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Extensive substrate profiling of cyclopentadecanone monooxygenase as Baeyer–Villiger biocatalyst reveals novel regiodivergent oxidations

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ABSTRACT

Cyclopentadecanone monooxygenase (CPDMO) is one of the latest additions to the established library of Baeyer–Villiger monooxygenases. Desymmetrizations of substituted cyclobutanones and -hexanones as well as kinetic resolutions of racemic cycloketones are efficiently catalyzed by CPDMO. Moreover the enzyme shows unprecedented preference in regiodivergent oxidations of terpenones and the bicyclic Geissman–Waiss lactone precursor giving access to the optical antipode of retronecine and other pyrrolizidine alkaloids.

1. Introduction

The Baeyer–Villiger reaction represents a distinct approach in synthesis of esters and lactones from carbonyl precursors [1,2]. The possibility of *de novo* generation of chirality upon transformation of prochiral cyclic ketones or the oxidative resolution of racemic starting materials calls for discovery of suitable stereoselective catalysts [3]. Although such catalytic entities have been developed on a transition metal base displaying moderate to reasonable chiral induction [4,5], Baeyer–Villiger monooxygenases (BVMOs) have attained more attention mostly due to their superior chemo-, regio-, and stereoselectivity [6–8] in combination with their ability to substitute potentially dangerous peroxide reagents with cheap and environmentally benign atmospheric oxygen [9].

Whereas the mentioned selectivities are generally mastered, the quest in biocatalysis remains to broaden the substrate scope and to gain access to both optical antipodes of a desired product. This can be achieved either by genetic modifications of already known enzymes or by discovery and isolation of new enzymes from natural resources. The comprehensive assessment of substrate profile, performance and selectivity of novel catalytic entities is therefore mandatory. Within the BVMO family examples for such enzymes are the first isolated BVMO cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 ($CHMO_{Acineto}$), cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 ($CPMO_{Coma}$) [10] and two CHMOs from *Brevibacterium* [11]. These enzymes have a remarkably broad substrate profile and a strong stereopreference producing enantiocomplementary lactones. We have shown earlier that $CHMO_{Acineto}$ and $CPMO_{Coma}$ represent prototypes of two predominant subgroups of BVMOs: members of these enzyme clusters usually have a high protein sequence identity (Fig. 1). Moreover, they generally exhibit a striking overlap in substrate scope and enantiopreference [12]. At present, a remarkably broad collection of BVMOs has become available with some notable differences in substrate acceptance. In particular, recent contributions outlined several aryl-ketone converting enzymes [13–15], as well as biocatalysts operating on linear precursors [16–19].

Whenever new BVMOs are discovered, it is reasonable to put their activity, performance, and selectivity into relation with the defined enzyme clusters.

CPDMO was first isolated from *Pseudomonas* sp. strain HI-70 by Lau et al. in 2006 [20]. The authors have demonstrated that this biocatalyst only shows a 29–50% sequence identity to any other known BVMO. The enzyme displayed the highest catalytic efficiency towards cyclopentadecanone, hence its name. Their initial findings included high activity not only with other large ring ketones (C_{11}–C_{13}) but also substituted and fused cyclohexanones. In a follow-up publication by the same group [21] the enzyme's activity on ketosteroids was tested and its high selectivity was

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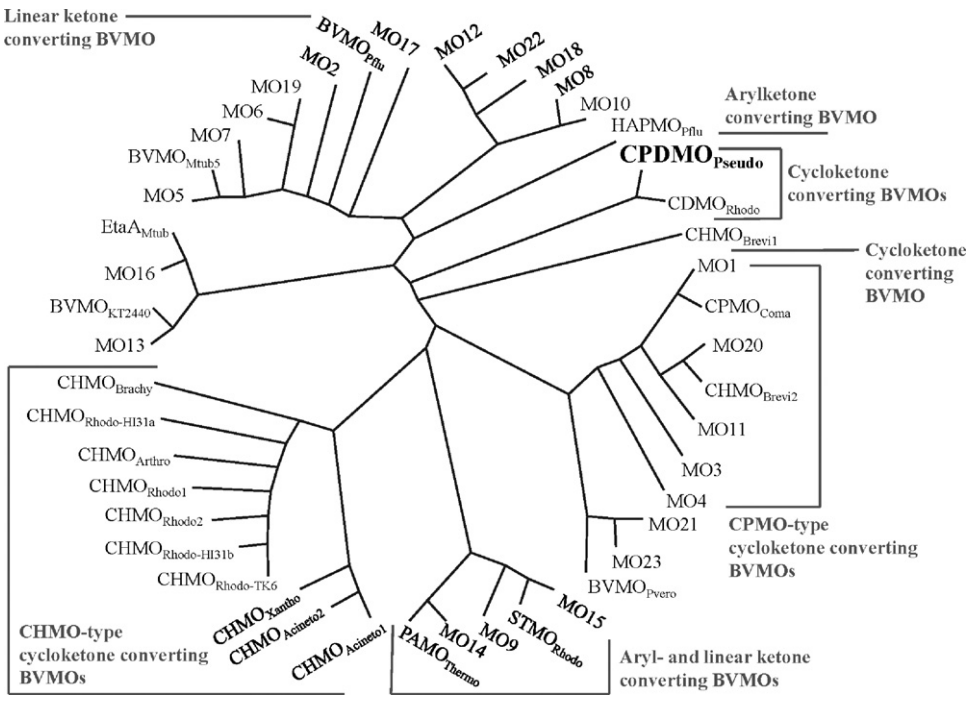


Fig. 1. Phylogenetic tree of BVMOs (not completely shown) visualizing the CHMO and CPMO clusters as well as the CPDMO branch (CHMO: cyclohexanone monooxygenase, CPMO: cyclopentanone monooxygenase, CDMO: cyclododecanone monooxygenase, HAPMO: *p*-hydroxyacetophenone monooxygenase, PAMO: phenylacetone monooxygenase, STMO: steroid monooxygenase, MO: monooxygenase).

confirmed. The unusual position of CPDMO in the phylogenetic tree of BVMOs and these intriguing first results initiated our in-depth profiling of this new biocatalyst.

