Enantiomeric biocatalytic hydrolysis of β-aminonitriles to β-aminoamides using *Rhodococcus rhodochrous* ATCC BAA-870

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Keywords: Nitrile hydratase, Amidase, β-aminonitrile, biocatalysis, biotransformation, *Rhodococcus*, Kinetic resolution.

Abstract

A range of β-aminonitriles (3-amino-3-phenylpropanenitrile and derivatives) were synthesised by reduction of the unsaturated precursor and subsequently hydrolysed to the corresponding amide using the nitrile biocatalytic activity of *Rhodococccus rhodocrous* ATCC BAA-870. Results showed that the nitrile hydratase enzyme was enantioselective for these compounds, in particular 3-amino-3-*p*-tolylpropanenitrile and 3-amino-3-(4-methoxyphenyl) propanenitrile. The stereoselectivity was facilitated by a nitrile hydratase, and hence could provide enatiomeric excess of both the nitrile or the amide (up to 85% in one case). The reactions were performed at pH9 after initial attempts at pH 7.0 were unsuccessful, most likely as a result of protonation of the 3-amino group at the lower pH.

1. Introduction:

β-Amino amides and acids can be used as building blocks for the synthesis of pharmaceutical intermediates. This includes biologically active peptides and small molecule pharmaceuticals (Hsiao et al 2004) [1]. β-Amino acids are constituents of compounds such as the antitumour drug Taxol [Holton et al 1994] [2], the antifungal antibiotic Cispentacin [Davies et al 1994] [3], and the antidiabetic drug Sitagliptin [Steinhuebel et al 2009] [4]. Another pharmaceutical application is for inclusion in peptidomimetics that may be of use as protease inhibitors against retroviruses such as HIV [Aguilar et al. (2007) [5], Steer et al. (2002) [6], Gademann et al. 1999 [7]].

New potential applications for these compounds are being discovered continually. Wolin et al (2004) [8] found that β-amino acid derivatives act as glycine transport inhibitors, while Zhu et al (2010) [9] have discovered that others are proteasome inhibitors. Armour (2004) [10] incorporated them into oxytocin inhibitors, while Angelaud et al (2004) [11] synthesised peptidase inhibitors and Imbriglio included them in niacin receptor agonists for treating atherosclerosis and dyslipidemias [Imbriglio et al 2007] [12].

Many applications require the β-amino substituted compounds as single enantiomers [Hsiao et al 2004] [1]. There are a number of chemical synthesis methods for the preparation of racemic β^3-amino acids [Liljeblad and Kanerva, 2006 [13], Weiner et al 2010 [14]]. These methods include homologation of α-amino acids, Curtius rearrangement, Michael addition to unsaturated carboxylic acids, β-lactam ring opening, modified Knoevenagel condensation, reductive amination of 3-keto-carboxylic acids, nitrile reduction, nitrile hydrolysis, oxidation of 3-amino alcohols, and amido-methylation of carboxylic esters [Liljeblad and Kanerva 2006] [13]. Racemates can be resolved by various methods. The classical method of resolving amino acids is through transformation of the racemate into diastereomeric salts via complexation of the carboxylic acids with a chiral base, usually followed by multistep fractional recrystallisation.

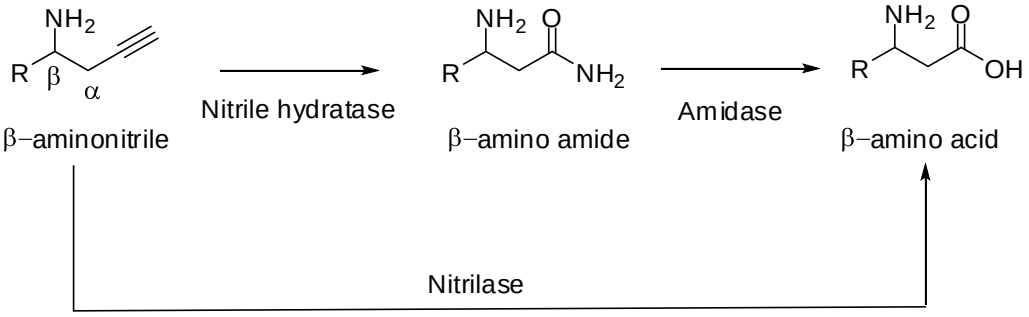
Resolution may be achieved more simply through the application of biocatalysts, such as enzymatic resolution of β-amino esters and N-acyl derivatives [Liljeblad and Kanerva 2006] [13]. Biocatalytic resolution of single enantiomer β^3-amino acids includes kinetic resolution

