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Org. Process Res. Dev., **Just Accepted Manuscript** • DOI: 10.1021/acs.oprd.7b00179 • Publication Date (Web): 30 Jun 2017

ACS Publications

Organic Process Research & Development is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Development of an enzymatic process for the production of (*R*)-2-butyl-2-ethyloxirane

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Abstract

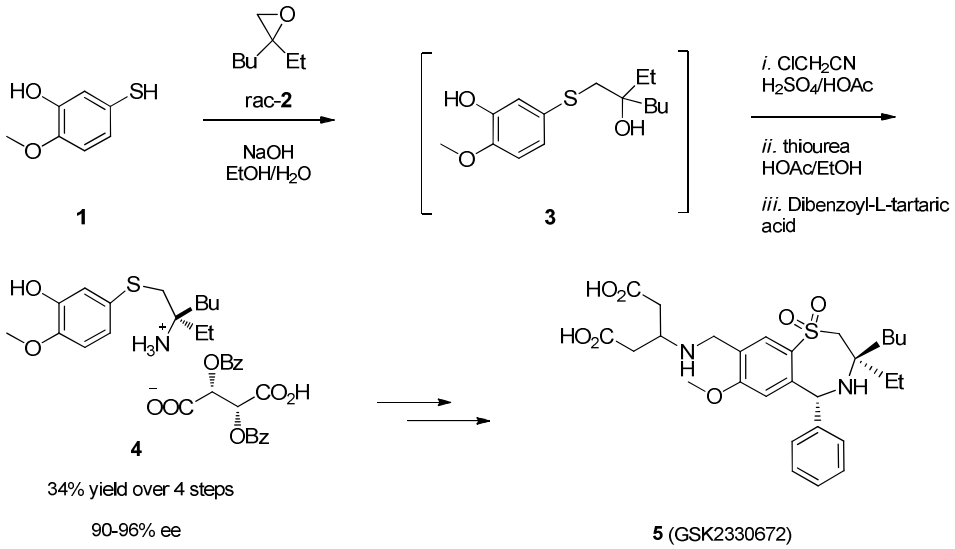
An epoxide resolution process was rapidly developed that allowed access to multi-gram scale quantities of (*R*)-2-butyl-2-ethyloxirane **2** at greater than 300 g/L reaction concentration using an easy to handle and store lyophilised powder of epoxide hydrolase from *Agromyces mediolanus*. The enzyme was successfully fermented on a 35L scale and stability increased by downstream processing. Halohydrin dehalogenases also gave highly enantioselective resolution, but were shown to favour hydrolysis of the (*R*)-**2** epoxide, whereas epoxide hydrolase from *Aspergillus niger* instead provided (*R*)-**7** via an unoptimized, enantioconvergent process.

Keywords: Biocatalysis, Biotransformations, Epoxide Hydrolase, Halohydrin dehalogenase, Enzyme

1. INTRODUCTION

GSK2330672 (**5**), an ileal bile acid transport (iBAT) inhibitor indicated for diabetes type II and cholestatic pruritus, is currently under Phase IIb evaluation in the clinic.[1] The API is a highly complex molecule containing two stereogenic centres, one of which is quaternary. A previous medicinal chemistry route to a related molecule had introduced the quaternary stereocentre using an enantiopure activated aminoalcohol or aziridine.[2] However, these building blocks themselves required a lengthy synthesis starting from a quaternary amino acid, which was resolved using pig

liver esterase.[3] In addition to the fact that the use of mammalian sourced enzymes should be avoided unless absolutely necessary due to transmissible spongiform encephalopathies (TSE) risks,[4] a more concise route, via ring opening of the racemic epoxide **2** with thiophenol **3** and conversion of the resulting alcohol to amine via a Ritter reaction was therefore developed (Scheme 1).[1]

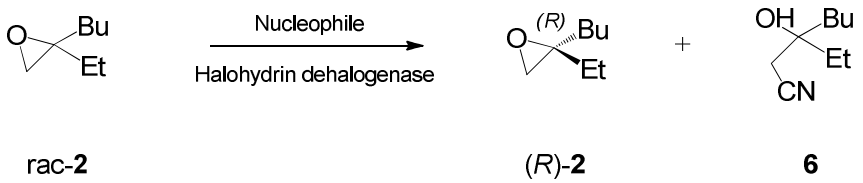


Scheme 1. Current route to chiral intermediate 4 in the synthesis of GSK2330672

To avoid the need for a wasteful classical resolution to produce the tartrate salt **4**, we wanted to access the enantioenriched epoxide (*R*)-**2** directly. Numerous chemocatalytic[5] and biocatalytic methods[6] have been reported to access chiral epoxides by asymmetric or kinetic resolution approaches from either unactivated alkenes or aldehydes, but few are capable of efficiently producing chiral 2,2-disubstituted epoxides. Perhaps the most elegant chemical approach is via asymmetric Corey-Chaykovsky reaction using a heterobimetallic La-Li3-BINOL complex (LLB) as reported by the Shibasaki group.[7] However, whereas excellent yields and enantioselectivities

are achieved for the synthesis of various aryl or alkyl-2-methyloxiranes, 2-aryl-2-ethyloxiranes are produced with only moderate enantioselectivity which was deemed too low for our needs.

We first turned our attention to halohydrin dehalogenases (HHDHs) to access (*R*)-**2**. HHDHs are lyases that catalyze the ring-closure of halohydrins to produce epoxide rings.[8] They are also capable of catalyzing epoxide ring-opening with numerous nucleophiles allowing the enantioselective kinetic resolution of epoxides. We were interested in assessing the possibility of accessing (*R*)-**2**, prepared readily in racemic form by achiral Corey-Chaykovsky reaction from cheap starting materials,[9] using a resolution approach based on the ring opening of racemic epoxide with cyanide using halohydrin dehalogenases (HHDHs; EC 4.5.1) (Scheme 2).



