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Published in:
ChemBioChem

DOI:
10.1002/cbic.201700427

Publication date:
2018

A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

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To be cited as: *ChemBioChem* 10.1002/cbic.201700427

Link to VoR: http://dx.doi.org/10.1002/cbic.201700427

A Journal of

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Exploring the substrate scope of Baeyer-Villiger monooxygenases with branched lactones as entry towards polyesters

Marie A. F. Delgove,[a] Maximilian J. L. J. Fürst,[b] Marco W. Fraaije,[b] Katrien V. Bernaerts,[a] and Stefaan M. A. De Wildeman*[a]

Abstract: Baeyer-Villiger monooxygenases (BVMOs) are biocatalysts able to convert cyclic ketones to lactones by the insertion of oxygen. The aim of this study was to explore the substrate scope of several BVMOs with (biobased) cyclic ketones as precursors for the synthesis of branched polyesters. The product structure and the degree of conversion of several biotransformations was determined after conversions using self-sufficient BVMOs. Full regioselectivity towards the normal lactone of jasmatone and menthone was observed, while the oxidation of other substrates such as α,β-thujone and 3,3,5-trimethylcyclohexanone resulted in mixtures of regio-isomers. This exploration of the substrate scope of both established as well as newly discovered BVMOs towards biobased ketones contributes to the development of branched polyesters from renewable resources.

Introduction

The chemical Baeyer-Villiger oxidation is a well-established reaction for the synthesis of esters and lactones from linear and cyclic ketones, respectively.[1] Biocatalysts, and in particular Baeyer-Villiger monooxygenases (BVMOs), offer an opportunity for a greener alternative for the synthesis of lactones since molecular oxygen is used as oxidant and water is formed as by-product.[2]

BVMOs have attracted growing attention since the discovery of a cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (AcCHMO; EC 1.14.13.22).[3] This enzyme catalyzes the oxidation of cyclohexanone to ε-caprolactone which is a widely used monomer for the synthesis of polyesters *via* ring opening polymerization.[4] The main advantage of BVMOs over chemical Baeyer-Villiger oxidation is their regio-, enantio- and stereoselectivity.[5] BVMOs are capable of regioselectivity towards either of the two possible regio-isomeric lactones, referred to as "normal" or "abnormal". Abnormal products have been reported for example on *rac*-bicyclo[3.2.0]heptanone[6] or on terpenone precursors,[7] and stand in contrast to the chemical Baeyer-Villiger oxidations which typically yield either the normal lactone (*i.e.* the lactone with substituents next to the ester group) or a mixture of both lactones.[1b] In the past decades, numerous BVMOs have been discovered and described, thereby broadening the substrate scope of this family of enzymes with macrocyclic ketones,[8] bi- or tri-cyclic compounds,[9] steroids,[8a, 10] heteroatom containing ketones,[11] and substituted cyclic ketones derivatives.[3, 5, 8b, 12]

Polyesters from branched lactones, and in particular from terpene-based lactones, are of growing interest for application as sustainable materials because they can be synthesized from renewable resources.[13] Menthide, obtained from the oxidation of menthone, has been used for both the synthesis of triblock copolymers, which exhibit a behavior similar to thermoplastic elastomers,[14] as well as for the preparation of pressure sensitive adhesives.[15] The potential of building blocks derived from the oxidized products of carvone has been explored to elaborate shape-memory polymers,[16] cross-linked polymers,[17] and polyurethane films.[18] However, these branched lactones are often prepared *via* chemical Baeyer-Villiger oxidation which usually gives the normal lactone, therefore influencing the polyester's properties. Additionally, the lactone ring size, the number of substituents and their size has an influence on the thermodynamics of the monomer, in particular on the reaction temperature required for polymerization.[19] Moreover, the position of the substituent is of importance because it can hinder transesterification reactions, thus influencing copolyester structure. This was demonstrated for ω-pentadecalactone-based copolymers using menthide[20] or a mixture of β,δ-trimethyl-ε-caprolactones as co-monomers.[21] Finally, the ratio of regio-isomeric lactones can result in polymeric backbones that have other properties in their applications. Thus, biocatalysis can become a differentiating technology in steering the final properties of the lactone-derived materials. Predictive power in this structure-property relationship is still limited. We chose to screen for regioselective BVMOs to empirically evaluate the resulting polymeric materials from their products.