by hydrolysis of carboxylic esters of N-acylated β^3-amino acids using lipases such as CalB and the protease α-chymotrypsin; or by transesterification using lipase or acylase I [Liljeblad and Kanerva 2006] [13]. Pousset et al (2004) [15] demonstrated the application of *Burkholderia cepacia* lipase for hydrolytic resolution of heterocyclic β-amino acids, achieving greater than 99% ee. Tasnádi et al (2008, 2009, 2010 [16. 17. 18]) achieved ee of 98-99% for both enantiomers of β-aryl-β-amino, β-arylalkyl-β-amino and β-heteroaryl-β-amino esters by enantioselective hydrolysis of the carboxylic acid ester using a commercial preparation of *B. cepacia* lipase (Amano PS) to hydrolyse the *S*-enantiomer. Li and Kanerva (2005) [19] used the lipase CAL-A to selectively acylate 3-amino-4-indolin-3-yl-butanenitrile, providing a 99% ee. A second option is to resolve the enantiomers through modification of the β-amine. N-acylation or deacylation can be performed by Acylase I [Liljeblad and Kanerva 2006] [13]. Peptide deformylase and Penicillin G-amidase can also N-deacylate [Liljeblad and Kanerva 2006] [13] 2006 [20] and Tasnádi et al 2007 [21] demonstrated enantioselective ring opening of β-lactams to provide single enantiomers of β-amino acids with ee of 99% using the lipase CAL-B. Penicillin G-amidase can also perform stereoselective N-acyl hydrolysis. β-Lactamses and lipases can stereoselectively open lactams to yield β-amino acids. Aspartase can form β-amino acids through Michael addition. Hydantoinase hydrolysis of hydantoins, reductive amination by aminotransferase, and isomerisation by 2,3-aminomutase add yet more options [Liljeblad and Kanerva 2006] [13]. Recently phenylalanine aminomutase (PAM) has been applied in the enzymatic synthesis of (R)-β-arylalanines with high ee (>99%) [Wu et al 2010] [22].

There are also methods available to generate the single enantiomers, such as asymmetric hydrogenation of enamines to yield β-amino substituted amides and esters at up to 97 and 96% enantiomeric excess (ee) (Hsiao et al 2004) [1]. Kawanaska recently developed a new enantioselective Mannich reaction for synthesis of N-Tosyl β-amino acid esters at up to 95% ee using an N-heterocyclic carbine as a catalyst [23].

Single enantiomer β-amino amides and acids can also be obtained through the enzymatic hydrolysis of the related nitrile (Scheme 1). Nitrile hydrolysing enzymes (nitrilase or a combination of nitrile hydratase and amidase) provide a mild approach for the synthesis of carboxylic acids from nitriles. β-Alanine, the only naturally occurring beta amino acid, can be derived from β-aminopropionitrile using whole cell biocatalysts *Alcaligenes* sp. OMT-MY14, *Aminobacter aminobrance* ATCC 23314 (Toshihiro et al 1998) [24] and

Rhodococcus sp (Liang et al 2008) [25]. Although β-alanine is achiral, nitrile and amide biocatalysts also demonstrate enantioselectivity, and this has been successfully applied to the resolution of N-protected cyclic β-amino acids [Primel et al 2003, 2004, Winkler et al 2005] [26, 27, 28]. Veitía, et al 2009 [29] demonstrated synthesis of N-protected β^3-amino amino acids using the Codexis range of nitrilases to hydrolyse the single enantiomer nitrile precursors. Biotransformation of alicyclic N-tolylsulfonyl- and N-butyloxycarbonyl protected β-amino nitriles to the trans amides and carboxylic acids using two strains of *Rhodococcus* (*Rhodococcus* sp. R312 and R, erythropolis NCIMB 11540) has been demonstrated [Preiml et al 2004] [27]. *Rhodococcus erythropolis* AJ270 cells 96.4% ee was achieved where the [Bn] N-protected β-amino acid β-substituent was a c-Pr group [Ma et al 2008]. [30]



Scheme 1: Biocatalytic conversion of β-amino nitriles to β-amino acids.

β-Amino nitriles themselves can be prepared through ring opening of aziridines. Preiml et al (2003) [26] used this method to generate trans-configured alicyclic β-amino acids. González et al (2010) [31] used a Mannich-type reaction to synthesise enantio-enriched R-unsubstituted β-amino nitriles through the organocatalytic addition of β-phenylsulfonylacetonitrile to either N-Boc-protected R-amido sulfones or imines. Enantiomeric excesses (ee) of 76, 83, 64, and 55% were obtained for the N-Boc-protected parent, *p*-methyl -methoxy, -chloro compounds respectively at conversions of about 80%.

Herein we demonstrate enantioselective hydrolysis of unprotected β-amino nitriles to the corresponding amide using *Rhodococcus rhodochrous* ATCC BAA-870, an organism isolated from soil [Brady et al 2004, 2006]. [32, 33]

2 Experimental

2.1 General

Reactions were monitored by thin layer chromatography (TLC) on aluminium-backed Merck silica gel 60 F_{254} plates using an ascending technique. The plates were visualized both by UV at $\lambda = 254$ nm and then visually by colour development after treatment with ninhydrin spray. Gravity column chromatography was carried out on Merck silica gel 60 F254 aluminium sheets and a mixture of ethyl acetate and hexane (or DCM and methanol) was used as eluent unless otherwise specified.. Organic layers were dried over anhydrous $MgSO_4$ or anhydrous Na_2SO_4 before evaporation on a Büchi rotary evaporator RE 111 with a bath temperature of 40°C or below as required

Nitrile and amide analysis was performed using a liquid chomatography (HPLC) system composed of Waters 2690 separation module coupled with Waters Diode Array Detector 996 (210 nm - 400 nm), a Waters X-Terra MS18 3.5 µm, 3.0 x 50 mm (ID x L) column (25°C), with the isocratic eluent of 0.1% v/v triflouroacetric acid in ultrapure water combined with acetonitrile (composition was varied according to the compound analysed) at a flow rate of 0.3 ml/min – 0.5 ml/min. The run time was 15 - 20.0 minutes. All data handling was by Empower 2 Software.

