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Regio- and stereoselective hydroxylation of *N*-substituted piperidin-2-ones with *Sphingomonas* sp. HXN-200

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Abstract—High activity, excellent regioselectivity, and opposite enantioselectivity were achieved in the hydroxylation of *N*-benzyl- and *N*-*tert*-butoxycarbonylpiperidin-2-one with *Sphingomonas* sp. HXN-200. High yield preparations of 4-hydroxypiperidin-2-ones were demonstrated in a bioreactor and in a shaking flask by use of the frozen/thawed cells as biocatalyst. The absolute configuration for the bioproducts was established.

1. Introduction

The regio- and stereoselective hydroxylation of non-activated carbon atoms is a great challenge in organic chemistry.[1] Nature has found a general solution for this reaction via oxygenases. Several biological systems have been successfully applied in the hydroxylation of steroids and several other alicyclic compounds.[2–11] Thus far, soluble cytochrome P450 monooxygenases such as P450 cam[12] and P450 BM-3,[13] soluble methane monooxygenase (sMMO),[14] and membrane-bound alkane hydroxylase (AlkB) of *P. putida* GPo1[15] have been well investigated. In practice, the filamentous fungus *Beauveria bassiana* ATCC 7159 has been widely used in organic laboratories for the hydroxylation of a broad range of substrates,[3,5–9,10c,11] although the activity and product concentration are rather low. We have recently discovered that *Sphingomonas* sp. HXN-200, an alkane-degrading bacterium containing a soluble monooxygenase, is a highly active and regio- and stereoselective catalyst for hydroxylations.[16] The easily handled frozen/thawed cells of *Sphingomonas* sp. HXN-200 have been successfully used in the high yield preparation of several pharmaceutical intermediates such as *N*-substituted (*S*)- and (*R*)-3-hydroxypyrrolidines, (*S*)-4-hydroxypyrrolidin-2-ones, 3-hydroxyazetidines, and 4-hydroxypiperidines.[16] We wanted to explore the synthetic application of this catalytic system further in the hydroxylation of other alicyclic compounds.

Optically active 4-hydroxypiperidin-2-one is a useful synthon for preparing antibiotics such as Blasticidin S[17a,b] and penem derivatives,[17c] 4-aminopiperidin-2-ones which are β-amino acid equivalents,[17d] and peptidomimetics with *Homo-Freidinger* lactam substructure.[17e,f] The chemical preparation of 4-hydroxypiperidin-2-ones, including the oxidation of *N*-substituted 4-piperidinopiperidines,[18a] 1,2,3,6-tetrahydropyridines,[18a] or 4-hydroxypiperidines,[18b] resulted in product mixtures. Preparation via biohydroxylation is also difficult: hydroxylation of *N*-phenyl-, *N*-benzyl-, or *N*-benzoyl-4-piperidin-2-ones with *Beauveria bassiana* ATCC 7159 gave the corresponding 4-hydroxypiperidin-2-ones in low concentration with a yield of 7,[6] 10,[11e] and 27%,[11e] respectively. Herein, we report our results on the hydroxylation of *N*-substituted piperidin-2-ones with *Sphingomonas* sp. HXN-200, preparative biotransformations and the stereochemistry of the hydroxylation products.

2. Results and discussion

For hydroxylation studies, cells of *Sphingomonas* sp. HXN-200 were grown on *n*-octane vapor in 30 L E2 medium, as previously described.[16a,b] The cells were harvested and the cell pellets were stored at −80°C. Frozen/thawed cells were used for biohydroxylation.

2.1. Small-scale biohydroxylation of 1–2

Based on the previous results from hydroxylation with *Sphingomonas* sp. HXN-200,[16] the hydrophobic *N*-ben-

