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Biocatalytic resolution of 2-methyl-2-(aryl)alkyloxiranes using novel bacterial epoxide hydrolases

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Abstract: Biocatalytic resolution of alkyl- and (arylalkyl)-oxiranes was accomplished by employing the epoxide hydrolase activity of lyophilized whole cells of seven bacterial strains belonging to the genera *Rhodococcus*, *Mycobacterium* and *Nocardia*. Whereas no suitable biocatalyst was found for 1-octene oxide, excellent selectivities were obtained with 2-methyl-2-substituted epoxides bearing an alkyl- or (aryl)alkyl side chain. © 1997, Elsevier Science Ltd. All rights reserved.

Introduction

Recently we have shown that whole bacterial cells of *Rhodococcus* sp. NCIMB 11216 can be used as a convenient source for epoxide hydrolases for the biocatalytic resolution of oxiranes on a preparative scale[1,2]. The products obtained – optically active epoxides and vicinal diols – can be employed as high-value intermediates for the synthesis of bioactive target molecules such as flavor and fragrance compounds[3] as well as pheromones[4]. For 2,2-disubstituted oxiranes, the reaction was shown to proceed *via* a classic kinetic resolution pattern, *i.e.* it involves an attack of a formal [OH^-] at the less hindered carbon atom while the absolute configuration at the chiral carbon center at C-2 is retained. As a consequence, the selectivity of the reaction can be accurately described by using the Enantiomeric Ratio[5] (E-value)[6,7]. In order to broaden the applicability of this method, we extended our study in two directions: (i) a search for other bacteria possessing highly selective epoxide hydrolase activity, and (ii) the investigation of substrates bearing an additional functional group. Since we knew from our study[8] on *Rhodococcus* sp. NCIMB 11216, that more polar functional groups are not tolerated by the enzyme[9], we envisaged that an aryl group might serve as a lipophilic masked carboxylate functionality[10] which would be tolerated by the enzyme(s).

In eukaryotes, epoxide hydrolases play a key role in the metabolism of xenobiotics, in particular of aromatic systems[11,12]. On the contrary, the natural substrates for these enzymes in procaryotes (bacteria) are unknown and it can only be speculated that they are involved in the utilization of alkenes as carbon-sources (Scheme 1). Thus, an alkene is epoxidized (by a mono-oxygenase) to yield an epoxide, which can be degraded in two ways: (i) hydrolysis by an epoxide hydrolase yields an innocuous vicinal diol[1], or alternatively, (ii) rearrangement of the oxirane catalyzed by an epoxide isomerase[13] furnishes an aldehyde or ketone. In presence of CO_2, carboxylation leads to the formation of a β-keto acid[35]. As may be deduced from our extensive screening for bacterial epoxide hydrolase activity, epoxide-hydrolysis seems to be more associated within the genus *Rhodococcus*, *Nocardia*, *Mycobacterium* and *Arthrobacter*, whereas epoxide-isomerization is associated with the *Pseudomonas* family[14].

Results and discussion

We initiated our screening along the following considerations. Due to the fact that enzymatic epoxide hydrolysis constitutes not only an important step in the provision of carbon (catabolism) but is also a detoxification reaction required for survival of the cell, the chance of finding highly enantioselective

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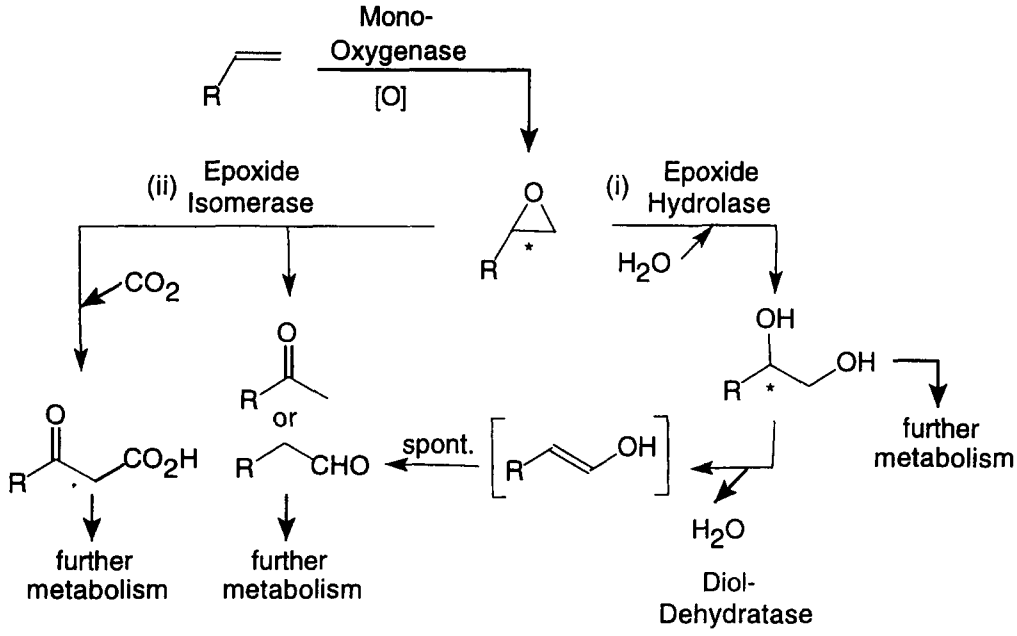