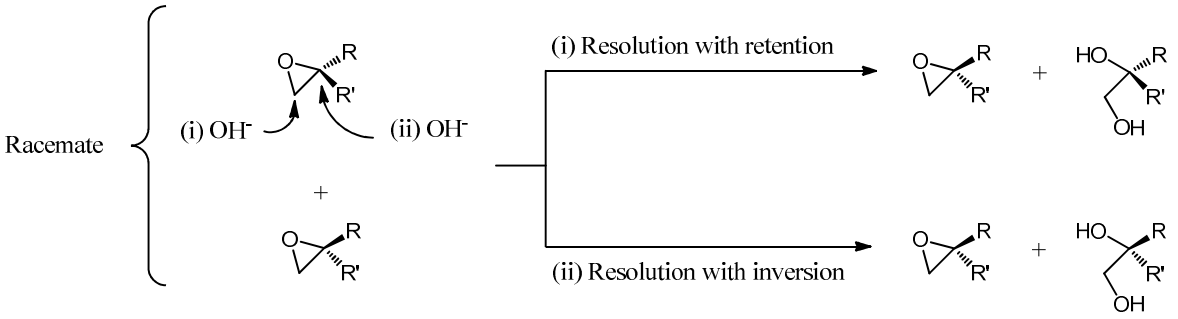
Scheme 2. Halohydrin dehalogenase catalysed enantioselective ring opening.

Although this approach is relatively new, a broad range of halohydrin dehalogenases have recently become available that we were keen to apply and investigate for this methodology.[10]

We also set out to investigate a similar resolution approach based on the ring opening of racemic epoxide with epoxide hydrolase enzymes (EHs), which can only ring open epoxides using water as the nucleophile (EHs; EC 3.3.2.3). It is well known that a variety of epoxides, including geminally disubstituted derivatives, can be hydrolytically cleaved with high enantiospecificity using EHs under extremely mild and benign aqueous conditions.[6b]

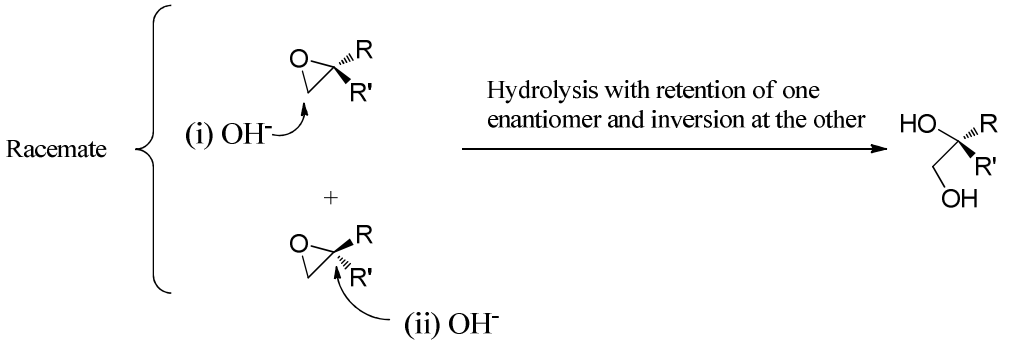
Depending on the orientation of the epoxide within the enzyme active site, nucleophilic attack may occur at different ends of the epoxide resulting in retention or inversion to afford diol and

residual epoxide of opposite or the same absolute configuration (Scheme 3). Either through acid hydrolysis,[11] or primary alcohol activation and ring closure,[12] it is then possible to access >50 % yields of diol or epoxide respectively.



Scheme 3. Hydrolytic kinetic resolution using (i) retaining and (ii) inverting enantioselective epoxide hydrolases (R, R' = alkyl).

In a few limited examples, some epoxide hydrolases have also been reported to selectively hydrolyse both epoxide enantiomers simultaneously at different carbon centres respectively to afford >50 % yields of enantiopure diol in an "enantioconvergent" fashion (Scheme 4).[13]



Scheme 4. Hydrolytic deracemisation using an enantioconvergent epoxide hydrolase.

Although a close literature example reported by Mitchitz *et al* gave only moderate enantioselectivity (E=7.2) from a hydrolytic resolution of a structurally related epoxide to compound **2** using EH from *Rhodococcus* sp. NCIMB 11216,[14] we hoped that by testing a small range of EH homologues, we could find a good starting hit for the preparation of (*R*)-**2** that could be further improved through directed evolution, as successfully applied towards this enzyme class for the synthesis of other epoxides.[15]

2. RESULTS AND DISCUSSSION

Screening of two Codexis HHDH panels identified variant HHDH-P1H08 as the top hit in terms of conversion and selectivity. Initial efforts toward process development uncovered that at higher substrate loading (20 mg mL^{-1}), the rac-**2** is immiscible in the Tris-SO_4 buffer system used as solvent. From this observation, we chose to focus on the use of biphasic reaction media using either immobilised enzyme or whole cells. Enzyme immobilization and the use of enzymes in whole cells are both known to increase enzyme stability, including in organic solvents. Several co-solvents such as toluene, *tert*-butyl methyl ether (TBME), 2-methyl tetrahydrofuran, isopropyl acetate, cyclohexane and ethyl acetate have been tested. We found that, on a small scale (<1 mL), HHDH-P1H08 immobilized on Sepabeads EC-EP/M (90 wt%) could resolve 40 mg mL^{-1} of rac-**2** to 69% ee with 43% conversion, using 0.6 equiv NaCN in 20% v/v of 200 mM Tris-SO_4 in ethyl acetate. However, when increasing the scale of this reaction to 30 mL, the conversion decreased significantly with a concomitant drop in ee. This could have resulted from the reduced surface area between phases to volume, resulting in decreased rate of substrate/product transfer between phases, but was not further investigated. This unfavourable result led us to focus on the second strategy of whole cells in a biphasic system. Indeed, we

found we could resolve 8 g of rac-**2** to 95% ee with 52% conversion as determined by GC. Unoptimized purification by extraction and distillation allowed us to produce 1.85 g of (*S*)-**2** in 95% ee. This enantioenriched epoxide was subsequently found to afford the unwanted enantiomer of a known intermediate in the current route to GSK2330672, indicating that the epoxide (*S*)-**2** had been obtained from the HHDH resolution.[16] Unfortunately, no good HHDH hits were found that produce the opposite enantiomer in sufficient enantioselectivity.

Turning our attention to EHs, we initially intended to select and order genes for a range of homologues that are known to accept geminally disubstituted epoxides or are known to perform enantioconvergent hydrolyses. We were surprised to find that the sequences of epoxide hydrolases commonly reported for the resolution of geminally disubstituted epoxides or the enantioconvergent hydrolysis of epoxides, such as the epoxide hydrolases from *Rhodococcus* or *Nocardia* sp., have not been reported in the literature.[17]

In spite of the diversity in sequence space within the largest, α/β-hydrolase fold family of epoxide hydrolases, it is well known that their core structure is actually extremely similar.[18] We therefore decided to select a range of genes from this family to primarily include epoxide hydrolases that are reported to express well (Table 1).