2. Experimental

Unless noted otherwise, all reagents were purchased from commercial suppliers and used without further purification. DCM and toluene intended for water-free reactions were desiccated over Al_2O_3 columns. All other solvents were distilled prior to use. Ketones for substrate acceptance screening were either purchased from commercial suppliers or synthesized according to literature known protocols. Column chromatography was performed on a Büchi Sepacore Flash System using silica gel 60 and distilled solvents. General conversion determination was conducted *via* GC using an achiral capillary column (BGB5, 30 m × 0.25 mm ID, 0.25 μm film). Enantiomeric excess values were measured on modified cyclodextrin GC columns BGB 175 (30 m × 0.25 mm ID, 0.25 μm film) or BGB 173 (30 m × 0.25 mm ID, 0.25 μm film). Specific rotation was measured on an Anton Paar MCP500 polarimeter. LB medium was used without pH correction; ampicillin concentration was set to 200 mg/L (LB_{amp}). For expression of CPDMO a codon-optimized synthetic gene was cloned into the recently developed pCRE2 expression vector [22].

Experimental procedure for substrate acceptance screening: A baffled Erlenmeyer flask was charged with LB_{amp} medium (10 mL), inoculated with a bacterial single colony from an Agar plate and incubated at 37 °C in an orbital shaker o/n. The fermentation medium (LB_{amp}) was then inoculated with 2% (v/v) of the preculture and incubated for ca. 1–2 h under the same conditions until an optical density of 0.2–0.6 was reached. L-Arabinose (0.02%, w/v) and β-cyclodextrin (4 mM) were added, the mixture was thouroughly mixed and split in 1.0 mL aliquots into 24-well plates. Substrates were added as 0.8 M solutions in 1,4-dioxane to a final concentration of 4 mM. The plates were sealed with adhesive film and incubated at 24 °C in an orbital shaker for up to 24 h. Analytical samples were prepared by extraction of 0.5 mL of fermentation broth with 1.0 mL EtOAc (supplemented with 1 mM methyl benzoate as internal standard) after centrifugal separation of the cell mass.

General procedure for preparative fermentations: LB_{amp} fermentation medium was inoculated, induced, and charged with additives as described above. The substrate was then added directly to the shakeflask as approx. 10% (w/v) solution in 1,4-dioxane. Incubation at 24 °C was carried out until the desired degree of conversion was determined *via* GC control. The aqueous solution was then centrifuged, the supernatant was extracted with EtOAc or Et_2O and concentrated. The crude compounds were purified by chromatography on silica using LP/Et_2O or LP/EtOAc mixtures.

*(−)-2-Allylcycloheptanone (**23a**) and (+)-8-allyloxocan-2-one (**23b**):* The fermentation was carried out with rac. **23a** (100 mg, 657 μmol) according to the general procedure. Purification on 15 g silica gel, LP/Et_2O 10:1 gave compounds **23a** (34 mg, 68%) and **23b** (33 mg, 60%) as colorless oils. (−)-**23a** ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.20–1.87 (m, 10H), 1.98–2.12 (m, 1H), 2.35–2.62 (m, 2H), 4.96–5.05 (m, 2H), 5.61–5.82 (m, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 24.3 (t), 28.7 (t), 29.5 (t), 30.6 (t), 36.3 (t), 43.1 (t), 51.6 (d), 116.5 (t), 136.3 (d), 215.6 (s); $\alpha_D^{25} = -43.4$ (c = 0.68, $CHCl_3$); *ee* (GC) = 85%. (+)-**23b** ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.32–1.98 (m, 8H), 2.22–2.60 (m, 4H), 4.53–4.66 (m, 1H), 5.06–5.16 (m, 2H), 5.81 (ddt, 1H, J_1 = 17.08 Hz, J_2 = 10.11 Hz, J_3 = 6.97 Hz); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 23.7 (t), 26.2 (t), 28.7 (t), 32.3 (t), 36.7 (t), 39.8 (t), 78.0 (d), 117.7 (t), 133.6 (d), 176.5 (s); $\alpha_D^{25} = +28.3$ (c = 0.66, $CHCl_3$); *ee* (GC) = 93%.

*(−)-6-Methyloxepan-2-one (**27b**) and (−)-4-methyloxepan-2-one (**27c**):* The fermentation was carried out with rac. **27a** (180 mg, 1.60 mmol) according to the general procedure. Purification on 45 g silica gel, LP/Et_2O 3:1 gave an inseparable mixture of compounds **27b** and **27c** (ratio 50:50 as determined by GC) as colorless oil (160 mg, 78%). ^{1}H NMR and ^{13}C NMR spectra were in accordance with published values [23]; **27b** *ee* (GC) = 76%; **27c** *ee* (GC) = 74%.

*(4R,7S)-7-Isopropyl-4-methyloxepan-2-one (**29b**):* The fermentation was carried out with (−)-menthone **29a** (100 mg, 648 μmol) according to the general procedure. Purification on 20 g silica gel, LP/Et_2O 3:1 gave compound **29b** as a colorless oil (72 mg, 65%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 0.94 (d, 3H, J = 6.8 Hz), 0.96 (d, 3H,

J = 6.8 Hz), 1.02 (d, 3H, J = 6.6 Hz), 1.10–1.99 (m, 6H), 2.38–2.60 (m, 2H), 4.02 (dd, 1H, J_1 = 9.0 Hz, J_2 = 4.4 Hz); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 17.1 (q), 18.4 (q), 24.0 (q), 30.5 (d), 31.0 (t), 33.4 (d), 37.5 (t), 42.6 (t), 84.8 (d), 175.1 (s); $\alpha_D^{20} = -19.7$ (c = 0.72, $CHCl_3$); *ee* (GC) = >99%.

