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Chemoselective Hydrolysis of Nitriles by *Rhodococcus rhodochrous* NCIMB 11216

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Summary

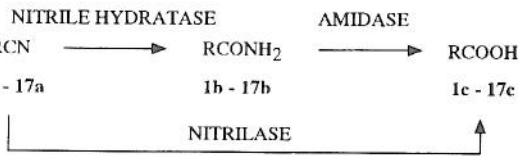
The formation of acids from nitriles was achieved using resting cells from **Rhodococcus rhodochrous** *NCIMB 11216. The hydrolysis of the nitrile group could be accomplished in a selective manner in the presence of acetal and ester groups in the molecule. While the former functional moieties remain unaffected during the hydrolysis, the latter ones were concurrently hydrolysed. Attempts to suppress the esterase activity of this strain by modification of the growth media remained unsuccessful for aliphatic substrates.*

Keywords: nitrile hydratase, nitrilase, chemoselective hydrolysis of nitriles, *Rhodococcus rhodochrous*

Introduction

The enzymatic hydrolysis of nitriles to amides and carboxylic acids, although well known, has recently found increasing interest in organic synthesis *(1)*. The mild reaction conditions regarding pH and temperature permit selective nitrile transformations in the presence of acid or base labile functional groups in contrast to the chemical conditions of nitrile hydrolysis. Moreover, a number of examples of stereoselective transformations, such as the hydrolysis of prochiral dinitriles to chiral acids and amides *(2,3)*, the preparation of enantioenriched 2-arylpropionic acids as well as other chiral compounds *(4)* have been reported to proceed with good selectivity.

Various bacterial and fungal systems with the ability to hydrolyse nitriles have been described. They can be distinguished by their two main pathways of nitrile hydrolysis owing to different enzymes involved *(Scheme 1)*.



Nitrilases, nitrile hydratases and amidases are inducible enzymes, but constitutive examples are also known, e. g. the constitutive nitrile hydratase of *Brevibacterium* sp. R-312 *(5)*. It has been reported that a number of enzymes with different substrate specificities are active, depending on the inducer used *(6)*. Microbial systems containing nitrilases have been reported in *Nocardia* sp. *(7)*, *Rhodococcus rhodochrous* J-1 *(8)*, *Rhodococcus* sp. ATCC 39484 *(9)*, *Alcaligenes faecalis* JM-3 *(10)* and others. They hydrolyse the nitrile directly into the carboxylic acid. No nitrilases that act on aliphatic nitriles have been reported, except the nitrilase from *Rhodococcus rhodochrous* K--22, which acts on acrylonitrile and glutaronitrile *(11)*. In contrast, strains containing nitrile hydratase hydrolyse nitriles to the amide, which is usually further hydrolysed to the carboxylic acid by an amidase. However, the intermediate amide can not be detected in all cases. Such nitrile hydratase/amidase containing microbial systems have been described in *Corynebacterium nitrilophilus (12)*, *Rhodococcus equi* TG 328 *(13)*, *Rhodococcus rhodochrous* ATCC 21197 *(14)*, *Rhodococcus rhodochrous* J-1 *(15)*, *Pseudomonas chlororaphis* B-23 *(16)* and *Rhodococcus rhodochrous* NCIMB 11216. Resting cells of this bacterium *(Rhodococcus rhodochrous* NCIMB 11216*)* were reported to hydrolyse a number of aliphatic and aromatic mono- and

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dinitriles *(17)*. For some dinitriles, the reaction proceeded in a regioselective manner, since it stopped at the stage of the monohydrolysis.

Recently, work on stereoselective *(18)* as well as chemoselective nitrile transformations *(19)* has been accomplished using an immobilised crude enzyme preparation. However, this enzyme is not commercially available. Therefore, the use of a microbial system, generally accessible, appears to be of some advantage. In this publication we wish to report on the chemoselective hydrolysis of various functionalised nitriles by using whole cells of *Rhodococcus rhodochrous* NCIMB 11216.