Table 1. Selected epoxide hydrolase constructs.

EH No.	Construct	Description	Reference
1	S sp K8 EH	*Sphingomonas sp*. K8 ID: ZP_09141165.1/ WP_010127425	19
2	S sp MC1 EH	*Sphingopyxis sp* MC1 (53% identity to *Sphingomonas sp*. K8, but higher similarity to *Sphingomonas sp* HXN-200)	20
3	NaDSM 12444	*Novosphinbium aromaticivorans* DSM 12444 ID:	21

	EH	ABD26703	
4	An LCP 631 EH	*Aspergillus niger* LCP 631 I: AJ238460	22
5	Am ZJB120203EH	*Agromyces mediolanus* ZJB1202030ID: JX467176	23
6	Ar AD1 echA	*Agrobacterium radiobacter* AD1 (echA) ID: O31243	20
7	MalE-Ar AD1 echA	MalE fused *Agrobacterium radiobacter* AD1	24
8	Cm Ch34 EH	*C. metallidurans* Ch34 ID:YP_583993	25

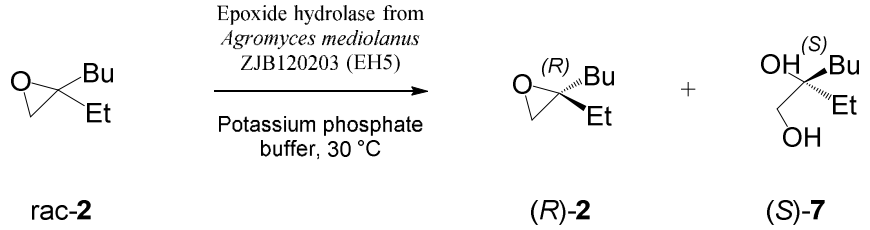
The EH proteins were expressed from BL21(DE3) cells transformed with pET28a vectors containing *N*-terminal His6-tagged genes. All EHs expressed well as expected, but most displayed poor enantioselectivity or activity towards rac-**2** in small scale scouting experiments using whole cells (**Table 2**).

Table 2. Initial screening data for EH 1-8.[a)]

Epoxide hydrolase	Time (min)	Conv. (%)	**2** e.e. [%]	**2** Config.	**7** e.e. [%]	**7** Config.	Selectivity (E)
EH1 [b]	60	10	3	(*R*)	30	(*S*)	2
EH2 [c]	10	58	8	(*S*)	5	(*R*)	1
EH3 [c]	5	92	4	(*R*)	0	-	n.d.[d]
EH4 [b]	15	55	98	(*S*)	70	(*R*)	25
EH5 [b]	60	65	87	(*R*)	76	(*S*)	20
EH6 [c]	15	63	13	(*S*)	8	(*R*)	1
EH7 [c]	60	53	6	(*S*)	25	(*R*)	2
EH8 [b]	60	40	35	(*R*)	82	(*S*)	14

a) Reaction conditions: rac-**2** (1–20 μL) was added to whole cell paste (10-15 mg) containing expressed epoxide hydrolase in potassium phosphate buffer 100 mM, pH 7.4 (1.5 mL) and the mixtures shaken at 1300 rpm at 30°C using a thermomixer. Aliquots (100 μL) were extracted into ethyl acetate (500 μL) and analysed by chiral GC (for GC conditions check supplementary information). Negative controls containing empty pET28a vector only or buffer, suggested low substrate hydrolysis in the buffer (2-3% in 20 h). b) substrate concentration of 4.3 mM. c) Substrate concentration of 86.5 mM. Absolute configuration assignment determined after derivatizing the chiral epoxide to intermediate **4** and chiral HPLC analysis. d) n.d.= not determined.

EH1 exhibited low activity when reactions were run at 4.3 mM, whereas EHs2-3 were approximately 20 fold more active, although the enantioselectivities for the epoxide **2** were very low in all cases. The EH4 from *Aspergillus niger* produced the diol (*R*)-**7** with moderate enantioselectivity (70% ee) at 55% conversion. A follow-up experiment at the same scale, but allowing the reaction to progress further (92 % conversion to the (*S*)-diol over 23 h) afforded the (*S*)-diol in 88% ee, demonstrating that the enzyme operates towards rac-**2** in an enantioconvergent fashion. Although the resulting epoxide had the undesired stereochemistry for our purposes, to the best of our knowledge, enantioconvergency had not previously been reported for this enzyme. In contrast, EH5 favoured hydrolysis of (*S*)-**2** with retention of stereochemistry to afford (*R*)-**2** and (*S*)-**7** (Scheme 5).[26] EHs6 and 7 provided poor enantioselectivities, whereas EH8 performed similarly to EH5, but with slightly reduced activity and enantioselectivity.



rac-**2** (*R*)-**2** (*S*)-**7**

Scheme 5. Epoxide hydrolase from *Agromyces mediolanus* catalysed enantioselective ring opening.

Cost analysis of the potential resolution and enantioconvergent approaches to (*R*)-**2** demonstrated little overall difference, whereas the latter would require an additional chemistry step to generate epoxide from diol. We therefore only progressed the resolution approach using the epoxide hydrolase from *Agromyces mediolanus* (EH5).

To optimize the reaction, several experiments were performed to improve parameters which are typically known to influence enzyme enantioselectivity and activity: catalyst loading, co-solvents, pH and temperature. Addition of higher enzyme amounts provided the desired enantioselectivity but the resulting faster reaction rate could impede easy monitoring and quenching if inappropriately controlled. As a consequence, we found that the addition of sufficient enzyme to allow the reaction to reach the selected end point (epoxide 60% conversion or 95% epoxide ee – see below) in approximately 15h allowed sufficient time to monitor reaction progress.

A reaction temperature of 25°C resulted in a slower rate and reduced selectivity, whereas reaction at 35°C increased the rate of background hydrolysis over the enzyme catalysed reaction. As a consequence, further reactions were performed at 30°C. Water immiscible co-solvents were next evaluated, to assess EH5 enantioselectivity/activity using a substrate concentration of 125 mg/mL (8 volume process) using two buffer/organic solvent ratios: 1:4 and 4:1 (v/v) respectively. However, none of the conditions tested led to an improvement over buffer alone.

