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Haloalkane dehalogenase catalysed desymmetrisation and tandem kinetic resolution for the preparation of chiral haloalcohols

Alja Westerbeek [a], Jan G.E. van Leeuwen [a], Wiktor Szymański [a,b], Ben L. Feringa [b], Dick B. Janssen [a,*]

[a] Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands
[b] Center for Systems Chemistry, Stratingh Institute for Chemistry and Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

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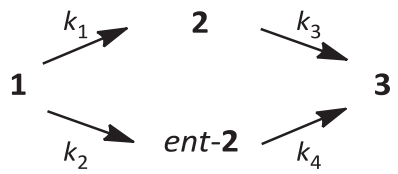
ABSTRACT

Six different bacterial haloalkane dehalogenases were recombinantly produced in *Escherichia coli*, purified, and used to catalyse the conversion of prochiral short-chain dihaloalkanes and a *meso* dihaloalkane, yielding enantioenriched haloalcohols. A two-reaction one-enzyme process was established in which the desymmetrisation of a dihaloalkane is followed by kinetic resolution of the chiral haloalcohol that is produced in the first step. In case of 1,3-dibromo-2-methylpropane and 1,3-dibromo-2-phenylpropane, an increase of the enantiomeric excess of the respective haloalcohol was observed in time, leading to ee values of >97%, with analytical yields of 24 and 52%, respectively. The results show that haloalkane dehalogenases can be used for the production of highly enantioenriched haloalcohols and that in some cases product enantiopurity can be improved by allowing a two-step one-enzyme tandem reaction.

1. Introduction

Enantiopure 1,2-haloalcohols (halohydrins) and 1,3-haloalcohols are chiral intermediates for the preparation of several important classes of compounds. Halohydrins can be converted into lactones[1] and epoxides,[2,3] which are versatile building blocks in organic synthesis.[4–7] For example, indene bromohydrin is a precursor for the anti-HIV protease inhibitor Indinavir,[4] and 1,3-haloalcohols are precursors of β-amino acids[8] and 1,3-aminoalcohols,[9] which are used in the synthesis of bioactive compounds and as chiral ligands,[10] respectively. Current biocatalytic routes towards enantiopure 1,2- and 1,3-haloalcohols include haloperoxidase-mediated conversion of unsaturated compounds to vicinal halohydrins,[11] kinetic resolution of halohydrins using a *Pseudomonas* strain,[12] and lipase-catalysed resolution of racemic alcohols and esters.[13–15] We are interested in exploring the possibility of employing isolated enzymes for the preparation of these compounds, using enantioenrichment methods that do not solely rely on kinetic resolution, e.g., by taking prochiral or *meso* compounds as starting materials.

Since haloalcohols can in principle be produced by enzymatic hydrolysis of dihaloalkanes, their preparation could be facilitated by the use of a tandem desymmetrisation/kinetic resolution process (Scheme 1). In such a system, the change of the enantiomeric excess of intermediate **2** over time depends on the ratios of the kinetic constants.[16] If one enantiomer of the intermediate is formed faster in the first step ($k_1 > k_2$), and consumed slower in the second reaction ($k_4 > k_3$), an increase of enantiomeric excess over time is expected,[17] unlike in a normal kinetic resolution, where product ee decreases over time, especially when the *E*-value is not very high. In the opposite case ($k_3 > k_4$), the enantiomeric excess of the intermediate will decrease, and it is possible that towards the end of the conversion the enantiomer of **2** that is not the preferred product in the first step predominates.[18]



Scheme 1. Reaction scheme for the tandem conversion of a prochiral or *meso* dihaloalkane **1** to chiral haloalcohol **2** and prochiral or *meso* diol **3**.

Applications of such single-enzyme tandem reactions were predominantly investigated using hydrolases.[16–18] Wang and coworkers[16] have shown that both porcine pancreatic lipase (PPL) and pig liver esterase (PLE) can perform a conversion of prochiral diesters with subsequent enhancement of the enantiomeric excess

* Corresponding author. Nijenborgh 4, 9747 AG, Groningen, The Netherlands. Fax: +31 50 363 4165; e-mail address: d.b.janssen@rug.nl (D.B. Janssen).

