Preparation of (*R*)- and (*S*)-*N*-Protected 3-Hydroxypyrrolidines by Hydroxylation with *Sphingomonas* sp. HXN-200, a Highly Active, Regio- and Stereoselective, and Easy to Handle Biocatalyst

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Hydroxylation of *N*-benzylpyrrolidine **8** with resting cells of *Sphingomonas* sp. HXN-200 gave *N*-benzyl-3-hydroxypyrrolidine **15** in 53% ee (*S*) with an activity of 5.8 U/g CDW. By changing the "docking/protecting group" in pyrrolidines, hydroxylation activity and enantioselectivity were further improved and the enantiocomplementary formation of 3-hydroxypyrrolidines was achieved: hydroxylation of *N*-benzoyl-, *N*-benzyloxycarbonyl-, *N*-phenoxycarbonyl-, and *N*-*tert*-butoxycarbonyl-pyrrolidines **9**−**12** gave the corresponding 3-hydroxypyrrolidines **16**−**19** in ee of 52% (*R*), 75% (*R*), 39% (*S*), and 23% (*R*), respectively, with an activity of 2.2, 16, 14, and 24 U/g CDW, respectively. Simple crystallizations increased the ee of **16**−**18** to 95% (*R*), 98% (*R*), and 96% (*S*), respectively. Hydroxylation of pyrrolidines **8**−**12** with soluble cell-free extracts of *Sphingomonas* sp. HXN-200 and equimolar NADH gave 3-hydroxypyrrolidines **15**−**19** in nearly the same ee as the products generated by whole cell transformation, suggesting that this strain possesses a novel soluble alkane monooxygenase. Cells of *Sphingomonas* sp. HXN-200 were produced in large amounts and could be stored at −80 °C for 2 years without significant loss of activity. The frozen cells can be thawed and resuspended for biohydroxylation, providing a highly active and easy to handle biocatalyst for the regio- and stereoselective hydroxylation of nonactivated carbon atoms. These cells were used to prepare 1.0−3.2 g (66.4−93.5% yield) of 3-hydroxypyrrolidines **16**−**19** by hydroxylation of pyrrolidines **9**−**12** on 0.9−2 L scale. Preparative hydroxylation was also achieved with growing cells as biocatalysts; hydroxylation of pyrrolidine **11** on 1 L scale gave 1.970 g (79.7% yield) of 3-hydroxypyrrolidine **18**.

Introduction

Regio- and stereoselective hydroxylation of alicyclic compounds is of great interest, since this could generate cyclic alcohols that are useful synthons or pharmaceutical intermediates. While chemical hydroxylation remains a challenge,[1] biohydroxylation can be a useful alternative, with successes in the hydroxylation of steroids[2] and some progress for the hydroxylation of other cyclic compounds.[3−13] Thus far, biohydroxylation of pyrrolidines to the corresponding 3-hydroxypyrrolidines, which has been attempted with *Beauveria bassiana* ATCC 7159,[9d,14] a fungus with well-known hydroxylation activity, and with *Cunninghamella verticillate*[15] and other *Cunninghamella* strains,[5a] has been difficult.

Nevertheless, (*R*)- and (*S*)-*N*-protected 3-hydroxypyrrolidines are interesting targets, since they are useful

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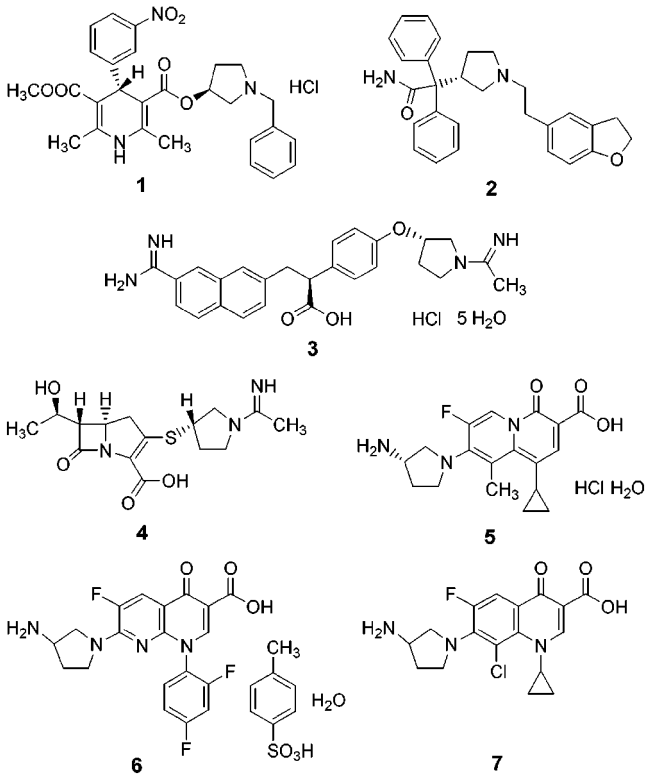
Scheme 1



