

Highly enantioselective hydrolysis of alicyclic meso-epoxides with a bacterial epoxide hydrolase from *Sphingomonas* sp. HXN-200: simple syntheses of alicyclic vicinal *trans*-diols

Dongliang Chang, Zunsheng Wang, Maarten F. Heringa, Renato Wirthner, Bernard Witholt and Zhi Li*

Institute of Biotechnology, Swiss Federal Institute of Technology Zurich, ETH-Hoenggerberg, CH-8093 Zurich, Switzerland. E-mail: zhi@biotech.biol.ethz.ch; Fax: +41 1 633 1051; Tel: +41 1 633 3811

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Hydrolysis of *N*-benzyloxycarbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide with the epoxide hydrolase of *Sphingomonas* sp. HXN-200, respectively, gave the corresponding vicinal *trans*-diols in high ee and yield, representing the first example of enantioselective hydrolysis of a meso-epoxide with a bacterial epoxide hydrolase.

Epoxide hydrolase (EH), an enzyme that catalyses the enantioselective addition of water to an epoxide forming the corresponding vicinal diol, has been extensively investigated,¹ and several microbial EHs have been applied to prepare enantiopure epoxide *via* kinetic resolution.² In principle, EH could also catalyse the enantioselective hydrolysis of a meso-epoxide to give the corresponding *trans*-diol in high ee and 100% theoretical yield, providing a simple and green synthesis in addition to the other known chemical methods.^{3,4} Mammalian epoxide hydrolase (mEH) is known to hydrolyse meso-epoxides such as cycloalkene oxides^{5,6} and acyclic 1,2-disubstituted epoxides^{6,7} with high enantioselectivity. However, synthetic applications are limited due to the poor availability of mEH. Microbial epoxide hydrolases can easily be produced in large amounts, but examples of hydrolysis of meso-epoxides with such EHs are rare: only a membrane-associated yeast EH from *Rhodotorula glutinis* was found to enantioselectively hydrolyse cyclohexene- and cyclopentene oxide;⁸ low enantioselectivity was observed in hydrolysis of cyclohexene oxide with a fungal EH;⁹ and thus far no bacterial EH was found to accept a meso-epoxide as substrate.¹⁰ Here we report the first example of highly enantioselective hydrolysis of a meso-epoxide catalysed by a bacterial EH from *Sphingomonas* sp. HXN-200 and the high yield preparation of the corresponding vicinal *trans*-diols.

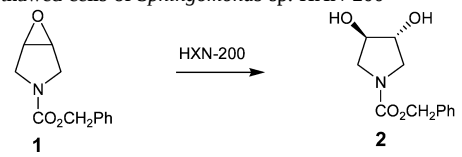
Sphingomonas sp. HXN-200 is an alkane-degrading strain and known to regio- and stereoselectively catalyse the hydroxylation of a series of aliphatic heterocycles.¹¹ Cells of *Sphingomonas* sp. HXN-200 were produced by growth on *n*-octane in 2 L E2 medium,¹¹ the harvested cell pellets were stored at -80°C , and the frozen/thawed cells were used for hydrolysis. 3,4-Epoxy-pyrrolidine **1** was chosen as substrate since the *trans*-diol product is a useful synthetic intermediate for the preparation of antibiotics,¹² Sialyl Lewis X mimetics¹³ and aza-sugars.¹⁴ Substrate **1** was prepared by epoxidation of *N*-benzyloxy-carbonyl-3-pyrroline with mCPBA,¹⁴ and small-scale hydrolysis of **1** (10–20 mM) were performed with frozen/thawed cells (10 g L⁻¹) of *Sphingomonas* sp. HXN-200 in 10 ml 50 mM K-phosphate buffer (pH = 8.0) containing glucose (2%) at 30 °C. Aliquots (0.1–0.2 ml) were taken from the bioconversion mixture at predetermined time points, diluted in MeOH, and the cells were removed by centrifugation. The samples were analyzed by reverse phase HPLC to follow the reaction directly in the aqueous phase. The conversion was quantitated by comparing the integrated peak areas at 210 nm of the samples with the product standard which was prepared by hydrolysis of **1** with TFA. In all the cases, the desired diol **2** was generated without formation of any byproduct. The conversion was 98–99% for the hydrolysis of 10–15 mM of substrate (Table 1).

The activity reached 17–18 U g cdw⁻¹. The ee of diol **2** was determined by HPLC analysis with a chiral column. It remained unchanged during the biotransformation at 95%. To our knowledge, this is the first example of highly enantioselective hydrolysis of *N*-containing meso-epoxide catalysed by either a chemical or a biological catalyst.

