Communication

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Polycyclic Ketone Monooxygenase from the Thermophilic Fungus *Thermothelomyces thermophila*: A Structurally Distinct Biocatalyst for Bulky Substrates

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Supporting Information Placeholder

ABSTRACT: Regio- and stereoselective Baeyer-Villiger oxidations are difficult to achieve by classical chemical means, particularly when large, functionalized molecules are to be converted. Biocatalysis using flavin-containing Baeyer-Villiger monooxygenases (BVMOs) is a well-established tool to address these challenges, but known BVMOs have shortcomings either in stability or substrate selectivity. We characterized a novel BVMO from the thermophilic fungus *Thermothelomyces thermophila*, determined its three-dimensional structure, and demonstrated its use as a promising biocatalyst. This fungal enzyme displays excellent enantioselectivity, acts on various ketones, and is particularly active on polycyclic molecules. Most notably we observed that the enzyme can perform oxidations on both the A and D ring when converting steroids. These functional properties can be linked to unique structural features, which identify enzymes acting on bulky substrates as a distinct subgroup of the BVMO class.

The Baeyer-Villiger oxidation is a widely-used reaction in organic synthesis to break carbon-carbon bonds through the insertion of a single oxygen atom adjacent to a carbonyl moiety, yielding esters or lactones from ketones. Since the inserted oxygen is usually derived from a peroxide or peracid, industrial scale processes raise environmental and safety concerns.[1] Baeyer-Villiger monooxygenases (BVMOs) represent an attractive biocatalytic alternative, which uses molecular oxygen as oxidant and often displays superior chemo-, regio-, and enantioselectivity.[2] The prototype BVMO, cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (CHMO) was shown to oxidize hundreds of small aromatic, linear, and cyclic ketones with high enantioselectivity.[3] Furthermore, one major hurdle for industrial application of BVMOs, the requirement of stoichiometric amounts of NADPH as reducing cofactor, has been successfully addressed.[4] What remains problematic, however, is the poor solvent tolerance and thermostability of most BVMOs. Even though close to a hundred BVMOs have been recombinantly produced and characterized so far, only two enzymes stand out by their stability: phenylacetone monooxygenase from *Thermobifida fusca*[5] (PAMO) and the recently discovered CHMO from *Thermocrispum municipale*.[6] Yet, these biocatalysts have a restricted substrate scope, being mainly active on small aromatic or cyclic aliphatic ketones. From an application point of view, it would be attractive to have access to a robust BVMO acting on structurally demanding compounds, as it is the case for many pharmaceuticals.

In an effort to exploit the genome of thermophiles for the discovery of new enzymes with interesting catalytic properties, we considered *Thermothelomyces thermophila* ATCC 42464. This genome-sequenced, thermophilic fungus efficiently degrades cellulose and other compounds derived from plant biomasses and is becoming an attractive organism for large-scale protein production.[7] We identified a gene encoding for a protein containing the typical BVMO fingerprint.[8] The gene is located in a cluster of hypothetical genes with unknown functions. A BLAST search in the UniProt database revealed ten characterized proteins with significant sequence identities (37-44%). Four are monooxygenases involved in the biosynthesis of meroterpenoids in *Aspergillus*.[9-11] Three are BVMOs from *Streptomyces* taking part in the processing of the antibiotic pentalenolactone.[12-13] One is a BVMO forming the carbonate functionality in the mycotoxin cytochalasin.[14] The remaining two are the biocatalytically characterized BVMOs from *Dietzia sp*.[15] and *S. coelicolor*.[16] A correlation between sequence similarity and substrate scope has been proposed for BVMOs,[17] and indeed, a feature shared by these enzymes is the activity on bulky, polycyclic ketones. Consistently, a phylogenetic analysis assigns the *T. thermophila* protein to a cluster containing versatile enzymes active on large substrates, such as cyclododecanone monooxygenase from *Rhodococcus ruber*[18] (CDMO) and cyclopentadecanone monooxygenase from *Pseudomonas sp.* HI-70[19] (CPDMO) (Figure S1). Collectively, these data led us to hypothesize that the *T. thermophila* protein is a BVMO that combines two attractive properties: (i) thermostability and (ii) activity on relatively large molecules.