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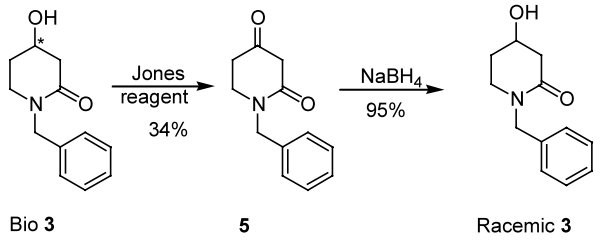
zyl- and *N*-*tert*-butoxycarbonyl-piperidin-2-one **1** and **2** were chosen as substrates. Compounds **1** and **2** were prepared according to established procedures.[19,20] Small-scale hydroxylations were performed with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in 10 ml of 50 mM K-phosphate buffer (pH 7.0–8.0) at a cell density of 4.0 g cdw/L. Glucose (2%, w/v) was added to improve the conversion via regeneration of the cofactor, and the mixture was shaken at 200 rpm and 30°C. Aliquots (0.1–0.2 ml) were taken from the bioconversion mixture at predetermined time points, diluted with MeOH, and the cells were removed by centrifugation. The samples were analyzed by reverse phase HPLC to follow the reaction in the aqueous phase. No extraction was needed, which is advantageous in comparison with GC analysis. Biohydroxylation of **1** and **2** afforded the desired products **3** and **4**, respectively. The conversion was quantified by comparing the integrated peak areas at 210 nm of the samples with the substrate and product standards. The results are given in Table 1.

High activity (12–15 U/g cdw) was observed in the hydroxylation of *N*-benzyl-piperidin-2-one, **1**. Biotransformation of a 10 mM solution of **1** gave 90% of *N*-benzyl-4-hydroxypiperidin-2-one **3** at pH 8.0. Changing the 'docking/protecting' group to a *tert*-butoxycarbonyl group resulted in a slightly lower hydroxylation activity (7.7–9.0 U/g cdw). Nevertheless, hydroxylation of a 10 mM solution of **2** produced 69–75% of *N*-*tert*-butoxycarbonyl-4-hydroxypiperidin-2-one **4**. While higher activity and conversion were achieved at pH 8.0 for the reaction of **1** to **3**, no significant pH effect was observed for the transformation of **2** to **4**. In both cases no other hydroxylation products were formed, indicating the excellent regioselectivity of the alkane monooxygenase of *Sphingomonas* sp. HXN-200. Moreover, no overoxidation products were detected, suggesting that the bioconversion is rather clean. Obviously, *Sphingomonas* sp. HXN-200 is significantly better than *Beauveria sulfurescens* ATCC 7159 in hydroxylation of *N*-substituted piperidin-2-one.[6,11e]

2.2. Determination of the ee for bioproduct 3 and 4

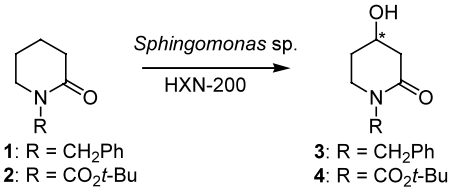
For the determination of ee, a racemic standard of **3** was prepared chemically: oxidation of the bioproduct **3** with Jones reagent gave the corresponding ketone **5** in 34% yield; reduction of **5** with $NaBH_4$ afforded the racemic **3** in 95% yield (Scheme 1). The two enantiomers were separated by HPLC on a chiralpak AS column with retention times of 8.7 and 10.4 min. Similarly, a pure sample of the bioproduct **4** was obtained by HPLC on a chiralcel OB-H column with retention times of 28.3 and 30.8 min for the two enantiomers.

As shown in Table 1, the ee of the bioproducts **3** and **4** are about 30–36 and 65–71%, respectively. No significant influence of pH on the ee was observed. While the ee of bioproduct **4** remained nearly unchanged during the biotransformation, there was a slight decrease in the ee of **3** (Fig. 1). This is probably due to trace alcohol dehydrogenase activity in the whole cells. While excel-



Scheme 1.

Table 1. Hydroxylation of *N*-substituted piperidin-2-ones **1**–**2** with frozen/thawed cells of *Sphingomonas* sp. HXN-200 (4.0 g cdw/L)