*Ethyl (4,4-diethoxybutyl)carbamate (**33**):* Amine **32** (5.64 g, 35 mmol) and NEt_3 (9.8 mL, 70 mmol, 2.0 equiv) were dissolved in dry DCM (100 mL) and cooled to 0 °C under an argon atmosphere. Then ethyl chloroformate (10.0 mL, 70 mmol, 2.0 equiv) was added quickly causing local reflux and clouding. The mixture was kept in the ice/water bath and stirred o/n. After 19 h the reaction composite was washed with saturated NH_4Cl solution (2× 50 mL) and brine, then dried over Na_2SO_4 and concentrated under reduced pressure yielding carbamate **33** as a pale yellow liquid (9.40 g, 90%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.06–1.33 (m, 9H), 1.51–1.72 (m, 4H), 3.13–3.23 (m, 2H), 3.40–3.71 (m, 4H), 4.03–4.20 (m, 2H), 4.47 (t, 1H, J = 5.2 Hz), 4.79 (br s, 1H).

*Ethyl 2-hydroxypyrrolidine-1-carboxylate (**34**):* Carbamate **33** (9.00 g, 36.6 mmol) was dissolved in THF (65 mL) before aqueous HCl (ca. 4%, w/v, 65 mL) was added. The mixture was stirred at rt. After 10 min complete conversion was determined *via* TLC. The solution was extracted with Et_2O (3× 150 mL), the pooled organic extracts were then washed with 10% K_2CO_3 solution (100 mL) and brine, dried over Na_2SO_4 and concentrated. Heterocycle **34** was obtained as colorless oil in >95% purity according to GC–MS as a mixture of isomers (5.80 g, quant.).

*Ethyl 7,7-dichloro-6-oxo-2-azabicyclo[3.2.0]heptane-2-carboxylate (**35**):* Hydroxypyrrolidine **34** (3.00 g, 18.85 mmol) was dissolved in toluene and refluxed using a Dean-Stark trap o/n under an Ar atmosphere. GC–MS reaction control after 17 h showed selective formation of the desired ethyl 2,3-dihydro-1*H*-pyrrole-1-carboxylate intermediate but incomplete conversion of starting material. After 29 h the reaction had progressed further, but starting material could still be detected. The Dean-Stark trap was then replaced by a reflux condenser and triethylamine (7.88 mL, 56.54 mmol, 3.0 equiv) was added at reflux temperature in one shot *via* syringe. Then dichloroacetyl chloride (5.44 mL, 56.54 mmol, 3.0 equiv) was quickly added with submersed syringe tip. The pale yellow solution turned brown immediately under heavy reflux and formation of ammonium chloride. The oil bath temperature was reduced from 135 to 120 °C. TLC reaction control after 17 h showed both the alcohol **34** as well as the intermediate elimination product to be fully consumed and indicated formation of two new products, confirmed by GC–MS as chlorine-containing molecules. The reaction mixture was cooled to rt, washed with H_2O (300 mL), 2 N HCl (2× 150 mL), saturated $NaHCO_3$ solution (3× 150 mL) and brine. The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. Careful column chromatography on 500 g silica gel under gradient elution from 10:1 to 1:1 LP/EtOAc yielded the desired dichloroketone **35** as a mixture of rotamers as brown oil (1.75 g, 37%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.93–1.34 (m, 6H), 1.93–2.16 (m, 2H), 2.29 (dd, 2H, J_1 = 13.3 Hz, J_2 = 6.7 Hz), 3.23–3.41 (m, 2H), 3.94 (q, 2H, J = 7.3 Hz), 4.09–4.35 (m, 6H), 4.81 (d, 1H, J = 7.3 Hz), 4.93 (d, 1H, 7.4 Hz); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 14.3/14.6 (q), 26.2/27.0 (t), 46.4/46.7 (t), 60.0/61.1 (d), 62.0 (t), 64.8/65.2 (d), 88.3 (s), 154.5 (s), 196.6 (s).

*Ethyl 6-oxo-2-azabicyclo[3.2.0]heptane-2-carboxylate (**26a**):* Cu/Zn couple was prepared as described by Krepski and Hassner [24]. Dichloroketone **35** (1.57 g, 6.23 mmol) was dissolved in MeOH (150 mL) saturated with NH_4Cl (approx. 40 g/L). The mixture was set under Ar atmosphere before freshly prepared Cu/Zn couple (2.00 g, 31.10 mmol, 5.0 equiv) was added slowly at rt. The resulting suspension was stirred at rt for 17 h. Full conversion was determined by TLC. The mixture was filtered through a pad of Celite® and washed with MeOH (200 mL). The solvent was evaporated and the residue was partitioned between EtOAc and H_2O (200 mL each). The aqueous phase was extracted with EtOAc (2× 200 mL) and the pooled organic layers were dried over Na_2SO_4 and concentrated, yielding the crude product as a brown liquid. Column chromatography on 70 g silica gel eluting with LP/EtOAc 1:1 gave pure **26a** as a mixture of rotamers (615 mg, 54%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.22 (t, 3H, J = 7.1 Hz), 1.82–2.03 (m, 1H), 2.12–2.24 (m, 1H), 2.76–2.86 (br m, 1H), 3.30–3.39 (br m, 2H), 3.75–3.85 (br m, 2H), 4.12 (q, 2H, J = 7.1 Hz), 4.53 (br s, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 14.8 (q), 25.3/26.0 (t), 46.1/46.5 (t), 48.2/48.8 (d), 53.7/53.8 (t), 61.4 (t), 63.7/64.8 (d), 155.0 (s), 210.0 (s).

