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Microbial deracemisation of *N*-(1-hydroxy-1-phenylethyl)benzamide

Gareth J. Cardus,[a] Andrew J. Carnell,[a,*] Harald Trauthwein[b] and Thomas Riermeir[b]

[a]*Department of Chemistry, Robert Robinson Laboratories, University of Liverpool, Liverpool L69 7ZD, UK*
[b]*Degussa AG—Project House Catalysis, Industriepark Höchst st. G 830, Frankfurt am Main D-65926, Germany*

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Abstract—Microbial deracemisation of racemic *N*-(1-hydroxy-1-phenylethyl)benzamide to give the (*R*)-enantiomer is described using whole cells of *Cunninghamella echinulata* NRRL 1384. The deracemisation involves fast highly (*S*)-selective oxidation followed by slower partially (*S*)-selective reduction of the intermediate ketone. The yield and ee of (*R*)-*N*-(1-hydroxy-1-phenylethyl)benzamide were optimised (82%, 98% ee) by removal of a competing extracellular amidase/protease activity by using static cells at pH 5. Use of the protease inhibitor PMSF with growing cells leads to a slower deracemisation (82% ee) but a very high (98%) overall recovery of alcohol and ketone.

1. Introduction

Chiral amino alcohols are widely used in synthesis as ligands for asymmetric catalysis and as chiral auxiliaries and are important structural elements in many pharmaceuticals such as β-blockers and HIV inhibitors.[1–3] Homochiral 2-amino-1-alkanols are easily obtained by the reduction of the corresponding amino acids.[4] However, the regioisomeric 1-amino-2-alkanols are not as easy to access in enantiomerically pure form. A number of approaches to their precursors have been developed including a catalytic asymmetric nitro-aldol reaction using rare earth Binol complexes[5] and chiral hydrocyanation[6,7] of aldehydes leading to optically active cyanohydrins. Chiral amino alcohols can be synthesised by ring opening of cyclic ester derivatives of chiral diols derived from Sharpless ADH reactions and more directly using the aminohydroxylation,[8–10] asymmetric hydrogenation of amino ketones using chiral ruthenium complexes[11] and the highly regio- and enantioselective ring opening of terminal epoxides using $TMSN_3$ and a (salen)Cr complex.[12,13]

In cases where the above methods produce materials of low ee or where the chiral reagent or catalyst ligands are prohibitively expensive, a method for deracemisation of the racemic or enriched amino alcohol or its synthetic precursor would be advantageous. We and others have previously reported the use of whole cell microorganisms[14–17] to catalyse the deracemisation of secondary alcohols and diols. The cells contain redox (dehydrogenase) enzymes that carry out the oxidation and reduction steps and the cofactors are recycled internally. It can be speculated that the driving force for such deracemisations derives from the cellular compartmentalisation of the enzymes catalysing each step. In addition only one of these steps needs to be enantioselective for a successful deracemisation. It is also possible to carry out dynamic kinetic resolutions of secondary alcohols using a lipase in combination with either a transition metal catalyst[18] or a racemase.[19]

2. Results and discussion

In order to extend our whole cell methodology to the more synthetically useful amino alcohols, we screened a number of microorganisms for the ability to deracemise the model substrate amidoalcohol **1**. This substrate was chosen in preference to the free amino alcohol since we envisaged that the solubility of the latter in aqueous buffer would prohibit efficient extraction from the biotransformation media. From 12 strains of filamentous fungi initially screened, one strain, *Cunninghamella echinulata* NRRL 1384 gave a promising deracemisation

* Corresponding author. Tel.: +44-151-794-3531; fax: +44-151-794-3588; e-mail: acarnell@liv.ac.uk

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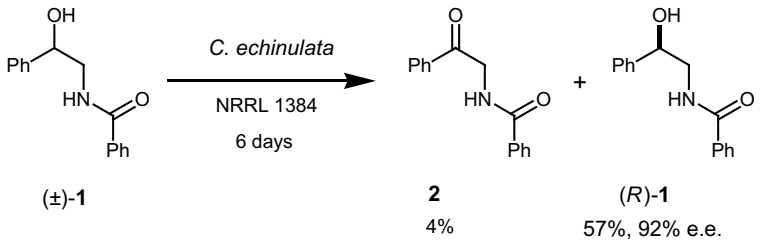


Scheme 1.

affording (*R*)-amidoalcohol **1** in 57% isolated yield and 92% ee (Scheme 1).