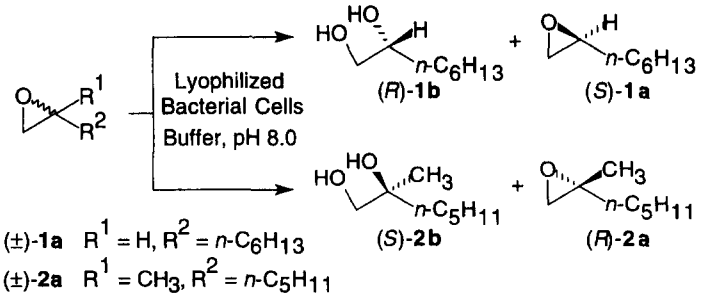
Scheme 1. Biodegradation of alkenes by procaryotes (bacteria).

epoxide hydrolases does not seem to be very high, bearing in mind that both enantiomers of an epoxide are about equally toxic to a living cell. On the other hand, it seems to be more likely that enantioselective epoxide hydrolases can be found in those strains, where the preceding step, *i.e.* alkene epoxidation, proceeds in a highly selective manner by producing predominantly one oxirane-enantiomer. As a consequence, the cell has no toxicity problem as long as the selectivities of the formation and degradation of the epoxide are matching.

There are numerous reports on the biocatalytic asymmetric epoxidation of alkenes[15,16] and this activity seems to be mainly associated with alkane- and alkene-utilizing bacteria[17–20]. Due to the fact that these reactions cannot be performed on a preparative scale with isolated enzymes owing to their complex nature and their dependency on a cofactor, such as NAD(P)H, in general whole microbial cells have been used instead[21]. Among the various biocatalysts, particularly bacteria have been found to epoxidize alkenes in an highly enantioselective manner. As a result of an intense search of the literature, our attention focussed on *Rhodococcus equi* IFO 3730[22], *Rhodococcus* sp. NCIMB 11216[8], *Rhodococcus ruber* DSM 43338[23], *Mycobacterium paraffinicum* NCIMB 10420[24] and *Nocardia* H8[25].

A set of seven bacterial strains was investigated in more detail for possible enantioselectivity using substrates (±)-**1a** through (±)-**4a** (Schemes 2 and 3). Disappointingly, negligible to low selectivity was observed in all cases for the monosubstituted oxirane (±)-**1a**, with selectivities not exceeding E ~6. On the other hand, the sterically more demanding epoxide (±)-**2a** turned out to be a much better substrate, in particular with *Nocardia* spp.[26] the selectivities were virtually absolute. As a consequence, the reactions stopped and did not proceed beyond a conversion of 50%. Interestingly, the enantiopreference was found to depend on the substrate structure, but not on the strain used. Thus, the (*R*)-epoxide was preferred for substrate (±)-**1a** (albeit with low selectivities), but the (*S*)-enantiomer was the faster transformed enantiomer for (±)-**2a**.

A more complex pattern emerged from substrates bearing a synthetically useful phenyl moiety at the ω-position of the alkyl chain. Thus substrate (±)-**3a** was resolved with varying success with selectivities ranging from low (*Rhodococcus equi* IFO 3730) to excellent (*Rhodococcus* sp. NCIMB



