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First Enantiodivergent Baeyer–Villiger Oxidation by Recombinant Whole-Cells Expressing Two Monooxygenases from *Brevibacterium*

Marko D. Mihovilovic,* Florian Rudroff, Bernhard Müller and Peter Stanetty

Vienna University of Technology, Institute of Applied Synthetic Chemistry, Getreidemarkt 9/163, A-1060 Vienna, Austria

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Abstract—Microbial Baeyer–Villiger oxidations of representative mesomeric ketones with recombinant *Escherichia coli* cells expressing two monooxygenases from *Brevibacterium* were investigated. The two enzymes displayed enantiodivergent biotransformations on an array of structurally diverse substrates, allowing access to some key lactone intermediates in natural compound synthesis.

Enzyme mediated Baeyer–Villiger oxidations offer a 'green chemistry' approach for the production of chiral lactones. Several organisms have been identified to catalyze this reaction in the course of their metabolic pathways. Apart from their natural substrates, a large variety of non-natural precursors are accepted by flavin dependent monooxygenases involved in this biotransformation. All these aspects taken together established microbial Baeyer–Villiger oxidations as one of the representative domains of biocatalysis for the production of valuable chiral intermediates.[1–6]

While chiral catalysis using organometallic compounds allows access to both enantiomeric products in a rather simple manner by inverting the chirality of the inducing ligands, this aspect represents one of the key challenges for biotransformations. Nature uses a code of 20 commonly found L-amino acids to construct enzymes consisting of hundreds of such building blocks, hence, the chiral induction of such catalytic entities is usually strong. However, inversion of the enantioselectivity of an enzyme with well defined substrate profile is not feasible by changing to D-amino acids, for no cellular machinery is available to accept these compounds to produce proteins. To circumvent this obstacle, screening and identification of alternative enzymes with opposite enantioselectivity becomes of increasing interest. Nevertheless, one has to consider that such novel enzymes will display also different substrate acceptance patterns. Consequently, the characterization of biotransformation profiles for pairs of enzymes with divergent enantioselectivity has to become a key activity in biocatalysis.

Substantial progress in molecular biology, led to the recent identification and cloning of two new Baeyer–Villiger monooxygenases (BVMOs) from an environmental *Brevibacterium* isolate by Rouviere and coworkers.[7] Expanding our ongoing acceptance profiling program of recombinant whole-cell expression systems,[8–11] we started to investigate biooxidations of representative mesomeric ketones by recombinant strains expressing these novel cyclohexanone monooxygenases $CHMO_{BreviI}$ and $CHMO_{BreviII}$.

The utilization of living cells circumvents the obstacle to recycle the cofactors (NADH and NADPH) required by BVMOs.[12–14] The construction of artificial regeneration cycles can be avoided, hence simplifying the application of such biocatalysts in synthetic chemistry. Production of high concentrations of the required protein in recombinant strains is usually accomplished by strong promoters. Potent overexpression systems minimize the chance of unwanted side reactions caused by competing enzymes with overlapping substrate acceptance in a living cell.[15,16] We and others have successfully demonstrated

*Corresponding author. Fax: +43-1-58801-15499; e-mail: mmihovil@pop.tuwien.ac.at

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this concept for the production of CHMO from *Acinetobacter* NCIMB 9871 in *Saccharomyces cerevisiae*[17–19] and *E. coli* hosts[20,21] and the subsequent application of the engineered cells for the production of chiral lactones.

Table 1 summarizes our results for the Baeyer–Villiger type biotransformations of representative mesomeric ketone substrates **1a–e** to the corresponding lactones **2a–e**. In contrast to kinetic resolutions of racemic starting compounds, asymmetrization of prochiral products allows for the generation of several chiral centers with a theoretical yield of 100%, which makes this strategy especially appealing to synthetic chemists.

