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Enantioselective biocatalytic hydrolysis of β-aminonitriles to β-amino-amides using *Rhodococcus rhodochrous* ATCC BAA-870

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ABSTRACT

A range of β-aminonitriles (3-amino-3-phenylpropanenitrile and derivatives) were synthesised by reaction of various benzonitriles with acetonitrile and subsequent reduction of the resulting acrylonitrile products. These compounds were hydrolysed to the corresponding amides using the nitrile biocatalytic activity of *Rhodococccus rhodochrous* 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 and the corresponding amides (up to 85% in one case). The reactions were performed at pH 9.0 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 [1]. β-Amino acids are constituents of compounds such as the antitumour drug Taxol [2], the antifungal antibiotic Cispentacin [3], and the antidiabetic drug Sitagliptin [4]. Another pharmaceutical application is for inclusion in peptidomimetics that may be of use as protease inhibitors against retroviruses such as HIV [5–7].

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

There are a number of chemical synthesis methods for the preparation of racemic β^3-amino acids [13,14]. However, many applications require the β-amino substituted compounds as single enantiomers [1], and racemates thus need to be resolved. The classical method of resolving amino acids is through transformation of the racemate into a mixture of 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 [13] using lipases like CAL-B and the protease α-chymotrypsin; or by transesterification using lipase or acylase I [13]. Pousset et al. [15] demonstrated the application of *Burkholderia cepacia* lipase for hydrolytic resolution of heterocyclic β-amino acids, achieving greater than 99% ee. Tasnádi et al. [16–18] achieved ee values 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 *B. cepacia* lipase (Amano PS) to hydrolyse the *S*-enantiomer. Li and Kanerva [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 and other enzymes [13]. Alternatively Forró et al. [20] and Tasnádi et al. [21] demonstrated enantioselective ring opening of β-lactams to provide β-amino acids with ee values of 99% using the lipase CAL-B. Aspartase can form β-amino acids through Michael addition. Hydantoinase hydrolysis of hydantoins, reductive amination by aminotransferase, and isomerisation by 2,3-aminomutase add

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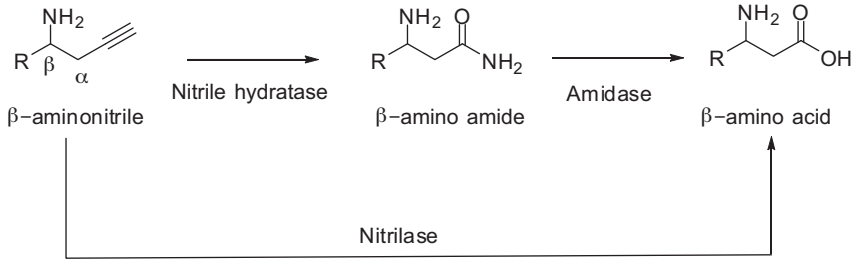


Scheme 1. Biocatalytic conversion of β-aminonitriles to β-amino amides and acids.

yet more options [13]. Recently phenylalanine aminomutase (PAM) has been applied in the enzymatic synthesis of (*R*)-β-arylalanines with high ee (>99%) [22].

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 [23]. β-Alanine, the only naturally occurring beta amino acid, can be derived from β-aminopropanenitrile using whole cell biocatalysts *Alcaligenes* sp. OMT-MY14, *Aminobacter* sp ATCC 23314 [24] and *Rhodococcus* sp [25]. β-Alanine is an achiral substrate, however nitrile and amide biocatalysts may act enantioselectively on chiral substrates, and this has been successfully applied to the resolution of *N*-protected cyclic β-amino acids [26–28]. Veitía et al. [29] demonstrated synthesis of *N*-protected β^3-amino acids using the Codexis range of nitrilases to hydrolyse the single enantiomer nitrile precursors. Biotransformation of alicyclic *N*-*p*-toluenesulfonyl- and *N*-butyloxycarbonyl protected β-aminonitriles to the *trans* amides and carboxylic acids using two strains of *Rhodococcus* (*Rhodococcus* sp. R312 and *R. erythropolis* NCIMB 11540) has been demonstrated [27]. With *Rhodococcus erythropolis* AJ270 cells 96.4% ee was achieved where the *N*-protected β-amino acid β-substituent was a c-Pr group [30].