Materials and Methods

Microorganism and Media

Rhodococcus rhodochrous NCIMB 11216 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK. The strain was maintained on medium 1 agar slants at 4 °C and was subcultured every two months. For cell growth rich media (medium 1 and medium 2) were employed. Medium 1 (pH = 7.0) consisted of 6 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4$, 1 g of $(NH_4)_2SO_4$, 2.5 g of yeast extract, 2.5 g of peptone, 6 g of glucose, 1 mL of an aqueous solution of $CaCl_2$ (0.1 mol/L) and a solution of trace elements (1 mL) per liter of distilled water *(20)*. Medium 2 consisted of 6 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4$, 2 g of $(NH_4)_2SO_4$, 0.5 g of yeast extract, 6 g of glucose, 1 mL of an aqueous solution of $CaCl_2$ (0.1 mol/L) and 1 mL of a solution of trace elements per liter of distilled water. For induction of nitrilase, nitrile hydratase and amidase a minimal medium (medium 3) was used. Medium 3 (pH = 7.0) contained 6 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4$, 1 mL of an aqueous solution of $CaCl_2$ (0.1 mol/L) and 1 mL of a solution of trace elements per liter of distilled water. As the source of carbon and nitrogen either propionitrile (70 mmol) or benzonitrile (20 mmol) was added.

Growth conditions

Starting with two agar slants, cells were used to inoculate a 1L Erlenmeyer flask containing 250 mL of medium 1 and incubated at 28 °C on a rotary shaker (110 rpm) for 24 hours. Growth was measured by the increase in absorbance at 690 nm ($A = 1 \triangleq 0.24$ mg dry wt. cells/mL) on a Perkin Elmer 550 SE UV/Vis spectrophotometer. The cultures were centrifuged at 5000 *g* for 15 minutes at 4 °C and washed with cold buffer A (6 g of K_2HPO_4 and 3 g of KH_2PO_4 per liter, pH = 7.0). The bacteria were then resuspended in medium 2 and cultivated further as described above. After 24 hours the cells were harvested by centrifugation and washed twice with cold buffer A.

For induction of the nitrile hydratase cells were transferred into flasks containing medium 3. Propionitrile was added and the flasks were shaken for 24 hours. Measurement of the cell dry weight gave an average of 4.8 g/L. Concentrations of nitrile and propionic acid were determined by gas chromatography (Shimadzu 14A) using a packed glass column 1 m length, 3.5 mm internal diameter) containing Porapak QS (100–120 mesh). The operating conditions were: oven temperature 200 °C (isothermal); injector temperature 150 °C; detector (FID) temperature 250 °C; N_2, carrier gas (200 mL/min). Retention times for propionitrile and propionic acid were 2.2 and 3.3 min, respectively. For induction of the nitrilase benzonitrile was added to medium 3. The flasks were shaken for 24 hours. To determine degradation of benzonitrile and production of benzoic acid a reversed phase HPLC (Merck/Hitachi 655A-11) equipped with a 7 μm Lichrosorb RP18 column (4 × 250 mm) was used. Acetonitrile/water mixtures (1/1, volume ratio) were used as the mobile phase with a flow rate of 1.0 mL/min. The retention times of benzonitrile and benzoic acid were 1.9 and 4.3 min, respectively. For detection a UV monitor (Merck/Hitachi 655A) was used at 254 nm.

Bioconversion with resting cells

The experiments were carried out in medium 3 after the inducer had been completely degraded. The substrate was added in a concentration of 10–25 mmol/L. The flasks were shaken at 28 °C at 110 rpm on a rotary shaker. At regular intervals samples were taken, centrifuged and subjected to analysis by TLC or GC.

Isolation and purification of products

After all the starting material had disappeared or no further change in concentration took place, the cells were removed by centrifugation. The resulting supernatant was evaporated to dryness under reduced pressure, resuspended in toluene and evaporated again. The residue was stirred in anhydrous methanol at 50 °C. The suspension was filtered off and the filtrate concentrated under reduced pressure, followed by chromatographic purification on silica gel.

Analytical methods

TLC was carried out on precoated alumina plates silica gel F-254 from Merck. Mobile phases consisted of chloroform/methanol/acetic acid (9/1/0.1) or toluene/dioxane/methanol/25% aqueous ammonia (2/5/2/1). Detection was accomplished by either UV light or by staining with a solution of cerammonium sulfate in concentrated sulfuric acid or by iodine. Column chromatography was performed on Merck 60 silica gel, 230–400 mesh.

Synthesis of substrates

Compounds **1a, 3a, 4a, 5a, 13a** and **14a** were purchased from Aldrich. Nitriles **2a, 6a–9a** *(19)*, **10a–12a** and **17a** *(21)* were prepared according to literature procedures. The structures of all products were verified by ^{13}C and ^{1}H NMR spectroscopy. These spectral data were previously reported *(19–21)*.

