Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 3011

www.rsc.org/obc

PAPER

Probing the enantioselectivity of a diverse group of purified cobalt-centred nitrile hydratases

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Received 23rd November 2010, Accepted 26th January 2011
DOI: 10.1039/c0ob01067g

In this study a diverse range of purified cobalt containing nitrile hydratases (NHases, EC 4.2.1.84) from *Rhodopseudomonas palustris* HaA2 (HaA2), *Rhodopseudomonas palustris* CGA009 (009), *Sinorhizobium meliloti* 1021 (1021), and *Nitriliruptor alkaliphilus* (iso2), were screened for the first time for their enantioselectivity towards a broad range of chiral nitriles. Enantiomeric ratios of >100 were found for the NHases from HaA2 and CGA009 on 2-phenylpropionitrile. In contrast, the Fe-containing NHase from the well-characterized *Rhodococcus erythropolis* AJ270 (AJ270) was practically aselective with a range of different α-phenylacetonitriles. In general, at least one bulky group in close proximity to the α-position of the chiral nitriles seemed to be necessary for enantioselectivity with all NHases tested. Nitrile groups attached to a quaternary carbon atom were only reluctantly accepted and showed no selectivity. Enantiomeric ratios of 80 and >100 for AJ270 and iso2, respectively, were found for the pharmaceutical intermediate naproxennitrile, and 3-(1-cyanoethyl)benzoic acid was hydrated to the corresponding amide by iso2 with an enantiomeric ratio of >100.

Introduction

Nitriles are widely used in industry as intermediates and building blocks. Nitrile hydrolysing enzymes can be used to produce a wide spectrum of higher value carboxylic amides and acids from nitrile intermediates. Along with the potential for high chemoselectivity and especially mild reaction conditions, enzymatic nitrile hydrolysis also offers the possible advantage of regio-, and enantioselectivity.[1] Two different pathways for the enzymatic hydrolysis of nitriles to the corresponding carboxylic acids exist in Nature.[2] A nitrilase (EC 3.5.5.1) catalyses the direct hydrolysis of a nitrile to the corresponding carboxylic acid and ammonium ion in the first pathway. In the second pathway a nitrile hydratase (NHase, EC 4.2.1.84) catalyses the hydration of a nitrile into the carboxylic amide and subsequently this amide can be hydrolysed to form the carboxylic acid and ammonium ion by an amidase (EC 3.5.1.4). Nitrile hydratases are metalloproteins containing either an iron(III) or cobalt(III) centre in the active site.

In contrast to nitrilases, early experiments suggested that NHases are relatively unspecific with respect to the chirality of the nitrile substrate and that any enantiodiscrimination in the nitrile hydrolysis pathway occurs during the hydrolysis of the intermediate amide by the amidase.[3] However, it was later shown that results of experiments with whole cells on arylaliphatic nitriles could be rationalized by the existence of enantioselective NHases.[4,5] The first reported enantioselective NHases were purified from *Pseudomonas putida* strain 5B,[6] *Agrobacterium tumefaciens* strain d3,[7,8] and *Rhodococcus equi* strain A4.[9] The enantioselective NHase from *P. putida* was expressed successfully in *Escherichia coli*[10] as well as in *Pichia pastoris.*[11] All of these purified enzymes show mainly a preference for (*S*)-2-arylpropionitriles and (*S*)-2-arylbutyronitriles leading to arylaliphatic amides of pharmaceutical interest. For instance, single enantiomer (*S*)-2-arylpropionamides can be useful as precursors for non-steroidal anti-inflammatory drugs like (*S*)-ibuprofen and (*S*)-naproxen, because these are much more active than the (*R*)-enantiomers.

Recently, the enantioselective NHase from *Rhodococcus erythropolis* strain AJ270 was purified[12] and expressed in *E. coli.*[13] This NHase shows enantioselectivity in the hydration of several racemic *trans*- and *cis*-2-arylcyclopropanecarbonitriles. A recent patent application and paper describe the enantioselectivity and heterologous expression of the NHases from *Raoultella terrigena*, *Pantoea* sp., *Brevibacterium linens*, and *Klebsiella oxytoca.*[14,15] Besides the preference of these NHases for the (*S*)-enantiomer of 2-phenylpropionitrile, they also show a preference for the (*S*)-enantiomers of mandelonitrile and phenylglycine nitrile, although

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Table 1 The five NHases probed for their enantioselectivity

Organism	Subunit ID number	Metal
Rhodococcus erythropolis AJ270[a] (AJ270)[12,13,17]	CAC08205/6	Fe(III)
Rhodopseudomonas palustris HaA2[a] (HaA2)	YP_486317/8	Co(III)
Rhodopseudomonas palustris CGA009[a] (009)[18]	NP_948148/9	Co(III)
Sinorhizobium meliloti 1021[a] (1021)	NP_386211/2	Co(III)
Nitriliruptor alkaliphilus[b] (iso2)[19]	n/a	Co(III)

[a] After overexpression in *E. coli* these NHases were purified by His-tag affinity chromatography. [b] All amidase and esterase activity was removed by partial purification using an ammonium sulfate cut in combination with anion exchange chromatography.

to a lesser extent. In the commercial arena, BioVerdant described a process for the production of levitiracetam in which a NHase, modified using directed evolution, is used to produce (*S*)-2-(2-pyrrolidon-1-yl)-butyramide from the related racemic nitrile.[16]

Despite a recent increase in literature reports describing enantioselective NHases, the scope of substrates and enzymes tested is still narrow and often the data available is not sufficient to calculate the enantiomeric ratio (*E*) values. In this study four diverse purified cobalt containing NHases from different bacterial strains and one purified example of an iron containing NHase were probed for the nature of their enantioselection towards a group of twelve different chiral nitriles (Fig. 1). Two of the cobalt containing NHases have never been analysed before (from *Rhodococcus palustris* HaA2 and from *Sinorhizobium meliloti* 1021) and the other two have never been analysed for enantioselectivity before (from *Rhodococcus palustris* CGA009 and *Nitriliruptor alkaliphilus*). Whilst the enantioselectivity of *Rhodococcus erythropolis* AJ270 has been probed previously almost all the published work has been on whole cell preparations in the host organism. Here we use it as a recombinant form enabling a more direct contrast to be made to one well-established synthetically useful iron-centred NHase, and to show the scope of the diversity within the class of NHases in general.

