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Hydrolysis of D,L-phenylglycine nitrile by new bacterial cultures

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Abstract

Bacterial cultures were screened for nitrile hydratase/amidase activity towards phenylglycine nitrile. Various strains were obtained, which harboured a nonselective nitrile hydratase and an extremely L-selective amidase. A highly active strain, identified as a *Rhodococcus* sp. was cultured with different nitriles as the sole source of nitrogen. The growth rate of the cells was not influenced by the structure of the nitriles, but the effect on the specific activity of the nitrile hydratase was significant. The best result was obtained with the cells grown on 2-methyl-3-butenenitrile. Hydrolysis of D,L-phenylglycine nitrile catalysed by this culture gave D-phenylglycine amide in 48% yield and $>99\%$ *ee* and L-phenylglycine in 52% yield and 97% *ee*.

Keywords: Nitrile hydratase; Amidase; Nitrile hydrolysis; D-Phenylglycine amide

1. Introduction

D-Phenylglycine amide (D-**2**) is a key intermediate in the industrial enzymatic synthesis of β-lactam antibiotics [1]. A convenient method for its synthesis would be the enzymatic hydrolysis of D,L-phenylglycine nitrile (**1**)[1] catalysed by a nitrile hydratase/amidase system, such as those found in, e.g. *Rhodococcus* sp. [2–4]. These generally comprise a non-stereoselective nitrile hydratase and an L-specific amidase [5]. Hence, we envisionised hydration of **1** to racemic phenylglycine amide (**2**) followed by hydrolysis of L-**2** to L-phenylglycine (**3**) (see Scheme 1). Published reports along these lines involved hydration of α-amino nitriles at minute concentrations (0.5–1 mM) [6,7] and the rate of the second step — hydrolysis of the amide — was generally quite low. Owing to the propensity of **1** for decomposition into benzaldehyde and ammonia via a retro-Strecker reaction [2,8], it is necessary to achieve high rates for the enzymatic reactions, preferably at high substrate concentrations.

We reasoned that these shortcomings could be remedied by screening nitrile hydrolysing microorganisms for activity and enantioselectivity in the transformation of **1** into D-**2**. We now report that a

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[1] Chemical Abstr. name: 2-aminophenylacetonitrile.

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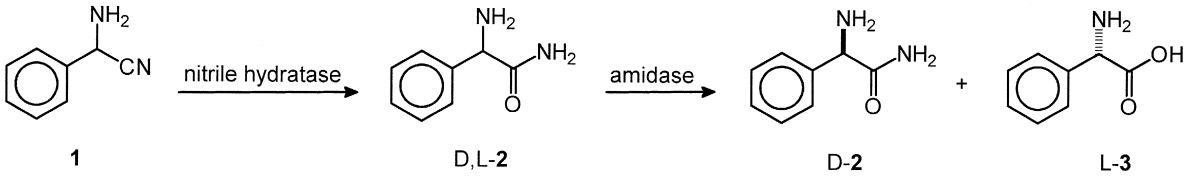
PII: S1381-1177(00)00089-8



Scheme 1. Hydration and subsequent hydrolysis of D,L-phenylglycine nitrile.

Rhodococcus sp. readily converts **1** into enantiopure D-**2** and L-**3** in an essentially quantitative yield.

2. Experimental

2.1. Materials

Racemic phenylglycine and racemic phenylglycine nitrile hydrochloride (technical grade) were purchased from Acros. The latter was purified by neutralisation of the α-aminonitrile hydrochloric acid salt with base followed by an extraction with dichloromethane. The combined organic layers were acidified with 1 N HCl and the nitrile hydrochloric salt was re-extracted in the aqueous phase, which was concentrated in vacuo. Enantiomerically pure D-phenylglycine, D-phenylglycine amide and D-phenylglycine nitrile were kindly donated by DSM (Geleen, The Netherlands). (*S*)-ketoprofennitrile was a gift from Bayer (Leverkusen, Germany). Benzonitrile, 2-methylbutanenitrile and phenylpropionitrile were purchased from Aldrich. Naphthalene-2-carbonitrile was obtained from Lancaster Synthesis. 2-Methyl-3-butenenitrile was obtained from Fluka. All other chemicals used for mineral salts media and buffer solutions were purchased from Aldrich, E. Merck or Fluka. Racemic phenylglycine amide was synthesised chemically by ammonolysis of the corresponding methylester [9].

2.2. Analytical methods

The reaction mixtures were analysed by chiral HPLC on a Daicel Chemical Industries 4.6×150 mm 5 μ Crownpak CR (+) column using a Waters 625 LC pump and a Waters 486 UV detector. The eluent was aqueous $HClO_4$, pH 1.0 at a flow of 0.6 ml/min, the column temperature was 18°C.

