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PAPER

Manipulating the stereoselectivity of the thermostable Baeyer–Villiger monooxygenase TmCHMO by directed evolution†

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Baeyer–Villiger monooxygenases (BVMOs) and evolved mutants have been shown to be excellent biocatalysts in many stereoselective Baeyer–Villiger transformations, but industrial applications are rare which is partly due to the insufficient thermostability of BVMOs under operating conditions. In the present study, the substrate scope of the recently discovered thermally stable BVMO, TmCHMO from *Thermocrispum municipale*, was studied. This revealed that the wild-type (WT) enzyme catalyzes the oxidation of a variety of structurally different ketones with notable activity and enantioselectivity, including the desymmetrization of 4-methylcyclohexanone (99% ee, *S*). In order to induce the reversal of enantioselectivity of this reaction as well as the transformations of other substrates, directed evolution based on iterative saturation mutagenesis (ISM) was applied, leading to (*R*)-selectivity (94% ee) without affecting the thermostability of the biocatalyst.

Introduction

The Baeyer–Villiger oxidation of ketones or aldehydes is a valuable transformation in synthetic organic chemistry in which a single oxygen atom is inserted into the respective C–C bond adjacent to a carbonyl moiety with formation of esters or lactones.[1] Many different reagents such as peracids or hydroperoxides have been used as stoichiometric oxidants, sometimes in the presence of chiral transition metal catalysts[2] or organocatalysts[3] for obtaining enantiomerically enriched or pure products. The C–C activating process can also be catalyzed by Baeyer–Villiger monooxygenases (BVMOs),[4] which require only dioxygen from air, thereby avoiding the use of potentially explosive reagents. Mechanistically, O$_2$ reacts with the reduced form of the enzyme-bound flavin (FAD), brought into this redox state by NADPH, with formation of an intermediate hydroperoxide. This intermediate in the anionic form adds to

the carbonyl function leading to the so-called Criegee intermediate. In the final step, one of the alkyl or aryl groups next to the original carbonyl function migrates. The product is then released, after which NADP$^+$ is also released. Being NADPH-dependent, BVMOs require a cofactor regeneration system such as glucose dehydrogenase or a whole-cell system.

By using BVMOs from different sources and numerous structurally different ketones as non-native substrates, high stereoselectivity has been observed numerous times.[4] Moreover, directed evolution[5] can be applied in those cases in which stereoselectivity proves to be moderate to poor, or if inversion of the enantioselectivity is required.[6] For example, cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (AcCHMO) has been subjected to protein engineering a number of times.[7] Nevertheless, a major obstacle for wide industrial applications appears to persist, namely the lack of robustness of most BVMOs under operating conditions.[8] The discovery of the thermostable phenylacetone monooxygenase from *Thermobifida fusca* (PAMO) set a milestone in BVMO research, although the respective substrate scope proved to be limited.[9] Protein engineering of PAMO, especially by means of directed evolution, was applied several times with formation of variants that show moderate to excellent activity and stereoselectivity, depending upon the nature of the substrate.[10]

A different approach would be to apply protein engineering for achieving significant thermostability of AcCHMO without a

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ob02692g

trade-off in its broad substrate scope. Alternatively, the recently discovered CHMO from *Thermocrispum municiple* DSM 44069 (TmCHMO) offers yet another option.[11] This BVMO is characterized by good thermostability, and it has been shown to accept ketones such as cyclohexanone, acetophenone, phenylacetone and 2-octanone, but stereoselectivity has not been studied to date.[11] Furthermore, the crystal structure of TmCHMO has been solved, providing beneficial information for guiding enzyme engineering and for developing a theory for explaining the evolved catalytic profiles. Therefore, we consider TmCHMO to be an attractive starting point for exploring and possibly engineering such properties as regio- and stereoselectivity as well as substrate acceptance.