An *Escherichia coli* codon-optimized copy of the gene was cloned in two translational fusion variants: a SUMO tagged construct and a cofactor-recycling phosphite dehydrogenase (PTDH) fusion.[20] Both constructs yielded very high expression levels in *E. coli* and the proteins were easily obtained in high purity and yield using standard metal affinity chromatography by exploiting the N-terminal 6xHis tag (Figure S2A). The purified yellow protein exhibited a typical flavoprotein spectrum with absorbance peaks at 371 and 454 nm (Figure S2B). SDS treatment revealed non-covalently bound FAD as cofactor and the extinction coefficient ε_{454} of the holoenzyme was estimated to be 10.2 $mM^{-1}\,cm^{-1}$. Upon mixing the enzyme with 100 µM NADPH and the BVMO model substrate bicyclo[3.2.0]hept-2-en-6-one (**13**), rapid consumption of NADPH was detected (Figure S3). By monitoring NADPH consumption in the absence of substrate, we determined an uncoupling rate of only 0.02 s^{-1}. We also compared the catalytic behavior of the two recombinant protein variants and determined the activity of the enzyme fused to PTDH and without any tag (obtained by cleaving SUMO). As previously observed, the effect of the dehydrogenase fusion was very modest and, in fact, resulted in a slightly higher activity (Figure S3) and unchanged uncoupling rate. Since the resulting self-sufficient biocatalyst can be used in reactions without constant addition of NADPH, as long as phosphite is added to drive the coenzyme regeneration reaction, all subsequent experiments were performed with the PTDH-fused enzyme. The preliminary analysis was completed by determining basic enzyme characteristics for optimal catalytic conditions. The pH profile highlighted good stability in a range of 6.5 - 9 with a slight preference for moderately alkaline conditions (Figure 1A).

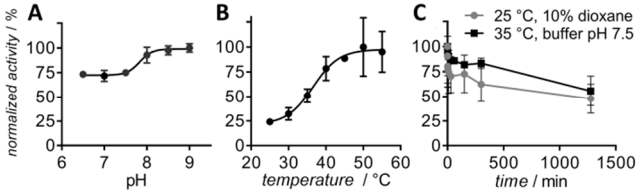
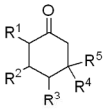
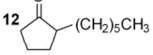
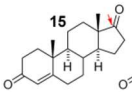
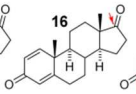
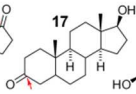
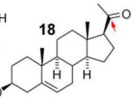


Figure 1. Effects of pH, temperature and organic solvents on the activity of *T. thermophila* BVMO (PockeMO). 2 µM of PTDH-PockeMO and 0.1 mM NADPH were mixed with **13**.

We next sought to probe the enzyme's thermostability. We found the activity to be highest at 50 °C, where it was four times faster than at room temperature (Figure 1B). Using the ThermoFAD method[21], we further observed that both native as well as PTDH-fused enzyme have an apparent melting temperature (T_m) of 47 °C, the second highest reported for a BVMO after PAMO. This T_m is sustained in a pH range of 7-9 and is increased a further 2 °C upon exposing the protein to 100 µM $NADP^+$, consistent with a tight binding of this dinucleotide cofactor (Figure S4). In parallel, we evaluated the effect of organic solvents which was probed by adding DMSO, 1,4-dioxane, ethanol, acetone or 1-propanol. The resulting reductions in T_m ranged from 1 - 5 °C to 2 - 8 °C with 5% and 10% solvent, respectively (Figure S4). The enzyme seemed to exhibit a better tolerance towards polar solvents, with DMSO having the least and 1-propanol the highest impact on T_m. Because DMSO can act as a substrate, and the effect of the remaining solvents was comparable, we chose dioxane as preferred cosolvent. We monitored the enzymatic activity as a function of time and observed a satisfactory half-life of approximately 24 hours when incubating the enzyme in buffer at 35 °C, or at room temperature with 10% dioxane (Figure 1C). These experiments indicated that the BVMO features a considerable degree of thermostability and only moderate sensitivity to organic solvents.