*(3aS,6aS)-Ethyl 2-oxotetrahydro-2H-furo[3,2-b]pyrrole-4(5H)-carboxylate (**26b**) and (3aR,6aS)-ethyl 4-oxohexahydro-1H-furo[3,4-b]pyrrole-1-carboxylate (**26c**):* The fermentation was carried out with rac. **26a** (100 mg, 546 μmol) according to the general procedure. Purification on 25 g silica gel eluting with LP/EtOAc 1:1 gave compounds **26b** (34 mg, 63%) and **26c** (51 mg, 93%) as yellow oils. **26b** ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.25 (t, 3H, J = 7.1 Hz, H-4′), 1.95–2.10 (m, 1H, H-3), 2.31 (dd, 1H, J_1 = 14.2 Hz, J_2 = 6.0 Hz, H-3), 2.71–2.89 (m, 2H, H-6), 3.33–3.45 (m, 1H, H-2), 3.79 (dt, 1H, J_1 = 39.6 Hz, J_2 = 9.8 Hz, H-2), 4.13 (q, 2H, J = 7.0 Hz, H-3′), 4.41–4.52 (m, 1H, H-6a), 5.02–5.12 (m, 1H, H-3a); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 14.7/14.8 (q, C-4′), 30.3/30.8 (t, C-3), 35.8/36.6 (t, C-6), 44.2/44.5 (t, C-2), 57.8/58.4 (d, C-6a), 61.6/61.7 (t, C-3′), 83.1/84.1 (d, C-3a), 154.2/154.7 (s, C-1′), 175.4/175.8 (s, C-5); $\alpha_D^{25} = +132.7$ (c = 0.50, $CHCl_3$); Lit. +144.1 (c = 0.52, $CHCl_3$) [25]; *ee* (GC) = 98%; HR-MS: calc. for [M+H] 200.0917; found 200.0917 (Δ = 0.00 ppm). **26c** ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.25 (t, 3H, J = 7.1 Hz, H-4′), 2.06–2.23 (m, 1H, H-3), 2.31–2.41 (m, 1H, H-3), 3.17–3.30 (m, 2H, H-2/H-3a*), 3.74 (dt, 1H, J_1 = 40.2 Hz, J_2 = 9.8 Hz, H-2), 4.08–4.24 (m, 2H, H-3′), 4.39 (dd, 1H, J_1 = 10.6 Hz, J_2 = 4.5 Hz, H-6), 4.45–4.60 (m, 2H, H-6/H-6a*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 14.7/14.8 (q, C-4′), 27.1/27.6 (t, C-3), 44.0/44.8 (d, C-3a), 45.3/45.6 (t, C-2), 57.8/58.4 (d, C-6a), 61.6/61.7 (t, C-3′), 72.7/73.3 (t, C-6), 154.0/154.8 (s, C-1′), 177.8/178.1 (s, C-4); $\alpha_D^{25} = -165.1$ (c = 0.42, $CHCl_3$) *ee* (GC) = 89%; HR-MS: calc. for [M+H] 200.0917; found 200.0922 (Δ = 2.50 ppm).

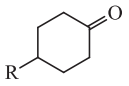
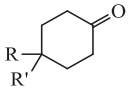
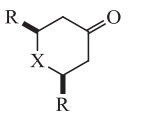
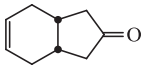
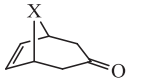
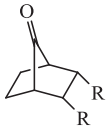
3. Results and discussion

In order to generate a complete picture of the catalyst performance, CPDMO was used to oxidize a large library of substituted cyclobutanones, cyclohexanones, fused and bridged bi- and tricyclic ketones. The experiments were conducted on analytical scale as described in Section 2. For novel transformations the fermentations were scaled up to 100 mg preparative scale. Results are referenced to published values obtained from fermentations with enzymes from the CHMO- and/or CPMO-cluster. The substrates are thematically grouped into desymmetrizations, kinetic resolutions and regiodivergent oxidations. CPDMO appears to be a CHMO-type enzyme except for the regiodivergent oxidations as compiled in Section 3.3, where the main potential for novel biotransformations of this enzyme was identified.

3.1. Desymmetrization reactions

A series of 20 symmetrical ketones was screened in desymmetrization biooxygenations (Table 1, Scheme 1). 3-Substituted cyclobutanones (substrates **1a**–**4a**) were all converted by CPDMO, usually showing full conversion to the desired lactones with high enantiomeric excesses. The optical purity of product **1b** was even slightly higher than previously published values (91 vs. 88% *ee*). Only 4-chlorophenyl compound **3a** showed poor performance as well as a rather low selectivity of 25%. The enantiomeric preference in this series goes in line with representatives of the CHMO-type enzyme cluster.

Table 1
Desymmetrizations of substituted cyclobutanones, cyclohexanones and bi-/tricyclic ketones (substrates **1**–**20**).

Substrate	R, R′, X	Compound No.	CPDMO		Reference biotransformation			
			% Conv.[a]	% *ee*[b]	% Conv.[a]	% *ee*[b]	Biocatalyst	Reference
	Bn	**1**	+++	91 (−)	+++ +	88 (−) 31 (−)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	3,4,5-triMeO-Bn	**2**	+++	82 (−)	+++ n.c.	95 (−) n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	4-Cl-Ph	**3**	++	25 (+)	+++ ++	85 (+) 44 (+)	$CHMO_{Acineto}$ $CPMO_{Coma}$	[26]
	3,4-(OCH_2O)-Bn	**4**	+++	80 (−)	+++ ++	99 (−) 40 (−)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	CO_2Et	**5**	+++	>99 (−)	+++ ++	98 (−) 64 (+)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	Ph	**6**	+++	99 (−)	+++ n.a.	98 (−) n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	*t*Bu	**7**	+++	>99 (−)	+++ n.a.	99 (−) n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	R = Me R′ = Me	**8**	+++	n.a.	+++ +	n.a. n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	R = Me R′ = Ph	**9**	n.c.	n.a.	+++ n.c.	95 (−) n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = CH_2 R = Me	**10**	+++	99 (−)	+++ ++	97 (−) 99 (+)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = O R = Me	**11**	+	>99 (−)	+++ n.c.	>99 (−) n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = O R = H	**12**	n.c.	n.a.	+++ ++	n.a. n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = S R = H	**13**	n.c.	n.a.	+++ n.a.	n.a. n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = NMe R = H	**14**	n.c.	n.a.	+++ n.a.	n.a. n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = NCO_2Me R = H	**15**	n.c.	n.a.	+++ n.a.	n.a. n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
		**16**	++	>99 (−)	+++ ++	88 (−) >99 (+)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = CH_2	**17**	n.c.	n.a.	++	>99 (−) n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	X = O	**18**	n.c.	n.a.	n.c.[c] ++	n.a. 95 (+)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	CO_2Me	**19**	n.c.	n.a.	n.c. n.c.	n.a. n.a.	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]
	$-(CH_2)_3-$	**20**	+++	64 (−)	+++ +	97 (−) 91 (+)	$CHMO_{Xantho}$ $CPMO_{Coma}$	[26]

n.a.: not available, n.c.: no conversion, rac.: racemic.

