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Nitrile Hydratase Activity of a Recombinant Nitrilase

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Abstract: Appreciable amounts of amide are formed in the course of nitrile hydrolysis in the presence of recombinant nitrilase from *Pseudomonas fluorescens* EBC 191, depending on the α-substituent and the reaction conditions. The ratio of the nitrilase and nitrile hydratase activities of the enzyme is profoundly influenced by the electronic and steric properties of the reactant. In general, amide formation increased when the α-substituent was electron-deficient; 2-chloro-2-phenylacetonitrile, for example, afforded 89% amide. We found, moreover, that (*R*)-mandelonitrile was hydrolysed with 11% of amide formation whereas 55% amide was formed from the (*S*)-enantiomer; a similar effect was found for the *O*-acetyl derivatives. A mechanism that accomodates our results is proposed.

Keywords: amide formation; enantiomer selectivity; mechanism; nitrilase; *Pseudomonas fluorescens* EBC 191; selectivity

Introduction

Nitriles are important intermediates in synthetic chemistry because they are simple to prepare and give access to a wide variety of interesting carboxylic acid derivatives. Chemical hydrolysis of nitriles is well established but requires harsh conditions that may necessitate the protection of any sensitive functional groups and leads to the generation of large amounts of waste. A preferred option would be to conduct the hydrolysis of the nitriles enzymatically, at mild reaction conditions with regio- and/or enantioselectivity as an additional advantage.

It is generally accepted that Nature employs two enzymatic pathways for nitrile hydrolysis (see Figure 1).[1] In the direct pathway a nitrilase (NLase, E.C. 3.5.5.1) catalyses the addition of two molecules of water to give the carboxylic acid. Nitrilases are widely found in Nature and have been isolated from microbes and plants. The second pathway is mainly present in microbes and involves a hydration step in the presence of a nitrile hydratase (NHase, E.C. 4.2.1.84), which yields the corresponding amide, followed by amidase-catalysed hydrolysis of the latter.

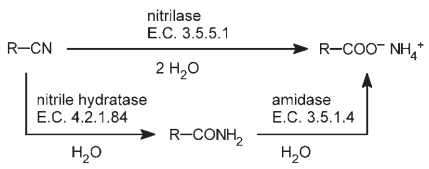


Figure 1. Pathways of enzymatic nitrile hydrolysis.

In contrast with common wisdom, which dictates that NLases transform nitriles into the carboxylic acids exclusively, reports can be found, scattered in literature as far back as the 1960s, of modest amounts of amides being formed in the presence of nitrilases.[2–4] This side-activity, which is not accounted for by the commonly accepted nitrilase mechanism,[3] has largely been neglected. Presumably, amide formation often was ascribed to the presence of a contaminating nitrile hydratase. In many cases, amide formation may have gone unnoticed, either because a contaminating amidase hydrolysed the amide by-product into the acid or because the detection of the catalytic activity or the selection of catalysts depended on ammonia release, which only occurs upon full hydrolysis into the acid.

In a recent characterisation study of a purified, recombinantly expressed NLase from *Arabidopsis thaliana* (AtNIT4), >60% amide was formed in the course of the hydrolysis of 3-cyano-L-alanine, the presumed natural substrate.[5] A different recombinant NLase from *A. thaliana* (AtNIT1) has been the subject of the most complete study up to date, which showed the effects of electron-withdrawing substitu-

ents on amide formation.[6,7] Thus, hydrolysis of 2-crotonitrile in the presence of AtNIT1 afforded 99% crotonic acid, whereas 95% amide was formed from the electron-deficient 3-nitroacrylonitrile.[7] It is worth noting in this respect that the cysteine-dependent protease papain has been transformed, by changing one amino acid residue, into a modestly active nitrile hydratase.[8] We hypothesise, on the basis of the experimental evidence, that the nitrilase and nitrile hydratase activities in NLases are intimately related.

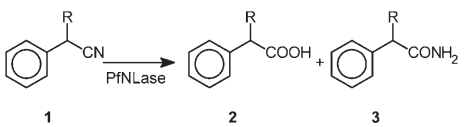
Nitrilase-catalysed amide formation deserves to be studied in detail as it may have serious implications regarding the yield and purity of the desired product and complicates the downstream processing. Furthermore, highly selective NLase-mediated amide synthesis, assuming that this latter activity can be controlled, would afford a synthetic route to enantiomerically pure amides. As such, it could be a valuable addition to the synthetic repertoire as NLases are often enantioselective, whereas it is an exception with NHases.