A small amount of the ketone **2** (ca. 4%) was also detected in the reaction mixture, indicating that the deracemisation is catalysed by redox enzymes. The absolute configuration of the product was determined by comparison (chiral HPLC) of a sample of the (*R*)-configured product prepared from commercially available amino alcohol (*R*)-2-amino-1-phenylethanol. When the (*R*)-amidoalcohol **1** was incubated as the substrate it showed no conversion to the ketone **2** or the (*S*)-amidoalcohol **1** after 6 days, suggesting that the deracemisation involves (*S*)-enantioselective oxidation of the racemic substrate. In this case, the recovered yield (72%) of (*R*)-**1** was substantially higher than when racemic-**1** was used (57%). This suggests that (*S*)-**1** or the ketone **2** are being preferentially depleted by another competing process, either spontaneous or enzymatic. Incubation of the ketone **2** showed an unexpected reaction profile (Table 1).

Table 1. Biotransformation of ketone **2**

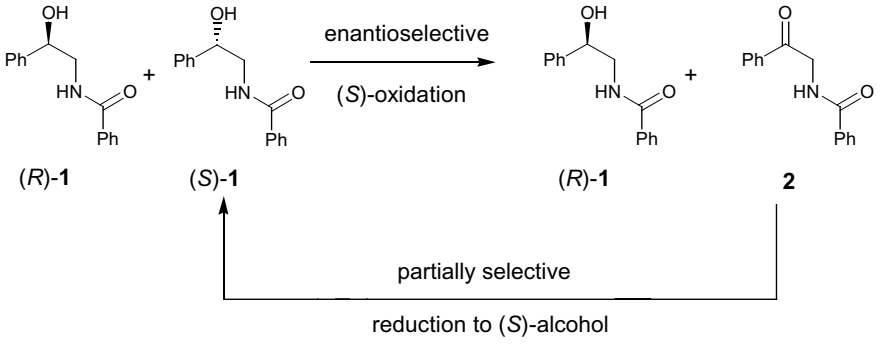
Reaction time (days)	Amidoalcohol **1**		Ketone **2**
	% Ee (conf.)	% Yield	% Yield
2	26 (*S*)	17	8
4	58 (*R*)	17	9
6	72 (*R*)	27	7

Microorganisms were grown in 50 cm^3 of liquid nutrient media (2% glucose, 2% corn steep liquors) in 250 cm^3 flasks at 28 °C in an orbital incubator shaker at 200 rpm. Ketone **2** was added at a concentration of 0.5 mg/cm^3 in 0.5 cm^3 of DMF, after 2 days of growth. Chiral HPLC, Chiralcel OJ column, eluent IPA–*n*-hexane 0.8:9.2, flow rate 1 cm^3/min, wavelength 210 nm, retention time for each enantiomer of amidoalcohol **1** 13.2 and 15.1 min.

After 2 days the ketone **2** was reduced with slight selectivity (26% ee) in favour of formation of the (*S*)-amidoalcohol **1**. However after 4 and 6 days of biotransformation the amidoalcohol **1** became increasingly enriched in the (*R*)-enantiomer. The overall yields of amidoalcohol **1** and ketone **2** were particularly low, again indicating the presence of a competing degradation process. These results collectively suggest that one enzyme carries out enantioselective oxidation of the (*S*)-amidoalcohol **1** to the ketone **2**, leaving the (*R*)-amidoalcohol **1** untouched. A second enzyme reduces the ketone **2** with partial selectivity to the (*S*)-amidoketone-**1**. However because the oxidation step is highly selective the overall result, after several cycles, is a deracemisation (Scheme 2).

