing systems.[10]

2. Results and discussion

The aim of this work was to hydrolyze a nitrile function of (*S*)-3-(thiophen-2-ylthio)butanenitrile **1** into the corresponding (*S*)-acid **3**. This molecule represents an important intermediate in the synthesis of MK-0507, a carbonic anhydrase inhibitor marketed by Merck under the name Dorzolamine (**5**).[11] It is used in the topical treatment of glaucoma. The synthesis of **1** has recently

* Corresponding author. Tel.: +33 4 72 89 69 88; fax: +33 4 72 89 67 21; e-mail: mirjana.gelo-pujic@eu.rhodia.com

0040-4039/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2006.09.038



Scheme 2.

been patented and will be published elsewhere.[12] A wide variety of different chemical and biochemical conditions have been studied in the hydrolysis of (*S*)-3-(thiophen-2-ylthio)butanenitrile **1** into its corresponding (*S*)-acid **3** (Scheme 2).

Different conditions of chemical hydrolysis have been applied. Moreover, these conditions were supposed to favour a one pot hydrolysis and cyclization into a ketosulfide **4**, thus affording the cyclic intermediate of Dorzolamine (**5**).

The behaviour of (*S*)-3-(2-thiophenyl)sulfanylbutyronitrile **1** in acidic conditions was studied using several mineral acids in the presence (or absence) of various Lewis acids. The conditions screened were

Acids:
HCl (37%), H_2SO_4 (20%), H_2SO_4 (98%), AcOH, Ac_2O/AcOH, HCl (37%)/$ZnCl_2$, HCl (37%)/$AlCl_3$, HCl (37%)/$BF_3 \times Et_2O$, $HCl \times Et_2O$, toluene + Lewis acid, TFA + Lewis acid, $HCl \times Et_2O$ + Lewis acid, HCl gas, HCl gas + Lewis acid ($AlCl_3$, $FeCl_3$, $TiCl_4$, $ZnCl_2$, $BF_3 \times Et_2O$, $MeSiCl_3$).

Unfortunately, under all conditions assayed no desired product was obtained. A large number of side products resulting from degradation of the starting material were obtained, but were not further characterized.

To circumvent this obstacle, we have screened microorganisms well known for their ability to grow and assimilate nitriles and amides as a carbon source in the hydrolysis of (*S*)-3-(thiophen-2-ylthio)butanenitrile **1** (Scheme 2). The aim was to identify microorganisms capable of hydrolyzing (*S*)-nitrile directly into (*S*)-acid and to optimize the biotransformation step. As the chirality of the substrate has been introduced early in the synthesis,[12] we did not insist on the enantioselectivity of the hydrolysis step. However, the enantioselectivity and the diastereoselectivity were determined after the transformation of acid **2** into ketosulfide **4** as already published by Blacker and Holt.[13]

Under the screening conditions, which are only general conditions without any optimization, 28 strains did not hydrolyze the substrate, 13 strains showed a low to moderate activity (substrate conversions between 10% and 50%), 12 strains displayed a high activity giving

Table 1. Results of screening in the hydrolysis of (*S*)-3-(thiophen-2-ylthio)butanenitrile **1**

Strain	Inducer	Time (h)	Nitrile (*S*)-**1** (%)	Amide (*S*)-**2** (%)	Acid (*S*)-**3** (%)
Brevibacterium strain R312	*N*-Methylacetamide	48	25	0	75
Brevibacterium strain ACV2		24	0	0	100
Brevibacterium imperiale	Isobutyronitrile	48	47	10	43
Brevibacterium strain C211		48	5	0	95
Bacteridium strain R341		48	4	0	96
Corynebacterium strain N771		48	27	0	63
Acinetobacter sp.		48	9	5	86
Brevibacterium pYG811a[a]		48	11	0	89
Brevibacterium pYG811b[a]		48	7	0	93
Pseudomonas fluorescens CRTL1		48	57	6	37
Pseudomonas fluorescens CRTL2		48	72	0	28
Rhodococcus sp.		48	65	5	30
Brevibacterium A4		24	0	100	0
Bacillus megaterium CRTL1	Benzonitrile	24	0	0	100
Rhodococcus sp. 4028		48	66	6	28
Rhodococcus sp. ATCC 39484		24	0	0	100
Bacillus megaterium CRTL2	Adiponitrile	48	19	0	81
Comamonas testosteroni		48	56	0	44
Corynebacterium N774	Isobutyramide	48	89	0	11
Achromobacter sp.		24	5	0	95
Brevibacterium lactofermentum		48	14	0	86
Blank (substrate stability)	w/o	48	100	0	0