The synthetic relevance of amide formation in the presence of NLases prompted us to study this latter side-activity in more detail, to get a better understanding of the factors that influence the amide formation, such as steric requirements and electron density. For this purpose we employed a recombinantly produced nitrilase originating from *Pseudomonas fluorescens* EBC 191 (PfNLase), which is known to be prone to amide formation.[9] It is worth noting that this latter enzyme is an arylacetonitrilase and tolerates quite bulky groups on the α-position, in contrast to the NLases from *Arabidopsis thaliana*. The use of an enzyme expressed in *E. coli* ensures that a single nitrile hydrolysing enzyme is present as the non-transformed *E. coli* strain shows no nitrile hydrolysing activity.

Results and Discussion

Hydrolysis of 2-Phenylacetonitrile

Hydrolysis of 2-phenylacetonitrile (**1a**, see Figure 2), a simple model substrate, in the presence of PfNLase afforded, besides the acid **2a**, a minor amount of 2-phenylacetamide (**3a**). From the time-course of the reaction (see Figure 3), it becomes apparent that **2a** and **3a** are formed concurrently. Temporary accumulation of amide and subsequent hydrolysis are not observed. Hence, **3a** is not an intermediate in the hydrolysis of **1a** into **2a**, as with nitrile hydratase/amidase systems. It is pertinent to note that PfNLase does not hydrolyse **3a**, neither does it mediate the formation of **3a** from **2a** and ammonia.



a R = H
b R = CH_3
c R = Cl
d R = OH
e R = OAc

Figure 2. Hydrolysis of nitriles using a nitrilase.

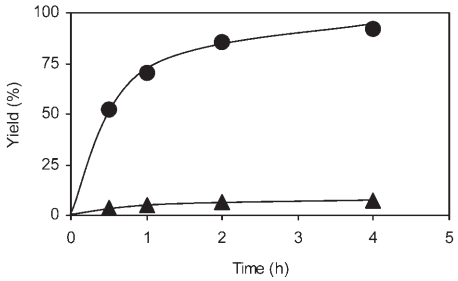


Figure 3. Hydrolysis of 2-phenylacetonitrile (**1a**, 10 mM) in the presence of PfNLase (35.5 μg mL^{-1}) in 100 mM phosphate buffer pH6 at 10 °C; acid (●), amide (▲).

Summarising, amide and acid are both *bona fide* reaction products that are formed directly from the nitrile in a ratio of 23:1. This latter parameter, the acid/amide ratio (Ac/Am), will be used throughout the paper to characterise the selectivity of PfNLase.

We found that the reaction pH, when varied from 5 to 9, influenced the acid/amide ratio only slightly (Figure 4a), with lower pH slightly reducing the amount of amide. The effect of the reaction tempera-

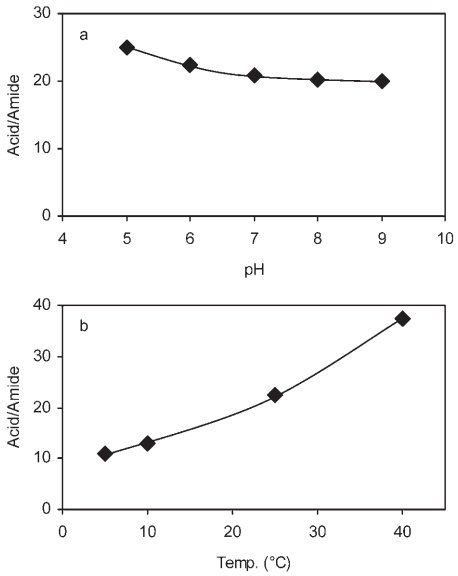


Figure 4. Acid/Amide ratio for PfNLase (35.5 μg mL^{-1}) in the hydrolysis of **1a** (10 mM) in 100 mM phosphate buffer; **a**: effect of pH at 25 °C; **b**: temperature effect at pH 6.

ture was more pronounced (see Figure 4b) and Ac/Am changed from 11 at 5 °C to 38 at 40 °C. Measurements of the initial rates of formation of **2a** and **3a** (data not shown) showed that acid formation is more temperature-dependent, which indicates a higher activation barrier for the formation of acid. These results suggest that elevated temperature and low pH steer the reaction towards acid, whereas a low temperature and increased pH bend the selectivity towards the amide.