The well-established AcCHMO has been shown to be active on (+)-menthone as well as on jasmatone, with full regioselectivity towards the normal lactone in both cases and enantioselectivity for the former.[12a, 22] Another cyclohexanone monooxygenase, from *Rhodococcus* sp. HI-31 (RhCHMO; EC 1.14.13.22), was reported to be active on jasmatone to give the normal lactone exclusively. RhCHMO can also oxidize 3,3,5-trimethylcyclohexanone to give a mixture of both regio-isomers (50:50).[12a] The newly discovered thermostable cyclohexanone monooxygenase from *Thermocrispum municipale* DSM 44069 (TmCHMO; EC 1.14.13.22) can oxidize small sized cyclic ketones as well as linear ketones.[23] The substrate scope of TmCHMO towards branched cyclic ketones remains, however, unknown. Cyclopentadecanone monooxygenase from

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Pseudomonas sp. strain HI-70 (PsCPDMO; EC 1.14.13) is active towards both (+)- and (–)-menthone with high conversions and yields the normal lactone only.[8b] 3,3,5-Trimethylcyclohexanone is also accepted as substrate by the enzyme.[8b]

The activities of BVMOs towards some terpene-based substrates such as α,β-thujone, isophorone, (+)-camphor, (+), (–)-menthone have been less explored. Additionally, the activities of the most recently discovered BVMOs (TmCHMO and PsCPDMO) towards branched cyclic ketones and terpene-based substrates in particular remain unknown. While some BVMOs have been shown to be active towards some terpene-based substrates,[24] no comparative study with such substrates has been performed. The successful substrate/biocatalyst combinations reported have been performed with various reaction conditions (pH, temperature, substrate and enzyme concentration, co-solvent, enzyme format – whole cells *vs.* purified biocatalyst). However, it is well known that these conditions can influence the kinetics of the bioconversions. Additionally, it has been demonstrated that enantio- and regioselectivity of BVMOs can be influenced by the presence of co-solvent,[23] as well as by the substrate concentration.[26]

In this article, we aim to explore the substrate scope of several BVMOs (AcCHMO, RhCHMO, TmCHMO and PsCPDMO) with (mostly) terpene-based branched substrates in order to evaluate their potential for the biocatalyzed oxidations of branched lactones as precursors for branched polyesters. The activity of these BVMOs in the presence of a co-solvent, 1,4-dioxane, was evaluated using a NADPH-based spectrophotometry assay. Product profiling and regioselectivity assessment was performed with bioconversions employing self-sufficient NADPH-recycling fusion enzymes.

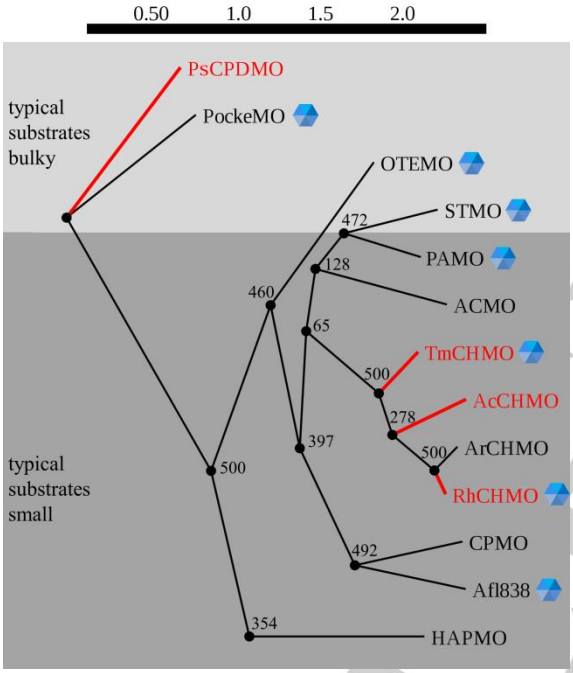


Figure 1. Representatives of different BVMO subclasses and their phylogenetic relationships are shown. Available crystal structures are indicated by a blue crystal symbol and the BVMOs chosen for this study are marked in red. The tree was generated using PhyML, applying a maximum-likelihood method with 500 bootstraps (support values shown at the nodes).[25] Genbank accession number, enzyme name (organism), full name: BAA86293, AcCHMO (*Acinetobacter* sp. NCIMB9871), Cyclohexanone MO; AAN37479, ArCHMO (*Arthrobacter* sp. BP2), Cyclohexanone MO; BAH56677, RhCHMO (*Rhodococcus* sp. HI-31), Cyclohexanone MO; WP_028849141, CHMO (*Thermocrispum municipale* DSM 44069), Cyclohexanone MO; WP_011291921, PAMO (*Thermobifida fusca* YX), Phenylacetone MO; BAA24454, STMO (*Rhodococcus rhodochrous*), Steroid MO; BAF43791, ACMO (*Gordonia* sp. TY-5), Acetone MO; BAN13280, OTEMO (*Pseudomonas putida* ATCC 17453), 2-Oxo-3-4,5,5-Trimethylcyclo-pentenylacetyl-Coenzyme A MO; AF355751_1, HAPMO (*Pseudomonas fluorescens* ACB), 4-Hydroxyacetophenone MO; BAC22652, CPMO (*Comamonas* sp. NCIMB 9872), Cyclopentanone MO; XP_002375657, Afl838 (*Aspergillus flavus* NRRL3357), *Aspergillus flavus* MO 838; BAE93346, CPDMO (*Pseudomonas* sp. HI-70), Cyclopentadecanone MO; XP_003661890, PockeMO (*Thermothelomyces thermophila* ATCC 42464), Polycyclic ketone MO.