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of the monoester product by conversion of one enantiomer to the diol. In another study, lipase from *Pseudomonas cepacia* was used for the preparation of a cyclic monoester in a tandem reaction from the diester.[17] Other tandem desymmetrisation reactions[19] were based on the use of two dehydrogenases or two transaminases with complementary enantioselectivities. By enzymatically converting one enantiomer of an alcohol (or amine) to a prochiral ketone, followed by back-conversion of this achiral intermediate to the alcohol (or amine) using an enzyme with opposite product enantioselectivity, full conversion to obtain enantiopure alcohol[20,21] or amine[22] could be achieved.

The potential of haloalkane dehalogenases in stereoselective biocatalytic reactions was recently recognised and was employed in the preparation of chiral esters,[23] bromoalkanes[24] and haloamides[25,26] via kinetic resolution. In the current study, we explored the possibility of using haloalkane dehalogenases in a tandem reaction as described in Scheme 1. We tested the activity of haloalkane dehalogenases from five different sources for the activity and stereoselectivity with selected prochiral and *meso* compounds **1a**–**1e** (Fig. 1).

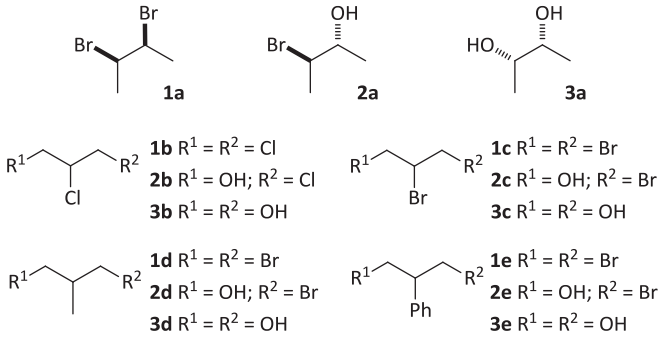

Fig. 1. Prochiral and *meso* substrates (**1a**–**1e**) together with the products of their single and double dehalogenation (**2** and **3**, respectively).

2. Results and discussion

2.1. Enzyme properties

Haloalkane dehalogenases used in this study were from *Xanthobacter autotrophicus* GJ10 (DhlA),[27] *Rhodococcus rhodochrous* NCIMB13064 (DhaA),[28] *Sphingomonas paucimobilis* UT26 (LinB),[29] *Bradyrhizobium japonicum* USDA110 (DbjA)[30] and *Mesorhizobium loti* MAFF303099 (DmlA).[30] DhaA31 is a mutant of DhaA with enhanced catalytic activity for 1,2,3-trichloropropane.[31] Haloalkane dehalogenase genes were expressed with an N-terminal His-tag under control of an L-arabinose inducible promoter in *E. coli* TOP10. Typically, around 50 mg of enzyme was obtained from a 1 L of overnight induced culture after purification by FPLC using Ni-NTA columns (GE Healthcare). The purity of the isolated enzymes was >95% as judged by SDS-polyacrylamide gel electrophoresis.

The kinetic parameters k_{cat} and K_M and the catalytic efficiency (k_{cat}/K_M) for the haloalkane dehalogenase catalysed conversions of compounds **1a**–**1e** were determined (Table 1). Due to the low substrate solubility, measurement of accurate values of K_M and k_{cat} values was not possible for all of the substrates, since saturation of the reaction rate could not be obtained with some compounds. In such cases, only the catalytic efficiency is reported. For all substrates, activity with at least one of the tested enzymes was found.

meso-2,3-Dibromobutane (**1a**) was converted by four of the six tested enzymes. Of these, LinB possessed the highest catalytic efficiency. The catalytic efficiency found with DhaA31 is 5-fold higher than with DbjA and DhaA, yet still an order of magnitude lower than with LinB.

Table 1
Kinetic parameters for conversion of prochiral/*meso* compounds with purified dehalogenases, measured in 50 mM Tris–SO_4, pH 8.2, at room temperature