Table 3. Hydrolysis of rac-2 using EH5 in the presence of various organic co-solvents.[a)]

Entry	Co-solvent		Conv[b)]	(*R*)-**2**	(*S*)-**7**	Selectivity
		(%)	(%)	e.e. (%)	e.e. (%)	(E)
1	None	-[b]	63	98	n.d.[c)]	16
2	Ethyl acetate	20	28	39	82	15

3	Ethyl acetate	80	2	n.d.	n.d.	n.d.
4	TBME	20	52	84	73	17
5	TBME	80	3	n.d.	n.d.	n.d.
6	Heptane	20	59	90	68	16
7	Heptane	80	7	n.d.	n.d.	n.d.
8	Toluene	20	50	73	77	17
9	Toluene	80	4	n.d.	n.d.	n.d.

a) Aqueous/organic solvent / 100 mM potassium phosphate buffer pH 7.4, 4/1 and 1/4 (v/v), EH5, 125 mg/mL rac-**2**, final volume 1 mL, 30°C. Two conical centrifuge tubes of EH5 containing 1.05 g cell paste dissolved in 8 mL 100 mM potassium phosphate buffer pH 7.4 in total and then split equally to all reactions. b) Conversion time point taken after 20h, initial experiments showed activity did not change when reaction times >20h. c) n.d.= not determined.

Experiments run at elevated pH (8.0 and 8.5) with EH5 displayed an increased rate of background hydrolysis and therefore lower pH conditions (7.0 and 7.5) were selected in order to obtain a higher enantioselectivity

To isolate the desired enantioenriched epoxide, several solvents such as heptane, *tert*-butyl methyl ether, hexane, diethyl ether, toluene and ethyl acetate were tested in order to effect selective extraction. Heptane was found to preferentially extract epoxide, resulting in a 64/36 mixture of epoxide to diol in the combined organic phases after 3 x 1 vol extractions and only <0.1% of residual epoxide in the aqueous phase. Further washing of the combined heptane phase with water (2 x 4 vols) led to further enrichment, resulting in a 92:8 epoxide/diol mixture. In contrast, extraction of both epoxide and diol into ethyl acetate (2 x 5 vols) led to a more practical approach. The resultant combined ethyl acetate portions could then be readily concentrated under reduced pressure and the epoxide isolated by vacuum distillation at 20±5 mbar with the jacket temperature set to 90°C.

With an isolation method in hand and before further scale-up, we needed to ensure that the stereochemistry remained intact in the subsequent Ritter reaction, determine the required enantiopurity of (*R*)-**2** to meet the API specification and determine whether vacuum distillation of the epoxide is necessary or whether the crude epoxide/diol mixture could be used directly. Samples of (*R*)-**2** (90% ee and 95% ee respectively) were thus transformed into the free amine of compound **4** under the standard Ritter conditions without any observable deterioration in enantiopurity. Isolation of compound **4** as the tartrate salt by crystallisation resulted in an enhancement in enantiopurity to 96% ee and 98% ee respectively, the latter of which was successfully converted through to API of acceptable enantiopurity. The minimum target for the enantiopurity of (*R*)-**2** was therefore set at 95% ee for subsequent process optimisation.

To assess whether vacuum distillation of the epoxide was necessary, the crude epoxide/diol mixture was used as input for the Ritter transformation.. The desired product **4** was obtained in typical purity with no deterioration in enantiomeric excess while the low molecular weight diol is dicarded as waste. The telescoped conditions provided a more robust and streamlined sequence moving forward.

For further scale-up work, larger quantities of EH5 enzyme were required. A large scale fermentation process using recombinant EH5 in pET28a vector and expressed in competent *E coli* BL21(DE3) cells, was developed, producing 1.43 kg of cell paste from a 35L growth. This cell paste gave significantly higher activity per gram than had been observed previously from cells produced in shake flasks when the same conditions and scale were tested, due to a better protein expression.

Having larger quantities of EH5 in hand, further process development work was performed using 22 g (97% purity) quantities of racemic **2** in 250 mL controlled laboratory reactors (CLRs).

Initially, we intended to test several enzyme forms in parallel reactions, using a final reaction volume of 200 mL at, 30°C and stirring at 300 rpm in a dish-bottomed CLR using a PTFE retreat curve impeller. However, from initial trial reactions using fresh cell paste, we quickly found that in contrast to our findings on a smaller scale, they could be performed in just 3 vols of buffer media (338g/L substrate concentration) (Table 4, entry 1) without any impact on enantioselectivity as long as the appropriate loading of cell paste was charged to keep the reaction rate sufficiently high to allow completion times of 15-22 h. The reaction was also successfully run over shorter reaction times such as 10 h, slow enough to allow monitoring whilst also avoiding deterioration in enantioselectivity to maximise yield. Reaction sampling was initially performed using achiral GC analysis. However, significant variation in the results were obtained due to the biphasic nature of the reaction mixture and the condensation of some of the volatile epoxide on the lid of the CLR. To avoid these two issues, reaction progression was instead followed by monitoring changes in ratio of the two epoxide enantiomers by chiral GC, and work-up commenced once the target enantiomeric excess of the epoxide had been achieved.

We also investigated the practicality of longer term storage and shipping of the enzyme. Therefore, a batch of frozen cell paste was tested for stability after 8 weeks, with no significant change in performance, which suggested that the cell paste could be stored at -80°C prior to processing if required (Table 4, entry 2). The (*R*)-**2** could be isolated by extraction into ethyl acetate (1 x 2.4 vols). However, phase separation was not ideal, presumably due to cell debris, with a persistent emulsion layer being observed. Slow stirring (50 rpm) did not break the emulsion, although quick filtration through wet Celite grade 545 and subsequent washing with ethyl acetate (1 x 1.4 vols) allowed the removal of the cell debris which was noticed to favour emulsion formation.

However, the longer-term need for low temperature storage of large volumes of frozen cell paste, the potential need to ship large volumes of biocatalyst and the emulsion formation on extraction made for a less than ideal process. We therefore looked towards the use of lyophilised whole cell and clarified lysate powders as more convenient, low-cost alternative. Enzyme immobilisation was also considered at this stage and discounted based on the negligible impact of biocatalyst on epoxide cost of goods and the high cost of supports. Hence, we decided that it is best to discard the enzyme after each use and not immobilise.

