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Preparation of optically active *N*-benzyl-3-hydroxypyrrolidine by enzymatic hydroxylation

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Abstract

Hydroxylation of *N*-benzylpyrrolidine **2** with *Pseudomonas oleovorans* GPo1 afforded 62% of (*R*)-*N*-benzyl-3-hydroxypyrrolidine **3** in 52% e.e. This reaction was catalyzed by the alkane hydroxylase system in this strain, which was demonstrated by hydroxylation of **2** with *Escherichia coli* GEc137 (pGEc47), a recombinant strain that carries the genes for the alkane hydroxylase system of *P. oleovorans* GPo1. In a set of 70 alkane-degrading microorganisms, 12 were found to catalyze the biotransformation of **2** into **3** by screening with a microtiter plate technique. Hydroxylation of **2** with isolates HXN-1100 and HXN-200 gave 67% of (*R*)-**3** in 70% e.e. and 62% of (*S*)-**3** in 53% e.e., respectively.

1. Introduction

We are interested in the enzymatic hydroxylation of *N*-benzylpyrrolidine **2** to prepare optically active *N*-benzyl 3-hydroxypyrrolidine **3**. (*S*)-*N*-Benzyl-3-hydroxypyrrolidine **3** is an intermediate in the preparation of a calcium antagonist.[1] Optically active *N*-benzyl-3-hydroxypyrrolidine **3** is also used practically as the protected form of optically active 3-hydroxypyrrolidine. While (*S*)-3-hydroxypyrrolidine is an intermediate for the preparation of an antibacterial agent[2] and a *k*-receptor agonist,[3] (*R*)-3-hydroxypyrrolidine is an intermediate in the synthesis of carbapenem antibiotics[4,5] and a 5-HT_{1Da} receptor agonist.[6]

Many synthetic routes to optically active 3-hydroxypyrrolidines have been developed, but each has one or more drawbacks: (i) (*R*)-3-hydroxypyrrolidine has been prepared by decarboxylation of the expensive (2*S*,4*R*)-4-hydroxy-L-proline;[7–9] (ii) (*S*)-3-hydroxypyrrolidine has been prepared by multistep syntheses from L-malic acid[3,10] or L-glutamic acid;[11] (iii) reduction of optically active 4-hydroxy-2-pyrrolidinones[12,13] or 3-hydroxy-2,5-pyrrolidindiones,[14] or cyclization of the optically active 4-halogenated-3-hydroxybutanes[15] or 4-halogenated-3-hydroxy butyl nitriles[16] gave the corresponding 3-

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hydroxypyrrolidines, respectively, but in each case it is necessary to prepare optically active precursors; (iv) asymmetric hydroboration of *N*-substituted 3-pyrroline[17,18] is elegant but not suitable for large scale preparation; (v) enzymatic reduction of *N*-benzyl-3-pyrrolidinone[19] needs synthesis of the substrate; and (vi) resolution by classical methods,[20–22] enzymatic hydrolysis,[23,24] and enzymatic esterification[25] could be practical, but the maximum theoretical yield is only 50%.

It is clear that the regio- and stereoselective hydroxylation of pyrrolidines should provide a more direct synthetic route to optically active 3-hydroxypyrrolidines. Although regio- and stereoselective insertion of oxygen into an unactivated carbon–hydrogen bond is very difficult when using classical methods, this type of reaction can be successfully carried out with enzymes,[26,27] as reported for the regio- and stereoselective hydroxylation of steroids[28–33] and terpenes.[34–39] Enzymatic hydroxylation of alicyclic compounds containing benzamide functions has been studied extensively with *Beauveria sulfurescens*, and has resulted in several predictive models.[27] Enzymatic hydroxylation of alicyclic compounds containing other 'anchoring' substituents has been reported recently, including the hydroxylation of *N*-substituted bridgehead azabicycloalkanes,[40,41] *N*-substituted azidoadamantanes,[42] and cycloalkylbenzoxazoles.[43]

Based on these previous findings, we have examined the biocatalytic hydroxylation of substituted pyrrolidines and we report here on the preparation of optically active *N*-benzyl-3-hydroxypyrrolidine **3** by enzymatic hydroxylation of *N*-benzylpyrrolidine **2**.

