Selective biotransformation of substituted alicyclic nitriles by *Rhodococcus equi* A4

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Abstract: Nitrile hydratase from *Rhodococcus equi* A4 discriminated between geometric isomers of substituted alicyclic nitriles. The enzyme transformed *trans*-4-benzoyloxycyclohexanecarbonitrile (*trans*-**1a**), *cis*-3-benzoyloxycyclohexanecarbonitrile (*cis*-**2a**), *trans*-2-hydroxycyclohexanecarbonitrile (*trans*-**3a**), and *trans*-2-hydroxycyclopentanecarbonitrile (*trans*-**4a**) into the corresponding amides. On the contrary, *cis*-2-hydroxycyclohexanecarbonitrile (*cis*-**3a**) and *cis*-2-hydroxycyclopentanecarbonitrile (*cis*-**4a**) were not converted to a significant extent. *cis*-4-Benzoyloxycyclohexanecarbonitrile (*cis*-**1a**) was also a substrate of the enzyme but reacted slowly. Diequatorial arrangement of the substituents in *trans*-**1a**, *cis*-**2a**, and *trans*-**3a** appears to positively influence the activity of the nitrile hydratase.

Key words: nitrile hydratase, substituted cyclohexanecarbonitriles, substituted cyclopentanecarbonitriles.

Résumé : L'hydratase des nitriles obtenue à partir de *Rhodococcus equi* A4 donne lieu à de la discrimination entre des isomères géométriques de nitriles alicyliques substitués. L'enzyme a transformé les *trans*-4-benzoyloxycyclohexanecarbonitrile (*trans*-**1a**), *cis*-3-benzoyloxycyclohexanecarbonitrile (*cis*-**2a**), *trans*-2-hydroxycyclohexanecarbonitrile (*trans*-**3a**) et *trans*-2-hydroxycyclopentanecarbonitrile *(trans*-**4a**) en amides correspondants. Au contraire, le degré de conversion des *cis*-2-hydroxycyclohexanecarbonitrile (*cis*-**3a**) et *cis*-2-hydroxycyclopentanecarbonitrile (*cis*-**4a**) est faible. Comme substrat de cet enzyme, le *cis*-4-benzoyloxycyclohexanecarbonitrile (*cis*-**1a**) ne réagit que lentement. L'arrangement diéquatorial des substituants dans les isomères *trans*-**1a**, *cis*-**2a** et *trans*-**3a** semble influencer positivement l'activité de l'hydratase des nitriles.

Mots clés : hydratase des nitriles, cyclohexanecarbonitriles substitués, cyclopentanecarbonitriles substitués.

[Traduit par la Rédaction]

Introduction

The use of bacterial nitrile-converting enzymes for enantio-, regio-, and chemoselective hydration and hydrolysis of nitriles has been described in numerous works (1). This work reports the resolution of geometric isomers using a nitrile hydratase.

Different affinities of this enzyme for geometric isomers of *trans*-1,4-dicyanocyclohexane (DCC) were shown using *Corynebacterium* sp. C5 cells containing both nitrile hydratase and amidase. The relative specific activity of the biocatalyst for the *cis* isomer of DCC was only 6% of that for the *trans* isomer (2). The conversion of *trans*-DCC to *trans*-4-cyanocyclohexane-1-carboxylic acid was involved in the chemoenzymatic synthesis of the pharmaceutically important *trans*-4-aminomethylcyclohexanecarboxylic acid (tranexamic acid) (3).

The hydrolysis of alicyclic mono- and dinitriles and amides of *cis* configuration was reported to be catalyzed by *Rhodococcus rhodochrous* IFO 15564 with enantioselectivity in some cases (4), but the biotransformation of the corresponding *trans* isomers was not studied.

Whole-cell biocatalyst SP 409 (Novo Industri, not available commercially) hydrolyzed both *cis*- and *trans*-2-hydroxycyclohexanecarbonitrile and *cis*-2-hydroxycyclopentanecarbonitrile (5).