2.3. Screening of bacterial cultures

Bacterial cultures were grown in a nitrogen-free mineral medium [10] with succinate (10 mM) as sole source of carbon and energy. Phenylpropionitrile or 2-methyl-3-butenenitrile was added as sole source of nitrogen (0.5 mM from a stock solution in methanol). After 1-day incubation, cultures were transferred (1:33 v/v) to fresh medium with the same composition. The cells were harvested by centrifugation.

2.4. Optimisation of growth conditions for strain MAWA

To find the best nitrogen source for optimal cell yield, strain MAWA was grown in a mineral medium with naphthalene-2-carbonitrile, benzonitrile, ketoprofennitrile, 2-methyl-3-butenenitrile or 2-methylbutanenitrile as nitrogen sources (1 mM each). Succinate (10 mM) was used as sole source of carbon and energy.

2.5. Growth measurement

The growth of bacterial cultures was either monitored with a Klett-Summerson Colorimeter (model 800-3; Klett MFG, New York, USA) or monitored spectrophotometrically by measuring the absorbance at 546 nm with a Kontron Uvikon 820 spectrophotometer (Kontron, Eching, Germany). The protein content of the whole cells was determined by the method of Schmidt et. al [11]. $A_{546\ nm} = 1$ corresponded to 0.19 mg protein/ml for strain MAWA and 0.29 mg protein/ml for strain MAWD.

2.6. Initial taxonomic characterisation and classification of the cultures

Strain MAWA was characterised by using the Biolog test system (GP plates; Biolog 3938 Trust

Way, CA, USA). For a more reliable taxonomic description, the almost complete 16S rDNA was amplified by PCR; the resulting fragment was cloned and partially sequenced using the universal primer 519r [12].

2.7. Standard assay for hydrolysis of D,L-phenylglycine nitrile with resting cells

10 μmol D,L-phenylglycine nitrile (10 mM stock solution in 54 mM sodium phosphate buffer pH 7.4) and cells with an absorbance ($A_{546\ nm}$) of 20 in 1 ml 54 mM sodium phosphate buffer pH 7.4 were shaken at 30°C. Samples were periodically withdrawn, the cells were removed by centrifugation and the supernatant was analysed by HPLC.

3. Results

3.1. Screening for bacterial strains with nitrile hydratase / amidase activity

Enrichments were performed with 2-methyl-3-butenenitrile and phenylpropionitrile as the sole source of nitrogen and succinate as the carbon source. From the ca. 60 cultures tested, five strains were able to convert D,L-phenylglycine nitrile (**1**). We focused our attention on those strains with the ability to catalyse fast hydration of the substrate (to avoid undesired decomposition of **1**).

Strains MAWA and MAWB synthesised a nonselective nitrile hydratase, which converted **1** under the standard conditions within 5 min to the corresponding amide **2**. Subsequently, accumulated **2** was rapidly hydrolysed to L-acid **3** by an L-specific amidase. When other strains were used, the hydration of **1** was rather slow, which resulted in partial degradation of **1** into benzaldehyde and ammonia. Furthermore, strains MAWD and MAWE hydrolysed **2** only slowly to the corresponding acid L-**3** (Table 1).

HPLC analysis showed that the nitrile hydratases did not exhibit any chiral discrimination; the observed enantioselectivity was a consequence of the L-specificity of the amidase as was found previously [5]. However, the enantiomeric ratio E[2] [13] for the amidase-catalysed hydrolysis was much lower for strain MAWC and MAWE than for the other strains.

Table 1
Nitrile hydrolysis by several bacterial strains[a]

Strain	Amide D-**2**		Acid L-**3**		E
	Yield (%)	*ee* (%)	Yield (%)	*ee* (%)	
MAWA	48	> 99	52	97	> 100
MAWB	43	85	38	> 99	> 100
MAWC	39	93	41	84	20
MAWD	46	96	14	> 99	> 100
MAWE	40	35	24	88	20

[a]Reaction conditions: D,L-phenylglycine nitrile **1** (0.01 mmole, 10 mM) and resting cells ($A_{546\ nm}$ 20) in 54 mM phosphate buffer pH 7.4 (1 ml) were shaken at 30°C for 4 h.

3.2. Initial taxonomic characterisation and classification of the cultures

All strains were gram-positive organisms according to the Gram and KOH test [14]. Strain MAWA was further identified using the Biolog test. This strain was tentatively identified by this system as *Corynebacterium pseudodiphteriticum* (SIM value 0.786). A part of the 16S rDNA was determined and the sequence was compared with the NCBI data bank. The sequence obtained from strain MAWA showed more than 99% identity (451 from 453 bp) to the corresponding sequence from the type strain of *R. globerulus*.