Results and discussion

Purified NHases

In order to accurately determine the enantioselectivity of the NHases, it was necessary to work with cell-free purified enzymes isolated from other interfering enzyme activities. Whole cells or crude cell-free extracts can contain a variety of enzymes such as nitrilases, amidases, and esterases that can have an influence on the enantiomeric excess (*ee*) of the nitrile substrate as well as the amide product. The NHases used in this study (Table 1) were purified using polyhistidine tag (His-tag) affinity chromatography following their expression in *E. coli* or by anion exchange chromatography after induction in the original host. The recombinantly expressed NHases are of known amino acid sequence, and represent a diverse group as shown by the sequence identity comparisons shown in Table 2 where amino acid sequence identities range between 82 and 36%.

Table 2 Comparison of the percentage identity of the amino acid sequences of the recombinantly expressed NHases

Enzyme	Enzyme	% identity
CGA009	1021	59
CGA009	HaA2	82
CGA009	AJ270	40
1021	HaA2	59
1021	AJ270	36
HaA2	AJ270	41

Non-chiral nitriles

Due to the difference in the methods of preparation and isolation of the purified NHases, relative rather than absolute activities with different substrates were compared by setting the activity with hexanenitrile at 100% for each enzyme. Although the enzyme preparations from recombinant production in *E. coli* had a higher purity than the partially purified iso2 enzyme, the activity of the latter was substantially higher.

Among the recombinant NHases, the Fe-containing AJ270 was most active in hydrating hexanenitrile (**1**) to the corresponding amide (Table 3). However, this Fe-containing NHase exhibited poorer conversion of nitrile to amide product than the Co-containing NHases (Fig. 2). The hydration of **1** using AJ270 halted at a concentration of approximately 35 mM of hexanamide. Similar levels of incomplete reaction were also observed for this enzyme during the hydration of other substrates. After the addition of another aliquot of AJ270 to the hexanenitrile experiment, the reaction continued, indicating that either the enzyme was less stable or deactivated in the course of the reaction, or that it may be more susceptible to product inhibition. Higher stability of Co-containing NHases has been demonstrated before and has been ascribed to the formation of more stable heterodimers because of the existence of an extra helix in the β-subunit that interacts with the α-subunit.[21,22,23] Furthermore, a comparison of the solvent stability of Co-containing 009 with that of AJ270 showed a greater stability for the former.[18]

The differences in hydration activity on benzonitrile (**2**) and phenylacetonitrile (**3**) between the example of an Fe-containing enzyme and the four Co-containing enzymes were remarkable. The Fe-containing NHase, AJ270, clearly preferred the arylaliphatic substrate, while the Co-containing NHases preferred the aromatic one. However, this substrate preference of AJ270 did not apply to the other arylaliphatic substrates.

α-Substituted phenylacetonitriles

The Co-containing NHases from both *R. palustris* strains (HaA2 and 009) showed a very high (*S*)-selectivity for 2-phenylpropionitrile (**4**, Table 3, Fig. 3a). The Co-containing NHases from *S. meliloti* and *N. alkaliphilus* (1021 and iso2) also converted **4** with a preference for the (*S*)-enantiomer, albeit with a lower selectivity (Fig. 3 b). The Fe-containing NHase from *Rh. erythropolis* (AJ270) was aselective for this substrate. *E*-values of higher than 100 for **4** have so far only been reported for the NHase of *A. tumefaciens* d3.[1,7]

2-Phenylbutyronitrile (**5**) proved to be a challenging substrate for these NHases with the activities of all the NHases on this

Table 3 The relative activities and enantiomeric ratios (E) of the biocatalytic hydration of a group of different nitriles using five different NHases

	Relative activity (%)[a,b]					E[c,d] (–)				
Substrate	AJ270	HaA2	009	1021	iso2	AJ270	HaA2	009	1021	iso2
1	100	100	100	100	100	—	—	—	—	—
2	2.6	110	177	131	60.7	—	—	—	—	—
3	11	1.0	0.91	1.0	1.7	—	—	—	—	—
4	0.69	6.0	10	2.4	28	1	>100	>100	18	49
5	2×10^{-3}	0.030	0.045	0.028	0.10	7	53	95	5	37
6[e]	0.030	0.40	0.32	0.14	0.22	2	12	11	10	10
7[e]	0.011	1.2	0.82	0.48	0.014	2	5	4	4	7
8[f]	0.14	0.85	0.77	0.41	0.092	4	7	6	5	1(*R*)
9[g]	2.5	19	18	52	69	2.5(*R*)	6.6	6.6	1.8	2.1(*R*)
10[g]	8×10^{-4}	3×10^{-3}	3×10^{-3}	6×10^{-3}	6×10^{-5}	1	1(*R*)	1	3(*R*)	2
11	1.59	60	59	100	26	1.9[h]	2.1[h]	1.7[h]	3.3[h]	2.7[h]
12	67	34	25	28	5.4	1.1	1.4	1.4	1.8	1.3
13	0.23	0.018	0.012	0.013	0.047	80	8.2	8.1	8.0(*R*)	>100
14	0.016	75	68	38	0.020	6.5[h]	32[h]	34[h]	11[h]	>100[h]
15	0.58	0.30	0.36	0.69	0.45	8.7[h]	21[h]	17[h]	12[h]	15[h]