Here we report the discrimination of *trans* and *cis* isomers of the 3- and 4-benzoyloxy- and 2-hydroxy derivatives of cyclohexanecarbonitriles as well as 2-hydroxycyclopentanecarbonitriles by the nitrile hydratase from *Rhodococcus equi* A4.

Received 4 September 2001. Published on the NRC Research Press Web site at http://canjchem.nrc.ca on 4 June 2002.

Dedicated to Professor J. Bryan Jones on the occasion of his 65th birthday.

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Results and discussion

Hydrolysis of alicyclic nitriles, 2-hydroxycyclohexanecarbonitrile, 2-hydroxycyclopentanecarbonitrile (5), cyclopentanecarbonitrile (6), and 2-arylcyclopropanecarbonitriles (7), or the regioselective hydrolysis of DCC (2, 3, 8), *trans*-cyclohexane-1,4-diacetonitrile (9), and other saturated and unsaturated dinitriles (4) was the subject of few reports regarding enzymatic nitrile conversion. The present work demonstrated that the nitrile hydratase from *R. equi* A4, which showed a broad substrate specificity for aromatic,

 DOI: 10.1139/V01-205

Scheme 1.

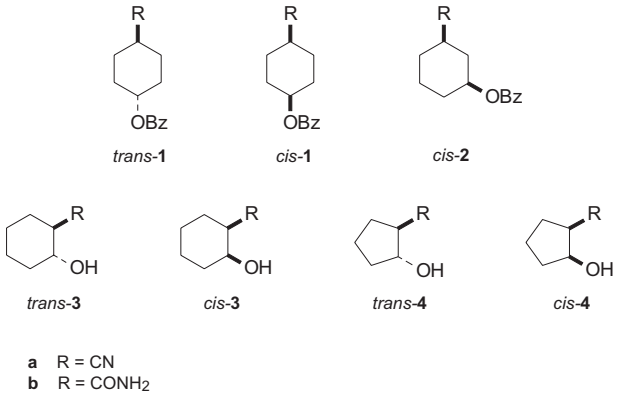

Table 1. Biotransformations of 2-hydroxycyclohexanecarbonitriles, 2-hydroxycyclopentanecarbonitriles, and 3-, 4-benzoyloxycyclohexanecarbonitriles by the nitrile hydratase of *Rhodococcus equi* A4.

Substrate[a]	Reaction time (h)	Product	Conversion (%)[b]	Isolated yield (%)
trans-**1a**	2	*trans*-**1b**	100	91
cis-**1a**	48	*cis*-**1b**	85	74
cis-**2a**	5	*cis*-**2b**	86	79
trans-**3a**	3.5	*trans*-**3b**	100	48
cis-**3a**	24	*cis*-**3b**	<5[c]	—
trans-**4a**	3	*trans*-**4b**	100	51
cis-**4a**	24	no reaction	—	—

[a]The structures (see Scheme 1) represent single enantiomers of *trans*-**1a**, *cis*-**1a**, *cis*-**2a**, *trans*-**3a**, *cis*-**3a**, *trans*-**4a**, and *cis*-**4a**, whereas the biotransformations were carried out with racemates.
[b]Determined by TLC (for compound **4a**) or HPLC (for other compounds).
[c]Traces of amide detected by NMR.

arylaliphatic, and aliphatic nitriles (10), is also useful for the hydration of alicylic nitriles. The use of the purified enzyme afforded 2-, 3-, and 4-substituted (hydroxy-, benzoyloxy-) alicyclic amides. On the contrary, whole-cell biocatalyst SP 409 exhibiting nitrile hydratase and amidase activities gave 2-hydroxycyclohexane- and 2-hydroxycyclopentanecarboxylic acids as the only products (5).