Preparative hydrolysis was performed with 15 mM of **1** in 100 ml cell suspension (10 g L⁻¹) of *Sphingomonas* sp. HXN-200 (Fig. 1).† 97% conversion was reached after 5 h, and 289.1 mg (81.3%) of **2** was isolated with 98.9% purity, 95% ee and an $[\alpha]_{\text{D}}^{25} +7.6$ ($c = 1.80$, CHCl₃). The bio-product **2** is identical with the authentic sample synthesized chemically, with respect to MS, ¹H- and ¹³C-NMR, UV, and IR spectra.

The configuration of bioproduct (+)-**2** was established by chemical correlation: deprotection of bioproduct (+)-**2** by hydrogenation with 20% Pd(OH)₂/C gave 94% of 3,4-dihydroxypyrrolidine with an $[\alpha]_{\text{D}}^{25}$ of -18.6 ($c = 0.80$, in MeOH). Since (3*S*, 4*S*)-3,4-dihydroxypyrrolidine has an $[\alpha]_{\text{D}}^{26}$ of $+20.7$ ($c = 0.30$, in MeOH),¹⁵ the configuration of bioproduct (+)-**2** can be deduced to be (3*R*, 4*R*).

Table 1 Hydrolysis of *N*-benzyloxycarbonyl-3,4-epoxy-pyrrolidine **1** with frozen/thawed cells of *Sphingomonas* sp. HXN-200

							
Con. of 1 /mM	Activity ^a /U g cdw ⁻¹	Conversion to 2 (%) ^b					
		0.5 h	1 h	2 h	3 h	4 h	5 h
10.0	17	52	72	88	98	99	99
15.0	18	37	53	70	86	96	98
20.0	18	27	43	56	70	79	85

^a Activity was determined over the first 30 min. ^b Conversion was determined by HPLC analysis; error limit: 2% of the stated values.

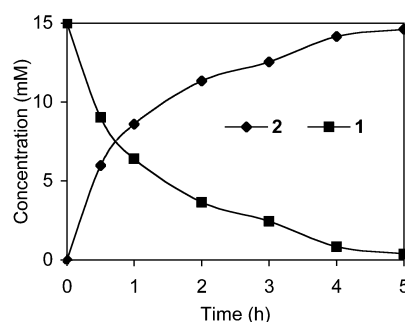
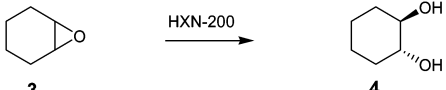


Fig. 1 Preparation of **2** by hydrolysis of **1** (15 mM) with frozen/thawed cells (10 g L⁻¹) of *Sphingomonas* sp. HXN-200.

Table 2 Hydrolysis of cyclohexene oxide **3** with frozen/thawed cells and soluble cell-free extracts of *Sphingomonas* sp. HXN-200

							
3/mM	Cells/g cdw L ⁻¹	CFE ^a /g prot. L ⁻¹	Scale/ ml	Activity ^b / U g ⁻¹	Time/h	Conv. ^c (%)	ee ^d of 4 (%)
10	13		20	1.8	7	99	87
20	13		12	3.1	7	96	86
10		20	5	1.0	7	95	86
20		20	5	1.4	14	91	85

^a Cell free extract. ^b Average activity for the whole reaction period. U g⁻¹ cdw⁻¹ and U g protein⁻¹ for whole cell and cell-free transformation, respectively. ^c Conversion was determined by GC analysis; error limit: 2% of the stated values. ^d Ee was determined by GC analysis with a chiral column; error limit: 2% of the stated values.

To further explore the hydrolysis potential, cyclohexene oxide **3** was selected as the second substrate, since it represents a carbocyclic meso-epoxide and the product **4** is an useful synthon. The enzymatic reaction was performed at 25 °C instead of 30 °C to reduce the non-enzymatic hydrolysis rate. Hydrolysis of **3** (10–20 mM) was examined with frozen/thawed cells (13 g L⁻¹) of HXN-200 in 50 mM Tris-HCl (pH = 7.5) and followed by GC analysis of samples that were prepared by taking aliquots (0.2 ml) at predetermined time points, removing the cells, and extracting with ethyl acetate (1:2).[‡] The conversion was quantitated by comparison of the integrated peak areas of the samples and the product standard and correction with the extraction efficiency. Here again, only the desired *trans*-diol **4** was formed, and the conversion reached > 95% at 7 h (Table 2). Hydrolysis of **1** (20 mM) under the same conditions with cells which were boiled for 20 min revealed that the non-enzymatic hydrolysis was only about 1.2% at 7 h. The ee of the product **4** was determined as 86–87% by GC analysis with a chiral column, and the configuration was established as (1*R*,2*R*) by comparison of the retention time with those of (1*R*,2*R*)- and (1*S*,2*S*)-**4**. This stereochemistry outcome is similar to the hydrolysis with mEH^{5,6} and the membrane-associated yeast EH from *R. glutinis*.⁸

Different from the *R. glutinis* EH, the HXN-200 EH was found to be a soluble enzyme. The preparation of the soluble cell-free extracts of HXN-200 involved suspending the frozen/thawed cells in 50 mM Tris-HCl (pH 7.5) to a cell density of 31 g L⁻¹, passing them through the French press to open the cells and removing the cell wall fragments, membranes, and membrane-associated proteins by ultracentrifugation at 244 000 g at 4 °C for 45 min. Hydrolysis of **3** (10–20 mM) with these soluble cell-free extracts (20 g protein L⁻¹) gave the corresponding diol (1*R*,2*R*)-**4** in 85–86% ee and > 90% conversion.

