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Comparing the Stereoselective Biooxidation of Cyclobutanones by Recombinant Strains Expressing Bacterial Baeyer–Villiger Monooxygenases

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Abstract: Microbial Baeyer–Villiger oxidation of representative prochiral ketones with a cyclobutanone structural motif was investigated using a collection of eight monooxygenases of different bacterial origin. This platform of enzymes is able to perform stereoselective biotransformations on an array of structurally diverse substrates. With several ketone precursors, biooxidations yielded enantiocomplementary butyrolactones as key intermediates for the synthesis of natural products and bioactive compounds. The microbial Baeyer–Villiger oxidation allows a facile and rapid entry to several compound classes in a desymmetrization reaction upon *de novo* generation of chirality.

Keywords: asymmetric catalysis; Baeyer–Villiger oxidation; biocatalysis; butyrolactones; cyclobutanones; whole-cell biotransformation

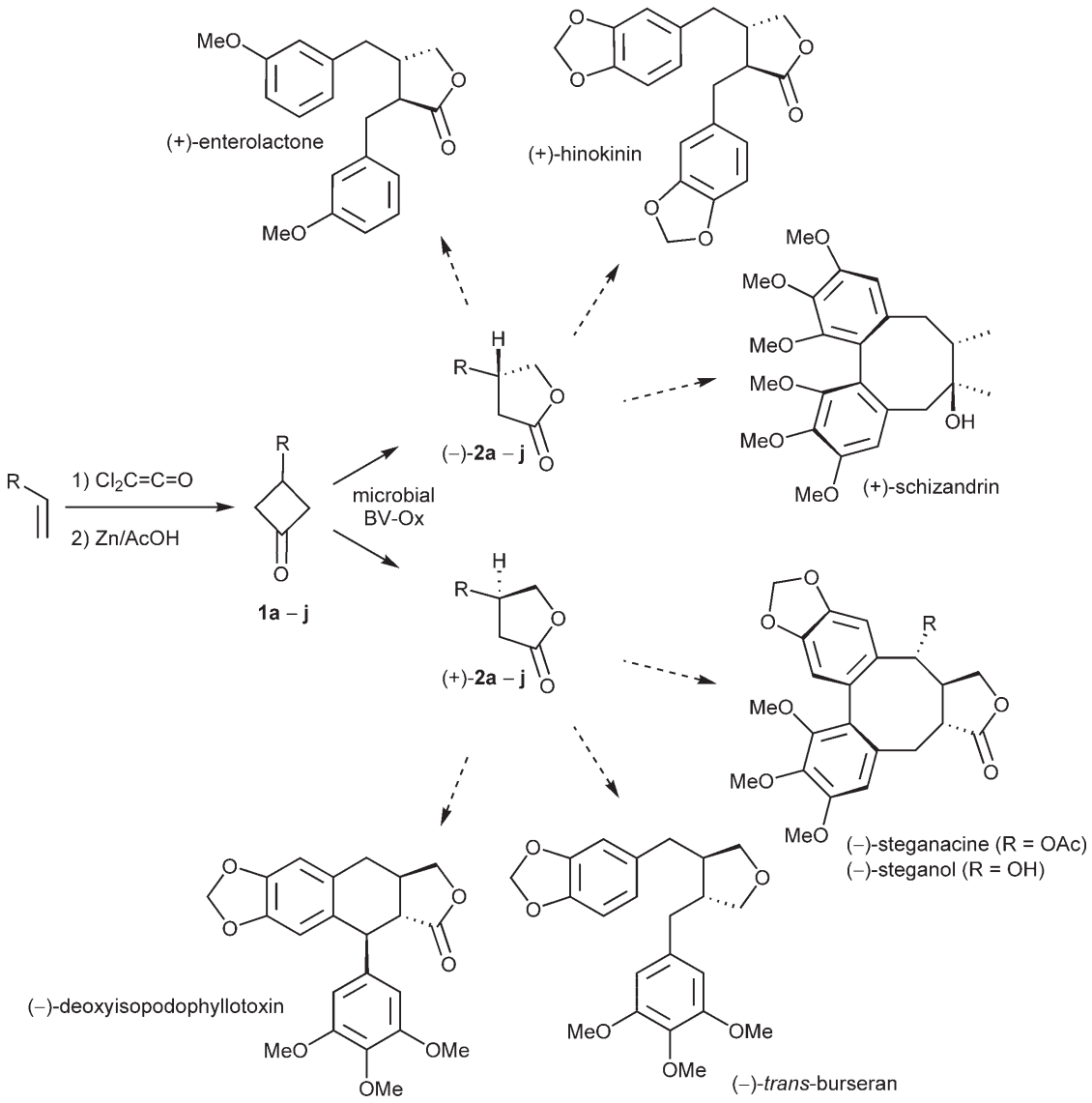
Introduction

Oxidations still represent a key challenge for the development of "green chemistry" processes and simultaneously implementing highest safety standards. The microbial Baeyer–Villiger oxidation fulfills several such requirements and has received substantial attention in recent years as a valuable tool for such transformations,[1] in particular when the biooxidation is carried out in stereoselective manner.[2]

Due to the versatility and potential of microbial Baeyer–Villiger oxidations recent research efforts are focused on the transformation of prochiral and racemic ketones into optically pure lactones as valuable and attractive intermediates in asymmetric synthesis. During the past years of progress in genome deciphering and advances in molecular biology, an increasing diversity of novel Baeyer–Villiger monooxygenases (BVMOs) originating from various natural sources has been identified and has become available as versatile catalysts for stereoselective biooxidation.[3,4] Additionally, modern methods in molecular biology and gene technology like directed evolution or gene shuffling offer strategies to modify, influence and optimize the performance of a biocatalytic entity.[5]