We next investigated the enzyme's substrate scope and selectivity (fully listed in Table S1). The closely related CPDMO and CDMO are highly active on large cyclic ketones (Figure S1). Consistently, we found conversion of **10** and **11** (Figure S5). The fifteen membered cyclopentadecanone (**8**) was fully converted and kinetic parameters were determined: k_{cat} = 0.1 s^{-1} and K_M = 144 µM (Figure S6). Of special interest, we observed efficient conversions of structurally demanding compounds, including several steroids. In particular, we tested stanolone (**17**), which has only one carbonyl group positioned at C3 of the A-ring and observed complete conversion after 24 h (Figure S5). As a reference, we performed the conversion with CPDMO, for which the 3-keto-4-oxa-4a-homo isomer was reported as the only product.[22] The MS spectrum of the resulting products with the expected mass shift of +16 Da were the same for both enzymes (Figure S5) and we confirmed the structure by NMR (Figure S7). With pregnenolone (**18**), a steroid harboring a carbonyl group only at the C17 sidechain, we found close to full conversion after 24 h (Figures 2 and S5). We again observed the +16 Da mass shift and the product was determined by NMR to be the corresponding acetate (Figure S8). When the enzyme was presented with androstenedione or androstadienedione (**15** and **16**), steroids with keto groups in both the A and D rings, it selectively oxidized the D-ring to yield the pharmaceutically relevant testo(lo)lactone (Figure S5). Again, complete conversion was observed after 24 hours.

Table 1. Substrate scope of PockeMO

Category	Structure
Linear ketones	 **1:** $R^1 = CH_3(CH_2)_3$, $R^2 = CH_2$ **2:** R^1 = Cyclohexane, R^2 = H **3:** $R^1 = CH_2$-Phe, R^2 = H
cyclic ketones	 **4:** n = 1 **7:** n = 7 **5:** n = 3 **8:** n = 10 **6:** n = 6
Substituted cyclic ketones	 **9:** R^1 = Phe, $R^{2,3,4,5}$ = H **10:** R^3 = Phe, $R^{1,2,4,5}$ = H **11:** $R^{2,4,5} = CH^3$, $R^{1,3}$ = H **12** 
Bicyclic ketones	**13**  **14** 
Steroids	**15**  **16**  **17**  **18** 

To our knowledge, this is the first report of an isolated BVMO that is able to oxidize the keto functionalities at these different positions. Steroid monooxygenase of *Rhodococcus rhodochrous* (STMO) can only convert steroidal carbonyls positioned on the C17 sidechain and not in the rings.[23] A homolog from *Cylindrocarpon radicicola* in addition is able to lactonize the D ring.[24] CPDMO accepts some steroids with the keto group on the 3 and 17 position, but is inactive on open chain ketones.[22] The *T. thermophila* BVMO represents a biocatalyst combining these abilities (Table 1, red arrows) and we therefore named it *polycyclic ketone monooxygenase* (PockeMO).

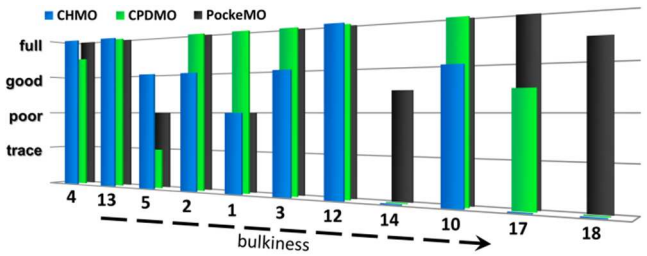


Figure 2. Conversion efficiencies of PockeMO, CPDMO and CHMO (all PTDH fused) of a mix of substrates. Efficiencies are scaled semi-quantitatively according to analysis of GC peaks of substrates and products into: full, good or poor conversion, or trace amounts detectable.