[a] The activity of the NHases on hexanenitrile was set to 100%. These activities are 189, 20, 22, 29, and 358 μmol min^{-1} mg^{-1} respectively for AJ270, HaA2, 009, 1021, and iso2. [b] Unless stated otherwise, the reactions are carried out at pH 8 and 21 °C. [c] $E = \frac{\ln[1-\xi(1+ee_P)]}{\ln[1-\xi(1-ee_P)]}$, when the enantiomeric excess of the product is used (ee_P)[20] and $E = \frac{\ln[1-\xi(1-ee_S)]}{\ln[1-\xi(1+ee_S)]}$, when the enantiomeric excess of the substrate is used (ee_S).[20] [d] The E-value is the average of at least 4 E-values calculated at different points of conversion. Unless stated otherwise the NHases are (*S*)-selective. [e] Reactions carried out at pH 5 and 5 °C. [f] Reactions carried out at pH 7 and 21 °C. [g] Reactions carried out at pH 6 and 21 °C. [h] Enantiopure standards to determine the enantiopreference of the enzymes for these substrates were not available. All enzymes produced the same enantiomer.

substrate two to three orders of magnitude lower than with **4**. The enantioselectivities for **5** followed the same trend as for **4** although in all cases, except for AJ270, the E-values were lower. The NHase from *A. tumefaciens* d3 showed a similar dip in enantioselectivity when the methyl group on the alpha position is replaced by an ethyl group.[1,7] AJ270 demonstrated low selectivity for **5**, mirroring the aselective hydration of the Fe-containing NHase from *Rh. equi* A4 for this substrate.[9]

The biocatalytic hydration of mandelonitrile (**6**) was carried out at low pH and low temperature in order to completely suppress its chemical decomposition into benzaldehyde and HCN, an equilibrium-based process resulting in the racemisation of the substrate that would complicate the calculation of E-values (see Scheme 1).

The introduction of a hydroxyl group at the α-position of phenylacetonitrile instead of an alkyl group reduced the enantioselectivity of the Co-containing NHases to an E of 10–12 (Table 3).

If the racemisation rate of the reversible decomposition reaction of mandelonitrile is high enough, compared to the reaction rate of the enzymatic nitrile hydration, the combined process would become a dynamic kinetic resolution (DKR, Scheme 1). A product yield of 100% is then theoretically possible and the *ee* of the product would remain constant during the reaction. However these enzymes proved unsuitable to this form of DKR, because besides the low E-values for **6**, they exhibited high sensitivity to the presence of cyanide released during the racemisation of **6** at pH 7 and 21 °C. Only small amounts of mandelamide can be produced under these conditions (Fig. 4b, Table 4).

The Fe-containing NHase from AJ270 showed remarkably higher activity in the presence of cyanide (Fig. 4a, Table 4). Unfortunately, this enzyme is practically chirally aselective for **6**, similar to **4** and **5**. This Fe-containing enzyme loses a substantial amount of its activity when the pH is decreased from 7 to 5 (Fig. 4c, Table 3). However, the negative effect of cyanide on the activity of the Co-containing enzymes was decreased by carrying out the reaction at low pH and low temperature (Fig. 4d, Table 4).

For the same reasons as for **6** the hydration reaction of phenylglycinonitrile (**7**) had to be carried out at low pH and low temperature. Unfortunately an amino group on the α-position of phenylacetonitrile had an even more negative effect on the enantioselectivity of the NHases than a hydroxyl group. Such a decrease of the enantioselectivity of the Co-NHases when an alkyl group is replaced by an amino or hydroxyl group on the α-carbon has been observed previously.[14]

α-Acetoxyphenylacetonitrile (**8**) is not sensitive to HCN elimination but the spontaneous hydrolysis of the ester bond is significant at pH >7. Racemisation can also occur *via* deprotonation on the α-position, although this process is slow in aqueous media at pH <7. When the reaction was carried out at pH 7 with a high enzyme loading, only trace amounts of mandelonitrile and benzaldehyde were detected and no apparent racemisation took place. The presence of the relatively bulky acetate group on the α-position did not improve the enantioselectivity of these NHases (Table 2).

2-Chloro-2-phenylacetonitrile (**9**) is very sensitive to racemisation *via* deprotonation on the α-position. In order to investigate the extent of racemisation, the hydration reaction was first carried out at different pH values with HaA2 (Fig. 5). The rate of racemisation clearly decreases with decreasing pH. A dynamic kinetic resolution could be performed by using a pH of 9 or by using a pH of 8 with a lower enzyme concentration, since a small decrease in *ee* could still be detected in the experiment at pH 8. Of all the α-substituted