Results and Discussion

Specificities of BVMOs

Four biocatalysts were selected for this study in order to explore their substrate scope: three CHMOs, as representatives of versatile BVMOs active on small molecules, and PsCPDMO, a member of a subclass of BVMOs most active on more bulky substrates (Figure 1, Table 1). While the typical substrate of AcCHMO, RhCHMO, and TmCHMO is cyclohexanone,

Table 1. Characteristics of the BVMOs used in this study (AcCHMO, RhCHMO, TmCHMO and PsCPDMO) with typical substrate, pH and temperature range, melting temperature T_m, solvent resistance, and thermostability.

Abbre-viation	AcCHMO	RhCHMO	TmCHMO	PsCPDMO
Full name	*Acinetobacter calcoaceticus* NCIMB 9871	*Rhodococcus* sp. HI-31	*Thermo-crispum municipale* DSM 44069	*Pseudo-monas* sp. strain HI-70
Reference	[22], this work	[12a], this work	[23], this work	[8c]
Typical substrate	Cyclo-hexanone	Cyclo-hexanone	Cyclo-hexanone	Bulky substrates
pH range	pH optimum 8.5-9[27]	Not determined	Not determined	Optimum at pH 9
Tempera-ture range	Not determined	Not determined	Not determined	Optimum at 40 °C
T_m (°C)	37 (at pH 7-9)[23]	37 (at pH 7-9)[12a]	48 (at pH 7.0)[23]	Not available
Crystal structure	Not available	Known[12a]	Known[23]	Not available
Solvent resistance	Poor[8a, 23]	Very poor ($t_{1/2}$ < 1 min in 14 % v/v acetonitrile at 20 °C)	Very robust (79 % residual activity after 20 h in 14% v/v acetonitrile at 20 °C)	Not determined
Thermo-stability	Poor (no more residual activity after a few minutes at 45 °C[23] and 70 % residual activity after 5.5 h at 30 °C)	Very poor (2 % residual activity after 30 min at 45 °C and 33 % residual activity after 5.5 h at 30 °C)	Good (58 % residual activity after 5.5 h at 45 °C)[23]	Not determined

PsCPDMO is active on bulkier substrates such as steroids and macrocycles.[8c, 10] AcCHMO is the most studied BVMO but its tolerance towards organic solvents is quite poor (no activity was reported after 25 min in 14 % v/v acetonitrile at 20 °C and full deactivation within 24 h was reported in 5 % methanol at 25 °C).[23, 28] This is accompanied by a poor thermostability, since all activity is lost after a few minutes at 45 °C.[23] RhCHMO became the prototype CHMO in structural studies since its crystal structure was solved, but it was reported to be very similar to AcCHMO in its substrate scope, covering a broad range of alkyl substituted cyclohexanone derivatives.[12a] This enzyme's tolerance towards solvents was previously not assessed. We determined a particularly short half-life of $t_{1/2} < 1$ min in 14 % v/v acetonitrile at 20 °C, despite the melting temperature of the protein being the same as that of AcCHMO (T_m = 37 °C). By contrast, TmCHMO, a recently discovered BVMO, presents better temperature and solvent stability, while its substrate acceptance of branched cyclic ketone has not been studied.[23]

Study of the effect of 1,4-dioxane on the activity with typical substrates of the BVMOs

Because the water solubility of the substrates is limited, 1,4-dioxane was chosen as co-solvent. The effect of the co-solvent on the activity of the BVMOs on their typical substrate (cyclohexanone for AcCHMO, RhCHMO, and TmCHMO, and cyclopentadecanone for PsCPDMO) was evaluated after 20 minutes in the presence and absence of co-solvent (except for cyclopentadecanone whose water solubility is too limited) (Figure 2). Activities were measured by spectrophotometric assay by monitoring the decrease of absorbance at 340 nm due to the consumption of NADPH.

