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Investigation of a Novel Type I Baeyer-Villiger Monooxygenase from *Amycolatopsis thermoflava* Revealed High Thermodynamic- but Limited Kinetic Stability

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Abstract

Baeyer-Villiger monooxygenases (BVMOs) are remarkable biocatalysts but due to their low stability, their application in industry is hampered. Thus, there is a high demand to expand the diversity and increase the stability of this class of enzyme. Starting from a known thermostable BVMO sequence from *Thermocrispum municipale* (TmCHMO), we identified a novel BVMO from *Amycolaptosis thermoflava* ($BVMO_{Flava}$) that was successfully expressed in *Escherichia coli* BL21(DE3). We investigated the activity and stability of the purified enzyme and assigned the substrate profile for structurally different cyclohexanones and cyclobutanones. The enzyme showed lower activity in comparison to cyclohexanone monooxygenase ($CHMO_{Acineto}$) from *Acinetobacter* sp. as the prototype BVMO but indicated higher kinetic stability by showing 2-fold increased half-life at 30 °C. The thermodynamic stability, represented by the melting temperature, resulted in a T_m value of 53.1 °C for $BVMO_{Flava}$ that is comparable to the T_m of TmCHMO ($\Delta Tm = 1°C$) and significantly higher than the T_m value for $CHMO_{Acineto}$ (($\Delta Tm = 14.6°C$)). We observed a strong deviation between the thermodynamic and the kinetic stability of $BVMO_{Flava}$, which might has a major impact for future enzyme discovery of BVMOs and their synthetic applications.

Keywords: BVMO, In silico, Substrate profile, Enzyme stability, Enzyme discovery

Introduction

Baeyer-Villiger Monooxygenases (BVMOs) have been identified, isolated and characterized in the late 1960s and since then became highly versatile biocatalysts for the oxidation of ketones and aldehydes into the corresponding esters or lactones (Baeyer-Villiger reaction).[1] These enzymes utilize molecular oxygen, operate at ambient temperatures and under slightly basic conditions, while the conventional chemical reactions often require explosive and hazardous oxidants like peracids.[2] Based on the type of cofactor accepted by the enzyme, two different BVMO types can be classified. Type I is tightly bound to flavin adenine dinucleotide (FAD) as a cofactor and uses NADPH as a source of electrons, while type II relies on flavin mononucleotide (FMN) and uses NADH as an electron donor.[3] Type I BVMOs catalyze the oxidation of ketones into esters or lactones [4, 5] with exceptional high regio-, chemo- and enantioselectivity for the production of fine chemicals or chiral building blocks.[2a, 6] Based on these features, many industrial applications[7] have been suggested but due to low operational stability[8] under given reaction conditions, exploitation on a large scale is still challenging.[9] Researchers attempted to overcome this limitation applying different approaches, such as reaction engineering[10], protein engineering[8, 11] and metagenome mining[12]. For example, Goncalves and co-workers could increase the kinetic stability of $CHMO_{Acineto}$ 1000-fold by performing reaction engineering. They used a combination of redox cofactors (NADPH and FAD) and natural catalytic antioxidants like superoxide dismutase and catalase to stabilize the enzyme. The only industrial application for BVMOs was published by Bong et al.[13], which is catalyzing the final step of esomeprazole synthesis by a heavily mutated BVMO variant (41 mutations). A different strategy is based on *in silico* methods by sequence similarity analysis. By exploring metagenomes, it is possible to find new BVMOs, which may show higher stability and a broad substrate acceptance while avoiding tedious protein engineering. One of the most stable BVMOs to date, phenylacetone monooxygenase (PAMO) from thermophilic actinomycete *Thermobifida fusca* was found by this method.[12b] Recently, genome

mining also guided Mattevi and co-workers to find two other thermostable cyclohexanone monooxygenases (TmCHMO & PockeMO), which were isolated from thermophilic bacteria.[12a, 14]

Intrigued by the work of Romero et al, we aimed to find a novel BVMO with altered thermodynamic stability but maintained high activity and substrate acceptance based on sequence similarity *in silico* approach. We envisaged exploiting the sequence space of thermophilic bacteria by using the TmCHMO sequence as a starting point. Among sequences found in the NCBI databank, a new putative BVMO sequence from the thermophilic organism *Amycolatopsis thermoflava,* which was isolated from a heat-treated soil,[15] was selected. The enzyme was cloned and expressed successfully, enzyme activity and stability (kinetic and thermodynamic) were measured, and the substrate profile of this novel BVMO was investigated.