Enzyme activity was unaffected upon lyophilisation suggesting that the enzyme is very robust (Table 4, entry 3). However, emulsion formation on product extraction still persisted (Figure 1). A batch stored at -20°C tested 3 weeks after lyophilisation again remained unchanged (Table 4, entry 4).

Table 4. Enantioselectivities from the hydrolysis of rac-2 using different forms of EH5.[a)]

Entry	Enzyme type	Enzyme loading		Conv.[c)]	Time	(*R*)-**2**
		(g)	(%w/w)	(%)	(h)	e.e. (%)
1	Fresh cell paste	8	36	66.4	15	96.4
2	Frozen cell paste[b)]	8	36	67.2	15	96.7
3	Lyophilised whole cells	2[e)]	9.1	68.7	15	96.6
4	Lyophilised whole cells[d)]	2[e)]	9.1	65.7	15	95.0
5	Lyophilised unclarified lysate	2.2[f)]	10	62.3	22	92.0
6	Lyophilised clarified lysate	2.1[f)]	9.5	58.9	20	91.1

a) Reactions were run in a 250 mL CLR, final reaction volume 65 mL, 22g substrate (loading 338 g/L) and 100 mM potassium phosphate buffer pH 7.4 at 30°C. All enzyme forms originate from the same enzyme batch. b) Batch retested after 8 weeks of storage at -80°C. c) Conversion after 15 h, Product was not isolated but in these cases GC yield was reported. d) Batch retested after 3 weeks of storage at -20°C. e) Amount of lyophilised enzyme was produced from 8 g of cell paste (using the 35 L fermentation procedure 41 g of cell paste were obtained / 1 L of fermentation broth). No buffer was added prior to lyophilisation. f) Amount of lyophilised

enzyme was produced from 8 g of cell paste. Prior to lysis, cell paste was resuspended in 32 mL 25 mM potassium phosphate buffer, pH 7.4.

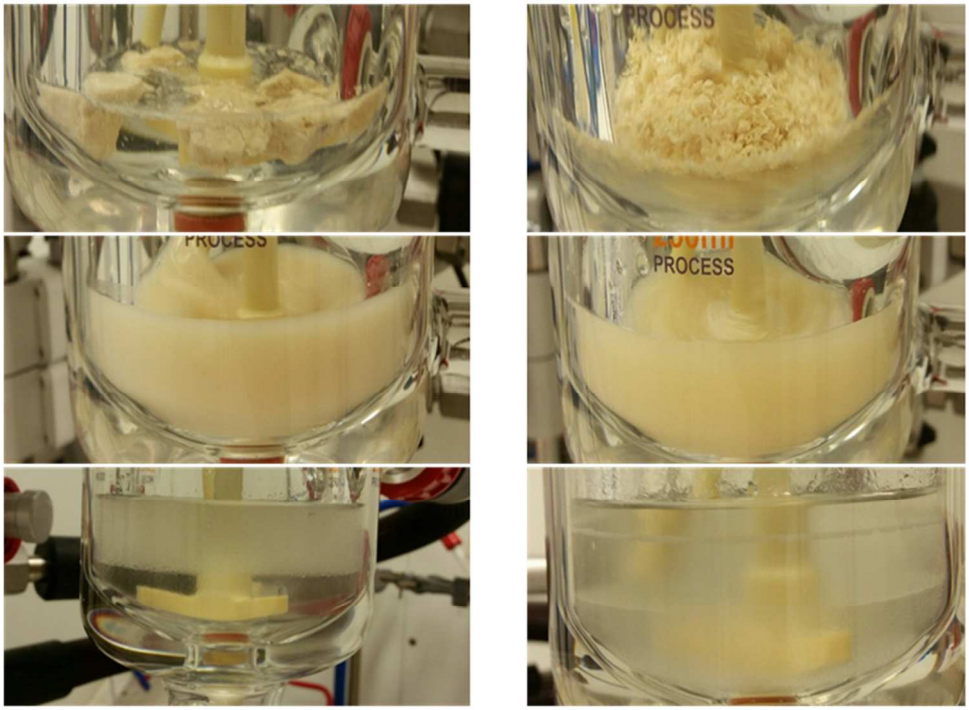


Figure 1. CLR screening (22 g)– lyophilised whole cells (left hand side) *vs* lyophilised clarified lysate (right hand side). From top to bottom (time 0 = lyo powder addition, t=10 min and bottom after ethyl acetate and brine addition).

Initial attempts to produce lyophilised unclarified lysate (from 8 g of cell paste) by using sonication or cell disruption resulted in a small loss of activity during lysis (92% ee after 22 h) (**Table 4**, entry 5). However, the high activity of lyophilised unclarified lysate suggested that the enzyme is stable upon cell disruption, although emulsion formation was still observed during reaction workup. Attempts to break the emulsion by increasing jacket temperature to 40-45°C combined with stirring failed to improve separation. Higher amounts of ethyl acetate (5-8 vols) provided a better layer separation, whereas water addition proved to be detrimental.

Ultimately, lyophilised clarified lysate powder (9.5% w/w) was tested and gave comparable activity to unclarified lyophilised lysate. The reaction occurred with very good enantioselectivities, and the drop in activity was no higher than 5% (**Table 4**, entry 6). Furthermore, we noticed a visible reduction in emulsion formation during workup, with layer separation occurring after approximately 2 min, compared to >5-10 min in the previous cases. After ethyl acetate extraction, brine addition (1.2 vols), and stirring at 50 rpm for 3 min, complete layer separation was achieved within 5 min (**Figure 1**, right hand side). Biocatalyst robustness experiments, showed only a slight drop in activity (<15%) on storage at -20°C over a 5 month period.

The wild-type EH5 provided very good activity at high substrate loading. As such, there was no significant value in engineering an improved enzyme. However, several mutants of EH5, previously developed for improved enantioselectivity towards epichlorohydrin by Xue et al,[26] were selected for further evaluation to gauge whether improvements could be gained through further enzyme modification if desired.