Enzyme	Kinetic parameters	1a	1b	1c	1d	1e
DhaA	K_M	>2.0	4.8	0.34	<0.15	N.D.
	k_{cat}	>0.07	0.047	1.6	0.36	<0.015
	k_{cat}/K_M	0.031	0.01	4.7	>2.3	N.D.
DbjA	K_M	>2.2	N.D.	>1.0	0.17	N.D.
	k_{cat}	>0.05	k_{obs}=0.005	>0.9	0.76	N.D.
	k_{cat}/K_M	0.025	N.D.	0.87	4.5	<0.002
LinB	K_M	0.41	N.D.	0.19	0.17	N.D.
	k_{cat}	0.97	N.D.	1.4	1.4	≥ 0.8
	k_{cat}/K_M	2.4	<0.002	7.3	8.4	N.D.
DhaA31	K_M	>1.1	1.7	0.021	<0.15	N.D.
	k_{cat}	>0.19	0.47	1.6	0.22	≥ 0.4
	k_{cat}/K_M	0.14	0.28	77	>1.5	N.D.
DhlA	K_M	N.D.	N.D.	>1.1	>1.3	N.D.
	k_{cat}	N.D.	N.D.	>0.017	0.06	N.D.
	k_{cat}/K_M	<0.002	<0.002	0.015	0.050	<0.002
DmlA	K_M	N.D.	N.D.	>1.0	>1.3	N.D.
	k_{cat}	N.D.	N.D.	>0.015	>0.4	N.D.
	k_{cat}/K_M	<0.002	<0.002	0.015	0.34	<0.002

K_M in mM, k_{cat} and k_{obs} in s^{-1} and k_{cat}/K_M=mM^{-1} s^{-1}. K_M and k_{cat} values had an RSD of ≤20%. For compound **1e**, reactions were performed in 20% DMSO at maximum solubility of 0.5 mM. N.D.: not determined because saturation was not obtained due to low substrate solubility.

1,2,3-Trichloropropane (TCP, **1b**) is a toxic xenobiotic compound that is used in chemical synthesis and occurs as a side-product in the synthesis of epichlorohydrin and 1,3-dichloropropene. Improper disposal of TCP led to several cases of spreading of this pollutant into water.[32] TCP occurs mainly as a waste compound, but its enantioselective conversion would yield a valuable chiral building block.[33] Hydrolysis of **1b** using DhaA was improved using directed eviolution,[34] which led to a DhaA variant with six mutations (DhaA31).[31] This variant possesses a 28-fold higher catalytic efficiency towards compound **1b** than wild-type DhaA (Table 1), in agreement with data reported by Damborsky and co-workers.[31] The activity of the other enzymes with **1b** was very low or undetectable (Table 1), although low activity of LinB for compound **1b** was reported earlier under different conditions.[35]

The DhaA31 mutant also has by far the highest catalytic efficiency and affinity for tribromopropane **1c**. The catalytic rates of DhaA and LinB are in the same range, whereas the other enzymes are much less active with **1c**. Tribromopropane **1c** is a better substrate than trichloropropane **1b** for all the haloalkane dehalogenases, in agreement with the lower stability of the carbon-bromine bond.

With 1,3-dibromo-2-methylpropane (**1d**), high affinities and catalytic efficiencies were found for DhaA, DbjA, LinB and DhaA31. Again both DmlA and DhlA possessed much lower activity. To increase the solubility (0.5 mM) of 1,3-dibromo-2-phenylpropane (**1e**) in the reaction medium DMSO (20% v/v) was added. Compound **1e** was only converted by LinB and DhaA31, in agreement with the observation that LinB reacts best with bulkier compounds, like its original substrate 1,3,4,6-tetrachloro-1,4-cyclohexadiene.[29]

In summary, LinB and DhaA31 are the best enzymes for conversion of compounds **1a**–**1e**. Under the experimental conditions, DhaA31 converts all of the tested substrates. LinB shows measurable conversion of all compounds with rates of about 1 s^{-1}, except for 1,2,3-trichloropropane (**1b**), for which the enzyme has extremely low activity. DhaA and DbjA also convert 3–4 substrates, but DmlA and DhlA both have poor activity with all tested compounds.

2.2. Enantioselectivity

The enantioselectivity of the conversion of haloalkanes **1** to haloalcohols **2** was determined by measuring the enantiomeric excess of **2** after a certain reaction time (Table 2 and Table 4 in Experimental section).

Table 2
Enantiomeric excess of products **2** obtained in haloalkane dehalogenase catalysed conversion of substrates **1**

Enzyme/product	**2a**[a]	**2b**	**2c**	**2d**	**2e**
DhaA	88%	18% (*R*)	42% (*R*)	41% (*R*)	N.D.
DbjA	85%	39% (*R*)	15% (*R*)	70% (*S*)	N.D.
LinB	4.4%	52% (*R*)	24% (*R*)	30% (*R*)	79% (*S*)
DhaA31	64%	17% (*R*)	6% (*S*)	19% (*R*)	57% (*S*)
DmlA	N.D.	6% (*S*)	18% (*R*)	39% (*R*)	N.D.
DhlA	N.D.	49% (*R*)	3% (*R*)	49% (*R*)	N.D.