Electronic Effects of the α-Substituent

It has been shown that the Ac/Am ratio of a nitrilase from *Arabidopsis thaliana* (AtNIT1) was highly sensitive to electronic effects.[7] We wished to investigate the behaviour of PfNLase in this respect by substituting the α-position in **1a** with similarly sized groups of opposing electronic character. Thus, we compared 2-phenylpropionitrile (**1b**, see Figure 2) and 2-chloro-2-phenylacetonitrile (**1c**) and found that the electron-deficient **1c** reacts faster by a factor of 2.4.[10] The methyl-substituted reactant **1b** was converted into >99% acid (**2b**), whereas 90% amide was formed from **1c** (see Table 1). Hence, it would seem that the extent of amide increases with increasing electronegativity of the α-substituent: $CH_3 < H < Cl$. A comparable effect of α-fluoro substitution has been reported for *Arabidopsis* nitrilases.[6]

Table 1. Electronic effects on the acid/amide ratio.[a]

Reactant	**1b**	**1c**
Initial rate [μmol $min^{-1} mg^{-1}$]	0.81	2.13
Acid [%]	>99	11
ee [%]	n.d.	82 (*R*)
Amide [%]	<1	89
ee [%]	n.d.	>94 (*S*)
Ac/Am	>99	0.1

[a] *Reaction conditions:* 10 mM nitrile in 100 mM phosphate buffer pH 6 at 25 °C.

Interestingly, the formation of acid (**2c**) from racemic **1c** was strongly biased towards the (*R*)-enantiomer (see Table 1), whereas the amide (**3c**) was nearly enantiomerically pure (*S*). This result strongly indicates that the absolute configuration of the α-carbon strongly influences the Ac/Am ratio but, unfortunately, this effect could not be investigated in detail as **1c** racemised under the reaction conditions.

Effects of the α-Substituent Size and the Absolute Configuration

Enantiomerically pure mandelic acid (**2d**) is industrially produced as a chiral intermediate in the synthesis of fine chemicals and as a chiral auxiliary in industrial resolutions. (*R*)-**2d** is produced[11] on a multiton scale *via* enantioselective hydrolysis of mandelonitrile (**1d**) in the presence of a nitrilase.[12] The pure enantiomers of **1d** are readily accessible *via* hydrocyanation of benzaldehyde in the presence of the appropriate oxynitrilase;[13] hence, they are, in principle, good model compounds for investigating possible effects of the absolute configuration of the reactant on the Ac/Am ratio.

The modest stability of **1d** in neutral aqueous medium is an obstacle that requires careful consideration. Compound **1d** decomposes into benzaldehyde and hydrogen cyanide; the process is reversible and is the major racemisation pathway of enantiomerically pure **1d**. The nitrile becomes stable at pH <4–5 and lower temperature but such conditions also have a strong deleterious effect on nitrilase activity. We compromised by investigating the hydrolysis of enantiomerically pure (*R*)- and (*S*)-**1d** in the presence of PfNLase at pH 6 and 0 °C. Under these conditions the racemisation of the nitrile is much slower than the enzymatic hydrolysis. Both enantiomers reacted with comparable rates, but with widely diverging product selectivity (see Table 2). While the hydrolysis of (*R*)-**1d** yielded only 11% amide (Ac/Am = 8.1), the amide (*S*)-**3d** was the major product from the hydrolysis of (*S*)-**1d** (Ac/Am = 0.8). Hence, it appears that Ac/Am is influenced by electronic as well as by steric factors. Here, the study of enantiopure compounds gives an insight into the system that is never possible by the use of racemates.

A more stable derivative of **1d** was required to measure the effects of the pH on amide formation. The ester derivative, 2-acetoxy-2-phenylacetonitrile

Table 2. Hydrolysis **1d** and **1e** in the presence of PfNLase.[a]

	(*R*)-**1d**	(*S*)-**1d**	(*R*)-**1e**	(*S*)-**1e**
Initial rate [μmol $min^{-1} mg^{-1}$]	0.62	0.62	0.23	1.73
Acid [%]	89	45	67	38
Amide [%]	11	55	33	62
Ac/Am	8.1	0.8	2	0.6

[a] *Reaction conditions:* 10 mM reactant, 100 mM phosphate buffer pH 6 at 0 °C (**1d**) or 10% methanol at 25 °C (**1e**).