BVMOs are flavin- and NADPH-dependent enzymes. The dependence on a nicotine amide redox cofactor and the low thermostability complicate the utilization of isolated BVMOs. Our approach to overcome these obstacles utilizes whole-cell mediated fermentations. By overexpression of the desired BVMOs in *Escherichia coli*[6] highly productive and "easy to handle" biocatalysts have become available to synthetically oriented chemists.[7]

One of the key challenges in asymmetric synthesis and especially in biocatalysis is the aspect of enantiodivergence. Whereas artificial catalytic entities can be easily modified to produce antipodal forms of the required products by inverting the chirality of the inducing ligand field, this strategy cannot be applied in enzyme-mediated transformations. Hence, identification and characterization of enzymes possessing overlapping substrate specificity and yielding antipodal products is one of the key aspects to establish biocatalytic methods in synthetic chemistry. Desymmetrization reactions are a particularly powerful approach for the *de novo* generation of chirality in biotransformations.[8] We have recently outlined some formal total syntheses of natural products based on the application of this strategy in microbial Baeyer–Villiger



Scheme 1. Microbial Baeyer–Villiger oxidation of prochiral cyclobutanones for the synthesis of antipodal butyrolactones (–)- and (+)-**2a–j** as a platform for the synthesis of various lignans.

oxidation establishing absolute configurations for up to four stereogenic centers in a single biotransformation.[9]

Within these studies, we have introduced a collection of BVMOs of various bacterial origin with overlapping substrate specificity, which enables access to enantiocomplementary lactone products with a significant number of ketone precursors.[10] In this contribution, we apply this methodology to the biooxidation of cyclobutanones to provide chiral butyrolactones. This compound class has been demonstrated as highly versatile platform to access natural products and bioactive compounds of great structural diversity, in particular due to the straight-forward access of the substrates *via* a [2+2] cycloaddition reaction (Scheme 1).[11] Very early, desymmetrizations by BVMOs were recognized as an interesting alternative to more traditional entries to this compound class.[12] However, previous studies were limited to only very few biocatalysts and antipodal products were not accessible readily.