Chiral nitrile analysis was performed using a liquid chromatography system composed of a Waters 600-MS Separation Module equipped with Waters 717 Autosample, Waters 2486 UV/Visible Detector (210 nm) and Empower 2 software. Columns used were a Chiralpack AD-H, 250 x 4.6 mm, 5 µm and a Chiracel OD-H, 250 x 4.6 mm, 5 µm (Daicel Chemical Industries Ltd.) (25°C). The eluent was n-hexane : isopropanol (both HPLC Grade) 90:10, %v/v and an isocratic flow rate of 1.00 ml/min with a run time of 35 min. The chromatographic system was conditioned for 1 h before the injection of samples. 3-Amino-3-phenylpropionitrile enantiomers were eluted under these conditions at 12 and 13.6 minutes; 3-Amino-3(4-methylphenyl)propionitrile at 11 and 12.7 min; 3-Amino-3(4-

chlorophenyl)propionitrile at 9 and 12.5; and 3-Amino-3(3-bromophenyl)propionitrile at 19 and 29.8 min.

Chiral amide analysis was performed using a liquid chromatography system composed of a Waters 2690 Separation Module equipped with Photodiode Array Detector 996, and Empower 2 software. The column was a Crownpak CR (+), 150 x 4 mm (10°C). The eluent used was 16.3 g/L perchloric acid, pH 2.00 in nano-pure water (MilliQ) at a flow rate of 0.25 ml/min over a run time of 20.0 – 60.0 minutes. The chromatographic system was conditioned for at least 1 hour in advance with a column flow rate of 0.25 ml/min before the injection of samples. 3-Amino-3-phenylpropionamide (210 nm) enantiomers eluted at 10.9 and 12.9 min. 3-Amino-3(4-methoxy)phenylpropionamide (225 nm) at 20 and 23.4 min; 3-Acetylamino-3(4-chlorophenyl)propionamide (220 nm) at 36.8 and 45.7; and 3-Acetylamino-3(4-methylphenyl)propionamide (210 nm) at 24.5 and 30.6 min.

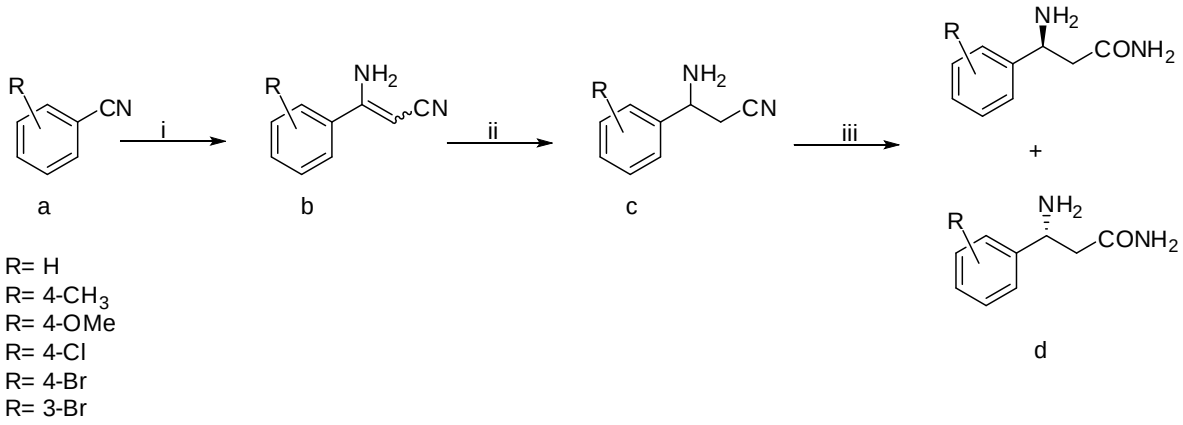
Uncorrected melting points were determined using a Reichert-Jung Thermovar hot-stage microscope.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded as either $CDCl_3$ or CD_3OD solutions with tetramethylsilane as an internal standard. Both a 400 MHz Varian Unity spectrophotometer as well as a 200 MHz spectrophotometer were used for all substrate and product analysis. The ^{13}C NMR spectra were recorded on the same instruments using tetramethylsilane as an internal standard. All chemical shifts were reported in ppm.

2.2 Overall Synthetic route for preparation of β-amino compounds

The preparation of unprotected β-amino amides (Scheme 2) was achieved by reacting benzonitrile (**1a**), and substituted derivatives (**2a** – **6a**) with acetonitrile and KO^{t}Bu. The resulting 3-amino-3-phenylacrylonitrile (**1b)** and substituted derivatives (**2b** – **6b**) were then reduced to yield 3-amino-3-phenylpropanenitriles (**1c** – **6c**) by using $NaCNBH_3$. These β-aminonitriles were subsequently hydrolysed to the amide (**1d** – **6d**) by the *Rhodococcus rhodochrous* ATCC BAA-870 biocatalyst. Reaction progression was monitored by TLC and HPLC. After purification compounds were analysed by ^{1}H and ^{13}C NMR to confirm the structure. Chiral HPLC was used to determine the enantiomeric ratio of the purified

products. For analytical purposes some material was *N*-Boc protected using the method of Kumar et al (2005) [34].



Scheme 2: Racemic synthesis of β-aminonitriles *i*. 2 eq. CH_3CN, 4 eq KO^tBu, benzene or toluene, 24 h. *ii*. $NaBH_3CN$, HCl, EtOH, 2 h. *iii*. *R. rhodochrous* sp., MeOH in Tris buffer, pH 9.0 (1 : 10 v/v), at 5^oC or 30^oC.

