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Biotransformation of nitriles using the solvent-tolerant nitrile hydratase from *Rhodopseudomonas palustris* CGA009

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ABSTRACT

A study has been carried out into the biocatalytic hydration of nitriles using the nitrile hydratase enzyme from *Rhodopseudomonas palustris* CGA009. It has been shown that this nitrile hydratase can hydrate aliphatic, aromatic and heterocyclic nitriles under very mild conditions, in mixtures of pH 7 buffer and a range of organic solvents, often with excellent chemoselectivity. The major determinant of hydration occurring is the degree of steric hindrance around the nitrile moiety and/or size of the substrates.

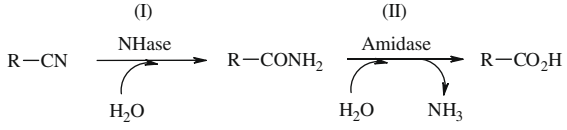
1. Introduction

Traditional abiotic conditions which are used for nitrile hydration to primary amides (I) are generally highly acidic or basic, and require high temperatures. These forcing conditions can often cause the amides to undergo hydrolysis of the C–N bond to give the corresponding carboxylic acid (II) (Scheme 1), as well as being incompatible with many other functional groups.[1]

Nitrile hydratases (NHases) are a class of metalloenzyme which selectively hydrate nitriles into the corresponding primary amide. They are commonly found in prokaryotes which also exhibit nitrilase (nitrile to carboxylic acid hydrolysis) and amidase (primary amide to carboxylic acid hydrolysis) activities, and are part of nitrogen catabolism. They are two-subunit enzymes and the metal anchored in their active site can be either a non-haem iron (Fe-type NHase) or a non-corrinoid cobalt (Co-type NHase).[2]

Hydration of nitriles using an NHase however, can be carried out in aqueous media, without using metal catalysts and at ambient temperatures making a biocatalytic approach a significantly 'greener' option.[3] A further advantage to a biocatalytic approach is the inherent regio- and stereoselectivity that hydration via an NHase has been shown to exhibit.[4–7]

Despite these advantages there are three major limitations which have hindered the more widespread use of NHases as purified enzymes in synthesis. First, they have been described as relatively unstable and hence problematic to work with.[8] Second, NHases have a highly conserved three-dimensional structure, and



Scheme 1.

it has been proposed that the bottleneck en route to the binding site is only 7 Å, which could limit the range of possible substrates and make NHases very sensitive to steric factors.[9] Third, aqueous media would appear to limit their applicability in the hydration of inherently hydrophobic nitriles, though organic co-solvents can be used to aid substrate availability to the enzyme.[10]

The nitrile hydratase from *Rhodopseudomonas palustris* CGA009 can be determined from primary sequence data as being a cobalt-centred NHase from the presence of the amino acid sequence VCTLCSC in the active site on the alpha subunit. It has been overexpressed in *Escherichia coli* BL21(DE3) giving a microbial source which is free of competing nitrilase and nitrile hydratase activities. Here we report on its performance and regioselectivity during hydration of nitriles under mild conditions, and probe its limitations with regards to stability, steric demand of substrates and the use of a mixed aqueous/organic media.

1.1. Effect of organic co-solvents on enzyme performance

As one of the drawbacks of using enzymatic methods for hydration of nitriles is the relative insolubility of the substrate in pH 7 buffer, conversion of 4-hydroxybenzonitrile **1** in a range of mixed

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aqueous/organic media was investigated using the NHase as a cell-free extract (CFE). This substrate was chosen because at the concentration used in this experiment, it was fully soluble in pH 7 buffer and all the mixed media investigated. It can be seen from the results that the NHase was very robust to significant proportions of DMSO and methanol (Table 1). This robustness compares very favourably with a cell-free extract of the Fe-type NHase from *Rhodococcus erythropolis* AJ270, which under the same conditions showed negligible tolerance for methanol as a co-solvent and could only tolerate a 20% v/v mixture with DMSO before performance decreased significantly. In all cases, the only reaction outcome was hydration and a hydrolysis product was not observed by any analytical technique.

1.2. Hydration of heterocyclic nitriles

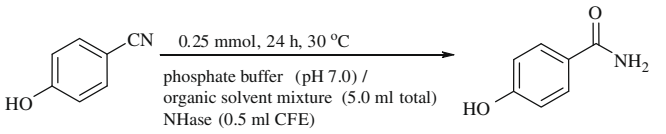
Having identified that this enzyme was able to operate in mixed aqueous/organic media as a CFE, the solvent conditions of 3:1 pH 7 phosphate buffer/DMSO were used with a purified enzyme (PE) preparation, and complete hydration of **1** was again observed.[11] A panel of nitriles **3a–h** containing either a benzene or heterocyclic moiety was subjected to hydration using this NHase, with all except **3b** and **3e** showing complete reaction with no remaining starting material present as indicated by TLC or GC–MS (Table 2). For reasons which may be related to mechanism and the shape of the active site, both compounds which hydrate poorly contained a methylene linker between the nitrile and the aromatic groups. It is interesting to note that this Co-type NHase is unaffected by a 2-pyridyl substitution pattern, unlike the Fe-type *R. erythropolis* AJ270 NHase.[9]

1.3. Hydration of substituted benzonitriles

To examine the sensitivity of this enzyme to steric crowding around the nitrile, and its chemoselectivity, PE NHase was used to hydrate a range of substituted benzonitriles with various degrees of steric hindrance offered by groups at the *ortho* and *para* positions. The results show that this enzyme is very sensitive to the steric bulk of *ortho* substitution and that even a methyl group *ortho* to the nitrile in **5c** is enough to decrease the percentage conversion. The *ortho*-substituted bromine in **5d** also inhibits hydration although *ortho* fluorine and *ortho* chlorine substitution (**5a** and **5b**) does not. *Para*-substituted benzonitriles **5h–p** were hydrated with very high conversion although the bulky ester groups in **5o–p** do not lead to almost quantitative conversion as with other substituents (Table 3).

