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Discovery and Characterization of a Baeyer-Villiger Monooxygenase Using Sequence Similarity Network Analysis

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Baeyer-Villiger monooxygenases (BVMOs) are important flavin-dependent enzymes which perform oxygen insertion reactions leading to valuable products. As reported in many studies, BVMOs are usually unstable during application, preventing a wider usage in biocatalysis. Here, we discovered a novel NADPH-dependent BVMO which originates from *Halopolyspora algeriensis* using sequence similarity networks (SSNs). The enzyme is stable at temperatures between 10 °C to 30 °C up to five days after the purification, and yields the normal ester product. In this study, the substrate scope was investigated for a broad range of aliphatic ketones and the enzyme was biochemically characterized to identify optimum reaction conditions. The best substrate (86% conversion) was 2-dodecanone using purified enzyme. This novel BVMO could potentially be applied as part of an enzymatic cascade or in bioprocesses which utilize aliphatic alkanes as feedstock.

Introduction

In chemical Baeyer-Villiger (BV) oxidations linear and cyclic ketones are oxidized into esters and lactones, respectively, with the incorporation of oxygen leading to a carbonyl C–O linkage.[1] The same type of reaction is catalyzed by the enzyme class of Baeyer-Villiger monooxygenases (BVMOs). These are flavin-dependent enzymes and they incorporate one atom from molecular oxygen into the substrate, while the second oxygen atom is reduced to water and NAD(P)H serves as the hydride donor.[2–4]

Besides the oxidation of aliphatic and cyclic ketones, BVMOs exhibit a wide substrate scope as they have been shown to catalyse the oxidation of heteroatoms,[5] *N*-oxidations[6] epoxidations,[7] oxidation of steroids,[2,7,8] aldehydes, bicyclic ketones, amines or α,β-unsaturated ketones.[9,10] The most well-studied BVMO so far is the cyclohexanone monooxygenase from *Acinetobacter calcoaceticus*,[11–18] which oxidises cyclic ketones. Previous studies have demonstrated that BVMOs also catalyse the oxidation of aliphatic ketones of varying chain length resulting in normal or abnormal products.[19,20] The interest in the BV oxidation of aliphatic compounds has also led to protein engineering campaigns of the thermostable BVMO phenylacetone monooxygenase (PAMO) with the aim to expand its limited substrate scope to include various aliphatic substrates.[21,22] Despite the strong interest in these enzymes, the generally low stability of BVMOs is a major limiting factor for their application at an industrial level.

Motivated to discover stable BVMOs with broad substrate scopes, we describe here the discovery of a novel BVMO derived from a marine metagenomic dataset of *Gammaproteobacteria* using sequence similarity networks (SSNs)[23] enriched by characterized BVMOs.[24] In order to characterize the substrate profile of the enzyme, a broad range of substrates were studied including aromatic compounds, such as a halogenated phenylacetone, cyclohexanone and aliphatic ketones of varying chain length. Particular focus was placed on aliphatic ketones as they can be derived from a prior oxidation step of *n*-alkanes. Medium chain (C_{12}–C_{16}) and long chain alkanes (C_{18}–C_{30}) have been reported to be either barely soluble or insoluble in water, making them inaccessible to biotransformation or biodegradation and therefore they tend to remain in the environment for a long time.[25] Therefore, scientific attention focused on efficient methods to achieve *n*-alkane conversion and degradation.[26,27] In previous studies, long-chain alkane biodegradation has been reported by microorganisms such as *Acinetobacter* sp., *Burkholderia cepacia*, *Acinetobacter calcoaceticus, Pseudomonas putida*,[28] *Alcanivorax borkumensis*, *Thalassolituus* sp., *Oleiphilus* sp. and *Oleispira gen.*,[29] with different hydroxylase genes (like *almA* and *alkB*) dominating the degradation process.[29–33] BVMOs also play a pivotal role in several enzymatic cascades, comprising an alcohol dehydrogenase, a BVMO and an esterase in *in vitro*[8,34–36] and *in vivo* reactions.[37]