β-Aminonitriles themselves can be prepared by a number of methods. Preiml et al. [26] used cyanide mediated ring opening of aziridines. González et al. [31] used a Mannich-type reaction to synthesise enantio-enriched (*R*)-unsubstituted β-aminonitriles through the organocatalytic addition of β-phenylsulfonylacetonitrile to either *N*-Boc-protected (*R*)-amido sulfones or imines.

Herein we synthesise various aryl substituted unprotected 3-amino-3-phenylpropanenitriles and demonstrate the enantioselective hydrolysis of these β-aminonitriles to the corresponding amides using *Rhodococcus rhodochrous* ATCC BAA-870, a nitrile hydrolysing organism isolated from soil [32,33], that expresses a benzamide induced cobalt type nitrile hydratase.

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 λ = 254 nm and then visually by colour development after treatment with ninhydrin spray. Gravity column chromatography was carried out on Merck silica gel 60 (70–230 mesh) 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.

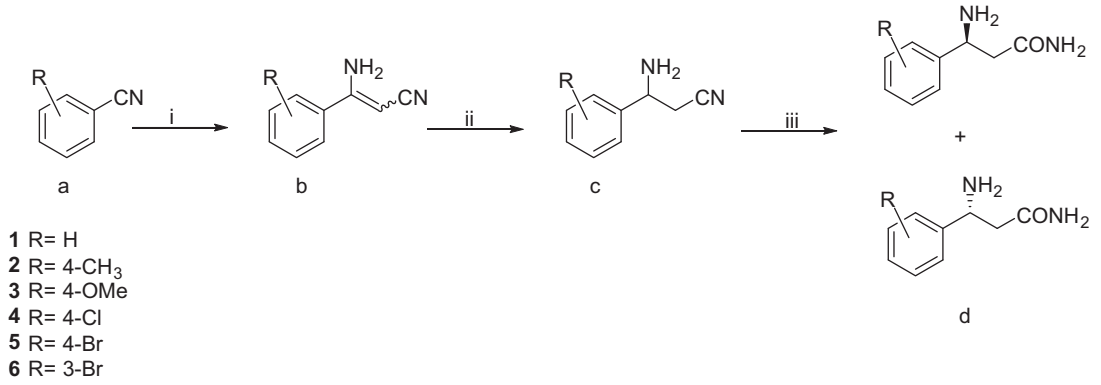
Nitrile and amide analysis was performed using a liquid chomatography (HPLC) system composed of a Waters 2690 separation module coupled with Waters diode array detector 996 (210–400 nm), a Waters X-Terra MS18 3.5 μm, 3.0 mm × 50 mm (ID × L) column (25 °C), with the isocratic eluent of 0.1% (v/v) trifluoroacetic acid in ultrapure water combined with acetonitrile (composition was varied according to the compound analysed) at a flow rate of 0.3–0.5 ml/min. The run time was 15–20.0 min, and 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 Autosampler, Waters 2486 UV/Visible Detector (210 nm) and Empower 2 software. Columns used were a Chiralpack AD-H, 250 mm × 4.6 mm, 5 μm and a Chiracel OD-H, 250 mm × 4.6 mm, 5 μm (Daicel Chemical Industries Ltd.). The eluent was n-hexane: isopropanol (both HPLC Grade) 90:10, % v/v and an isocratic flow rate of 1.00 ml/min at 25 °C with a run time of 35 min. The chromatographic system was conditioned for 1 h before the injection of samples. 3-Amino-3-phenylpropanenitrile (**1c**) enantiomers were eluted under these conditions at 12 and 13.6 min; 3-amino-3-*p*-tolylpropanenitrile (**2c**) at 11.0 and 12.7 min; 3-amino-3-(4-methoxyphenyl)propanenitrile (**3c**) at 19.0 and 23.0 min; 3-amino-3-(4-chlorophenyl)propanenitrile (**4c**) at 9.0 and 12.5 min; 3-amino-3-(4-bromophenyl)propanenitrile (**5c**) at 19.0 and 29.0 min; and 3-amino-3-(3-bromophenyl)propanenitrile (**6c**) at 19.0 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 mm × 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 min. The chromatographic system was conditioned for at least 1 h in advance with a column flow rate of 0.25 ml/min before the injection of samples. 3-Amino-3-phenylpropanamide (**1d**) enantiomers eluted at 10.9 and 12.9 min (210 nm); 3-amino-3-*p*-tolylpropanamide (**2d**) at 24.5 and 30.6 min (210 nm); 3-amino-3-(4-methoxy)phenylpropanamide (**3d**) at 20.0 and 23.4 min (225 nm), 3-amino-3-(4-bromophenyl)propanamide (**5d**) at 22.9 and 28.5 min (230 nm); and 3-amino-3-(3-bromophenyl)propanamide (**6d**) at 13.8 and 28.1 min (230 nm).