The N-Boc and N-tosyl substrates were also prepared to compare their activity with that of the unprotected substrates towards the biocatalyst.

2.3 Microorganism and cell cultivation

Strain ATCC BAA-870 was first cultured in a rich media (TSB) prior to nitrile hydratase induction by transfer into a defined media. The composition of the defined media was as follows: K_2HPO_4 4.97 g/L, KH_2PO_4 0.05 g/L, $CaCl_2.2H_2O$ 0.05 g/L, $FeSO_4.7H_2O$ 0.09 g/L, $MgSO_4$ 0.02 g/L and 1 mL trace element solution), based on that by Layh *et al*. [32] Glucose (1.8 g/L) and inducer (30 mM benzamide) addition into the defined media was done after autoclaving. The cellular pellet was water washed twice prior to it being re-suspended in 100 mM phosphate buffer, pH 7.0 (average cell wet weight was 1.0 mg/mL).

2.4 Biotransformation using whole cells

Wet weight cells (1.0 g) were added to a solution containing 100 mg of the respective 3-amino-3 phenylpropionitrile substrate (solubilised in 1 mL methanol) in 100 mM Tris buffer (either at pH 7 or at pH 9). The resulting mixture was then stirred at either 5°C or 30°C with amide generation being monitored by TLC analysis. The reactions were quenched by centrifugation at 10 000 rpm x 15 minutes with the supernatant being decanted and extracted using three volumes of ethyl acetate. The cell pellet was also extracted in a separating funnel with three volumes of ethyl acetate with product recovery from the sediment by methanol washing. The extracted supernatant fraction as well as the cellular pellet extract were combined and then water and brine washed before drying over anhydrous magnesium sulphate. Concentration was performed under reduced pressure. Product purification was done by flash column purification using varied ratios of hexane/ethylacetate or methanol/DCM solvent systems. Sample analysis was by HPLC.

2.5 General synthetic procedures

General method for synthesis of 3-amino-3-phenylacrylonitriles (compounds 1b-6b) exemplified by compound 1b

Benzonitrile **1a** (4.00 g, 38.8 mmol) was dissolved in dry toluene (100 ml) and acetonitrile (3.20 g, 78.0 mmol) was added. Potassium tert-butoxide (10.00 g, 89.2 mmol) was added in portions. The reaction was allowed to stir at room temperature overnight. Water (100 ml) and diethyl ether (100 ml) were added to the reaction mixture and the organic layer was separated. The aqueous layer was extracted again with diethyl ether (100 ml) and the combined organic layers were washed with brine and dried over $MgSO_4$. Solvent was removed *in vacuo* and to the brown oil was added a small volume of ether and sufficient hexane to make the mixture cloudy. After overnight cooling, crystals were collected and washed with ice-cold hexane to yield 3-amino-3-phenylacrylonitrile (3.68 g, 66%) as white crystals.

2.5.1 3-Amino-3-phenylacrylonitrile 1b (66% yield).; White crystals, m.p. 82.9°C (lit m.p. 81-84°C [35]); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 7.40 (m, 5H), 4.97 (s, 2H), 4.25 (t, *J* = 0.8, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.48, 135.27, 130.91, 128.95, 125.94, 119.49, 63.61 lit [36]. HRMS (ESI): m/z 145.08 $(M+H)^+$; calc. for $C_9H_{19}N_2$: 145.08.

2.5.2 3-Amino-3-*p*-tolylacrylonitrile 2b (1.0 g, 50%). Yellow crystals, m.p. 103.5°C (lit m.p. [37] 107-110°C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (dd, *J* = 1.1, 7.3, 2H), 7.25 – 7.20 (m, 2H), 4.96 (s, 2H), 4.22 (t, *J* = 0.8, 1H), 2.39 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.48, 141.31, 132.35, 129.58, 125.79, 119.73, 62.89, 21.29 lit [36]. HRMS (ESI): m/*z* 159.09 $(M+H)^+$; calc. for $C_{10}H_{11}N_2$: 159.09.

2.5.3 3-Amino-3-(4-methoxyphenyl)acrylonitrile 3b (36% yield). Yellow- brown crystals, m.p. 119.4°C (lit m.p. 117-118°C [37]); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 – 7.42 (m, 2H), 6.95 – 6.90 (m, 2H), 4.98 (s, 2H), 4.17 (s, 1H), 3.83 (s, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 161.60, 161.17, 133.87, 127.36, 119.94, 114.12, 62.07, 55.34 lit [36, 38]. HRMS (ESI): m/*z* 175.09 $(M+H)^+$; calc. for $C_{10}H_{11}N_2O$: 175.09.

2.5.4 3-Amino-3-(4-chlorophenyl)acrylonitrile 4b (56% yield). Beige powder, m.p. 124.8°C (lit m.p [38]. 140-144°C [38]); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 – 7.39 (m, 5H), 4.89 (s, 2H), 4.24 (t, *J* = 0.9, 1H).^{13}C NMR (101 MHz, $CDCl_3$) δ 160.26, 136.97, 133.70, 129.22, 127.33, 119.13, 64.27 lit [36]. HRMS (ESI): m/*z* 179.04 $(M+H)^+$; calc. for $C_9H_8N_2Cl$: 179.04.