Butyrolactones (Scheme 1) derived from prochiral 3-substituted cyclobutanones (**1a–j**) represent a most interesting and versatile class of biooxidation products. Accessibility of antagonistic analgesics like eptazocine,[13] GABA receptor inhibitors,[12b] the synthesis of β-amino acids like β-proline,[12c] and structurally related lignans[14] are within their area of application.

Scheme 1 gives a summary of the importance of antipodal benzyl butyrolactones **2f–i** in the synthesis of various lignans. This class of compounds and their derivatives possess significant pharmacological activities

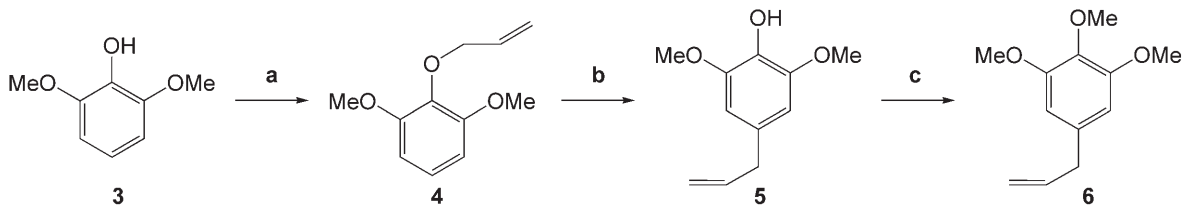
to cover a broad range and include especially antiviral and antineoplastic [e.g., (−)-deoxypodophyllotoxin[15] obtained from (+)-**2h**], antitumor [e.g., enterolactone[16] starting from (*S*)-**2f**, (−)-*trans*-burseran from (+)-**2h**, and (−)-steganes[17] obtained from lactone (+)-**2h**], cytostatic [e.g., (+)-hinokinin accessible by lactone (−)-**2h**] and antifungal properties. Furthermore, they show the ability to specifically inhibit enzymes [dibenzocyclooctadiene lignans like (+)-schizandrin[18] accessible by (−)-**2i**], and display toxicity to fungi, insects [e.g., (+)-codigerine starting from (−)-**2i**], and invertebrates.

In the present study we utilized a library of BVMOs expressed in recombinant *E.coli* for the biooxidative desymmetrization of cyclobutanone precursors. The enzymes originated from the following organisms: *Acinetobacter* ($CHMO_{Acineto}$),[19] *Arthrobacter* ($CHMO_{Arthro}$),[4d] *Brachymonas* ($CHMO_{Brachy}$), *Brevibacterium* ($CHMO_{Brevi1}$, $CHMO_{Brevi2}$),[4f] *Comamonas* ($CPMO_{Coma}$)[20] and *Rhodococcus* ($CHMO_{Rhodo1}$, $CHMO_{Rhodo2}$).[4d]

Results and Discussion

Prochiral cyclobutanones **1a–j** were synthesized according to the literature either by Cu/Zn couple mediated [2+2] cyclization under classical thermal[11a] or ultrasonic conditions[11b] from the corresponding alkenes. Access to compound **6** was optimized by re-visiting previous protocols (Scheme 2): Williams' ether synthesis of **3** with allyl bromide in the presence of sodium and methanol led to compound **4**. Subsequent Claisen-rearrangement under neat microwave conditions gave substance **5** after 75 min quantitatively compared to classical thermal heating for 7 days, as reported previously.[21] Methylation of **5** with dimethyl sulfate completed this sequence giving the required allyltrimethoxybenzene **6** in 77% yield after three steps, which could then be utilized in the above cyclization protocols to access **1i**.

Table 1 summarizes the results for stereoselective biotransformations by whole-cells expressing all BVMOs of the enzyme collection with respect to enantioselectivity in order to provide an overview of the stereopreference of individual biocatalysts and to



3 4 5 6

Scheme 2. *Conditons:* a) allyl bromide, Na/MeOH, room temperature, 83%; b) MW, 250 W, 180°C, in substance, quant.; c) Me_2SO_4, KOH, 93%.