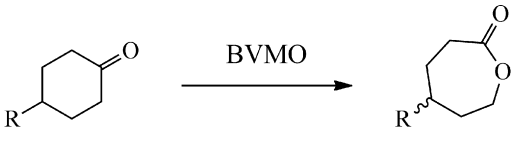
[a] Relative conversion (Conv.) of starting material determined by chiral phase GC after 24 h: +++ > 90%, ++ 50–90%, + < 50%.

[b] Enantiomeric excess values determined by chiral phase GC; sign of optical rotation or absolute configuration is given in parentheses and assigned on the basis of reference biotransformations.

[c] Mainly epoxide was formed.

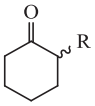
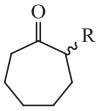
Substituted cyclohexanones **5a**–**9a** provide a more diverse picture of the substrate scope of CPDMO. It was reported before that the model CHMO from *Acinetobacter* performs rather poorly on bulky 4-phenyl- and 4-*tert*-butylcyclohexanone, however, these precursors are readily transformed by $CHMO_{Xantho}$ with excellent selectivity. The allegedly large active site of CPDMO can accommodate those sterically demanding compounds, but not 4-methyl-4-phenyl substituted substrate **9a** in contrast to reference enzymes. Nevertheless, the achieved optical purities are 99% or greater and align with the CHMO cluster preference. This also holds true for 3,5-dimethyl substrates **10a** and **11a**, although conversion of the oxa-heterocycle was rather low. Generally, CPDMO does not accept heteroatom-containing 6-rings. This could be substantiated with substrates **12a**–**15a** where only starting material was recovered after 24 h.

The enantioselectivity on fused ring system **16a** could be improved from 88% as obtained with $CHMO_{Xantho}$ to >99%, although with lower conversion. Only one other polycyclic system was converted (substrate **20a**) with moderate *ee*. Summarizing this section, it can be concluded that, although CPDMO's active site accepts substrates as large as cyclopentadecanone and smaller rings bearing bulky substituents, it is not as flexible as $CHMO_{Xantho}$. Enantioselectivities though are in the same region as other well-established BVMOs.



Scheme 1. Desymmetrization of prochiral cycloketones with BVMOs.

Table 2
Kinetic resolutions of 2-substituted six and seven-membered cycloketones (substrates **21**–**24**).

Substrate	R	Compound No.	CPDMO			Reference biotransformation				
			% Conv.[a]	% ee_S, % ee_P[a]	E[b]	% Conv.[a]	% ee_S, % ee_P[a]	E[b]	Biocatalyst	Reference
	Me	**21**	57	*97 (+)*, 82 (−)	41	24	*29 (+)*, 62 (−)	6	$CHMO_{Xantho}$	[30]
	Ph	**22**	100	rac.	n.a.	48	*>99 (+)*, 76 (−)	>200	$CHMO_{Xantho}$	[30]
	allyl	**23**	n.c.	n.a.	n.a.	53	*85 (−)*, 93 (+)	74	$CHMO_{Acineto}$	This work
	Bn	**24**	n.c.	n.a.	n.a.	20	*13*, 45	3	$CHMO_{Xantho}$	This work

n.a.: not available, n.c.: no conversion, rac.: racemic.
[a] Relative conversion (Conv.) of starting material and enantiomeric excess values determined by chiral phase GC; sign of optical rotation or absolute configuration is given in parentheses and assigned on the basis of reference biotransformations; ee_S (substrate) in italics, ee_P (product) in normal font.
[b] Enantiomeric ratio E was calculated using the Selectivity software developed by Faber.

3.2. Kinetic resolutions

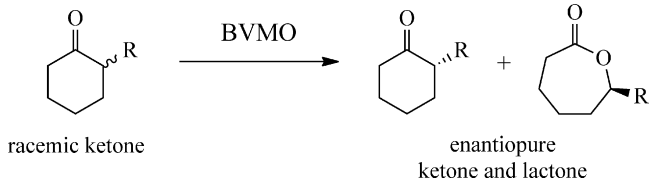
After dealing with desymmetrizations of various cyclic ketones we were interested in the performance of CPDMO in kinetic resolutions of racemic ketones (**21a**–**24a**).

Enantiomeric excess values are a function of conversion in kinetic resolution reactions, thus progress of these biotransformations was monitored over time in order to determine the 50% mark where a compound optimum of selectivity and yield is reached. For individual purposes where only enantiomerically pure ketone or lactone is desired, it may be necessary to shift the point of stopping the reaction: for maximal optical purity of the starting material the conversion should significantly exceed 50%. For highly enantioenriched product collection on the other hand it is advisable to stop at ca. 40%. In order to assess the stereoselectivity of an enzyme for a particular substrate, the enantiomeric ratio E was introduced as it is independent from conversion [27].