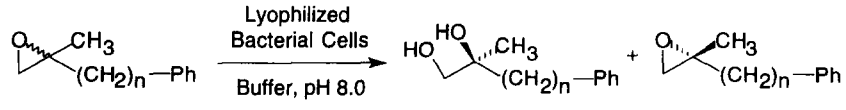
Biocatalyst	Substrate	Conv.	Products				Selectivity
		[%]	Diol	e.e. [%]	Epoxide	e.e. [%]	(E)
Rhodococcus equi IFO 3730	(±)-**1a**	~5	(*R*)-**1b**	3	**1a**	~0	~1
Rhodococcus NCIMB 11216	(±)-**1a**	35	(*R*)-**1b**	39	(*S*)-**1a**	21	2.8
Mycobacterium paraffinicum NCIMB 10420	(±)-**1a**	8	(*R*)-**1b**	36	(*S*)-**1a**	3	2.2
Rhodococcus ruber DSM 43338	(±)-**1a**	43	(*R*)-**1b**	30	(*S*)-**1a**	23	2.3
Nocardia H8	(±)-**1a**	35	(*R*)-**1b**	57	(*S*)-**1a**	31	4.9
Nocardia TB1	(±)-**1a**	41	(*R*)-**1b**	57	(*S*)-**1a**	40	5.3
Nocardia EH1	(±)-**1a**	50	(*R*)-**1b**	54	(*S*)-**1a**	54	5.6
Rhodococcus equi IFO 3730	(±)-**2a**	21	(*S*)-**2b**	95	(*R*)-**2a**	25	49
Rhodococcus NCIMB 11216	(±)-**2a**	41	(*S*)-**2b**	96	(*R*)-**2a**	71	105
Mycobacterium paraffinicum NCIMB 10420	(±)-**2a**	40	(*S*)-**2b**	98	(*R*)-**2a**	65	194
Rhodococcus ruber DSM 43338	(±)-**2a**	32	(*S*)-**2b**	>99	(*R*)-**2a**	46	>200
Nocardia H8	(±)-**2a**	44	(*S*)-**2b**	>99	(*R*)-**2a**	79	>200
Nocardia TB1	(±)-**2a**	50	(*S*)-**2b**	>99	(*R*)-**2a**	>99	>200
Nocardia EH1	(±)-**2a**	50	(*S*)-**2b**	>99	(*R*)-**2a**	>99	>200

Scheme 2. Resolution of 2-mono- and 2,2-dialkyloxiranes.

11216). Unexpectedly, *Mycobacterium paraffinicum* NCIMB 10420, *Rhodococcus ruber* DSM 43338 and all of the *Nocardia* spp., which were the biocatalysts of choice for 2-methyl-1,2-epoxyheptane (**2a**) were less useful in this case. It is quite likely that the reduced selectivities can be attributed to the presence of multiple epoxide hydrolases possessing various (or even opposite) stereochemical preferences bearing in mind that the most selective strain in this series (*Rhodococcus* sp. NCIMB 11216) has only a single epoxide hydrolase acting on substrates of this type[27]. Alternatively, insufficient chiral recognition of a single enzyme cannot be excluded. This problem is currently being studied by using purified enzymes.

When the carbon-chain was extended by an additional CH_2-unit, the selectivity declined in general. In analogy to the dialkyl substrate (±)-**2a**, the *Nocardia* spp. gave best selectivities (E up to 13). This observation is in contrast to our previous finding[8], that (at least for *Rhodococcus* sp. NCIMB 11216) the selectivity depends on the relative difference in size of the two alkyl groups, *i.e.* 2-methyl-1,2-epoxynonane gave a higher selectivity than 2-methyl-1,2-epoxyheptane. It should be mentioned, that the α-methylstyrene oxide was not suitable as substrate because it was only slowly accepted and that the reaction was impeded by a significant fraction of spontaneous (non-selective) hydrolysis[28].

Diol products were identified by comparison with material obtained by chemical hydrolysis of the epoxide. The absolute configuration of compounds was determined as follows: comparison of optical rotation values with literature data[8] revealed the absolute configuration of **1a,b**[29], **2a,b**[30,31], **3b**[32]. A