In order to optimise the yield and ee of the alcohol from this biotransformation and gain further insight into the mechanism, we examined the reaction in greater detail. A time-course study (Fig. 1) revealed that mass balance was indeed poor, with only ca. 40% material remaining after 120 h. This could result from retention by the biomass, metabolism by the cells or bioconversion by an extracellular enzyme.

During the first 32 h of the biotransformation there is a slow decrease in the concentration of (*R*)-amidoalcohol **1** with a much faster decrease in the amount of (*S*)-**1** concomitant with a rise in the amount of ketone **2**. The ketone level then falls off after 32 h, with a sharp rise in the level of (*R*)-**1**. These results suggest a competing process that consumes the (*S*)-amidoalcohol **1** enantioselectively and/or the ketone **2**. The addition of glucose



Scheme 2.

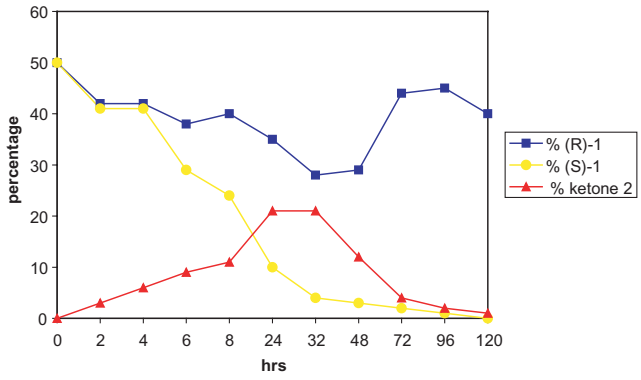


Figure 1. Time course study for deracemisation of (±)-**1** with *C. echinulata*.

Table 2. Glucose addition study showing the affect of addition of glucose (2 g/L) throughout the biotransformation of (±)-**1**

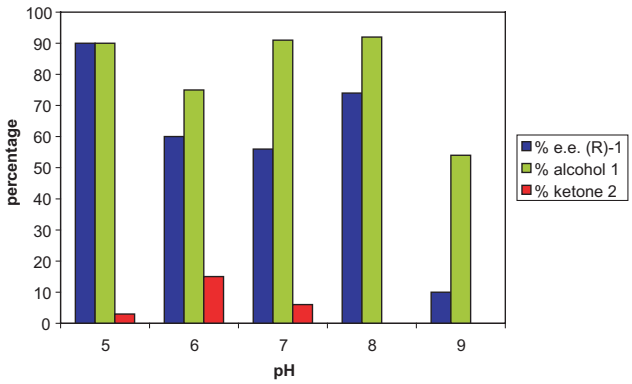
Glucose addition points (days)	% Ee (*R*)-**1**	% Alcohol **1**	% Ketone **2**
0, 2, 4	44	76	10
2, 4	74	51	1
4	100	43	1

Reactions were run over 6 days according to Section 4.5 of the experimental with the addition of glucose supplements as shown in Col-

to fermentations can provide additional reducing equivalents, leading to improved kinetics in bioreductions. We reasoned that, if the reduction component of the deracemisation is rate limiting and facilitates a competing process, additional reducing equivalents might speed up the reduction thus reducing loss of material. We therefore investigated the effect of supplementing the bioconversion with glucose (2 g/L) at various points throughout the reaction (Table 2). All reactions were run for 6 days and glucose supplements were added at 0, 2 and 4 days, 2 and 4 days or at 4 days only.

