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Microbial Baeyer–Villiger Oxidation: Stereopreference and Substrate Acceptance of Cyclohexanone Monooxygenase Mutants Prepared by Directed Evolution

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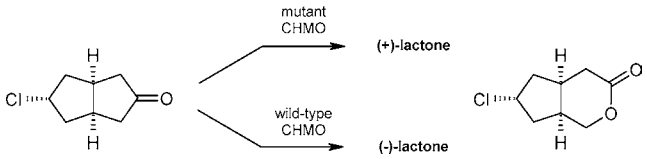
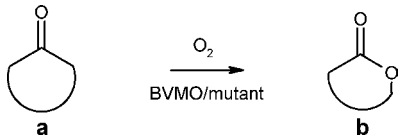
ABSTRACT



An array of random mutants of cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 was screened against a library of structurally diverse ketones for modifications in the substrate acceptance profile and stereopreference of the enzymatic Baeyer–Villiger biooxidation. While the set of mutant biocatalysts was initially evolved for the enantiocomplementary oxidation of 4-hydroxycyclohexanone, improved and/or divergent stereoselectivities were observed for several substrates. In addition, expanded substrate acceptance to facilitate biotransformation of sterically demanding ketones was found.

The microbial Baeyer−Villiger oxidation (Scheme 1) has received substantial attention in recent years as a valuable tool for the transformation of prochiral or racemic ketones into optically pure lactones which are attractive intermediates in organic synthesis. Thanks to progress in genome deciphering during the past years, an increasing diversity of Baeyer−Villiger monooxygenases (BVMOs) has become available as versatile catalysts for such stereoselective transformations which fulfill the requirements of sustainable and green chemistry strategies.[1] Until recently, widespread application of such catalytic entities was limited by a general obstacle of biocatalysis: While access to both antipodal forms of a product can be achieved with de novo designed synthetic catalysts, no generally applicable strategy is available to provide enantiocomplementarity in biocatalysis.

Scheme 1. General Scheme of Baeyer−Villiger Oxidation



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Two approaches to overcome this limitation were previously successfully implemented in the area of enzymatic Baeyer−Villiger oxidation by our two groups. Taking advantage of nature's biodiversity, a platform of recombinant *E. coli* strains overexpressing BVMOs of various microbial origin was introduced[2] with overlapping substrate profiles for the conversion of various ketones. These catalysts display enantiocomplementary stereospecificity[3] and regiodivergent biotransformations.[4]

Another strategy is based on the modification of a particular biocatalyst of known characteristics. The combination of recent protocols from molecular biology[5] with efficient screening methods[6] under a directing evolutionary pressure (directed evolution) represents a powerful approach toward obtaining new asymmetric catalysts.[7] In the field of enzymatic Baeyer−Villiger oxidation, a random strategy has been successfully applied to modify the stereopreference of cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871,[8] and enantiodivergent biocatalysts were evolved within two generations.[9] In this process, a limited number of amino acids become exchanged, and the genes of the most promising mutants are used as templates for subsequent rounds of mutagenesis/screening. In a knowledge-based approach, a region within the active site of phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* (ZP_57328)[10] was modified to alter the substrate specificity of the enzyme.[11] This was possible on the basis of the 3-dimensional structure of PAMO constituting the first crystal structure of a BVMO.[12]

While these previous studies aimed at the modification of a specific property of the enzyme with respect to the transformation of a particular substrate, in this contribution the impact of mutations on a variety of substrates is investigated. We selected a number of CHMO mutants that displayed enantiocomplementary biooxidations of 4-hydroxycyclohexanone.[9] These mutant enzymes were screened against a library of structurally diverse ketones of different polarity and electronic properties. In this contribution, we present the most interesting results of a comparative study of biotransformations by mutant enzymes and wild-type CHMO (Scheme 1 and Table 1).

Recombinant whole-cell expression systems were utilized to implement easy-to-use cofactor recycling (NADPH) and biocatalyst production within living *E. coli* cells.[9] We used a screening methodology outlined recently based on a 24-well plate format with a fermentation volume of 1 mL (0.5 mg of substrate).[13] This multidish format reflects fermentation characteristics of shake-flask cultures to a significant extent, hence allowing also an assessment of biocatalyst efficiency and performance on larger scale. In combination with chiral-phase GC analysis, this screening method allows the investigation of diverse ketones with respect to conversion and stereoselectivity within a relatively short period of time.