AcCHMO and RhCHMO display high activity towards cyclohexanone in the absence of co-solvent (k_{obs} = 13.0 s^{-1} and 9.7 s^{-1} respectively). However, a significant decrease of activity in the presence of 10 % v/v 1,4-dioxane was observed for both enzymes after 20 minutes (70 % and 50 % decrease respectively), therefore demonstrating the poor solvent resistance of both enzymes. Although the activity of TmCHMO towards cyclohexanone was lower compared to the other CHMOs (k_{obs} = 1.2 s^{-1}), it was decreased by only 15 % after 20 minutes with 10 % v/v 1,4-dioxane, which is in accordance with the robustness of TmCHMO reported by Romero *et al.*[23] Cyclopentadecanone was chosen as substrate to measure the activity of PsCPDMO. Because of the high hydrophobicity and very low water solubility of this macrocyclic ketone (113 ± 21 µM),[29] the activities could only be measured in the presence of co-solvent. Although the activity of PsCPDMO was low (k_{obs} = 2.2 s^{-1}), it is in the same order of magnitude as the activities of the CHMOs in the presence of the co-solvent.

Self-sufficient biotransformations

In this study, for testing of the substrates, we chose branched cyclic ketones with some of them terpene-based. Terpenes are a family of compounds which can be extracted from wood

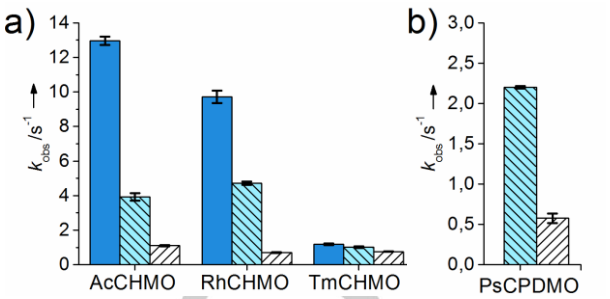


Figure 2. Activity of BVMOs (k_{obs} in s^{-1}) on their model substrate without co-solvent (blue bars), with 10 % v/v 1,4-dioxane (blue stripped bars) and control with 10 % v/v 1,4-dioxane without substrate (white stripped bars), measured after 20 minutes with a) 5 mM cyclohexanone, and b) 1 mM cyclopentadecanone (the activity in the absence of co-solvent was not measured on cyclopentadecanone for PsCPDMO due to the limiting water solubility of the substrate).

rosin.[30] They are also a major constituent of some essential oils. In particular, menthone is derived from menthol which can be found in the essential oils of *Mentha arvensis* (wild mint) and *Mentha piperita* (peppermint).[31] Another terpene-based cyclic ketone, thujone, can be sourced from cedar wood[32] and is present in its α- and β- forms in more than 50 % of the essential oil of *Salvia officinalis L.* (dalmatian sage).[33] Dalmatian sage also contains non-negligible amounts of camphor.[33] This bi-cyclic ketone has been synthesized from turpentine at an industrial scale.[34] (–)-Carvone can be obtained from the extraction and purification of the essential oil of *Mentha spicata* (spearmint) while (+)-carvone can be found in the essential oils of *Carum carvi* (caraway) and *Anethum graveolens* (dill) oils.[35] Jasmatone, a cyclopentanone derivative with a $–C_6H_{13}$ alkyl substituent, is present in the essential oil of *Anisomeles indica Kuntze*.[36] Lastly, two other branched substrates with similar structures were tested on the selected BVMOs: isophorone which is prepared from the aldol condensation of acetone,[37] and its hydrogenated counterpart 3,3,5-trimethylcyclohexanone. These substrates can also be considered as produced from renewable resources since acetone is a by-product of the synthesis of bio-ethanol by acetone-butanol-ethanol fermentation of lignocellulose.[38]

After confirming the activity of the enzymes on their respective model substrates, we screened a wide range of (branched) cyclic ketones, namely a mixture of (+),(–)-menthone ***1***, α,β-thujone ***2***, (+)-camphor ***3***, isophorone ***4***, 3,3,5-trimethylcyclohexanone ***5***, jasmatone ***6*** and (–)-carvone ***7***. Preliminary results from spectrophotometric screening showed that the activities of the tested BVMOs were comparable for most of the branched ketones. The highest activities were observed for AcCHMO, RhCHMO and PsCPDMO on jasmatone, with an observed rate $k_{obs} > 1$ s^{-1}.