pharmaceutical intermediates. The (*S*)-enantiomers, for example, are used in the synthesis of calcium antagonist *Barnidipine* **1**,[16] agent for irritable bowel syndrome, *Darifenacin* **2**,[17] and anticoagulant DX-9065a **3**[18] (Scheme 1). The (*R*)-enantiomers are intermediates for the preparation of carbapenem antibiotics RS-533 **4**[19] and antibacterial drug ABT-719 **5**.[20] Even racemic 3-hydroxypyrrolidines are useful intermediates in the synthesis of antibacterial drugs *Tosuxacin* **6**[21] and *Clinafloxacin* **7**.[22] The current syntheses of 3-hydroxypyrrolidines are not efficient: (*R*)-3-hydroxypyrrolidine has been prepared by decarboxylation of the expensive (2*S*,4*R*)-4-hydroxy-L-proline;[23] (*S*)-3-hydroxypyrrolidine has been synthesized from L-malic acid[24] or L-glutamic acid[25] in low overall yield or from special optically active precursors such as 4-halo-3-hydroxybutanes[26] or 4-halo-3-hydroxy butyl nitriles;[27] and asymmetric syntheses including hydroboration of *N*-substituted 3-pyrroline[28] and enzymatic reduction of *N*-benzyl-3-pyrrolidinone[29] require either special reagents or expensive starting materials. (*R*)- or (*S*)-3-Hydroxypyrrolidines have also been prepared by chemical[30] or enzymatic resolution,[31] but the theoretical maximum yield is only 50% and the preparation of the racemates requires multistep syntheses.

It is clear that regio- and stereoselective hydroxylation of pyrrolidines should give the simplest synthesis of optically active 3-hydroxypyrrolidines, and we have therefore sought new biocatalysts for these reactions. Screening of 70 *n*-alkane-degrading bacteria yielded 12 strains that catalyze the regio- and stereoselective hydroxylation of *N*-benzylpyrrolidine **8**, giving optically active *N*-benzyl 3-hydroxypyrrolidine **15**.[5a] Among these strains, *Sphingomonas* sp. HXN-200[32] showed high activity and relatively high enantioselectivity and was chosen for further investigation. Here, we describe the hydroxylation of *N*-substituted pyrrolidines with this strain: the improvement of hydroxylation activity and enantioselectivity by introduction of new "docking/protecting" groups into pyrrolidine; the hydroxylation with soluble cell-free extracts; the development of frozen/thawed cells of *Sphingomonas* sp. HXN-200 as easy to handle catalysts; and the first gram scale preparation of optically active 3-hydroxypyrrolidines by biohydroxylation with frozen/thawed cells or growing cells as biocatalysts.

Results and Discussion

Hydroxylation of Pyrrolidines 8−14 with Resting Cells of *Sphingomonas* sp. HXN-200. Previously, we found that hydroxylation of *N*-benzylpyrrolidine **8** with *Pseudomonas putida* (*oleovorans*) GPo1[33] and the isolate HXN-1100 gave 62% of (*R*)-**15** in 52% ee and 67% of (*R*)-**15** in 70% ee, respectively.[5a] However, the activity is only about 0.1−0.3 U/g CDW (U = μmol/min, CDW = cell dry weight). On the other hand, hydroxyla-

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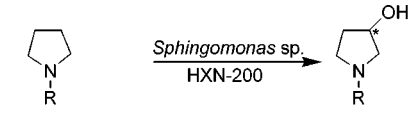
Table 1. Hydroxylation of Pyrrolidines 8−12 to 3-Hydroxypyrrolidines 15−19 with Resting Cells of *Sphingomonas* sp. HXN-200

substrate (mM)	cell density[a] (g/L)	glucose (%)	product	activity[b] (U/g CDW)	conversion (%)[c] 0.5 h	1 h	2 h	3 h	5 h
8 (5.0)	5.5	2	**15**	5.8	19				62
9 (2.0)	3.9	0	**16**	2.2	13	17	19	19	20
9 (2.0)	3.9	2	**16**	2.2	13	21	33	43	56 (70[d])
10 (3.0)	4.0	2	**17**	16	66	90			
10 (4.0)	4.0	2	**17**	10	31	57	80	91	
11 (5.0)	4.5	2	**18**	14	37	63	71	73	77
11 (10)	4.5	2	**18**	13	18	30	39	39	40
12 (5.0)	5.3	2	**19**	22	71	93	98	98	
12 (10)	5.3	2	**19**	24	38	65	72	77	85

[a] Cells were suspended in 20 mL 50 mM K-phosphate buffer (pH=7.5). [b] Activity was determined over the first 30 min. [c] Conversion was determined by HPLC analysis; error limit: 2% of the stated values. [d] Conversion at 24 h.

Scheme 2



		ee	ee after crystallization
8: R = CH_2Ph	**15**: R = CH_2Ph	53% (*S*)	
9: R = COPh	**16**: R = COPh	52% (*R*)	95% (*R*)
10: R = CO_2CH_2Ph	**17**: R = CO_2CH_2Ph	75% (*R*)	98% (*R*)
11: R = CO_2Ph	**18**: R = CO_2Ph	39% (*S*)	96% (*S*)
12: R = CO_2t-Bu	**19**: R = CO_2t-Bu	23% (*R*)	
13: R = COMe			
14: R = CO_2Me			

tion of **8** with *Sphingomonas* sp. HXN-200 afforded 62% of (*S*)-**15** in 53% ee with an activity of 5.8 U/gCDW (Table 1), significantly better than the hydroxylation with *P. putida* GPo1, the isolate HXN-1100, or *Cunninghamella verticillate*.[15]