(**1e**),[14] does not suffer from HCN elimination but racemisation still occurs, *via* deprotonation on the α-position. Fortunately, this latter process is slow in aqueous media at pH < 7 (data not shown). Spontaneous hydrolysis of the ester bond in **1e** (with formation of **2d** and **3d**), which is significant at pH > 7, remained restricted to < 5% but often much less, depending on the pH.

Much to our surprise, the ester group profoundly influenced the course of the enzymatic hydrolysis. The reaction rates (see Table 2) should not be compared directly as different temperatures were used, but it is noteworthy that (*R*)-**1e** reacted three times slower than (*R*)-**1d**, the 25 °C temperature difference notwithstanding. The enantiomeric kinetic bias shifted from pro-(*R*), with **1d**, to pro-(*S*). Both enantiomers produced more amide than the corresponding enantiomers of the free cyanohydrin; the Ac/Am ratio of the hydrolysis of (*R*)-**1e** was even four times lower than that of (*R*)-**1d**. The progress curve for the separate hydrolysis of (*R*)- and (*S*)-**1e** (Figure 5) illustrates well that the amide is not a reaction intermediate and that the two enantiomers are differently hydrolysed.

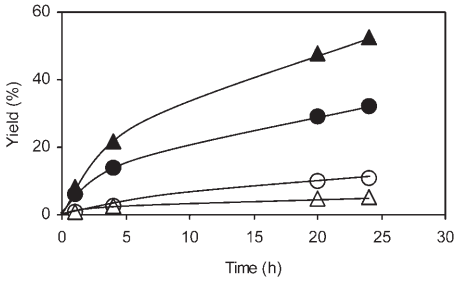


Figure 5. Hydrolysis profiles for (*R*)- and (*S*)-**1e**: (*S*)-**2e** (●), (*S*)-**3e** (▲), (*R*)-**2e** (○), (*R*)-**3e** (△) at pH 6.

We conclude, on the basis of these results, that enantiomers of chiral reactants should be treated as separate entities, and that unambiguous conclusions as regards selectivity issues cannot be based on racemate conversions. Thus, for example, in the hydrolysis of (*R*,*S*)-**1e**, (*S*)-**2e** and (*S*)-**3e** are the major products initially, with Ac/Am = 0.7, whereas at total conversion (*R*)-**2e** and (*S*)-**3e** predominate (Ac/Am = 1.1, data not shown).

A pH profile of the hydrolysis of **1e** (Figure 6) revealed widely diverging effects on the propensity of the two enantiomers to produce amide. Amide formation from (*R*)-**1e** was found to increase when the medium was turned acidic, whereas the Ac/Am ratio of the (*S*)-enantiomer passed through a minimum at pH 6. Both patterns are completely different from what is seen with **1a**. Changes in charge distribution obviously affect (*R*)- and (*S*)-**1e** in different ways,

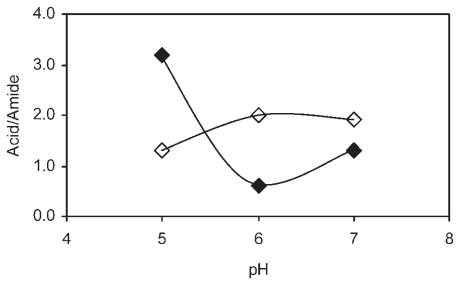


Figure 6. pH profile for the hydrolysis of (*R*)-**1e** (◇) and (*S*)-**1e** (◆); *reaction conditions:* 10 mM reactant, 100 mM phosphate buffer, 25 °C.