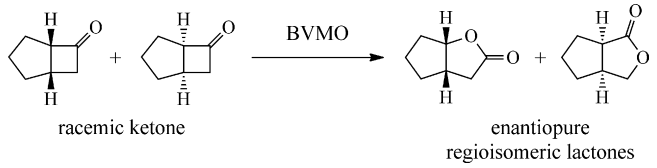
Usually consumption of one enantiomer is completed within 8 h for normal ring sizes when employing recombinant whole-cell based CPDMO fermentations. Medium and larger rings may take up to 24 h to reach completion. Enantiomeric ratio E was calculated by fitting the experimental data to a mathematical equation using the *Selectivity* software developed by Faber et al. [28]. All values are compiled in Table 2.

It has been reported that CPDMO catalyzes kinetic resolutions of five and six-membered cycloketones (Scheme 2) [20]. While substituted cyclopentanones are fully converted to racemic lactones, it was possible to selectively oxidize only the (−)-enantiomer of 2-methylcyclohexanone **21a** to the normal lactone in 82% *ee* (E = 41). The only other BVMO known to surpass this is a cyclododecanone monooxygenase (CDMO) from *Rhodococcus* SC1 (E > 200) [29]. The best result from the CHMO-cluster so far was obtained with $CHMO_{Xantho}$ producing **21b** in 62% *ee* (E = 6). When the methyl substituent is replaced with the more sterically demanding and electronically different phenyl residue (substrate **22a**), no selectivity at all could be observed and only racemic lactone **22b** was isolated. To our surprise 2-substituted cycloheptanones **23a** and **24a** were not accepted by CPDMO. For reference reasons these substrates were screened with $CHMO_{Acineto}$ and $CHMO_{Xantho}$ and for the allyl compound **23a** a preparative fermentation was carried out (see Section 2). Due to poor selectivity and conversion substrate **24a** and product **24b** were not isolated and characterized.

Although CPDMO proficiently catalyzes the difficult kinetic resolution of 2-methylcyclohexanone **21a**, it did not emerge as a suitable biocatalyst for this reaction type, in general.



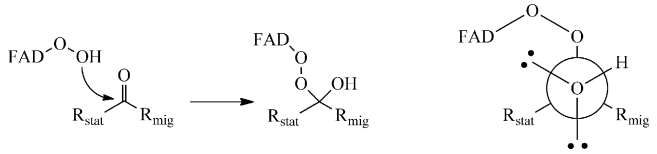
Scheme 2. Schematic view of a kinetic resolution of 2-substituted cyclic ketones catalyzed by BVMOs.



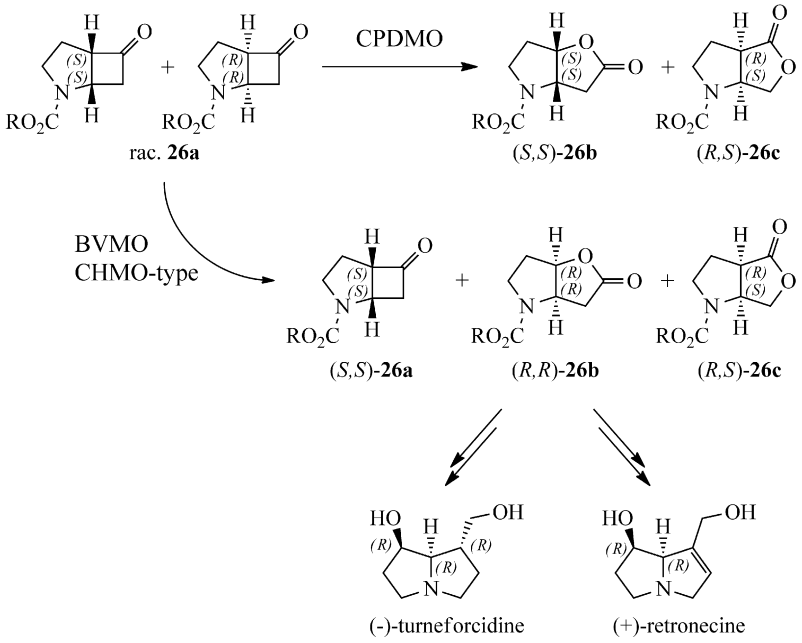
Scheme 3. Regiodivergent Baeyer–Villiger oxidations of fused bicyclic cyclobutanones.

3.3. Regiodivergent bio-oxidations

In general, fused bicyclic cyclobutanones are good substrates for BVMOs due to highly favored alleviation of ring strain by insertion of an oxygen atom. Moreover, they highlight another level of selective Baeyer–Villiger oxidation: regiodivergent transformations (Scheme 3). It was found that the mechanism of the reaction allows for prediction of product formation not only based upon nucleophilicity of carbonyl-adjacent carbon atoms but also *via* stereoelectronic requirements of the rearrangement [31]. For a successful migration the migrating bond must adopt a conformation *antiperiplanar* to the bond between the two oxygens of the peroxy-reagent **and** *anti* to one of the two non-bonding electron pairs of the carbonyl oxygen. Only when these prerequisites are met, the oxygen insertion can take place (Scheme 4). Based on these two effects, formation of the "normal" lactone as product of the nucleophilicity driven rearrangement as well as the "abnormal" lactone



Scheme 4. Left: attack of the flavin adenine dinucleotide (FAD) peroxo species and formation of the Criegee intermediate; right: required conformation for the Baeyer–Villiger rearrangement (R_{stat} = static residue, R_{mig} = migrating residue).



Scheme 5. Comparison of CHMO-catalyzed kinetic resolution and CPDMO-catalyzed regiodivergent oxidation of **26a** to Geissman–Waiss lactone **26b** and the role of **26b** in the total synthesis of the necine bases (+)-retronecine and (−)-turneforcidine.

as result of the rearrangement governed by stereoelectronic effects may be expected.

While the quite fast auto-oxidation of these starting materials with atmospheric oxygen leads to only one pair of racemic enantiomers, BVMOs are able to stabilize the Criegee intermediates in a way that two optically pure regioisomers can be obtained in many cases. It must be stressed that this reaction type is not a kinetic resolution as both enantiomers are converted to different products with comparable rates. In contrast to stereoselective desymmetrizations or kinetic resolutions of racemic ketones which were also conducted under metal-assisted catalysis with a wider substrate scope, such asymmetric and regiodivergent chemical Baeyer–Villiger oxidations are only reported on compound **25a** [3]. Consequently, they represent a unique feature of BVMOs.