The syntheses of *cis*- and *trans*-2-hydroxycyclohexanecarboxamide (11), 2-hydroxycyclopentanecarboxamide (11), as well as *cis*- (12, 13) and *trans*-4-hydroxycyclohexanecarboxamide (13) have been published previously. Hydroxycyclohexanecarboxamides, both the *cis*- and *trans*-3-isomers, were reported as the respective *O*-acetyl derivatives only (12); however, no NMR data were given for these compounds.

All examined *trans* isomers of 2- and 4-substituted alicyclic nitriles (Scheme 1) were readily converted into the corresponding amides by the nitrile hydratase from *R. equi* A4 (Table 1). *trans*-4-Benzoyloxycyclohexanecarbonitrile (*trans*-**1a**) was transformed at a substantially higher rate than *cis*-**1a**. The reaction rate of *cis*-3-benzoyloxycyclohexanecarbonitrile (*cis*-**2a**) was somewhat lower than that of *trans*-**1a** (Table 1). The *trans* isomer of **2a** was not available.

An even more striking difference than between *trans*-**1a** and *cis*-**1a** was observed when comparing the activities of the nitrile hydratase towards *trans* and *cis* isomers of 2-hydroxycyclohexanecarbonitriles (*trans*-**3a** and *cis*-**3a**) or 2-hydroxycyclopentanecarbonitriles (*trans*-**4a** and *cis*-**4a**) (Table 1). In both pairs of nitriles, the compounds bearing *trans*-substituents were totally converted within 3 h to afford corresponding amides *trans*-**3b** and *trans*-**4b**, respectively. On the contrary, only traces of *cis*-**3b** were formed from *cis*-**3a**, and nitrile *cis*-**4a** was not transformed by the enzyme at all.

It is apparent that the enzyme prefers an equatorial position for both substituents, which can be adopted by the *trans*-1,2- and *trans*-1,4-isomers of the investigated substituted cyclohexanecarbonitriles. Nevertheless, this diequatorial arrangement of the substituents is also in accordance with *cis*-3-benzoyloxycyclohexanecarbonitrile (*cis*-**2a**), which is readily converted to the amide. The two large coupling constants J_{aa} = 9.2 and 9.7 Hz and J_{aa} = 8.7 and 8.8 Hz for H-1 and H-3, respectively, and the ^{1}H coupling pattern clearly reveal a diequatorial substituent position in *cis*-**2a**.

Impairment of the activity of the nitrile hydratase from *R. equi* A4 by steric effects, for example by nitriles with bulky groups such as 2-(3-benzoylphenyl)propionitrile, 2,6-dichlorobenzonitrile, and some 2-substituted benzonitriles, was previously observed (10). Its inefficient hydration of 2- and 4-substituted cyclohexane(pentane)carbonitriles with a *cis*-configuration suggested that this enzyme is more sensitive to steric hindrances than biocatalyst SP 409.

Experimental

NMR spectra of substrates were recorded on a Varian Gemini 200 spectrometer (200 and 50.3 MHz for ^{1}H and ^{13}C, respectively) in $CDCl_3$. NMR spectra of products of the biotransformation were recorded on a Unity Inova 400 MHz spectrometer (399.90 MHz for ^{1}H, 100.57 MHz for ^{13}C) in DMSO-d_6 at 30°C. The assignment was based on COSY, HMQC, and HMBC experiments performed using the manufacturer's software. Residual solvent signals (DMSO: δ_H 2.50 ppm, δ_C 39.60 ppm; $CDCl_3$: δ_H 7.27 ppm, δ_C 77.27 ppm) were used as internal standards.

