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Biotransformation of 3-substituted methyl (*R*,*S*)-4-cyanobutanoates with nitrile- and amide-converting biocatalysts

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Abstract

Whole cells of *Rhodococcus equi* A4 chemoselectively hydrolyzed methyl (*R*,*S*)-3-benzoyloxy-4-cyanobutanoate and methyl (*R*,*S*)-3-benzyloxy-4-cyanobutanoate into monomethyl (*R*,*S*)-3-benzoyloxyglutarate and monomethyl (*R*,*S*)-3-benzyloxyglutarate, respectively. The intermediates of the biotransformations were the corresponding amides which were also obtained using the purified nitrile hydratase from the same microorganism.

Keywords: *Rhodococcus equi*; Nitrile hydratase; Amidase; Cyanobutanoates; Monomethyl glutarates

1. Introduction

Biocatalysts have been used for hydrolysis of nitriles containing other labile groups (for a review, see [1]). However, esterase activity makes many of these agents unsuitable for chemoselective transformations of cyano esters. Nitrile-hydrolyzing biocatalyst, nitrilase Novo SP 409 produced dihydroxy nitriles, dihydroxy amides and dihydroxy acids from diacetoxy nitriles [2]. Hemiesters were obtained from ethyl and methyl cyanobenzoate by whole cells of *Rhodococcus rhodochrous* NCIMB 11216 but cleavage of the ester groups (leading to cyano acids and diacids) reduced the product yields [3].

Excellent chemoselectivity towards the cyano group was found for hydration and hydrolysis of cyanobenzoates catalyzed by whole cells of *Rhodococcus equi* A4 [4]. Here, we used the same microorganism, *Rhodococcus equi* A4, as well as the purified nitrile hydratase [5] from this strain to prepare amides and carboxylic acids from 3-substituted ω-cyano butanoates (**1a**, **2a**; Scheme 1).

2. Experimental

NMR spectra were recorded on a Varian Gemini 200 spectrometer at 200 and 50.3 MHz and on a Unity Inova — 400 MHz spectrometer at 399.90 and 100.57 MHz in d_6-DMSO 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 2.5 ppm, δ_C 39.6 ppm)

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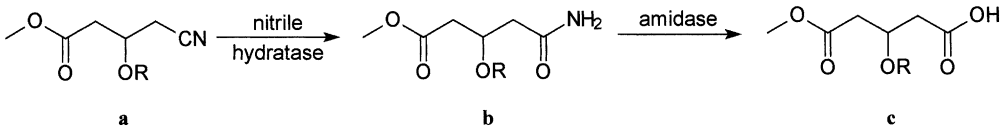

1 R = Bz

2 R = Bn

Scheme 1.

were used as an internal standard. Compounds **1a** or **2a** were prepared by literature-reported procedures [6,7]. **1a**: ^{1}H-NMR (399.905 MHz, d_6-DMSO, 30°C): 2.875 (dd, 1H, J = 7.4, 16.2 Hz, H-2a), 2.921 (dd, 1H, J = 5.6, 16.2 Hz, H-2b), 3.096 (dd, 1H, J = 5.9, 17.4 Hz, H-4a), 3.154 (dd, 1H, J = 4.8, 17.4 Hz, H-4b), 3.598 (s, 3H, CH_3O), 5.547 (m, 1H, J = 4.8, 5.6, 5.9, 7.4 Hz, H-3), 7.560 (m, 2H, H-*meta*), 7.696 (m, 1H, H-*para*), 7.957 (m, 2H, H-*ortho*). ^{13}C-NMR (100.566 MHz, d_6-DMSO, 30°C): 22.3 (C-4), 37.4 (C-2), 51.7 (CH_3O), 66.0 (C-3), 117.3 (C-5), 128.2 (C-*ipso*), 128.8 (C-*meta*), 129.3 (C-*ortho*), 133.7 (C-*para*), 164.6 (Ph–COO), 169.6 (C-1). **2a**: ^{1}H-NMR (399.905 MHz, d_6-DMSO, 30°C): 2.642 (dd, 1H, J = 7.1, 15.7 Hz, H-2a), 2.684 (dd, 1H, J = 5.8, 15.7 Hz, H-2b), 2.827 (dd, 1H, J = 5.2, 17.1 Hz, H-4a), 3.010 (dd, 1H, J = 4.8, 17.1 Hz, H-4b), 3.605 (s, 3H, CH_3–O), 4.082 (m, 1H, J = 4.8, 5.2, 5.8, 7.1 Hz, H-3), 4.515 (d, 1H, J = 11.6 Hz, Ph–CH_2–O), 4.623 (d, 1H, J = 11.6 Hz, Ph–CH_2–O), 7.271–7.377 (m, 5H, aromatic protons). ^{13}C-NMR (100.566 MHz, d_6-DMSO, 30°C): 21.8 (C-4), 38.6 (C-2), 51.5 (CH_3O), 70.7 (Ph–CH_2–O), 71.0 (C-3), 118.1 (C-5), 127.6, 128.1, 128.3 (5 × –CH=), 137.8 (C-*ipso*), 170.4 (C-1).

