Biotransformations

Family Clustering of Baeyer–Villiger Monooxygenases Based on Protein Sequence and Stereopreference**

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Since its discovery by Adolf von Baeyer and Victor Villiger in 1899,[1] the oxidation process later named after the two scientists has become a powerful tool in synthesis to break carbon–carbon bonds in an oxygen-insertion process.[2] The regiochemistry of the reaction is governed by predictable

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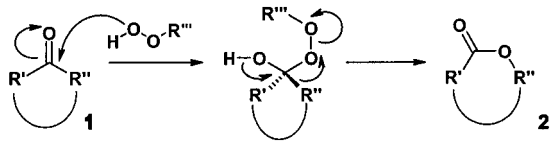
conformational, steric, and electronic effects,[3] and the rearrangement process of the tetrahedral peroxo Criegee intermediate proceeds with strict retention of configuration.[4] These factors are key prerequisites for performing the Baeyer–Villiger oxidation in an enantioselective manner.

The conversion of cyclic ketones into optically pure lactones (Scheme 1), in particular, allows access to highly



Scheme 1. Baeyer–Villiger oxidation of cyclic ketones **1** to form lactones **2**.

flexible compounds as platforms for the subsequent synthesis of bioactive compounds and natural products. Consequently, enantioselective Baeyer–Villiger oxidations have become a highly active field in asymmetric chemistry in recent years.[5] Currently, two major strategies are being developed with implementation of the "green-chemistry" concept aimed at sustainable, environmentally benign, and atom-efficient processes. Metal-based, de novo designed chiral catalysts have been continuously improved and are becoming promising candidates for industrial-scale applications.[6] By taking advantage of the vast catalytic repertoire of enzymes in nature, biocatalysis offers alternative entities for stereoselective oxidation processes with molecular oxygen utilized as the oxidant.[7] An increasing number of flavin-containing Baeyer–Villiger monooxygenases (BVMOs) have been identified during the past decade, and several such proteins display a remarkably broad acceptance profile for nonnatural substrates.[8]

Our approach to overcome some of the BVMO limitations, which have hampered the widespread utilization of oxidizing enzymes by synthetic chemists, utilizes living whole cells that are genetically engineered to express the required protein in high concentration.[9] This concept simplifies the problem of cofactor recycling, which arises because BVMOs require nicotinamide adenine dinucleotide (phosphate) in the reduced form (NAD(P)H) in the initial step of the catalytic cycle.[10] In addition, the tedious process of protein isolation is overcome and enzyme stability is not a limiting factor. As a result of the genetic modifications, the overexpressed BVMO becomes the major fraction in the organism's proteome, and side reactions by competing enzymes can be essentially avoided. This strategy was successfully optimized[11] and scaled up in pilot-plant industrial fermentation facilities[12] very recently.

The second major challenge in biocatalysis in general is the aspect of enantiodivergence. Artificial catalytic entities can be readily tailored to produce antipodal forms of the required products by inverting the chirality of the inducing ligand field. This strategy cannot be transferred to biotransformations, as there is no efficient process available to yield D-amino acid based proteins. Consequently, the identification and characterization of enzymes with overlapping substrate specificity that yield antipodal products is a key issue for the further implementation of biocatalytic methods in synthetic chemistry.

Recently, we and others have observed the formation of antipodal lactones by some representatives of the BVMO enzyme family.[13] This study compares the stereopreference, with respect to enantio- and regiodivergence, of cyclohexanone (CH) and cyclopentanone (CP) monooxygenases originating from *Acinetobacter* ($CHMO_{Acineto}$),[14] *Arthrobacter* ($CHMO_{Arthro}$),[15] *Brachymonas* ($CHMO_{Brachy}$),[16] *Brevibacterium* ($CHMO_{Brevi1}$, $CHMO_{Brevi2}$),[17] *Comamonas* ($CPMO_{Coma}$),[18] and *Rhodococcus* ($CHMO_{Rhodo1}$, $CHMO_{Rhodo2}$)[15] species in recombinant whole-cell-mediated Baeyer–Villiger oxidations with *Escherichia coli* as the host organism.