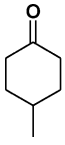
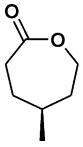
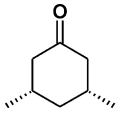
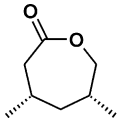
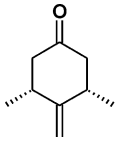
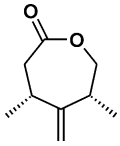
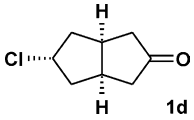
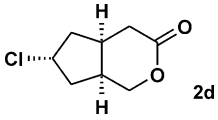
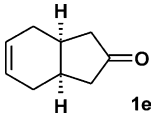
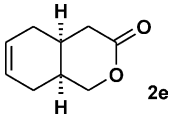
Biotransformation of 4-methylcyclohexanone **1a** gave the expected lactone **2a** with both protein expression strains in good yields. Comparison of the specific rotation with literature data[11,17,18,22] showed, that $CHMO_{BreviI}$ produced (−)-*S*-**2a** in excellent optical purity as determined by chiral phase GC, while $CHMO_{BreviII}$ generated the antipodal (+)-*R*-enantiomer, however, at moderate enantioselectivity. We observed the same enantiodivergence for prochiral disubstituted ketone **1b**. Here, oxidations with the two recombinant strains generated two novel chiral centers with excellent e.e. values for both optical isomers **2b**. The absolute configuration 4*S*,6*R* was assigned for the (−)-lactone produced by the $CHMO_{BreviI}$ expressing strain DPR#14 based on reported data.[22]

Olefin **1c** was chosen as model substrate to demonstrate the compatibility of the Baeyer–Villiger process with functional groups susceptible to oxidation reactions. Both enzymes gave Baeyer–Villiger oxidation to lactones **2c**[23] and no undesired epoxidation of the double bond was observed. Enantiodivergence of the two recombinant expression systems was excellent and assignment of the absolute configuration of lactones **2c** is currently carried out in our laboratory.

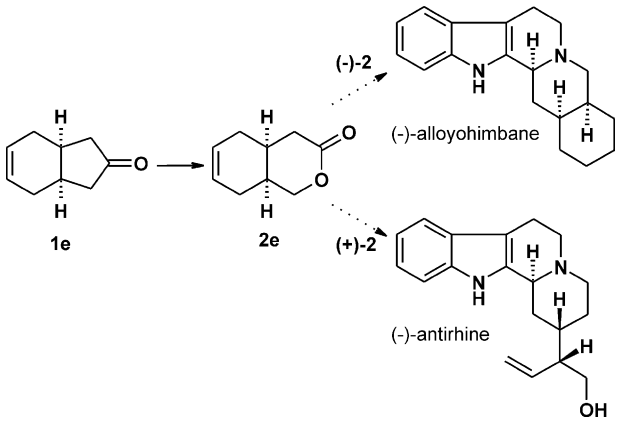
Recently, we have identified fused ketones as excellent probes to investigate structural influence on the stereoselectivity of BVMOs.[9,24] In the case of chloro substituted compound **1d** we observed formation of antipodal lactones **2d** by the two enzymes originating from *Brevibacterium*. By comparison with our previous results with an overexpression system for CHMO from *Acinetobacter* NCIMB 9871,[9] we assigned the absolute configuration for the lactone isolated after fermentation with the $CHMO_{BreviI}$ expressing strain DPR#14 as 4a*R*-(4aα,6α,7aα).

Lactone **2e** represents a key intermediate for a series of indole alkaloids, such as alloyohimbane[25] and antirhine.[26] Asymmetrization of ketone **1e** allows for a straight forward biocatalytic entry into this class of natural products (Scheme 1).[8] Both expression strains displayed high chemoselectivity for the Baeyer–Villiger transformation. Fermentations of this substrate

Table 1. Microbial Baeyer–Villiger oxidations to lactones **2a–e** using recombinant whole-cells expressing monooxygenases from *Brevibacterium*