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Results and Discussion

In silico analysis

A dataset derived from a marine metagenomic analysis[23] was mined for novel BVMOs (Figure 1) using sequence similarity networks. Initially, the dataset was clustered into different functional categories via the inclusion of well characterized enzymes such as cycloalkanone monooxygenase ($CHMO_{Acineto}$[18] and $BVMO_{Parvi}$[24]), phenylacetone monooxygenase (PAMO),[38] 4-hydroxyacetophenone monooxygenase (HAPMO)[39] and a steroid monooxygenase (SMO)[7] (Figure 1a). This allowed for the clustering of the characterized BVMOs with enzymes present in the dataset. Enzymes belonging to this cluster contain a BVMO consensus sequence and share approximately 55% sequence identity with the characterized enzymes. One of these genes was selected and its sequence was used as a template to search for other enzymes with sequence similarities higher than 50%. The retrieval of new sequences was achieved with a second SSN, (Figure 1b). From the new SSN generated, several genes were selected, including the one of a BVMO originating from *Halopolyspora algeriensis* (accession code: DFQ14_11046) (Figure 1b). Only this gene expressed recombinantly in *E. coli* (Figure S2) and therefore, we subsequently investigated this enzyme to determine its substrate profile (Scheme 1, Table S1).

As expected, two Rossman fold domain motifs (GxGxxG), were identified as well as the canonical; Type I BVMOs motif, (FxGxxxHxxxW[P/D]) and also the conserved motif [A/G]GxWxxxx[F/Y]P[G/M]xxxD.[21,37,42,43] The phylogenetic tree (Figure 2) includes sequences of BVMOs of known functions which were filtered to a sequence length 401 to 600 amino acids. $BVMO_{Halo}$ is clustered closer to aromatic compound and cycloalkanone converting monooxygenases.

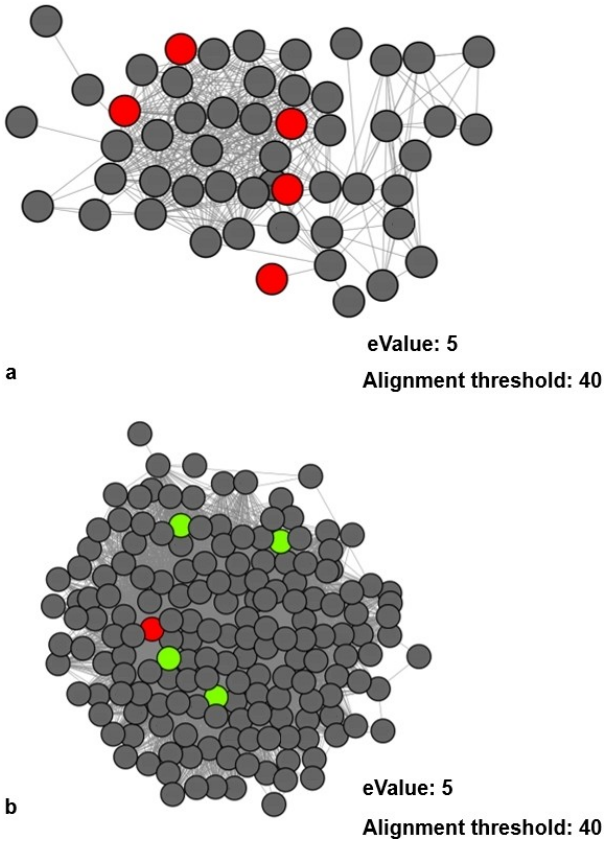
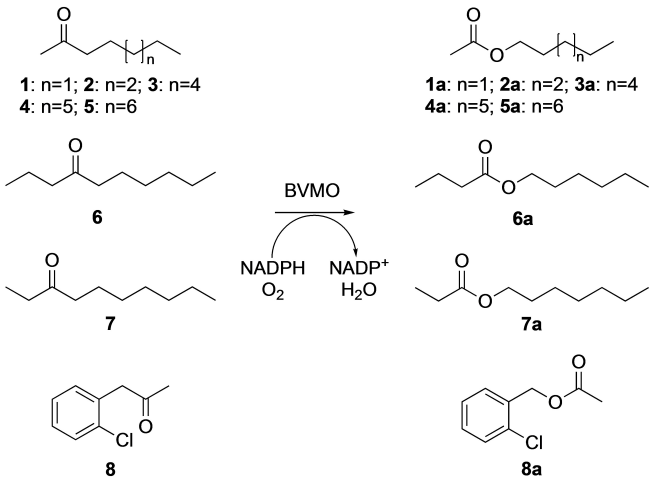