Entry	Substrate (mM)	Prod.	pH	Activity[a] (U/g cdw)	Conv.[b] (ee[c]) (%)					
					0.5 h	1 h	2 h	3 h	4 h	5 h
1	**1** (8.0)	**3**	7.5	12	18 (36)	36 (33)	55 (31)	68 (31)	77 (31)	79 (30)
2	**1** (10.0)	**3**	7.5	12	14 (35)	27 (33)	40 (31)	47 (33)	51 (31)	52 (31)
3	**1** (10.0)	**3**	8.0	15	18 (36)	34 (34)	58 (33)	77 (33)	83 (31)	90 (30)
4	**2** (10.0)	**4**	7.0	9.0	11 (71)	25 (66)	45 (66)	59 (65)	68 (64)	69 (69)
5	**2** (10.0)	**4**	7.5	8.6	10 (66)	24 (66)	47 (65)	64 (65)	74 (63)	75 (65)
6	**2** (10.0)	**4**	8.0	7.7	9.2 (68)	21 (68)	40 (67)	55 (68)	66 (68)	71 (68)

[a] Activity was determined over the first 30 min.
[b] Conversion was determined by HPLC analysis; error limit: 2% of the stated values.
[c] Number in brackets is the ee determined by HPLC analysis with a chiral column.

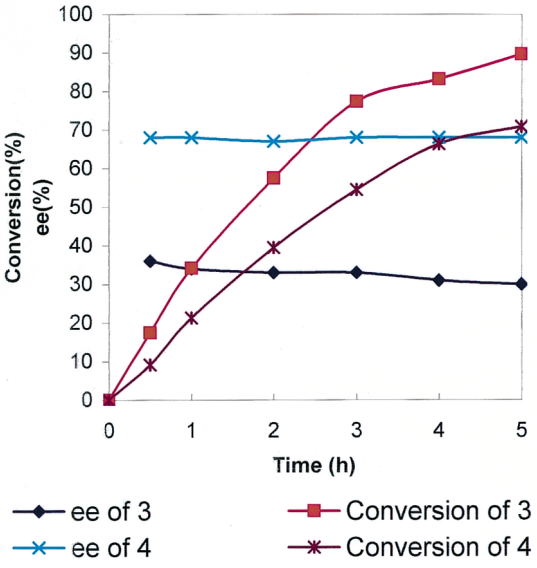


Figure 1. Biotransformation of **1** (10mM) to **3** and of **2** (10 mM) to **4** with frozen/thawed cells of *Sphingomonas* sp. HXN-200 (4 g cdw/L) at pH 8.0.

lent enantioselectivity was observed in hydroxylations of *N*-benzyl- and *N*-*tert*-butoxycarbonyl piperidin-2-one,[16c] hydroxylation of **1**–**2** occurred with only moderate to good enantioselectivity.

2.3. Preparative biohydroxylation

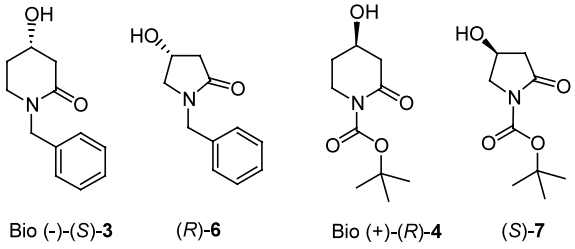
As an example of preparation on a gram scale, bioconversion of *N*-benzyl-piperidin-2-one **1** was carried out in a 3 L bioreactor. Biotransformation of substrate **1** (1.52 g, 8.04 mmol) in a 1 L suspension of the frozen/thawed cells in 50 mM of K-phosphate buffer (pH 8.0) containing glucose (2%, w/v) at a cell density of 10 g cdw/L for 5 h gave 75% conversion to **3**. Work-up and chromatographic purification afforded 1.168 g (71%) of the pure **3** as white powder with an $[\alpha]_D^{25}$ of −6.5 (*c* 1.10, $CHCl_3$) and an ee of 31% (Fig. 2a).

The preparation of **4** was demonstrated by biotransformation with a shaking flask. Biohydroxylation of **2** (199 mg) in 100 ml of 50mM K-phosphate buffer (pH 8.0) containing glucose (2%, w/v) at a cell density of 4.0 g cdw/L gave 71% of conversion to **4** at 5 h. Extraction of the product with *n*-butanol/ethyl acetate (1:1) and purification by flash chromatography afforded 96.2 mg (45%) of **4** in 68% ee (Fig. 2b) with an $[\alpha]_D^{25}$ of +6.7 (*c* 1.50, $CHCl_3$). The low isolated yield is probably caused by the incomplete extraction of the product **4** due to its hydrophilic nature. Continuous extraction would increase the yield.