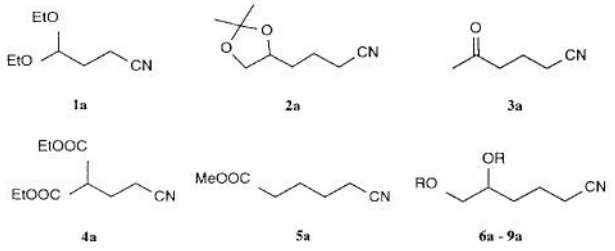
Results and Discussion

Various aliphatic and aromatic nitriles with additional functional groups in the molecule were subjected to biotransformations using resting cells of *Rhodococcus rhodochrous* NCIMB 11216. Such functional groups are

either subject of hydrolysis themselves (e. g. acetals, esters) or their presence might cause unwanted side reactions in the molecule (ketones, esters) under the conditions of chemical nitrile hydrolysis. Thus, the mild reaction conditions using the enzyme system of *Rhodococcus rhodochrous* NCIMB 11216 should be a useful method of hydrolysing nitrile functional groups selectively.

Complete substrate conversion to the desired products could be achieved with nitriles **1a–4a** using propionitrile as the inducer. Among the other substrates listed in Table 1, compound **1a** and **3a** were hydrolysed without any side products. Acid **4c** was accompanied by 20% of 2-(2-cyanoethyl) malonic acid monoethylester. However, the unwanted ester hydrolysis product 5-cyanovaleric acid was isolated from all attempts to transform nitrile **5a** to the desired product.

Table 1. Conversion of aliphatic nitriles



Compound **1a–9a**		Reaction time/h	Yield acid **1c–9c**[a]
1a		112	50 (100)
2a		31	16 (100)
3a		42	44 (100)
4a		48	56 (100)
5a		9	0 (100)
6a	R = Pivaloyl	7	0 (100)
7a	R = Acetyl	70	0 (100)
8a	R = Benzoyl	72	0 (0)
9a	R = H	149	15 (50)

[a] isolated yield in % of starting material; number in parenthesis represents conversion monitored by TLC

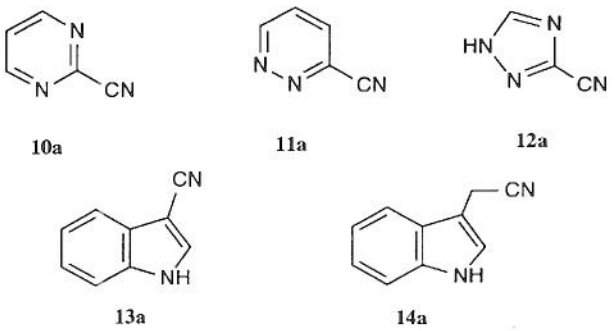
Microscopical examination of *Rhodococcus rhodochrous* has shown that the strain is capable of storing poly-(*R*)-hydroxybutyrate (PHB) granulates intercellularly as a reserve material for carbon and energy production. When PHB is degraded esterase activities are usually found in the soluble enzyme fraction of such cells *(22,23)*. In order to minimize the influence of such rather unspecific esterases on the products wanted and in order to come to higher biomass concentration to carry out the experiments, growth conditions for the strain have been optimised by applying the rather rich media 1 and 2 for the growth of the strain. The strain grew well on these media, but even after such measures esterase activities could not be completely avoided and resting cells of *Rhodococcus rhodochrous* always showed some esterase activity.

In the same manner, the desired acids **6c–9c** could not be obtained from the corresponding nitriles. The acetyl and pivaloyl group is cleaved off by the esterase activity present after induction with propionitrile and 5,6-dihydroxyhexane nitrile **9a** was isolated in both cases. This polar product was only a poor substrate for the enzyme, since no further transformation to the acid occurred. An attempt to hydrolyse this compound (**9a**) *per se* resulted in a 15% yield of acid **9c**. Dibenzoylated substrate **8a** did not react at all and unchanged starting material was recovered. Hydrolysis of 4-(3-cyanopropyl)-2,2-dimethyl dioxolane (**2a**) afforded only a low yield of the desired acid **2c**. The main product isolated from this transformation was the diol **9a** with the nitrile group unchanged. An explanation for the cleavage of the isopropylidene acetal can be proposed as follows: Microbial hydroxylation at carbon 5 of the 1,3-dioxolane heterocycle is leading to the hemiacetal, which hydrolyses under subsequent loss of acetone to the diol.