[a] *Brevibacterium* pYG811a and pYG811b are two recombinant *Brevibacterium* R312 expressing the amidase (Amd) gene of *Rhodococcus* IBN20 under its natural promoter (EP 433117, Rhône-Poulenc Santé). The molecular identification of *Brevibacterium* R312 showed >99% of homology with *Rhodococcus erythropolis*.

acid **3** as the hydrolysis product (substrate conversions >75%), one strain among these 12 gave a high conversion (91%) with predominantly acid as the product and small quantities (5%) of the amide, while one strain lacked the amidase activity and resulted in the amide as the only hydrolysis product. The results are summarized in Table 1.

Brevibacterium R312 pYG811b was chosen to explore the influence of the inducer on its growth rate and the specific activity. The choice of microorganism for further study was based on its industrial property (*Brevibacterium* R312 pYG811b is covered by Rhône-Poulenc Santé patent[14]). In order to verify the need of *Brevibacterium* R312 pYG811b for the inducer, very simple tests were performed: the microorganism was grown without any inducer and in the presence of either its standard inducer (isobutyronitrile 2.5 g/L) or substrate **1**. At regular time intervals, a sample of microbial culture was analyzed for the cell density at 600 nm and the same sample was washed with double distilled water (ddH_2O) and dried at 120 °C for 24 h in order to obtain the information on dry cell mass.

As results show, *Brevibacterium* R312 pYG811b grows faster without any inducer, while the substrate significantly inhibits the cell growth. At the same time, the biomass grown in the presence of the substrate has a better activity, as shown by the comparative assays of biohydrolysis (Table 2). The substrate is a more efficient inducer of nitrilase production than isobutyronitrile. The assays were performed on a small scale (2 mL of phosphate buffer pH 7 and 3% (v/v) of acetonitrile). The samples were incubated at 30 °C and shaken in a thermomixer at 1300 rpm.

Table 2. Biohydrolysis of **1** with *Brevibacterium* R312 pYG811b

Inducer	Substrate/ dry biomass (w/w)	Time (h)	Conversion (%)	Conversion/ dry biomass (%/mg)
None	0.6	6	15.7	1.9
		25	44.7	5.4
1	0.6	6	78	9.4
		25	100	12
Isobutyronitrile	0.6	6	21	2.6
		25	52.5	6.3

The next series of experiments was undertaken with a wild type *Brevibacterium* R312 in order to determine the concentration of the substrate tolerated by the enzymatic system of *Brevibacterium* R312, that is, which concentration of substrate inhibits biohydrolysis. Again, the assays were performed on a small scale (2 mL of phosphate buffer pH 7 and 3% v/v of acetonitrile). The samples were incubated at 30 °C and shaken in a thermomixer at 1300 rpm. The results (Fig. 1) indicate that a concentration of 20 g/L of **1** is well tolerated, while high concentrations such as 100 g/L inhibit the enzymatic system and slow down the kinetics. For that high concentration a conversion of only 40% was achieved after 1 week of incubation.

These conditions were scaled up and reproduced with 1 g and 2.5 g of (*S*)-3-(thiophen-2-ylthio)butanenitrile **1** (examples given in Section 4).

A search of strain data bases like ATCC or DMSZ reveals that many of the strains deposited as *Brevibacterium* and *Bacteridium* have changed the genus and have been renamed. For that reason, we have undertaken the approach of molecular screening by sequencing the 16S rRNA and aligning the sequences by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The results obtained for the three strains (*Brevibacterium* ACV2, *Brevibacterium* C211 and *Bacteridium* R341) showed a high homology (>99%) to the full identity with *Rhodococcus erythropolis* (16S rRNA sequences are available upon request).