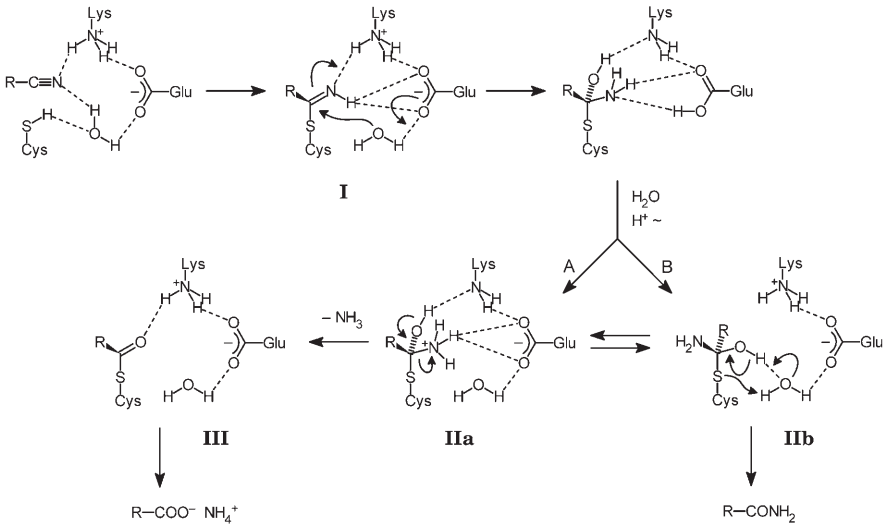
which leads us to the conclusion that the enantiomers bind in very different ways.

Mechanism of Action

Any discussion of the catalytic mechanism of nitrilases is hampered by a lack of crystal structures. It is commonly accepted, nevertheless, that nitrilases harbor a Cys-Glu-Lys triad in the active site[15] and that the reaction takes place *via* a thioimidate intermediate (**I**, see Scheme 1).[2,3,16] The active site triad has been identified in the crystal structures of the worm NitFhit fusion protein[17] and in a putative nitrile hydrolase from yeast.[18] This nitrile hydrolysis mechanism is depicted in Scheme 1. A bridging water molecule has been added because it has been identified in the yeast protein,[18] as well as in a structurally closely related D-carbamoylase (*N*-carbamoyl-D-amino acid amidohydrolase).[19]

Elimination of ammonia from the tetrahedral intermediate (pathway **A**), with formation of the acyl-enzyme intermediate **III**, requires a positive charge on the nitrogen atom in the reactant, stabilised by the Glu residue. If, in contrast, the positive charge is not on the reactant but, for example, on the Lys residue (tetrahedral intermediate **IIb**), formal thiol elimination is expected to prevail (pathway **B**). Such a charge distribution could be expected, for example, when an electron-demanding R group destabilises the positive charge on the reactant N. Otherwise, steric interactions could force the N atom away from the stabilising Glu (see Scheme 1, pathway **B**). Summarising, NLase-mediated nitrile hydrolysis into the carboxylic acid and water addition to give the amide are two branches of the nitrilase mechanism, as originally suggested by Hook and Robinson.[2] We propose that the charge distribution in the tetrahedral intermediate, depending, in turn, on the stereochemical and electronic properties of the R group acts as a mechanistic switch.

We note that amide elimination from the tetrahedral intermediate is amply supported in the chemical



Scheme 1. Proposed nitrilase mechanism for the formation of acid (**A**) and amide (**B**).

literature. It is the major pathway in the hydrolysis of thioimidate esters[20] and mercaptoethanol-catalysed nitrile hydrolysis[21–23] at neutral pH. In both cases the product selectivity shifted towards acid at pH <3. We found a similar pH effect, although under near-neutral conditions and of smaller magnitude, in the enzymatic hydrolysis of **1a**.

The active site does not contain bound amide at any time, according to our mechanism and amide, once formed, is not hydrolysed any further. It is commonly observed that NLases do not hydrolyse amides, although there are strong indications that NLases, aliphatic amidases and D-carbamoylases have very similar active site architectures.[24] A very modest amidase activity (6000 times less than the NLase activity), mainly due to a reduced V_{max}, has been measured with the nitrilase from *Rhodococcus rhodochrous* J1.[25] If this factor also applies to PfNLase, hydrolysis of **3e** would be in the order of 0.01% over 24 h.

Why amides cannot react *via* **IIa** into the thiol ester intermediate (**III**) is not clear but an obvious explanation is that **IIa** is too high in energy, relative to the amide. As regards the amidase from *Pseudomonas aeruginosa*, it has been suggested that only one amide molecule can be bound per hexameric amidase molecule,[26] possibly because conformational changes in the entire hexamer are required to smooth the energetic pathway towards the acyl-enzyme intermediate.

As regards nitrilase catalysis, it would seem that the enzyme conserves the chemical energy in the nitrile triple bond, maintaining **IIa** and **IIb** well above ground-state level, to transform the reactant smoothly into acid and amide.