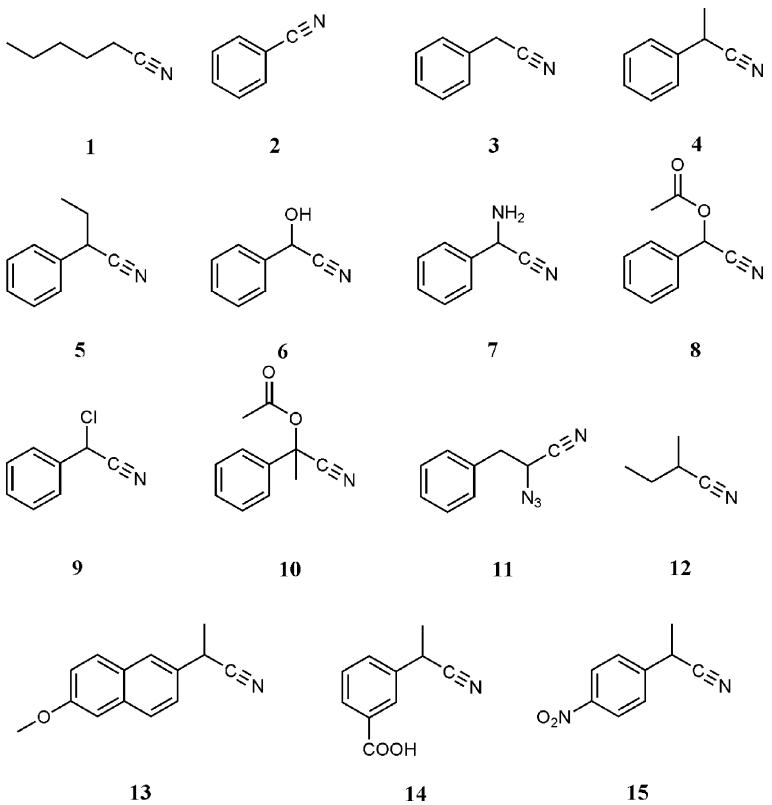
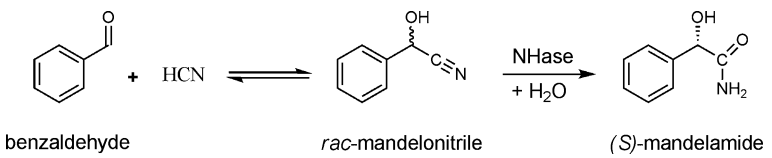


Fig. 1 The achiral and chiral nitriles which were subjected to biocatalytic hydration by NHases.



Scheme 1 Dynamic kinetic resolution of *rac*-mandelonitrile. Racemisation takes place through the reversible decomposition of mandelonitrile into benzaldehyde and cyanide.

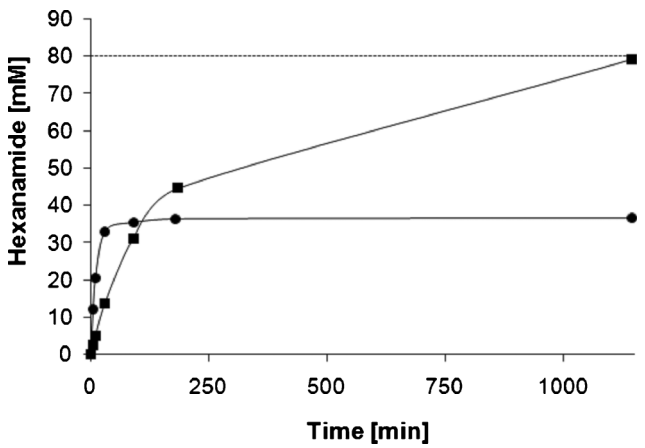


Fig. 2 The hydration of hexanenitrile (80 mM) using 12.8 mg L^{-1} AJ270 (●) and 23.6 mg L^{-1} HaA2 (■) at pH 8 and 21 °C.

phenylacetonitriles, **9** is converted with by far the highest activity, which is probably due to the strong electron withdrawing effect of the chloride group, which would make the nitrile carbon more susceptible to nucleophilic attack by an activated water molecule.[24] Interestingly, the reaction rate seemed to increase with decreasing pH. Since there was practically no racemisation at pH 6, the reactions for the calculation of *E* were carried out at this pH.

Unfortunately no beneficial effect of the chloride substituent was observed regarding the enantioselectivity of the NHases (Table 3) though AJ270 and iso2 reversed their enantiopreference for this substrate from (*S*) to (*R*).

Other structurally different nitriles

The hydration of 1-cyano-1-phenylethylacetate (**10**) was carried out at pH 6 to avoid the chemical hydrolysis of the acetate group. An additional methyl group at the α-carbon of **8** has a strongly negative effect on the rate of hydration (Table 3). The tertiary nitrile group of **10** is probably difficult to access by the NHases for steric reasons. The additional steric bulk also caused a decrease in enantioselectivity.

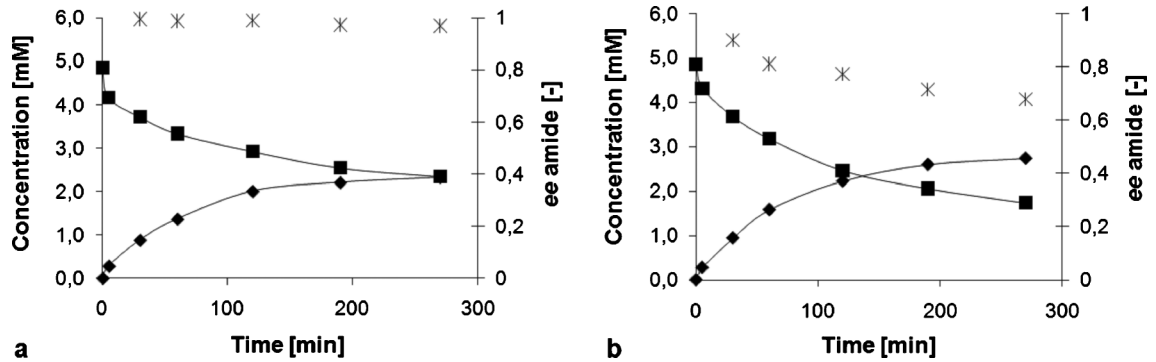


Fig. 3 The conversion of (**4**) into the corresponding amide by 009 (a) and 1021 (b) at pH 8 and 21 °C, nitrile (■), amide (◆), and *ee* of the amide product (×).