Initially, desymmetrization of prochiral ketones **1a–i** to the corresponding lactones **2a–i**, in part potential precursors in natural product synthesis, was investigated (Table 1). In a series of monocyclic ketones with prochiral substitution patterns, a significant clustering into two groups was observed: while the majority of BVMOs ("CHMO type") gave (−)-**2a–d** and (+)-**2e** lactones, $CPMO_{Coma}$ and $CHMO_{Brevi2}$ ("CPMO type") gave the antipodal products with moderate to excellent enantiomeric excess. This general trend is only violated by the enzyme $CHMO_{Brevi1}$, which displays the stereopreference of a CHMO-type BVMO but does not accept 4,4-disubstituted ketone **1c**. As observed previously for similar hydroxy compounds,[19] the oxidation of **1c** does not yield the expected seven-membered-ring lactone but rearranges under biotransformation conditions to give the more stable five-membered-ring system.

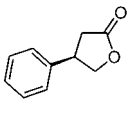
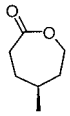
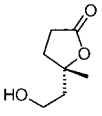
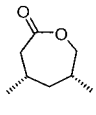
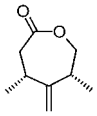
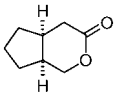
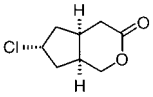
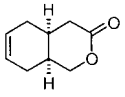
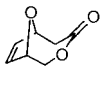
The enantiodivergent trend in biooxidation was also observed for fused bicycloketones **1f–h**. Generally, moderate to excellent stereoselectivity was obtained upon biooxidation with CHMO-type enzymes. CPMO-type BVMOs gave lactones with chirality consistent with the two-enzyme-groups hypothesis but with lower selectivity for **2f**.

Bridged bicyclo precursor **1i** is only oxidized by CPMO-type enzymes to form lactone **2i**. Together with previous studies of classical kinetic resolutions,[20] this is to some extent an exception of the hypothesis of stereodivergent biotransformations. However, the clearly differentiated substrate acceptance again supports the classification of the studied BVMOs into two groups.

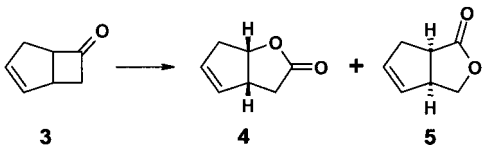
Representatives of this enzyme library exhibited superior enantioselectivities for desymmetrizations with all ketones compared to those observed in previously reported bacterial BVMO oxidations. The potential of enantiodivergent biocatalysts with overlapping substrate acceptance for natural product synthesis is demonstrated by accessing various indole alkaloids such as alloyohimbane[21] from (−)-**2h** and antirhine[22] from the antipodal (+)-**2h**.

Intrigued by the significantly different behavior of the two BVMO clusters in the desymmetrization reactions, we investigated the regiodivergent transformation of fused ketones bearing a cyclobutanone structural motif (Scheme 2). This conversion is considered to be one of the "benchmark" reactions for asymmetric Baeyer–Villiger oxidations[23] and

Table 1: Enantiodivergent Baeyer–Villiger oxidation of prochiral ketones **1** by recombinant *E. coli* cells producing BVMOs of bacterial origin.[a]