The existence of an OH group in products **3** and **4** was evidenced by the absorption at 3382 and 3446 cm^{-1} in the IR spectra and the signal at 2.64 and 2.60–2.30 ppm in the ^1H NMR spectra, respectively. This was further confirmed by the MS signal at 206 and 215 (16 higher than the corresponding substrate), the resonance for H-C-OH at 4.64 and 4.21 ppm in the ^1H NMR spectra, and the signal for C-OH at 65.23 and 65.17 ppm in the ^{13}C NMR spectra, respectively. The existence of the signals of the NCH_2 and $NCOCH_2$ for both **3** and **4** in the ^1H NMR spectra exclude the possibility of OH groups at positions 6 or 3. The couplings of H-C-OH with $NCOCH_2$ and not with NCH_2 in the ^1H and (H,H) COSY NMR spectra suggest clearly that the OH group in **3**–**4** is at the 4-position.

2.4. Stereochemistry of the bioproducts 3–4

For determination of the absolute configuration of **3**–**4**, (*R*)- and (*S*)-*N*-benzyl-4-hydroxypyrrolidin-2-one **6** and (*R*)- and (*S*)-*N*-*tert*-butoxycarbonyl-4-hydroxypyrrolidin-2-one **7** were prepared chemically according to our previously published procedures.[16c] In the CD spectrum shown in Fig. 2(c), a negative Cotton effect for (−)-**3** at 245 nm and a negative Cotton effect at 240 nm for (*R*)-**6** was observed, suggesting that (−)-**3** has the same configuration at the C4-position as (*R*)-**6** (Scheme 2). According to the CIP nomenclature, this configuration is (*S*) for (−)-**3**. Similarly, a negative Cotton effect was observed for bioproduct (+)-**4** and (*S*)-**7** at 260 and 266 nm, respectively, in Fig. 2(d). This indicates the same configuration at the C4-position for (+)-**4** and (*S*)-**7**. Thus, according to the CIP principle, the configuration of (+)-**4** is established as (*R*) (Scheme 2).



Scheme 2.

The opposite enantioselectivity in the hydroxylation of *N*-benzyl- and *N*-*tert*-butoxycarbonyl-piperidin-2-one is very interesting. This provides the possibility of preparing both enantiomers of 4-hydroxypyrrolidin-2-ones by biohydroxylation with *Sphingomonas* sp. HXN-200. The enantioselectivity might be further improved by changing the docking/protecting groups or modifying the enzyme.

3. Conclusion

The hydroxylations of *N*-substituted pyrrolidin-2-ones **1**–**2** with *Sphingomonas* sp. HXN-200 occur with high activity and regioselectivity. Preparative transformations of **1** and **2** with the frozen/thawed cells afforded the corresponding 4-hydroxypyrrolidin-2-ones **3** and **4** in high concentration and good yield. Opposite enantioselectivity was observed for hydroxylation of **1** and **2**, giving the corresponding product **3** and **4** in 31% ee (*S*) and 68% ee (*R*), respectively.