3. Materials and methods

The microorganisms used in screening are available from culture collections (ATCC, DSMZ) or isolated at the Rhodia site and characterized previously. Columbia broth was supplied by Difco. All chemicals and solvents were used without any further purification if not stated otherwise. The standard samples of

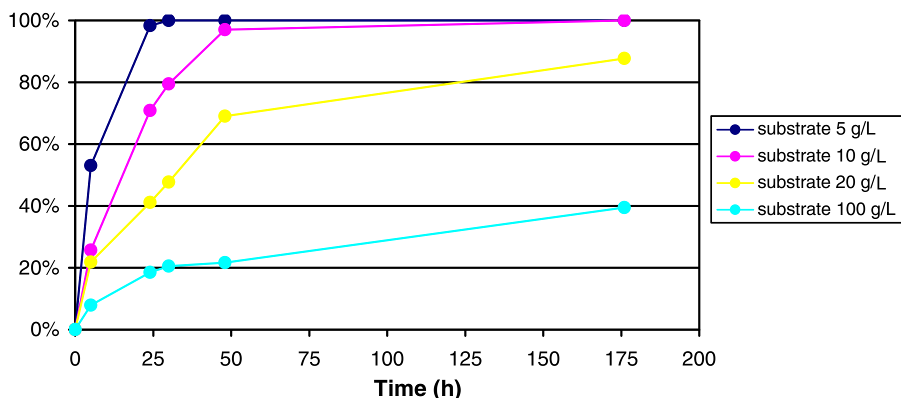


Figure 1. Kinetics of biohydrolysis for different substrate concentrations.

substrate and products were prepared as described in the patent.[12] NMR spectra were recorded in $CDCl_3$ solutions on a Bruker instrument operating at 300 MHz. Chemical shifts (δ) are expressed in ppm relative to HMDS.

4. Analytical techniques

The reactions were followed by HPLC/UV on a Waters Alliance 2790 separation module equipped with 996 PDA detector using 125/4 Kromasil 100-5 C18 column thermostated at 30 °C under the following conditions: flow rate 1 mL/min; solvents: A: H_2O/0.1% HCOOH and B: acetonitrile/0.1% HCOOH. The gradient was programmed from A/B 90/10 to 10/90 in 8 min followed by 2 min of re-equilibration under initial conditions. The retention times (t_R in minutes) were 6.67 (nitrile), 5.80 min (acid) and 4.65 min (amide). The reaction medium (0.5 mL) was withdrawn, centrifuged and 0.25 mL were diluted with 0.75 mL of H_2O/CH_3CN (1/1 v/v). For the analysis, 10 µL were injected. The substrate and products were quantitatively determined from the calibration curves. Enantioselective HPLC has been run according to a published procedure.[13] Acetic acid was measured by using the enzymatic kit purchased from Roche.

4.1. Microbial culture conditions and biotransformations

Microbial strains were cultured in 24-deep well plates in Columbia medium at 25 °C and 130 rpm for 48 h (Infors thermostated shaker). Known inducers of nitrile hydratase activity were added: *N*-methylacetamide (20 mM), isobutyronitrile (2.5 g/L), isobutyramide (3.5 g/L), benzonitrile (0.5 mM) or adiponitrile (10 mM) as indicated in Table 1. After 48 h of growth period, the cultures were centrifuged (1000 rpm, 30 min at 4 °C) in order to remove the culture medium and the cell pellets were re-suspended in 3 mL of the 50 mM phosphate buffer pH 7.0. These suspensions were frozen at −30 °C for at least 72 h. Cell lysis was enabled upon de-freezing by incubating the cell suspensions with gentle shaking at 37 °C for 2 h. This heat shock provokes the cell wall to break down and to liberate the intracellular enzymes.

The activity test was performed with 5 mg/mL of (*S*)-nitrile **1** as the substrate. Acetonitrile (100 µL per deep well corresponding to 3.3% v/v) was added in order to obtain a homogeneous reaction medium. It was shown that acetonitrile is not the substrate of nitrilases by measuring the pH and by following the eventual formation of acetic acid by the enzymatic method from Roche.