Strain	Product								
	 **2a**	 **2b**	 **2c**	 **2d**	 **2e**	 **2f**	 **2g**	 **2h**	 **2i**
$CHMO_{Acineto}$	53%	61%[9b]	59%	65%	54%	50%	78%	33%	n.c.[b]
	62% *ee* (−)	98% *ee* (−)	86% *ee* (−)	>99% *ee* (−)	92% *ee* (+)	89% *ee* (−)	>99% *ee* (−)	5% *ee* (−)	n.a.[c]
$CHMO_{Arthro}$	54%	50%	42%	35%	38%	66%	55%	46%	n.c.[b]
	87% *ee* (−)	>99% *ee* (−)	92% *ee* (−)	>99% *ee* (−)	96% *ee* (+)	82% *ee* (−)	>99% *ee* (−)	60% *ee* (−)	n.a.[c]
$CHMO_{Brachy}$	45%	69%	48%	40%	51%	71%	45%	56%	n.c.[b]
	93% *ee* (−)	>99% *ee* (−)	97% *ee* (−)	99% *ee* −)	94% *ee* (+)	91% *ee* (−)	>99% *ee* (−)	85% *ee* (−)	n.a.[c]
$CHMO_{Brevi1}$	73%	65%	n.c.[b]	61%	70%	21%	55%	10%	n.c.[b]
	98% *ee* (−)	>99% *ee* (−)	n.a.[c]	97% *ee* (−)	>99% *ee* (+)	65% *ee* (−)	95% *ee* (−)	71% *ee* (−)	n.a.[c]
$CHMO_{Brevi2}$	50%	59%	37%	56%	44%	42%	59%	92%	19%
	39% *ee* (+)	44% *ee* (+)	61% *ee* (+)	99% *ee* (+)	>99% *ee* (−)	0% *ee*	60% *ee* (+)	94% *ee* (+)	93% *ee* (+)
$CPMO_{Coma}$	66%	68%[13a]	54%	58%	63%	89%	92%	76%	53%
	37% *ee* (+)	46% *ee* (+)	76% *ee* (+)	*91*% *ee* (+)	99% *ee* (−)	9% *ee* (+)	*48*% *ee* (+)	>99% *ee* (+)	95% *ee* (+)
$CHMO_{Rhodo1}$	58%	72%	59%	59%	75%	62%	51%	47%	n.c.[b]
	52% *ee* (−)	>99% *ee* (−)	94% *ee* (−)	96% *ee* (−)	96% *ee* (+)	85% *ee* (−)	98% *ee* (−)	73% *ee* (−)	n.a.[c]
$CHMO_{Rhodo2}$	63%	67%	47%	64%	60%	75%	71%	51%	n.c.[b]
	50% *ee* (−)	95% *ee* (−)	94% *ee* (−)	90% *ee* (−)	96% *ee* (+)	75% *ee* (−)	95% *ee* (−)	73% *ee* (−)	n.a.[c]

[a] Yields are given for products isolated after flash column chromatography; *ee* values were determined by chiral-phase gas chromatography; the sign of specific rotation is given. [b] n.c. = no conversion. [c] n.a. = not applicable.



3 **4** **5**

Scheme 2. Regiodivergent Baeyer–Villiger oxidation of fused ketone **3** to "normal lactone" **4** and "abnormal lactone" **5**.

has been studied in detail with $CHMO_{Acineto}$.[24] Racemic compound **3** is transformed into two types of regioisomeric lactones in a resolution process: migration of the more-substituted carbon atom generates the expected "normal" lactone **4**, while "abnormal" lactone **5** is formed by migration of the less-substituted carbon atom.

Again, we observed a divergent trend for the two enzyme groups (Table 2).[25] CHMO-type proteins displayed a clean resolution that led to the formation of regioisomeric lactones **4** and **5** in approximately 1:1 ratio and with high optical purities. By contrast, $CPMO_{Coma}$ and $CHMO_{Brevi2}$ (CPMO-type enzymes) yielded predominantly "normal" lactone **4** in nearly racemic form. Trace amounts of "abnormal" product **5** were obtained in good enantiomeric excess.

To rationalize this significantly different biocatalytic activity, we compared the results from the biooxidations with sequence-analysis data for the genes and proteins of all eight BVMOs. While only minor correlation was observed on the DNA level of the structural genes, a significant trend in enzyme similarity was found at the amino acid level. Phylogenetic tree analysis of the representatives of the BVMO enzyme family vis-à-vis a remote reference sequence also resulted in clustering into two groups, which to a high degree reflected the reaction profiles of the biocatalysts (Figure 1). $CPMO_{Coma}$ and $CHMO_{Brevi2}$ form a distinctly different cluster while the other six enzymes form the

Table 2: Regiodivergent Baeyer–Villiger oxidation of racemic fused ketone **3** by recombinant *E. coli* cells producing BVMOs of bacterial origin.

