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Preparation of (*S*)-piperazine-2-carboxylic acid, (*R*)-piperazine-2-carboxylic acid, and (*S*)-piperidine-2-carboxylic acid by kinetic resolution of the corresponding racemic carboxamides with stereoselective amidases in whole bacterial cells

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Abstract: Whole bacterial cells containing stereospecific amidases were used for the kinetic resolution of racemic piperazine-2-carboxamide and piperidine-2-carboxamide to (*S*)- and (*R*)-piperazine-2-carboxylic acid, and (*S*)-piperidine-2-carboxylic acid, respectively. (*S*)-Piperazinecarboxylic acid dihydrochloride produced with the biocatalysts *Klebsiella terrigena* DSM 9174 had an ee value of 99.4% (41% yield), and the ee value of (*R*)-piperazinecarboxylic acid dihydrochloride obtained with *Burkholderia sp.* DSM 9925 was 99.0% (22% yield). Using *Pseudomonas fluorescens* DSM 9924 (*S*)-piperidine-2-carboxylic acid with an ee value of 97.3% (20% yield) was isolated. © 1997 Elsevier Science Ltd

Introduction

Non-proteinogenic amino acids such as piperidine-2-carboxylic acid (pipecolic acid), **6** and piperazine-2-carboxylic acid **3** are precursors of numerous bio-active compounds. Derivatives of pipecolic acid include the immunosuppressant FK506,[1] local anesthetics,[2] an antipsychotic drug,[3] and a K-opioid analgesic.[4] (*S*)-Piperazine-2-carboxylic acid can be used for the synthesis of the HIV protease inhibitor Crixivan from Merck[5], an *N*-methyl-D-aspartate antagonist,[6] and a cardioprotective nucleoside transport blocker.[7] The best current route to the pure enantiomers of these amino acids is classical resolution of the racemate by fractional crystallization of diastereomeric salts.[8,9] Asymmetric syntheses for (*S*)-pipecolic acid have been developed[10] as well as a kinetic resolution of (*R,S*)-*n*-octyl-pipecolate using a partially purified *Aspergillus niger* lipase.[11] (*S*)-Piperazine-2-carboxylic acid has also been prepared by kinetic resolution of racemic 4-(*tert*-butoxycarbonyl)piperazine-2-carboxamide with leucine aminopeptidase.[7] Both of these biocatalytical processes have practical disadvantages in that the preparation of the racemic starting materials is complicated and the availability of the biocatalysts for large scale production is limited.

In this paper we report the efficient kinetic resolution of racemic piperazine-2-carboxamide **2** and racemic piperidine-2-carboxamide **5** to the corresponding enantiomerically pure carboxylic acids with whole cells of wild-type micro-organisms that contain stereospecific amidases.[12]

Results and discussion

A primary screening for micro-organisms with amidase activity was done starting from soil samples using growth media containing one of the racemic carboxamides **2** and **5** as sole nitrogen source. Micro-organisms forming stereospecific amidases were identified as follows. Individual clones were grown for 3 days in media containing 2 gL^{-1} of one of the racemic carboxamides **2** or **5** as N-source. Samples of the cell-free growth medium were then analysed on TLC plates to estimate the

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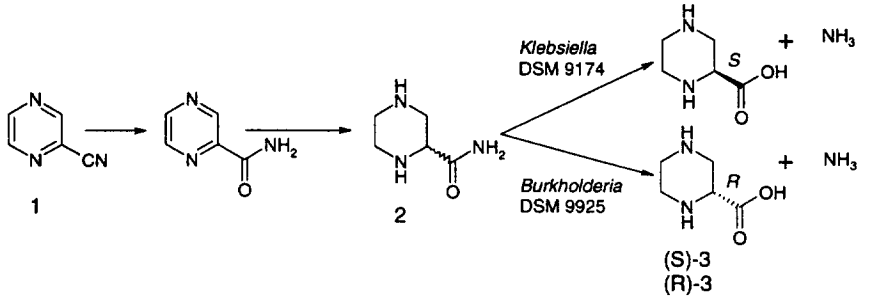
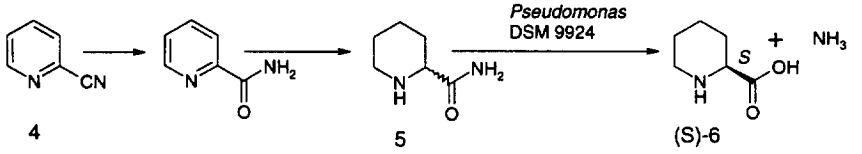
Table 1. Substrate specificity, stereospecificity and reaction conditions of isolated micro-organisms