From **1a** and **2a**, the corresponding amides and carboxylic acids were obtained using the nitrile hydratase (44 μg ml^{-1}) [5] or the whole cells (2 mg of dry cell weight ml^{-1}) of *R. equi* A4 [4], respectively, in Na_2HPO_4/KH_2PO_4 buffer (54 mM, pH 7.5) at 30°C and shaking (850 rpm, Thermomixer Compact Eppendorf). Compounds **1b** and **1c** were identified by the comparison of their retention times in the reversed-phase and chiral HPLC (see below) with those of authentic standards prepared chemically. **1b**: ^{1}H-NMR (399.905 MHz, d_6-DMSO, 30°C): 2.536 (dd, 1H, J = 6.0, 14.9 Hz, H-4a), 2.613 (dd, 1H, J = 7.1, 14.9 Hz, H-4b), 2.791 (dd, 1H, J = 7.3, 15.7 Hz, H-2a), 2.854 (dd, 1H, J = 5.2, 15.7 Hz, H-2b), 3.574 (s, 3H, CH_3O–), 5.580 (m, 1H, J = 5.2, 6.0, 7.1, 7.3 Hz, H-3), 6.900 (br s, 1H, $CONH_2$), 7.440 (br s, 1H, $CONH_2$), 7.523 (m, 2H, H-*meta*), 7.656 (m, 1H, H-*para*), 7.908 (m, 1H, H-*ortho*). ^{13}C-NMR (100.566 MHz, d_6-DMSO, 30°C): 38.1 (C-2), 39.1 (C-4), 51.5 (CH_3O–), 68.5 (C-3), 128.7 (C-*meta*), 129.1 (C-*ortho*), 133.3 (C-*para*), 128.1 (C-*ipso*), 164.8 (COOPh), 170.4 (C-1), 170.5 (C-5). **1c**: ^{1}H-NMR: 2.711–2.807 (m, 2H, H-2), 2.830–2.889 (m, 2H, H-4), 3.582 (s, 3H, CH_3O–), 5.580 (m, 1H, H-3), 7.525 (m, 2H, H-*meta*), 7.660 (m, 1H, H-*para*), 7.905 (m, 2H, H-*ortho*). ^{13}C-NMR: 38.0 (C-2), 38.2 (C-4), 51.6 (CH_3O–), 67.9 (C-3), 128.7 (C-*meta*), 129.1 (C-*ortho*), 133.4 (C-*para*), 129.6 (C-*ipso*), 164.8 (COOPh), 170.2 (C-5), 171.2 (C-1).

