DOI: 10.1002/adsc.200700027

Stereo-Complementary Two-Step Cascades Using a Two-Enzyme System Leading to Enantiopure Epoxides

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Received: January 16, 2007

Supporting information for this article is available on the WWW under http://asc.wiley-vch.de/home/.

Abstract: A novel one-pot, two-step, two-enzyme cascade is described. Pro-chiral α-chloro ketones are stereoselectively reduced to the corresponding halohydrins as an intermediate by a biocatalytic hydrogen transfer process. The intermediate is transformed to the corresponding epoxide by a non-enantioselective halohydrin dehalogenase. Thus, by combining a Prelog- or anti-Prelog alcohol dehydrogenase with a non-selective halohydrin dehalogenase, enantiopure (*R*)- as well as (*S*)-epoxides were obtained.

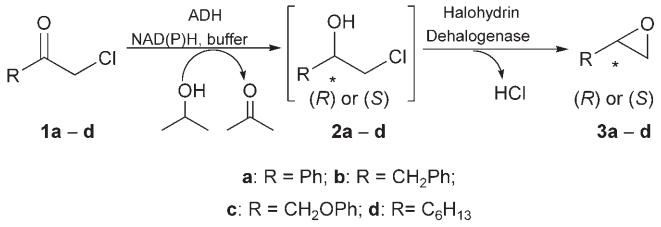
Keywords: asymmetric catalysis; cascade reactions; enzyme catalysis; epoxides; halohydrins; reduction

Introduction

Cascade or domino reactions[1,2] involve two or more sequential transformations without isolation of intermediates using one or more catalysts. Due to their elegance, but also due to the more complex system, cascade reactions recently gained significant attention.[1–5] We have recently described a cascade where in a first step α-chloro ketones were reduced to the corresponding halohydrins employing wild-type cells of *Rhodococcus ruber*. The halohydrins were transformed further in a second step to the epoxides under very basic conditions (pH 12).[6] Unfortunately, these reaction conditions applied with the wild-type cells of *Rhodococcus ruber* are not suitable either for the pure enzyme ADH-'A' or for *E. coli* cells containing overexpressed ADH-'A'. Therefore an alternative was required for an up-scaling of the cascade employing overexpressed/pure enzyme leading to enantiopure epoxides. The latter are important building blocks for the synthesis of biologically active compounds and pharmaceuticals[7] since they can easily react with several types of nucleophiles, affording β-substituted alcohols. Several chemical approaches have been designed in order to obtain enantiopure epoxides,[8] such as the Sharpless[9] or Jacobsen[10] epoxidation. Furthermore, biocatalytic methods have been employed, for example, mono-oxygenation of alkenes[11,12] or resolution of epoxides and precursors thereof.[12,13] Among them, transformations employing halohydrin dehalogenases are very promising.[12–15] These enzymes catalyse the opening of epoxides with various nucleophiles[16] such as azide,[14] cyanide[17] and nitrite[18] as well as the reversible ring-closure *via* dehalogenation of vicinal halohydrins to yield the corresponding epoxides.[19–21] These transformations usually go in hand with a kinetic resolution, which is limited to 50% yield for each enantiomer. To overcome this drawback, it seemed reasonable to employ them in a one-pot cascade process starting from the corresponding prochiral α-chloro ketones employing an alcohol dehydrogenase combined with a halohydrin dehalogenase.

Results and Discussion

To access both enantiomers of the epoxide, we planned to use either an (*R*)- or (*S*)-selective alcohol dehydrogenase (ADH) for the stereoselective reduction of the ketone moiety to the corresponding chiral alcohol. The latter represents an intermediate in the cascade, which is transformed by ring closure to the corresponding epoxide catalysed by a halohydrin dehalo-



Scheme 1. Cascade reaction to synthesise enantiopure epoxides.

genase (Scheme 1). In order to facilitate the NAD(P)H recycling required for the reduction step, we used ADHs which are able to catalyse the ketone reduction and the cofactor recycling at the same time.[22] In this 'coupled substrate' approach, 2-propanol was employed as a reducing agent *via* hydrogen transfer.