2.5.5 3-amino-3-(4-bromophenyl)acrylonitrile 5b (42% yield). Yellow powder, m.p. 135°C (lit m.p.147-148°C [35]); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 – 7.54 (m, 2H), 7.41 – 7.35 (m, 2H), 4.90 (s, 2H), 4.25 (d, *J* = 0.8, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.35, 134.17, 132.17, 127.54, 125.23, 119.13, 64.21 lit [36]. HRMS (ESI): m/*z* 222.99 $(M+H)^+$; calc. for $C_9H_8N_2Br$: 222.99.

2.5.6 3-amino-3-(3-bromophenyl)acrylonitrile 6b (33% yield). Yellow powder, m.p. (lit m.p. [35]); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (t, *J* = 1.8, 1H), 7.59 (ddd, *J* = 1.0, 2.0, 8.0, 1H), 7.44 (ddd, *J* = 1.1, 1.8, 7.8, 1H), 7.30 (t, *J* = 7.9, 1H), 5.05 (s, 2H), 4.22 (t, *J* = 0.9, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.99, 137.24, 133.74, 130.44, 129.07, 124.66, 122.91, 119.02, 64.30. HRMS (ESI): m/*z* 222.98 $(M+H)^+$; calc. for $C_9H_8N_2Br$: 222.99.

General method for preparation of 3-Amino-3-phenylacrylonitriles (compounds 1c – 6c) exemplified by 1c.

3-Amino-3-phenylacrylonitrile (**1c**) (3.00 g, 20.8 mmol) were dissolved in absolute ethanol (50 ml). Sodium cyanoborohydride (1.44 g, 22.9 mmol) was added, followed by bromocresol green (1 drop of a 0.5% solution in ethanol). Concentrated HCl (32%) was added dropwise until a permanent yellow colour was obtained. The reaction was stirred at room temperature for 3 h. Water was added, followed by concentrated aqueous ammonia (25%) to about pH 11. The aqueous layer was extracted with diethyl ether (x 3) and the combined organic layers were washed with brine, dried over $MgSO_4$ and the solvent removed *in vacuo*. The resulting brown oil was purified by column chromatography (elution ethyl acetate) to yield 3-amino-3-phenylpropanenitrile (1.79 g, 59%) as colourless oil.

2.5.7 3-Amino-3-phenylpropanenitrile 1c (59% yield). Clear oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (m, 4H), 7.31 – 7.25 (m, 1H), 4.27 (dd, *J* = 5.8, 7.1, 1H), 2.68 – 2.55 (m, 2H), 1.73 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 142.17, 128.55, 127.85, 125.65, 117.80, 52.25, 28.06. ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.92, 129.89, 129.13, 127.46, 119.31, 53.82, 28.29. HRMS (ESI): m/*z* 147.09 $(M+H)^+$; calc. for $C_9H_{11}N_2$: 147.09.

2.5.8 3-Amino-3-*p*-tolylpropanenitrile 2c (53% yield). Clear oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 – 7.24 (m, 2H), 7.21 – 7.15 (m, 2H), 4.30 (dd, *J* = 5.8, 7.1, 1H), 2.71 – 2.58 (m, 1H), 2.35 (s, 3H), 1.73 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 139.37, 137.93, 129.48, 125.72, 117.96, 52.33, 28.45, 21.03. HRMS (ESI): m/*z* 161.11 $(M+H)^+$; calc. for $C_{10}H_{13}N_2$: 161.11.

2.5.9 3-Amino-3-(4-methoxyphenyl)propanenitrile 3c (53% yield). Clear oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.27 (m, 2H), 6.92 – 6.87 (m, 2H), 4.32 – 4.25 (m, 1H), 3.79 (s, 3H), 2.63 (dd, *J* = 5.5, 6.4, 2H), 1.74 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.21, 134.33, 126.94, 117.96, 114.02, 55.16, 51.93, 28.42. HRMS (ESI): m/*z* 177.10 $(M+H)^+$; calc. for $C_{10}H_{13}N_2O$: 177.10.

2.5.10 3-Amino-3-(4-chlorophenyl)propanenitrile 4c (52% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.30 (m, 4H), 4.37 – 4.29 (m, 1H), 2.73 – 2.58 (m, 2H), 1.99 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.45, 133.92, 128.98, 127.36, 117.56, 51.94, 28.23. HRMS (ESI): m/*z* 181.05 $(M+H)^+$; calc. for $C_9H_{10}N_2Cl$: 181.05.

2.5.11 3-Amino-3-(4-bromophenyl)propanenitrile 5c (126% yield). Yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 8.5, 2H), 7.25 (d, *J* = 8.5, 2H), 4.25 (t, *J* = 6.3, 1H), 2.70 –

2.53 (m, 2H), 1.80 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.07, 131.37, 127.44, 121.33, 117.48, 51.48, 27.74. HRMS (ESI): m/*z* 225.0027 $(M+H)^+$; calc. for $C_9H_{10}N_2Br$: 225.00

2.5.12 3-Amino-3-(3-bromophenyl)propanenitrile 6c (40% yield). Yellow oil, (lit m.p. not available); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (t, *J* = 1.8, 1H), 7.45 (ddd, *J* = 1.2, 1.9, 7.8, 1H), 7.35 – 7.29 (m, 1H), 7.29 – 7.21 (m, 1H), 4.31 (dd, *J* = 5.7, 7.1, 1H), 2.67 (m, 2H), 1.85 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.45, 131.26, 130.40, 129.03, 124.65, 122.81, 117.50, 52.05, 28.20. HRMS (ESI): m/*z* 225.00 $(M+H)^+$; calc. for $C_9H_{10}N_2Br$: 225.00.