	Yield **4**+**5**[a] [%]	Ratio **4**:**5**[b]	*ee* (1*S*,5*R*)-**4** [%]	*ee* (1*R*,5*S*)-**5** [%]
$CHMO_{Acineto}$	74	51:49	95	>99
$CHMO_{Arthro}$	86	53:47	88	>99
$CHMO_{Brachy}$	73	50:50	94	>99
$CHMO_{Brevi1}$	85	51:49	96	>99
$CHMO_{Brevi2}$	61	98:2	0	99
$CPMO_{Coma}$	61	97:3	0	>99
$CHMO_{Rhodo1}$	83	50:50	99	>99
$CHMO_{Rhodo2}$	81	50:50	97	>99

[a] Combined yields are given for the mixture of **4** and **5** after single flash column chromatography. [b] Ratio and *ee* values were determined by chiral-phase gas chromatography.

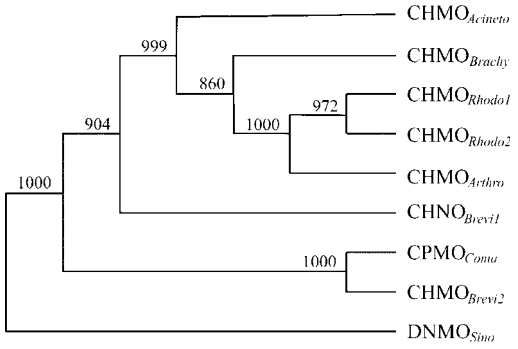


Figure 1. Phylogenetic tree of BVMOs originating from *Acinetobacter*, *Arthrobacter*, *Brachymonas*, *Brevibacterium*, *Comamonas*, and *Rhodococcus* species with the N^4-diaminopropane monooxygenase from *Sinorhizobium meliloti* (DNMO$_{Sino}$) as the outgroup (1000 bootstraps).

"CHMO-type" group. CHMO$_{Brevi1}$ is located at the borderline of the two clusters closer to the CHMO group, a fact which again agrees with its stereopreference and slightly modified substrate-acceptance profile.

A related phylogenetic tree analysis with biomolecular interpretation has been reported previously for a large general set of monooxygenases.[26] However, we consider our results to be the first connection of primary protein sequence with biocatalyst performance for BVMOs.

When the alignment of protein sequences of BVMOs included in this study is compared with the recently described point mutations in CHMO$_{Acineto}$,[27] two striking similarities can be identified. The Leu 143 Phe mutation exactly follows the separation in the two main enzyme groups. The CPMO type in both cases has a phenylalanine, whereas the CHMO type, with the exception of CHMO$_{Brevi1}$, has a leucine residue in this position, a fact again that reflects the borderline position of the latter enzyme. Phenylalanine is conserved in position 432 throughout the studied sequences with two exceptions: CHMO$_{Brevi1}$ and CHMO$_{Arthro}$. The Phe 432 Tyr mutation exactly mimics the amino acid composition of CHMO$_{Brevi1}$ at this position. This mutation has been indicated to significantly increase stereoselectivity and, interestingly, a similar trend was observed in this study.

Recently, the first X-ray structure for a moderately related BVMO from an extremophilic microorganism was solved.[28] This work gave valuable suggestions for the molecular mechanism of the enzymatic oxidation. However, the enzyme structure was determined in the absence of NADPH and no cocrystallization with a substrate is available, so far. As the authors suggest that the protein undergoes extensive conformational changes in the biocatalytic cycle, further conclusions for distant members of the BVMO family, such as those included in this study, seem rather speculative. However, when we consider the information together with recent results in modifying the enantioselectivity of CHMO$_{Acineto}$ by random mutagenesis,[27] we can begin to identify BVMO regions with major impact on biocatalytic behavior and stereopreference. Further structural and biotransformation studies on BVMOs more closely related to the two clusters outlined herein and on a larger set of ketones—currently being addressed in our laboratory—seem necessary for the development of a comprehensive and predictive model for this enzyme family to successfully expand the biocatalytic armament in the field of Baeyer–Villiger oxidations.

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