Result and Discussion

First, we blasted against the NCBI database using TmCHMO as search query, which is one of the most thermostable BVMOs to date. The most similar sequence to TmCHMO among thermophilic bacteria was selected and identified as a new putative BVMO from *Amycolaptosis thermoflava* ($BVMO_{Flava}$). The sequence similarity between $BVMO_{Flava}$ and TmCHMO was 83 % and contained the conserved consensus (G/AGxWxxxxF/YPG/MxxxD and FxGxxxHxxxWP/D) of the Type I BVMO family. Moreover, both Rossmann-fold motifs (GxGxxG/A), which are responsible for dinucleotide binding were identified in $BVMO_{Flava}$. The full alignment is depicted in the supporting information (Fig S1).

Furthermore, we performed a phylogenetic tree analysis with $BVMO_{Flava}$. The phylogenetic tree was constructed by PhyML (Fig 1) and visualized by TreeDYN to find the position of $BVMO_{Flava}$

between different groups of BVMOs (Fig 1). The mid-point rooted maximum likelihood phylogram shows the diversity of different BVMOs from different groups, I to VII.[21] As can be seen in the maximum likelihood phylogram (Fig 1), the sequence of $BVMO_{Flava}$ is close to the sequence TmCHMO with a strong bootstrap statistical support of 100 %. The tree also shows that $BVMO_{Flava}$ is placed in the clade of the CHMO-family and especially it is a close neighbour of $CHMO_{Acineto.}$ This suggests $BVMO_{Flava}$ to display a similar substrate profile compared to $CHMO_{Acineto}$. A closer look at the structure of $BVMO_{Flava}$ (based on the sequence homology towards TmCHMO) revealed a high similarity to TmCHMO, whereas for $CHMO_{Acineto}$ small differences especially in the outer regions and some loops were observed (see SI Fig S2). This could be an indication that the flexibility of $BVMO_{Flava}$ is hampered and therefore structural stability could be increased.