Conversion of 5,5′-bicycloketone **1a** with native CHMO gave (−)-lactone **1b** in very good yield and good enantioselectivity. Exchange of phenylalanine by serine at position 432 resulted in a slight increase in stereoselectivity compared to wild-type CHMO, whereas mutations L426P and A541V showed a significant decrease of enantiopreference. Remarkably, the more lipophilic chloro-bicyclic compound **2a** was biooxidized to enantiocomplementary lactone **2b** with good stereoselectivity, depending on the particular mutant used. Again, mutation F432S resulted in the same stereoselectivity as in wild-type CHMO, and mutations at positions 426 and 541 in this case led to formation of the antipodal lactone.

In the cyclobutanone series (substrates **3a**, **4a**) a related trend was observed, although the degree of enantioselectivity turned out to be lower. The mutation F432S for the more polar precursor **3a** led to the antipodal lactone **3b** compared to wild-type CHMO. A different mutation at position 432 (Phe → Ile) gave a slight increase in the enantioselectivity compared to the wild-type enzyme. Similar results were obtained with phenylcyclobutanone **4a**. The mutation F432S (together with K78E) increased the stereoselectivity significantly compared to wild-type CHMO, while the mutation F432I had the opposite effect resulting in almost racemic lactone **4b**.

Based on previous results for the enantiocomplementary biooxidation of 4-hydroxycyclohexanone,[9] we investigated different substituted prochiral cyclohexanones **5a**−**7a**. Surprisingly, no enantiodivergence was observed, and neither a change of hybridization (**6a**) nor modifications in polarity (**7a**) of substituents at C-4 showed substantial influence on the stereopreference. Stereoselectivity was slightly improved for lactones **6b** and **7b**.[17] The given results are representative for a larger set of ketones investigated.

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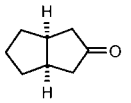
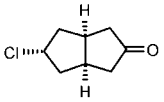
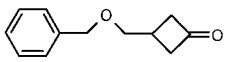
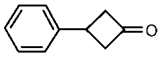
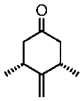
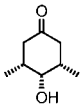
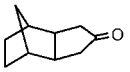
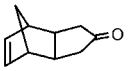
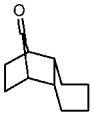
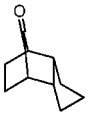
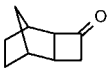
Table 1. Screening Results of CHMO Mutants Produced by Directed Evolution[9]

substrate		mutant	mutation	+/-[a]	ee [%]	conv[b]
1a		wild type	-	-[14]	89	+++
		1-K2-F5	F432S	-	94	+++
		1-H7-F4	L426P, A541V	-	17	+++
2a		wild type	-	-[14]	>99	+++
		1-K2-F5	F432S	-	99	+++
		1-H7-F4	L426P, A541V	+	60	+++
3a		wild type	-	+[15]	53	+++
		1-K2-F5	F432S	-	83	+++
		1-E12-B5	F432I	+	66	+++
4a		wild type	-	-[16]	62	+++
		1-K6-G2	K78E, F432S	-	96	+++
		1-E12-B5	F432I	-	8	+++
5a		wild type	-	-[17]	>99	+++
		1-K2-F5	F432S	-	>99	+++
		1-H7-F4	L426P, A541V	-	>99	+++
6a		wild type	-	+[3a]	92	+++
		1-K2-F5	F432S	+	>99	+++
		1-H7-F4	L426P, A541V	+	>99	++
7a		wild type	-	-[17]	96	+++
		1-K2-F5	F432S	-	>99	+++
		1-H7-F4	L426P, A541V	-	>99	+++
8a		wild type	-	na[c]	na	nc[d]
		1-C2-B7	F432Y, K500R	+	92	+
		1-H7-F4	L426P, A541V	+	12	++
9a		wild type	-	na	na	nc
		1-K2-F5	F432S	-	ca 90[e]	++
		1-H7-F4	L426P, A541V	+	ca 60[e]	++
10a		wild type	-	-[13]	96	+++
		1-K6-G2	K78E, F432S	-	94	+++
		1-E12-B5	F432I	-	48	+++
11a		wild type	-	-[13]	96	+++
		1-K2-F5	F432S	-	93	+++
		1-E12-B5	F432I	-	48	+++
12a		wild type	-	-[f]/-[g]	44[e]/>99[f]	+++ / (70/30)[h]
		1-K2-F5	F432S	-[f]/-[g]	65[e]/>99[f]	+++ / (58/42)[h]
		1-H7-F4	L426P, A541V	-[f]/-[g]	94[e]/>99[f]	+++ / (47/53)[h]