Lyophilised cells of *E. coli* containing the overexpressed ADH-'A' from *Rhodococcus ruber* DSM 44541 (*E. coli*/ADH-'A')[23,24] were chosen as a suitable catalyst to perform the first part of the cascade sequence since ADH-'A' has shown impressive operational stability in the presence of high concentrations of 2-propanol.[25] Excess of 2-propanol is not only used as hydride donor for cofactor recycling but also to solubilise the substrate in the aqueous phase.[22] Among several types of functionalised ketones, ADH-'A' reduces α-chloro ketones with excellent stereoselectivity affording the Prelog alcohols.[6] On the other hand, *Lactobacillus brevis* alcohol dehydrogenase (LBADH) was chosen to access the stereo-complementary products.[26]

For the second step of the cascade reaction, a halohydrin dehalogenase which possesses the matching enantioselectivity for the halohydrin produced in the first step was required. In a first approach, the halohydrin dehalogenase from *Mycobacterium* sp. GP1 (HheB)[27,28] was investigated.

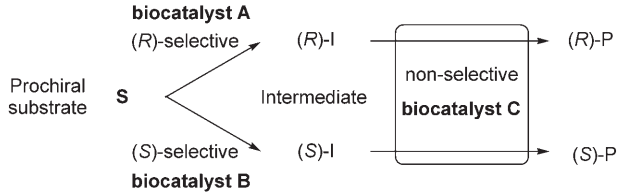
For determination of the enantioselectivity E of the halohydrin dehalogenase, racemic halohydrins **2a–d** were subjected to the biocatalytic ring closure. E values were determined from the *ee* of the halohydrin and the epoxide (Table 1).

The results showed that the halohydrin dehalogenase HheB converted both enantiomers at almost the same rate as indicated by the low E value (Table 1). Although this enzyme is obviously not suitable for a kinetic resolution of these substrates, HheB is an excellent candidate for cascade reactions in which the stereochemistry is created in the first step by an (*R*)- or (*S*)-selective biocatalyst (**A** or **B**, Scheme 2) because no enantiodiscrimination is needed in the second step. Actually, low or no enantiospecificity is an advantage, since a single dehalogenase (**C**) is sufficient and can be combined with ADH-'A' as well as LBADH leading to either the (*R*)- or (*S*)-epoxide.

Table 1. Enantioselectivities of the HheB-catalysed epoxide formation from halohydrins **2a–d**.

Substrate	Halohydrin		Epoxide		E
	[%][a]	*ee* [%]	[%][a]	*ee* [%]	
2a	91	16 (*R*)[b]	9	34 (*S*)[b]	2.3
2b	61	2 (*S*)[c]	39	5 (*R*)[c]	1.1
2c	65	2 (*S*)[c]	35	6 (*S*)[c,d]	1.1
2d	87	8 (*R*)[b]	13	7 (*S*)[b]	1.2

[a] GC yield after 5 h.
[b] Determined by enantioselective GC.
[c] Determined by enantioselective HPLC.
[d] Switch in CIP sequence priority.



Scheme 2. Cascade process using one enantioselective and one non-selective enzyme to obtain both product enantiomers.

However, it was not known whether HheB would tolerate the presence of 2-propanol and the co-product acetone from the first step. Fortunately, a test experiment showed that HheB lost only 25% of activity in up to 10% (v v^{-1}) of 2-propanol using (*R*)-2-chloro-1-phenylethanol (*R*)-**2a** as substrate (Figure 1).

A similar picture was observed for the activity of HheB in the presence of acetone. Subsequently, 5% (v v^{-1}) 2-propanol was the concentration of choice for the cascade reaction.