Results and discussion

As the model reaction for assessing the enantioselectivity of TmCHMO, we chose the BV-oxidation of 4-methylcyclohexanone (**1a**) with formation of (*R*)- and (*S*)-**2a** (Scheme 1). We discovered that in whole-cell reactions, WT TmCHMO shows excellent enantioselectivity and conversion in favour of (*S*)-**2a** (99% ee; conversion 100%/24 h). Thus, in this particular transformation, the result is comparable to the use of AcCHMO, the most prominent prototype enzyme of this family, which is likewise (*S*)-selective (>98% ee). However, TmCHMO has the advantage that it is more thermally stable, the melting temperature (T_m) of the wildtype being 49.8 °C as determined here and reported earlier,[11] compared to AcCHMO ($T_m = 37$ °C).

We then set out to invert stereoselectivity by protein engineering. Lactone (*R*)-**2a** is a chiral compound of particular interest because it is a precursor of (*R*)-β-methyl-substituted adipic acid, which in turn is an important intermediate in the synthesis of an effective inhibitor of acetylcholinesterase (AChE)[12] as shown in Scheme 2. In earlier work, a mutant of cyclopentanone monooxygenase from *Comamonas* sp. was shown to be (*R*)-selective (96% ee) in this reaction,[13] but this BVMO, as in
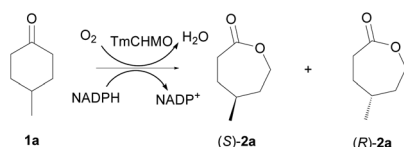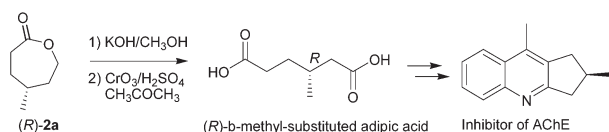
the case of AcCHMO, lacks sufficient thermostability and robustness under operating conditions to be of practical significance. A PAMO variant was also shown to be (*R*)-selective (98% ee), but activity proved to be poor.[10b]

We started our investigations with directed evolution based on saturation mutagenesis at sites surrounding the binding pocket (CAST-sites; Combinatorial Active-site Saturation Test)[14] and iterative saturation mutagenesis (ISM).[14] Utilizing the X-ray structure of TmCHMO (pdb accession code 5M10),[11] we docked the substrate **1a** in the active site. Subsequently, 11 amino acids within 5 Å from the docked substrate were chosen for saturation mutagenesis (L145, L146, F248, F279, R329, F434, T435, N436, L437, W492 and F507) (Fig. 1). These positions were subjected individually to NNK-based randomization in which all 20 canonical amino acids are used as building blocks, requiring in each case the screening of about 96 transformants for 95% library coverage (one microtiter plate).[14] Such exploratory experiments are fast and deliver valuable information for subsequent mutagenesis steps.

In the mini-libraries generated by saturation mutagenesis at positions 146, 434, 435, 437 and 507, several mutants were discovered showing decreased (*S*)-selectivity, namely L146E, F434I, T435F, T435Y, T435W, L437G, L437T, L437A and F507W, thereby pointing the way towards reversal of enantioselectivity. The libraries created at the other six positions failed to harbor any positive variants (Table S1†). At this stage we explored two mutagenesis strategies in parallel. In one approach, saturation mutagenesis at a relatively large 5-residue randomization site defined by the above hot spots was performed. This time, appropriately reduced amino acid alphabets, individually designed for each of the five residues were used.[14] The respective design was based on the information that the initial positive mutants provide (Table S2†). Accordingly, 146E/L, 434I/F, 435F/Y/W/T, 437T/A/G/L and 507W/F, including the amino acid alphabets encoding wildtype and positive mutations at individual positions, were chosen for creating a 5-residue saturation mutagenesis library.

Scheme 1 Baeyer–Villiger oxidation of 4-methycyclohexanone (**1a**) catalyzed by TmCHMO.