(±)-**3 a** n = 1 (*S*)-**3b** (*R*)-**3 a**

(±)-**4 a** n = 2 (*S*)-**4b** (*R*)-**4 a**

Biocatalyst	Substrate	Conv.	Products				Selectivity
		[%]	Diol	e.e. [%]	Epoxide	e.e. [%]	(E)
Rhodococcus equi IFO 3730	(±)-**3a**	4	(*S*)-**3b**	25	(*R*)-**3a**	~1	1.7
Rhodococcus NCIMB 11216	(±)-**3a**	11	(*S*)-**3b**	>98	(*R*)-**3a**	12	111
Mycobacterium paraffinicum NCIMB 10420	(±)-**3a**	3	(*S*)-**3b**	75	(*R*)-**3a**	2	7.1
Rhodococcus ruber DSM 43338	(±)-**3a**	3	(*S*)-**3b**	87	(*R*)-**3a**	3	14
Nocardia H8	(±)-**3a**	36	(*S*)-**3b**	90	(*R*)-**3a**	51	31
Nocardia TB1	(±)-**3a**	45	(*S*)-**3b**	89	(*R*)-**3a**	72	36
Nocardia EH1	(±)-**3a**	45	(*S*)-**3b**	90	(*R*)-**3a**	75	42
Rhodococcus equi IFO 3730	(±)-**4a**	19	(*S*)-**4b**	66	(*R*)-**4a**	15	5.6
Rhodococcus NCIMB 11216	(±)-**4a**	38	(*S*)-**4b**	72	(*R*)-**4a**	45	9.5
Mycobacterium paraffinicum NCIMB 10420	(±)-**4a**	9	(*S*)-**4b**	82	(*R*)-**4a**	8	10
Rhodococcus ruber DSM 43338	(±)-**4a**	4	(*S*)-**4b**	74	(*R*)-**4a**	3	6.9
Nocardia H8	(±)-**4a**	23	(*S*)-**4b**	82	(*R*)-**4a**	25	12
Nocardia TB1	(±)-**4a**	36	(*S*)-**4b**	80	(*R*)-**4a**	45	13
Nocardia EH1	(±)-**4a**	29	(*S*)-**4b**	80	(*R*)-**4a**	33	12

Scheme 3. Resolution of 2-methyl-2-(aryl)alkyloxiranes.

sample of (*S*)-**4b** was independently synthesized by Li_2CuCl_4-catalyzed addition of benzyl magnesium chloride onto commercially available (*R*)-2-methylglycidol following the procedure of Hosokawa *et al.*[8,33].

Summary

Six novel bacterial strains possessing a highly selective epoxide hydrolase activity were identified, which can be conveniently employed in lyophilized form for the resolution of 2-methyl-2-alkyl- and 2-methyl-2-(aryl)alkyloxiranes. Due to the fact that no enzyme induction is required, ample supply of the biocatalysts allows the reactions to be performed on a preparative scale. An asymmetric synthesis of (*R*)-mevalonolactone by using one of these synthons will be reported in due course.

Experimental

General

Reactions were monitored by TLC (silica gel Merck 60 F_{254}), compounds were visualized by spraying with vanillin/H_2SO_4 conc. (5 g/l) or Mo-reagent [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (1.1 g/l), $Ce(SO_4)_2\cdot 4H_2O$ (4 g/l) in H_2SO_4 (10%)]. Preparative chromatography was performed on silica gel Merck 60 (40–63 μm). ^1H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ solution on a Bruker MSL 300 (300 MHz and 75.47 MHz, resp.). Chemical shifts are reported in δ from TMS as internal standard. Optical rotation values were measured on a Perkin-Elmer polarimeter 341 at 589 nm (Na-line) in a 1 dm cuvette.

Synthesis of substrates and reference materials

(±)-**1a** was obtained from Aldrich, (±)-**2a** (±)-**3a** and (±)-**4a** were synthesized from the corresponding ketones and trimethylsulfoxonium iodide following a general procedure as previously described[8]. (±)-**2a**:[8]. (±)-**3a**: 75% yield, bp_{10} 90°C. ^{1}H-NMR: δ=1.6 (s, 3H, CH_3), 2.6–2.69 (m, 2H, Ph–CH_2), 2.83 (d, J=12 Hz, $1H_A$, CH_2), 2.91 (d, J=12 Hz, $1H_B$, CH_2), 7.19–7.36 (m, 5H, Ar–H). ^{13}C-NMR: δ=20.97 (CH_3), 43.17 (Ph–CH_2), 53.38 (C–CH_2–O), 57.31 (quart. C), 126.66 (Ar-*p*), 128.42 (Ar-*m*), 129.65 (Ar-*o*), 137.31 (Ar-*i*). (±)-**4a**: 63% yield, bp_{13} 150°C. NMR-data[34]. Racemic diols were obtained by chemical hydrolysis of the corresponding epoxide under acidic (H_2O/THF 1:1, cat. H_2SO_4, r.t.) or alkaline conditions (DMSO/H_2O 6:1, KOH ~3 eq., 100°C) and subsequent column chromatography. (±)-**3b**: Alkaline hydrolysis, 90% yield, NMR-data[31]. (±)-**4b**: Acidic hydrolysis, 85% yield. ^{1}H-NMR: δ=1.20 (s, 3H, CH_3), 1.75–1.85 (m, 2H, C–CH_2–C), 2.67 (d, J=7 Hz, $1H_A$, CH_2–O), 2.72 (d, J=7 Hz, $1H_B$, CH_2–O), 3.48 (m, 2H, Ph–CH_2), 7.19–7.32 (m, 5H, Ar–H). ^{13}C-NMR: δ=23.18 (CH_3), 30.15 (Ph–CH_2), 40.49 (C–CH_2–C), 69.79 (quart. C), 73.11 (CH_2–OH), 125.92 (Ar-*p*), 128.37, 128.42 (Ar-*o,m*), 142.32 (Ar-*i*).