Substrate	Product	Enzyme	Yield[a]	e.e.[b]	$[\alpha]_D^{20}$
 **1a**	 **2a**	$CHMO_{BreviI}$	65%	>99%[c]	−46.1 (*c* 2.4, $CHCl_3$)
		$CHMO_{BreviII}$	60%	44%[c]	+18.4 (*c* 2.6, $CHCl_3$)
 **1b**	 **2b**	$CHMO_{BreviI}$	61%	97%	−10.7 (*c* 2.2, $CHCl_3$)
		$CHMO_{BreviII}$	56%	99%	+9.6 (*c* 2.0, $CHCl_3$)
 **1c**	 **2c**	$CHMO_{BreviI}$	70%	>99%	+8.3 (*c* 0.36, $CHCl_3$)
		$CHMO_{BreviII}$	44%	>99%	−8.4 (*c* 0.64, $CHCl_3$)
 **1d**	 **2d**	$CHMO_{BreviI}$	55%	>99%	−39.5 (*c* 1.2, $CHCl_3$)
		$CHMO_{BreviII}$	59%	>99%	+36.2 (*c* 1.0, $CHCl_3$)
 **1e**	 **2e**	$CHMO_{BreviI}$	10%	71%	n.d.
		$CHMO_{BreviII}$	92%	94%	+21.0 (*c* 1.0, $CHCl_3$)

[a]Isolated yield after purification by flash column chromatography. Yields are not optimized.
[b]e.e. determined by chiral phase gas chromatography; racemic reference material was prepared by mCPBA oxidation of ketones **1a**, **1b**, **1d**.
[c]e.e. determined after LAH reduction of lactone **2a** to the corresponding diol by chiral phase gas chromatography.



Scheme 1. (+)- and (−)-lactones **2e** as key intermediates for indole alkaloids.

revealed, that the spatial limitations of the active site for $CHMO_{BreviI}$ seem to be reached, since biotransformations proceeded very sluggishly to (−)-(4a*S*,8a*S*)-**2e** in a moderate 71% ee (Note: the chloro substituent in **2d** requires a change in priority numbering which leads to a reversal of the *R*/*S* assignment; in both cases the sense of chirality was the same for the lactones). In contrast, $CHMO_{BreviII}$ gave excellent conversion to (+)-(4a*R*,8a*R*)-**2e** (94% ee).[8]

Recently, we observed a similar trend of enantiodivergent oxidations for CHMO from *Acinetobacter* NCIMB 9871 and cyclopentanone monooxygenase from *Pseudomonas* NCIMB 9872, both expressed in whole-cells of *E. coli*.[8,24] With the current results we present the first substrate acceptance study on two recombinant CHMOs from a single native organism. Whole-cell expression systems display good to excellent enantiodivergence on some substrate ketones. The substrate profiles of both enzymes seem to overlap to a significant extent, based on the results available so far. Further acceptance profiling is currently on the way in our laboratories to characterize scope and limitations of the enantiodivergence.

General Procedure

Fresh LB-amp medium (1% Bacto-Peptone, 0.5% Bacto-Yeast Extract, 1% NaCl supplemented by 200 ppm ampicillin) was inoculated with 1% of an overnight preculture of recombiant *E. coli* strains DPR#14 or DPR#399 (constructs transformed with the corresponding expression plasmid for $CHMO_{BreviI}$ and $CHMO_{BreviII}$, respectively) in a baffled Erlenmeyer flask. The culture was incubated at 120 rpm at 37 °C on an orbital shaker for 2 h, then IPTG was added to a final concentration of 0.025 mM. The substrate **1a–e** (3–5 mM) was added neat along with β-cyclodextrin (1 equiv). The culture was incubated at room temperature until GC showed complete conversion of the ketone (24–96 h). The biomass was removed by centrifugation (3500 rpm, 10 min), the aqueous layer was passed through a bed of Celite®, and the product was isolated by repeated extraction with Et_2O or EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated. Lactones **2a–e** were purified by flash column chromatography.

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$CDCl_3$) 16.9 (q), 20.8 (q), 35.8 (d), 40.9 (t), 41.0 (d), 73.0 (t), 109.0 (t), 154.1 (s), 174.4 (s).
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