Uncorrected melting points were determined using a Reichert-Jung Thermovar hot-stage microscope or a Mettler FP62 capillary melting point apparatus.

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 spectrometer as well as a 200 MHz spectrometer 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.



Scheme 2. Synthesis of β-amino amides. Reagents *i*. 2 equiv. CH_3CN, 4 equiv. KO^tBu, 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 °C or 30 °C.

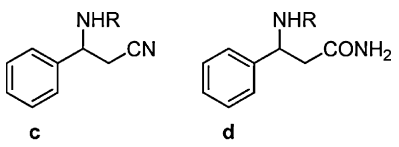
2.2. Overall synthetic route for preparation of β-amino compounds

The preparation of unprotected β-amino-amides (Scheme 2) was achieved by reacting benzonitrile (**1a**), or substituted derivatives (**2a**–**6a**) with acetonitrile and potassium *tert*-butoxide (KO^tBu) as described by de Paulis et al. [34]. The resulting 3-amino-3-phenylacrylonitrile (**1b)** or substituted derivatives (**2b**–**6b**) were then reduced with $NaBH_3CN$ [35] to yield the corresponding 3-amino-3-phenylpropanenitriles (**1c**–**6c**). These β-aminonitriles were subsequently hydrolysed to the amides (**1d**–**6d**) by the *R. rhodochrous* ATCC BAA-870 biocatalyst. Reaction progression was monitored by TLC and HPLC. After purification compounds were analysed by ^1H 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 prior to analysis using the method of Kumar et al. [36].

The *N*-Boc and *N*-tosyl derivatives of **1c** were also prepared by standard methods in order to compare their activity with that of the unprotected substrate towards the biocatalyst (Fig. 1).

2.3. Microorganism and cell cultivation

Strain ATCC BAA-870 was first cultured in a rich medium (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, $CaCl_2{\cdot}2H_2O$ 0.05 g/l, $FeSO_4.7H_2O$ 0.09 g/l, $MgSO_4$ 0.02 g/l and 1 ml trace element solution, as previously described [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).



7 R=acetyl
8 R= *p*-toluensulfonyl
9 R= Boc

Fig. 1. *N*-protected β-aminonitriles.

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-phenylpropanenitrile substrate (solubilised in 1 ml methanol) in 100 mM Tris buffer (either at pH 7 or at pH 9) to make the reaction volume up to 10 ml. The resulting mixture was then stirred at either 5 °C or 30 °C with amide generation being monitored by TLC analysis. The reactions were stopped by centrifugation at 10 000 rpm × 15 min with the supernatant being decanted and extracted using three volumes of ethyl acetate. The cell pellet was also washed with 5 ml of ethyl acetate. 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 column chromatography using varied ratios of hexane/ethyl acetate or methanol/DCM solvent systems. Sample analysis was by HPLC.

2.5. General synthetic procedures

2.5.1. 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.

*2.5.1.1. 3-Amino-3-phenylacrylonitrile **1b** (66% yield).* White crystals, m.p. 82.9 °C (lit m.p. 81–84 °C [37]); ^1H 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 [38]). HRMS (ESI): m/z 145.0785 $(M+H)^+$; calc. for $C_9H_{19}N_2$: 145.0766.

*2.5.1.2. 3-Amino-3-p-tolylacrylonitrile **2b** (50% yield).* Yellow crystals, m.p. 103.5 °C (lit m.p. 107–110 °C [39]); ^1H 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 [38]). HRMS (ESI): m/z 159.0896 $(M+H)^+$; calc. for $C_{10}H_{11}N_2$: 159.0922.