Growth of bacteria

Bacteria were obtained from culture collections, except for the *Nocardia* spp., which were a kind gift of J. de Bont (Wageningen, NL) and C. Syldatk (Stuttgart). The following strains were grown as previously described: *Rhodococcus* sp. NCIMB 11216[8], *Rhodococcus equi* IFO 3730 and *Mycobacterium paraffinicum* NCIMB 10420[4]. *Rhodococcus ruber* DSM 43338, *Nocardia* TB1, H8, and EH1 were grown in shake-flask cultures on the following medium: peptone (10 g/L), yeast extract (10 g/L), glucose (10 g/L), $MgSO_4 \cdot 7H_2O$ (147 mg/L), NaCl (2 g/L), K_2HPO_4 (4.4 g/L), NaH_2PO_4 (1.3 g/L). At the late exponential growth phase (~25–40 h) the cells were harvested by centrifugation (3000·g), resuspended in Tris-buffer (0.05 N, pH 8.0), centrifuged again and lyophilized. Typical yields of dry cells ranged from 3–5 g/L. The cells could be stored over several months at +5°C without significant loss of activity.

General procedure for the asymmetric hydrolysis of epoxides

Lyophilized microbial cells (100 mg) were rehydrated in Tris-buffer (5 mL, 0.05 M, pH 8.0) for 30 min on a rotary shaker (180 rpm, r.t.). Substrate (100 mg) was then added and the mixture was agitated at room temperature while the reaction was monitored by TLC or GLC. The specific activity of all biotransformations was in the range of ~1000–2000 μmol/mg·h (with respect to pure protein). After an appropriate degree of conversion was reached, acetone was added (3 mL), the cells were centrifuged, and the formed diols and remaining epoxides were extracted with ethyl acetate from the buffer medium and the pellet in ~90% overall yield. With more water soluble short-chain 1,2-diols, product recovery was considerably improved using the Extrelut-system (Merck)[8].

Specific rotation values

Compound	Specific Rotation	Concentration, Solvent	E.e. [%]
(*S*)-**1a**	$[\alpha]_D^{21}$ -2.82	4.35, EtOH	21
(*R*)-**1b**	$[\alpha]_D^{21}$ +6.78	5.0, EtOH	46
(*R*)-**2a**	$[\alpha]_D^{20}$ -6.35	3.43, $CHCl_3$	71
(*S*)-**2b**	$[\alpha]_D^{20}$ -3.25	3.6, $CHCl_3$	96
(*S*)-**3b**	$[\alpha]_D^{20}$ -11.7	0.56, EtOH	75
(*S*)-**4b**	$[\alpha]_D^{20}$ -0.52	0.52, EtOH	82

E.e.-determination

Enantiomeric excesses were analyzed by GLC (Shimadzu GC-14A equipped with FID) on a CP-Chirasil-DEX CB column (25 m, 0.32 mm, 0.25 μm film, H_2). The enantiomeric excess of **1a,b** and

2a,b was determined by GLC on a chiral stationary phase as previously described[8]. **3a**: 90°C iso, 0.5 bar (*R*) 15.9 min, (*S*) 16.4 min. **3b**: 140°C iso, 1 bar (*S*) 7.0 min, (*R*) 7.3 min. **4a** was transformed into 1-methoxy-2-methyl-4-phenylbutan-2-ol by treatment of the **4a** with NaOMe prior to analysis: 115°C iso, 1 bar (*R*) 11.6 min, (*S*) 12.0 min. **4b** was analyzed as the corresponding acetonide (2,2-dimethoxypropane, cat. ion exchange resin IR 120, H^+-form, r.t.; alternatively NaOH, MeI, DMSO): 110°C iso, 1 bar (*S*) 12.6 min, (*R*) 13.5 min.

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