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Microbial Baeyer–Villiger oxidation of 4,4-disubstituted cyclohexan- and cyclohexenones by recombinant whole-cells expressing monooxygenases of bacterial origin

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Abstract

Screening of 4,4-disubstituted and 3,4,5-polysubstituted cyclohexan- and cyclohexenones with eight different overexpression systems of microbial monooxygenases in recombinant *Escherichia coli* provided valuable information about substrate acceptance and enantioselectivity of this enzyme family, which are responsible for the stereoselective Baeyer–Villiger biooxidation of ketones. For this purpose whole-cell mediated biotransformations were realized to overcome some limitations in the application of cofactor dependent biocatalysts. The different behavior of various enzymes reflects a recent hypothesis about two distinct clusters of biooxidation catalysts. In contrast to isolated enzyme biooxidations, recombinant cells did not yield unsaturated lactone products derived from cycloalkenones. They rather displayed reductase activity to reduce such precursors to saturated ketones, which were subsequently oxidized to the corresponding Baeyer–Villiger products in a sequential two-step biotransformation.

Keywords: Baeyer–Villiger oxidation; Baeyer–Villiger monooxygenase; Stereoselective oxidation; Whole-cell biotransformation; Sequential biotransformation

1. Introduction

In recent years biocatalytic methods, using whole cells or isolated enzymes as catalytic agents, have received increasingly widespread application, particularly in the pharmaceutical industry with a demand for optically pure building blocks [1]. Out of more than 300 such processes [2], the microbial Baeyer–Villiger oxidation represents a particularly useful reaction for biotransformations in asymmetric mode [3–5].

Recent advances in molecular biology enabled access to an increasing number of novel Baeyer–Villiger monooxygenases (BVMOs) originating from various natural sources [6–8]. Together with recent attempts to modify the stereoselectivity of such enzymes [9], a toolbox of such biooxidation catalysts is becoming available for the transformation of a large variety of structurally diverse substrates to high-value chiral lactones. In this context, the characterization of stereopreference and substrate acceptance of these enzymes is becoming a key aspect in order to evaluate their potential as biocatalysts in organic synthesis [10].

Utilization of strains engineered to overexpress a particular BVMO instead of wild-type organisms minimizes problems with unwanted side reactions and low monooxygenase activity. Production of the required enzyme at high level is accomplished by the use of strong promoters and is highly controlled [11–13]. Simultaneously, whole-cell mediated biotransformations offer a solution to obstacles associated with cofactor regeneration, as BVMOs belong to an NADPH-dependent flavoenzyme family. Finally, overexpression of enzymes in a suitable non-pathogenic strain such as *Escherichia coli* provides "easy-to-use" catalytic systems, which require a minimum of special laboratory equipment and microbiological expertise and can consequently be provided to preparative chemists for subsequent applications in organic synthesis.

Recently, we had established various 4,4-disubstituted cyclohexanones (**1**) as substrates for cyclohexanone monooxygenase ($CHMO_{Acineto}$) from *Acinetobacter* NCIMB 9871 [14] as the most abundantly utilized BVMO in enzyme mediated Baeyer–Villiger oxidations [15]. Extending our previous substrate acceptance screenings of $CHMO_{Acineto}$ and seven novel BVMOs of bacterial origin ($CHMO_{Arthro}$ from *Arthrobacter* sp. [16], $CHMO_{Brachy}$ from *Brachymonas* sp. [17], $CHMO_{Brevi1}$ and $CHMO_{Brevi2}$ from *Brevibacterium* sp. [18], $CPMO_{Coma}$ from *Comamonas* sp. [19], $CHMO_{Rhodo1}$ and $CHMO_{Rhodo2}$ from

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Rhodococcus sp. [16]), we became interested in a comparative study of such substrates on this small library of enzymes. Recent work has revealed some interesting differences in biotransformations by representatives of this group of proteins, in particular with respect to substrate acceptance, enantiocomplementary stereopreference and regiodivergent oxidations [10,20].