Selected mutants (S207V, N240D, W128F, S207V/N240D, W128F/S207V, S207V/N240D/W182F) were also screened (data not shown). We found that the N240D (EH15) mutant gave a slight improvement in enantioselectivity (E=23) towards racemic epoxide **2** compared to wild-type (E=20), and at reduced enzyme loading compared to wild type (5-8% w/w). Small scale experiments and further reaction optimisation performed in the same manner as EH5, and initial process development showed that using EH15 the enzyme loading could be reduced to 5-8% w/w.

Given that EH15 appeared to offer significant advantages over EH5, fermentation and downstream processing to produce a lyophilised, clarified lysate powder form of the biocatalyst

was evaluated. A 10 litre fed batch fermentation was performed to generate high cell density material and assess the efficacy of an industrial process. In comparison to the batch fermentation the fed system achieved a higher biomass with maximum OD_{600} >150. The intracellularly over-expressed protein was recovered by mechanical cell lysis of the fermentation broth or resuspended cell paste, followed by clarification by centrifugation in the presence or absence of a flocculating agent. The broth lysate or resuspended cell lysate treated with polyethylenimine acetate solution (PEI) as flocculating agent prior to lyophilisation (clarified lysate), was observed to have a lower turbidity upon resuspension of resultant lyophilised protein powder within the reaction mixture than lysates without flocculent (non-clarified lysate). Furthermore, this had a positive impact on the extraction step. In summary, a high activity clarified powder was produced that could be readily stored refrigerated at 4°C for months with minimal activity loss. The PEI treated lysates were observed to improve phase separation during product extraction as less suspended cell debris were present at the recovery step.

3. CONCLUSIONS

Multi-gram scale quantities of (*R*)-2-butyl-2-ethyloxirane **2** were successfully prepared by the kinetic resolution of racemic **2** at 338 g/L reaction concentration using an easy to handle and store lyophilised powder of epoxide hydrolase from *Agromyces mediolanus*. The enzymes EH5 and EH15 were successfully fermented on a 35L scale. EH15 was also produced using high cell density fermentation with the enzyme stabilized by using several downstream processing conditions. EH15, a variant of EH5 gave improved activity and enantioselectivity and so it is likely that enzyme engineering would result in further improvements. As we had tested only a small set of EHs selected almost at random one would not be surprised if screening of further

natural enzymes would also lead to even better hits towards either resolution or enantioconvergent approaches to the asymmetric synthesis of geminally disubstituted epoxides. Epoxide hydrolase from *Aspergillus niger* provided instead (*R*)-2-butyl-2-ethyloxirane **2** and shown to be enantioconvergent. On the same note HHDHs were also shown to favour the (*S*)-**2** formation.

4. EXPERIMENTAL SECTION

General Remarks: General Remarks: The genes encoding EHs1-8 and EH15 containing N-terminal His_6-tags were codon optimised for expression in *E. coli* BL21(DE3), synthesised, and cloned into pET28a at GenScript. Double strand sequence confirmation was performed at GenScript. Rac-**2** was prepared using the Corey-Tchaikovsky reaction and used without further purification. Unless otherwise stated, the reactions were performed in test tubes (1.5 mL), conical centrifuge tubes (50 mL), conical flasks (120 mL) and controlled laboratory reactors (250 mL). 1 Wt is defined as the weight of 2-butyl-2-ethyloxirane **2** charged to the reactor in grams. All other weights, volumes and equivalents given are calculated relative to this figure. The reaction progress was monitored by chiral GC and products were assigned by comparison with authentic samples. Conversion values determined by GC were calculated based on rac-**2** consumption and formation of the alcohol **7**. NMR spectra were recorded on a Bruker Advance 400 (^{1}H: 400 MHz, ^{13}C: 101 MHz) spectrometer using TMS as internal standard (d=0). Small amounts of cell paste were lysed using a Fisher Scientific sonicator model FB705 equipped with a specific probe depending on the lysis volume (up to 100 mL). Higher amounts of cell paste were disrupted using a microfluidiser M110Y from analytikLtd. Lyophilisation was performed using a BPS VirTris SP Scientific Advantage Pro lyophiliser.

For the enzyme fermentation a 50 L Fermenter SOP Stuart Pope (BPES) 07957 125 829 was used. Run parameters: 35 L working volume. 30 L/min air, 200 rpm, 0.2 bar. For the second fermentation procedure production stage was carried out in 12 L Braun bioreactors. The batch fed enzyme fermentation was carried out in 12 L Braun bioreactors with Siemens control system, 10 L working volume employing rushton turbine impellors. Dissolved oxygen was monitored using Hamilton Visiferm DO sensors and ph monitored using Hamilton Easyferm 325 pH sensors. Feed solutions were introduced via Watson Marlow 502S peristaltic pumps with feedback control via the Siemens control system. OD_{600} measurements were recorded using a Cecil Spectrophotometer.

Small scale reactions. Two conical centrifuge tubes (2 x 50 mL) containing Luria Broth medium (2 x 10 mL) and kanamycin (2 x 10 µL, 1000x, final conc: 50 µg/mL) were pre-inoculated with a colony of *Agromyces mediolanus* ZJB120203 (EH5) in *E. coli* BL21(DE3) cells (grown previously on an agar plate containing kan 50 µg/mL and 1% glucose) and incubated for 5 h at 37°C with shaking in an orbital shaker at 220 rpm (O.D. after 5 hours = 0.4). This preculture was used to inoculate Terrific broth medium TB (2 x 1 L) containing kan (final conc: 50 µg/mL) and allowed to grow at 37°C (in a 2.8 L baffled shake flask) until O.D. of 0.6–0.8 at 600 nm was reached (after approximately 3 hours), then IPTG was added (final conc: 0.5 mM) and the culture grown at 30 °C for 19 h keeping the same shaking (Kuhner shaker 220 rpm). Cells were pelleted by centrifugation (2 x 50 mL and 2 x 950 mL) using 50 mL conical centrifuge tubes (20 min, 4000 rpm, 4°C) and 1L centrifuging bottles (20 min, 4000 rpm at 4°C). The pellet was stored overnight in the freezer at -20°C. Pellet obtained from 50 mL fermentation broth (cell wet mass =1.05 and 1.08) and 950 mL fermentation broth (cell wet mass =16.61 and 16.87) was resuspended in potassium phosphate buffer (pH 7.4, 100 mM), mixture being

transferred to an Erlenmeyer flask (see above for sizes). Reactions were started by addition of 2-butyl-2-ethyloxirane (**2**) and were carried out in an Kuhner shaker at 220 rpm at 30°C. Reaction evolution was monitored via chiral GC, (50 µL samples were taken each hour and extracted with ethyl acetate 600 µL).