N.D.: not determined, because of low activity.
[a] For compound **2a** the absolute configuration was not determined.

Table 3
Kinetic parameters of conversion of 3-bromo-2-methylpropan-1-ol (**2d**) with DbjA

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)	*E*-value
(*R*)-Enantiomer	>0.7	>2.5	0.30	6
(*S*)-Enantiomer	>0.11	>2.3	0.047	

Table 4
Incubation times, amount of enzyme and substrate concentrations used in incubations

Enzyme (amount)	**1a** (1 mM)	**1b** (2 mM)	**1c** (1 mM)	**1d** (1 mM)	**1e** (0.5 mM)[a]
DhaA (0.64 mg)	30 min	90 min	20 min	60 min	—
DbjA (0.56 mg)	5 min	90 min	20 min	60 min	—
LinB (0.48 mg)	30 min	90 min	20 min	60 min	60 min[b]
DhaA31 (0.64 mg)	30 min	20 min	3 min	60 min	60 min[c]
DmlA (1.3 mg)	—	90 min	60 min	90 min	—
DhlA (0.62 mg)	—	90 min	60 min	90 min	—

[a] DMSO (20% v/v) was added to enhance the solubility.
[b] Divergent amount of LinB: 0.24 mg.
[c] Divergent amount of DhaA31: 0.32 mg.

The enantiomeric excess of **2a** was high in the reactions catalysed by DbjA and DhaA. The ee value measured for LinB product was very low. For the dichloropropanol **2b**, moderate ee values were found; here the highest value was obtained with LinB. The ee values for **2c** were low to moderate, with the highest value of 42% found with DbjA. This enzyme provided also the best result for **2d**. The enantiomeric excess of **2e** was only determined for LinB and DhaA31, which both gave values above 50%.

2.3. Tandem conversions for kinetic resolution of haloalcohols

We proceeded to study the possibility of using haloalkane dehalogenases in a tandem desymmetrisation/kinetic resolution process (Scheme 1), taking advantage of the fact that haloalkane dehalogenases have been described to convert haloalcohols to diols.[36] Based on the results presented in Table 2, we chose the most promising combinations of enzymes and substrates to perform reactions in which the concentrations of **1** and **2** and the enantiomeric excess of **2** were monitored in time.

Fig. 2A shows the DbjA-catalysed conversion of **1a** to **2a**, which was converted further to the diol **3a**. The enantiomeric excess of **2a** started at 85%, but this value decreased as the predominantly formed enantiomer of product **2a** is also the preferred substrate for the second reaction. This makes this process unattractive for a tandem desymmetrisation and kinetic resolution of **2a**.

The graph in Fig. 2B shows the conversion of **1b** by LinB, which was chosen because it provided the product **2b** with the highest enantiomeric excess (Table 2). The conversion of **1b** to **2b** is relatively slow (Table 1). The enantiomeric excess of **2b** is 58% at the beginning, and slightly decreases to 50% during the course of the reactions. Enantiopure dichloropanol **2b** is not obtained, which renders LinB unsuitable for its production. Next, the conversion of **1b** by DbjA (Fig. 2C) and DhlA (Fig. 2D) was attempted. When DbjA was used, the enantiomeric excess of **2b** stayed the same over the whole reaction time, while the concentration of **2b** was decreasing. For DhlA, a small kinetic resolution effect was observed, as the e.e. of **2b** slightly raised from 50 to 58%, but the enantioselectivity was too low and the conversion proceeded very slowly.

During the conversion of **1c** to **3c** by DhaA, the first reaction was very fast (Fig. 2E). (*R*)-**2c** is the preferred product of the desymmetisation step, but is also the preferred enantiomer for the second reaction leading to the diol **3c**. This second reaction even inverts the enantiomeric excess as conversion proceeds, since (*S*)-**2c** dominated at the end of the reaction. The unfavourable ratio of the kinetic constants (Scheme 1) makes this reaction unsuitable for production of enantiopure **2c**.

Fig. 2F shows the changes of the concentrations of **1d** and **2d** during the course of conversion by DbjA. The desymmetrisation of tribromopropane **1d** was fast, but gave an enantiomeric excess of only 28% for (*S*)-**2d**. During the course of the second reaction, this value went up slowly to 97% after 300 min, as the non-preferred enantiomer (*R*)-**2d** is the preferred substrate for the reaction to diol **3d** by DbjA. This confirms the beneficial effect of the enzyme-catalysed kinetic resolution on the e.e. of **2d**, even though the analytical yield at the e.e. of 97% is low, only 24%.