In this context, we intended to also investigate the potential of recombinant whole-cell expression systems for BVMOs on the biooxidation of α,β-unsaturated cycloketones (**4**). So far, only $CPMO_{Coma}$ was reported to accept such substrates in an early work using isolated protein [21], while whole-cell transformations with wild type *Pseudomonas* NCIMB 10007 resulted in a complex mixture of both saturated and unsaturated lactones and ketones [22]. The only other BVMO accepting unsaturated ketones is $HAPMO_{Pseudo}$ from *Pseudomonas fluorescence*, however, the enone substrate specificity is limited to acyclic aryl ketones [23]. An added incentive for studying the biotransformation of cyclic alkenones was to gain access to higher functionalized lactone products, as the equivalent chemical reaction with *m*-CPBA proceeds with concomitant epoxidation dominating over Baeyer–Villiger oxygenation.

2. Experimental

2.1. General

Unless otherwise noted, chemicals and microbial growth media were purchased from commercial suppliers and used without further purification. All solvents were distilled prior to use. Shake flask fermentations were performed in a Gerhard THO5 orbital thermoshaker. Preparative MPLC was carried out on a Büchi 681/684 system with silica gel packed columns. *Kugelrohr* distillation was carried out using a Büchi GKR-51 apparatus. Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected. Enantiomeric excess was determined via GC using a BGB 173 or BGB 175 column (30 m × 0.25 mm i.d., 0.25 μm film) on a ThermoQuest Trace GC 2000 chromatograph and a FID detector (240 °C), biotransformation progress and conversion control was performed with a standard capillary column DB5 (30 m × 0.32 mm i.d.). The NMR spectra were recorded from $CDCl_3$ solutions on a Bruker AC 200 (200 MHz) or Bruker Avance UltraShield 400 (400 MHz) spectrometer and chemical shifts are reported in ppm using Me_4Si as internal standard. Peak assignment is based on correlation experiments. Specific rotation $[\alpha]_{20}^{D}$ was determined using a Perkin Elmer Polarimeter 241 by the following equation: $[\alpha]_{20}^{D} = 100 \times \alpha/(c \times l)$; c [g/100 mL], l [dm].

2.2. Substrates and reference material

The disubstituted and trisubstituted substrates **1a**, **1b**, **1c**, **1f**, **1g**, **1i** were synthesized according to literature procedures [14,24] *via* Robinson annulation from appropriate aldehyde and methylalkyl ketone. α,β-Unsaturated compounds were used either directly for biotransformations as substrates (**4f**, **4g**, **4i**) or were catalytically hydrogenated using Pd/C [14] leading to the required products (**1a**, **1b**, **1c**). Ketone **1d** was prepared from commercially available 1,4-cyclohexanedione monoethylenacetal after addition of methyl lithium and subsequent acid-catalyzed hydrolysis of the ketal bond [25]. Substrate **1e** was performed *via* a recently published route by our group involving Wittig and modified Simmons–Smith protocols [20a].

Chemical Baeyer–Villiger oxidation of ketones was carried out for reference purposes wherever possible prior to biotransformation experiments utilizing *m*-chloroperbenzoic acid [26]. Pure substrates and products were obtained after flash column chromatography or *Kugelrohr* distillation.

2.3. Cultivation of bacterial strains

Cultivation of recombinant *E. coli* strains for the expression of the corresponding BVMO was carried out in baffled Erlenmayer flasks on an orbital shaker (120 rpm, 37 °C) using LB medium [1% bacto-peptone, 0.5% bacto-yeast extract, 1% NaCl, supplemented with ampicilin (200 μg/mL)].

Frozen stocks (1 mL portions stored at −80 °C) were prepared by adding glycerol (final concentration 15%) to a culture grown overnight. Fresh plates were streaked weekly from frozen stocks and grown overnight at 37 °C on ampicillin supplemented LB plates containing 1.5% Bacto-Agar.