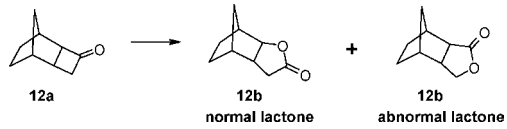
[a] Sign of specific rotation. [b] Conversion: +++: >90%, ++: 50−90%, +: <50%. [c] Not applicable. [d] No conversion. [e] No baseline separation. [f] "Normal" lactone. [g] "Abnormal" lactone. [h] Ratio between "normal" and "abnormal" lactones **12b**.

In a number of previous studies it became apparent that wild-type CHMO does not accept structurally demanding ketones. Therefore, we focused our study on this class of substrates and obtained very interesting results with exo tricyclic ketones (**8a**, **9a**). Due to multiple mutations, an expansion in substrate acceptance was observed. The most interesting mutations are L426P and A541V in mutant 1-H7-F4 and various amino acid exchanges at position 432. The saturated exo compound **8a** gave 92% enantioselectivity with mutant 1-C2-B7 and essentially racemic lactone with mutant 1-H7-F4 (12% ee). Interesting results were obtained with the unsaturated exo-tricyclic ketone **9a**, whereas the mutant 1-K2-F5, with a mutation at position 432, produced (−)-lactone **9b** with high enantioselectivity, and biooxidation with mutant 1-H7-F4 resulted in formation of the antipodal (+)-product.

In the endo-tricyclic series (**10a**, **11a**), again mutations at position 432 showed the strongest effects. The exchange of phenylalanine by isoleucine resulted in a significant decrease in enantioselectivity, whereas mutation F432S had no effect on the stereopreference.

Finally, the regiodivergent biooxidation of a racemic fused bicycloketone was investigated (**12a**) (Scheme 2). In general, compound **12a** was converted to both regioisomers ("normal"

Scheme 2. Regiodivergent Baeyer−Villiger Oxidation of Ketone **12a**: Formation of "Normal" and "Abnormal" Lactone **12b**



and "abnormal" lactone; ratio 70/30) by wild-type CHMO in very high optical purity for the abnormal lactone and moderate stereoselectivity for the normal lactone. The two mutants 1-H7-F4 and 1-K2-F5 displayed an increase in the enantioselectivity of the "normal" lactone and by improving the regioselectivity of the biooxidation toward the optimal 50:50 distribution.

In summary, we have demonstrated the potential of random mutagenesis for the wide-spread modification of a Baeyer−Villiger monooxygenase. This tool offers a powerful methodology to optimize several properties of biocatalysts, ultimately overcoming limitations which hampered the widespread application of enzymes in synthetic chemistry in the past.

Within this study, we could demonstrate that a limited number of modifications within a protein can significantly alter both substrate acceptance and stereopreference compared to the wild-type enzyme. In a number of cases, new substrates were identified and enantiocomplementary biooxidations were realized.

The results obtained also confirm the existence of certain "hot spots" within CHMO of paramount influence to both substrate specificity and stereoselectivity. In particular, mutations at position 432 have a significant impact on the characteristics of mutant biocatalysts. The identification of such hot spots offers the option of modifications of the enzyme in subsequent rounds of biocatalyst optimization either by rational design or directed evolution.[7,18]

However, as opposed to naturally evolved BVMOs,[2] no explicit trend was identified for the effect of specific amino acid exchanges on both substrate acceptance and stereopreference. This study accumulated a multitude of novel data on structurally diverse substrates, which may be compiled into a more comprehensive model on molecular level. So far, this remains a major challenge, as previous studies suggested major conformational changes of such enzymes during the complete catalytic cycle.[12] A homology model of CHMO[11] made possible by the X-ray structure of PAMO[12] forms the basis of a future study aimed at understanding the source of enhanced or inverted stereoselectivity.

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Supporting Information Available: Details of the screening method and corresponding chiral GC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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