Strain	Substrate	Product	Reaction conditions	% ee	isolated yield*
Klebsiella terrigena DSM 9174	(*RS*)-piperazine-2-carboxamide	(*S*)-piperazine-2-carboxylic acid	40°C -47°C pH 8.0	99.4%	41%
Burkholderia sp. DSM 9925	(*RS*)-piperazine-2-carboxamide	(*R*)-piperazine-2-carboxylic acid	40°C-47°C pH 8.0	99.0%	22%
P. fluorescens DSM 9924	(*RS*)-piperidine-2-carboxamide	(*S*)-piperidine-2-carboxylic acid	30°C pH 7.0	97.3%	20%

*: Piperazine-2-carboxylic acids were isolated as dihydrochloride salts.

amount of carboxylic acid formed. Micro-organisms hydrolysing approximately 50% of the racemic carboxamides were chosen for further investigation to determine the stereoselectivity of the amidases. As a result of this screening the strains *Klebsiella terrigena* DSM 9174, *Burkholderia sp.* DSM 9925, and *Pseudomonas fluorescens* DSM 9924 were used for the preparation of (*S*)-piperazine-2-carboxylic acid, (*R*)-piperazine-2-carboxylic acid, and (*S*)-pipecolic acid, respectively (Table 1).





For the production of samples smaller than 10 g, the micro-organisms were grown on the appropriate medium, harvested by centrifugation, and washed in physiological salt solution. The cells were then re-suspended for various periods of time in buffer typically containing 10–20 gL^{-1} of the corresponding racemic carboxamide. The ee-values of piperazine-2-carboxylic acid and pipecolic acid were analyzed by capillary electrophoresis and HPLC, respectively. Piperazine-2-carboxylic acid was isolated from the cell free solution as the dihydrochloride salt. Pipecolic acid was isolated using anion chromatography (adsorbance on Duolite A 147 and subsequent elution with 10% acetic acid). No attempts were made to purify the carboxamides after the bioconversion. However, following the biotransformation with *Klebsiella terrigena*, the carboxamide remaining in aqueous solution after the (*S*)-piperazine-2-carboxylic acid had been precipitated with HCl, was hydrolysed for 4 h at 80°C, and after cooling (*R*)-piperazine-2-carboxylic acid was isolated. The ee-value of the recrystallized (*R*)-**3** was 99.2% $\{[\alpha]^{20}_{589}=+4.74$ (c 1.2; $H_2O)\}$.

Larger samples of (*S*)-piperazine-2-carboxylic acid were produced in a 1 L fermenter by growing cells of *Klebsiella terrigena* in the presence of 22 gL^{-1} of racemic **2**.

The kinetic resolutions presented in this paper are particularly attractive because the starting materials **2** and **5** can be easily prepared from 2-cyanopyrazine **1** and 2-cyanopyridine **3** via the

corresponding aromatic carboxamides. Furthermore, the micro-organisms can be grown in fermenters on the racemic carboxamides at the same time as the biotransformations are taking place.

Experimental

(RS)-Piperazine-2-carboxamide 2

A solution of pyrazinecarboxamide (1200 g, 9.747 moles) in absolute ethanol (6 L) was hydrogenated at 85°C under 20 bars of pressure in the presence of 240 g of 10% Pd/C. A considerable exotherm was observed at the beginning. After completion of the reaction (5 h), the warm solution was concentrated to 2.5 L, whereupon the product began to crystallise. The stirred suspension was cooled to 2°C and filtered. The filter cake was washed with 1 L of cold ethyl acetate and dried in vacuum at 50°C. A yield of 1035 g of racemic piperazine-2-carboxamide was obtained as a white crystalline powder, m.p. 143–144°C (82.2% of theoretical yield). A second crop could be obtained from the mother liquor.