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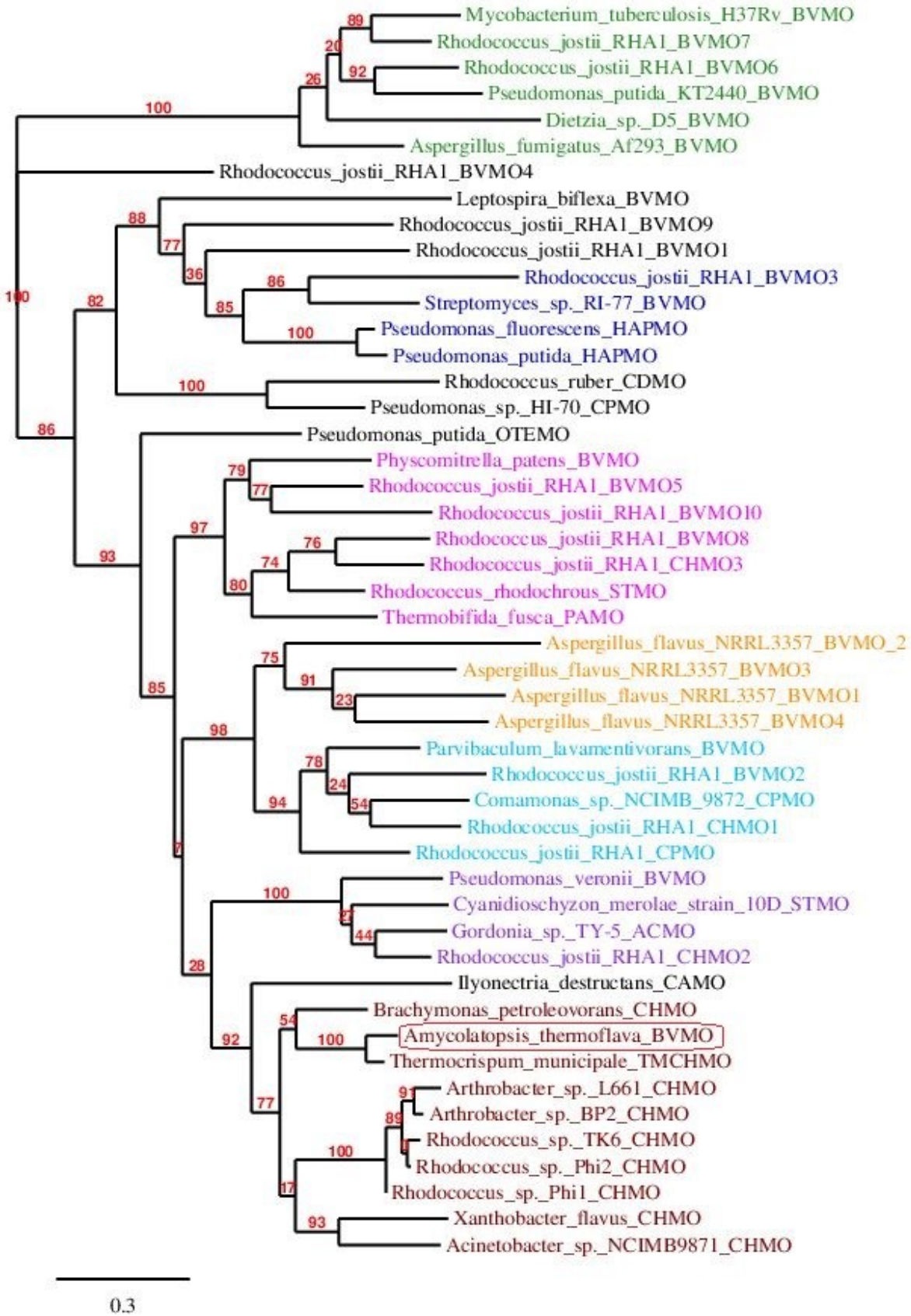

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Fig 1 The sequences of recombinantly expressed BVMOs have been used to make the phylogenetic tree, which was constructed by PhyML and visualized by Inkscape. Different BVMOs are color-coded based on their group they belong. Group 1 (light blue), group 2 (pink), group 3 (maroon), group 4 (blue), group 5 (green), group 6 (orange), and group 7 (violet). $BVMO_{Flava}$ is located in group 3 (red). The accession code of the sequences can be found in the supporting information (Table S1).

Expression and Purification

With this novel putative type I BVMO sequence in hand we ordered the synthetic gene already cloned into a pET22b(+) expression vector with a His-Tag on the C-terminus. Subsequently *E. coli* BL21 DE(3) was transformed and protein expression was performed in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG, 50 μM) at 20 °C for 20-22 h. Successful expression was analysed by SDS-PAGE analysis (Fig S3). A 59.5 kDa band of the purified enzyme was found that belong to the new $BVMO_{Flava}$. Purification was performed by standard His-Trap affinity column.