2.5.1.3. 3-Amino-3-(4-methoxyphenyl)acrylonitrile 3b. (36% yield). Yellow–brown crystals, m.p. 119.4 °C (lit m.p. 117–118 °C [39]); ^{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 [38,40]). HRMS (ESI): m/z 175.0880 $(M+H)^+$; calc. for $C_{10}H_{11}N_2O$: 175.0871.

2.5.1.4. 3-Amino-3-(4-chlorophenyl)acrylonitrile 4b (56% yield). Beige powder, m.p. 124.8 °C (lit m.p. 140–144 °C [40]); ^{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 [38]). HRMS (ESI): m/z 179.0357 $(M+H)^+$; calc. for $C_9H_8N_2Cl$: 179.0376.

2.5.1.5. 3-Amino-3-(4-bromophenyl)acrylonitrile 5b (42% yield). Yellow powder, m.p. 135 °C (lit m.p.147–148 °C [37]); ^{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 [38]). HRMS (ESI): m/z 222.9867 $(M+H)^+$; calc. for $C_9H_8N_2Br$: 222.9871.

2.5.1.6. 3-Amino-3-(3-bromophenyl)acrylonitrile 6b (33% yield). Yellow powder; ^{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 222.9822 $(M+H)^+$; calc. for $C_9H_8N_2Br$: 222.9871.

*2.5.2. General method for preparation of 3-amino-3-phenylpropanenitriles (compounds **1c–6c**) exemplified by **1c***

3-Amino-3-phenylacrylonitrile (**1b**) (3.00 g, 20.8 mmol) was 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 (×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 a colourless oil.

*2.5.2.1. 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 147.0909 $(M+H)^+$; calc. for $C_9H_{11}N_2$: 147.0922.

*2.5.2.2. 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 161.1083 $(M+H)^+$; calc. for $C_{10}H_{13}N_2$: 161.1079.

*2.5.2.3. 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 177.1027 $(M+H)^+$; calc. for $C_{10}H_{13}N_2O$: 177.1028.

*2.5.2.4. 3-Amino-3-(4-chlorophenyl)propanenitrile **4c** (52% yield).* Clear oil; ^{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 181.0522 $(M+H)^+$; calc. for $C_9H_{10}N_2Cl$: 181.0533.

2.5.2.5. 3-Amino-3-(4-bromophenyl)propanenitrile 5c. (26% 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 $C_9H_{10}N_2Br$: 225.0027.

*2.5.2.6. 3-Amino-3-(3-bromophenyl)propanenitrile **6c** (40% yield).* Yellow oil; ^{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 225.0007 $(M+H)^+$; calc. for $C_9H_{10}N_2Br$: 225.0027.

*2.5.3. General method for biotransformation of β-aminonitriles to β-amino-amides (compounds **1d–6d**)*

3-Amino-3-phenylpropanenitrile (100 mg) was incubated with resting cells of *R. 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 h).

*2.5.3.1. 3-Amino-3-phenylpropanamide **1d** (23% yield).* White powder; m.p. 110.1 °C; ^{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 165.1042 $(M+H)^+$; calc. for $C_9H_{13}N_2O$: 165.1028.

*2.5.3.2. 3-Amino-3-p-tolylpropanamide **2d** (22% yield).* Yellow powder; ^{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 179.1287 $(M+H)^+$; calc. for $C_{10}H_{14}N_2O_2Na$: 179.1284.

*2.5.3.3. 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 217.0926 $(M+H)^+$; calc. for $C_{10}H_{14}N_2O_2Na$: 217.0953.

*2.5.3.4. 3-Amino-3-(4-bromophenyl)propanamide **5d** (28% 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 243.0150 $(M+H)^+$; calc. for $C_9H_{12}N_2OBr$: 243.0133.

2.5.3.5. 3-Amino-3-(3-bromophenyl)propanamide ***6d*** *(6% 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* 243.0140 $(M+H)^+$; calc. for $C_9H_{12}N_2OBr$: 243.0133.