Chemical hydrolysis of **2a** was not feasible, since, the strong acidic or basic conditions required would cleave the methyl ester. Therefore, amide **2b** and acid **2c** were extracted from supernatants of the reaction mixtures with ethyl acetate at pH 8.5 (NaOH) and pH 2 (HCl), respectively. **2b**: ^{1}H-NMR (399.905 MHz, d_6-DMSO, 30°C): 2.284 (dd, 1H, J = 6.1, 14.5 Hz, H-4a), 2.473 (dd, 1H, J = 6.8, 14.5 Hz, H-4b), 2.532 (dd, 1H, J = 8.0, 15.3 Hz, H-2a), 2.630 (dd, 1H, J = 4.7, 15.3 Hz, H-2b), 3.584 (s, 3H, CH_3O), 4.185 (m, 1H, J = 4.7, 6.1, 6.8, 8.0 Hz, H-3), 4.459 (d, J = 11.6 Hz, Ph–CH_2–O), 4.535 (d, J = 11.6 Hz, Ph–CH_2–O), 6.858 (br s, 1H, $CONH_2$), 7.237–7.346 (5H, aromatic protons), 7.375 (br s, 1H, $CONH_2$). ^{13}C-NMR (100.566 MHz, d_6-DMSO, 30°C): 39.3 (C-2), 40.0 (C-4), 51.3 (CH_3O–), 70.7 (Ph–CH_2–O), 73.2 (C-3), 127.3, 127.5, 128.1 (5 × –CH=), 138.6 (C-*ipso*), 171.2 (C-1), 171.7 (C-5). **2c**: ^{1}H-NMR (399.905 MHz, d_6-DMSO, 30°C): 2.519 (dd, 1H, J = 5.8, 15.7 Hz, H-2a), 2.578 (dd, 1H, J = 6.7,

15.7 Hz, H-2b), 2.604 (dd, 1H, J = 7.0, 15.5 Hz, H-4a), 2.650 (dd, 1H, J = 5.5, 15.5 Hz, H-4b), 3.592 (s, 3H, CH_3O), 4.169 (m, 1H, J = 5.5, 5.8, 6.7, 7.0 Hz, H-3), 4.487 (d, 1H, J = 11.6 Hz, Ph–CH_2–O), 4.529 (d, 1H, J = 11.6 Hz, Ph–CH_2–O), 7.241–7.349 (5H, aromatic protons). ^{13}C-NMR (100.566 MHz, d_6-DMSO, 30°C): 38.7 (C-4), 38.8 (C-2), 51.3 (CH_3O), 70.4 (Ph–CH_2–O), 72.3 (C-3), 127.2, 127.4, 128.0 (5 × –CH=), 137.9 (C-*ipso*), 171.1 (C-5), 172.0 (C-1). As hydroxyl protons were not detected in the NMR spectra of acids **1c** and **2c**, the structures were proven by mass spectra. Mass spectrum (EI) was recorded on a HP 6890 MSD. **1c**: MS (EI) *m/z* 105 (100), 77 (40), 122 (34), 143 (13), 161 (6). Positive ion FAB mass spectrum was recorded on a Finnigan MAT 95 double focusing instrument using *m*-nitrobenzyl alcohol as a matrix. The saddle field FAB gun (TonTech, Teddington, UK) was operated at 2 mA current and 6 keV energy, xenon (1×10^{-5} bar) was used as a bombarding gas. The mass range calibration was performed with Ultramark 1600F (PCR Inc., USA) as a standard. **2c**: MS (FAB): 275 $[M+Na]^+$ ($C_{13}H_{16}O_5$).

Analytical HPLC was performed using HPLC Millenium Chromatography Manager 2.0, equipped with an HPLC solvent delivery system 600 and a PDA detector 996 (Waters Associates, Milford, MA, USA) and a Nova-Pak C_{18} column (5 μm, 3.9 mm × 150 mm, Waters). The compounds **1a**, **1b**, **1c** and benzoic acid (detected spectrophotometrically at 230 nm) eluted at 23.0, 4.9, 7.3, and 4.4 min, respectively, with 22% (v/v) acetonitrile plus 0.1% (v/v) H_3PO_4 at a flow rate of 0.9 ml min^{-1}. The compounds **2a**, **2b, 2c** and benzyl alcohol (detected spectrophotometrically at 212 nm) eluted at 14.8, 3.4, 7.1 and 5.3 min, respectively, with 25% (v/v) acetonitrile plus 0.1% (v/v) H_3PO_4 at a flow rate of 0.9 ml min^{-1}.