To further increase the activity and enantioselectivity in hydroxylation with *Sphingomonas* sp. HXN-200, we modified the substrates by introducing new types of "docking/protecting" group. *N*-Benzoyl-, *N*-benzyloxycarbonyl-, *N*-phenoxycarbonyl-, *N*-*tert*-butoxycarbonyl-, *N*-acetyl-, and *N*-methoxycarbonyl-pyrrolidine **9**−**14** were chosen as new substrates (Scheme 2), since compounds containing amide and carbamate functions are good substrates for hydroxylation with several fungi.[4,6] The substrates **9**,[34] **10**,[35] **11**,[36] **13**,[37] and **14**[38] and the standard (*R*)- and (*S*)-3-hydroxypyrrolidines **16**, **17**,[31a] and **19**[39] were prepared according to known procedures. (*R*)- and (*S*)-*N*-Phenoxycarbonyl-3-hydroxypyrrolidine **18** were prepared in 24% and 34% yield, respectively, by treatment of (*R*)- or (*S*)-3-hydroxypyrrolidine with phenyl chloroformate. The characterization of the structure of **18** was straightforward by ^1H and ^{13}C NMR spectra. Two sets of signals for carbons on the ring and C=O in the ^{13}C NMR spectrum and two signals for OH in the ^1H NMR spectrum were observed, evidencing the existence of two rotamers due to restricted rotation about the *N*−CO bond.

Small scale biohydroxylations were performed with resting cells of *Sphingomonas* sp. HXN-200 as biocatalyst on a 20 mL scale. Pyrrolidine **8**−**14** (2−10 mM) was added to a cell suspension (3.9−5.5 g/L) in 50 mM K-buffer (pH = 7.5), and the mixture was shaken at 200 rpm and 30 °C. The reaction was followed by HPLC analysis. Samples were prepared by taking aliquots (0.1−0.2 mL) from the bioconversion mixture at predetermined time points, diluting in MeOH, and removing the cells by centrifugation. Comparison of sample retention times and integrated peak areas at 210 nm with substrate and product standards allowed confirming and quantitating bioconversions. Hydroxylation of **9**−**12** afforded the desired 3-hydroxypyrrolidines **16**−**19**. No products were detected in the bioconversion of **13** and **14**. The results are summarized in Table 1.

As shown in Table 1, the conversion of *N*-benzoylpyrrolidine **9** to *N*-benzoyl-3-hydroxypyrrolidine **16** was increased from 20% to 56% by addition of 2% glucose, presumably as a result of the regeneration of the cofactor via intracellular metabolism. The activity during the first 30 min of about 2.2 U/g CDW was lower than that for the hydroxylation of **8**. This is probably caused by steric hindrance of the *N*-benzoyl substituent. Remarkably, cells were active for quite a long time in the hydroxylation of **9**: 70% of **16** was obtained in 24 h. Much higher activity and conversion were obtained in the hydroxylation of *N*-benzyloxycarbonyl pyrrolidine **10**, *N*-phenoxycarbonyl pyrrolidine **11**, and *N*-*tert*-butoxycarbonyl pyrrolidine **12**. Hydroxylation of **10** gave 90−91% of **17** with an activity of 10−16 U/g CDW. Hydroxylation of **11** afforded 40−77% of **18** and an activity of 13−14 U/g CDW. Hydroxylation of **12** formed 85−98% of **19** with an activity of 22−24 U/g CDW, the highest among the hydroxylations of **8**−**12**.

Compounds **16**−**19** were isolated by solvent extraction followed by chromatography on silica gel or aluminum oxide. The structures were confirmed by their ^1H and ^{13}C NMR and MS spectra. The purity of **16**−**19** was >99%, as established by GC analysis. The ee was determined

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by analytical HPLC with a chiralcel OD-H, chiralcel OJ, or Chiralpak AS column. It is worth mentioning that attempts to determine the ee of **16** by a reported method[31a] failed. In comparison with the hydroxylation of **8**, opposite enantioselectivities were observed in the hydroxylations of **9** and **12**, giving **16** in 52% ee (*R*) and **19** in 23% (*R*), respectively. Higher and opposite enantioselectivity was obtained in the hydroxylation of **10**, affording **17** in 75% ee (*R*), while hydroxylation of **11** gave **18** in 39% ee (*S*). Thus, the enantiocomplementary formation of 3-hydroxypyrrolidines was achieved with the same catalyst by changing the "docking/protecting group" in pyrrolidines. Simple crystallization from ethyl acetate and *n*-hexane increased the ee of (*R*)-**17** to 98% in 66% yield, the ee of (*R*)-**16** to 95% in 40% yield, and the ee of (*S*)-**18** to 96% in 25% yield, respectively.

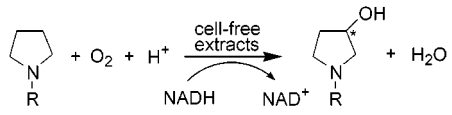
The positive results in the hydroxylation of hydrophobic substrates **8**−**12**, while the hydrophilic substrates **13** and **14** were not hydroxylated, suggested that the hydroxylation enzyme, an alkane monooxygenase, contains a hydrophobic substrate binding pocket. The enzyme is induced in *n*-octane grown cells and is responsible for hydroxylation of *n*-octane to 1-octanol during growth on *n*-octane, thus preferring to take hydrophobic substrates. Hydrophobic carbamates are clearly excellent "docking/protecting" groups for the hydroxylation with *Sphingomonas* sp. HXN-200. Pyrrolidines with substituents containing a phenyl moiety such as **8**−**11** are good substrates, possibly because of the hydrophobic interaction between the phenyl moiety and the enzyme substrate binding pocket. The activity depends strongly on the steric effect and the distance between phenyl moiety and C−(3) position.