Synthesis of substrates

Nitriles *trans*-**1a** (14), *cis*-**1a** (14), *cis*-**2a** (15), *trans*-**3a** (16), *cis*-**3a** (17), *trans*-**4a** (18), and *cis*-**4a** (17) were prepared according to literature reported procedures. ^{13}C and ^{1}H NMR data were previously reported for *trans*-**1a** (19), *cis*-**1a** (19), *trans*-**3a** (16, 20), *cis*-**3a** (20–22), *trans*-**4a** (18), and *cis*-**4a** (20–22) but no ^{1}H–^{1}H coupling constants were given.

trans-*1a*

^{1}H NMR δ: 1.70–1.84 (m, 4H, H-2, H-3, H-5, H-6), 2.06–2.16 (m, 4H, H-2, H-3, H-5, H-6), 2.71 (tt, *J* = 7.8, 4.4 Hz, 1H, H-1), 5.11 (tt, *J* = 7.6, 3.7 Hz, 1H, H-4), 7.44 (m, 2H, H-*meta*), 7.56 (m, 1H, H-*para*), 8.02 (m, 2H, H-*ortho*).

cis-*1a*

^{1}H NMR δ: 1.83–2.11 (m, 8H, H-2, H-3, H-5, H-6), 2.77 (tt, *J* = 7.4, 3.7 Hz, 1H, H-1), 5.10 (tt, *J* = 7.4, 3.7 Hz, 1H,

H-4), 7.46 (m, 2H, H-*meta*), 7.58 (m, 1H, H-*para*), 8.05 (m, 2H, H-*ortho*).

cis-*2a*

^{1}H NMR δ: 1.40–2.10 (m, 7H), 2.31 (m, 1H), 2.72 (tt, *J* = 3.5, 5.9, 9.2, 9.7 Hz, 1H, H-1), 5.04 (tt, *J* = 3.5, 5.0, 8.7, 8.8 Hz, 1H, H-3), 7.44 (m, 2H, H-*meta*), 7.53 (m, 1H, H-*para*), 8.06 (m, 2H, H-*ortho*). ^{13}C NMR δ: 21.68 (C-5), 26.22 (C-6), 28.95 (C-4), 30.56 (C-2), 33.98 (C-1), 70.36 (C-3), 121.88 (CN), 128.62 (C-*meta*), 129.95 (C-*ortho*), 130.32 (C-ipso), 133.33 (C-*para*), 165.98 (C=O).

trans-*3a*

^{1}H NMR δ: 1.10–1.44 (m, 4H, H-3, H-4, H-5, H-6), 1.51–1.84 (m, 3H, H-4, H-5, H-6), 2.06 (m, 1H, H-3), 2.42 (ddd, *J* = 11.5, 9.3, 3.8 Hz, 1H, H-1), 3.03 (br s, 1H, OH), 3.69 (dt, *J* = 9.4, 4.2 Hz, 1H, H-2).

cis-*3a*

^{1}H NMR δ: 1.13–2.20 (m, 8H, H-3/3, H-4/4, H-5/5, H-6/6), 3.01 (q, *J* = 3.1 Hz, 1H, H-1), 3.10 (br s, 1H, OH), 3.75 (dt, *J* = 8.4, 4.1, Hz, 1H, H-2).

trans-*4a*

^{1}H NMR δ: 1.48–2.30 (m, 6H), 2.65 (m, Σ *J* = 13.2 Hz, 1H, H-1), 3.68 (d, *J* = 3.5 Hz, 1H, OH), 4.37 (m, Σ *J* = 11.9 Hz, 1H, H-2).

cis-*4a*

^{1}H NMR δ: 1.46–2.10 (m, 6H), 2.71 (dt, *J* = 8.3, 4.8 Hz, 1H, H-1), 3.65 (br s, 1H, OH), 4.35 (dt, *J* = 5.3, 3.5 Hz, 1H, H-2).