Bioconversions were performed using self-sufficient phosphite dehydrogenase (PTDH)-fused BVMOs to regenerate the NADPH co-factor (Figure 3). The degree of conversion was measured by GC-FID and the oxidized products were analyzed using GC-MS (Table 1). Since the substrates have asymmetric

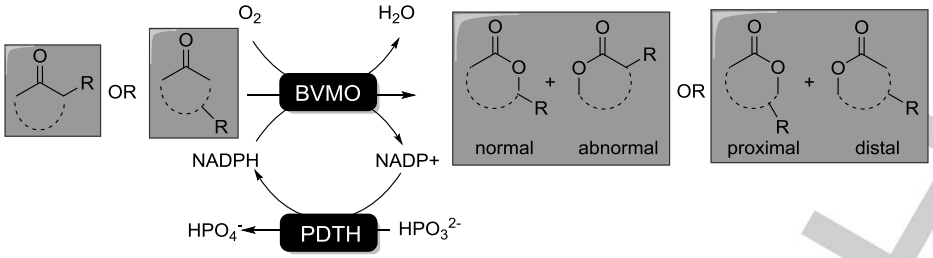
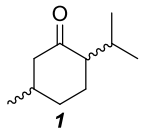
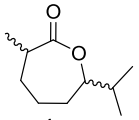
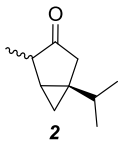
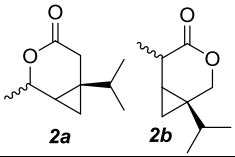
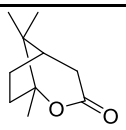
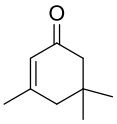
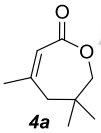
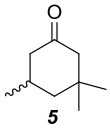
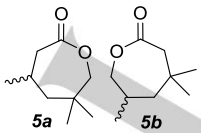
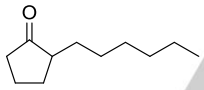
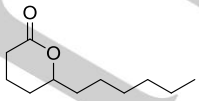
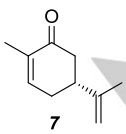
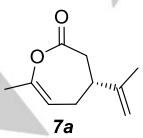


Figure 3. Reaction scheme of bioconversions of cyclic ketones with BVMOs fused to PTDH for NADPH regeneration with formation of the normal and abnormal lactone when the substituent is next to the ketone group and of the proximal and distal lactone when the substituent is located further.

Table 1. Products obtained from the bioconversions of the substrates using AcCHMO, RhCHMO, TmCHMO or PsCPDMO.

Substrate	Products[a]	BVMO	Degree of conversion[b]	Normal: abnormal[c]	Reported as substrate?
 1	 1a	AcCHMO	+	n.a.	Yes, (+)-menthone only[39]
		RhCHMO	+	n.a.	This work
		TmCHMO	+	n.a.	This work
		PsCPDMO	++	100:0	Yes[8b]
 2	 2a 2b	AcCHMO	+	n.a.	This work
		RhCHMO	+	50:50	This work
		TmCHMO	+	70:30	This work
		PsCPDMO	+	84:16	This work
 3	 3a	AcCHMO	-	n.a.	Not a substrate*[8a]
		RhCHMO	-	n.a.	This work
		TmCHMO	-	n.a.	This work
		PsCPDMO	-	n.a.	Not a substrate*[8a]
 4	 4a	AcCHMO	-	n.a.	This work
		RhCHMO	-	n.a.	This work
		TmCHMO	-	n.a.	This work
		PsCPDMO	-	n.a.	This work
 5	 5a 5b	AcCHMO	+	64:36	This work
		RhCHMO	++	44:56	Yes [12a]
		TmCHMO	+++	54:46	This work
		PsCPDMO	+	38:62	Yes[8b]
 6	 6a	AcCHMO	++	100:0	Yes[12a]
		RhCHMO	++	100:0	Yes[12a]
		TmCHMO	+++	100:0	This work
		PsCPDMO	+++	100:0	Yes[8a]
 7	 7a	AcCHMO	-	n.a.	Yes[12a] / not a substrate*[22]
		RhCHMO	-	n.a.	Yes[12a]
		TmCHMO	-	n.a.	This work
		PsCPDMO	-	n.a.	This work

[a]If no conversion was observed, the expected normal or proximal lactone product is given. [b]Degree of conversion determined by GC-FID with 100 %, +++; > 50 %, ++; 1-50 %, +; 0 %, -.[c] The structure of the product was determined by comparison with a mixture of lactones synthesized by chemical Baeyer-Villiger oxidation ((+),(–)-menthone and 3,3,5-trimethylcyclohexanone) or by comparison with commercially available lactone (δ-undecalactone). n.a.: no oxidized product could be detected with GC-MS.* This substrate was reported as not belonging to the substrate scope of the enzyme.

alkyl substituents, two regio-isomers can be formed. In the case of the substituent directly located next to the ketone, the biocatalyst can show regioselectivity towards either the normal (most substituted) or abnormal (least substituted) lactones. When the substituent is positioned further on the cyclic ketone, either the proximal (substituent close to ester) or the distal lactones (substituent far from the ester) are expected.