Enantiomers of **1a**, **1b**, **2a** and **2b** were separated using a Chiral-AGP column (5 μm particles, 100 mm × 4.0 mm, ChromTech, Hägersten, Sweden) and a mobile phase consisting of 10 mM Na-phosphate buffer, pH 7.0, with 1 mM DMOA at a flow rate of 0.9 ml min^{-1}. Elution times of individual enantiomers were 10.5 and 14.4 min for **1a**, 4.5 and 6.5 min for **1b**, 6.9 and 9.8 min for **2a** and 6.9 and 10.8 min for **2b**. Enantiomers of **1c** and **2c** did not separate under the above conditions.

3. Results and discussion

3.1. Bioconversions using resting cells of *R. equi* A4

Conversions of ω-cyano butanoates **1a** and **2a** (2.5 mM each) were catalyzed by whole cells of *R. equi* A4 producing a nitrile hydratase and an amidase (Figs. 1 and 2).

Nitrile **1a** was readily converted into amide **1b**. The biocatalyst showed benzoyl esterase activity which was, however, significantly lower than that of the nitrile hydratase (approx. 30 times). Therefore, the nitrile hydration was not accompanied by significant

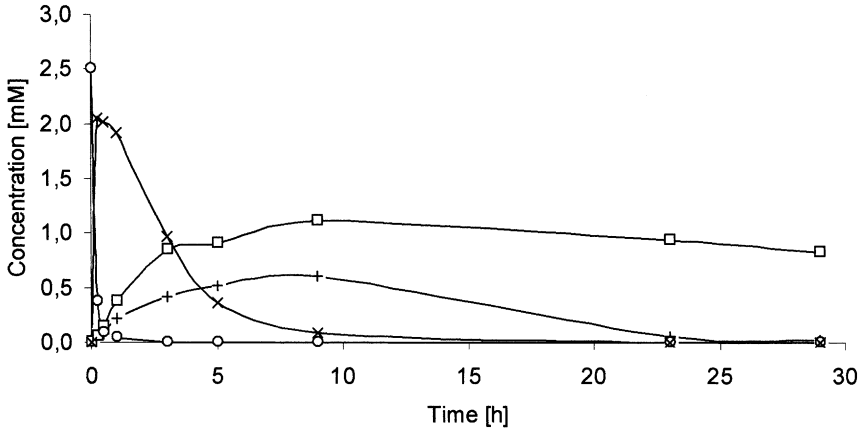

Fig. 1. Biotransformation of **1a** (○) by whole cells of *Rhodococcus equi* A4 into **1b** (×), **1c** (□) and benzoic acid (+).

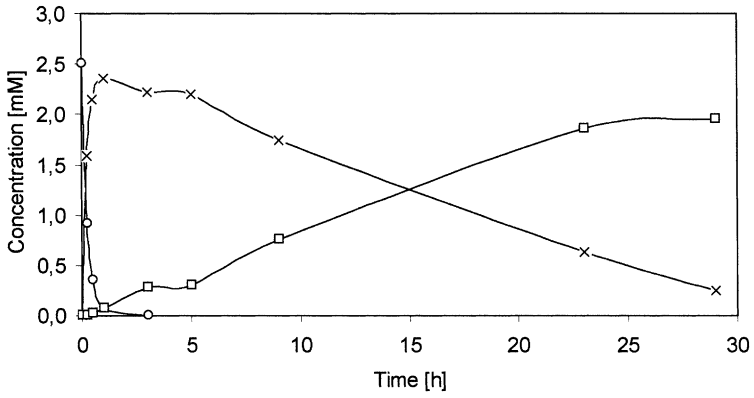

Fig. 2. Biotransformation of **2a** (○) by whole cells of *Rhodococcus equi* A4 into **2b** (×) and **2c** (□).

ester hydrolysis and the conversion of the nitrile into the amide was high.