Preparation of (*R*)-2-butyl-2-ethyloxirane (2)

Lyophilized EH5 from clarified lysate (12 wt%) was charged to the reaction vessel. Potassium phosphate buffer pH 7.4 (100 mM, 1.9 vol) was then charged to the same reaction vessel and the agitation adjusted to 300 rpm. When suspended the reaction was started by the addition of racemic 2-butyl-2-ethyloxirane (22.6 g, 176.3 mmol, 1 wt). The reaction mixture was stirred at 30°C and reaction monitored by chiral GC until the enantiomeric excess (ee) of (*R*)-2-butyl-2-ethyloxirane **2** reached a value ≥ 95% (*R*) (typically conversion is around ≥ 62±2% over a maximum 15 hr time period). The reaction was quenched by adding ethyl acetate (2.4 vol). The resultant biphasic solution was then filtered over Celite. Additional ethyl acetate (1.2 vol) was used to wash the Celite cake. The layers were then separated. The aqueous layer was discarded. The organic layer was washed with brine (1.2 vol). The organic layer was then concentrated by distillation under reduced pressure to afford a neat mixture of the desired epoxide (*R*)-2-butyl-2-ethyloxirane (**2**) and diol by-product (*S*)-2-ethylhexane-1,2-diol **7**. The mixture was distilled at 90°C and 20±5 mbar to give the desired epoxide (*R*)-2-butyl-2-ethyloxirane (4.58 g, 20% yield, 99.2% purity, 95% ee). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.61 (d, J = 4.9 Hz, 1H), 2.59 (d, J = 4.9 Hz, 1H), 1.72−1.46 (m, 4H), 1.42−1.26 (m, 4H), 0.99−0.87 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 60.2, 52.2, 33.7, 27.0, 26.9, 22.9, 14.0, 8.9.

Preparation of (*R*)-5-((2-amino-2-ethylhexyl)thio)-2-methoxyphenol (4)

A reaction vessel was charged with 3-hydroxy-4-methoxythiophenol (564 mg, 3.61 mmol), (*R*)-2-butyl-2-ethyloxirane (**2**) (509 mg, 3.97 mmol) and EtOH (3.4 mL). The mixture was treated with a solution of NaOH (318 mg, 7.94 mmol) in water (2.3 mL). The mixture was stirred at ambient temperature for 20 h under dry nitrogen gas. The mixture was treated with toluene (4 mL) and stirred for 2 min. The layers were separated and the organic layer was discarded. The aqueous layer was neutralized with 2N HCl and extracted with toluene. The extract was washed successively with saturated aqueous Na_2CO_3 solution and water and concentrated in vacuum to give intermediate **3** as an oil. The oil intermediate **3** was dissolved in chloroacetonitrile (5.5 mL) and HOAc (2 mL). The mixture was cooled to 0^{o}C. H_2SO_4 (0.96 mL, 18.05 mmol), pre-diluted with 0.33 mL of water) was added at a rate maintaining the temperature below 5^{o}C. After stirring at below 10^{o}C for 0.5 h, the reaction mixture was treated with water, extracted with MTBE. The extract was washed with saturated aqueous $NaHCO_3$ and concentrated in vacuo to give intermediate chloroacetamide protected amine as an oil. The oil intermediate chloroacetamide protected amine was then dissolved in EOH (9.1 mL) and treated with HOAc (1.8 mL) and thiourea (0.412 g, 5.42 mmol). The mixture was heated at reflux until completion, and then cooled to ambient temperature. The solids were removed by filtration The filtrate was concentrated in vacuo to give an oil. The oil was treated with ethyl acetate, washed successively with saturated aqueous Na_2CO_3 solution and water, and then concentrated in vacuo to give intermediate **4** (851mg, 83% yield over 3 steps, 79% purity) as an oil. 425 mg of the intermediate **4** was further purified by silica gel chromatography to give intermediate **4** (223 mg, 100% purity, 94.8% ee). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (d, *J* = 2.2 Hz, 1H), 6.85 (dd, *J* = 8.4, 2.2 Hz, 1H), 6.67 (d, *J* = 8.4 Hz, 1H), 5.23 (s, 1H), 3.78 (s, 3H), 2.87 (s, 2H), 1.46−1.28 (m, 4H), 1.25−1.05 (m, 4H), 0.81 (t, *J* = 6.9 Hz, 3H), 0.76 (t, *J* = 7.45 Hz, 3H). ^{13}C NMR (125 MHz,

$CDCl_3$) δ 146.8, 146.0, 129.1, 122.9, 117.7, 111.2, 56.0, 54.8, 47.5, 38.6, 31.8, 25.8, 23.3, 14.1, 8.0.

Enzyme expression in fermenter (50 L)

Seed stage: A sterile Corning Erlenmeyer flask (500 mL) containing Luria Broth medium (100 mL, LB) and kanamycin final conc: 50 µg/mL) was inoculated with 10 µL of glycerol stock cells and incubated overnight at 37°C with shaking in a Kuhner shaker at 200 rpm. A secondary seed stage was inoculated using a 1% inoculum from the above primary seed into 8 sterile glass Erlenmyer flasks (500 mL) containing Luria Broth medium (100 mL, LB), kanamycin final conc: 50 µg/mL) and glucose (final conc 1%) and incubated at 30°C at 200 rpm in a Kuhner shaker overnight

Production stage: Production fermentations were carried out in a 50 L Pierre Guerin Biolaftte bioreactor using 35 L of TB medium, 1% glycerol, 2 mL/L antifoam (DC1520), 50 µg/mL kanamycin and 2% inoculum from the secondary seed stage. The fermenter was incubated at 37°C, agitated at 200 rpm with air supplied at 30 L/min with a pressure of 0.2 bar. At inoculation the culture was at OD_{600} = 0.2. The culture was incubated at 37ºC for ~90 min, with continuous OD monitoring until OD_{600} = 2.0. Temperature was reduced to 25ºC and then induced by the addition of IPTG to a final concentration 0.5 mM. Induction period was 23 h.