3.3. Optimisation of growth conditions

Because strain MAWA gave the best results with regard to reaction rate and L-specificity of the amidase, further optimisation was carried out with this strain. To find the best nitrogen source for an optimal cell yield and for highest enzyme activities,

[2] The enantiomeric ratio (E) for the acid was calculated from the conversion (c) and the enantiomeric excess (ee) of the acid as follows:

$$E = \frac{1n[1 - c(1 + ee)]}{1n[1 - c(1 - ee)]}.$$

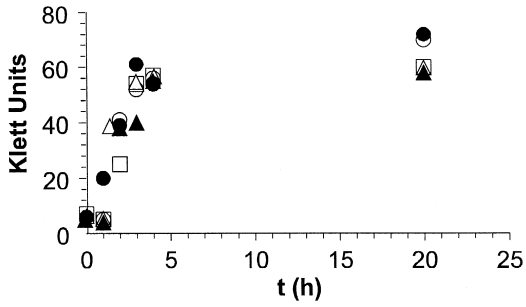


Fig. 1. Growth of strain MAWA on different *N*-sources: (□) naphthalene-2-carbonitrile, (○) benzonitrile, (△) ketoprofennitrile, (▲) 2-methyl-3-butenenitrile, (●) 2-methylbutanenitrile.

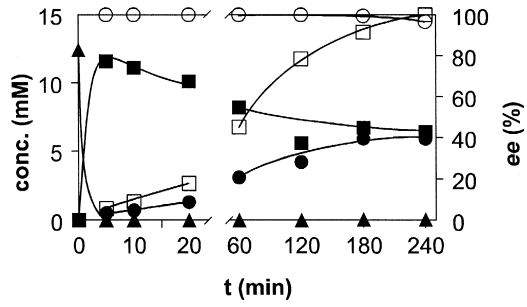


Fig. 2. Hydrolysis of D,L-phenylglycine nitrile **1** by strain MAWA grown on 2-methyl-3-butenenitrile; (▲) phenylglycine nitrile, (■) phenylglycine amide, (□) *ee* D-phenylglycine amide, (●) phenylglycine, (○) *ee* L-phenylglycine.

strain MAWA was grown in mineral medium with different aliphatic as well as aromatic nitriles (0.5 mM) as the sole nitrogen source and succinate (10 mM) as the sole source of carbon and energy. Strain MAWA showed rapid growth with all tested nitrogen-sources (Fig. 1).

The growth rate of the cells was not influenced by the structure of the nitriles used. In contrast, the activity of the nitrile hydratase and amidase was markedly affected by the addition of different nitriles (Table 2).

After growth with naphthalene-2-carbonitrile as sole nitrogen source only a slow hydration of 1 was observed. With all other nitriles used considerably, higher rates were observed; the best result was obtained with 2-methyl-3-butenenitrile. **1** was hydrated within 5 min to the corresponding amide **2** and subsequent hydrolysis of L-**2** was complete within 4 h (Fig. 2), affording L-**3** in 52% yield and 97% *ee* together with D-**2** in 48% yield and $>99\%$ *ee*. This corresponds to $E > 100$ for the amidase-catalysed hydrolysis.

4. Conclusion

Several new bacterial cultures were found, which harboured nitrile hydratase/amidase activity. Strain MAWA identified as a *Rhodococcus* sp. gave D-phenylglycine amide in high yield and high *ee*. Further research is currently in progress to exploit

Table 2
Activity of strain MAWA grown on various nitriles[a]

Nitrile[b]	Amide D-**2**		Acid L-**3**		*E*	Relative activity[c]	Relative activity[d]
	Yield (%)	*ee* (%)	Yield (%)	*ee* (%)			
NN	27	83	28	98	> 100	26	100
KP	79	4	4	89	18	55	10
BN	50	> 99	48	92	65	91	165
MeBuN	71	27	21	76	9	60	24
$MeBu^{\Delta}N$	48	> 99	52	97	> 100	100[e]	100[f]

[a]Reaction conditions: D,L-phenylglycine nitrile **1** (0.01 mmole, 10 mM) and resting cells ($A_{546\ nm}$ 20) in 54 mM phosphate buffer pH 7.4 (1 ml) were shaken at 30°C for 4 h.

[b]NN: napthalene-2-carbonitrile, KP: ketoprofennitrile, BN: benzonitrile, MeBuN: 2-methylbutanenitrile, $MeBu^{\Delta}N$: 2-methyl-3-butenenitrile.

[c]Activity of nitrile hydratase at optical density 20 relative to 2-methyl-3-butenenitrile ($MeBu^{\Delta}N$) used as nitrogen source.

[d]Activity of amidase at optical density 20 relative to 2-methyl-3-butenenitrile ($MeBu^{\Delta}N$) used as nitrogen source.

[e]Specific activity of the nitrile hydratase: 692 $\mu mol\ g^{-1}\ min^{-1}$.

[f]Specific activity of the amidase: 17 $\mu mol\ g^{-1}\ min^{-1}$.

the stereoselectivity of this nitrile hydratase/amidase system for the synthesis of a wide range of optically active amino acid derivatives.

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