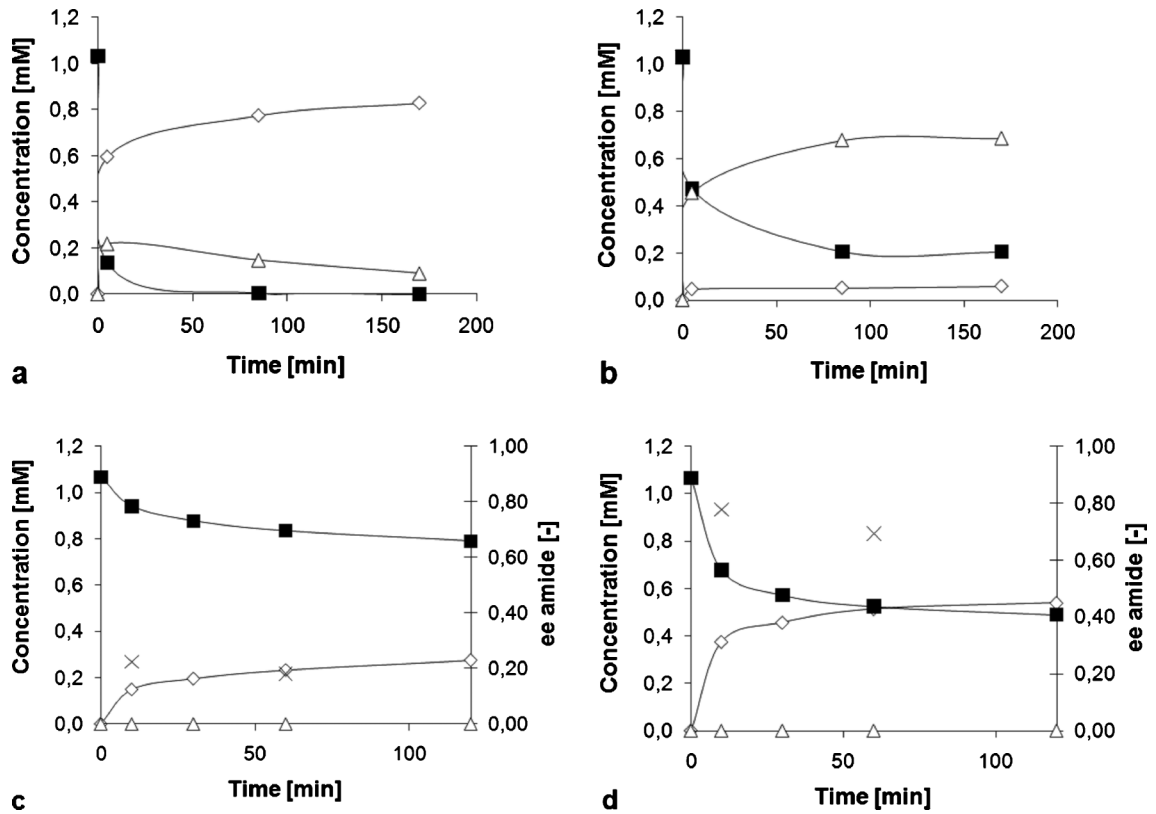


Fig. 4 The conversion of **6** into the corresponding amide by AJ270 (a, c) and HaA2 (b, d) under racemisation conditions (pH 7 and 21 °C, a, b) and under acidic conditions with low temperature (pH 5 and 5 °C, c, d), nitrile (■), amide (◇), benzaldehyde (△), and *ee* of the amide product (×).

Table 4 Activities of the NHases in the hydration of **6** under different reaction conditions

	Protein concentration (mg mL^{-1})			Activity (μmol min^{-1} mg^{-1})		
NHase	pH 7, 21 °C	pH 5, 21 °C	pH 5, 5 °C	pH 7, 21 °C	pH 5, 21 °C	pH 5, 5 °C
AJ270	63[a]	63	248[c]	1.88[a]	0.02	0.06[c]
HaA2	117[b]	117	457[d]	0.08[b]	0.11	0.08[d]
CGA009	63	63	248	0.09	0.13	0.07
1021	154	154	600	0.07	0.06	0.04
iso2	n.d.	n.d.	22.3	n.d.	n.d.	0.69

[a] Fig. 4a. [b] Fig. 4b. [c] Fig. 4c. [d] Fig. 4d. n.d. = not determined.