In order to tune the system for the cascade process, further parameters like enzyme or substrate concen-

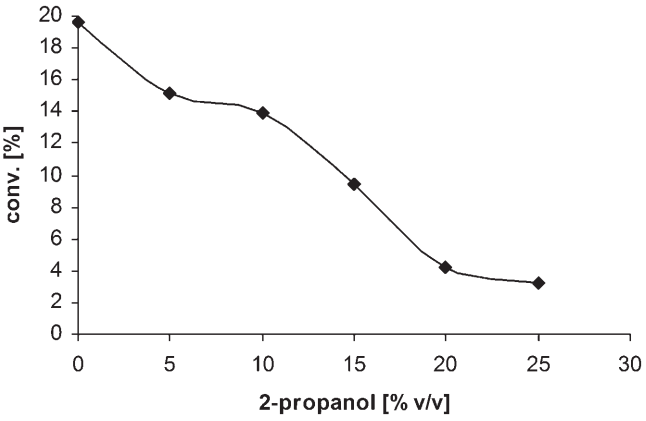


Figure 1. Conversion of (*R*)-**2a** to (*R*)-**3a** employing HheB (t = 3 h) at varied concentrations of 2-propanol.

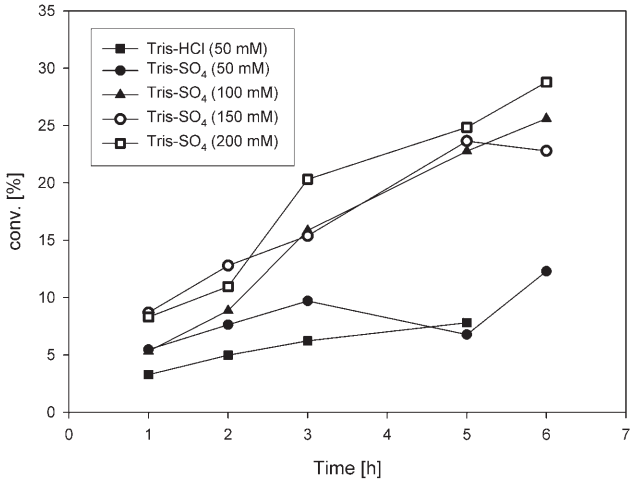


Figure 2. Effect of counter ion and buffer concentration on the conversion of the enzymatic ring-closure of (*R*)-**2a** catalysed by HheB at pH 7.5.

Table 2. Optimisation of reaction conditions for HheB catalysed ring-closure of (*R*)-**2a** (conversions after 3 h).

Entry	Enzyme conc. [mg mL^{-1}]	*T* [°C]	(*R*)-**2a** conc. [mM]	Conv.[a] [%]
1	0.04	30	75	11
2	0.08	30	75	15
3	0.04 + 0.04[b]	30	75	13[c]
4	0.04	r.t.	75	8
5	0.04	30	60	14
6	0.04	30	45	16
7	0.04	30	30	29
8	0.04	30	15	33
9	0.04	40	15	34

[a] Determined by GC.
[b] 'Fresh' enzyme added after 3 h.
[c] Conversion at 6 h.

tration, temperature and buffer were optimised for the formation of (*R*)-**3a** from halohydrin (*R*)-**2a** using HheB. Since biotransformations catalysed by halohydrin dehalogenases are reversible, the concentration of the halide ion should be kept low to minimise the back reaction. For instance, employing Tris-HCl buffer (50 mM) conversions lower than 10% were obtained (Figure 2). With a non-nucleophilic anion like sulphate as a counter ion for the buffer (Tris-SO_4), higher concentrations of buffer salt led to enhanced conversions. For the combined process, 150 mM Tris-SO_4 buffer (pH 7.5) seemed to be appropriate. In another approach, Ag_2SO_4 was added in the reaction mixture to bind the chloride and shift the equilibrium, however in this case no reaction was observed, probably due to inactivation of the enzyme.