General procedure of the biotransformation

The nitrile hydratase from *Rhodococcus equi* A4 (10) was diluted with KH_2PO_4–Na_2HPO_4 buffer (54 mM, pH 7.5) to a concentration of 22 μg of protein mL^{-1} (42 μg of protein mL^{-1} for *cis*-**2a**) and the substrates were added from stock solutions in methanol to make up final concentrations of 1.25 mM of *trans*-**1a**, *cis*-**1a**, and *cis*-**2a** and 5 mM of *trans*-**3a**, *cis*-**3a**, *trans*-**4a**, and *cis*-**4a**. Methanol did not exceed 5% (v/v) of the reaction mixture. The reactions were carried out in shaken vessels (850 rpm, Thermomixer Compact Eppendorf) at 30°C. At intervals, samples were withdrawn, the reaction was quenched with 1 M HCl (0.1 mL per 1 mL of sample) and the precipitated protein was removed by centrifugation. The supernatants were analyzed by HPLC as described below or by TLC on silica gel plates (Merck) developed with chloroform–ethyl acetate (10:1) (for substrates *trans*-**3a**, *cis*-**3a**, *trans*-**4a**, and *cis*-**4a**); the spots were visualized by charring with 5% sulphuric acid in ethanol.

Analytical HPLC

The concentrations of substrates and products were determined using an HPLC system consisting of a solvent-delivery system 600 (Waters), a photo-diode array detector 996 (Waters), and a refractive-index detector 2410 (Waters) and a Nova-Pak C_{18} column (5 μm, 3.9 × 150 mm, Waters). As mobile phases, 50% (v/v) acetonitrile (for compounds *trans*-**1a**, *trans*-**1b**, *cis*-**1a**, *cis*-**1b**, *cis*-**2a**, and *cis*-**2b**) or 10% (v/v) acetonitrile (for other compounds) in water plus 0.1 % (v/v) H_3PO_4 were employed at a flow rate of 1.0 mL min^{-1}. Compounds *trans*-**1a**, *trans*-**1b**, *cis*-**1a**, *cis*-**1b**, *cis*-**2a**, and *cis*-**2b** (retention times 5.6, 2.2, 5.0, 1.8, 5.5, and 2.1 min, respectively) were detected at 230 nm. Compounds *cis*-**3a**, *trans*-**4a**, and *cis*-**4a** (retention times 5.4, 3.5, and 2.9 min, respectively) were detected by the refractive-index detector.

Isolation and identification of products

Amides *trans*-**1b**, *cis*-**1b**, and *cis*-**2b** were extracted from supernatants of the reaction mixtures with ethyl acetate at pH 8.5–8.8 (NaOH). The reaction mixtures from the biotransformations of substrates *trans*-**3a** and *trans*-**4a** were lyophilized and extracted with methanol to give the respective amides *trans*-**3b** and *trans*-**4b**.

trans-*1b*

^{1}H NMR δ: 1.41–1.55 (m, 4H, H-2a, H-3a, H-5a, H-6a), 1.85 (m, 2H, H-2b, H-6b), 2.05 (m, 2H, H-3b, H-5b), 2.14 (m, Σ *J* = 29.7 Hz, 1H, H-1), 4.82 (m, Σ *J* = 29.7 Hz, 1H, H-4), 6.71 (br s, 1H, NH), 7.25 (br s, 1H, NH), 7.52 (m, 2H, H-*meta*), 7.64 (m, 1H, H-*para*), 7.94 (m, 2H, H-*ortho*). ^{13}C NMR δ: 27.17 (t, C-2 and C-6), 30.60 (t, C-3 and C-5), 42.44 (d, C-1), 73.01 (d, C-4), 128.82 (d, C-*meta*), 129.19 (d, C-*ortho*), 130.25 (s, C-ipso), 133.33 (d, C-*para*), 165.29 (s, O*C*OPh), 176.80 (s, $CONH_2$).