No other hydroxylation products were detected, demonstrating the excellent regioselectivity of the alkane monooxygenase. As this enzyme is probably responsible for the specific terminal hydroxylation of *n*-octane during growth, the hydroxylation at the C(3)-position in pyrrolidines, a terminal-like position, is preferred. The relationship between the enantioselectivities and the structures is currently under investigation.

A frequently encountered problem in whole cell bioconversion is the side reactions catalyzed by other enzymes in the cells. Surprisingly, no byproducts could be detected in biotransformations of **9** and **11** with resting cells of *Sphingomonas* sp. HXN-200. Only a small amount of 3-ketones was formed in hydroxylation of **10** and **12**. This overoxidation may be caused by the alcohol dehydrogenase that is responsible for the oxidation of 1-octanol to octyl aldehyde during the metabolism of *n*-octane. Apparently, this alcohol dehydrogenase has a relatively low activity for the oxidation of the cyclic alcohols to ketones. Further studies revealed that pH has significant influence on the resting cell biotransformation of **10** and **12**.[40] The conversion of **12** to **19** increased from 50% to 60% when the pH increased from 6.4 to 8.0, while the formation of ketone increased from 0 to 1.0% and the ee of (*R*)-**19** dropped from 25% to 14%. A similar phenomenon was observed in the hydroxylation of **10**; an increase of pH from 6.5 to 8.0 improved the conversion to **17** from 29% to 86% and increased the formation of byproduct from 0 to 4.9%; the ee of (*R*)-**17** remained 73−75% at pH 6.5−7.5 and decreased to 69% at pH 8.0. Considering both activity and enantioselectivity, the best pH for hydroxylation of **10** and **12** is between 7.2 and 7.5.

(40) For details see Table S-1 in Supporting Information.

Table 2. Hydroxylation of Pyrrolidines 8−12 with Soluble Cell-Free Extracts[a] of *Sphingomonas* sp. HXN-200



8-12 **15-19**

substrate (mM)	NADH (mM)	product	conv (%)[b]	ee (%)[c]
8 (5.0)	5.0	**15**	36	53 (*S*)
9 (1.5)	1.5	**16**	34	52 (*R*)
10 (5.0)	5.0	**17**	22	76 (*R*)
11 (5.0)	5.0	**18**	80	39 (*S*)
12 (5.0)	5.0	**19**	72	9 (*R*)

[a] Cell-free extracts were prepared from a cell suspension (20 g/L) in Tris-HCL (pH 7.5). [b] Conversion to product at 1 h; error limit, 2% of the stated values. [c] ee of the isolated product after 1 h reaction.

Hydroxylation of Pyrrolidines 8−12 with Soluble Cell-Free Extracts of *Sphingomonas* sp. HXN-200. In comparison with *P. putida* GPo1, *Sphingomonas* sp. HXN-200 shows a much higher activity and opposite enantioselectivity in the hydroxylation of **8**. Thus, the alkane monooxygenase of *Sphingomonas* sp. HXN-200 could also be different from the well-known three-component alkane hydroxylase system, which contains a membrane monooxygenase AlkB, of *P. putida* GPo1.[41] Therefore, we investigated the hydroxylation with soluble cell-free extracts of *Sphingomonas* sp. HXN-200 as catalyst. The soluble cell-free extracts were prepared by passage of the cell suspension (20 g/L in Tris-HCl buffer (pH 7.5)) through the French press followed by removal of the cell debris and membranes by centrifugation at 245 000*g* for 45 min. Incubation of the soluble cell-free extracts with pyrrolidines **8**−**12** and equimolar NADH gave the corresponding 3-hydroxypyrrolidines **15**−**19** in nearly the same ee as the products generated with resting cells (Table 2). Incubation of **8**−**12** with cell-free extracts without addition of NADH gave only a small amount of products. These results indicate that *Sphingomonas* sp. HXN-200 contains a soluble NADH-dependent monooxygenase that is clearly different from the well-known membrane-bound alkane hydroxylase of *P. putida* GPo1. Enzyme purification and characterization are currently being carried out.

Preparation of Cells of *Sphingomonas* sp. HXN-200. To develop a practical biohydroxylation catalyst, we first optimized the growth of *Sphingomonas* sp. HXN-200 by using *n*-octane or glucose/*n*-octane as carbon sources in E2 medium. Typical growth curves are shown in Figure 1a. The alkane monooxygenase was found also to be inducible with dicyclopropyl ketone.[42] The cells grew faster on glucose/*n*-octane. In both cases, the harvested cells were found to possess a high hydroxylation activity. Large amounts of cells were easily produced in this way, and storage at −80 °C for 2 years did not result in a significant loss of activity.