The quantitative conversion of 2,6-difluorobenzonitrile **6** to 2,6-difluorobenzamide **7** (Scheme 2) could also be achieved without

Table 1
Effect of different organic co-solvents at different v/v percentages on the activity of the NHase towards 4-hydroxybenzonitrile[a]

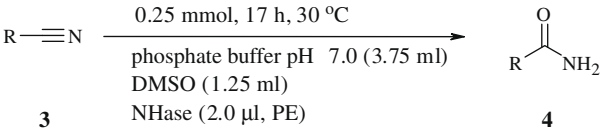


1 **2**

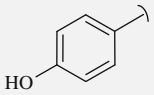
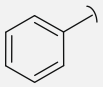
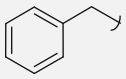
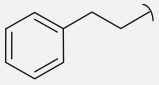
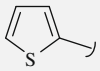
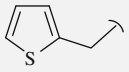
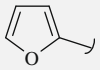
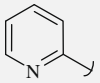
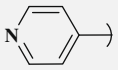
Entry	% Co-solvent (v/v)	% Conversion (DMSO)	% Conversion (MeOH)
1	0	>99.9	>99.9
2	10	>99.9	>99.9
3	20	>99.9	>99.9
4	30	>99.9	>99.9
5	40	>99.9	35
6	50	4	2

[a] Conversion was determined by correlation of the appropriate signals on GC–MS.

Table 2
NHase-mediated hydration of nitriles containing a benzene or heterocyclic moiety[a]

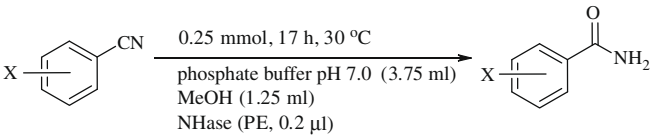


3 **4**

Entry	Substrate	% Conversion **4**
1	 **1**	>99.9
2	 **3a**	>99.9
3	 **3b**	28
4	 **3c**	>99.9
5	 **3d**	>99.9
6	 **3e**	89
7	 **3f**	>99.9
8	 **3g**	>99.9
9	 **3h**	>99.9

[a] Conversion was determined by correlation of the appropriate signals on GC–MS.

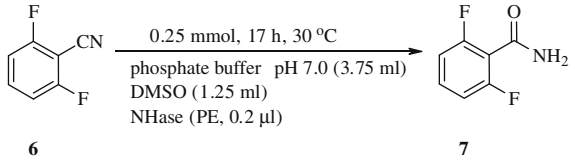
Table 3
NHase-mediated hydration of substituted benzonitriles[a]



4 **5**

Entry	X	*ortho*	% Conversion	*para*	% Conversion
1	F	**5a**	>99.9	**5h**	>99.9
2	Cl	**5b**	95	**5i**	>99.9
3	Me	**5c**	17	**5j**	>99.9
4	Br	**5d**	5	**5k**	>99
5	CF_3	**5e**	5	**5l**	>99.9
6	OMe	**5f**	3	**5m**	>99
7	COMe	—	—	**5n**	>99
8	CO_2Me	—	—	**5o**	98
9	CO_2Et	**5g**	0	**5p**	95

[a] Conversion was determined by correlation of the appropriate signals on GC–MS.



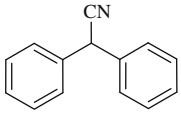
6 7

Scheme 2.

detectable amounts of products resulting from either cleavage of the amide bond or nucleophilic substitution of a fluoride. Highlighting the sensitivity of this enzyme to steric crowding in the *ortho* position, the equivalent dichloro-compound showed less than 5% hydration under similar conditions.

1.4. Hydration of larger aromatic nitriles

One of the acknowledged limitations of NHase methodologies in synthesis is the sensitivity of these enzymes to the overall dimensions of the substrate.[9] Under the reaction conditions used for the compounds in Table 3, this NHase was unable to hydrate the nitrile functionality in diphenylacetonitrile **8**, a lack of reactivity which can be ascribed to its steric bulk, as it was recovered from the reaction mixture, quantitatively.



8

It has been shown that NHase enzyme CGA009 is capable of hydrating a wide variety of nitriles (aryl, alkyl and heterocyclic), under very mild reaction conditions, and in a mixed aqueous/organic media which solubilise compounds that are relatively hydrophobic. Whilst its use is limited to nitriles with low steric demands and to smaller substrates, it shows good stability and good conversions and should prove a useful biocatalyst. Studies are currently in progress on the enantiospecificity of this enzyme with chiral nitriles.

2. General procedure for the biotransformation

The nitrile substrate was added to a mixture of organic co-solvent (as detailed in Tables 1–3) and 100 mM potassium phosphate buffer, pH 7.0 (giving a total reaction volume of 5.0 ml), and the enzyme (as CFE or PE as detailed below) was added. The solution was mixed using a standard laboratory magnetic stirrer for 17–24 h at 30 °C whilst following the progress of the reaction by TLC. The reaction medium was extracted with EtOAc (3×5.0 ml) and the organic extract dried with $MgSO_4$ and analysed by GC–MS. Compound identities were established by comparison with published NMR, IR and melting point data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.12.094.

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11. Both CFE and PE of this Co-type NHase are stable with this proportion of either DMSO or MeOH, and we believe, can be used interchangeably in these transformations.