We further explored the enzyme's substrate profile using several substrates frequently tested with other BVMOs (Tables 1 and S1). For this purpose, we used a highly efficient substrate screening method, based on the conversion of a mixture of compounds and subsequent separation and analysis via GC-MS. We tested a mixture of 16 linear, aromatic, cyclic, and polycyclic ketones (Figure S5) and could immediately identify 13 conversion products. Of the unsubstituted cyclic ketones, PockeMO converted cyclohexanone to 100%, but the conversion of cyclooctanone was poor in comparison. The monosubstituted cyclic ketones **9, 10** and **12** were converted with yields close to 100%. Approximately half of the disubstituted cyclic ketone **11** was converted, whereas the unsaturated variant of this molecule (isophorone) was not accepted. The aromatic ketone **3** was fully converted and also the bridged, bicyclic **14** was converted with moderate yield (Table S1, Figure 2). Notably, although being only a poorly accepted substrate, the linear 4-octanone (**1**) was exclusively converted to the abnormal product propyl pentanoate (Figure S9). These experiments elegantly demonstrated the activity on an array of substrates of varying degree of bulkiness. At the same time, none of the compounds seemed to exhibit inhibitory effects on the enzyme. To put this further into perspective, we performed the conversion of a mix of 15 ketones with purified CHMO, CPDMO and PockeMO. Figure 2 shows the semi-quantitative comparison of the conversion efficiency of the three enzymes and clearly shows PockeMO's excellent all-round performance as well as its particular superiority with larger compounds.

For BVMOs, enantio- and regioselectivity has frequently been studied by analyzing the conversion of racemic **13** into the respective lactones. A recent extensive study on BVMO activity in various fungi revealed diverse selectivities and only a moderate phylogenetic correlation.[25] We found that PockeMO (4 µM enzyme, 2 hours) completely converted 10 mM racemic **13** enantiodivergently, yielding two regioisomeric lactones, with *ee* values of 100% and 97%, respectively (Figure S10). Thus, the enzyme exhibited both high enantio- and regioselectivity as well as high activity towards this substrate. Kinetic analysis confirmed this notion as a k_{cat} of 3.3 s^{-1} was measured (K_m = 0.4 mM).

Given these valuable stability and catalytic properties as well as the fact that PockeMO belongs to the structurally uncharacterized CPDMO/CDMO subfamily of BVMOs (Figure S1), it was of interest to determine the enzyme's three-dimensional structure. After removal of the SUMO tag, the protein could be crystallized as a complex with FAD and $NADP^+$ and its structure solved at a resolution of 2.0 Å (Table S2). Most of the residues could be modelled, apart from loop 607-614. PockeMO exhibits the typical BVMO domain organization with an FAD-domain (residues 1-229 and 479-655), an NADP-domain (residues 230-314 and 417-478), and a helical domain (residues 315-416) (Figure 3). The closest structural homologs are STMO[26] and PAMO (rmsd of 1.91 and 1.75 Å for 523 and 533 equivalent Cα atoms, respectively, with 30% sequence identity to both). The location of the FAD and NADP cofactors is consistent with that of other BVMOs and the flavin shows no significant deviation from planarity (Figure S11).

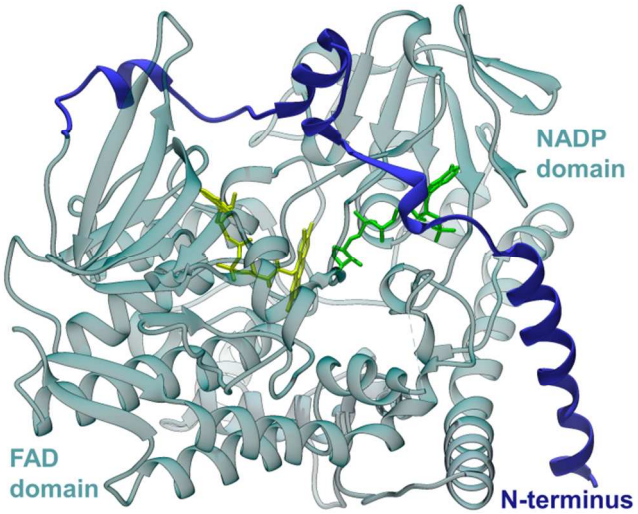


Figure 3. Overall structure of PockeMO (PDB entry 5MQ6). The N-terminal extension (residues 1-73; dark blue) is specific to this BVMO enzyme subclass (Figure S1). FAD is yellow and $NADP^+$ green (nicotinamide ring is disordered).