Firstly, with AcCHMO, RhCHMO, TmCHMO, and PsCPDMO, no product was observed for the biotransformations of the unsaturated ketones isophorone ***4*** and (–)-carvone ***7***. On the other hand, the hydrogenated counterpart of isophorone, 3,3,5-trimethylcyclohexanone ***5***, could be oxidized by all tested BVMOs. The absence of conversion for isophorone ***4*** and (–)-carvone ***7*** is attributed to the presence of the double bond, therefore confirming the inactivity of AcCHMO towards α,β-unsaturated cyclic ketones like most BVMOs, with very few exceptions.[40] AcCHMO is able to convert substituted cycloketones when the double bond is located on the substituents.[12b] In particular, an alternative route to prepare (–)-carvone-based lactones was reported by oxidation of dihydrocarvone with AcCHMO.[41] A synthesis approach from limonene *via* dihydrocarvone to carvolactone with full regioselectivity towards the normal lactone has been established using AcCHMO in a cascade reaction.[42] Biocatalysis would then be more advantageous than chemical Baeyer-Villiger oxidation since the latter can lead to epoxidation which results in cross-linking of the corresponding polymer under given conditions.[17] Alternatively, some type-II BVMOs are able to directly oxidize isophorone, avoiding the challenges of sequence reactions.[43]

3,3,5-Trimethylcyclohexanone ***5*** was successfully converted by AcCHMO, RhCHMO, TmCHMO, and PsCPDMO. This is the first report of the oxidation of ***5*** by AcCHMO and TmCHMO. The biotransformation reached full conversion with the latter enzyme. The tested BVMOs did not seem to exhibit strong regioselectivity for this substrate since mixtures of proximal and distal lactones ***5a*** and ***5b*** were obtained with ratios close to 50:50. Since the substituents are relatively small and they are located one position further away from the ketone group, they seem to have little effect on the side of oxygen insertion during oxidation unlike other substituted ketones such as (+),(–)-menthone ***1*** and jasmatone ***6***. The biotransformation of (+)-camphor ***3*** did not result in lactone formation, maybe due to the sterical hinderance of the bridged-substrate. PsCPDMO demonstrated excellent regioselectivity towards a mixture of (+)- and (–)-menthone ***1*** with formation of the normal lactone ***1a*** exclusively with full conversion. This is in agreement with the general preference of PsCPDMO for bulky cyclic ketones,[8b, 8c] although this enzyme showed limited acceptance for the smaller substrates α,β-thujone ***2*** and 3,3,5-trimethylcyclohexanone ***5***. With AcCHMO, RhCHMO, and TmCHMO as biocatalysts, (+),(–)-menthone did not result in significant conversions. PsCPDMO also showed excellent regioselectivity with jasmatone ***6*** thereby producing exclusively the normal lactone. Although this branched ketone had already been reported as a substrate by AcCHMO and RhCHMO,[12a] this work shows that it can also be converted to δ-undecalactone ***6a*** exclusively with TmCHMO and PsCPDMO, with full substrate conversion in both cases.

The mixture of α,β-thujone ***2*** could be oxidized by AcCHMO, RhCHMO, TmCHMO, and PsCPDMO although the biotransformations resulted in low degrees of conversion for all enzymes. This ketone has not been reported yet as a substrate for the tested CHMOs. Interestingly, while RhCHMO did not show regioselectivity (50:50 of both regio-isomers), TmCHMO and PsCPDMO exhibited a preference for the normal lactone ***2a*** over the distal lactone ***2b*** (70:30 and 84:16 respectively). Although it has been demonstrated that the regioselectivity of RhCHMO is dictated by the tight-binding structure of the enzyme,[44] it is not yet possible to predict regioselectivity depending on the structure of the substrate. Similarly, although TmCHMO displays a compact ligand-binding site cavity which is consistent with the enzyme's preference for small sized substrates,[23] it is not yet possible to foresee if one of the regio-isomeric products will be favored. Steering the regioselectivity to one of the oxidized regio-isomer by careful choice of the co-solvent should make this lactone a novel renewable building block for the synthesis of branched polyesters. Additionally, rational protein design is a useful tool in directing regioselectivity of BVMOs,[45] thus making biocatalysis a remarkable technology for monomer synthesis.

Conclusions

The aim of this article was to explore the substrate scope of several BVMOs with branched cyclic ketones since branched lactones are of interest for the synthesis of branched polyesters. Three CHMOs (AcCHMO, RhCHMO, and TmCHMO) as well as PsCPDMO were selected as biocatalysts. Substrates were branched cyclic ketones, including some terpene-based substrates. After evaluating the effect of 1,4-dioxane as co-solvent on the activity of the BVMOs by spectrophotometric assay, bioconversions were performed using self-sufficient PTDH-fused biocatalyst. TmCHMO was shown to accept α,β-thujone, 3,3,5-trimethylcyclohexanone as well as jasmatone as substrates, with full regioselectivity towards the normal lactone δ-undecalactone for the latter. Additionally, PsCPDMO could also convert jasmatone with full regioselectivity and full conversion. These results therefore suggest that BVMOs have potential for the synthesis of branched lactones as precursors for polyesters. In particular, the full conversion of jasmatone with PsCPDMO towards the normal lactone exclusively is very promising. This comparative study shows that BVMOs have potential for the synthesis of (terpene-based) lactones as precursors for polyesters with tuned functionalities.