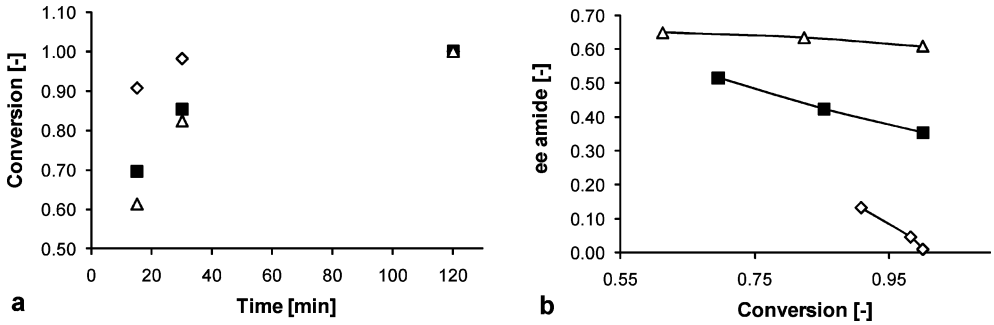


Fig. 5 The effect of the pH on the conversion (a) and the *ee* (b) in the hydration of **9** using 451 mg L^{-1} HaA2 at 21 °C, pH 6 (◇), pH 7 (■) and ph 8 (△).

The azide functionality in 2-azido-3-phenylpropionitrile (**11**) is bulky and contains a strong internal dipole and is therefore an interesting substituent to test to expand knowledge of the factors behind the enantioselectivity of the NHases. The activities of the NHases for **11** were relatively high (Table 3), but their enantioselectivity was low, both of which can be explained by the combined effect of the azido group and the added distance between the aromatic ring and the α-carbon giving a less congested but less directed access to the nitrile.

Replacing the aromatic ring in **4** by an ethyl group as in substrate **12** causes an almost complete loss of enantioselectivity, indicating that it is necessary to have at least one aromatic or a bulky group attached to the α-position of the nitrile compound to induce enantioselectivity in the Co-containing enzymes.

The relative increase in bulkiness of the aromatic ring in naproxennitrile (**13**) compared to **4** had a positive effect on the enantioselectivity of AJ270 and iso2. In contrast, the Co-containing NHases HaA2, 009, and 1021 lost enantioselectivity when the bulkiness of the aromatic ring was increased, although intriguingly 1021 switched its enantioselectivity from (*S*) to (*R*). A switch in enantioselectivity was also reported to occur when using the NHase from *P. putida*.[1,5,25] The low activity for the hydration of **13** is probably caused by a combination of its bulkiness and the extremely low aqueous solubility of this nitrile.

The introduction of a nitro group at the *para*-position of the aromatic ring of **4** reduced the rate as well as the enantioselectivity of the hydration reaction, except in the case of AJ270 (Table 3). The introduction of a carboxylic acid group at the *meta*-position of **4**, in contrast, caused some remarkable differences in activity. Both AJ270 and iso2 became substantially less active while the other Co-containing NHases increased their activity. The *E*-values for the hydration of 3-(1-cyanoethyl)benzoic acid (**14**) follow the same trend as for 2-(4-nitrophenyl)propanenitrile (**15**) compared to **4** with the remarkable exception of iso2 which increased in enantioselectivity to an $E > 100$ (Table 3). The origin of these effects requires further study.

Conclusion

A diverse panel of four Co-containing NHases (HaA2, 009, 1021 and iso2) was active with a wide range of aliphatic, aromatic, and arylaliphatic nitriles, and had intriguing differences to the selectivities shown by the well-studied Fe-containing AJ270 NHase which has been shown to be effective in a number of chiral syntheses.[26] All of these NHases, except 1021, showed high ($E > 50$) enantioselectivity with at least one nitrile from the test library. Nitriles bearing an α-methyl group were, in general, much more efficiently resolved than α-oxygen or α-nitrogen substituted ones and at least one bulky group close to the α-position of the chiral nitrile seemed to be necessary to induce enantioselectivity in these enzymes. This study has offered further proof that NHase enzymes are not fundamentally poorly enantioselective as each of the NHases described here exhibit *E* values of at least 18 when paired with an appropriate substrate, though levels of activity are low in the cases described here.

Work is continuing to produce other NHases from all three kingdoms informed by genome sequence searching and correlation, and in optimizing conditions for their use as biocatalytic reagents. As further X-ray crystal structures are published of this class of enzyme, analysis of 3D structures may further elucidate the origins of enantioselectivity in Co-centred NHases.

Experimental

General

All the purified NHases were stored at 4 °C as ammonium sulfate precipitates in the presence of 30–40 mM butyric acid. Reactions were carried out in Eppendorf tubes using a ThermoTWISTER comfort shaker from QUANTIFOIL Instruments. Protein concentrations were determined using the Bradford assay.[27] For experiments at pH 7-8 a 0.01 M Tris-HCl buffer was used, while for experiments at pH 5–6 a 0.01 M citrate buffer was applied.

Chemicals

Hexanenitrile (98%, Aldrich), hexanamide (98%, Aldrich), benzonitrile (≥99%, Fluka), benzamide (99%, Aldrich), phenylacetonitrile (≥99%, Aldrich), *rac*-2-phenylpropionitrile (96%, Aldrich), (*S*)-2-phenylpropionic acid (97%, Acros), 2-phenylbutyronitrile (95%, Aldrich), (*R*)-2-phenylbutyric acid (99%, Aldrich), *rac*-mandelonitrile (90%, Fluka), *rac*-mandelamide (97%, Alfa Aesar), (*R*)-mandelamide (≥97%, Aldrich), benzaldehyde (99.5%, Acros), *rac*-phenylglycinonitrile·HCl (technical grade, Acros), 2-hydroxy-2-phenylpropionamide (Sigma), phenylacetone (99%, Aldrich), 2-methylbutyronitrile (>80%, Lonza quality, Aldrich), (*S*)-2-methylbutyronitrile (98%, Aldrich), (*S*)-naproxen (98%,

Sigma), 3-(1-cyanoethyl)benzoic acid (98%, Aldrich), 2-(4-nitrophenyl)propanenitrile (97%, Aldrich) were used in the experiments as received without additional purification. 1-Cyano-1-phenylethylacetate was produced as described previously.[28] Racemic naproxennitrile and racemic naproxenamide were a gift from Andreas Stolz of the Institut für Mikrobiologie der Universität Stuttgart and (*R*)-phenylglycinoamide (>99%) was a gift from DSM. *rac*-2-Azido-3-phenylpropionitrile and *rac*-2-azido-3-phenylpropionamide were a gift from the Institute of Chemical and Engineering Sciences (ICES) Singapore.