The results from this group of biotransformations show a simple trend. The addition of glucose at regular intervals (0, 2 and 4 days) throughout the biotransformation results in a dramatic decrease in the enantiomeric excess observed for (*R*)-amidoalcohol **1**. However the more glucose addition points there are, the higher the yield of recovered alcohol. These findings provide additional evidence that the reductase activity is selective for formation of the (*S*)-amidoalcohol **1** and when provided with more reducing equivalents, it is able to over-ride the effect of (*S*)-selective oxidation and the competing process that is responsible for loss of material. The results also point to the ketone being the main substrate for further metabolism. As would be predicted, incubation of the ketone **2** with glucose supplementation at 0, 2 and 4 days gave the (*R*)-amidoalcohol **1** in higher yield (34% vs 27%) but lower ee (12% vs 72%) (when compared to the same reaction without glucose supplements).

Table 3. Resting culture biotransformations of (±)-**1** at various pH's



Reactions were carried out according to Section 4.5 of the Experimental over 6 days using resuspended cells in the appropriate pH phosphate buffer (100 mM).

With the possibility of improving product recovery and to examine the effect of pH, we also used resting cultures *C. echinulata* for the biotransformation. The cells were spun down from the normal growth media (pH 5) and resuspended in 100 mM phosphate buffer at a range of pH's (5–9) (Table 3). Surprisingly the results show that *C. echinulata* 1384 has a broad tolerance to pH. The yields of amidoalcohol **1** from all the biotransformations, with the exception of pH 9, were significantly increased when compared to the growing cell biotransformations. However the result at pH 5 was the most striking, with yields of 90% alcohol **1** (90% ee) and 3% ketone **2**. This result is significant as it confirms that there is extracellular activity in the supernatant of the normal growing cell biotransformation that is responsible for the lower yields observed. The resting culture screening was carried out on 25 mg of substrate (0.5 mg/mL). Subsequently the resting culture biotransformation at pH 5 was scaled-up to 200 mg of substrate to afford an increased 98% ee and an isolated yield of 82% for the amidoalcohol **1**. The slight decrease in yield when compared to the small scale biotransformations can be attributed to isolation and purification procedures.

Obviously there is a limitation in scale for use of resting cells in this type of biotransformation, since cofactor regeneration is limited. We therefore investigated the possibility of inhibiting the competing activity in the growing cell biotransformations. We strongly suspected the presence of an amidase/protease in the supernatant, which may cleave the amide bond of the ketone **2** or the amidoalcohol **1** leading to the more polar amino alcohol, which would not extract into organic solvent. We therefore ran the biotransformation in the presence of the known protease inhibitor phenylmethylsulfonyl fluoride (PMSF). This inhibitor has a half-life of approximately 5 h so needed to be added to the reaction daily. In the presence of this inhibitor, the biotransformation gave excellent yields of the amidoalcohol **1** (86%) and ketone **2** (12%) (overall recovery 98%) and an 86% ee for the amidoalcohol after 5 days. In the absence of PMSF, the amidase/protease may be selective for the

(*S*)-amidoalcohol, thereby making the apparent rate of deracemisation faster. However, when PMSF is used, the competing enzyme is suppressed, thereby increasing the total alcohol yield, but decreasing the enantiomeric excess.

3. Summary

In summary, we have shown that whole cells of the fungus *C. echinulata* NRRL 1384 can deracemise the model substrate amidoalcohol **1** by sequential (*S*)-selective oxidation and (*S*)-selective reduction. The first oxidation step appears to be highly selective and relatively fast. The second reduction step is slower and only partially selective, allowing high levels of the (*R*)-amidoalcohol to accumulate over the course of the biotransformation. Supplementation of the bioconversion with glucose gives higher yields but lower ee for the (*R*)-amidoalcohol. Yield and selectivity can be further improved using static cells over the pH range 5–8, with the optimum pH being pH 5 giving the (*R*)-amidoalcohol **1** in 82%, 98% ee. An extracellular amidase/protease enzyme is likely to be responsible for reducing the recovered yields of amidoalcohol and ketone in the growing cell reactions and can be suppressed using the protease inhibitor PMSF, leading to a slower deracemisation but a very high overall recovery (98%) and a reasonable 86% ee for the amidoalcohol **1**.