Experimental Section

Starting materials: *L*-menthone (Sigma-Aldrich, > 96 %), jasmatone (Alfa Aesar, 97 %), (–)-carvone (Sigma-Aldrich, 98 **%**), (+)-camphor (Sigma-Aldrich, 97 %), isophorone (Acros, 98 %), α,β-thujone (Sigma-Aldrich**,** ~80 %), cyclododecanone (TCI, > 99 %) and 3,3,5-trimethylcyclohexanone (Sigma-Aldrich, 98 %) were used as received. Cyclopentadecanone was kindly supplied by Givaudan. GC-FID showed that the mixture of menthone isomers consisted of 83 % of (–)-menthone and 17 % of (+)-menthone. The mixture of α,β-thujone consisted of 70 % of (–)-α-thujone, 12 % of (+)-β-thujone, 15 % of fenchone and 3 % of (+)-camphor. NADPH (Alfa Aesar, 95 %) was stored at -20 °C and used as received. Phosphate buffer (25 mM) at pH 8.5 was prepared from stock solutions of K_2HPO_4 and KH_2PO_4.

Enzymes: AcCHMO, RhCHMO, TmCHMO, and PsCPDMO were prepared as purified His-PTDH-fusion proteins as previously described.[8a, 23] *E. coli* NEB 10 beta cells transformed with the pCRE-BVMO plasmid

were grown for 40 h at 24 °C shaking in Terrific Broth TB medium supplemented with ampicillin and 0.02 % *L*-arabinose. Cells were harvested, resuspended in Tris/HCl buffer at pH 7.5, and sonicated to create cell free extract. Purification of the enzyme was performed *via* Nickel-Sepharose affinity chromatography. Elution was achieved with 500 mM imidazole and the protein was subsequently applied on a desalting column before shock freezing in liquid nitrogen and storage at -80 °C.

GC-FID analysis: Gas Chromatography (GC) analyses were performed using a Shimadzu GC-2010 Plus Gas Chromatograph with a hydrogen flame-ionisation detector (FID), and a Supelco SPB-1 capillary column (30 m × 0.25 µm with 0.25 mm inner diameter). Helium was used a carrier gas. The temperature program of the oven was: temperature maintained to 60 °C for 2 minutes, increased to 200 °C at a heating rate of 10 °C/min, maintained at 200 °C for 2 minutes, increased to 320 °C at a heating rate of 20 °C/min, and maintained at 300 °C for 5 minutes. Samples were prepared by dissolving part of the reaction mixture in acetonitrile (1/1 v/v). Retention times of the products were identified with commercially available chemicals or lactones synthesized by chemical Baeyer-Villiger oxidation.

GC-MS analysis: Mass spectrometry analyses were performed on a Shimadzu GC-2010 Plus Gas Chromatograph system, equipped with an AOC-20i auto-injector system, a Shimadzu GC-MS-QP2010 ultra mass spectrometer detector, and a Supelco SPB-1 capillary column (30 m × 0.25 µm with 0.25 mm inner diameter). The temperature program of the oven was: temperature maintained to 60 °C for 2 minutes, increased to 200 °C at a heating rate of 10 °C/min, maintained at 200 °C for 2 minutes, increased to 320 °C at a heating rate of 20 °C/min, and maintained at 300 °C for 5 minutes. Samples were prepared by extracting the reaction mixture with the same volume of dichloromethane. A mass/charge (m/z) range of 35 to 750 Da was analyzed. Fragmentation patterns were compared to those of commercially available lactones (δ-undecalactone) or to lactones synthesized by chemical Baeyer-Villiger oxidation according to procedures reported in the literature (β,δ,δ-trimethyl-ε-caprolactone/ β,β,δ-trimethyl- ε-caprolactone mixture,[46] α,β-thujone lactone,[47] and menthide[47]).