Harvesting: Whole broth was harvested by centrifugation (6,427 rcf in Sorvall RC 12BP) for 20 min, pre-chilled to 4°C, and pellets frozen and stored at -80°C. At harvest the cell wet mass was 41 g/L. Total wet cell weight harvest was 1.43 Kg, OD_{600} = 35.0.

Cell lysis (small scale QC): Samples (1 mL) were centrifuged at 16,000 g for 1.5 minutes. Supernatant was discarded and pellets frozen. Samples were prepared by evaluating soluble

protein OD_{600} of Bugbuster Mastermix (Novagen) and centrifuged at 16,000g for 25 minutes at 4ºC. The lysate was analysed with an equal amount of SDS-Reduced (soluble).

Fed Batch Fermentation (10L)

Seed Stage: 1 L baffled conical flasks containing Luria Broth medium (300 mL), 2% glucose and 30 µg/mL kanamycin were inoculated with 0.6 mL of EH15 glycerol stock. Flasks were incubated for 12 h at 28°C and 220 rpm to an OD_{600} of between 1.0–2.0.

Production stage: 1% inoculum of the seed stage culture (described above) was added into a 12 L fermenter containing 5.5 L of growth medium, GF01B. This medium was sterilised at natural pH and adjusted to pH 7.0 with ammonium hydroxide solution (9.0 M) prior to inoculation. One-hour post inoculation kanamycin (30 µg/mL) was added. The fermentor was incubated at 27°C and agitated at 300–950 rpm with air supplied at 5.5–20 L/min to maintain dissolved oxygen levels at 20% saturation or greater. The pH was controlled at 6.90 by the addition of ammonium hydroxide solution (9.0 M) and growth maintained by the addition of a 50% w/w glucose monohydrate solution. This feed (50% w/w glucose monohydrate solution) was introduced exponentially over 12 hours to a maximum of 14.5 g/L/h initial volume from the point of batched glucose exhaustion, as indicated by a rise in pH and dissolved oxygen content (DO_2). After the culture reached an OD_{600} of 80 (+/- 10) expression of the epoxide hydrolase was induced by the addition of isopropyl-*β*-D-thiogalactosidase (IPTG) (final conc 1.0 mM). Feed rate was reduced to 11 g/L/Hr initial volume and the fermentation was harvested 40 hours post induction. At harvest the fermenter was chilled to 12°C and broth collected for downstream processing.

Harvesting: Two methods were used to isolate a clarified protein solution from 10 L fermentation broth.

Method 1: Biomass from the fermentation broth was isolated by centrifugation (40 min, 4500 rpm at 4°C) to recover cell paste. The cell paste was resuspended to a solid content of 25% (w/w) with a 100 mM potassium phosphate buffer (79 mM K_2HPO_4 and 21mM K_2HPO_4, pH 7–7.5). The resuspended cells were mechanically disrupted at 28-30 KPSI at a constant temperature of 10°C. Half of the lysate was clarified by addition of polyethylenimine (PEI, MW 750000) to a final concentration of 0.4% (w/v), mixing for 30 min, then centrifugation (30 min, 4500 rpm, 4°C) to recover a clarified supernatant. The other half was directly centrifuged (30 min, 4500 rpm, 4°C) to recover a turbid supernatant.

Method 2: The fermentation broth (pH 6.3) was pH adjusted (pH 6.8-7.0) by addition of 100 mM potassium phosphate buffer solution (79 mM K_2HPO_4 and 21 mM K_2HPO_4, pH 7-7.5). The cells were mechanically disrupted at 28-30 KPSI at a constant temperature of 10°C. Half of the lysate was clarified by addition of polyethylenimine (PEI, MW 750000) to a final concentration of 0.4% (w/v), mixing for 30 min, then centrifugation (30 min, 4500 rpm, 4°C) to recover a clarified supernatant. The other half was directly centrifuged (30 min, 4500 rpm, 4°C) to recover a turbid supernatant. All supernatants volumes were aliquoted in large square Petri dishes and frozen at –20°C. The frozen clarified protein solutions were lyophilised and milled. Protein concentrations were measured by Bradford assays.

Lyophilization

All supernatant volumes were aliquoted in large square petri dishes and frozen at -20°C. The frozen clarified protein solutions were lyophilised and milled and stored at -20°C.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Media and buffer preparation and fermenter data (file type, PDF)

The following files are available free of charge (file type, PDF)

Epoxide Hydrolase SDS-Page Chiral GC methods and GC chromatograms (file type, PDF)

Enzyme Lyophilisation recipe (file type, PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

We thank Angela Bridges and Anne Gronlund for ordering the epoxide hydrolase plasmids and Matt John for reviewing the manuscript and providing constructive comments.

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20. a) Sequence similarity (86.6%) to EH from *Sphingomonas sp* HXN-200 see: Wu, S.; Li, A.; Chin, Y. S.; Li, Z., Enantioselective Hydrolysis of Racemic and Meso-Epoxides with Recombinant Escherichia coli Expressing Epoxide Hydrolase from *Sphingomonas sp*. HXN-200: Preparation of Epoxides and Vicinal Diols in High ee and High Concentration. *ACS Catal.* **2013**, *3* (4), 752−759; b) Rink, R.; Fennema, M.; Smids, M.; Dehmel, U.; Janssen, D. B., Primary Structure and Catalytic Mechanism of the Epoxide Hydrolase from *Agrobacterium radiobacter* AD1. *J. Biol. Chem.* **1997**, *272* (23), 14650−14657.

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26. Absolute stereochemistry of the diol **7** was determined by subsequent mesylation and ring closure which afforded the (*S*)-**2**.

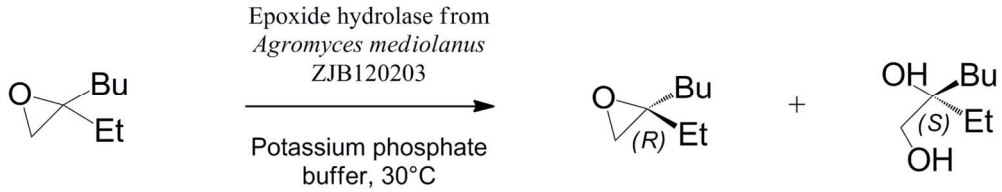

Table of Contents Graphic

134x26mm (300 x 300 DPI)