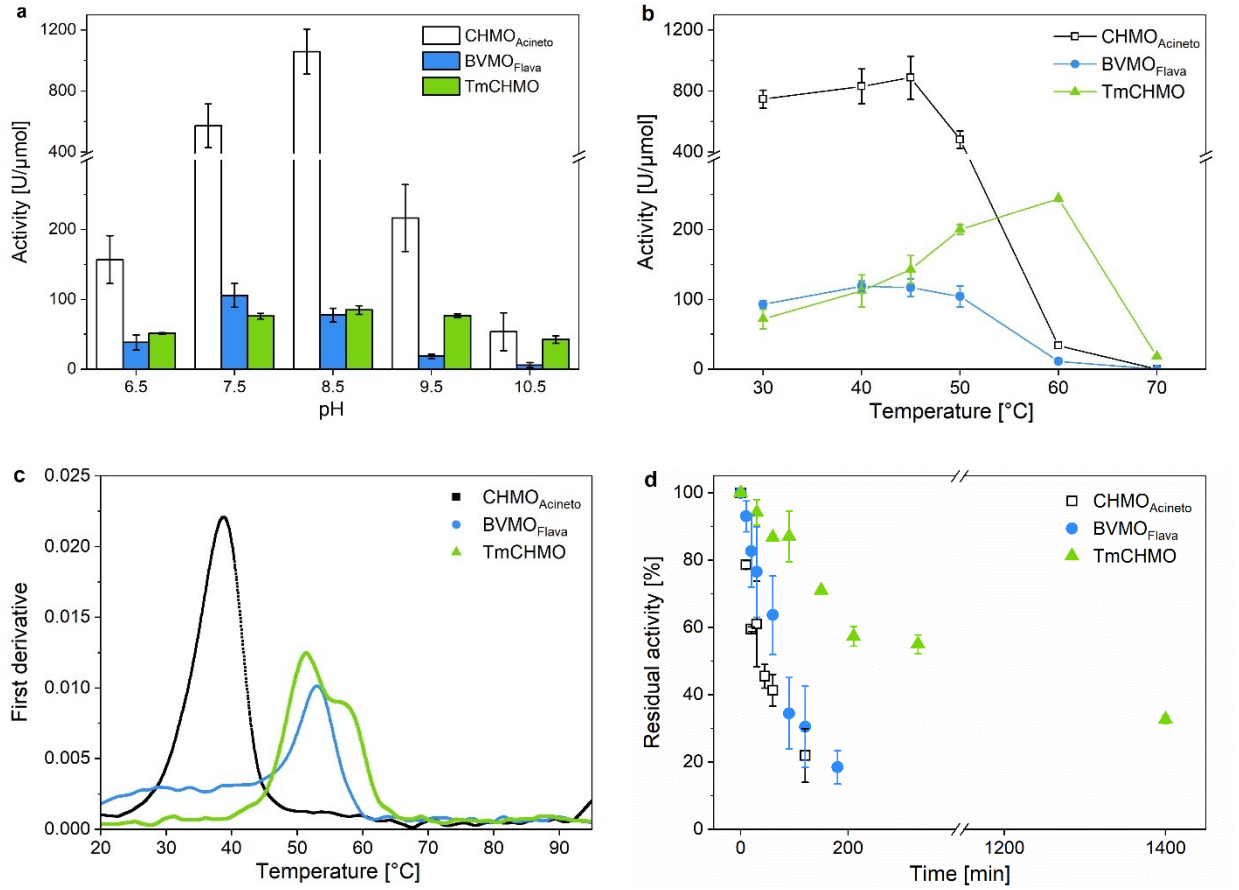
Activity and Stability measurement:

After successful soluble protein expression and purification, we investigated the activity and stability of $BVMO_{Flava}$. First, we had to find a suitable substrate, since the natural one was unknown. Based on the sequence similarity to $CHMO_{Acineto}$ we assumed a comparable substrate profile and tested cyclohexanone as a model compound. Indeed, cyclohexanone was converted to the corresponding ε- caprolactone. The activity was comparable to TmCHMO and approximately 10-fold lower than $CHMO_{Acineto}$ (Fig 2). Kinetic values have been studied for all three enzymes. The K_m value for cyclohexanone and $BVMO_{Flava}$ was 0.53±0.1 μM, for TmCHMO it was below <1 μM[22] and for $CHMO_{Acineto}$ it turned out to be one order of magnitude higher and gave 6.74±2 μM. Next, we determined k_{cat}, which resulted in the highest value for $CHMO_{Acineto}$ 15±1.3 s^{-1} in comparison to