Figure 1. a. Sequence similarity network analysis generated from the marine metagenome dataset bolstered by the addition of characterized enzymes of known function. BVMOs such as SMO, $CHMO_{Acineto}$, PAMO, HAPMO and $BVMO_{Parvi}$ shown in red share over 50% sequence identity with the genes displayed in grey. b. A sequence similarity network (SSN) of BVMOs was generated from further clustering of those originally selected from the marine metagenomic dataset as represented in Cytoscape 3.9.1. The node in red represents the input sequence, while the nodes in green represent the four other BVMO genes of which only one, $BVMO_{Halo}$ was suitable for recombinant expression.



Scheme 1. Substrates studied to determine the scope of the $BVMO_{Halo}$ in the conversion of **1**–**8** into their corresponding esters (**1a**–**8a**).

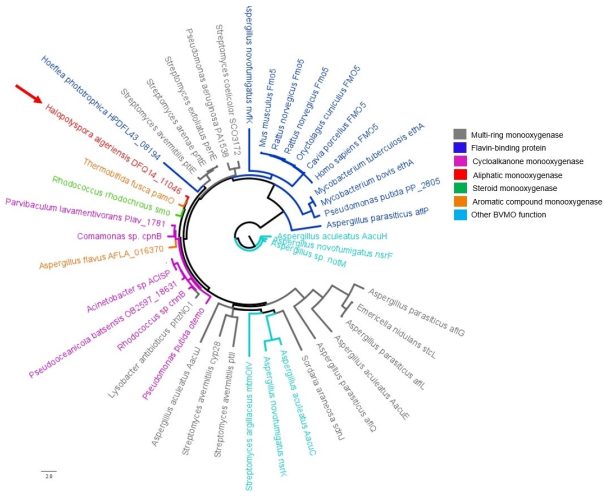


Figure 2. Phylogenetic tree of BVMOs identified in prior studies filtered to a length of sequence 400 to 600 amino acids. The $BVMO_{Halo}$ is highlighted with a red arrow. In total 44 sequences with defined functions were aligned with AliView 1.1.[40] The tree was calculated with the software IQ-TREE[41] and visualized via Figtree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). The scale bar represents 2.0 substitutions per site.

Expression and substrate scope of $BVMO_{Halo}$ in whole-cells biotransformations

The gene encoding the BVMO from *Halopolyspora algeriensis* ($BVMO_{Halo}$) was ordered already cloned into the pBAD vector and heterologously expressed in *E. coli* TOP10 cells. In order to fully characterize the substrate scope of $BVMO_{Halo}$, a variety of compounds were studied through biotransformations using resting cells, such as cycloalkanones, aromatic and various aliphatic ketones and aldehydes, bicyclic compounds and steroids (Scheme 1, Table S1). The $BVMO_{Halo}$ showed no activity on cycloalkanes, such as cyclohexanone, despite the fact that it shares approximately 55% sequence identity with cyclohexanone monooxygenases. Through further *in vivo* biotransformations[44] it was determined that the $BVMO_{Halo}$ exhibits preference towards aliphatic ketones and also converts 2-chlorophenylacetone (Scheme 1, Table S1).

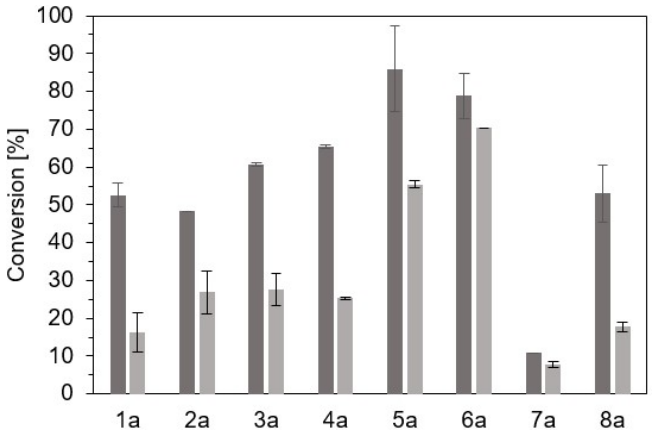


Figure 3. Conversion to the ester products **1a**–**8a** from the corresponding ketone substrates (**1**–**8**) after 20 h using $BVMO_{Halo}$ in an *in vitro* (dark grey) or in an *in vivo* whole cell *E. coli* system (light grey).