Changes in the temperature or in the amount of enzyme did not lead to a significant increase of conversion within three hours (entries 1–4, 8, 9, Table 2). Lower substrate concentrations led to better apparent conversions (entries 5–8, Table 2). As a compromise, a substrate concentration of 45 mM, a temperature of 30 °C as well as 150 mM Tris-SO_4 buffer (pH 7.5) were chosen for the combined set-up.

For epoxides **3b**–**3d** no spontaneous hydrolysis to the corresponding diols was found under the reaction conditions investigated. However, spontaneous chemical hydrolysis of styrene oxide **3a** occurred to a certain extent (33.5% after 24 h), but at reduced incubation time (6 h) hydrolysis was negligible.

Finally, the enzymatic reduction *via* hydrogen transfer was combined with the biocatalytic ring-closure in a single process. As shown in Table 3, enantiopure epoxides were successfully obtained from prochiral α-chloro ketones in a one-pot two-enzyme cascade. Depending on the ADH employed, the (*S*)- as well as the (*R*)-epoxide were obtained with *ee* > 99%.

Table 3. Results of cascade reactions using substrates **1a–d**.

Substrate	ADH	Ketone **1**	Halohydrin **2**		Epoxide **3**	
		[%][a]	Yield [%][a]	*ee* [%][b]	Yield [%][a]	*ee* [%]
1a[c]	ADH-'A'	0	90	>99 (*R*)	7	>99 (*R*)[b]
1b	ADH-'A'	1	66	>99 (*R*)	33	>99 (*R*)[d]
1c	ADH-'A'	0	55	>99 (*R*)	45	>99 (*S*)[d,e]
1d	ADH-'A'	0	56	>99 (*R*)	44	>99 (*R*)[d]
1b	LBADH	86	3	>99 (*S*)	11	>99 (*S*)[d]
1c	LBADH	20	26	>99 (*S*)	57	>99 (*R*)[d,e]
1d	LBADH	32	34	>99 (*S*)	34	>99 (*S*)[d]

[a] GC yield after 5 h.
[b] Determined by enantioselective GC or enantioselective HPLC.
[c] 3% of diol formed.
[d] In a separate experiment it was proven that ring closure of an enantiopure halohydrin always leads to an enantiopure epoxide. The known mechanism of a halohydrin dehalogenase cannot lead to a decrease of the optical purity.[29]
[e] Switch in CIP priority.

Conclusions

A novel biocatalytic cascade reaction with two enzymes was reported starting from prochiral α-halo ketones, which were asymmetrically reduced to the corresponding halohydrins. The latter were transformed further into the corresponding epoxides by a non-selective halohydrin dehalogenase. Although this enzyme is not suitable for a kinetic resolution, it is a perfect candidate for a follow-up step in a cascade process, where the stereoselectivity is fixed in a previous step. In the first step the biocatalytic asymmetric reduction *via* hydrogen transfer by stereoselective ADHs furnished either the (*R*)- or the (*S*)-halohydrins, giving the possibility to access both enantiomeric branches. The stereochemical outcome of the process was controlled by the choice of the appropriate alcohol dehydrogenase in the first step, while a single non-selective halohydrin dehalogenase was sufficient to complete the cascade.

Experimental Section

General Remarks

NMR spectra were recorded in $CDCl_3$ using a Bruker AMX 360 at 360 (^{1}H) and 90 (^{13}C) MHz or a Bruker DMX Avance 500 at 500 (^{1}H) and 125 (^{13}C) MHz, respectively. Chemical shifts are reported relative to TMS ($\delta = 0.00$) and coupling constants (*J*) are given in Hz. TLC plates were run on silica gel Merck 60 F_{254} and compounds were visualised either by spraying with Mo reagent [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (100 g L^{-1}), $Ce(SO_4)_2\cdot 4H_2O$ (4 g L^{-1}) in H_2SO_4 (10%)] or by UV. Optical rotation values ($[\alpha]_D$) were measured on a Perkin–Elmer polarimeter 341 at 589 nm (Na line) in a one-dm cuvette.