$BVMO_{Flava}$ 1.5±0.1 s^{-1} and TmCHMO 2.0 s^{-1} (see supporting information Table S2). In the following, we investigated the pH optimum, the temperature profile, thermodynamic- and kinetic stability of $BVMO_{Flava}$ in comparison to $CHMO_{Acineto}$ and TmCHMO. The optimum pH for the activity was measured at different pH values ranging from 7.5 to 10.5 with an interval of 1 (Fig. 2a). $BVMO_{Flava}$ showed the highest activity at pH 7.5, $CHMO_{Acineto}$ has its optimum at 8.5 whereas TmCHMO is equally active from 7.5-9.5 (Fig. 2a). Especially at higher pH values (10.5), TmCHMO outperforms $BVMO_{Flava}$ and $CHMO_{Acineto}$ maintaining 50% of its initial activity. Next, we determined the temperature optimum for all three enzymes, which turned out to be 45 °C for both $BVMO_{Flava}$ and $CHMO_{Acineto}$ while TmCHMO showed the highest activity at 60 °C (Fig 2b). This result is in contrast to our expectations, since the sequence of $BVMO_{Flava}$ originated from a thermophilic organism. A different picture was observed by comparison of the thermodynamic stability by their melting temperatures (T_m, Fig 2c). $BVMO_{Flava}$ showed the highest T_m (53.1±0.2 °C) whereas TmCHMO and $CHMO_{Acineto}$ showed a T_m of 52.1±0.6 °C and 38.5±0.1 °C, respectively (Fig 2c). This finding is in agreement with the origin of the sequence based on the thermostable TmCHMO. Interestingly, TmCHMO showed a second transition midpoint that might indicates an unfolding and deactivation process with two active native states. When the temperature exceeds the second limit, the enzyme goes into the unfolded and deactivated state.

Next, we investigated the kinetic stability (half-life = $t_{1/2}$) of all three BVMOs at 30 °C, 40 °C and 60 °C (for detailed reaction conditions see material and methods). At 30 °C, $BVMO_{Flava}$ is approximately 2-fold (73±10 min) more stable than $CHMO_{Acineto}$ (46±6 min) and 7.5-fold less stable than TmCHMO (549±51 min, Fig 2d). A similar picture was observed after incubation for 1 h at 40°C. $CHMO_{Acineto}$ showed a $t_{1/2}$ for 2.02±0.45 min, whereas $BVMO_{Flava}$ is 3-times more stable with a $t_{1/2}$ of 6.00±0.80 min. In contrast, TmCHMO still shows more than 60 % of its residual activity. We investigated the $t_{1/2}$ of TmCHMO at 60 °C and determined a half-life of 0.8±0.2 min

(supporting information figure S5A and S5B). These results confirmed also our previous finding that thermodynamic stability did not necessarily correlate to kinetic stability within BVMO biocatalysts.[10a] Moreover, we also investigated the stability of all three enzymes in presence of different organic solvents (5 % v/v, Fig 2e). All of them showed a decent stability in the presence of MeOH, whereas 5 % v/v ACN affects $CHMO_{Acineto}$ the most and resulted in almost complete loss of activity. The most destructive co-solvent for the investigated enzymes was THF, which led immediately to the complete deactivation of all three enzymes.



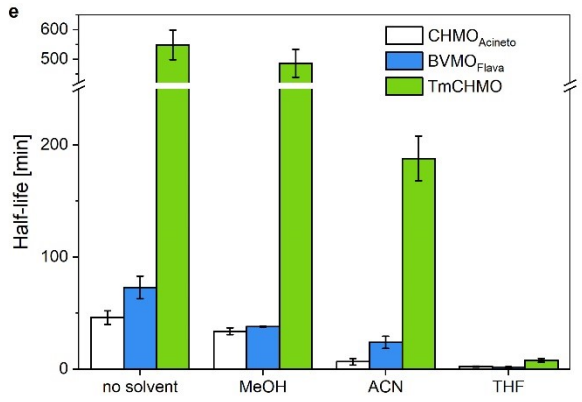


Fig 2a) Effect of pH on activity at 30 °C, 50 mM TrisHCl + 10 µM FAD, 0.5 mM cyclohexanone and 100 µM NADPH. b) Activity measurement at various temperatures from 30 to 70 °C (same conditions as for the pH study). c) Melting temperature determination was performed by nano differential scanning fluorimetry (nanoDSF): 50 mM TrisHCl, 10 µM FAD, 2 mg mL^{-1} enzyme. d) Half-life measurement: incubation at 30 °C, 10 µM enzyme, 50 mM TrisHCl, 10 µM FAD, pH 7.5. e) Determination of half-life in the presence of 5 % co-solvent (same condition as for half-life measurement).