Effects of pH, temperature and co-solvents on the enzymatic reaction

The characterization of the $BVMO_{Halo}$ regarding optimum buffer and pH was investigated with purified enzyme using **1** and **5** as reference compounds. Experiments were performed in phosphate buffer (50 mM, KH_2PO_4/K_2HPO_4 or NaH_2PO_4/Na_2HPO_4) or Tris/HCl buffer (50 mM) in the pH range 6 to 8. This revealed potassium phosphate buffer and pH 6.5 as the best condition (Figure S3a, b, c). Experiments at different temperatures indicated that the $BVMO_{Halo}$ is most active at temperatures between 10–25 °C (Figure S4). Solvent tolerance studies of $BVMO_{Halo}$ showed limited acceptance of DMF and *n*-hexane up to 40% (optimum: 3% v/v DMF, Figure S5). With respect to storage stability, the enzyme completely retained its initial activity after five days storage at 4 °C (Figure S6). Based on nano differential scanning fluorimetry (nanoDSF) measurements, $BVMO_{Halo}$ loses structural integrity around 25 °C, which does not seem to strongly effect the activity even at higher temperatures as Figure S6 illustrates. Comparing activities of purified enzyme versus whole cell biotransformations, purified $BVMO_{Halo}$ is more active (Figure 3).

In the sequentially related PAMO, the residues V54, I67, Q152, R331 and A435 play a critical role to substrate specificity, with R337 being especially important, as this arginine is thought to bind $NADP^+$ during the oxygenation step.[45] Overlaying the Alphafold generated structure of $BVMO_{Halo}$ with the PAMO structure indicates the residues T46, V59, C144, R331 and T428 as corresponding residues. R331 in $BVMO_{Halo}$ seems to have the same function as R337 in PAMO (Figure 4).[46] Despite high conservation between the sequences of these two enzymes, the active site architecture differs considerably and thereby serves as a likely explanation for the different substrate preferences we have observed.

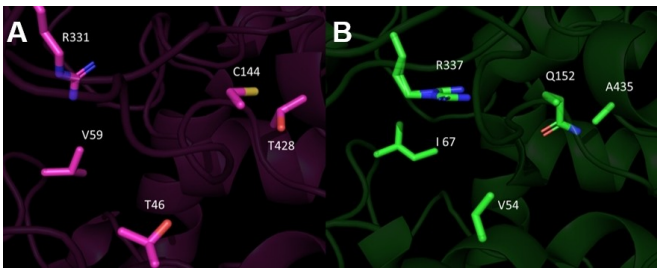


Figure 4. Comparison of the active site residues in the structures of $BVMO_{Halo}$ (A) and PAMO (B).

Conclusion

We identified a marine BVMO using sequence similarity networks to mine a marine metagenomic dataset compiled from *Gammaproteobacteria* originating from the North Sea.[23] This enzyme converts aliphatic substrates and shows a broad substrate scope towards various medium chain length aliphatic ketones and also converts 2-chlorophenylacetone preferentially between 10 °C to 30 °C. Interestingly, the activity at the lower temperature is comparable to that of the recently described enzyme, JsFMO, from *Janthinobacterium svalbardensis*, which has also demonstrated activity against aliphatic ketones.[47]

Experimental Section

Materials: Unless stated otherwise all chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), New England Biolabs (Ipswich, MA, USA) or Merck (Darmstadt, Germany).

Bacterial strains and plasmids: *E. coli* TOP10 cells were obtained from Invitrogen. The synthetic gene encoding the BVMO (accession code: DFQ14_11046) was already cloned in the pBAD vector and was obtained from BioCat.