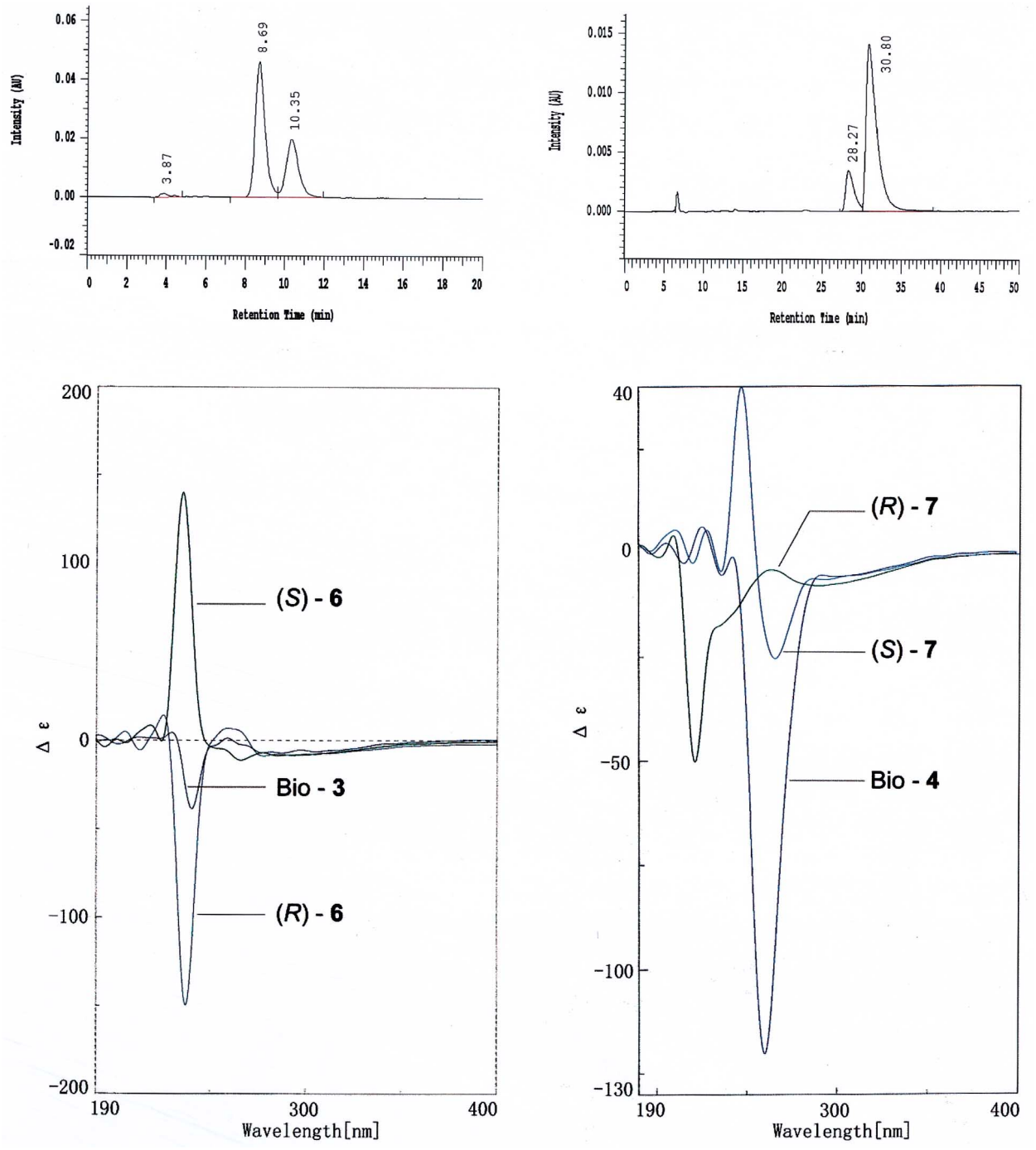


Figure 2. (a) HPLC analysis of ee for bioproduct (−)-**3**. (b) HPLC analysis of ee for bioproduct (+)-**4**. (c) CD spectra of bioproduct (−)-**3** and (*R*)- and (*S*)-*N*-benzyl-4-hydroxypyrrolidin-2-one **6** (in acetonitrile). (d) CD spectra of bioproduct (+)-**4** and (*R*)- and (*S*)-*N*-*tert*-butoxycarbonyl-4-hydroxypyrrolidin-2-one **7** (in acetonitrile).

4. Experimental

4.1. General methods

^{1}H and ^{13}C NMR spectra were determined at 300 K in $CDCl_3$, chemical shifts are reported in ppm relative to TMS, coupling constants (*J*) are reported in Hz. Mass spectra were obtained by Atmospheric Pressure Chemical Ionization at 40 eV with a Hewlett Packard 1100 LC-MS apparatus. Melting points are uncorrected. Analytical high-performance liquid chromatography (HPLC) was carried out with a Hewlett Packard 1050 instrument. IR spectra were measured in $CHCl_3$. Optical rotations were determined using a Perkin Elmer 241 polarimeter. CD spectra were measured in acetonitrile at 20°C on a JASCO J-710 spectropolarimeter. Bioconversion was assayed by HPLC. The purity of the products was established by GC analyses with a Chrompack

CP–Sil-5CB column (25 m×0.32 mm, temperature program: 60°C for 2 min, increase to 280°C at a rate of 25°C/min, then 280°C for 1 min).