2. Results and discussion

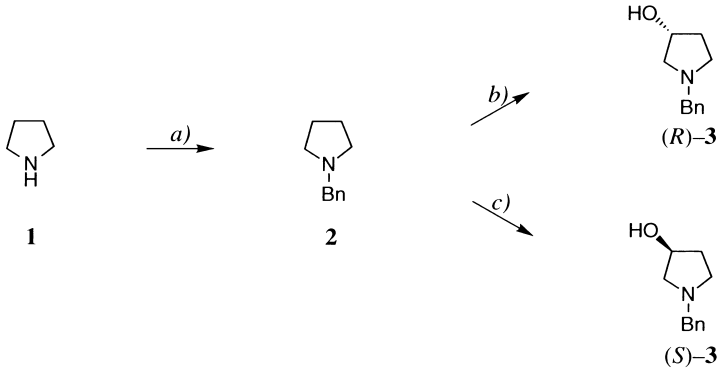
Enzymatic hydroxylation of pyrrolidine derivatives has been described in only one report: *N*-benzoylpyrrolidine was hydroxylated with *Cunninghamella verticillate* to give 38% of *N*-benzoyl-3-hydroxypyrrolidine with low e.e.,[44] in which the well-known benzamide function may be essential for substrate–enzyme binding, functioning as an 'anchoring' group. Our attempt to hydroxylate *N*-benzylpyrrolidine **2** with *B. sulfurescens* and fungi from the genus *Cunninghamella* failed, possibly because substrate **2** does not contain this type of 'anchoring' group.

*2.1. Preparation of (*R*)-*N*-benzyl-3-hydroxypyrrolidine **3** by hydroxylation of **2** with* P. oleovorans *GPo1 and* E. coli *GEc137(pGEc47)*

Microorganisms which are able to grow on *n*-alkanes are often able to carry out regio- and stereoselective hydroxylations of aliphatic compounds. We have previously reported that *P. oleovorans* GPo1, which grows on *n*-octane, also catalyzes hydroxylation of alicyclic compounds such as cyclopentane and cyclohexane.[45] When we used this interesting alkane hydroxylation system to hydroxylate our aliphatic heterocyclic substrate **2**, the desired optically active *N*-benzyl-3-hydroxypyrrolidine **3** was formed.

N-Benzylpyrrolidine **2** was prepared in 89% yield by alkylation of pyrrolidine **1** with benzyl bromide according to the known procedure.[46] Compound **2** was identified by comparing the data of GC, GC–MS, ^{1}H and ^{13}C NMR with the reported data (Scheme 1).

Hydroxylation of **2** with *P. oleovorans* GPo1 was carried out with resting cells. The strain was grown on *n*-octane, the cells were harvested at a cell density of 1–2 g/L and resuspended to 6–42 g/L in a K-phosphate buffer (50 mM, pH 7.0). *N*-Benzylpyrrolidine **2** (0.5–2 mM) was added and the mixture was shaken at 30°C for 2 days. The reaction was followed by analytical HPLC: substrate **2** elutes at 2.9 min, and product **3** at 4.6 min, which allows for fast analysis. Only one product could be detected, indicative of the excellent regioselectivity of the hydroxylation. The product was easily isolated by extraction with



Scheme 1. (a) BnBr, r.t., 89%. (b) *P. oleovorans* GPo1, 30°C, 62% (52% e.e.); HXN-1100, 30°C, 67% (70% e.e.) (c) HXN-200, 30°C, 62% (53% e.e.)

Table 1
Preparation of (*R*)-*N*-benzyl-3-hydroxypyrrolidine **3** by hydroxylation of *N*-benzylpyrrolidine **2** with resting cells of *P. oleovorans* GPo1

Entry	**2** (mM)	Cells (g/L)	**3** (%)				
			1 h	3 h	16 h	23 h	43 h
1	0.5	6	5	15		33	31
2	0.5	21	12	23	30		38
3	0.5	42	19	36	47		62
4	2	9	3	5		19	
5	2	21	8	18	24		31
6	2	42	15	29	39		49

ethyl acetate and subsequent chromatography on aluminum oxide. The data for GC, GC–MS, ^{1}H and ^{13}C NMR is identical to that of the authentic sample of *N*-benzyl-3-hydroxypyrrolidine **3**.