cis-*1b*

^{1}H NMR δ: 1.60–1.80 (m, 6H, H-2, H-6, H-3a, H-5a), 1.93 (m, 2H, H-3b, H-5b), 2.22 (m, Σ *J* = 28.6 Hz, 1H, H-1), 5.14 (m, Σ *J* = 13.9 Hz, 1H, H-4), 6.71 (br s, 1H, NH), 7.23 (br s, 1H, NH), 7.55 (m, 2H, H-*meta*), 7.66 (m, 1H, H-*para*), 7.99 (m, 2H, H-*ortho*). ^{13}C NMR δ: 23.96 (t, C-2 and C-6), 28.77 (t, C-3 and C-5), 42.10 (d, C-1), 69.64 (d, C-4), 128.76 (d, C-*meta*), 129.06 (d, C-*ortho*), 130.38 (s, C-ipso), 133.20 (d, C-*para*), 165.01 (s, O*C*OPh), 176.78 (s, $CONH_2$).

cis-*2b*

^{1}H NMR δ: 1.27 (m, 1H, H-6a), 1.37 (m, 1H, H-4a), 1.38 (m, 1H, H-5a), 1.52 (d, *J* = 12.2 Hz, 1H, H-2a), 1.74 (m, 1H, H-6b), 1.86 (m, 1H, H-5b), 2.01 (m, 1H, H-4b), 2.08 (m, Σ *J* = 23.6 Hz, 1H, H-2b), 2.30 (dd, *J* = 3.5, 11.9 Hz, 1H, H-1), 4.88 (dd, *J* = 4.4, 11.0 Hz, 1H, H-3), 6.73 (br s, 1H, NH), 7.25 (br s, 1H, NH), 7.52 (m, 2H, H-*meta*), 7.65 (m, 1H, H-*para*), 7.95 (m, 2H, H-*ortho*). ^{13}C NMR δ: 23.07 (t, C-5), 28.19 (t, C-6), 31.01 (t, C-4), 34.33 (t, C-2), 41.97 (d, C-1), 72.92 (d, C-3), 128.71 (d, C-*meta*), 129.10 (d, C-*ortho*), 130.13 (s, C-ipso), 133.22 (d, C-*para*), 165.11 (s, O*C*OPh), 175.75 (s, $CONH_2$).

trans-*3b*

^{1}H NMR δ: 1.01–1.24 (m, 3H, H-3a, H-4a, H-5a), 1.27 (m, 1H, H-6a), 1.54–1.72 (m, 3H, H-4b, H-5b, H-6b), 1.81 (m, 1H, H-3b), 1.94 (ddd, *J* = 3.7, 9.8, 12.2 Hz, 1H, H-1), 3.46 (m, 1H, H-2), 4.57 (d, *J* = 5.1 Hz, 1H, OH), 6.61 (br s, 1H, NH), 7.13 (br s, 1H, NH). ^{13}C NMR δ: 24.39 (t, C-4), 24.84 (t, C-5), 28.73 (t, C-6), 35.16 (t, C-3), 52.10 (d, C-1), 69.80 (d, C-2), 176.67 (s, $CONH_2$).

trans-*4b*

^{1}H NMR δ: 1.42 (m, 1H, H-3a), 1.50–1.64 (m, 3H, H-4a, H-4b, H-5a), 1.68–1.83 (m, 2H, H-3b, H-5b), 2.39 (m, 1H,

H-1), 4.08 (m, 1H, H-2), 6.67 (br s, 1H, NH), 7.20 (br s, 1H, NH). ^{13}C NMR δ: 22.50 (t, C-4), 27.90 (t, C-5), 34.96 (t, C-3), 53.01 (d, C-1), 75.42 (d, C-2), 176.54 (s, $CONH_2$).

Acknowledgments

Financial support of this work from the Grant Agency of the Academy of Sciences of the Czech Republic (project A4020802/1998), by the Grant Agency of the Czech Republic (project 524/00/1275), by the grant AKTION 17p12 from the Austrian and Czech Ministries of Education and by the Österreichische Nationalbank (project No. 7241) is gratefully acknowledged. Authors wish to thank Dr. Petr Sedmera for critically reading the manuscript and Ms. Magdalena Balvínová for her excellent technical assistance.

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