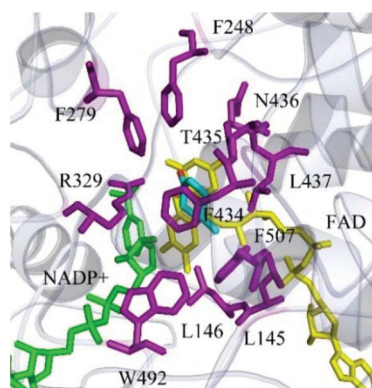**Scheme 2** Synthesis of (*R*)-β-methyl-substituted adipic acid, the precursor of an AChE-inhibitor.[12]

Fig. 1 TmCHMO structure model showing docked 4-methylcyclohexanone (**1a**) (in cyan) based on the crystal structure of wild-type TmCHMO (5M10),[11] which served as a guide for choosing amino acids for saturation mutagenesis (in purple).

A total of only 384 transformants were screened for 95% library coverage, and three (*R*)-selective variants were indeed identified (Table S3†) showing moderately reversed enantioselectivity (50%–66% ee).

According to the second strategy, the best hit identified in the exploratory NNK-based mini-libraries, mutant L437A, was chosen as a template for performing iterative saturation mutagenesis (ISM) at the remaining four hot spot residues (L146, F434, T435 and F507), which were grouped into two randomization sites: A (F434, T435) and B (L146, F507). Thereafter, several ISM pathways were explored using the respective best variants as templates and NDT codon degeneracy encoding only 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, and Gly) (Fig. 2). Two excellent (*R*)-selective mutants, which could not have been found in the above described 5-residue saturation mutagenesis library, were identified, LGY3-4-E5 (F434I/T435L/L437A/F507V) (91% ee) and LGY3-4-D11 (L146F/F434I/T435L/L437A/F507C) (94% ee). All results are summarized in Fig. 2 and in the ESI (Table S4†). In this ISM strategy, a total of only 768 transformants had to be screened for 95% library coverage. This is more than the first approach in which the 5-residue site was randomized by a single saturation mutagenesis experiment using optimally designed codons at each individual position (384 transformants). However, the screening effort involves only 8 microtiter plates which can easily be handled by automated GC. The superiority of the ISM-based approach has to do with the greater structural diversity at protein level.

By measuring the consumption of NADPH in the presence of varying amounts of 4-methylcyclohexanone (**1a**), the enzyme kinetic parameters were determined using a spectrophotometer.[11,15] Under the experimental conditions (150 µM NADPH, 5% methanol, pH 7.4), WT showed an uncoupling rate of 0.22 s^{-1}. The maximum turnover rate k_{cat} was determined to be 1.9 s^{-1}, while the affinity for 4-methylcyclohexanone was so high that a $K_m < 1 \text{ µM}$ could only be esti-

mated. For the LGY3-4-D11 mutant, a K_m could not be accurately determined, because the activities with concentrations lower than the apparent K_m were almost undistinguishable from the uncoupling rate (0.16 s^{-1}). The K_m of the LGY3-4-E5 mutant is approximately 5.5 mM and a lowered uncoupling rate in 5% methanol of 0.05 s^{-1} was observed. The k_{cat} was determined to be 8 times lower than WT for the LGY3-4-D11 mutant, while the LGY3-4-E5 mutant was close to WT with 1.23 s^{-1}. It is striking to see that all four mutants that show a high enantioselectivity (Fig. 2) have a mutation of F507 in common, while L146 is only mutated in two of these mutants. This suggests that the F507 mutations play a dominant role in equipping the enzyme with high enantioselectivity for the target substrate while the L146 mutations may only have a marginal effect and may even harm the kinetic performance as observed in the LGY3-4-D11 mutant.

Some degree of substrate inhibition, which is not unusual for BVMOs,[4] was observed in all cases. The WT is half-maximal inhibited at a concentration of 13 mM. In the LGY3-4-E5 mutant, inhibition is less strong, with a K_i roughly 3 times higher than that of WT. The fitting for the LGY3-4-D11 mutant suggests a 20 times higher K_i than WT, but has to be considered unreliable for the same reasons as the K_m determination (Table 1, Fig. S1†).