We are currently extending the biotransformation for deracemisation of a range of related amidoalcohols and exploring the possibility of replacing the (*S*)-selective dehydrogenase with an enzyme of opposite selectivity using recombinant methods in order to accelerate the deracemisation.

4. Experimental

4.1. General

Elemental microanalyses were performed using a Carlo Erba module 1106 elemental analyser. NMR spectra were recorded on a Gemini-300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) and a Gemini-400 (^{1}H, 400 MHz; ^{13}C, 75 MHz) spectrometer. Chemical shifts are described in parts per million downfield shift from $SiMe_4$ and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, sep = septet, m = multiplet and br = broad), coupling constant (*J*, Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). Infra-red spectra were recorded on a Perkin–Elmer 1710 FT-IR spectrometer in the range of 4000–600 cm^{-1}. The samples were prepared as either Nujol mulls, or neat, between sodium chloride discs. The frequencies (ν) as absorption maxima are given in wave numbers (cm^{-1}) relative to a polystyrene standard. Mass spectra and accurate mass measurements were recorded on a VG 70-070E, or a TRIO 1000. Major fragments were given as percentages of the base peak intensity (100%). Melting points were taken on a Gallenkamp melting point apparatus and are uncorrected. HPLC analysis was performed on a Waters 2690 separations module equipped with a Chiralcel OJ column. Samples were recorded using a Waters 996 photodiode array detector. Integration was performed using Waters Millenium^{32} software. All reagents were purchased from Aldrich, Lancaster and Fluka chemical companies and were used without purification. Flash column chromatography was performed using Sorbsil C 60 (40–60 μm mesh) silica gel. Analytical thin layer chromatography (TLC) was carried out on 0.25 mm pre-coated silica gel plates (Macherey–Nagel SIL g/UV$_{254}$) and compounds were visualised using UV fluorescence or permanganate. DCM was distilled from CaH_2 and diethyl ether and THF from sodium/benzophenone. Light petrol ether refers to that portion boiling between 40 and 60 °C.

4.2. (±)-*N*-(1-Hydroxy-1-phenylethyl)benzamide 1[20]

To a stirred solution of racemic 2-amino-1-phenylethanol (2.0 g, 14.60 mmol) in 1 M sodium hydroxide (50 cm^3) was added benzoyl chloride (2.1 cm^3, 18.09 mmol). The solution was stirred at rt for 3 h. whereupon the sticky residue was extracted into ethyl acetate (2 × 30 cm^3) and washed with 1 M hydrochloric acid (2 × 40 cm^3), water (2 × 40 cm^3), saturated aqueous sodium hydrogen carbonate (2 × 40 cm^3) and water (2 × 40 cm^3). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Purification by recrystallisation from ethyl acetate–petrol gave racemic benzamide **1** (2.52 g, 71%) as a white solid; R_f (ethyl acetate, 100%) 0.72; mp 143–145 °C (lit.[21] 146–147 °C); Found: C, 74.5; H, 6.2; N, 5.7. $C_{15}H_{15}NO_2$ requires C, 74.7; H, 6.2; N, 5.8%; ν_{max} (Nujol) cm^{-1} 3335 (NH), 3300 (br, OH), 1635 (CONH); δ_H (400 MHz, $CDCl_3$) 3.22 (1H, s, br, OH), 3.52–3.57 (1H, m, H-2$_a$), 3.90–3.96 (1H, m, H-2$_b$), 4.96–4.99 (1H, m, H-1), 6.57 (1H, s, br, NH), 7.26–7.76 (10H, m, Ar-H); δ_C (100 MHz, $CDCl_3$) 48.2 (CH_2), 74.2 (CH), 126.2, 127.4, 128.4, 129.0, 129.5 and 132.1 (Ar-H), 134.5 and 142.2 (*ipso* Ar), 169.0 (CO); m/z (CI) 242 (100%, $[M+H]^+$), 224 (87, $[M-H_2O+H]^+$). HPLC Chiralcel OJ, t_R 13.2 min, 15.1 min, eluent 9.2:0.8 hexane–isopropyl alcohol, flow rate 1 cm^3/min, λ 210 nm.