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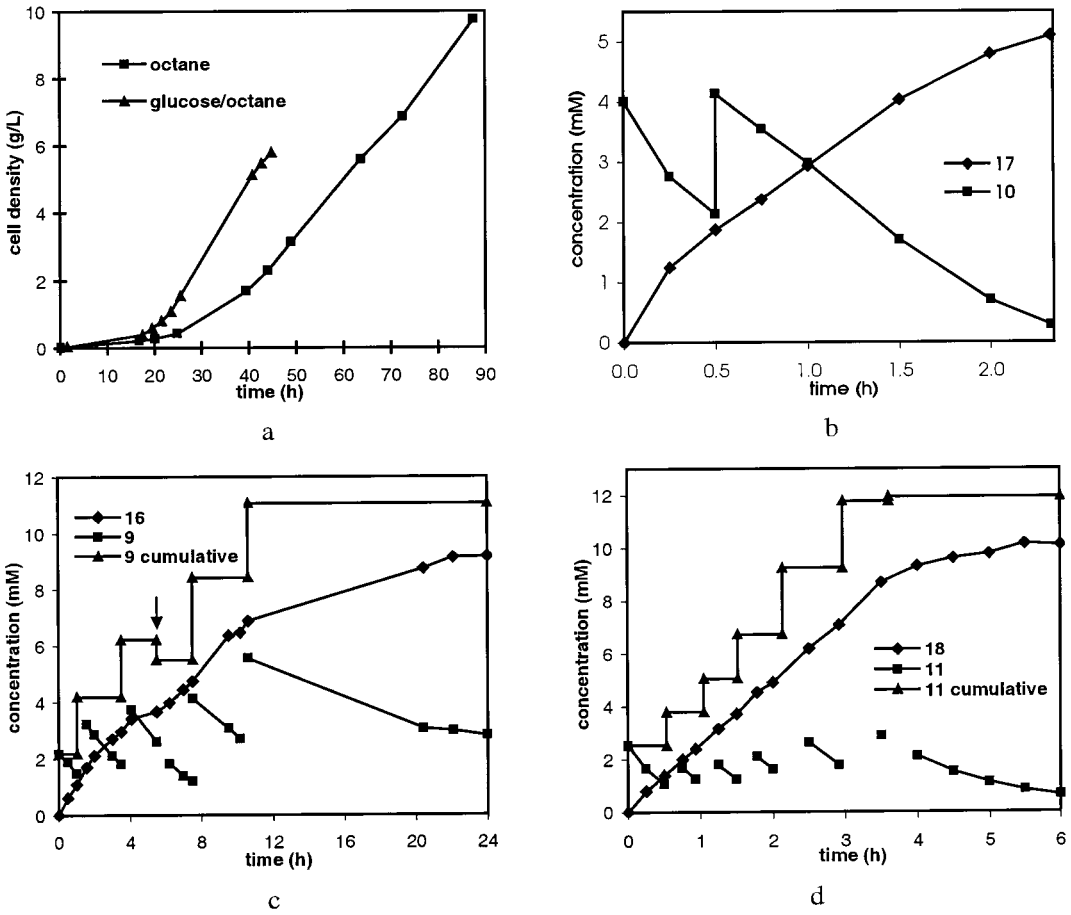

Figure 1. (a) Growth of *Sphingomonas* sp. HXN-200 on *n*-octane or glucose/*n*-octane in 2 L of E2 medium in a bioreactor. (b) Preparative hydroxylation of *N*-benzyloxycarbonyl-pyrrolidine **10** to **17** in 1 L of cell suspension (4.0 g/L) of frozen/thawed cells in 50 mM K-phosphate buffer (pH 8.0) containing 2% glucose. (c) Preparative hydroxylation of *N*-benzoyl-pyrrolidine **9** to **16** in 1 L of cell suspension (7.6 g/L) of frozen/thawed cells in 50 mM K-phosphate buffer (pH 8.0) containing 2% glucose. New cell suspension (130 mL) was added at 5.5 h to a final cell density of 13.1 g/L. (d) Preparative hydroxylation of *N*-phenoxycarbonyl-pyrrolidine **11** to **18** with growing cells of *Sphingomonas* sp. HXN-200 in 1 L of E2 medium (pH 7.2) with starting cell concentration of 6.2 g/L.

Gram Scale Preparation of 3-Hydroxypyrrolidines 16−19 with Frozen/Thawed Cells of *Sphingomonas* sp. HXN-200. The preparative hydroxylation of pyrrolidines **9**−**12** was carried out with frozen/thawed cells as biocatalysts. Hydroxylation of **10** at a cell concentration of 4.0 g/L is shown in Figure 1b. Pyrrolidine **10** was added at the beginning (4 mM) and after 30 min (2 mM). The reaction proceeded very fast during the first 15 min and continued at a nearly constant rate between 0.5 and 2.3 h; 85% of **17** was formed and 1.043 g (78.9%) of **17** was isolated. The product concentration was increased to 2.478 g/L (62.2% conversion) by use of higher cell density (14.4 g/L) (Table 3). Hydroxylation of **11** and **12** gave 96% of **18** and **19** with 93.5% and 85.4% isolated yield, respectively.

As shown in Figure 1c, biotransformation of **9** was carried out in a 1 L cell suspension (7.3 g/L). By the addition of substrate at different time points and a second addition of cells at 5.5 h, 75% of **16** was formed at 24 h and 1.588 g (66.4%) of the pure product was isolated. This demonstrated a practical hydroxylation with frozen/thawed cells even if the activity was only 2.7 U/gCDW.

Gram Scale Preparation of 3-Hydroxypyrrolidine 18 with Growing Cells of *Sphingomonas* sp. HXN-200. Preparative biohydroxylations can also be achieved with growing cells as biocatalysts. As an example, the hydroxylation of **11** is shown in Figure 1d. The cells were first grown on glucose in 1 L E2 medium (pH 7.2) and then on *n*-octane to a cell density of 6.2 g/L. Pyrrolidine **11** (2.52 mM) was added, while *n*-octane vapor was still introduced, and the cells grew during the bioconversion. Additional substrate was added at different time points and a linear increase of product concentration was observed during the first 4 h; 85% (2.097 g/L) of **18** was formed at 6 h and 1.970 g (79.7%) of pure **18** was obtained.