Despite this clear sequence and structural homology with other BVMOs and the conservation of the overall fold, PockeMO features some striking structural peculiarities. Firstly, a 70-residues N-terminal extension forms a long α-helix followed by an elongated stretch that wraps around the enzyme in the vicinity of the FAD-domain (Figure 3). The interactions between the 44 N-terminal residues and the rest of the protein are extensive and establish 24 H-bonds, 5 salt bridges, and many hydrophobic contacts. Notably, this elongated N-terminus is a feature of all BVMOs in the phylogenetic group of enzymes acting on complex molecules (Figures S1 and S12A). Secondly, there is a structural element ranging from residue 316 to 388 whose characteristic conformation reshapes the active site. This region has low sequence similarity to the canonical BVMOs (Figure S12B), in which this portion is structurally conserved (as exemplified by PAMO, Figure 4A). In PockeMO, this stretch forms a characteristic conformation of alternating loops and α-helices. As a result, the substrate-binding site of PockeMO is a long and wide tunnel-like cavity, which starts from the loop 587-624 and ends at the *re*-face of FAD (Figures 4B-C). The flexible loop delimited by residues 606 and 615 serves as a gate-like mechanism, constituting a longer element when compared to the same topological loop in PAMO (residues 495-515). Modeling shows that bulky molecules can readily be accommodated in the PockeMO's substrate tunnel (Figure S13). Thus, the enzyme exhibits characteristic structural features, which are fully consistent with the notion that it belongs to a distinct subgroup of BVMO enzymes (Figure S1).

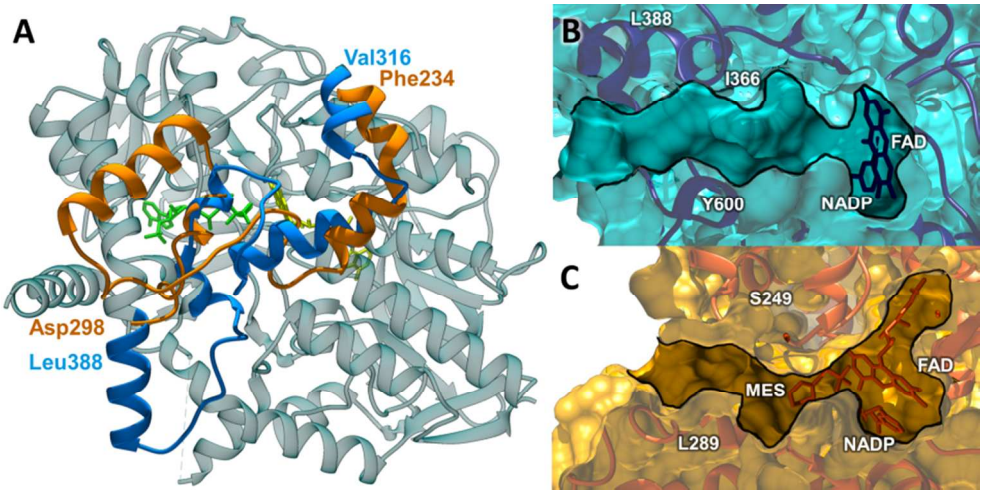


Figure 4. Characteristic structural features of PockeMO. (A) Residues 316-388 (bright blue) cover the active site and adopt a conformation not observed in other BVMO structures as exemplified by the superimposed equivalent residues of PAMO (PDB 2YLT, 234-298; orange; Figure S11). (B) PockeMO structure cut along the substrate entrance to outline the wide tunnel forming the active site. (C) PAMO active site features a narrower channel, ranging from the solvent exposed surface to the active site with MES bound.

In summary, we identified a novel, robust and versatile enzyme performing Baeyer-Villiger oxidations. PockeMO combines thermostability and organic solvent tolerance with a broad substrate profile. Specifically, the enzyme accepts bulky and complex substrates and converts them with high efficiency, making it a promising candidate for application as an industrial biocatalyst. In addition, the elucidated crystal structure not only lays the basis for enzyme engineering, but can also be regarded as a prototype of an evolutionary and structurally distinct group of BVMOs. Careful further analysis should contribute to a better understanding of the still largely unknown mechanism by which substrate selectivity in BVMO is tuned. The structural and sequence features of PockeMO can also be exploited to identify new and diverse BVMOs evolved to process relatively large substrates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:
Experimental procedures, Figures S1-S13, Tables S1, S2 (PDF)

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Notes

The authors declare no competing financial interests.

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