Construction of the SSN: A marine metagenomic dataset was used to generate a sequence similarity network (SSN) in conjunction with characterized BVMOs of known sequences and functions ($CHMO_{Acineto}$ (cyclohexanone monooxygenase from *Acinetobacter* sp.), $BVMO_{Parvi}$ (cycloalkanone monooxygenase from *Parvibaculum lavamentivorans*), PAMO (phenylacetone monooxygenase from *Thermobifida fusca*) SMO (steroid monooxygenase from *Rhodococcus rhodochrous*) and HAPMO (hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens*)). This resulted in an SSN of uncharacterized genes clustered with those of known function, thereby allowing us to identify uncharacterized enzymes. The parameters set for the construction of the networks were constrained to an eValue of 5 and an alignment score threshold of 40. The resulting SSN was visualized with Cytoscape 3.9.1. The cluster of the BVMOs was further refined to elucidate novel related BVMOs.

Expression and purification of the $BVMO_{Halo}$: The gene, codon-optimized for *E. coli*, encoding the $BVMO_{Halo}$ was already cloned into the pBAD vector with a C-terminal 6xHis-tag. *E. coli* TOP10 cells were transformed with this vector, precultured in 4 mL Lysogenic Broth (LB) supplied with ampicillin (100 $\mu g\,mL^{-1}$) and incubated at 37 °C and 180 rpm for 16 h. For larger scale cultivation, 400 mL LB supplemented with 100 $\mu g\,mL^{-1}$ ampicillin were inoculated with 1% of the preculture. The culture was incubated at 37 °C and 180 rpm until an OD_{600} of 0.6–0.8 was achieved and induction was performed by the addition of 0.2% arabinose. The culture was incubated at 37 °C for 4 h and afterwards it was transferred at 20 °C. The cells were harvested after approximately 16 h by centrifugation at 8000 x g, 4 °C for 5 min. The pellets were resuspended in 15 mL 100 mM KH_2PO_4/K_2HPO_4 buffer, pH 6.5, containing 500 mM NaCl followed by cell disruption performed at 2000 psi (MAXIMATOR, Nordhausen, Germany). The cell debris was separated from the crude cell extract by centrifugation at 8000 x g for 15 min. The supernatant was filtered using a 0.45 µm filter, loaded on a Co^{2+}-NTA column and purified via the 6xHis affinity tag. The column was washed with three volumes of the resuspension buffer followed by three volumes of the same buffer containing additional 20 mM imidazole. Elution of the enzyme was performed by using 6 mL resuspension buffer containing 500 mM imidazole. The purified enzyme was concentrated to 2.5 mL using 10 kDa cut off centricons and desalted by applying 2 mL enzyme to an EconoPac 10-DG desalting column. Subsequently, it was eluted in 3.5 mL KH_2PO_4/K_2HPO_4 buffer (50 mM, pH 6.5). The enzyme concentration was determined using the Nanodrop method based on the molecular weight (60416.42 Da) and the extinction coefficient (97540 $M^{-1}\,cm^{-1}$), including the C-terminal 6xHis-tag.

Biocatalysis: For the purposes of the substrate scope investigation, *in vivo* biotransformations were performed using freshly harvested pellets, resuspended in resting cell medium (200 mM KH_2PO_4/K_2HPO_4, 20 mM NaCl, 1% (w/v) glucose, 0.4% (w/v) glycerol, pH 7.4), and adjusted to a suspension of OD_{600} 15. Substrate was added to a final concentration of 5 mM from a 100 mM stock solution in DMF in 400 µL reaction volume in 2 mL air-tight glass vials. The reactions were incubated at 25 °C with shaking at 750 rpm for 20 h. For the optimization of the reaction conditions, biocatalysis with purified enzyme were performed in a reaction volume of 400 µL in air-tight 2 mL glass vials with 3 mM substrate, 3 mM NADPH and 4.2 µM desalted enzyme eluate. The reaction volume was adjusted to 400 µL with buffer (50 mM KH_2PO_4/K_2HPO_4, NaH_2PO_4/Na_2HPO_4 or Tris HCl buffer) with pH values ranging from 6 to 8. To improve substrate solubility, the reactions were supplemented with 1.25 mg/mL rhamnolipids.[27,48] In *in vivo* as well as *in vitro* biocatalysis, a 200 µL control sample (t_0) was immediately frozen at the start of the reaction. All biocatalysis reactions were performed mostly in triplicates (otherwise in duplicates).