Table 3. Preparation of 16−19 by Hydroxylation with Frozen/Thawed Cells of *Sphingomonas* sp. HXN-200

substrate (mM)	cells[a] (g/L)	scale (L)	product	time (h)	conv (%)[b]	yield (g)	yield (%)
9 (12.5)[c]	7.3 (13.1)[d]	1.0	**16**	24.0	75	1.588	66.4
10 (6.0)[c]	4.0	1.0	**17**	2.3	85	1.043	78.9
10 (18.0)[c]	14.4	0.9	**17**	3.0	62	1.660	46.1
11 (8.0)[c]	4.0	1.0	**18**	3.5	96	1.554	93.5
12 (10.0)	4.0	2.0	**19**	3.0	96	3.203	85.4

[a] Cells were suspended in 50 mM K-phosphate buffer (pH 8.0) containing 2% glucose. [b] Conversion was determined by HPLC analysis; error limit, 2% of the stated values. [c] Substrate was added in several portions at different time points. [d] New cells were added at 5.5 h to a final cell density of 13.1 g/L.

Conclusions

Sphingomonas sp. HXN-200 catalyzes the hydroxylation of pyrrolidines **9**−**12** to the corresponding 3-hydroxy-

pyrrolidines **16**−**19** in an ee of 53% (*S*) to 75% (*R*) with high activity, high conversion, and excellent regioselectivity. Improvement of the enantioselectivity and the enantiocomplementary formation of 3-hydroxypyrrolidines are achieved by changing the docking/protecting group in pyrrolidines. The enantioselectivity could be further improved by use of an appropriate docking/protecting group. Simple crystallizations increased the ee of bioproducts **16**−**18** to 95% (*R*), 98% (*R*), and 96% (*S*), respectively. The alkane monooxygenase of *Sphingomonas* sp. HXN-200 is a soluble enzyme, different from the well-known membrane-bound alkane hydroxylase of *P. putida* GPo1. Cells of *Sphingomonas* sp. HXN-200 can be easily prepared in large amounts, stored at −80 °C for 2 years without significant loss of activity, and the frozen cells can be thawed and resuspended for biohydroxylation. With high activity and broad substrate range, the easy-handling frozen/thawed cells of *Sphingomonas* sp. HXN-200 can be used as a routine biocatalyst for regio- and stereoselective hydroxylation in organic synthesis. Finally, we have developed the first practical biohydroxylation for preparing (*S*)- and (*R*)-3-hydroxypyrrolidines by use of either frozen/thawed or growing cells of *Sphingomonas* sp. HXN-200 as biocatalysts.

Experimental Section

General Methods. ^{1}H and ^{13}C NMR spectra were determined at 300 (^{1}H) and 75 (^{13}C) MHz, all in $CDCl_3$, with chemical shifts in ppm relative to TMS and coupling constants *J* in Hz. Mass spectra were obtained by atmospheric pressure chemical ionization (APCI) with LC-MS. IR spectra were measured in $CHCl_3$. Optical rotations were determined using a Perkin Elmer 241 polarimeter. Melting points are uncorrected. Bioconversion was analyzed 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). The ee of the products was determined by HPLC analysis with a chiral column.

Materials. Pyrrolidine (>99.5%) and (*R*)-3-hydroxypyrrolidine (>99%) were purchased from Fluka, (*S*)-3-hydroxypyrrolidine (97%) from Synthon Co., and *N*-*tert*-butoxycarbonylpyrrolidine **12** (97%), (*R*)- and *(S)*-*N*-benzyl-3-hydroxypyrrolidine **15** (98% and 99%, respectively) from Aldrich. Pyrrolidines **8**,[5a] **9**,[34] **10**[35], **11**,[36] **13**,[37] and **14**[38] and the standard (*R*)- and (*S*)-3-hydroxypyrrolidines **16**, **17**,[31a] and **19**[39] were prepared according to the published procedures.

***(R)*-*N*-Phenoxycarbonyl-3-hydroxypyrrolidine 18.** Phenyl chloroformate (0.37 mL, 2.15 mmol) was added dropwise at room temperature to a mixture of (*R*)-3-hydroxypyrrolidine (260 mg, 2.98 mmol) and $NaHCO_3$ (350 mg, 4.17 mmol) in THF (3 mL) and H_2O (3 mL), and the mixture was stirred for 2 h. CH_2Cl_2 (10 mL) was added; the organic phase was separated, washed with 5% Na_2CO_3 (5 mL), dried over Na_2SO_4, and filtered; and the solvent removed by evaporation. Column chromatography on silica gel gave 148 mg (24%) of (*R*)-**18**: R_f 0.12 (ethyl acetate/*n*-hexane 1:1); mp 74.3−75.7 °C; $[\alpha]^{20}_D$ −23.8 (*c* 1.04, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.35 (2 H, tt, J = 7.8, 2.1 Hz), 7.18 (1 H, tt, J = 7.4, 1.1 Hz), 7.12 (2 H, d, J = 8.3 Hz), 4.43 (1 H, br, s), 3.76−3.45 (4 H, m), 2.48 (0.5 H, br, s), 2.46 (0.5 H, br, s), 2.09−1.82 (2 H, m); ^{13}CNMR ($CDCl_3$) δ 153.47 (s), 153.38 (s), 151.32 (s), 129.27 (d), 125.26 (d), 121.76 (d), 70.91 (d), 70.04 (d), 54.83 (t), 54.62 (t), 44.37 (t), 34.09 (t), 33.52 (t); MS (40 eV) *m*/*z* 208 (M + 1, 100%); IR ($CHCl_3$) ν 3425, 1712 cm^{-1}.