For 3-bromo-2-methylpropan-1-ol (**2d**), the commercially available enantiomers were used to measure the catalytic efficiencies. The determined *E*-value with DbjA is only 6 (Table 3), which is in agreement with the time-course experiment (Fig. 2D) where (*R*)-**2d** is the preferred substrate for the formation of the diol **3d**.

Fig. 3 shows the changes in enantiomeric excess of **2e** and concentration of **1e** and **2e** during the course of the reaction catalysed by LinB. The first reaction of **1e** to **2e** is very fast and produced (*S*)-**2e** with an e.e. of 64%. In the course of the much slower second conversion of **2e** to the diol **3e** the e.e. of (*S*)-**2e** increased to 98% after 20 h, as (*R*)-**2e** is the preferred enantiomer for the second reaction, resulting in kinetic resolution of **2e**. The final analytical yield of the reaction was 52%.

In the literature, various yields and ees were described for tandem desymmetrisation/kinetic resolution processes, ranging from 15% (95% ee)[16] to 51% (95% ee)[17] or 55% (99% ee),[37] i.e., in the same range as the values we found here. Further improvement would require enzymes with higher enantioselectivity in the first step or better complementarity in the tandem conversion. The engineering of enzyme variants with enhanced enantioselectivity has been intensively explored.[38,39] Haloalkane dehalogenases belong to the α/β hydrolase fold family of proteins, and several members of this class of enzymes were subjected to engineering studies[40] aimed at enhancing enzyme enantioselectivity, including lipases,[41–44] esterases,[38] and epoxide hydrolases.[45,46] For haloalkane dehalogenases, it has been shown that the enantioselectivity can also be enhanced by structure-based protein engineering.[24] Furthermore, directed evolution has yielded complementary mutants with enhanced selectivity in asymmetric conversion of trichloropropane **1b** to the enantioenriched 2,3-dichloropropanols.[33] In addition to protein engineering, expression of putative dehalogenases identified by genome sequence analysis is providing dehalogenase variants with new