Substrate Profile of BVMO$_{Flava}$

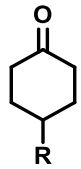
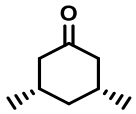
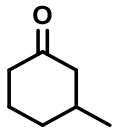
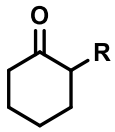
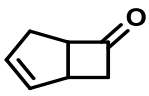
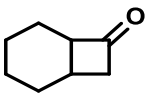
After full biochemical characterization of the novel BVMO$_{Flava}$ we elucidated its substrate profile for potential industrial applications and compared it to literature data from CHMO$_{Acineto}$ and TmCHMO. We applied whole-cell biotransformations under non-growing conditions and analysed the performance (conversion and enantiomeric excess) by chiral gas chromatography after 24 h reaction time. Positive control experiments were performed with cyclohexanone. First, Baeyer-Villiger oxidation of different substituted cyclohexanones and cyclobutanones were studied.

The substrate acceptance and enantiopreference of 4-substituted cyclohexanones **1a-d** are quite comparable for all three enzymes (Table 1). The only minor exception was found for the bulky substrate **1c** which gave full conversion in the presence of BVMO$_{Flava}$ and a very high optical purity (96 % ee) of the desired lactone (Table 1). A similar trend was observed for substrates **2** and **3**,

whereas all three enzymes showed the same catalytic performance with respect to conversion and enantiopreference.

The kinetic resolution of **4a** and **4b** resulted in formation of the *R* enantiomer in up to 99 % ee after almost 50 % conversion. Next, four different cyclobutanones were tested (**5**-**7**), whereas again same conversions and enantioselectivities were obtained. Compounds **5a** and **5b** were poorly accepted by $CHMO_{Flava}$: substrate **5a** gave almost racemic lactone whereas **5b** resulted in the desired lactone with 77 % ee optical purity. For the fused cyclobutanone **6** full conversion and perfect optical purities for both the normal and the abnormal lactone were achieved. The normal lactone is an intermediate in the synthesis of Corey lactone which is a building block for prostaglandin synthesis[23] and the abnormal product is a starting material of brown algae pheromone synthesis[2a].

Table 1 Baeyer-Villiger reaction of substituted cyclic ketones.

Substrate	R	$BVMO_{Flava}$ Conv (%)[A]	$BVMO_{Flava}$ *ee* (%)[B]	Reference reaction $CHMO_{Acineto}$ Conv (%)	Reference reaction $CHMO_{Acineto}$ *ee* (%)	Reference reaction TmCHMO Conv (%)	Reference reaction TmCHMO *ee* (%)[22]
	**1a R=Me**	>99	99 (*S*)	>99	98 (*S*)[24]	>99	99 (*S*)
	1b R=OH	83	8 (*R*)	81	10 (*R*)[25]	89	18 (*R*)
	1c R=*t*Bu	>99	96 (*S*)	17	>98 (*S*)[25]	>99	93 (*S*)
	1d R=Ph	34	89 (-)	30	60 (-)[26]	82	88 (-)
	**2**	59	99 (4*S*,6*R*)	85	99 (4*S*,6*R*)[27]	84	99 (4*S*,6*R*)
	**3**	>99	P:D/41:59[C] >99(-),96(-)	>99	P:D/49:51 99(-),99(-)[28]	>99	P:D/49:51 99(-),99(-)
	**4a R=Ph**	40	94 (*R*)	41	98 (*R*)[28]	49	97 (*R*)
	4b R=Bn	42	99 (*R*)	38	96 (*R*)[28]	48	98 (*R*)
	**5a R=Ph**	10	17 (*R*)	94	62 (*R*)[26]	>99	49 (*R*)
	5b R=Cl-Ph	16	77 (*S*)	83	81 (*S*)[26]	81	95 (*S*)
	**6**	>99	N:ABN/50:50[D] >99(-),>99(-)	>99	N:ABN/51:49 95(-),>99(-)[28]	>99	N:ABN/50:50 >99(-),>99(-)
	**7**	>99	N:ABN/59:41 72(-), >99(-)	>99	N:ABN/65:35 60(-),>95(-)[28]	>99	N:ABN/55:45 79(-),>98(-)