4.3. *N*-(1-Oxo-1-phenylethyl)benzamide 2[22]

To a stirred mixture of molecular sieves (1.0 g) and pyridinium dichromate (0.80 g, 2.10 mmol) in dry DCM (40 cm^3) was added acetic anhydride (0.65 cm^3, 6.82 mmol). Benzamide **1** (0.50 g, 2.07 mmol) was added and the reaction mixture was stirred at rt under nitrogen for 4 h. Ether (100 cm^3) was added and the reaction mixture was filtered through a small pad of silica. The filtrate was concentrated under reduced pressure to yield the crude product as a colourless liquid, which was redissolved in toluene and concentrated under reduced pressure to remove the excess acetic anhydride. This was repeated several times until a solid residue was obtained.

Purification by recrystallisation from petrol–ethyl acetate gave the title compound as a white solid (0.32 g, 60%); mp 122–124 °C (lit.[23] 125–126 °C); HRMS: found $[M+H]^+$ 240.10209. $C_{15}H_{14}NO_2$ requires 240.10245; ν_{max} (Nujol) cm^{-1} 3356 (NH), 1690 (CO), 1634 (CONH); δ_H (400 MHz, $CDCl_3$) 4.95 (2H, d, *J* 4.1 Hz, H-2), 7.26–7.66 (6H, m, Ar-H), 7.88 (2H, d, *J* 7.3 Hz, Ar-H), 8.03 (2H, d, *J* 7.54 Hz, Ar-H); δ_C (100 MHz, $CDCl_3$) 47.3 (CH_2), 127.5, 127.8, 128.7, 129.1, 130.0 and 130.4 (Ar-H), 134.6 and 134.8 (*ipso* Ar), 170.1 and 194.7 (CO); *m*/*z* (CI) 240 (100%, $[M+H]^+$), 139 (18), 122 (29), 105 (25). HPLC Chiralcel OJ, t_R 25.9 min, eluent 9.2:0.8 hexane–isopropyl alcohol, flow rate 1.0 cm^3/min, λ 220 nm.

4.4. (*R*)-*N*-(1-Hydroxy-1-phenylethyl)benzamide 1[20]

The (*R*)-benzamide **1** was synthesised in an identical manner to that described for (±)-**1** using (*R*)-2-amino-1-phenylethanol (0.30 g, 2.19 mmol) to yield the title compound after recrystallisation from petrol–ethyl acetate as a white solid (0.25 g, 47%). Spectral data was consistent with the data previously observed. Chiral HPLC showed a single enantiomer: Chiralcel OJ, t_R 13.2 min, eluent 9.2:0.8 hexane–isopropyl alcohol, flow rate 1.0 cm^3/min, λ 210 nm.

4.5. Biotransformation procedure for preparation of (*R*)-*N*-(1-hydroxy-1-phenylethyl)benzamide 1

Growth medium (200 cm^3) was prepared (20 g D-glucose and 20 g corn steep liquor in 1 L water, pH 5.0) and sterilised in an autoclave. The resultant sterile solution was inoculated with *C. echinulata* NRRL 1384 (stored on PDA slopes at 5 °C) and incubated in an orbital shaker at 30 °C at 200 rpm. After 2 days, (±)-benzamide **1** (100 mg) was added in DMF (2.0 cm^3). The solution was incubated for 6 days until 92% ee was observed for (*R*)-**1** by HPLC, whereupon the cells were removed by centrifugation (3000 rpm for 5 min), and the supernatant was extracted with ethyl acetate (3×150 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. Purification by flash column chromatography on silica (eluent 6:4, ethyl acetate–petrol) gave the title compound as a white solid (57%, 114 mg). The spectral data was consistent with the data previously observed. HPLC Chiralcel OJ, t_R = (*R*) 13.2 min, eluent 9.2:0.8 hexane–isopropyl alcohol, flow rate 1.0 cm^3/min, λ 210 nm.

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