For anhydrous reactions, flasks were dried and flushed with dry argon just before use. Standard syringe techniques were applied to transfer dry solvents and reagents in an inert atmosphere of dry argon. Anhydrous THF was distilled from potassium. Petroleum ether (bp 60–90 °C) and EtOAc used for chromatography were distilled prior to use.

α-Chloroacetophenone (**1a**), *rac*-(2,3-epoxypropyl)-benzene (**3b**), *rac*-(2,3-epoxypropyl) phenyl ether (**3c**), *rac*-1,2-epoxyoctane (**3d**), (*S*)-1,2-epoxyoctane (*S*)-**3d**, and 1-phenyl-1,2-ethanediol are commercially available.

Synthesis of Substrates and Reference Compounds

1-Chloro-3-phenyl-2-propanone (**1b**),[6] 3-chloro-1-phenoxy-2-propanone (**1c**,[6] 1-chloro-2-octanone (**1d**),[6] *rac*-2-chloro-1-phenylethanol (**2a**),[30] *rac*-1-chloro-3-phenyl-2-propanol (**2b**),[6] *rac*-3-chloro-1-phenoxy-2-propanol (**2c**)[31] as well as reference compounds (*R*)-**2a–2d** and (*R*)-**3a–3c** were synthesised as previously described.[6]

Epoxide Formation using HheB at Different Acetone and 2-Propanol Concentrations

HheB (20 μL, 1 mg mL^{-1}) and (*R*)-2-chloro-1-phenylethanol (3 μL, 22.5 μmol, 45 mM) were added to a Tris-SO_4 buffer (477 μL, 50 mM, pH 7.5). The appropriate amounts of acetone and 2-propanol were added (5–26 μL acetone or 2-propanol). The samples were incubated for 3 h at 30 °C and 120 rpm, extracted twice with ethyl acetate (2×350 μL), and dried (Na_2SO_4). The conversion was determined by GC.

Optimisation of Reaction Conditions for HheB

To elucidate the optimal conditions for reactions with HheB (20 μL, 1 mg mL^{-1}) several set-ups were investigated: Variation of substrate concentration [(*R*)-2-chloro-1-phenylethanol, 15 mM–75 mM], variation of HheB concentration and variation of reaction temperature. One sample (Table 2, entry 3) was incubated for 3 h at 30 °C and 130 rpm, then again 20 μL of HheB (1 mg mL^{-1}) were added and the sample was shaken for further 3 h. For all set-ups Tris-SO_4 buffer (50 mM, pH 7.5) was used. Samples were taken after 3 h, extracted twice with ethyl acetate (350 μL) and dried with Na_2SO_4. The conversion was determined using GC analysis.

Enzymatic Ring Closure of (*R*)-2-Chloro-1-phenylethanol: Optimisation of the Buffer

HheB (20 μL, 1 mg mL^{-1}) was added to Tris-HCl buffer (475 μL, 50 mM, pH 7.5). The reaction was started by adding (*R*)-2-chloro-1-phenylethanol (3 μL, 22.5 μmol, 45 mM) and incubated at 30 °C and 120 rpm. Samples were taken after 20 min, 40 min, 1 h, 2 h, 3.5 h, 4 h, 6 h and 24 h, extracted twice with ethyl acetate (2×350 μL) and dried (Na_2SO_4). The conversion was determined using GC analysis.

The experiment was repeated using (*R*)-2-chloro-1-phenylethanol (3 μL, 22.5 μmol, 45 mM) and Tris-SO_4 buffer (pH 7.5) at various concentrations (50 mM, 100 mM, 150 mM, 200 mM) and samples were taken hourly during a period of seven hours. The samples were extracted twice with ethyl acetate (350 μL) and dried (Na_2SO_4). The conversion was determined using GC analysis.