***(S)*-*N*-Phenoxycarbonyl-3-hydroxypyrrolidine 18.** In the same procedure, 150 mg (34%) of (*S*)-**18** was prepared from (*S*)-3-hydroxypyrrolidine (189 mg, 2.17 mmol), $NaHCO_3$ (302 mg, 3.59 mmol), and phenyl chloroformate (0.27 mL, 2.94 mmol): mp 74.0−74.7 °C; $[\alpha]^{20}_D$ 23.2 (*c* 1.01, $CHCl_3$).

General Procedure for Hydroxylation of Pyrrolidines 8−12 on a Small Scale. The cells of *Sphingomonas* sp. HXN-200[43] were resuspended into 20 mL of 50 mM K-phosphate buffer containing glucose (0 or 2% w/v) at pH 7.5 to a cell density of 3−6 g/L in a 100 mL Erlenmeyer flask. Pure substrates **8**−**10** and **12** and a solution of 10% **11** in MeOH were added to the mixture to a final concentration of 2−10 mM, respectively. The mixtures were shaken at 200 rpm at 30 °C for 1−24 h, and the reactions were followed by HPLC analysis of samples that were prepared by taking aliquots (0.1−0.2 mL) from the bioconversion mixtures at predetermined time points and mixing with equal volume of MeOH followed by removal of cells by centrifugation.

HPLC Analysis for Biohydroxylation of Pyrrolidines 8−12. The following conditions were used to analyze samples: Hypersil BDS-C18 (5 μm) column (125 mm × 4 mm), UV detection at 210 nm, flow rate of 1 mL/min; eluent A, acetonitrile, and eluent B, 10 mM K-phosphate buffer (pH 7.0). Retention times (min): t_R of **15**, 2.7; t_R of **8**, 4.6, A/B 20/80; t_R of **16**, 1.5; t_R of **9**, 3.2, A/B 30/70; t_R of **17**, 1.8; t_R of **10**, 4.2, A/B 50/50; t_R of **18**, 2.1; t_R of **11**, 5.9, A/B 35/65; t_R of **19**, 1.7; t_R of **12**, 5.5, A/B 40/60.

Standard Workup Procedure for Biohydroxylation of Pyrrolidines 8−12. The cells were removed from the bioconversion mixture by centrifugation, and the supernatant was adjusted to pH 11−12 by addition of KOH followed by extraction with ethyl acetate. The organic phase was separated and dried over $MgSO_4$, and the solvent was removed by evaporation. The product was purified by column chromatography on aluminum oxide or silica gel.

Determination of ee of Bioproducts 16−19 by HPLC Analysis. The following conditions were used to analyze samples: Chiral column (250 mm × 4.6 mm), UV detection at 210 and 254 nm, flow rate at 1.0 mL/min; eluent A, *n*-hexane, and eluent B, 2-propanol. Retention times (min): t_R of (*S*)-**16**, 19.5; t_R of (*R*)-**16**, 22.9, column, chiralcel OD-H, A/B 9/1; t_R of (*S*)-**17**, 13.5; t_R of (*R*)-**17**, 15.4, column, chiralpak AS, A/B 9/1; t_R of (*S*)-**18**, 15.1; t_R of (*R*)-**18**, 12.0, column, chiralcel OJ, A/B 8/2; t_R of (*S*)-**19**, 34.8; t_R of (*R*)-**19**, 39.0, column, chiralpak AS, A/B (99/1). ee of **16**−**19** from exploratory hydroxylation: 52% (*R*) for **16**; 75% (*R*) for **17**; 39% (*S*) for **18**; and 23% (*R*) for **19**.

General Procedure for Hydroxylation of Pyrrolidines 8−12 with Soluble Cell-Free Extracts. The cells of *Sphingomonas* sp. HXN-200 were suspended in 60 mL of Tris-HCl buffer (pH 7.5) to a density of 20 g/L. After three passages through the French press, the cell debris was removed by centrifugation at 245 000*g* for 45 min yielding soluble cell-free extracts containing no membrane proteins. To each 10 mL of extract was added the substrate **8**−**12** and equimolar NADH, respectively. The mixture was shaken at 200 rpm and at 30 °C for 1 h and the products **15**−**19** were isolated. The conversion and the ee of **15**−**19** were analyzed by HPLC and listed in Table 2.

General Procedure for Preparative Biohydroxylations with Frozen/Thawed Cells. Frozen cells of *Sphingomonas* sp. HXN-200[43] were thawed and suspended in 0.9−2 L of 50 mM of K-phosphate buffer (pH 8.0) containing glucose (2%, w/v) in a 3 L bioreactor, the substrate was added, and the mixture was stirred at 1500 rpm and at 30 °C under the introduction of air at 1 L/min. The biotransformation was followed by analytical HPLC. Workup according to the same procedure described above and purification by column chromatography on silica gel afforded the pure products.