Determination of enzyme long term stability and stability in acetonitrile: The long term stability and the stability in acetonitrile were determined based on the procedure from Romero *et al.*[23] The enzymes (10 µM) were incubated in Tris-HCl buffer (50 mM) at pH 7.0 for 16 h with a final volume of 400 µL. For long term stability determination, the enzymes were incubated at 30 °C or 45 °C. For the determination of the stability in acetonitrile, the enzymes were incubated at 20 °C in the presence of 14 % v/v acetonitrile. Aliquots were taken to determine the enzyme activity using the NADPH consumption assay described by Romero *et al.*[23] with cyclohexanone as substrate. Reaction mixtures (total volume of 1 mL) contained 0.1 µM of enzymes, 150 µM of NADPH and 30 µM of cyclohexanone in air-saturated Tris-HCl buffer (50 mM) at pH 7.0 and at 25 °C. Activities were measured in duplicate.

Screening of the activity of BVMOs: The activity of BVMOs on substrates was measured by spectrophotometry by monitoring NADPH consumption corresponding to a decrease of absorbance at 340 nm using a Multiskan GO microplate spectrophotometer from Thermo Scientific. Reaction mixtures were placed in a 96-well plate and consisted of [substrate] = 5 mM in 10 % v/v 1,4-dioxane, [NADPH] = 800 µM, [BVMO-PTDH] = 0.5 µM in TRIS/HCl buffer at pH = 8.5. Activity was measured at 22 °C except for PsCPDMO (28 °C). Initial reaction rates ($k_{obs.}$ in s^{-1}) were calculated as $k_{obs.} = (dA_{340}/dt)/(\varepsilon_{340}.[BVMO].\ell)$ in s^{-1} with $\varepsilon_{340,\ NADPH}$ = 6.22 $L.mmol^{-1}.cm^{-1}$ and ℓ = 0.5 cm. Initial reaction rates were calculated as an average of 3 measurements.

Small scale bioconversions: Bioconversions were performed with a total volume of 2 mL with [ketone] = 15 mM for substrate ***3*** and 50 mM for the other substrates, in 10 % v/v 1,4-dioxane, [NADPH or $NADP^+$] = 100 µM, [BVMO-PTDH] = 0.5 µM (AcCHMO, RhCHMO, TmCHMO or PsCPDMO), [HPO_3^{2-}] = 2.5 eq. The bioconversions were performed in KPi buffer (25 mM) at pH 8.0. The reaction mixtures were let to incubate at 20 °C (AcCHMO, RhCHMO) or 30 °C (TmCHMO, PsCPDMO) for 24h. Degrees of conversions were measured by GC-FID. The structure of the products was analyzed by GC-MS after extraction with dichloromethane.

GC-MS fragmentation patterns of the products (m/z): menthide ***1a***. ($C_{10}H_{18}O_2$): 166, 127, 99, 81, 69, 55, 41, 39. Thujone lactone ***2a*** ($C_{10}H_{16}O_2$): 154, 139, 121, 112, 97, 83, 69, 55, 41, 39. Thujone lactone ***2b*** ($C_{10}H_{16}O_2$): 154, 139, 121, 111, 97, 83, 69, 55, 41, 39. (β,δ,δ-Trimethyl-ε--caprolactone ***5a*** ($C_9H_{16}O_2$): 156, 126, 111, 99, 83, 69, 56, 41, 39. β,β,δ-Trimethyl- ε-caprolactone ***5b*** ($C_9H_{16}O_2$): 126, 111, 108, 93, 83, 69, 56, 41, 39. δ-Undecalactone ***6a*** ($C_{11}H_{20}O_2$): 166, 148, 133, 114, 99 (100 %), 84, 71, 55, 41, 39.

Acknowledgements

The research for this work has received funding from the European Union (EU) project ROBOX (grant agreement n° 635734) under EU's Horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1. The views and opinions expressed in this article are only those of the authors, and do not necessarily reflect those of the European Union Research Agency. The European Union is not liable for any use that may be made of the information contained herein. The authors would like to acknowledge Dr. Elvira Romero (University of Groningen) for the determination of long term stability and acetonitrile stability of the enzymes, and Dr. Iris Wauters (ChemStream) for the chemical synthesis of lactone standards.

Keywords: Baeyer-Villiger monooxygenases • lactones • oxidoreductases • branched cyclic ketones • polyester

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10.1002/cbic.201700427

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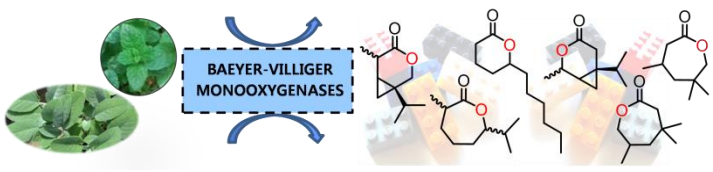

The substrate scope of several BVMOs was explored towards (terpene-based) branched ketones. This comparative study shows that BVMOs have potential for the synthesis of branched lactones as precursors for polyesters with tuned functionalities.

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Exploring the substrate scope of Baeyer-Villiger monooxygenases with branched lactones as entry towards polyesters