General method for Biotransformation of β aminonitriles (compounds 1d to 6d)

β-Aminonitriles (100 mg) were incubated with resting cells of *Rhodococcus rhodochrous* ATCC BAA 870 in a 100 mM Tris buffer, pH 9.0. Incubation was at 5°C or 30°C with agitation and reaction sampling/termination being done at varying time periods (0 min-25 hours).

2.5.13 3-Amino-3-phenylpropanamide 1d (23% yield). White powder; m.p. 110.1°C (lit m.p. not available); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.30 (m, 5H), 6.94 (s, 1H), 5.54 (s, 1H), 4.37 (t, *J* = 6.7, 1H), 2.59 – 2.54 (m, 2H), 2.04 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.38, 143.53, 128.65, 127.52, 125.97, 52.38, 43.90. HRMS (ESI): m/*z* 165.10 $(M+H)^+$; calc. for $C_9H_{13}N_2O$: 165.10

2.5.14 3-Amino-3-*p*-tolylpropanamide 2d (22% yield). Yellow powder (lit m.p. not available); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.22 (d, *J* = 8.1, 2H), 7.18 – 7.12 (m, 2H), 7.02 (s, 1H), 5.73 (s, 1H), 4.32 (t, *J* = 6.7, 1H), 2.53 (d, *J* = 6.5, 2H), 2.34 (s, 3H), 1.96 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.02, 141.98, 137.07, 129.38, 125.68, 52.54, 44.82, 21.02. HRMS (ESI): m/*z* 179.12 $(M+H)^+$; calc. for $C_{10}H_{14}N_2O_2Na$: 179.12.

2.5.15 3-Amino-3-(4-methoxyphenyl)propanamide 3d (16% yield). White powder;; ^{1}H NMR (400 MHz, CD_3OD) δ 7.63 (br s, 1H), 7.53 (br s, 1H), 7.35 – 7.27 (m, 2H), 6.92 – 6.85 (m, 2H), 4.55 – 4.49 (m, 1H), 3.71 (s, 3H), 2.83 (qd, *J* = 7.2, 15.8, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.21, 158.89, 127.23, 127.20, 114.02, 55.21, 51.88, 43.85. HRMS (ESI): m/*z* 217.09 $(M+H)^+$; calc. for $C_{10}H_{14}N_2O_2Na$: 217.10.

2.5.16 3-Amino-3-(4-bromophenyl)propanamide 5d (28.0% yield). Yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.4, 2H), 7.25 (d, *J* = 8.4, 2H), 4.43 – 4.35 (m, 1H), 2.75 – 2.39 (m, 2H), 1.26 (s, 4H).^{13}C NMR (101 MHz, $CDCl_3$) δ 173.83, 141.80, 131.72, 127.92, 121.41, 51.85, 43.24. HRMS (ESI): m/*z* 243.01 $(M+H)^+$; calc. for $C_9H_{12}N_2OBr$: 243.01.

2.5.17 3-Amino-3-(3-bromophenyl)propanamide 6d (6.2% yield). Yellow oil; ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.69, 145.30, 130.76, 130.33, 129.31, 124.90, 122.69, 51.97, 43.28. HRMS (ESI): m/*z* 243.01 $(M+H)^+$; calc. for $C_9H_{12}N_2OBr$: 243.01.

Procedure for *N*-acetylation of the β-amino position

To the amino substrate (1.23 mmol solubilised in 5 ml DCM), was added 5 eq acetic anhydride (6.13 mmol), 4 eq of pyridine (4.9 mmol) and a catalytic quantity of DMAP (~ 1 mg).

2.5.18 *N*-(2-cyano-1-phenylethyl)acetamide 7c (60% yield) cream crystals; (? ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.69 – 7.34 (m, 5H), 6.56 (s, 1H), 4.26 – 3.98 (m, 2H), 3.72 – 3.53 (m, 1H), 2.043 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.122, 149.15, 131.33, 129.23, 127.53, 118.84, 63.92, 24.14, 20.94. HRMS (ESI): m/*z* 187.09 $(M+H)^+$; calc. for $C_{11}H_{11}N_2O$: 187.09.

2.5.19 3-Acetamido-3-phenylpropanamide 7d (60% yield). white crystals; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.27 (m, 6H), 6.17 (dd, *J* = 5.1, 8.7, 1H), 5.53 (s, 1H), 5.46 (s, 1H), 2.87 (dd, *J* = 8.7, 14.9, 1H), 2.68 (dd, *J* = 5.1, 14.9, 1H), 2.08 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.02, 169.84, 139.33, 128.69, 128.36, 126.25, 72.53, 43.05, 21.13. HRMS (ESI): m/*z* 229.09 $(M+H)^+$; calc. for $C_{11}H_{14}N_2O_2Na$: 229.10.