The melting temperatures (T_m) of WT and both mutants were determined with a fluorescence-based thermal shift assay (ThermoFAD).[16] It was found that the thermostability of LGY3-4-D11 and LGY3-4-E5 mutants is maintained as compared to WT TmCHMO (Table 1).

In order to further explore the potential of WT TmCHMO and the two best mutants as catalysts in organic chemistry, we tested the BV-reaction of a number of structurally diverse ketones using recombinant whole cells. The reaction mixture was shaken at 30 °C (220 rpm) for 24 h in a 25 mL flask with sufficient oxygen, containing recombinant *E. coli* expressed cells ($OD_{600} = 35$) and 10 mM substrates in 1 mL PBS buffer (pH 7.4, 50 mM). As shown in Table 2, considerable improvement in stereoselectivity towards compound **1b** was achieved with both mutants relative to WT. Enantioselectivity was boosted from 18% to 99% ee, although reversal was not observed (Scheme 3 and Table 2). A similar trend was also found for substrate **5a**, the two mutants inducing pronounced enhancement of stereoselectivity from 49% ee (*R*) to 98–99% ee (*R*). It is also interesting to note that 4-*tert*-butylcyclohexanone (**1c**) was converted completely by WT TmCHMO (93% ee, *R*), but this sterically bulky substrate is hardly accepted by the two variants LGY3-4-D11 and LGY3-4-E5. A similar tendency was found for compound **1d**. In the case of substrate **3**, WT TmCHMO and both mutants showed essentially identical behaviour with excellent (4*S*,6*R*)-selectivity (99% ee), as in the case of the more sensitive AcCHMO.[17] Of special interest is the enzymatic oxidation product of **3**, which enables the ready synthesis of the C_{11}–C_{16} subunit of ionomycin, a naturally occurring polyether antibiotic.[18] Importantly, variants LGY3-4-D11 and LGY3-4-E5 lead to reversed stereoselectivity in the reaction of compound **5b** (94%–95%, *R*) relative to WT TmCHMO,
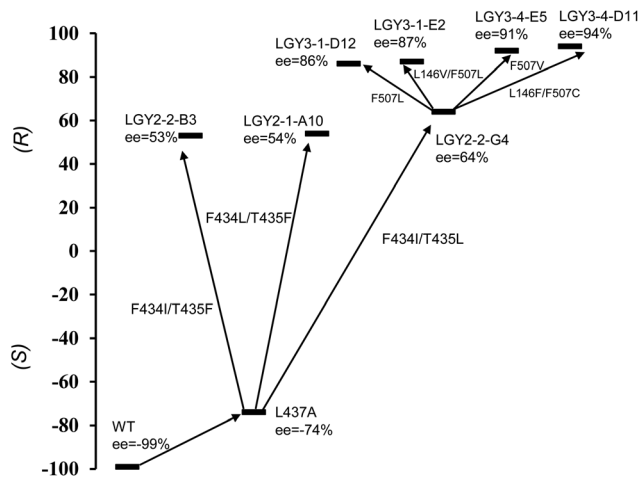

Fig. 2 Iterative saturation mutagenesis (ISM) exploration of TmCHMO as catalyst in the model reaction of ketone **1a** (Scheme 1) with focus on reversal of enantioselectivity.

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Table 1 Kinetic and thermostability data of WT TmCHMO, and mutants LGY3-4-D11 and LGY3-4-E5. 4-Methylcyclohexanone (**1a**) was used as substrate for the kinetic analysis

Entry	Mutations	k_{cat} (s^{-1})	K_m (µM)	K_i (mM)	k_{unc} (s^{-1})	T_m (°C)^b
WT		1.93 ± 0.03	< 1	13 ± 3	0.22 ± 0.08	49.8
LGY3-4-D11	L146F/F434I/T435L/L437A/F507C	0.2^a	n.d.^{a}	n.d.^{a}	0.16 ± 0.01	53.0
LGY3-4-E5	F434I/T435L/L437A/F507V	1.23 ± 0.09	5500 ± 750	47 ± 8	0.053 ± 0.002	51.5

^{a} n.d. = not determined, due to unreliable fitting only a maximal observed rate is given. All experiments were done in triplicates. ^{b} The standard deviation (triplicates) was <0.5 °C.