2.4. Biotransformations and product isolation

Fresh LB-ampicillin medium (250 mL) was inoculated with a 2.5 mL aliquot of an overnight preculture of appropriate *E. coli* strain in a 1000 mL Erlenmeyer flask. The culture was shaken at 120 rpm at 37 °C until OD_{600} between 0.2 and 0.4 was reached, then IPTG was added to a final concentration of 0.025 mM. The substrate (100 mg) was added neat and β-cyclodextrin (one equivalent) was supplemented if required. The culture was shaken at 150 rpm at 24 °C. Complete conversions required between 24 and 48 h, then the cells were removed by centrifugation and the supernatant was extracted repeatedly with ethylacetate after saturation with NaCl. The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated. The crude products were purified by flash column chromatography.

2.5. Physical properties of biotransformation products

Lactones were obtained in yields and enantioselectivities specified in Table 1. Spectroscopic properties of compounds **2a**, **2b** [14], **2d** [10] and **2e** [20a] matched to previously reported data. Reference material for lactone **2c** and **2i** was prepared by chemical *m*-CPBA oxidation of ketone **1c** and **1i** to give a yellow oil in both cases: **2c**: ^{1}H NMR ($CDCl_3$): 1.30 (s, 3H), 1.75–2.05 (m, 4H), 2.30–2.80 (m, 2H), 4.05–4.35 (m, 2H), 7.20–7.45 (m, 5H); ^{13}C NMR ($CDCl_3$): 30.6 (q), 31.0 (t), 33.9 (t), 40.3 (s), 46.6 (t), 65.3 (t), 125.9 (d), 126.4 (d), 128.9 (d), 145.7 (s), 175.9 (s). **2i**: ^{1}H NMR ($CDCl_3$): 0.98 (s, 6H), 1.17–1.21 (m, 3H), 1.48–1.53 (m, 4H), 2.43–2.47 (m, 1H), 4.41–4.55 (m, 2H); ^{13}C

Table 1
Biooxidation of ketones **1** to lactones **2** and **3**

	Lactone						
	2a	**2b**		**3d**		**2e**	
$CHMO_{Acineto}$	61%	56%	75% e.e. (−) −8.16 (c = 0.5)	59%	86% e.e. (−) −7.04 (c = 0.7)	57%	>99% e.e. (+) +71.9 (c = 0.8)
$CHMO_{Arthro}$	36%	62%	88% e.e. (−) −10.75 (c = 0.5)	42%	92% e.e. (−) −8.22 (c = 0.6)	62%	>99% e.e. (+) +72.2 (c = 3.1)
$CHMO_{Brachy}$	30%	56%	61% e.e. (−) −6.44 (c = 0.6)	48%	97% e.e. (−) −9.09 (c = 0.4)	56%	>99% e.e. (+) +71.9 (c = 0.8)
$CHMO_{Brevi1}$	11%	n.c.	n.a.	Traces	51% e.e. (−)	n.c.	n.a.
$CHMO_{Brevi2}$	29%	78%	43% e.e. (−) −2.74 (c = 0.4)	37%[a]	61% e.e. (+) +2.47 (c = 0.9)	n.c.	n.a.
$CPMO_{Coma}$	45%	56%	21% e.e. (−) −1.3 (c = 0.7)	54%	76% e.e. (+) +3.56 (c = 0.6)	n.c.	n.a.
$CHMO_{Rhodo1}$	58%	46%	84% e.e. (−) −9.56 (c = 0.4)	59%	94% e.e. (−) −8.63 (c = 0.7)	46%	>99% e.e. (+) +71.9 (c = 3.3)
$CHMO_{Rhodo2}$	46%	53%	83% e.e. (−) −10.08 (c = 0.5)	47%	94% e.e. (−) −8.96 (c = 0.6)	53%	>99% e.e. (+) +72.0 (c = 0.6)

Isolated yields after flash column chromatography; e.e. determined by chiral phase gas chromatography; sign of specific rotation given; value of specific optical rotation $[\alpha]_{20}^{D}$ measured in $CHCl_3$; n.c.: no conversion; n.a: not analysed.
[a] Yield based on consumed starting material.