Enzymatic Ring-Closure of (*R*)-2-Chloro-1-Phenylethanol in the Presence of Silver Sulphate

HheB (20 μL, 1 mg mL^{-1}) and Ag_2SO_4 (7.8 mg, 50 mM) were added to Tris-SO_4 buffer (477 μL, 150 mM, pH 7.5) in Eppendorf vials, which were wrapped with aluminium foil. The reaction was started by adding (*R*)-2-chloro-1-phenylethanol (3 μL, 22.5 μmol, 45 mM) and incubated at 30 °C and 130 rpm. Samples were taken after 2 h, 4 h, 6 h and 24 h, extracted twice with ethyl acetate (2×350 μL) and dried (Na_2SO_4). The conversion was determined using GC analysis.

Test for Spontaneous Hydrolysis of Styrene Oxide

Styrene oxide (5 μL, 37.5 μmol, 75 mM) and HheB (20 μL, 1 mg mL^{-1}) were added to Tris-HCl buffer (500 μL, 50 mM, pH 7.5) and incubated at 30 °C and 120 rpm. Samples were

taken after 20 min, 40 min, 1 h, 2 h, 3.5 h, 4 h, 6 h and 24 h, extracted twice with ethyl acetate (2×350 μL) and dried (Na_2SO_4). The conversion was determined using GC analysis.

General Procedure for the Enzymatic Ring Closure of Halohydrins using HheB

HheB (20 μL, 1 mg mL^{-1}) was added to Tris-SO_4 buffer (480 μL, 150 mM, pH 7.5). The reaction was started by adding substrate **1a–d** (22.5 μmol, 45 mM) and incubated at 30 °C and 130 rpm. Samples were taken every hour, extracted twice with ethyl acetate (2×350 μL) and dried (Na_2SO_4). The conversion was determined using GC analysis.

General Procedure for the Enzymatic Cascade Reaction of α-Halo Ketones to the Corresponding Epoxides using *E. coli* Tuner™ (DE3)/pET22b-,ADH-A' and HheB

Lyophilised cells of *E. coli* Tuner™ (DE3)/pET22b-,ADH-'A' (5 mg, 7.5 U) were rehydrated in Tris-SO_4 buffer (480 μL, 150 mM, pH 7.5, 1 mM NADH) in an Eppendorf vial (1.5 mL) for 1 h at 30 °C and 120 rpm. Substrates **1a–d** (22.5 μmol, 45 mM), 2-propanol (26 μL, 5% v v^{-1}) and HheB (20 μL, 1 mg mL^{-1}) were added and the sample was incubated at 30 °C and 120 rpm. Samples were taken every hour, extracted twice with ethyl acetate (2×350 μL) and dried (Na_2SO_4). The conversion was determined using GC analysis.

General Procedure for the Enzymatic Cascade Reaction of α-Halo Ketones to Epoxides using Alcohol Dehydrogenase of *Lactobacillus brevis* and HheB

A stock solution of LB-ADH (11.1 U mg^{-1}, 8 mg mL^{-1}) in Tris-SO_4 buffer (150 mM, pH 7.5, 1 mM NADPH) was prepared. Substrates **1a–d** (22.5 μmol, 45 mM,), 2-propanol (26 μL, 5% v v^{-1}) LB-ADH stock solution (50 μL, 4.44 U) and HheB (20 μL, 1 mg mL^{-1}) were added to Tris-SO_4 buffer (435 μL, 150 mM, pH 7.5, 1 mM NADPH). The sample was incubated at 30 °C and 120 rpm. Samples were taken every hour, extracted twice with ethyl acetate (2×350 μL) and dried (Na_2SO_4). The conversion was determined using GC analysis.

For chiral analytics of all compounds see Supporting Information. Absolute configurations were assigned by (i) comparison of elution order on enantioselective GC with published data or by (ii) co-injection with commercial available material or independent synthesized chiral reference material.

Acknowledgements

I. L. is funded by the Research Centre Applied Biocatalysis. Financial support by FFG and the Province of Styria is gratefully acknowledged.

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