The enantiomeric excess of product **3** was measured by analytical HPLC with a chiral column [Chiralcel OB-H (Daicel) 250 mm×4.6 mm; eluent hexane:isopropanol, 98:2; flow rate: 0.5 mL/min; detection wavelength: 210 nm; retention times: 26.1 min for (*R*)-**3** and 43.5 min for (*S*)-**3**]. Hydroxylation of **2** with *P. oleovorans* GPo1 gave (*R*)-**3** in 52% e.e.

The yield of *N*-benzyl-3-hydroxypyrrolidine **3** depended on the concentrations of cells and substrate used, as can be seen in Table 1, and increased with the cell concentration used. Hydroxylation of 2 mM and 0.5 mM of *N*-benzylpyrrolidine **2** with 42 g/L of cells afforded **3** in 49% yield and 62% yield, respectively.

P. oleovorans GPo1 has a three-component alkane hydroxylase system:[47] AlkB, an integral cytoplasmic membrane protein that transfers one oxygen atom from molecular oxygen to the substrate;[48] AlkT, an NADH-dependent rubredoxin reductase which supplies electrons to reduce the other oxygen atom of molecular oxygen;[49] and AlkG, the electron carrier rubredoxin.[50] In order to determine whether the hydroxylation of **2** with *P. oleovorans* GPo1 is in fact catalyzed by the alkane hydroxylase, we examined the hydroxylation of **2** with *E. coli* GEc137 (pGEc47), a recombinant strain which carries the genes for the three-component alkane hydroxylase in *P. oleovorans* GPo1 and produces a functional alkane

Table 2
Preparation of (*R*)-*N*-benzyl-3-hydroxypyrrolidine **3** by hydroxylation of *N*-benzylpyrrolidine **2** with cell extracts (CEs) of *P. oleovorans* GPo1

Entry	**2**	Cells for CE[1]	NADH	**3** (%)					
	(mM)	(g/L)	(mM)	0.5 h	1 h	1.5 h	2 h	3 h	4 h
1	0.5	9	0.5	7	10	14	14	13	13
2	0.5	21	0.5	13	21	29	32	40	44
3	0.5	42	0.5	17	30	36	40	46	50

[1] CE derived from a cell suspension of the indicated concentration

hydroxylation system.[51] Biotransformation of **2** with resting cells of *E. coli* GEc137 (pGEc47) afforded 7% of **3** in 52% e.e. of the (*R*)-enantiomer. Biotransformation with the host strain GEc137 containing no hydroxylase utilized no substrate **2** and produced no product **3**, proving that hydroxylation of **2** into **3** was catalyzed by the alkane hydroxylase of *P. oleovorans* GPo1.

Hydroxylation of **2** was also performed in vitro with crude cell extracts of *P. oleovorans* GPo1. The preparation of crude cell extracts involved harvesting the cells, suspending them in a Tris–HCl buffer (pH=7.5) to 8–50 g/L, and passing them through the French press to break open the cells and release intracellular enzymes. The cell wall fragments were removed from the crude extract by centrifugation at 4000*g* which leaves, not only the soluble components rubredoxin and rubredoxin reductase, but also the membrane monooxygenase AlkB in the cell free extract. As NADH concentration was low in such extracts, additional NADH was added. Table 2 shows that hydroxylation of **2** with cell extracts was faster than with the corresponding resting cells. However, the reaction stopped after 4 h. Similar to the resting cell experiments, the yield of **3** increased when higher cell concentrations were used for the preparation of the cell extracts. Hydroxylation of 0.5 mM of **2** with 0.5 mM of NADH and cell extracts from 42 g/L of cells in Tris–HCl buffer gave 50% of the *N*-benzyl-3-hydroxypyrrolidine **3**.

Hydroxylation of **2** with growing, rather than resting, cells of *P. oleovorans* GPo1 afforded only 12% of **3**. This low yield was probably due to the competitive hydroxylation of *n*-octane which served as a carbon source in the growth medium.

*2.2. Screening of alkane-degrading strains for the biotransformation of **2** into **3***

As many alkane-degrading microorganisms have different alkane hydroxylase systems which may give rise to better enantioselectivity and better reactivity for the hydroxylation of **2**, we screened alkane-degrading strains in our collection.