Product identification: For GC-FID analysis, 200 µL samples were collected, supplemented with 2 mM of an internal standard, and extracted with an equal volume ethyl acetate. For the aliphatic compounds, *n*-pentadecane was used as internal standard and for the halogenated phenylacetone substrate, acetophenone. After vortexing and centrifugation for 1 min, the upper organic phase was collected and dried with anhydrous Mg_2SO_4. The extraction was repeated and the organic phases were combined. The samples were analyzed using a GC-2010 (Shimadzu) by injecting 1 µL with an auto-injector and separated on a ZB 5MSi column (25 m × 0.25 mm ID, Macherey–Nagel). For the aliphatic compounds, the flow was set at 1.40 mL min^{-1}, the injector temperature to 320 °C, and a column temperature program at 40 °C for 3 min, 85 °C from 5 min, and 10 °C/min gradient up to 220 °C for 0.75 min hold. For the aromatic compound **8**, the flow was set at 1.33 mL/min, the injector temperature to 320 °C, and a column temperature program at 60 °C for 5 min and with 10 °C/min gradient up to 300 °C for 5 min, up to 320 °C for 0.75 min hold.

Chemical standard synthesis

2-Chlorobenzyl acetate and heptyl propanoate were synthesized for product identification.

2-Chlorobenzyl acetate: 2 g 2-chlorobenzyl alcohol were dissolved in 10 mL dichloromethane (DCM) supplemented with 850 µL pyridine and 660 µL acetyl chloride were added drop wise. The mixture was stirred at room temperature for 2 h and then washed twice with 100 mL water each time. After the extraction, the organic phase was washed with 100 mL 10% HCl (hydrochloride) and afterwards with NaCl saturated water.[49] Subsequently, the solvent was evaporated under reduced pressure to obtain 2-chlorobenzyl acetate as a colourless oil (159 mg quantified). The product formation was initially analysed by thin layer chromatography (TLC) and afterwards by GC. The ^{1}H NMR spectroscopic data of 2-chlorobenzyl acetate matched literature NMR data: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42–7.33 (m, 2H), 7.29–7.21 (m, 2H), 5.19 (s, 2H), 2.11 (s, 3H) (Figure S7).[50]

Heptyl propanoate: A 25 mL two-necked round bottom flask was heat dried with a heat gun and flushed with nitrogen using standard Schlenk-technique. Propionic acid (77 mg, 1.03 mmol, 1.20 equivalent), *n*-heptanol (100 mg, 0.86 mmol, 1.00 equivalent), 4-DMAP (11 mg, 0.86 mmol, 0.10 equivalent) and dry DCM (2 mL), were mixed and the resulting solution was cooled with an ice/water bath and stirred for 10 min. EDCI·HCl (182 mg, 0.95 mmol, 1.10 equivalent) was added in one portion and the reaction was allowed to slowly warm up to room temperature and stirred overnight. As TLC indicated that *n*-heptanol persisted in the solution, 0.5 equivalent *n*-heptanol and 0.5 equivalent EDCI·HCl were added, and the reaction was stirred at room temperature. After 3 h, all *n*-heptanol was consumed, and subsequently, the reaction mixture was diluted with DCM (30 mL) and the organic phase was washed with 1 M HCl (30 mL), saturated aqueous $NaHCO_3$ (30 mL), distilled water (30 mL), and brine (30 mL). The organic phase was further dried with anhydrous $MgSO_4$, filtrated and evaporated under reduced pressure to obtain crude heptyl propionate as slightly yellow liquid (148 mg).[51] The ^{1}H NMR spectroscopic data of heptyl propanoate matched literature NMR data: ^{1}H NMR (300 MHz, Chloroform-*d*) δ 0.82–0.96 (m, 3H), 1.14 (t, J = 7.6 Hz, 3H), 1.21–1.38 (m, 8H), 1.49–1.68 (m, 2H), 2.32 (q, J = 7.6 Hz, 2H), 4.06 (t, J = 6.7, 6.7 Hz, 2H ppm (Figure S8).[52]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: aliphatic ketones · Baeyer-Villiger monooxygenase · biocatalysis

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