NMR ($CDCl_3$): 22.8 (q), 24.9 (q), 30.5 (t), 32.6 (s), 35.7 (t), 42.7 (d), 67.6 (t), 177.7 (s).

3. Results and discussion

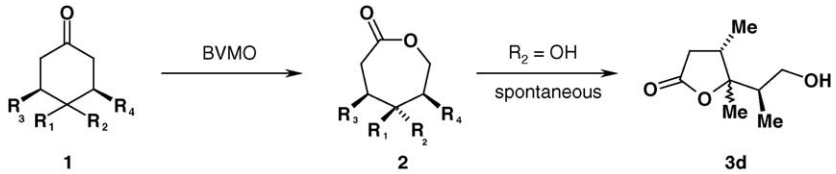
Based on our previous results on 4,4-disubstituted ketones [14], the aim of this study was to investigate the effect of non-polar and polar groups on the corresponding precursors on the substrate acceptance and stereoselectivity of the BVMO mediated biooxidation. Our data obtained with $CHMO_{Acineto}$ indicated that in the case of simple aliphatic substituents the stereoselectivity of the enzymatic transformation was predominantly governed by conformational effects within the substrate and the Criegee intermediate, respectively. In addition, our work on fused bicyclo precursors displayed an essential effect of the polarity of substituents on both substrate acceptance and stereopreference of the biooxidation [20a,f].

When extending our investigation of this substrate class to a small library of recently designed whole-cell expression systems of novel BVMOs, we were particularly interested in general trends caused by the polarity of substituents. In addition, restricting the flexibility of such substrate ketones was also expected to give additional insights, which ultimately could result in an increasingly fine-tuned active site model of this group of BVMOs (Schemes 1 and 2).

Very recently, the first structure of a comparably distantly related BVMO was reported, indicating also several conformational changes of the protein during the catalytic process of the enzymatic Baeyer–Villiger oxidation [27]. In particular, the incomprehensive understanding of these structural changes on molecular level complicates the development of a model of the enzyme's active site in sufficient quality to predict stereopreference. Consequently, substrate inspired models based on biotransformations of structurally diverse compounds represent an alternative approach to develop predictive models [28].

Initial biotransformations with ketone **1a** were realized with all novel overexpression systems, with recombinant *E. coli* producer for $CHMO_{Acineto}$ serving as reference. The oxidation of compound **1a** was complete after 24 h with every strain apart from $CHMO_{Brevi1}$. In this case, conversion stopped at approximately 20% after 24 h (determined by gas chromatography) and was not increased after prolonged incubation time. This established in principal acceptance of 4,4-disubstituted cyclohexanones by the novel BVMOs and isolated yields were not optimized (Table 1).

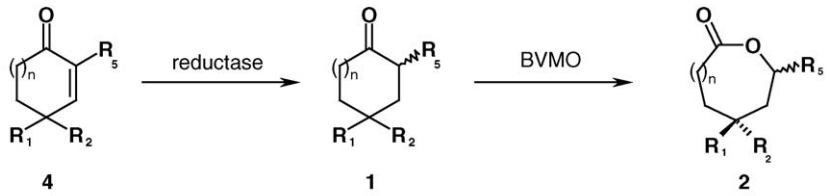
The biotransformation of prochiral ketone **1b** with all eight recombinant *E. coli* strains gave lactone **2b** in good yields, again



1 2 3d

Substate	R_1	R_2	R_3	R_4
1a	Me	Me	H	H
1b	Me	Et	H	H
1c	Me	Ph	H	H
1d	Me	OH	H	H
1e	-CH_2-CH_2-		Me	Me

Scheme 1.