Table 2 Substrate scope of WT TmCHMO and variants LGY3-4-D11 and LGY3-4-E5

	WT		LGY3-4-D11		LGY3-4-E5	
Substrate	Conversion	ee [%]	Conversion	ee [%]	Conversion	ee [%]
1a	+++	99(S)	+++	94(R)	+++	91(R)
1b	+++	18(R)	+++	99(R)	+++	99(R)
1c	+++	93(S)	n. c.	n. d.	n. c.	n.d.
1d	++	88(−)	+	16(−)	+	30(+)
3	++	99(4S,6R)	++	99(4S,6R)	++	99(4S,6R)
5a	+++	49(R)	+++	99(R)	+++	98(R)
5b	+++	95(S)	++	94(R)	+++	95(R)
7	+++	N : ABN = 50 : 50 ee% >99(−), >99(−)	+++	55 : 45 83(−), 99(−)	+++	50 : 50 98(−), 99(−)
9	+++	N : ABN = 55 : 45 ee% 79(−), 98(−)	+++	48 : 52 97(−), 97(−)	+++	48 : 52 98(−), 98(−)
11	+++	P : D = 45 : 55 ee% 99(−), 99(−)	+++	35 : 65 28(−), 44(−)	+++	30 : 70 86(−), 55(−)
13	++	97(R)	+	75(R)	+	96(R)
15	+	98(R)	+	66(R)	+	58(R)

Conversion: +++ >80%, ++ 50–80%, + <50%; P: proximal, D: distal; N: normal, ABN: abnormal.; n.d. not determined, n.c. not converted.
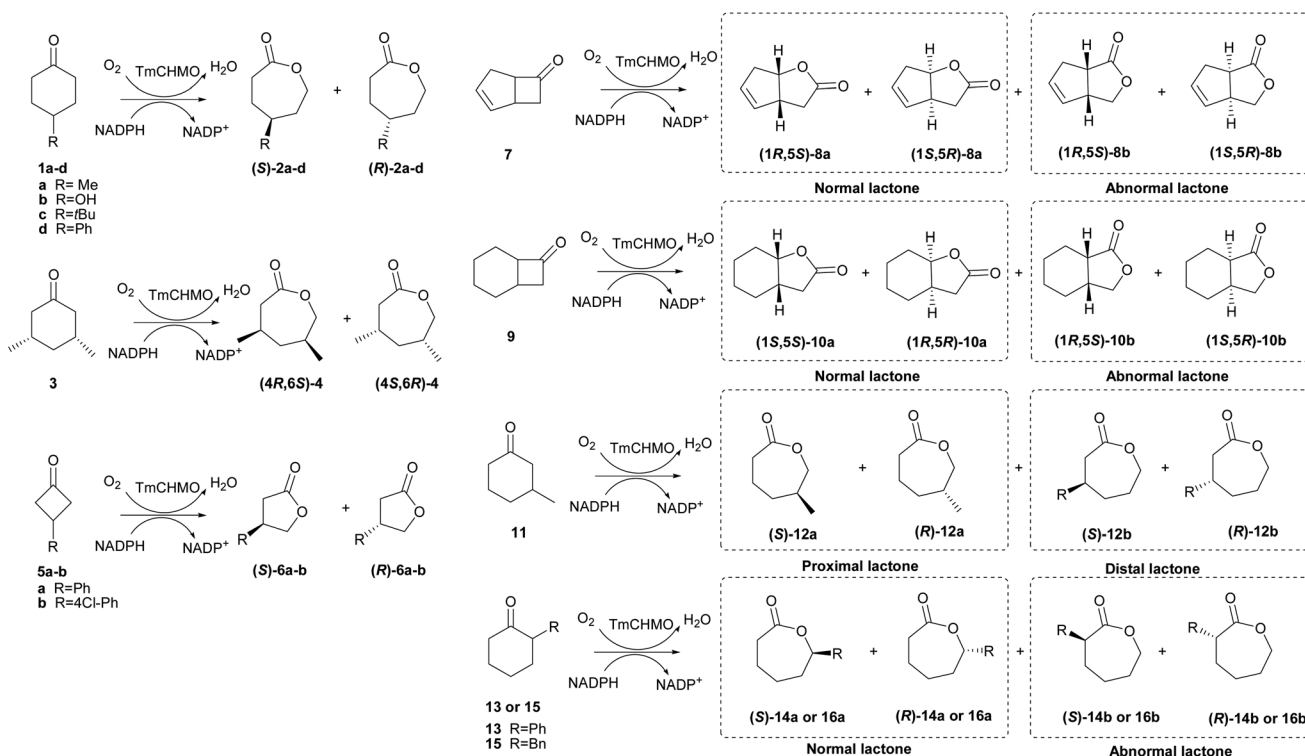
Scheme 3 Baeyer–Villiger oxidation of a series of structurally diverse substrates catalyzed by TmCHMO.