Seventy alkane-degrading microorganisms were grown in microtiter plates, which allowed for efficient screening on a microscale. The strains were grown on a mixture of *n*-octane, *n*-decane, and *n*-dodecane (20:30:50) in 750 μL of medium. Cells were harvested by centrifugation and resuspended in 70 μL of *N*-benzylpyrrolidine **2** (2 mM) in K-phosphate buffer (50 mM, pH 7.0), and after biotransformation for 24 h, the hydroxylation products were analyzed by HPLC. Table 3 lists 12 alkane-degrading microorganisms that catalyzed the biotransformation of *N*-benzylpyrrolidine **2** into *N*-benzyl-3-hydroxypyrrolidine **3**.

In order to investigate the enantioselectivity and activity of the hydroxylation of **2** with these strains, they were grown on *n*-octane on a 200 mL scale to a density of 0.6 g/L, and the biotransformations

Table 3
Enantioselectivity and activity of the hydroxylation of *N*-benzylpyrrolidine **2** to *N*-benzyl-3-hydroxypyrrolidine **3** with several alkane-degrading strains

Entry	Strain	e.e. of **3** (%)	Activity[1] (U/ g CDW)
1	HXN-1100	70 (*R*)	0.4
2	HXN-400	65 (*R*)	0.05
3	*P. putida* P1	62 (*R*)	0.1
4	*P. oleovorans* GPo1	52 (*R*)	0.1
5	BC20	40 (*R*)	0.1
6	HXN-1500	25 (*R*)	0.3
7	HXN-500	10 (*R*)	1.1
8	HXN-200	53 (*S*)	0.6
9	HXN-100	10 (*S*)	0.3
10	HXN-1900	< 10 (*S*)	1.0
11	HXN-1000	< 10 (*S*)	0.1
12	HXN-600	0	0.3

[1] Activity was determined over the first 30 min.

performed with resting cells (5.8 g/L). Each reaction was followed by analytical HPLC, the product was isolated, and optical purity was determined by analytical HPLC with a chiral column. The e.e. of product **3**, and average resting cell activities (U/g CDW, U=μmol/min, CDW=cell dry weight) during the first 30 min of the biotransformation, are given in Table 3. These hydroxylations were not optimized.

The strains HXN-500 and HXN-1900 gave the highest activity for the hydroxylation of **2**, but their enantioselectivities were very poor. Hydroxylation of **2** with HXN-1100 gave (*R*)-**3** with 70% e.e. Interestingly, hydroxylation of **2** with HXN-200 afforded (*S*)-**3** in 53% e.e. Both HXN-1100 and HXN-200 have higher activities than *P. oleovorans* GPo1.

2.3. *Preparation of (*R*)-*N*-benzyl-3-hydroxypyrrolidine **3** by hydroxylation of **2** with HXN-1100*

Hydroxylation of **2** with resting cells from HXN-1100 was carried out with 0.5–5 mM of *N*-benzylpyrrolidine **2** and 8.7–43.5 g/L of cells in K-phosphate buffer (50 mM, pH 7.0). The biotransformation was faster than that with *P. oleovorans* GPo1 and stopped after 24 h. The highest yields of **3** were obtained with the highest cell concentrations, as shown in Table 4. Hydroxylation of 0.5, 2 and 5 mM of *N*-benzylpyrrolidine **2** with 43.5 g/L of cells gave **3** in 67%, 49%, and 33% yields, respectively, with 70% e.e. for the (*R*)-enantiomer in each of these experiments.

2.4. *Preparation of (*S*)-*N*-benzyl-3-hydroxypyrrolidine **3** by hydroxylation of **2** with HXN-200*

The HXN-200 strain was grown on *n*-octane to a cell density of 4.6 g/L, and the cells were harvested and resuspended to a cell density of 8.7 g/L. Hydroxylation of **2** with these cells gave a high activity. As

Table 4
Preparation of (*R*)-*N*-benzyl-3-hydroxypyrrolidine **3** by hydroxylation of *N*-benzylpyrrolidine **2** with resting cells of HXN-1100

Entry	**2** (mM)	Cells	**3** (%)					
		(g/L)	0.5 h	1 h	2 h	3 h	5 h	24 h
1	0.5	8.7	5	9	15	19	27	32
2	0.5	21.8	9	20	29	38	48	55
3	0.5	43.5	14	30	45	56	66	67
4	2	8.7	3	5	8	10	12	16
5	2	21.8	6	12	18	22	27	30
6	2	43.5	10	21	31	38	47	49
7	5	8.7	2	3	4	5	5	7
8	5	21.8	3	7	10	12	16	19
9	5	43.5	7	14	20	23	30	33

shown in Table 5, the average activity in the first 30 min reached 3.4 and 5.0 U/g CDW, starting with 5 mM and 10 mM for **2**, respectively. Surprisingly, the activity was still quite high (5.4 U/g CDW) at 20 mM for **2**. The highest activity obtained was 6.3 U/g CDW, starting with 15 mM for **2**. Product **3** had 53% e.e. for the (*S*)-enantiomer in all cases. Harvested HXN-200 cells could be stored at −80° without significant loss of hydroxylation activity after 1 month storage.