***N*-Benzoyl-3-hydroxypyrrolidine 16.** Biohydroxylation of *N*-benzoylpyrrolidine **9** (380 mg, 2.17 mmol) was started in 1 L of cell suspension (7.3 g/L). Additional **9** were added to the mixture at 1 h (352 mg, 2.01 mmol), 3.5 h (357 mg, 2.04 mmol), 7.5 h (581 mg, 3.32 mmol), and 10.6 h (520 mg, 2.97 mmol). New cells suspension (130 mL) and glucose (30 mL, 50% w/v) were added at 5.5 h to a cell density of 13.1 g/L. Biotransformation for 24 h gave 75% of **16**. Workup with $CHCl_3$ as

(43) For preparation of cells of *Sphingomonas* sp. HXN-200 see Supporting Information.

extraction solvent and column chromatography on silica gel gave 1.588 g (66.4%) of **16** in 52% ee (*R*) as white powder. Crystallization of **16** from ethyl acetate increased the ee to 95% (*R*) in 40% yield: R_f 0.14 (ethyl acetate/$CHCl_3$ 8/2); mp 112.7−114.0 °C; $[\alpha]^{20}_D$ −79.9 (*c* 1.03, $CHCl_3$), lit.:[31a] $[\alpha]^{20}_D$ −84.6 (*c* 1.06, MeOH); purity 99.4 (GC t_R = 9.98 min).

***N*-Benzyloxycarbonyl-3-hydroxypyrrolidine 17.** (A) Biohydroxylation of *N*-benzyloxycarbonyl-pyrrolidine **10** (823 mg, 4.01 mmol) in 1 L of cell suspension (4.0 g/L) with addition of **10** (403 mg, 4.96 mmol) at 30 min afforded 85% of **17** at 2 h 20 min. Standard workup and column chromatography on silica gel afforded 1.043 g (78.9%) of **17** in 70% ee (*R*) as white powder. Crystallization of **17** from ethyl acetate/*n*-hexane (1/2−5) gave **17** in 98% ee with 66% yield: R_f 0.17 (ethyl acetate/*n*-hexane 1/1); mp 77.8−79.1 °C; $[\alpha]^{20}_D$ −21.9 (*c* 2.01, $CHCl_3$), lit.:[44] $[\alpha]^{22}_D$ −21.2 (*c* 1.0, $CHCl_3$); purity 99.3% (GC t_R = 10.31 min). (B) Biohydroxylation of **10** (1.850 g, 9.01 mmol) in 0.9 L cell suspension (14.4 g/L) with addition of **10** at 56 min (0.740 g, 3.61 mmol) and 93 min (0.750 g, 3.65 mmol) gave 62.2% of **17** at 185 min. Standard workup and column chromatography afforded 1.660 g (46.1%) of **17**.

***N*-Phenoxycarbonyl-3-hydroxypyrrolidine 18.** Biohydroxylation of pyrrolidine **11** (0.767 g in 2 mL MeOH, 4.01 mmol) in 1 L of cell suspension (4.0 g/L) with addition of **11** at 34 (0.383 g in 1 mL MeOH, 2.00 mmol), 67 (0.192 g in 1 mL MeOH, 1.00 mmol), and 110 min (0.192 g in 1 mL MeOH, 1.00 mmol) gave 96% of **18** at 3 h 27 min. Standard workup and column chromatography on silica gel gave 1.554 g (93.5%) of **18** in 39% ee (*S*). Crystallization from ethyl acetate (1/1−2) gave **18** in 96% ee (*S*) with 25% yield: R_f 0.17 (ethyl acetate/*n*-hexane 6/4); mp 74.5−76.1 °C; $[\alpha]^{20}_D$ 22.0 (*c* 1.01, $CHCl_3$); purity 99.3% (GC t_R = 9.95 min).

***N*-*tert*-Butoxycarbonyl-3-hydroxypyrrolidine 19.** Biohydroxylation of pyrrolidine **12** (3.424 g, 20.0 mmol) in 2 L cell suspension (4.0/L) gave 96% of **19** at 3 h. Standard workup and crystallization of the crude product from ethyl acetate and *n*-hexane (3/10) gave 3.033 g (80.9%) of **19** as white crystals. Recovery of the product from mother liquor and subsequent column chromatography on aluminum oxide gave additional 0.170 g (4.5%) of **19**. Total yield: 3.203 g (85.4%); ee 16% (*R*); R_f 0.35 (ethyl acetate/*n*-hexane 3/7); mp 61.2−62.5 °C; purity 99.4% (GC t_R = 7.11 min).

Preparative Biohydroxylation with Growing Cells. *N*-Phenoxycarbonyl-3-hydroxypyrrolidine 18. The cells of *Sphingomonas sp.* HXN-200 were grown in 1 L of E2 medium first on glucose and then on *n*-octane to a cell density of 6.2 g/L.[43] *N*-Phenoxycarbonyl-pyrrolidine **11** (3.00 mL of 0.840 M in MeOH) was added, the mixture was stirred at 1536 rpm at 30 °C with air introduction at 2 L/min. During bioconversion introduction of *n*-octane vapor was continued and the cells were still grown. Additional **11** was added at 32 (1.50 mL, 0.840 M), 63 (1.50 mL, 0.840 M), 91 (2.00 mL, 0.840 M), 128 (3.00 mL, 0.840 M), 178 (3.00 mL, 0.840 M), and 216 min (0.20 mL, 0.840 M); 85% of **18** were formed at 6 h and 1.970 g (79.7%) of **18** in 39% (*S*) ee were isolated. Purity 99.7% (GC t_R 9.95 min).

Acknowledgment. We thank Dr. Th. Plaggemeier and Prof. K. Engesser (University of Stuttgart) for supplying us with the HXN-200 strain.

Supporting Information Available: Preparation of cells of *Sphingomonas* sp. HXN-200; MS, IR, ^1H and ^{13}C NMR data of bioproducts **16**−**19**; ee determination for the bioproducts **16**−**18** after crystallization; and pH influence on hydroxylation of **10** to **17** and **12** to **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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