4.2. Hydrolysis with (*S*)-3-(thiophen-2-ylthio)butanenitrile (1) as an inducer

Brevibacterium R312 pYG811b was grown in the sterile Columbia broth medium supplemented with the substrate as an inducer (2.5 g/L) at 140 rpm for 48 h in the shaker thermostated at 30 °C. The culture was then centrifuged and the supernatant was discarded. The remaining cells (590 mg) were re-suspended in 60 mL of phosphate buffer (50 mM NaH_2PO_4 pH 7) and kept frozen at −30 °C for 72 h. The biomass was placed at 37 °C for 1 h in order to thaw and lyse the cells and then introduced in an Erlenmeyer containing 440 mL of 50 mM phosphate buffer pH 7. The substrate (2.5 g of **1** dissolved in 15 mL of acetonitrile) was then added and the reaction mixture was incubated for 5 days at 30 °C until the conversion reached 60%. The reaction mixture was acidified to pH 3 with 5 M HCl and the remaining substrate and the product were extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude oily brown product (2.5 g) was applied to SiO_2 and purified by flash chromatography by eluting the column with hexane/ethyl acetate 9/1. Acid **3** (1.2 g; 43% yield) was obtained as a light yellow product. Its structure was confirmed by ^1H NMR and ^{13}C NMR and the molar purity was determined to be 96% (presence of 2% of crotonic acid as the main impurity).

^1H NMR ($CDCl_3$, ppm): 7.35 (d, 1H, $J = 5.2$ Hz), 6.97 (dd, 1H, $J = 5.5$, 3.6 Hz), 7.10 (d, 1H, $J = 3.57$ Hz), 3.33 (m, 1H, $J = 8.25$ Hz), 1.29 (d, 3H, $J = 6.87$ Hz), 2.6 (dd, 1H, $J_1 = 16.2$ Hz, $J_2 = 6.6$ Hz), 2.4 (dd, 1H, $J_1 = 16$ Hz, $J_2 = 7.9$ Hz).

^{13}C NMR ($CDCl_3$, ppm): 189.4 (C_{IV}), 177.7 (C_{IV}), 136.6 (C_{arom}), 130.8 (C_{arom},C_{arom}), 41.4 (C_{II}, C_{III}), 20.6 (C_I).

4.3. Hydrolysis of (*S*)-racemic 3-(thiophen-2-ylthio)-butanenitrile (1) with isobutyronitrile as the inducer

The same culture conditions as described above were used except that isobutyronitrile (2.5 g/L) was used as the inducer. Biotransformation was performed on a 1 g scale under the same conditions of pH and temperature. HPLC conversion showed complete hydrolysis after 3 days. The crude product was obtained after acidification and extraction and was applied to SiO_2 and purified by flash chromatography by eluting the column with hexane/ethyl acetate 9/1. Acid **3** (0.84 g) was obtained in a 76% yield. ^1H NMR and ^{13}C NMR confirmed the expected product and purity.

References and notes

1. Nagasawa, T.; Yamada, H. *Pure Appl. Chem.* **1990**, *62*, 1441–1444.
2. Yamada, H.; Kobayashi, M. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1391–1400.
3. Mylerova, V.; Martinkova, L. *Curr. Org. Chem.* **2003**, *7*, 1–17.
4. Cowan, D. A.; Cramp, R. A.; Pereira, R. A.; Almatawah, Q. *Extremophiles* **1998**, *2*, 207–216.
5. Kobayashi, M.; Shimizu, S. *Curr. Opin. Chem.* **2000**, *4*, 95–102.
6. Nagasawa, T.; Nanba, H.; Yamada, H. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 1305–1312.

7. Nagasawa, T.; Nanba, H.; Ryuno, K.; Takenichi, K.; Yamada, H. *Eur. J. Biochem.* **1987**, *162*, 691–698.
8. Kato, Y.; Tsuda, T.; Asano, Y . *Eur. J. Biochem.* **1999**, *263*, 662–670.
9. Brandao, P. F. B.; Clapp, J. P.; Bull, A. T. *Appl. Environ. Microbiol.* **2003**, *69*, 5754–5766.
10. Brandao, P. F. B.; Bull, A. T. *Antonie Leeuwenhoek* **2003**, *84*, 89–98.
11. Baldwin, J. J.; Ponticello, G. S.; Christy, M. E. EP 0 296 879 (Merck, 1988).
12. Turconi, J.; Mauger, C.; Schlama, T. FR 05 10270 (Rhodia, 2005).
13. Blacker, A. J.; Holt, R. A. In *Chirality in Industry II: Developments in the Commercial Manufacture and Applications of Optically Active Compounds*; Collins, A. N., Gary, Sheldrake, Crosby, J., Eds.; John Wiley & Sons Ltd., 1997; pp 245–261.
14. Petré, D.; Carbelaud, E.; Mayaux, J.-F.; Yeh, P. EP 433117 (Rhône-Poulenc Santé, 1997).