4.2. Materials

Compounds **1**,[19] and **2**[20] were prepared according to published procedures. Cells of *Sphingomonas* sp. HXN-200 were produced by growth on *n*-octane in 30 L E2 medium,[21] as previously reported.[16a,b]

4.3. *N*-Benzyl-piperidine-2,4-dione 5

A solution of chromium(VI) oxide (30 mg, 0.30 mmol) in sulfuric acid (30 μl) and water (90 μl) was added dropwise at 0°C to a stirred solution of *N*-benzyl-4-hydroxypiperidin-2-one **3** (61 mg, 0.30 mmol) in acetone (5 ml). The mixture was stirred at rt for 5 min and the reaction was stopped by addition of isopropanol (2 ml). After evaporation, the residue was treated with water (2 ml) and the product was extracted into $CHCl_3$ (100 ml) and ethyl acetate (50 ml). Drying over Na_2SO_4, filtration, and evaporation afforded the crude product, which was purified by column chromatography on silica gel giving **5**[22] (20.7 mg, 34%). Purity: 95.7% (GC); R_f 0.32 (ethyl acetate), ^{1}H NMR (400 Hz) δ 7.36–7.25 (5H, m), 4.68 (2H, s), 3.48 (2H, t, $J=6.0$), 3.42 (2H, s), 2.53 (2H, t, $J=6.0$); ^{13}C NMR (100 MHz) δ 204.60 (s), 167.42 (s), 137.32 (s), 129.97 (d), 129.20 (d), 129.00 (d), 51.10 (t), 49.99 (t), 43.38 (t), 39.75 (t); MS (m/z): 204 (100%, M+1).

4.4. Racemic *N*-benzyl 4-hydroxypiperidin-2-one, 3

A mixture of *N*-benzyl-piperidin-2,4-dione **5** (6.5 mg, 0.032 mmol) and $NaBH_4$ (2.4 mg, 0.064 mmol) in CH_2Cl_2/acetic acid (9:1, 2 ml) was stirred at rt for 10 h. The product was extracted into $CHCl_3$ (50 ml). Drying over Na_2SO_4, filtration, and evaporation afforded the crude product that was purified by column chromatography on silica gel giving **3** (6.2 mg, 95%). Purity: 95.9% (GC); R_f 0.34 (ethyl acetate/MeOH, 9:1).

4.5. General procedures for small-scale hydroxylation

The frozen cells of *Sphingomonas* sp. HXN-200 were thawed and suspended to a cell density of 4.0 g cdw/L in 10 ml of 50 mM K-phosphate buffer containing glucose (2% w/v) at pH 7.0–8.0 in a 100 ml Erlenmeyer flask. Substrates were added directly to a final concentration of 2–10 mM. The mixture was shaken at 200 rpm at 30°C for 5 h. Aliquots (0.1–0.2 ml) were taken from the bioconversion mixture at predetermined time points and mixed with an equal volume of MeOH followed by centrifugation to remove the cells. The supernatant portion of the samples were analyzed by HPLC to quantify the conversion. At the same time, aliquots (0.5 ml) were taken from the bioconversion mixture, the cells were removed, and the product was extracted with ethyl acetate (0.5 ml). These samples were analysed by HPLC with a chiral column to determine the ee. All results are summarized in Table 1.

HPLC analysis. Column: Hypersil BDS-C18 (5 μm, 125×4 mm); eluent: a mixture of A (10 mM K-phosphate buffer, pH 7.0) and B (acetonitrile); flow: 1.0 ml/min; detection: UV at 210, 225, and 254 nm; t_R of **3**: 1.8 min, t_R of **1**: 4.4 min (A/B 70:30); t_R of **4**: 1.8 min, t_R of **2**: 4.3 min (A/B 70:30).

Determination of ee by HPLC. Chiral column (250×4.6 mm), UV detection at 210 and 254 nm, eluent A: *n*-hexane, and eluent B: isopropanol. For **3**: chiralpak AS column; flow rate 1.0 ml/min, A/B (75/25); t_R: 8.7 and 10.4 min. For **4**: chiralcel OB-H column; flow rate 0.5 ml/min, A/B (95/5); t_R: 28.3 and 30.8 min.