[A]Relative conversion (Conv) of substrate to product

[B]Enantiomeric excess (*ee*) of product

[C]P:D ratio of proximal to distal lactone

[D]N:ABN ratio of normal to abnormal lactone

Within our study, we identified a novel type I BVMO based on a sequence similarity search of the recently published and thermostable TmCHMO. The BVMO from *Amycolaptosis thermoflava* showed a high amino acid sequence similarity to TmCHMO with a molecular weight of 59.5 kDa.

Based on the phylogenetic tree analysis, $BVMO_{Flava}$ belongs to group III of type I BVMOs and is located in the same clade as $CHMO_{Acineto}$ and TmCHMO. As expected a similar substrate profile for all three enzymes was determined. Although $BVMO_{Flava}$ originates from a thermophilic organism the kinetic stability at slightly elevated temperatures dropped from 72 min (30 °C) to 6 min at 40 °C. In contrast, the thermodynamic stability (T_m-value) was quite comparable to TmCHMO and significantly higher than the T_m of $CHMO_{Acineto}$. The deviation between kinetic and thermodynamic stability is a major problem in the field, since often only T_m values are published without any context to the actual operational performance of the new catalyst. Within this study, we would like to emphasize how important it is to determine both stabilities for future comparison and putative industrial applications of BVMOs.

Material and Methods

Materials

All chemicals and reagents were from commercial sources (New England BioLabs, Ipswich, Massachusetts, United States; Promega Corp., Madison, WI, USA; Carl Roth, Karlsruhe, Germany; Lab M limited, Lancashire, United Kingdom; Sigma-Aldrich Corp., St. Louis, MO, USA; Merck KGaA, Darmstadt, Germany; Chem Lab, Zedelgem, Belgium). All substrates in this study either supplied commercially or synthesized in our lab. Distilled solvents have been used in this study.

Sequence analysis

Multiple sequence alignment has been performed by MUSCLE (Multiple Sequence Comparison by Log-Expectation).[29] The phylogenetic tree was generated by using *phylogeny.fr*.[30] The homology model was created by SWISS-MODEL[31] and protein 3D structure visualized by Swiss PDB viewer.[32] Multiple structure alignment has been carried out by PyMOL.

Plasmid construction, microbial strains and culture media

An optimized DNA fragment containing the selected BVMO genes from *A. thermoflava* (WP_027929099.1) and TmCHMO (WP_028849141.1) were synthesized and inserted into pET22b(+) by GeneScript with *Nde*I and *Not*I restriction sites, respectively. $CHMO_{Acineto}$ was obtained from G. Chen et al[33] The synthesized gene was confirmed by sequencing using T7 and T7term primers. *E. coli* strain BL21 (DE3) was transformed by heat-shock using standard procedures provided by neb transformation kit. Transformed cells were grown in an incubator operating at 37 °C in lysogeny broth–agar (LB-agar) medium supplemented with 100 μg mL^{-1} ampicillin.

Protein expression

E. coli strain BL21(DE3) was used as an expression host for all enzymes in this study ($BVMO_{Flava}$, $CHMO_{Acineto}$ and TmCHMO). LB medium (5 mL) supplemented with ampicillin to a final concentration of 100 μg mL^{-1} was inoculated with *E. coli* BL21(DE3) pET22b(+)_$BVMO_{Flava}$/$CHMO_{Acineto}$/TmCHMO and incubated in an orbital shaker at 37 °C, 200 rpm over-night. Pre-cultivated bacteria (2 % v/v) was transferred to a 1 L flask containing 250 ml LB with the same concentration of ampicillin as before. They were incubated at 37 °C, 200 rpm for 2 hours to reach an optical density between 0.6-0.8 at 590 nm. Then Isopropyl β- d- thiogalactopyranoside (IPTG) was added to the final concentration of 50 μM and the flask was transferred to 20 °C and incubated for 18-22 h.