2.5.4. Procedure for N-acetylation of β-amino compounds

To the 3-amino substituted compound (1.23 mmol solubilised in 5 ml DCM), was added 5 equiv. acetic anhydride (6.13 mmol), 4 equiv. of pyridine (4.9 mmol) and a catalytic quantity of DMAP (~1 mg). This was stirred at room temperature and monitored by TLC. After conversion, as evidenced by TLC, the reaction mixture was washed with 1 M HCl, followed by 1 M NaOH. The organic layer was washed with brine and dried over $MgSO_4$ and the solvent removed *in vacuo*. The product was purified by column chromatography.

2.5.4.1. N*-(2-cyano-1-phenylethyl)acetamide* ***7c*** *(60% yield) cream crystals.* ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.55–7.06 (*m*, 5H), 6.98 (s, 1H), 5.30–4.87 (*m*, 1H), 2.98–2.58 (*m*, 2H), 1.85 (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* 187.0871 $(M+H)^+$; calc. for $C_{11}H_{11}N_2O$: 187.0871.

2.5.4.2. 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* 229.0895 $(M+H)^+$; calc. for $C_{11}H_{14}N_2O_2Na$: 229.0953.

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

3-Amino-3-phenylpropanenitrile (**1c**) (1.62 g, 0.011 mol) was dissolved in dichloromethane (50 ml) and 4-dimethylaminopyridine (1.3 equiv., 1.76 g, 0.014 mol) and *p*-toluenesulfonyl chloride (1.2 equiv., 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 **8c** (1.66 g, 50%). White powder, ^{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* 323.0811 $(M+H)^+$; calc. for $C_{16}H_{16}N_2O_2$: 323.0830.

2.5.6. General procedure for preparation of N-Boc derivatives

The β-amino compound (~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 equiv., 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 (×4) and the combined organic layer was washed with brine, dried over $MgSO_4$, and the solvent removed *in vacuo* to yield product.

2.5.6.1. tert-Butyl N*-(2-cyano-1-phenylethyl)-carbamate* ***9c*** *(92% yield).* White powder, ^{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* 269.1261 $(M+H)^+$; calc. for $C_{14}H_{18}N_2O_2Na$: 269.1266.

2.5.6.2. tert-Butyl N*-(3-amino-3-oxo-1-phenyl-propyl)carbamate* ***9d*** *(45% yield).* 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* 287.1349 $(M+H)^+$; calc. for $C_{14}H_{20}N_2O_3Na$: 287.1372.

3. Results and discussion

Previous studies with unprotected β-hydroxynitriles showed that these compounds were hydrolysed by *R. rhodochrous* ATCC BAA-870 to the corresponding amides and acids [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-phenylpropanenitrile. Hence we investigated the use of *N*-protected β-substituted amino derivatives, specifically *N*-acetyl (**7c**), *N*-tosyl (**8c**) and *N*-Boc (**9c**) 3-amino-3-phenylpropanenitrile (Fig. 1). The conversion of the tosyl and Boc compounds was negligible, an observation also made by Preiml et al. [26] when investigating the hydrolysis of **8c** using other *Rhodococcus* strains as nitrile biocatalysts.

However, the acetyl compound yielded a modest 19% of the corresponding amide (reaction conditions 30 °C, pH 7.0, whole cell reaction, 7 days, high cell load) showing that this class of compound may be amenable to nitrile hydratase catalysed hydrolysis. 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*-acetyl compound was a better fit. However, based on this assumption (and previous results with the structurally similar β-hydroxynitriles [41]) the unprotected 3-amino-3-phenylpropanenitrile should fit into the active site. Therefore it was hypothesised that the lack of hydrolysis could be ascribed to protonation of the amino group at pH 7, resulting in a positive charge. Amino groups are protonated at pH below 9–10, but a pH > 10 could result in enzyme inactivation [42]. Hence we repeated the reactions at the intermediate pH 9.0 (Tris buffer) where there is a significant proportion of the unprotonated species. Under these conditions hydrolysis was indeed observed, and eventually **1c** was completely converted to **1d**. Confirmation that the reaction was catalysed by nitrile hydratase was obtained by use of purified enzyme in the reaction instead of whole cells.