Substate	n	R_1	R_2	R_5
4f	1	Me	Me	H
4g	1	Me	Et	H
4h	1	H	H	H
4i	1	Me	Me	Me
4j	0	H	H	H

Scheme 2.

with exception of $CHMO_{Brevi1}$, where only traces of lactone were detected according to GC after 24 h. Stereoselectivity was in a range of approximately 80% e.e. for those enzymes previously clustered as "CHMO-group". The calculated energy difference of transition states derived from such an enantioselectivity correlates to an interesting extent with the energy difference of the two conformers, with Me and Et alternatively adopting equatorial or axial positions. We had interpreted this observation on $CHMO_{Acineto}$ that conformational aspects within the Criegee intermediate seem to have a significant effect in generating the novel stereogenic center [14]. The enzyme itself only provides a ligand field to induce chirality but does not seem to prohibit formation of both conformational forms. This behavior leads to similar enantioselectivities within the "CHMO-group".

In case of the "CPMO-type" BVMOs ($CPMO_{Coma}$ and $CHMO_{Brevi2}$), substrate **1b** was converted with noticeably lower enantioselectivity, however, leading to lactone **2b** with the same absolute configuration.

When the bulkiness of the substrate was increased, 4-methyl-4-phenyl-cyclohexanone **1c** turned out to be no substrate for recombinant whole-cells producing BVMOs from this library. This is in good agreement with previous studies on 4-phenyl-cyclohexanone, which was only converted in reasonable yields with isolated $CHMO_{Acineto}$ in the presence of 5% glycol [29], while whole-cell mediated biotransformations gave no detectable amounts of the corresponding lactone product. Such observations can be interpreted in two ways: either the compounds are very poor substrates for the enzymes, or the bulkiness prevents penetration into the cells, consequently permitting access to the biocatalyst in whole-cell mediated conversions, in general.

A different situation was encountered, when biooxidations were carried out with 4-hydroxy-4-methyl-cyclohexanone **1d**, which can give polar interaction with hydrophilic groups within the active site of the BVMO. Oxidation by $CHMO_{Brevi2}$ and $CPMO_{Coma}$ gave enantiodivergent lactones compared to "CHMO-group" expression strains (determined by chiral GC). Again, $CHMO_{Brevi1}$ essentially did not accept this substrate (only trace amounts of lactone **2d** were detected by GC). Recombinant cells expressing the second BVMO from *Brevibacterium* ($CHMO_{Brevi2}$) did not give complete conversion of starting material after prolonged incubation time (approximately 70% after 48 h determined by GC) and the product was ultimately separated from remaining ketone by column chromatography. As observed previously for similar cyclohexanones bearing a 4-hydroxyl group [30], the oxidation of **1d** does not yield the expected 7-ring lactone but rearranges under biotransformation conditions to give the more stable 5-ring system.

Ketone **1e** was synthesized to investigate the spatial limitations on position 4 for biocatalytic Baeyer–Villiger oxidations using enzymes of the CHMO as well as CPMO family. It was somewhat surprising that $CPMO_{Coma}$ did not accept ketone **1e**, while it is efficiently transforming other substituted cyclohexanones. Consequently, this compound was also no substrate for the $CHMO_{Brevi2}$ expression system. The fact that this ketone was not transformed by $CHMO_{Brevi1}$ is consistent with results for the other 4,4-disubstituted cyclohexanones, which were also only very poorly biooxidized by this enzyme. All other "CHMO-type" enzymes transformed ketone **1e** in overall good yields and excellent enantioselectivites.

The oxidation of α,β-unsaturated cyclohexenones and cyclopentenones was carried out with $CPMO_{Coma}$ on the basis of results obtained from previous work [21]. With the previously discovered close relationship of $CPMO_{Coma}$ and $CHMO_{Brevi2}$ similar behavior of the latter enzyme was expected. In those compounds the carbonyl group possesses lower activity than in saturated ketones. After 48 h of biotransformation of **4g** with $CPMO_{Coma}$ a new product was isolated, purified *via* column chromatography and analyzed by NMR. The obtained product was 5-ethyl-5-methyl-2-oxepanone **2b**, a fully saturated lactone, converted with similar enantioselectivity as substrate **1b** (Table 2).