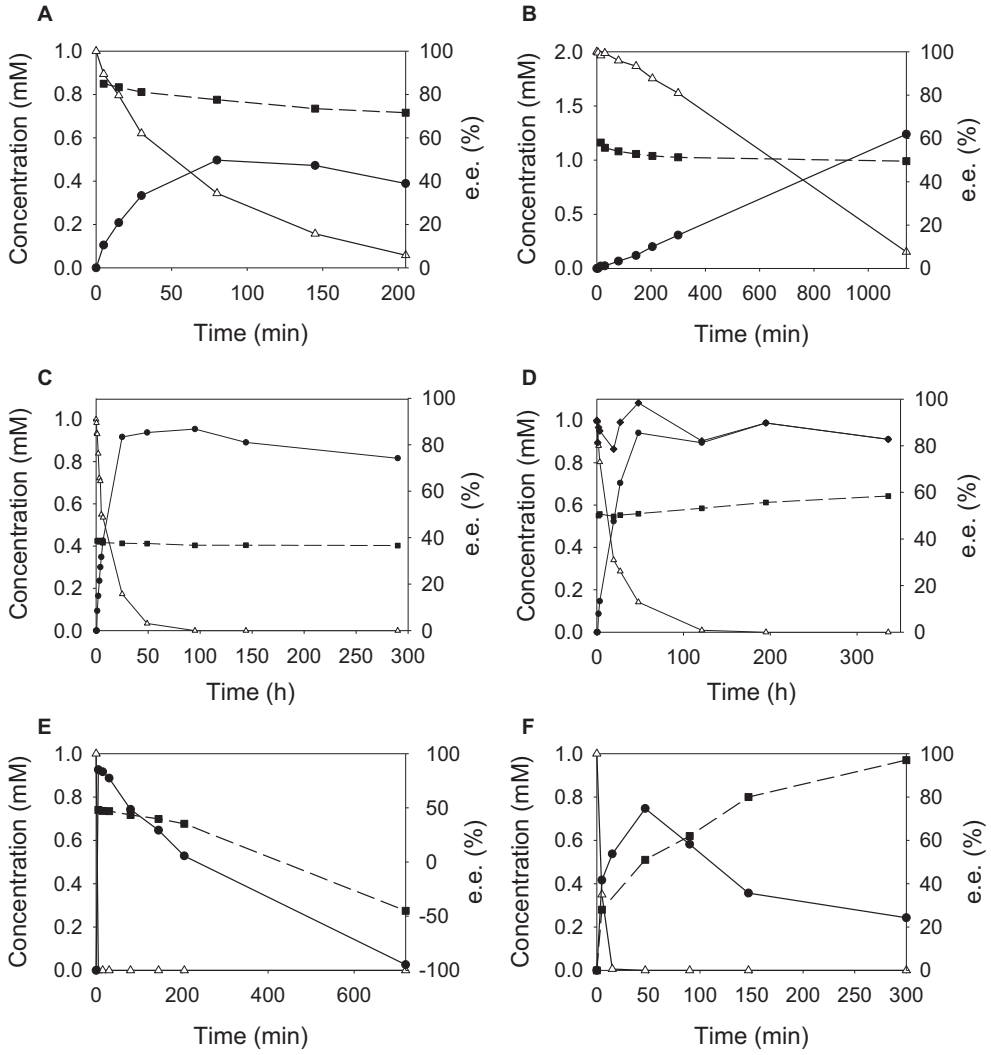

Fig. 2. Tandem conversion of **1a**–**1d** followed in time. Concentrations of substrates **1** (△) and haloalcohols **2** (●) are depicted, together with the enantiomeric excess of haloalcohols **2** (■, broken line). (A) Substrate **1a**, conversion with DbjA, (B) substrate **1b**, conversion with LinB, (C) substrate **1b**, conversion with DbjA, (D) substrate **1b**, conversion with DhlA, (E) substrate **1c**, conversion with DhaA, (F) substrate **1d**, conversion with DbjA.

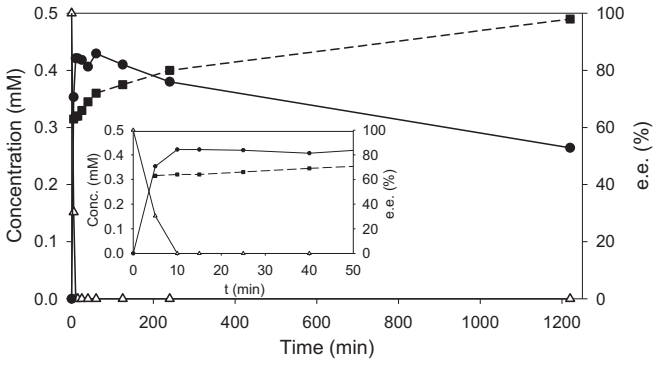

Fig. 3. Tandem conversion of *rac*-1,3,-dibromo-2-phenylpropane (**1e**) by LinB followed over time. The concentration of **1e** (△) and **2e** (●) are depicted, together with the enantiomeric excess of **2e** (■, dotted line). The inset shows the first 50 min of the conversion.

selectivities.[47–49] The combined use of genomics-based enzyme discovery and structure-based engineering would thus allow the development of a toolbox of dehalogenase variants tailored for specific biocatalytic processes including the type of asymmetric conversions and tandem reactions of *meso* and prochiral compounds explored here.

3. Conclusions

In this paper we report the activity of haloalkane dehalogenases towards prochiral and *meso* dihaloalkanes **1a**–**1e**. For four compounds, ee values from 52 to 88% were obtained in asymmetric conversions. The possibility of a sequential desymmetrisation and kinetic resolution, catalysed by the same enzyme, was demonstrated for products **2a**–**2e**. The activity of haloalkane dehalogenases in conversions of substrates **1** allowed the use of 6–23% (w/w) of the catalyst. Although the reported yields were modest, the results comprise the first use of haloalkane dehalogenases for such a tandem process.

4. Experimental section

4.1. General

^{1}H NMR spectra were recorded at 300 or 400 MHz with $CDCl_3$ as solvent. ^{13}C NMR spectra were obtained at 75.4 or 100.59 MHz in $CDCl_3$. Chemical shifts were determined relative to the residual

solvent peaks ($CHCl_3$, δ=7.26 ppm for ^1H, δ=77.0 for ^{13}C). The following abbreviations are used to indicate signal multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Enantiomeric excess determinations were performed by capillary GC analysis or HPLC analysis using a flame ionisation detector or UV-detection (all in comparison with racemic products, column and conditions further specified in relevant experiment descriptions). Optical rotations were measured in $CHCl_3$ on a polarimeter with a 10 cm cell (*c* given in g/100 mL). Flash chromatography was performed on silica gel. Solutions were dried with $MgSO_4$ or Na_2SO_4 and concentration steps were conducted with a rotary evaporator.