2.5.20 *N*-(2-cyano-1-phenylethyl)-4-methylbenzenesulfonamide 8c

3-Amino-3-phenylpropanenitrile (1.62 g, 0.011 mol) was dissolved in dichloromethane (50 ml) and 4-dimethylaminopyridine (1.3 eq., 1.76 g, 0.014 mol) and tosyl chloride (1.2 eq., 2. 53 g, 0.013 mol) were added. The reaction was allowed to stir at room temperature for 2 days. The organic layer was then washed with 1 M HCl (50 ml), dried over $MgSO_4$ and the solvent removed *in vacuo*. The crude product was purified by silica gel column

chromatography, elution hexane: ethyl acetate (from 7:3 to 5:5) to yield ***N*-(2-cyano-1-phenylethyl)-4-methylbenzenesulfonamide (***N*-tosyl-3-amino-3-phenylpropanenitrile).

A yield of 50% was obtained. White powder, m.p. (lit m.p. [39])^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 8.3, 2H), 7.33 – 7.12 (m, 5H), 7.12 – 7.04 (m, 2H), 5.56 (d, *J* = 7.3, 1H), 4.53 (td, *J* = 5.6, 7.2, 1H), 2.87 (dd, *J* = 3.9, 6.4, 2H), 2.35 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.95, 137.09, 136.41, 129.73, 129.09, 128.86, 127.07, 126.20, 116.47, 54.13, 26.29, 21.51. HRMS (ESI): m/*z* 323.08 $(M+H)^+$; calc. for $C_{16}H_{16}N_2O_2$: 323.08.

General procedure for preparation of *N*-Boc derivitives

The β-amino compounds (~ 1.20 mmol) was dissolved in dioxane (2.5 ml), water (1.2 ml) and 1 M NaOH (1.2 ml). This reaction was cooled in an ice-bath and di-*tert*-butyl dicarbonate (1.1 eq., 0.29 g, 1.33 mmol) was added. The reaction was stirred at room temperature for 2 - 6 h. Half of the solvent was removed in vacuo. Ethyl acetate (10 ml) was added, forming two layers. The aqueous layer was acidified to pH 2-3 using a solution of $KHSO_4$. The aqueous layer was extracted with ethyl acetate (x 4) and the combined organic layer was washed with brine, dried over $MgSO_4$, and the solvent removed *in vacuo* to yield product.

2.5.21 tert-butyl *N*-(2-cyano-1-phenylethyl)-carbamate 9c (92% yield). White powder, m.p. (lit m.p.109-113°C [40]) ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.50 – 7.23 (m, 5H), 5.22 – 5.03 (m, 1H), 5.03 – 4.83 (m, 1H), 3.12 – 2.75 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 154.73, 138.44, 129.13, 128.64, 126.17, 117.01, 80.56, 51.23, 28.28, 25.22. HRMS (ESI): m/*z* 269.13 $(M+H)^+$; calc. for $C_{14}H_{18}N_2O_2Na$: 269.13.

2.5.22 tert-butyl N-(2-carbamoyl-1-phenylethyl)carbamate 9d (44.9% yield). Prepared by Boc group addition to 3-amino-phenyl propanamide; white crystals; ^{1}H NMR (400 MHz, DMSO) δ 7.41 – 7.34 (m, 1H), 7.31 – 7.23 (m, 5H), 7.23 – 7.15 (m, 1H), 6.80 (s, 1H), 4.88 (q, *J* = 7.5, 1H), 2.48 – 2.37 (m, 2H), 1.35 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 171.68, 154.76, 143.70, 128.15, 126.71, 126.35, 77.84, 51.45, 42.43, 28.32. HRMS (ESI): m/*z* 287.14 $(M+H)^+$; calc. for $C_{14}H_{20}N_2O_3Na$: 287.13.

3. Results and Discussion

Previous studies with β-hydroxynitriles were successful in that the *R. rhodochrous* hydrolysed the compounds to the amides and the acids [Kinfe et al 2009] [41]. The first reaction, catalysed by nitrile hydratase, was relatively rapid, but not enantioselective. The subsequent hydrolysis of the amide to the corresponding carboxylic acid was slower, but yielded β-substituted phenoxy compounds with high enantiomeric excess (up to 99% ee).

In the current study we attempted to enantioselectively hydrolyse the nitrile group of β-amino substituted nitrile compounds. Surprisingly initial biocatalytic studies using *R. rhodochrous* failed to produce any hydrolysis products using the unprotected 3-amino-3-phenyl propanenitrile. Hence we investigated the use of *N*-protected β-substituted amino derivatives, specifically *N*-tosyl, N-Boc and N-acyl 3-amino-3-phenylpropanenitrile. Again the tosyl and Boc compounds failed to provide any hydrolysed material, but the acyl compound yielded 18.9% (reaction conditions 30°C, pH 7.0, whole cell reaction, 7 days, high cell load). It is possible that the *N*-tosyl and *N*-Boc protected compounds were not hydrolysed because they were too large for the nitrile hydratase active site, while the smaller N-acyl compound was a better fit. However, based on this assumption (and previous results with the structurally similar β-hydroxy nitriles [Kinfe et al 2009] [41] the unprotected 3-amino-3-phenylpropanonitrile should fit into the active site. On this basis it was hypothesised that the lack of hydrolysis could be ascribed to protonation of the amino group at pH 7. Amino groups are protonated at pH below 9 - 10, but this pH may influence the stability of the enzyme [Martinkova et al. 2009] [42]. Hence we repeated the reactions at pH 9.0 (Tris buffer) and under these conditions hydrolysis was indeed observed. The role of the nitrile hydratase in this reaction was confirmed using purified enzyme.