4.6. Preparation of *N*-benzyl-4-hydroxypiperidin-2-one 3 by biohydroxylation in a bioreactor

N-Benzyl-piperidin-2-one **1** (1.52 g, 8.04 mmol) was added to a 1 L suspension (10 g cdw/g) of frozen/thawed cells of *Sphingomonas* sp. HXN-200 in 50 mM of K-phosphate buffer (pH 8.0) containing glucose (2%, w/v) in a 3 L bioreactor, the mixture was stirred at 1500 rpm at 30°C under the introduction of air at 1 L/min. The biotransformation was followed by analytical HPLC and stopped at 5 h. After removing the cells, the supernatant pH was adjusted to 11–12 by addition of KOH followed by extraction with ethyl acetate. The organic phase was separated, dried over Na_2SO_4, and the solvent was removed by evaporation. The crude product was purified by flash chromatography on silica gel (methanol/ethyl acetate, 5:95, R_f 0.05) giving pure **3** as a white powder (1.168 g, 71% yield). ee: 31% (*S*); purity: 98.4% (GC), 99.2% (HPLC); $[\alpha]_D^{25}=-6.5$ (*c* 1.10, $CHCl_3$); mp 98.1–99.7°C, lit:[11e] 92–94°C; ^{1}H NMR (400 MHz) δ 7.33–7.22 (5H, m), 4.64 (1H, d, $J=14.4$), 4.55 (1H, d, $J=14.4$), 4.18 (1H, m), 3.41 (1H, m), 3.14 (1H, quin, $J=6.0$), 2.73 (1H, dd, $J=16.0$, 4.4), 2.64 (1H, s, br), 2.50 (1H, dd, $J=16.0$, 4.4), 1.95 (1H, m), 1.84 (1H, m); ^{13}C NMR (100 MHz) δ 169.87 (s), 137.85 (s), 129.68 (d), 128.93 (d), 128.45 (d), 65.23 (d), 51.03 (t), 44.15 (t), 42.01 (t), 31.33 (t); MS (m/z): 206 (100%, M+1), 188 (8%); IR ($CHCl_3$) ν 3382, 1632 cm^{-1}.

4.7. Preparation of *N*-*tert*-butoxycarbonyl-4-hydroxypiperidin-2-one 4 by biohydroxylation in a shaking flask

N-*tert*-Butoxycarbonyl-piperidin-2-one **2** (199 mg, 1.00 mol) was added to a 100 ml suspension (4.0 g cdw/L) of frozen/thawed cells of *Sphingomonas* sp. HXN-200 in 50 mM K-phosphate buffer (pH 8.0) containing glucose (2%) in a 500 ml shaking flask. The mixture was shaken at 200 rpm and 30°C for 5 h and the bioconversion was followed by analytical HPLC. The cells were removed from the bioconversion mixture by centrifugation and the pH of the supernatant was adjusted to 11–12 by addition of KOH followed by extraction with ethyl acetate/*n*-butanol (1:1). The organic phase was collected, water was removed by azeotropic distillation with MeOH, and the solvent was dried over Na_2SO_4 and removed by evaporation. The product was purified by column chromatography on silica gel (ethyl acetate, R_f 0.21), yielding **4** as a white powder (96.2 mg, 45%). ee: 68% (*R*); purity: 97.7% (GC), 99.9% (HPLC);

$[\alpha]_D^{25} = +6.7$ (c 1.50, $CHCl_3$); mp 61.9–63.6°C; ^1H NMR (400 MHz) δ 4.21 (1H, s), 3.83 (1H, dd, $J = 12.0$, 4.4), 3.57 (1H, dd, $J = 12.0$, 4.4), 2.74 (1H, d, $J = 15.6$), 2.53 (1H, d, $J = 15.6$), 2.60–2.30 (1H, s, br), 2.02 (1H, d, $J = 3.2$), 1.88 (1H, m), 1.50 (9H, s); ^{13}C NMR (100 MHz) δ 170.89 (s), 153.39 (s), 84.25 (s), 65.17 (d), 44.75 (t), 43.12 (t), 31.81 (t), 29.03 (q); MS (m/z): 215 (14%, M), 116 (100%), 100 (39%); IR ($CHCl_3$) ν 3446, 1766, 1715 cm^{-1}.

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