Compounds **1a**,**1c**, **3d** and **3e** were purchased from Sigma–Aldrich, compounds **1b** and **2b** were purchased at TCI Europe, and compound **2c** was purchased at Acros Organics. Racemic compound **2a** was obtained according to a literature procedure.[50]

4.2. General procedure 1: preparation of 1,3-dibromides from 1,3-diols

N-Bromosuccinimide (2.2 mmol) was added at 0 °C to a mixture of diol (1.0 mmol) and triphenylphosphine (2.2 mmol) in dichloromethane (10.0 mL). The cooling bath was removed and the reaction mixture was stirred at room temperature for 30 min. The solvent was evaporated and the product was purified by column chromatography (Silicagel, 40–63 μm, pentane).

Compound ***1d***: 1,3-dibromo-2-methylpropane. Yield: 71%. Colourless oil. ^1H NMR (300 MHz, $CDCl_3$): δ 1.15 (d, 3H, ^3J=7.2 Hz, CH_3), 2.22–2.44 (m, 1H, C*H*), 3.45 (dd, ^2J=10.2 Hz, ^3J=6.0 Hz, 2H, 2×CH_2Br), 3.53 (dd, ^2J=10.2 Hz, ^3J=5.1 Hz, 2H, 2×CH_2Br); ^{13}C NMR (50 MHz, $CDCl_3$): δ 18.1,37.2, 37.9; MS (EI^+) *m*/*z* 216 (M^+, 15), 135 (70), 55 (100).

Compound ***1e***: 1,3-dibromo-2-phenylpropane. Yield: 66%. Colourless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 3.38 (quintet, ^3J=6.6 Hz, 1H, C*H*), 3.73 (dd, ^2J=10.4 Hz, ^3J=6.4 Hz, 2H, 2×CH_2Br), 3.80 (dd, ^2J=10.4 Hz, ^3J=6.8 Hz, 2H, 2×CH_2Br), 7.22–7.38 (m, 5H, Ar*H*); ^{13}C NMR (50 MHz, $CDCl_3$): δ 35.8, 49.2, 127.8, 128.2, 129.0, 139.8; MS (EI^+) *m*/*z* 278 (M^+, 10), 197 (30), 183 (50), 117 (40), 104 (100), 91 (55).

Reference racemic compound *rac*-**2e** was obtained by performing general procedure 1 with 1 equiv of both *N*-bromosuccinimide and triphenylphosphine. The compound was obtained as a colourless oil. ^1H NMR (400 MHz, $CDCl_3$): δ 3.18–3.25 (m, 1H, C*H*), 3.66 (dd, ^2J=10.0 Hz, ^3J=6.4 Hz, 2H, CH_2Br), 3.95 (m, 2H, CH_2OH), 7.22–7.38 (m, 5H, Ar*H*), consistent with the literature.[51]

4.3. Determination of enantiomeric excess with single time point measurements

The enantiomeric excess values described in Table 1 were determined by adding enzyme (0.24–1.3 mg) to 10 mL of a solution of substrate (0.5–2 mM) in 50 mM Tris, pH 8.2. The mixture was incubated at 30 °C, for 3–90 min, depending on enzyme activity.

Ee values of the haloalcohols **2** were determined by chiral chromatography analysis. For compound **2a**: chiral GC, Chiraldex G-TA (1 mL/min, isothermal, 42 °C), retention times (min) 36.9 and 38.3. For compound **2b**: chiral GC, Hydrodex-β-TBDAc (1 mL/min, isothermal, 140 °C), retention times (min) 8.2 (*S*) and 8.6 (*R*). For compound **2c**: chiral GC, Hydrodex-β-TBDAc (1 mL/min, isothermal, 140 °C), retention times (min) 9.6 (*S*) and 9.9 (*R*). For compound **2d**: chiral HPLC, after transformation into *p*-nitrobenzoyl ester following literature procedure,[52] Chiralcel OJ-H (heptane/*i*-PrOH 97:3, v/v, flow 0.5 mL/min), retention times (min) 36.3 (*R*) and 39.0 (*S*). For compound **2e**: chiral HPLC, Chiralcel OD-H (heptane/*i*-PrOH, 93:7, v/v, flow 0.5 mL/min), retention times (min) 19.4 (*R*) and 20.2 (*S*).