The amidase-catalyzed hydrolysis of amide **1b** into acid **1c** proceeded at a lower rate than the nitrile hydration. Therefore, ester hydrolysis at position C-3 was competing with the amide hydrolysis. Benzoic acid, formed transiently as a side product, was probably metabolized by the cells. Therefore, carboxylic acid **1c** was the major product in the reaction medium after a reaction time of 24 h. *Rhodococcus rhodochrous* IFO 15564 used for enzymatic hydrolysis of methyl 3-benzoyloxypentanoate also showed hydrolytic activity towards the benzoate group. This reaction was catalyzed by a constitutive esterase and not by the amidase itself [8].

Detection at 215 nm showed five minor peaks with low retention times and lacking the spectrum maximum around 230 nm typical for benzoylated compounds. These peaks could correspond to side products lacking the benzoyl group or both the benzoyl and the methyl group. However, their concentrations could not be assayed because the authentic standards were not available. Therefore, it cannot be excluded that such compounds were partly metabolized. Cleavage of the methyl ester could also contribute to the formation of side products as two peaks which could correspond to demethylated compounds bearing the benzoyl groups as chromophores were detected. Neither were the authentic standards of these compounds available. However, a limited degree of demethylation might be suggested by comparison with substrate **2a** (see below). Lastly, spontaneous degradation of the products could also cause material losses. However, the nitriles, amides and acids characterized in this work proved to be stable under the mild reaction conditions used.

The conversion of nitrile **2a** also afforded the corresponding amide **2b** after a short incubation time (1 h). Benzyl alcohol arising from the cleavage of the ether bond was found only in small amounts (not exceeding 0.12 mM). Neither was the methyl ester hydrolyzed to a significant extent as judged from the high conversion of amide **2b** into acid **2c**.

3.2. Bioconversions using purified nitrile hydratase from R. equi A4

The purified nitrile hydratase from *R. equi* A4 converted nitriles **1a** and **2a** (0.6 mM each) at the rates of 0.43 and 0.22 μmol min^{-1} mg^{-1} of protein, respectively. The amides **1b** and **2b** (conversion 95%) were obtained within 30 min and 1 h, respectively. The enzyme showed a very low enantioselectivity for **1a** ($E = 2$) and no enantioselectivity for **2a**. Thus, a benzoyloxy group on C-3 was more suitable for the enantioselectivity of the nitrile hydratase than a benzyloxy group. A similar observation was made with enzymatic hydration of prochiral dinitriles [8,9].

Some nitrile hydratases [5,10,11] showed enantioselectivity towards 2-substituted nitriles. The enzyme from *R. equi* A4 exhibited a moderate preference for (*S*)-2-(6-methoxynaphthyl)propionitrile ($E = 15$–41) [5]. However, enantioselectivity of nitrile hydratases towards 3-substituted nitriles is less frequent: Only prochiral dinitriles, 3-benzoyloxyglutaronitrile and 3-benzyloxyglutaronitrile, were *S*-selectively hydrated

by the nitrile-hydrating biocatalysts, namely *Rhodococcus butanica* ATCC 21197 [12] (later reclassified as *Rhodococcus rhodochrous* IFO 15564 [8]), "nitrilase SP 361" from Novo [9] and *Brevibacterium* sp. R 312 [6]. The ω-cyano group is probably crucial for the enantioselectivity of the nitrile hydratases. Its replacements by an ω-methyl group (in 3-benzoyloxypentanenitrile) canceled the enantioselectivity of the enzyme from *R. rhodochrous* [8]. Neither cyanobutanoates **1a** and **2a** bearing an ω-methylcarboxy group were suitable for the enantioselective operation of the nitrile hydratase.

4. Conclusions

R. equi A4 appears to be a convenient chemoselective biocatalyst for the preparation of 3-substituted ω-amidobutanoates from the studied ω-cyanobutanoates. The respective glutaric acid hemiesters were also accessible using this biocatalyst. The nitrile hydratase showed little or no enantioselectivity for 3-*o*-substituted ω-cyanobutanoates but the enzyme system of *R. equi* A4 can be useful for the stereoretentive transformation of enantiopure ω-cyanobutanoates prepared, e.g. by the hydrolysis of prochiral dinitriles and subsequent esterification of the resulting cyano acids.

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