Although for the parent compound (3-amino-3-phenylpropanenitrile) there was relatively insignificant enantioselectivity, enantioselectivity was observed for the derivatives with substitution on the aromatic moiety. 3-Amino-3-*p*-tolylpropanamide, 3-amino-3-(4-methoxyphenyl) propanamide, 3-amino-3-(3-bromophenyl) propanamide, and 3-amino-3-(4-bromophenyl) propanamide were all generated with an enantiometic excess (Table 1). In particular the 3-amino-3-(4-methoxyphenyl) propanamide, 3-amino-3-(4-methoxyphenyl) propanenitrile, 3-amino-3-*p*-tolylpropanitrile and 3-amino-3-*p*-tolylpropanamide could be obtained with relatively high enantiometic excess. This was more evident when the reactions were run at 5ºC than at 30ºC. This improved enantioselectivity based on decreased

temperature is opposite to that of some other enzymatic reactions reported [Ewert et al 2008, Bauer, 1998] [43, 44]. The (S)-enantiomer of the β-amino amide was preferentially generated, according to assignment based on the elution times of 3-amino-3-phenylpropanamide enantiomers. Although these enantiomeric excesses are not as high as observed with the β-hydroxyl compounds [Kinfe et al 2009] [41] it is interesting that the nitrile was enantioselectively hydrolysed, and hence the enantioselectivity resided in the nitrile hydratase. Cobalt containing nitrile hydratases, such as the enzyme expressed by this organism, have previously been demonstrated to have enantioselectively towards α-amino nitriles, while the iron containing nitrile hydtatases do not show any enantioselectivity [van Pelt et al 2011] [45]. Enzyme enantioselectivity was not absolute, and eventually complete conversion of the starting nitrile occurred with time, defining this reaction as a kinetic resolution. Although some carboxylic acid was generated by the organism (as determined by an HPLC-MS method), the yield was low.

The bioconversion products of five-membered carbocyclic nitriles were mainly the respective acids whereas, similar to the results of the current study, the carbocyclic six-membered nitriles were accumulated at the stage of the amide (Preiml et al 2004) [27]. Liang et al (2008) [25] ran a reactor for the synthesis of the achiral β-alanine from the respective nitrile, and found that conversion proceeded better at pH 7.5 than pH 6.0, although higher a pH was not tested.

The aryl methyl substituted nitrile had a maximum enantiomeric ratio (E) of 7.7 and the amide of 4.3, while the methoxy substituted nitrile had an E of 9.7 and the amide an E of 5.9 at 5°C.

Table 1. Biotransformation of racemic aromatic β-aminonitriles.

Entry	Substrate	3-amino	R	Reaction temperature °C	Conversion (%)	Amide ee (%)	Nitrile ee (%)
1	**1 c**	H	4-H	30	46	7 (S)	8 (R)
				5	41	3 (S)	11 (S)
2	**2 c**	H	4-Me	30	39	3 (S)	21 (R)
				5	61	48 (S)	19 (R)
3	**3 c**	H	4-OMe	30	62	24 (S)	39 (R)
				5	33	62 (S)	15 (R)
				5	68	37 (S)	85 (R)
5	**4 c**	H	4-Cl	30	0	ND	ND
4	**5 c**	H	4-Br	30	54.5	43.3 (S)	31.5 (R)
5	**6 c**	H	3-Br	30	32.6	22 (S)	3.6 (R)
6	**7 c**	Acyl	3-H	30	19	ND	ND
7	**8 c**	Tosyl	3-H	30	0	0	0
8	**9 c**	Boc	3-H	30	0	0	0

(R) and (S) assignments based on elution times of **3-amino-3-phenylpropanamide (1d)** enantiomers compared to the (R)-**3-amino-3-phenylpropanamide** standard.

4. Conclusion

We have shown that asymmetric hydrolysis of unprotected β-amino nitriles to the corresponding amide can be achieved with ee of product and substrate above 50% using *R. rhodochrous* ATCC BAA-870. This was achieved by the nitrile hydratase, which

demonstrated enantioselective activity for substrates where there was substitution on the β-aromatic moiety. Higher enantiomeric excess may be achievable through enzyme engineering, reaction engineering, use of different substrates, or combination of the nitrile hydratase with an amidase with complementary enantioselectivity.

Boc, tosyl, and acyl functional groups covalently attached to the primary amine group provided insights into the selectivity of the catalyst based on the electronic charge and functional group size tolerance by the enzyme active site. Both steric hindrance from bulky functional groups and an inability to convert compounds that have a charged primary amine are evident as no reaction was observed with both the Boc and tosyl functional groups, or the parent compound with a free amine, while the smaller acetylated amine and protonated amine were both accepted.

Acknowledgements

We would like to thank the DST and the CSIR Thematic Programme for Funding. This research was presented in part at the 5th International Congress on Biocatalysis, (Hamburg, Germany, August 29 - September 2, 2010) and a COST Action CM0701 workshop (Vilnius, Lithuania. 8-11 September, 2010. We would like to thank, Prof Andreas Stolz (University of Stuttgart and Action CM0701 (Cascade Biocatalysis) working group leader, Prof Paul Steenkamp for additional analysis, Henok Kinfe and Jonathan Pantshwa (CSIR) and Prof N Klempier and Dr M Winkler (University of Graz) for technical inputs and discussions. We would also like to thank Dr Daniel Visser, Dr Petrus van Zyl and Joni Frederick for fermentation to bulk up the biocatalytic biomass and enzyme purification. Chemical structures were created using Chem Draw.

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