4.4. Determination of absolute configuration

For compounds **2b** and **2c**, the absolute configuration was determined by transformation into the epihalohydrin[18] and comparison with a commercial sample. For compound **2d**, this was done by comparison with a commercial sample. The absolute configuration of compound **2e** was determined by isolation of the enzymatic reaction product and comparison of optical rotation with a literature value ($[\alpha]_D$ +12.8 (*c* 0.67, $CHCl_3$) for 68% ee), lit.[52] ($[\alpha]_D$ −21.7 (*c* 1.8, $CHCl_3$) for the (*R*)-enantiomer).

4.5. Enzyme production

For construction of the haloalkane dehalogenase gene expression plasmid, the genes were cloned into a pBAD vector, with addition of a DNA segment encoding an N-terminal hexahistidine tag. Precultures were started by inoculating 5 mL of LB medium with *E. coli* TOP10 transformants from glycerol stocks and incubated overnight at 37 °C, while shaking. The next day, 2 L of LB_{amp} medium was inoculated with 1% of an overnight culture and incubated at 37 °C with shaking. Enzyme expression was induced by adding 0.002% (w/v) L-arabinose at when the OD_{600} reached 0.6. Cultures were incubated overnight with shaking at 30 °C, except for the culture aimed at expression of LinB, which was incubated at 17 °C. After growth, the cells were harvested, broken by sonication, centrifuged and the supernatant with the haloalkane dehalogenases was used for purification using three in line His-Trap Ni-NTA columns from GE Healthcare attached to an AKTA purifier system. The purified enzymes were concentrated using an Amicon ultrafiltration cell and stored in TMG buffer (10 mM Tris–SO_4, 1 mM β-mercaptoethanol and 10% (v/v) glycerol, pH 7.5). Protein concentrations were determined with Bradford reagent (Biorad) using bovine serum albumin as the standard. The purified enzyme was analysed using SDS-polyacrylamide gel electrophoresis.

4.6. Enzymatic reactions

Kinetic parameters were determined using a halide release assay. Assays were done at room temperature in 200 μL 50 mM Tris–SO_4, pH 8.2. For compound **1e**, 20% of DMSO was added to enhance the solubility to 0.5 mM. Samples were taken at different times and after adding mercuric thiocyanate and ferric ammonium sulfate the absorption was measured at 460 nm.[27,53] The initial rates of halide release at different substrate concentrations were plotted in a Michaelis–Menten graph in order to determine the kinetic parameters.

Time-course experiments were done as follows. To 10 mL of a solution of compound **1** in 50 mM Tris–SO_4, pH 8.2, enzyme was added and the mixture was incubated at 30 °C (for details, see Table 5). At various times, 1 mL samples were taken and extracted with a solvent containing dodecane as internal standard. The samples were dried with $MgSO_4$. The enantiomeric excess values for haloalcohols **2a**–**2c** were determined by Chiral GC and for **2d** and **2c** by chiral HPLC, as described above. For the reactions with

Table 5
Details of the tandem conversions of compounds **1**

Compound	**1a**	**1b**	**1b**	**1b**	**1c**	**1d**	**1e**
Enzyme (mg)	DbjA (0.56)	LinB (0.48)	DbjA (5.0)	DhlA (5.0)	DhaA (0.64)	DbjA (0.50)	LinB (0.24)
Solution (mL)	10	10	20	20	10	20	30
Substrate (mM)	1	2	1	1	1	1	0.5
Substrate (mg)	2.2	2.9	2.9	2.9	2.8	4.3	4.2
Extraction solvent (mL)	$CHCl_3$ (0.5)	$CHCl_3$ (0.5)	$CHCl_3$ (0.5)	$CHCl_3$ (0.5)	$CHCl_3$ (0.5)	CH_2Cl_2 (1.5)	Et_2O (2.0)
Sample vol. (mL)	1	1	1	1	1	2	2

compounds **1a**, **1b** and **1c**, the conversion was determined with the same GC program, whereas for **1d** and **1e** a different GC program was used. For **1d**: AT5 GC column (80 °C→130 °C (10 °C/min), 130 °C for 1 min, 130 °C→80 °C (20 °C/min), retention times 3.8 min (**2d**) and 4.4 min (**1d**)). For **1e**: AT5 GC column (80 °C→180 °C (10 °C/min), 180 °C for 1 min, 180 °C→80 °C (20 °C/min), retention times: 10.9 min (**2e**); 11.9 min (**1e**)).

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