Preparative hydrolysis was performed with 10 mM of **3** in 150 ml cell suspension (10 g L⁻¹) of *Sphingomonas* sp. HXN-200 at 25 °C. Higher than 99% conversion was reached after 22 h, and 159.3 mg (91.4%) of **4** was isolated in 99.6% purity and 87% ee.

In summary, the soluble epoxide hydrolase of *Sphingomonas* sp. HXN-200 has been found to catalyse the hydrolysis of *N*-benzyloxycarbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide with high enantioselectivity. The high yield preparation of the corresponding vicinal *trans*-diols in high ee has been demonstrated by simple enzymatic hydrolysis. This provides us with the first efficient bacterial EH for hydrolysis of a meso-epoxide.

Notes and references

[†] (3*R*,4*R*)-*N*-Benzyloxycarbonyl-3,4-dihydroxy-pyrrolidine **2** was prepared by hydrolysis of **1** (329 mg, 1.5 mmol) in a 100 ml cell suspension (10.0 g L⁻¹) of *Sphingomonas* sp. HXN-200 in 50 mM K-phosphate buffer (pH 8.0)

containing glucose (2%) in a 500 ml shaking flask at 200 rpm and 30 °C for 5 h. The reaction was stopped by removing the cells via centrifugation, and the product was extracted into ethyl acetate. The organic phase was separated, dried over Na₂SO₄, and the solvent was removed by evaporation. Purification by column chromatography on silica gel (*R*_f 0.27, ethyl acetate) afforded 289.1 mg (81.3%) of **2**: 95% ee; 98.9% purity; [α]_D²⁵ +7.6 (*c* = 1.80, CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ 7.34–7.26 (m, 5 H, Ph-H), 5.06 (s, 2 H, PhCH₂); 4.07 (s, *br*, 2 H, CHOH $\times$ 2); 3.86 (s, *br*, 1 H, OH), 3.63 (d, 2 H, *J* = 10.4 Hz, CH(H)N $\times$ 2), 3.35 (t, 2 H, *J* = 10.4 Hz, CH(H)N $\times$ 2), 2.63 (s, *br*, 1 H, OH); ¹³C NMR (CDCl₃, 100 MHz) δ 156.71 (s, C=O), 137.43 (s), 129.58 (d), 129.18 (d), 128.89 (d) (aromatic C), 76.27 (d), 75.63 (d) (CHOH $\times$ 2), 68.28 (t, PhCH₂), 52.85 (t), 52.51 (t) (CHN, $\times$ 2); APCI-MS(40eV) *m/z* 238 (30%, *M* + 1), 194 (100%); IR (CHCl₃) ν 3406 (*br*, OH), 1693 (C=O) cm⁻¹. The conversion was analysed by HPLC. Column: Hypersil BDS-C18 (5 μ m, 125 mm $\times$ 4 mm); eluent: 10 mM K-phosphate buffer (pH = 7.0)–acetonitrile (70:30); flow: 1.0 ml min⁻¹; detection: UV at 210, 225, and 254 nm; *t*_R of **2**: 2.0 min, *t*_R of **1**: 4.2 min. The ee of **2** was determined by HPLC analysis with a chiral column (Chiralpak AS, 250 mm $\times$ 4.6 mm). eluent: *n*-hexane–isopropanol (97:3); flow rate: 1.0 ml min⁻¹; UV detection at 210 nm; *t*_R: 114.1 and 130.4 min.

[‡] The biohydrolysis of cyclohexene oxide **3** to **4** was followed by GC analysis. Column: Optima-5–0.25 μ m (25 m $\times$ 0.32 mm); temperature program: 60 °C for 1 min, then to 140 °C at 15 °C min⁻¹, and finally to 260 °C at 49 °C min⁻¹; *t*_R of **3**: 2.46 min, *t*_R of **4**: 4.54 min. The ee of **4** was determined by GC analysis with a chiral column (lipodex-A, 25 m $\times$ 0.25 mm). Temperature program: 60 °C for 1 min, then to 120 °C at 2 °C min⁻¹, 120 °C for 1 min, and finally to 160 °C at 40 °C min⁻¹; *t*_R of (1*S*,2*S*)-**4**: 26.13 min, *t*_R of (1*R*,2*R*)-**4**: 26.45 min.

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