(*S*)-2-Phenylpropionamide, (*R*)-2-phenylbutyramide, and (*S*)-naproxenamide were prepared by reacting the corresponding enantiopure acids with $SOCl_2$ at 80 °C for 1 h. After the evaporation of excess $SOCl_2$, concentrated ammonium hydroxide solution (25 w% ammonia) was added to the formed acyl chloride. Amide crystals immediately formed on cooling the reaction mixture and the product was purified through recrystallisation in water. In a similar fashion *rac*-2-chloro-2-phenylacetonitrile was prepared by the reaction of *rac*-mandelonitrile with thionylchloride in chloroform and (*R*)-2-Chloro-2-phenylacetamide was synthesised from (*R*)-mandelic acid.[29]

The purification of technical *rac*-phenylglycinonitrile·HCl was carried out by the neutralisation of the hydrochloric acid salt with a 1 M NaOH solution followed by an extraction with dichloromethane. The combined organic layers were acidified with a 1 M HCl solution and the nitrile hydrochloric acid salt was re-extracted in the aqueous phase, which was concentrated in *vacuo*, resulting in pure white *rac*-phenylglycinonitrile·HCl crystals. **Warning:** during the neutralisation of *rac*-phenylglycinonitrile·HCl large amounts of HCN are liberated. This experiment should always be performed in a fume cupboard with a good draught and a well-calibrated HCN detector.

rac-α-Acetoxyphenylacetonitrile was synthesised by the acetylation of *rac*-mandelonitrile with acetic anhydride and pyridine in dichloromethane at room temperature. The same procedure was used for the acetylation of (*S*)-mandelonitrile to produce (*S*)-α-acetoxyphenylacetonitrile and for the acetylation of 2-hydroxy-2-phenylpropionamide to produce the amide of cyano-1-phenylethylacetate. (*S*)-Mandelonitrile was produced *via* the enzymatic hydrocyanation of benzaldehyde using the oxynitrilase from *Manihot esculenta*.[30]

Purified enzymes

***N. alkaliphilus* (iso2).** *N. alkaliphilus* was cultivated as described previously.[19] The whole cells were disrupted at a pressure of 2 Kbar (IKS Lab Equipment Constant Cell Disruption System). A pellet of precipitated protein from a 47–67% ammonium sulfate cut containing the NHase was redissolved in buffer A (40 mM HEPES-NaOH buffer of pH 7.4 containing 40 mM hexanoic acid) and the resulting extract was filtered (0.45 μm filter). The semi-purified extract was applied to a DEAE (diethylaminoethyl) Sepharose Fast Flow (GE Healthcare) column at 4 mL min^{-1} under the control of a Shimadzu LC-20AT FPLC. The column was equilibrated with buffer A and the same buffer was used to wash the column until no protein was detected in the flowthrough. The enzyme was eluted with a linear gradient of NaCl (0–1 M) in buffer A at a flowrate of 5 mL min^{-1}. Fractions of 10 mL were collected and the protein in these fractions was detected using a Shimadzu SPD-M20A UV-VIS detector at 280 nm. The active fractions eluted at 330–350 mM of NaCl and were pooled and concentrated using a 10 kDa centrifuge filter device.

The recombinant NHases. The recombinant NHase enzymes were produced from *E. coli* BL21 clones obtained from Nzomics Biocatalysis (www.nzomics.com), that over-express either the *Rh. erythropolis* AJ270 NHase (comprising α-subunit, CAC08205, and β-subunit, CAC08206), the *R. palustris* HaA2 NHase (comprising α-subunit, YP_486317, and β-subunit, YP_486318), the *R. palustris* CGA009 NHase (comprising α-subunit, NP_948148, and β-subunit, NP_948149) or the *S. meliloti* 1021 NHase (comprising α-subunit, NP_386211, and β-subunit, NP_386212). The protein production and purification protocol described by Charnock *et al.*[31] was followed, except that the cultures were supplemented with kanamycin (50 μg ml^{-1}) and ampicillin (100 μg ml^{-1}), and $CoCl_2$ (1 mmol L^{-1}), for the HaA2, CGA009 and 1021 NHases, was also added on induction of NHase production with isopropyl-thio-β-D-galactoside (1 mmol L^{-1}). Butyric acid was added to the cell free extract (CFE) and purified enzyme (PE) preparations of NHase at a concentration of 40 mmol L^{-1}.

Biotransformations

Reactions were run in 1 mL of buffer, using 0.04–80 mM solution of nitrile substrate. If the nitriles had poor solubility in water, they were dissolved in methanol, and this solution was then dissolved in the buffer, noting that the concentration of MeOH in the reaction mixture was kept < 5 v% to prevent rapid deactivation of the NHases. Special care was taken to carry out the reactions at a nitrile concentration where complete dissolution is assured, since incomplete dissolution would result in inaccurate *E*-values.

After taking a sample to determine the exact starting concentration of the reaction, the reaction was commenced by the addition of enzyme directly from the ammonium sulfate stock suspension. Periodically samples were withdrawn from the reaction and analysed on chiral and non-chiral HPLC as well as on chiral GC.