As discussed above, hydroxylation of **2** with alkane hydroxylase is NADH dependent. Therefore, for a high-yield whole-cell biotransformation NADH must be regenerated continuously via intracellular metabolism, which depends on the supply of an appropriate carbon source. Hence, we examined the biotransformation of **2** into **3** with resting cells in the presence of glucose (2%), glycerol (2%), and pyruvate (2% and 5%). While the addition of glycerol did not improve the biotransformation of **2** into **3**, the presence of either glucose or pyruvate caused nearly the same two-fold increase in the yield for **3**. Table 5 shows the results with 2% glucose: the yield for **3** at 5 h was improved from 38% to 62% starting with 5 mM (0.81 g/L) of **2**, from 28% to 48% starting with 10 mM (1.61 g/L) of **2**, from 26% to 35% starting with 15 mM (2.42 g/L) of **2**, and from 17% to 27% starting with 20 mM (3.22 g/L) of **2**, respectively.

3. Experimental

3.1. General

Analytical high-performance liquid chromatography (HPLC) was carried out with a Hewlett–Packard 1050 instrument [column: Spherisorb ODS2 (5 μm), 125 mm×4 mm; eluent acetonitrile:10 mM K-phosphate buffer (pH 4.6), 7:3; flow rate: 1.5 mL/min; detection wavelength: 210 nm; retention times: 2.9 min for **2**, 4.6 min for **3**]. The e.e. of **3** was determined by HPLC with a chiral column [Chiralcel OB-H (Daicel), 250 mm×4.6 mm; eluent hexane:isopropanol, 98:2; flow rate: 0.5 mL/min; detection

Table 5
Preparation of (*S*)-*N*-benzyl-3-hydroxypyrrolidine **3** by hydroxylation of *N*-benzylpyrrolidine **2** with resting cells (8.7 g/L) of HXN-200

Entry	**2**	Glucos	Activity[1]	**3** (%)						
	(mM)	(%)	(U/ g CDW)	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h	5 h
1	5		3.4	18		32		36		38
2	5	2	3.6	19		42		52		62
3	10		5.0	13		23		26		28
4	10	2	5.4	14		32		42		48
5	15		6.3	11	18		23		25	26
6	15	2	5.7	10	19		30		33	35
7	20		5.4	7	10		14		15	17
8	20	2	4.6	6	12		22		25	27

[1] Activity was determined over the first 30 min.

wavelength: 210 nm; retention times: 26.1 min for (*R*)-**3** and 43.5 min for (*S*)-**3**]. GC analyses were performed on a Fisons instrument HRGC MEGA2 series (column: Chrompack CP-Sil-5CB, temperature: 60°C for 2 min and then 25°C/min to 240°C). GC–MS analyses were carried out with a Fisons GC8000-MD800 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 300 (^{1}H) and at 75 MHz (^{13}C), respectively; chemical shifts in ppm relative to TMS, coupling constants *J* in hertz.

3.2. *Preparation of* N-*benzylpyrrolidine* ***2***

A solution of pyrrolidine **1** (0.400 g, 5.62 mmol), benzyl bromide (0.970 g, 5.67 mmol), and potassium hydroxide (0.320 g) in ethanol was stirred at room temperature for 12 h. The mixture was treated with ethyl acetate and brine. The organic layer was separated, dried over $MgSO_4$, filtered, and passed through a short column with Al_2O_3. Removal of the solvent afforded 0.803 g (89%) of *N*-benzylpyrrolidine **2** as a colorless liquid.[46] GC analysis: >99% purity. Data for **2**: ^{1}H NMR ($CDCl_3$): δ 7.35–7.20 (*m*, 5H, aromatic H), 3.61 (*s*, 2H, $PhCH_2$), 2.50 (*m*, 4H, 2 NCH_2), 1.78 ppm (*m*, 4H, 2 CH_2); ^{13}C NMR ($CDCl_3$): δ 139.42 (*s*), 128.91 (*d*), 128.19 (*d*), 126.84 (*d*) (aromatic C); 60.76 (*t*, $PhCH_2$); 54.18 (*t*, NCH_2); 23.46 ppm (*t*, CH_2); MS (70 eV): m/e 161 (25%, M^+), 91 (100%), 84 (60%), 70 (34%).