Although we had previously seen conversion of β-hydroxynitriles to the corresponding carboxylic acids [41], in the current study of unprotected aliphatic β-aminonitriles the amides were predominantly generated and the carboxylic acid yield was low (as determined by HPLC-MS analysis). The same result was observed by Preiml et al. for *N*-protected six-membered carbocyclic β-aminonitriles which were accumulated during *Rhodococcus* catalysed hydrolysis [27], even though the structurally related five-membered carbocyclic nitriles were converted mainly to the respective acids. This implies a difference in the substrate profiles of the nitrile hydratase and the amidase.

Table 1
Biotransformation of racemic aromatic β-aminonitriles.

Entry	Substrate	Aryl substitution	Reaction temperature (°C)	Conversion (%)	(*R*)-Nitrile % ee	(*S*)-Amide %ee	Enantiomeric ratio (E) nitrile amide	
1	**1c**	4-H	30	46	8	7	1.3	1.2
2	**2c**	4-Me	30	39	21	3	2.4	1.1
3	**2c**	4-Me	5	61	19	48	1.5	6.1
4	**3c**	4-OMe	30	62	39	24	2.3	2.3
5	**3c**	4-OMe	5[a]	33	15	62	2.2	5.7
6	**3c**	4-OMe	5[a]	68	85	37	5.8	4.8
7	**4c**	4-Cl	30	0	0	0	–	–
8	**5c**	4-Br	30	55	32	43	2.3	4.1
9	**6c**	3-Br	30	33	4	22	1.2	1.7

[a] At 2 and 24 h, entries 5 and 6, respectively.

In the current study of the parent compound **1c** there was relatively insignificant enantioselectivity (Table 1), however enantioselectivity was observed for the derivatives with substitution on the aromatic moiety, an exception being the chloro-substituted nitrile (**4c**) that did not yield the expected amide product. Compounds **2d**, **3d**, **5d** and **6d** were all generated with an enantiomeric excess (Table 1). In particular the amide **3d** and the nitrile **3c** could be obtained with relatively high enantiometic excess. This was more evident when the reactions were run at 5 °C than at 30 °C. This improved enantioselectivity based on decreased temperature is opposite to that of some other enzymatic reactions [43,44]. The (*S*)-enantiomer of the β-amino-amide was preferentially generated, according to assignment based on the elution times of the 3-amino-3-phenylpropanamide enantiomer standards. This observation was supported by the positive optical rotation value demonstrated by *N*-Boc derivatised 3-amino-3-*p*-tolylpropanenitrile remaining after biocatalytic conversion of 3-amino-3-*p*-tolylpropanenitrile, which according to González et al. [31] corresponds to the (*R*)-enantiomer. Although these enantiomeric excesses are not as high as observed with the β-hydroxyl compounds [41] it is interesting that, contrary to the case of β-hydroxynitrile compounds here the β-aminonitriles were enantioselectively hydrolysed to amides, and hence the enantioselectivity resided in the nitrile hydratase and not the amidase. Cobalt containing nitrile hydratases, such as the enzyme expressed by this organism, have previously been demonstrated to have enantioselectively towards α-aminonitriles, while the iron containing nitrile hydratases do not show any enantioselectivity [45].

Enzyme enantioselectivity was not absolute, and eventually complete conversion of the starting nitrile occurred with time, defining this reaction as a kinetic resolution. The *p*-tolyl (**2**) and the 4-methoxy derivatives (**3**) had the highest enantiomeric ratios (*E*) at 5 °C (Table 1). It also appears that *para* substitution of the bromine (**5c**) on the aromatic ring provided better enantioselectivity when compared to the *meta* position (**6c**) (Table 1). The stereoselective biocatalytic conversion of unprotected chiral aliphatic β-aminonitriles to the corresponding amides demonstrated here seems to be unprecedented.

4. Conclusion

We have shown that asymmetric hydrolysis of unprotected β-aminonitriles to the corresponding amides can be achieved with ee values 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 excesses may be achievable through enzyme engineering, reaction engineering, use of different substrates, or combination of the nitrile hydratase with an amidase with complementary enantioselectivity.

N-protection of the β-aminonitriles with Boc, tosyl, and acetyl groups provided insights into the selectivity of the nitrile hydratase active site. Both steric hindrance from bulky groups (Boc or tosyl) and an inability to convert compounds with a charged primary amine are suggested as no reaction was observed in any of these examples, while the smaller acetylated amine and unprotonated amine were both accepted.

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