After this observation the interest was concentrated on detailed monitoring of biotransformation by gas chromatography. It was found that the first step is the reduction of double bound, which gives saturated ketone followed by

Table 2
Biotransformation of alkenones **4** to saturated lactones **2** *via* reduced intermediates **1**

	Substrate						
	4f	**4g**		**4h**	**4i**		**4j**
$CHMO_{Acineto}$	88%	–	–	–	63%	40% e.e.	–
$CHMO_{Brevi2}$	–	81%	43% e.e. (−)	20%	–	–	75%
$CPMO_{Coma}$	25%	95%	18% e.e. (−)	52%	62%	28% e.e.	76%

Conversion determined by gas chromatography after 48 h of biotransformation; e.e. determined by chiral phase gas chromatography.

Baeyer–Villiger oxidation, which provides the appropriate lactone. This sequence was established by parallel experiments using recombinant cells expressing $CPMO_{Coma}$ and $CHMO_{Brevi2}$ on substrate **1g** with and without induction of BVMO production by addition of IPTG. Main target of those examinations was to confirm the sequence of enzymatic reactions. In every biotransformation reduction of the double bond of ketone was observed prior to oxidation to lactone (Scheme 2). Oxidation of substrates **4f**, **4h** and **4j** was carried out with $CPMO_{Coma}$ and $CHMO_{Brevi2}$ as GC experiments to validate the observed trend. In every case reduction of double bond was observed prior to Baeyer–Villiger oxidation.

While this reductase activity was somewhat unexpected, in particular at such efficiency as to compete with the overexpressed BVMO, we wanted to investigate the potential of this whole-cell mediated two-step biotransformation sequence. Consequently, substrate **4i** was selected as model compound to probe the enantioselectivity of both enzymes. In this case, a new stereogenic center in α-position of the carbonyl group is generated during the reduction step from a non-chiral precursor. Recombinant strains expressing $CHMO_{Acineto}$, $CPMO_{Coma}$ and $CHMO_{Brevi2}$ were used as biocatalysts. However, no biotransformation was observed, only starting material remained in the fermentation broth. In a reference experiment **4i** was converted to saturated substrate **1i** by chemical hydrogenation. Subsequent biooxidation was performed with $CHMO_{Acineto}$ and $CPMO_{Coma}$ as a GC experiment. In both cases substrate was converted to the appropriate lactone **2i** with moderate enantioselectivity of 40 and 28% e.e., respectively, in a kinetic resolution process. Probably, steric hindrance within the active site of the reductase compromised conversion of this unsaturated substrate to form the fully saturated compound.

4. Conclusion

In summary, substrate acceptance of functionalized 4,4-disubstituted cycloalkanone and cycloalkenone derivatives was investigated for eight expression systems of BVMOs of bacterial origin ($CHMO_{Acineto}$, $CHMO_{Arthro}$, $CHMO_{Brachy}$, $CHMO_{Brevi1}$, $CHMO_{Brevi2}$, $CPMO_{Coma}$, $CHMO_{Rhodo1}$ and $CHMO_{Rhodo2}$). Similarities in the substrates acceptance and partly in the enantioselectivity were observed for $CPMO_{Coma}$ and $CHMO_{Brevi2}$ as well as for $CHMO_{Acineto}$, $CHMO_{Arthro}$, $CHMO_{Brachy}$, $CHMO_{Rhodo1}$ and $CHMO_{Rhodo2}$, with $CHMO_{Brevi1}$ displaying only poor tolerance for such precursors. This reflects to a certain degree our previously reported clustering of BVMOs converting cycloketones into two groups (CHMO- and CPMO-type).

The *E. coli* based expression systems used in this study were not suitable to study the BVMO mediated biooxidation of cycloalkenones, as remarkable activity of a reductase was observed, which gives rapid reduction of the α,β-unsaturated substrates to the corresponding fully saturated ketones. Possible utilization of such a two-enzyme sequential biotransformation is currently investigated in our laboratory by coupling the Baeyer–Villiger process with reductases of different origins in a suitable host without intrinsic reductase activity.

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