(RS)-Piperidine-2-carboxamide 5

Pyridine-2-carboxamide (350 g, 2.866 moles) in 3 L of absolute ethanol was hydrogenated at 110°C and 20 bars in the presence of 14 g of 10% Pd/C. The product was isolated as described above and the yield of crystalline racemic piperidine-2-carboxamide, m.p. 147–148°C, was 337 g (91.7%).

Micro-organisms

The micro-organisms have been deposited at the Deutsche Sammlung für Mikroorganismen, Mascheroder Weg 1B, D-38124 Braunschweig, Germany.

Analytical methods

Thin layer chromatography: pre-coated silica gel 60 plates (Merck) were developed with ethanol:chloroform:25% NH_4OH (11:6:6). The compounds were detected with ninhydrin (R_f **2** and **5**≈0.8, R_f **3** and **6**≈0.2).

Capillary electrophoresis of (*R*)-**3** and (*S*)-**3**: aqueous solutions or isolated products were first derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate. The CE system (Hewlett-Packard HP ^{3D}CE, capillary column: HP G1600–61211) was operated at 20 kV and a current of 24–30μA at 20°C oven temperature. The buffer (pH 9.0) contained 10 mM Na_2HPO_4, 10 mM H_3BO_3, and 150 mM sodium dodecyl sulfate. The electrolyte was buffer:methanol (9:1). The UV detector was set at 210 nm. The migration times of (*S*)-**3** and (*R*)-**3** were 17.1 and 17.7 min, respectively.

HPLC of (*S*)-**6**: Samples were first derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate. The HPLC system (Hewlett-Packard 1090) was operated with a reverse phase column (Machery Nagel, Nucleosil 120-3 C_{18} AB) at 40°C. The UV detector was set at 250 nm. The solvents were A: H_2O, B: CH_3CN, C: 250 mM KH_2PO_4 (pH 2.3). Solvent C was kept constant at 20%. The compounds were eluted by increasing solvent B from 30% to 50%. The retention times of (*S*)-**6** and (*R*)-**6** were 9.2 and 9.9 min, respectively. Reference samples of (*R*)-**6** and (*S*)-**6** were obtained from Fluka Chemicals, Buchs, Switzerland.

Cultivation for small scale bioconversions

For the growth of *Klebsiella terrigena* DSM 9174 and *Burkholderia sp.* DSM 9925 a mineral salts medium[13] was supplemented with 500 mgL^{-1} yeast extract, 10 gL^{-1} glycerine, and 2 gL^{-1} **2**. The mineral salts medium for *Pseudomonas fluorescens* DSM 9924 was supplemented with 500 mgL^{-1} yeast extract, 4 gL^{-1} glucose, and 1 gL^{-1} **5**. Growth was at 30°C and pH 7.0.

Preparative scale formation of (S)-piperazine-2-carboxylic acid

Klebsiella terrigena DSM 9174 was grown in a 1.5 L fermenter (1 L working volume) in the mineral salts medium[13] supplemented with 500 mgL^{-1} yeast extract, 30 gL^{-1} glycerine, and 22 gL^{-1} **2** at 30°C, pH 7.0, and an aeration rate of 0.5 $Lmin^{-1}$ for 16 h. After this period the temperature was increased to 40°C and the pH adjusted to 8.0 to enhance the enzymatic hydrolysis rate. The

biotransformation was followed by TLC and capillary electrophoresis and stopped after 36–72 h by removing the cells by centrifugation. For the isolation of (*S*)-**3** the cell free solution was concentrated 10-fold at 60°C under reduced pressure and acidified with conc. HCl (pH 1.0) to precipitate the product. (*S*)-**3** was re-crystallized in 0.1 M HCl. ^{1}H-NMR (400 MHz, D_2O): 4.46 ppm (dd, 1H, H-2, $J_{2,3A}$=4.1 Hz, $J_{2,3B}$=11.0 Hz); 4.00 ppm (dd, 1H, H-3A, $J_{2,3A}$=4.1 Hz, $J_{3A,3B}$=14.2 Hz); 3.70–3.83 ppm (m, 2H, H-5A und H-6A); 3.56 ppm (dd, 1H, H-3B, $J_{2,3B}$=11.0 Hz, $J_{3A,3B}$=14.2 Hz); 3.40–3.56 ppm (m, 2H, H-5B und H-6B). $[\alpha]^{20}_{589}$=−4.85 (c 1.2; H_2O).

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