For reversed-phase HPLC the enzyme in the sample was denatured by the addition of 1 M HCl, after which denatured NHase was spun down (13 000 rpm, 5 min). When necessary the sample was diluted with MilliQ and subsequently directly injected on HPLC. For normal phase HPLC, a reaction sample was added to a mixture of hexane–isopropanol 80 : 20 (v/v). After adding magnesium sulfate to dry the sample, the sample was centrifuged (13 000 rpm, 5 min) and the supernatant was injected on HPLC. The same sample methodology was used for chiral GC but the sample was added to diethylether instead of hexane–isopropanol.

High Performance Liquid Chromatography

Achiral methodology. All achiral analyses were carried out on a 4.6 × 50 Merck Chromolith SpeedROD RP-18e with different eluent compositions containing 0.1 v% TFA as organic modifier at 1 mL min^{-1} and a column temperature of 21 °C. Compounds were detected using a Shimadzu SPD-10A VP UV-VIS detector

at different wavelengths and a Shimadzu RID 10A refractive index detector. The following conditions allowed for baseline separation of the mentioned nitriles and their corresponding amides as well as any occurring side products like aldehydes: compound **1–3** (H_2O–AcN 90 : 10 (v/v)), compound **4–5**, **8–9**, **10–11**, **14** (H_2O–AcN 20 : 80 (v/v)), compound **6** (H_2O – AcN 97.5 : 2.5 (v/v)), compound **12** (H_2O – AcN 99 : 1 (v/v)), compound **13** (H_2O – MeOH 60 : 40 (v/v)), and compound **15** (H_2O – MeOH 70 : 30 (v/v)). For all compounds a UV detection wavelength of 210 nm was used, except for **2–3** (230 nm) and **12** (205 nm).

Chiral methodology. Unless stated otherwise, the compounds were detected using a Waters 486 Tunable Absorbance Detector at 210 nm.

The enantiomers of the amide of **4** were separated on a 250 × 4.6 mm Chiralcel OD column, eluent hexane–isopropanol, 80 : 20 (v/v) containing TFA (0.1%, v/v) at 0.5 mL min^{-1} and a column temperature of 21 °C. The (*S*)-enantiomer eluted before (*R*). The same analysis method was used for the amide of **5** ((*S*) elutes before (*R*)) and **13** ((*S*) elutes before (*R*)). The enantiomers of the amide of **14** were separated on the same column using heptane–isopropanol, 90 : 10 (v/v). Although no enantiomerically pure standard was available for this compound, extrapolation of the previous results might indicate that the (*S*)-enantiomer elutes before (*R*).

The enantiomers of the amide of **6** were separated using a Nucleodex β-OH column, eluent H_2O–MeOH, 90 : 10 (v/v) at 0.7 mL min^{-1} and a column temperature of 21 °C. The compounds were detected using a Shimadzu SPD-10A VP UV-VIS detector at a wavelength of 210 nm. (*R*)-mandelamide eluted from the column after (*S*)-mandelamide.

The enantiomers of **7** and the enantiomers of the amide of **7** were separated on a Daicel Chemical Industries Ltd. 4.6 × 150 mm; 5 μm Crownpak CR (+) column. The eluent was aqueous $HClO_4$, pH 1, at a flowrate of 0.6 mL min^{-1}. The column temperature was 18 °C. In the case of both the nitrile and the amide, the (*R*)-enantiomers eluted before the (*S*). The compounds were detected using a Shimadzu SPD-10A VP UV-VIS detector at a wavelength of 210 nm.

The enantiomers of the amides of **8** and **9** were separated on a 250 × 4.6 mm Daicel ChiralPak AD-H column, eluent hexane–isopropanol, 92 : 8 (v/v) containing TFA (0.1%, v/v) at 0.5 mL min^{-1} and a column temperature of 21 °C. In both cases the (*R*)-enantiomer elutes before (*S*). The same analysis method was used for the separation of the enantiomers of the amide of **10**. To determine the elution order of the (*R*)- and (*S*)-amide, a previously described chiral GC method was used first to determine the configuration of the remaining nitrile.[27] Using this method it was found that the (*S*)-amide eluted before the (*R*)-amide. The enantiomers of the amides of **11** and **15** were separated on the same column using hexane–isopropanol, 97 : 3 (v/v) containing TFA (0.1%, v/v) for the amide of **11** and heptane–isopropanol, 85 : 15 (v/v) containing TFA (0.1%, v/v) for the amide of **15**.

The enantiomers of **12** were separated using chiral GC on a Chiradex GTA column of 50 m × 0.25 mm, d_f 0.12 μm with an isothermal temperature program of 50 °C for 10 min and a gas flow of 7 mL min^{-1}. (*R*)-**12** elutes before (*S*)-**12**.

Acknowledgements

The authors wish to thank Andreas Stolz of the Institut für Mikrobiologie der Universität Stuttgart for supplying them with naproxennitrile and naproxenamide. Haiyan Yang of ICES Singapore is gratefully acknowledged for *rac*-2-azido-3-phenylpropionitrile and *rac*-2-azido-3-phenylpropionamide and DSM for (*R*)-phenylglycinoamide. The authors wish to thank Tom Moody and Rachel Agnew of Almac Sciences for valuable advice. Financial support of the Delft Research Center of Life Sciences and Technology (DRC-LST) and the Dutch National Research School Combination Catalysis Controlled by Chemical Design (NRSC-C) is also gratefully acknowledged.

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