which is (*S*)-selective (95% ee) (Scheme 3 and Table 2). This reversal is a synthetically significant result because the standard BVMO, AcCHMO, is (*S*)-selective (81% ee),[19] while the (*R*)-product constitutes a chiral precursor in the synthesis of the therapeutically useful γ-aminobutyric acid receptor (GABA$_B$) agonist (*R*)-baclofen.[20] As shown in Table 2, all three enzymes convert substrate **7** with a fast reaction rate and almost the same ratio of normal lactone to abnormal lactone (N : ABN %) and high ee value, which is similar for AcCHMO [N : ABN = 51 : 49, 95(−), 95(−)].[21] The expected "normal" lactone can be used as an intermediate in the synthesis of the Corey lactone,[4h] a versatile building block in the synthesis of prostaglandins. Furthermore, the "abnormal" lactone was used as a starting material in the chemoenzymatic total synthesis of various brown algae pheromones.[22] The conversion results of 3-methyl-cyclohexanone (**11**) indicate that the mutations improve the tendency of the enzyme to produce more of the distal product. Protein engineering of other BVMOs resulting in a change of the normal/abnormal lactone ratio has been reported earlier.[23] However, the introduced mutations have little effect in reactions of compound **13** and a negative effect in the reaction of **15** (Scheme 3 and Table 2).

Conclusions

This study shows that the recently discovered thermally stable Baeyer–Villiger monooxygenase TmCHMO, and the two variants LGY3-4-D11 and LGY3-4-E5, are excellent biocatalysts in the asymmetric transformation of a variety of structurally different ketones. The two mutants were generated by directed evolution utilizing a special version of iterative saturation mutagenesis (ISM). As demonstrated by the determination of the T_m values, the thermostability of the two TmCHMO mutants is retained relative to WT. In some cases the high enantioselectivity in desymmetrization reactions is similar to those reported for the prototype CHMO, AcCHMO, but this well-known BVMO is only moderately stable. Moreover, reversal of enantioselectivity as reported herein allows ready access to several chiral compounds of particular synthetic value. The overall results of this study show that TmCHMO is an attractive scaffold for future mutagenesis. It will be interesting to see how TmCHMO and the two generated variants perform in BV-reactions of other ketones, and as biocatalysts in enantioselective sulfoxidations. Further ISM studies starting from WT or the two mutants may also be a rewarding task in future work. Finally, a complete theoretical study, hopefully unveiling the molecular basis of stereoselectivity, would not only be of fundamental interest, it could also guide future directed evolution of this promising BVMO.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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