Enzyme purification

All further steps were carried out at 4 ºC to protect the enzyme against inactivation. The overnight culture containing expressed recombinant cells were centrifuged at 8000 x g, 4 ºC for 10 min and cells were collected. Cell pellets were resuspended in 50 mM TrisHCl, pH 7.5 containing 100 μM FAD and 100 μM of PMSF (phenylmethylsulfonyl fluoride). The crude cell extract was sonicated by a Bandelin KE76 sonotrode connected to a Bandelin Sonoplus HD 3200 in 9 cycles (5s pulse, 55s break, amplitude 50 %). Cell debris and aggregates were removed by centrifugation (25000 x g, 25 min, 4 ºC, JA-17 Beckmann rotor). Supernatant was filtered using a 0.25 μm filter, equilibrated with 50 mM TrisHCl, pH 7.5, 0.5 M NaCl, 100 μM FAD and applied on 1 mL of Ni- sepharose column (1 mL, GE Healthcare Bioscience). The unwanted non-attached proteins were washed by using 5 column-volumes of 50 mM TrisHCl, 0.5 M NaCl, 40 mM imidazole, 100 μM FAD, at pH 7.5. Elution step has been performed by applying 5 column-volumes of 50 mM TrisHCl, 0.5 M NaCl at pH 7.5 containing 400 mM imidazole and 100 μM FAD. The eluted

enzymes were washed by 50 mM TrisHCl, 100 μM FAD, at pH 8 and concentrated with an ultracentrifugal tube with a cut-off of 10 kDa.[10a]

Activity and Stability measurement

Enzyme activity was measured by monitoring the decrease of NADPH absorbance at 340 nm. Standard assays contained the enzyme (0.05 μM), cyclohexanone (0.5 mM) and NADPH (100 μM) in 50 mM TrisHCl, adjusted to the desired pH. All the measurement have been carried out at 30 °C [10a]. The reaction was started immediately after enzyme addition by mixing 4 μL NADPH (25 mM stock solution) to the cuvette (final volume 1 mL). Oxidation of NADPH was followed at 30 °C in a Lambda 35 spectrophotometer (Perkin–Elmer, Waltham, MA, USA) for 120 seconds. Stability measurement was performed by incubating 10 μM enzyme at 30 °C in 50 mM TrisHCl, 10 μM FAD, pH 7.5. Samples were taken at different time points and added to a cuvette containing 100 μM NADPH and 0.5 mM substrate to test for catalytic activity. The stability in presence of different co-solvents (MeOH, ACN and THF) with final concentration of 5 % was measured under the same reaction conditions as described before. The experimental data were fitted to an exponential decay equation using Origin Pro software (Origin 9.1 for Windows). The regression data are depicted in the SI.

Melting temperature measurement

The melting temperatures (T_m) of all three enzymes were measured by Prometheus NT.48. The samples were prepared in TrisHCl 50 mM pH 7.5, 10 μM FAD with a final enzyme concentration of 2 mg mL^{-1} and the samples run from 20 to 95 °C.

Biotransformations

Recombinant protein expression was performed in LB medium, supplemented with ampicillin (100 μg mL^{-1}). The enzyme expression was induced by IPTG (final concentration of 50 μM) at

20 °C. Cells were centrifuged (8000 x g, 4°C, 10 min) and resuspended and washed in 50 mM PBS buffer pH 7.4. After washing, the cells were centrifuged (8000 x g, 4 °C, 10 min) and resuspended again with the same buffer to reach OD 30. 1 mL (OD_{590}=30) of recombinant expressed cells suspended in PBS buffer (pH 7.4, 50 mM), and 10 mM substrate final concentration, (methanol as co-solvent (5 % of total volume)) The components of the reaction (1.02 mL in total) were added into a 25 mL flask, and the reaction was performed at 30 °C by shaking (220 rpm) for 24 h.[12a] The product was extracted with ethyl acetate containing 0.1 mM methyl benzoate as the internal standard for the GC analysis. The product analysis was performed with GC (Thermo Scientific Trace or Focus GC, Thermo Fisher Scientific, Waltham, MA, USA) using chiral/achiral column. Product validation was performed by literature known reference biotransformations. The information of columns and methods for the GC experiments are listed in the supporting information.

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