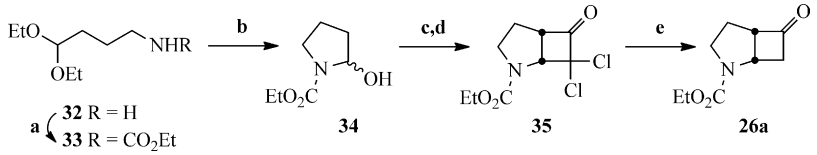
Two examples were chosen to demonstrate the ability of CPDMO to catalyze this reaction type (Table 3). Commercially available bicyclic ketone **25a** is transformed to a 51:49 mixture of regioisomeric lactones with high selectivity (86 and 85% *ee*). Again, the enzyme groups with the CHMO-type stereopreference domain. Previously, different behaviors were identified for CPMO-type biocatalysts converting both antipodal substrates into racemic normal lactones [32] as well as for a BVMO from *Mycobacterium tuberculosis* giving predominant access to optically enriched abnormal lactones [33].

An exception to this clustering of CPDMO and CHMO was observed in the Baeyer–Villiger oxidation of N-heterocyclic bicyclic ketone **26a**.

In 2005 Alphand et al. published BVMO-mediated access to a protected Geissman–Waiss lactone **26b** (R = Bn) by two different CHMOs from *Acinetobacter* sp. [34]. This compound is an important intermediate in the synthesis of (+)-retronecine [35] and other pyrrolizidine alkaloids [36]. By switching between the two catalysts, they were able to facilitate a preferential formation of normal lactone **26b** (R = Bn) with (*R*,*R*)-configuration or of abnormal **26c** (R = Bn) with (*R*,*S*)-configuration. Both enzymes catalyzed the oxidation in a kinetic resolution fashion, leaving behind the (*S*,*S*)-enantiomer of the ketone precursor (Scheme 5). In contrast to this, CPDMO provided access to the antipodal Geissman–Waiss lactone (*S*,*S*)-**26b** (R = Et) as well as the abnormal product (*R*,*S*)-**26c** (R = Et) in a perfect 50:50 mixture *via* regiodivergent oxidation in line with carbocyclic analogues of this compound class. A preparative fermentation on 100 mg scale yielded (*S*,*S*)-**26b** in 63% yield (98% *ee*) and (*R*,*S*)-**26c** in 93% yield (89% *ee*). This in turn means that through the different behavior of CPDMO the non-natural enantiomer of retronecine and other necine bases are accessible *via* this chiral intermediate (Scheme 5). Moreover, in a detailed screen using 10 BVMOs of various microbial origin ($CHMO_{Acineto}$, $CHMO_{Xantho}$, $CHMO_{Brevi1}$, $CHMO_{Brevi2}$, $CHMO_{Brachy}$, $CHMO_{Arthro}$, $CHMO_{Rhodo1}$, $CHMO_{Rhodo2}$, $CPMO_{Coma}$, CPDMO) it was revealed, that only CPDMO performed this reaction with ideal regioselectivity (unpublished results; for further information on the strains used see [6] and references cited therein).

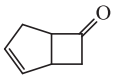
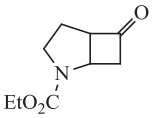
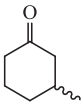
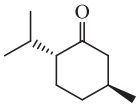
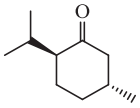
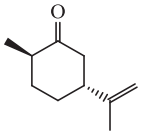
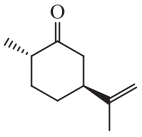
Ketone **26a** was synthesized according to the protocol for the benzyl carbamate published by Alphand et al. [34]. Since a detailed experimental procedure was not reported there, full experimental description was disclosed in this work (Scheme 6).

3-Methylcyclohexanone **27a** is another model substrate for the demonstration of CPDMO's regioselectivity. Here the difference in nucleophilicity between the carbonyl substituents is less pronounced as in 2-substituted cycloketones and a mixture of proximal



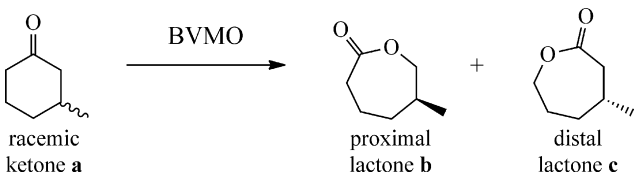
Scheme 6. Synthetic route to rac. **26a**, the ketone precursor for the N-protected Geissman–Waiss lactone **26b** (a: $ClCO_2Et$, NEt_3, DCM; b: HCl, THF; c: toluene, Dean-Stark trap; d: Cl_2HCOCl, NEt_3, toluene; e: Cu/Zn couple, NH_4Cl, MeOH).

Table 3
Regiodivergent bio-oxidations of racemic (substrates **25–27**) and optically pure ketones (substrates **28–31**).