Contrary to these conversions of aliphatic nitriles which are reported *(4)* to occur by the subsequent action of a nitrile hydratase and an amidase (in our case the intermediate amide could not be detected nor isolated), the hydrolysis of aromatic nitriles using resting cells of *Rhodococcus rhodochrous* NCIMB 11216 has been reported to proceed by a different mechanism *(7)*. Incubation with a simple aromatic nitrile (e. g. benzonitrile) results in the inductive production of a single enzyme, a nitrilase, capable of hydrolysing nitriles to the corresponding acid via a one step mechanism.

Table 2 shows the application of this nitrile transforming pathway to nitrogen heterocyclic aromatic nitriles **10a–14a**. Hydrolysis to the corresponding acids could be achieved for all compounds except for triazole-3-carbonitrile, which turned out to be unreactive. The hydrolysis to the acid using such mild reaction conditions is useful for compounds such as pyrimidine-2-carbonitrile and pyridazine-3-carbonitrile, since their corresponding carboxylic acids **10c** and **11c** easily decarboxylate in acidic media, even at moderate temperature *(24)*. The incomplete conversion of nitriles **13a** and **14a** is reflected in their lower isolated yields, compared with acids **10c** and **11c**.

Table 2. Hydrolysis of nitrogen heterocyclic aromatic nitriles

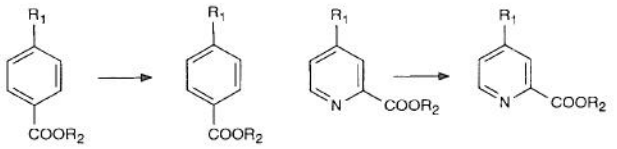


Compound **10a–14a**	Reaction time/h	Yield acid **10c–14c**[a]
10a	58	95 (100)
11a	120	81 (100)
12a	48	0 (0)
13a	24	30 (80)
14a	72	23 (30)

[a] isolated yield in % of starting material; number in parenthesis represents conversion of the substrate estimated by TLC

The results of the chemoselective hydrolysis of aromatic nitriles with an alkoxycarbonyl group as second substituent is depicted in Table 3.

Table 3. Chemoselective hydrolysis of aromatic nitriles



15a, 16a **15c - 15e** **16c - 16e** **17a** **17c**

Starting Compound	Reaction time/ h	Products	Yield[a]
15a (R_1 = CN, R_2 = Me)	7	**15c** (R_1 = COOH, R_2 = Me)	79 (100)
		15d (R_1 = CN, R_2 = H)	16
		15e (R_1 = COOH, R_2 = H)	
16a (R_1 = CN, R_2 = Et)	13	**16c** (R_1 = COOH, R_2 = Et)	42 (100)
		16d (R_1 = CN, R_2 = H)	48
		16e (R_1 = COOH, R_2 = H)	
17a (R_1 = CN, R_2 = Et)	40	**17c** (R_1 = COOH, R_2 = Et)	27 (100)

[a] isolated yield in % of starting material; number in parenthesis represents conversion of the substrate as monitored by TLC

As with the nitrilase/amidase system induced by propionitrile, the nitrilase activity is accompanied by the action of hydrolytic enzymes. Thus, the hydrolysis of methyl and ethyl 4-cyanobenzoate (**15a, 16a**) was accompanied by formation of 4-cyanobenzoic acid (**15d/16d**) and terephthalic acid (**15e/16e**). Ethyl 5-cyano-2-pyridinecarboxylate (**17a**) was converted smoothly into the corresponding 2-ethoxycarbonylpyridine-4-carboxylic acid.

In summary, the application of resting cells of *Rhodococcus rhodochrous* to perform chemoselective transformations turned out to be successful for substrate bearing no ester functional group in the molecule. However, attempts to carry out the hydrolysis in the presence of a methyl/ethyl ester functionality remained unsuccessful for aliphatic model substrates like compounds **4a** and **5a** as well as for structures **6a** and **7a**. Aromatic esters, such as compounds **15a–17a**, remained in part unaffected probably for reasons mentioned above.

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Kemoselektivna hidroliza nitrila s pomoću *Rhodococcus rhodochrous* NCIMB 11216

Sažetak

Nastajanje kiselina iz nitrila uspjelo je koristeći stanice **Rhodococcus rhodochrous** *NCIMB 11216 u mirovanju. Postignuta je selektivna hidroliza nitrilne skupine ako su u molekuli bile acetalne i esterske skupine. Ako su tijekom hidrolize nitrilne funcionalne skupine ostale nepromijenjene, konkurentno su se hidrolizirale acetalna i esterska skupina. Neuspješni su bili pokušaji da se modifikacijom hranjive podloge potisne esterazna aktivnost tog spoja za alifatske supstrate.*