Conclusions

We have shown that *Pseudomonas fluorescens* nitrilase converts nitriles into the carboxylic acid as well as the amide. The relative formation of acid and amide is subject to the pH and the temperature. Electron-withdrawing substituents at the α-position favour amide formation.

We have shown, for the first time, that the absolute configuration at the α-position in the reactant exerts a dramatic influence on the extent of amide formation.

Experimental Section

Chemicals

Soluble, His_6-tagged *Pseudomonas fluorescens* EBC 191 nitrilase (PfNLase) was recombinantly produced in *E. coli* JM109(pIK9).[9] The stock solution contained approx. 48 mg mL^{-1} of protein, according to a Bradford test. Its activity in the hydrolysis of racemic mandelonitrile was 3.1 μmol (mg protein)$^{-1}$ min^{-1} at 0 °C and pH 6. 2-Phenylpropionitrile (**1b**) from Acros was purified by distillation under reduced pressure in a Kugelrohr apparatus (Büchi) to remove an unknown contaminant that inhibited PfNLase. Unless otherwise stated, the chemicals were obtained from normal commercial sources and used without further purification.

(*R*)- and (*S*)-mandelonitrile [(*R*)- and (*S*)-**1d**] were synthesised *via* enzymatic hydrocyanation of benzaldehyde in the presence of the appropriate oxynitrilase.[27,28] Enantiomeric purities were verified by chiral HPLC to be 99% (*R*) and 98% (*S*).

(*R*)-2-Acetoxy-2-phenylacetonitrile [(*R*)-**1e**] was synthesised by acylation of (*R*)-**1d** with acetic anhydride and pyridine in 1,4-dioxane at room temperature. The mixture was

diluted with ether and consecutively washed with water, 1 M hydrochloric acid, saturated sodium carbonate and brine, dried over $MgSO_4$ and concentrated under vacuum. The product was isolated as a colourless liquid with *ee* >99% according to chiral GC. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.16 (s, 3H, CH_3), 6.41 (s, 1H, CH), 7.26–7.55 (m, 5H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 20.4 (CH_3), 62.8 (CH), 11.6 (CN), 127.8 (C-2,6), 129.2 (C-3,5), 130.3 (C-4), 131.7 (C-1), 168.9 (C=O).

(*S*)-2-Acetoxy-2-phenylacetonitrile [(*S*)-**1e**] was obtained using a published procedure[29] with enantiomeric purity 93.7% according to chiral GC.

Analysis

Conversions and yields were determined by reversed phase HPLC analysis, using a Waters 590 pump, a 50×4.6 mm Merck Chromolith SpeedROD RP-18e column and a Waters 486 UV detector. Mobile phase ACN-H_2O-TFA 10:90:0.1 (**1a**–**3a**), 20:80:0.1 (**1b**–**e**–**3b**–**e**) at 1 mL min^{-1}.

Enantiomeric purities were determined by chiral HPLC, using a Waters 510 pump, a 4.6×250 mm Chiracel AD-H column and a Waters 486 UV detector. Mobile phase hexane-isopropyl alcohol-TFA = 95:5:0.1 (**2c**, **3c**) or 92:8:0.1 (**2e**, **3e**).

Chiral GC of **1c** and **1e** was performed with a Shimadzu GC-17 A instrument equipped with a Shimadzu Auto Injector AOC-20i, a 25 m×0.25 mm Varian CP-Chirasil-Dex CB column and an FID detector. Column temperature: 140 °C (**1c**), 145 °C (**1e**).

Enzymatic Hydrolysis

Enzymatic hydrolysis of nitriles (**1a–e**) was carried out at 1–2 mL scale in magnetically stirred Eppendorf tubes immersed in a water-bath at the desired temperature. Stock solutions of the substrate and internal standard (in methanol), PfNLase and buffer (in water) were used. Final concentrations were 10 mM of substrate, 1 mM internal standard, 10% methanol and 100 mM phosphate buffer at the desired pH.

The reaction was started by the addition of the enzyme solution (all enzyme weights refer to the total protein content). Samples were taken periodically, diluted with acid (to stop the reaction) and filtered over a Microcon YM-10 centrifugal filter device prior to analysis. Samples for chiral analysis were further extracted with ether and dried over $MgSO_4$. The solvent was evaporated and residue was redissolved in the appropriate solvent.

Reported Ac/Am ratios were measured when these had stabilised and stayed constant until full conversion.

Acknowledgements

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