Substrate	Compound No.	CPDMO		Reference biotransformation			
		% Conv.[a]/ratio[b]	% *ee*[c]	% Conv.[a]/ratio[b]	% *ee*[c]	Biocatalyst	Reference
	**25**	+++ 51:49	*86 (−)*, 85 (−)	+++ 52:48	*91 (−)*, >99 (−)	$CHMO_{Xantho}$	[26]
	**26**	+++ 50:50	*98 (+)*, 89 (−)	+[d] 78:22[d]	*96 (−)*, 93 (−)[d]	$CHMO_{Acineto}$	[34]
	**27**	+++ 50:50	*76 (−)*, 74 (−)	+++/50:50 +++/100:0	*95 (−)*, 94 (−) rac.	$CHMO_{Acineto}$ $CPMO_{Coma}$	This work
	**28** (+)-Menthone	+++ 100:0	>*99 (+)*[e]	+++ 100:0	>*99 (+)*[e]	$CHMO_{Acineto}$	[30]
	**29** (−)-Menthone	+++ 100:0	>*99 (−)*[e]	n.c. n.c.	n.a. n.a.	$CHMO_{Xantho}$ $CHMO_{Acineto}$	[30]
	**30** (+)-*trans*-Dihydrocarvone	+++ 0:100	>99 (−)[e]	+++ 0:100	>99 (−)[e]	$CHMO_{Xantho}$	[30]
	**31** (−)-*trans*-Dihydrocarvone	+++ 100:0	>*99 (+)*[e]	+++ 100:0	>*99 (+)*[e]	$CHMO_{Xantho}$	[30]

n.a.: not available, n.c.: no conversion, rac.: racemic.
[a] Relative conversion (Conv.) of starting material determined by chiral phase GC after 24 h: +++ > 90%, ++ 50–90%, + < 50%.
[b] Ratio of regioisomers (normal:abnormal).
[c] Enantiomeric excess values determined by chiral phase GC; sign of optical rotation or absolute configuration is given in parentheses and assigned on the basis of reference biotransformations; ee_{normal} in italics, $ee_{abnormal}$ in normal font.
[d] The reference biotransformation proceeded in a kinetic resolution fashion (see text).
[e] Starting material was optically pure; no epimerization observed.

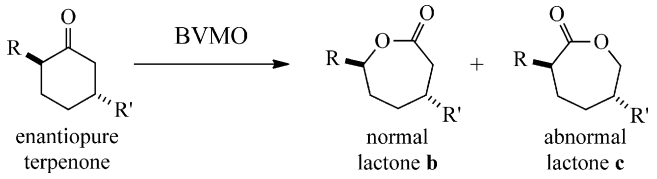
and distal lactones is to be expected (Scheme 7). Studies with isolated enantiomers of **27a** have shown that BVMOs from both the CHMO and the CPMO cluster behave highly regio- and stereoselectively [23]. For comparison fermentations were repeated with racemic starting material using $CHMO_{Acineto}$ and $CPMO_{Coma}$. In a 200 mg preparative biotransformation CPDMO produced both proximal and distal lactones **27b** and **27c** in good enantiomeric purities ($ee_{\mathbf{27b}}$ 76%, $ee_{\mathbf{27c}}$ 74%) as a clean 50:50 mixture in 78% yield (isomers are inseparable by preparative chromatography). This once again complies with the CHMO-cluster, as CPMO-type enzymes usually produce proximal lactones in racemic form only. It is noteworthy that the transformation by CPDMO is significantly slower (4 h vs. 20 h) and that our results deviate from the reported values (ratio distal/proximal 3:1, both 96% *ee*, 62% yield) [20].



Scheme 7. Regiodivergent oxidation of β-substituted cycloketones to proximal and distal lactones.

The last substrate class in this series consists of four terpenones (substrates **28a**–**31a**). The biocatalytic Baeyer–Villiger oxidation of terpenones yields both the expected normal migration products **b** as well as the abnormal lactones **c**. As was shown by our group in 2007 [37] enzymes from the CHMO cluster usually perform these reactions as enantiodivergent transformations. These starting materials are commercially available in their diastereomerically and enantiomerically pure forms, hence, the fermentations lead to a single product when employing optically pure precursors. In this case, formation of either normal or abnormal lactone depends on the absolute configuration of the starting material (Scheme 8).

CPDMO follows this trend among BVMOs, as could be shown with the completely selective oxidation of *trans*-dihydrocarvones **30a** and **31a** to abnormal lactone **30c** and normal lactone **31b**. Unexpectedly, an unprecedented biotransformation was found when menthone was tested: (−)-menthone **29a** was accepted by a BVMO for the first time and was converted to normal lactone **29b**, confirmed by a preparative biotransformation on 100 mg scale (65% yield, >99% *ee*, 100:0 **29b**:**29c**). It is intriguing that CPDMO does



Scheme 8. Regiodivergent transformations of optically pure terpenones **28a**–**31a**.

not show any regiodivergence in this case, but oxidizes both enantiomers of menthone to optical antipodes of the same regioisomer.

Summarizing this section, CPDMO shows a genuinely different selectivity profile in the case of regiodivergent Baeyer–Villiger oxidations compared to other known BVMOs and does not group with the previously defined enzyme clusters (Table 3).

4. Conclusion

In this study we have reported on the substrate scope and performance of cyclopentadecanone monooxygenase as a whole-cell biocatalyst overexpressed in *Escherichia coli*. It was clearly shown that CPDMO performs well as a biocatalyst in the Baeyer–Villiger oxidation of commercially available and synthesized cycloketones serving as model substrates and intermediates in natural compound synthesis. Despite CPDMO's rather low sequence homology with members of the CHMO cluster, it shows a similar substrate scope and identical stereopreference within desymmetrizations and kinetic resolutions. Although a versatile enzyme, the conversions and enantioselectivities in general do not surpass other high performing BVMOs like $CHMO_{Xantho}$ and its ability to oxidize large compounds seems not to be a general feature of this enzyme but rather restricted to particular substrates. As was pointed out in the last section, a number of novel features of CPDMO could be identified within regiodivergent biooxidations: with the oxidation of (−)-menthone **29a** an unprecedented biotransformation was achieved and the (*S*,*S*)-Geissman–Waiss lactone **26b** was obtained through a regiodivergent reaction pathway, enabling access to optical antipodes of the naturally occurring alkaloids (+)-retronecine and (−)-turneforcidine. Consequently, CPDMO enables a number of interesting novel biooxygenations to complement the already established portfolio of highly selective transformations by this enzyme class. The particular behavior of the enzyme in regiodivergent reactions also represents the major distinction of this enzyme relative to the previously identified enzyme clusters based on substrate profile, stereopreference, and phylogenetic relationship.

Acknowledgement

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