3.3. *Preparation of (*R*)-*N*-benzyl-3-hydroxypyrrolidine* ***3*** *with resting cells of* P. oleovorans *GPo1*

P. oleovorans GPo1 was inoculated in E2 medium[52] with octane vapor as the sole carbon and energy source and grown at 30°C for 10 h. The cells were harvested at a cell density of 1–2 g/L and resuspended to 6–42 g/L in 50 mM K-phosphate buffer (pH 7.0). *N*-Benzylpyrrolidine **2** was added to a final concentration of 0.5–2 mM, and the mixture was shaken at 30°C for 2 days. Samples were taken from the reaction mixture at different times, the cells were removed by centrifugation and the supernatants were analyzed by analytical HPLC.

The product was isolated as follows: the reaction mixture was adjusted to pH=12 by the addition of KOH and extracted with ethyl acetate. The organic phase was dried over $MgSO_4$ and the solvent evaporated. The residue was subjected to chromatography on aluminum oxide with a short column. The unreacted substrate **2** was eluted with ethyl acetate first, and then the product was eluted with methanol. GC and GC–MS analysis: identical with the authentic sample, >99% purity. The results are listed in Table 1. Data for **3**: ^{1}H NMR ($CDCl_3$): δ 7.33–7.21 (*m*, 5H, aromatic H), 4.30 (*m*, H-3), 3.61 (*s*, 2H, $PhCH_2$), 2.83 (*dt*, *J*=5.0, 8.8 Hz, H_A-5), 2.77 (*s*, OH), 2.64 (*dd*, *J*=2.3, 10.1 Hz, H_A-2), 2.53 (*dd*, *J*=5.3, 10.1 Hz, H_B-2), 2.31 (*dd*, *J*=6.4, 8.8 Hz, H_B-5), 2.23–2.11 (*m*, H_A-4), 1.77–1.66 ppm (*m*, H_B-4); ^{13}C NMR ($CDCl_3$): δ 138.68 (*s*), 128.87 (*d*), 128.27 (*d*), 127.05 (*d*) (aromatic C); 71.24 (*d*, C-3); 62.92 (*t*, C-2); 60.22 (*t*, $PhCH_2$); 52.40 (*t*, C-5); 34.95 ppm (*t*, C-4); MS (70 eV): m/e 177 (61%, M^+), 133 (60%), 100 (52%), 91 (100%), 86 (57%), 65 (60%), 42 (97%).

The enantiomeric excess of *N*-benzyl-3-hydroxypyrrolidine was measured by analytical HPLC with a chiral column. The *N*-benzyl-3-hydroxypyrrolidine **3** obtained had 52% e.e. of the (*R*)-form.

*3.4. Preparation of (*R*)-N-benzyl-3-hydroxypyrrolidine **3** with crude cell extracts of* P. oleovorans *GPo1*

P. oleovorans GPo1 was inoculated in E2 medium with octane vapor as the sole carbon and energy source at 30°C and shaken for 10 h. The cells were harvested at a cell density of 1–2 g/L and resuspended in a Tris–HCl buffer (pH=7.5) to a concentration of 9–42 g/L. After passage through the French press, the cell debris was removed by centrifugation at 4000*g*. *N*-Benzylpyrrolidine **2** (0.5 mM) and NADH (0.5 mM) were added to this crude cell extract containing the complete alkane hydroxylation system, and the mixture was shaken at 30°C for 24 h. Procedures for analysis and isolation were as described above. The results are given in Table 2.

*3.5. Preparation of (*R*)-N-benzyl-3-hydroxypyrrolidine **3** with growing cells of* P. oleovorans *GPo1*

P. oleovorans GPo1 was inoculated in E2 medium with octane vapor as a carbon source and grown at 30°C to a cell density of 0.6 g/L. *N*-Benzylpyrrolidine **2** (0.5 mM) was added and the cells were allowed to grow for a further 3 days. About 12% of *N*-benzyl-3-hydroxypyrrolidine **3** was formed.

*3.6. Preparation of (*R*)-N-benzyl-3-hydroxypyrrolidine **3** with resting cells of* E. coli *GEc137 (pGEc47)*

E. coli GEc137 (pGEc47)[51] was inoculated in M9 medium[53] with glucose as a carbon source and grown at 37°C for 10 h to a cell density of 0.2 g/L. The alkane hydroxylase system was then induced by adding DCPK (dicyclopropylketone) to 2 mM. Cells were harvested at a cell density of 0.6 g/L, and resuspended to 6 g/L in 50 mM K-phosphate buffer (pH 7.0). *N*-Benzylpyrrolidine **2** (0.5 mM) was added and the mixture was shaken at 30°C for 16 h. Procedures for analysis and isolation were as described above. The results showed that 7% *N*-benzyl-3-hydroxypyrrolidine **3** was obtained. Product **3** has a 52% e.e. of the (*R*)-enantiomer.

*3.7. Screening of alkane-degrading strains for the biotransformation of **2** into* N-*benzyl-3-hydroxypyrrolidine **3***

Seventy alkane-degrading microorganisms in a deep well microtiter plate were grown at room temperature in 750 μL of medium which consisted of 50% concentration of all nutrients from the Evans medium[54] with nitrilotriacetic acid as a complexing agent, 20 mM glucose, 20 mM L-aspartate and

100 mM K-phosphate buffer (pH=7.0). After 3 days, vapor of a mixture of *n*-octane, *n*-decane, and *n*-dodecane (20:30:50) was supplied as a carbon source. The cells were grown for an additional 3 days and harvested by centrifugation. Seventy microliters of the *N*-benzylpyrrolidine **2** (2 mM) in a K-phosphate buffer (50 mM, pH 7.0) were added to the cells, and the mixture was shaken at 30°C for 24 h. The biotransformation was analyzed by HPLC. Twelve alkane-degrading microorganisms, listed in Table 3, were found to catalyze the biotransformation of *N*-benzylpyrrolidine **2** into *N*-benzyl 3-hydroxypyrrolidine **3**.

3.8. Biotransformation of N-*benzylpyrrolidine* ***2*** *into* N-*benzyl-3-hydroxypyrrolidine* ***3*** *with selected alkane-degrading microorganisms*

Strains were inoculated individually in E2 medium with octane vapor as a carbon source and grown at 30°C to a cell density of 1–2 g/L. The cells were harvested and resuspended to 5.8 g/L in 50 mM K-phosphate buffer (pH 7.0). *N*-Benzylpyrrolidine **2** (2 mM) was added and the mixture was shaken at 30°C for 24 h. Procedures for analysis and isolation were as described above. The e.e. of **3** was determined by analytical HPLC with a chiral column. The results are listed in Table 3.

*3.9. Preparation of (*R*)-*N*-benzyl-3-hydroxypyrrolidine* ***3*** *in vivo with resting cells of HXN-1100*

HXN-1100 was inoculated in E2 medium with octane vapor as a carbon source and grown at 30°C for 10 h. The cells were harvested at a cell density of 1–2 g/L and resuspended to 8.7–43.5 g/L in 50 mM K-phosphate buffer (pH 7.0). *N*-Benzylpyrrolidine **2** was added to a final concentration of 0.5–5 mM, and the mixture was shaken at 30°C for 24 h. Procedures for analysis and isolation were as described above. The results are shown in Table 4. The e.e. of product **3** was 70% (*R*).

*3.10. Preparation of (*S*)-*N*-benzyl-3-hydroxypyrrolidine* ***3*** *with resting cells of HXN-200*

HXN-200 was inoculated in 2 L of E2 medium with octane vapor as a carbon source and grown at 30°C. The cells were harvested at a cell density of 4.6 g/L and stored at −80°C. *N*-Benzylpyrrolidine **2** (5–20 mM) was added to a suspension of 8.7 g/L of the cells in 50 mM K-phosphate buffer (pH 7.0), and the mixture was shaken at 30°C for 5 h. Procedures for analysis and isolation were as described above. The results are listed in Table 5, with product **3** showing 53% e.e. for the (*S*)-enantiomer.

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