Table 1. Multi-well plate screening of prochiral cyclobutanones with recombinant *E.coli* strains producing different BVMOs.

R	*n*-Bu	*i*-Bu	$C_6H_5CH_2$	Ph	*p*-Cl-C_6H_4	*m*-MeO-$C_6H_4CH_2$	*p*-MeO-$C_6H_4CH_2$	piperonyl	3,4,5-$(MeO)_3$-$C_6H_2CH_2$	$C_6H_5CH_2OCH_2$
	2a	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**2i**	**2j**
$CHMO_{Acineto}$	17%[a]	rac.	88%	62%	81%	99%	97%	97%	90%	53%
	(−)[b]		(−)	(−)	(+)	(−)	(−)	(−)	(−)	(+)
$CHMO_{Arthro}$	32%	74%	93%	87%	87%	93%	97%	98%	94%	58%
	(−)	(−)	(−)	(−)	(+)	(−)	(−)	(−)	(−)	(−)
$CHMO_{Brachy}$	14%	77%	84%	93%	68%	93%	90%	90%	98%	rac.
	(−)	(−)	(−)	(−)	(+)	(−)	(−)	(−)	(−)	
$CHMO_{Brevi1}$	99%	99%	93%	98%	87%	35%	26%	75%	79%	55%
	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(+)	(+)	(+)
$CHMO_{Brevi2}$	69%	22%	59%	39%	42%	45%	24%	37%	n.c.[c]	62%
	(−)	(−)	(−)	(+)	(+)	(−)	(+)	(−)		(−)
$CPMO_{Coma}$	76%	76%	31%	37%	44%	45%	24%	40%	n.c.	63%
	(−)	(−)	(−)	(+)	(+)	(−)	(+)	(−)		(−)
$CHMO_{Rhodo1}$	rac.	79%	87%	52%	95%	98%	80%	98%	95%	6%
		(−)	(−)	(−)	(+)	(−)	(−)	(−)	(−)	(+)
$CHMO_{Rhodo2}$	rac.	45%	87%	50%	95%	98%	95%	98%	92%	9%
		(−)	(−)	(−)	(+)	(−)	(−)	(−)	(−)	(+)

[a] Enantiomeric excess determined by chiral GC.
[b] Sign of optical rotation based on published results for biooxygenation using $CHMO_{Acineto}$.
[c] n.c. = no conversion.

Table 2. Desymmetrization of prochiral cyclobutanones **1a–j** on a preparative scale.

R	Product	Enzyme	Yield	*ee*	Absolute configuration	$[\alpha]_D^{20}$:
n-Bu	**2a**	$CHMO_{Acineto}$	62%	17%	(*S*)[12d]	−2.05 (*c* 2.10, $CHCl_3$)
		$CHMO_{Brevi1}$	65%	>99%	(*S*)	−5.95 (*c* 2.20, $CHCl_3$)
		$CPMO_{Coma}$	72%	76%	(*S*)	−4.46 (*c* 2.42, $CHCl_3$)
i-Bu	**2b**	$CHMO_{Acineto}$	53%	*rac.*	n.d.[12a]	n.a.
		$CHMO_{Brevi1}$	30%	>99%	(*S*)	−1.47 (*c* 1.16, $CHCl_3$)
		$CPMO_{Coma}$	63%	76%	(*S*)	−1.02 (*c* 2.46, $CHCl_3$)
$C_6H_5CH_2$	**2c**	$CHMO_{Arthro}$	56%	93%	(*S*)[12a]	−5.35 (*c* 1.31, $CHCl_3$)
		$CHMO_{Brevi1}$	30%	93%	(*S*)	−12.8 (*c* 0.70, $CHCl_3$)
		$CPMO_{Coma}$	37%	31%	(*S*)	−2.20 (*c* 0.86, $CHCl_3$)
Ph	**2d**	$CHMO_{Brachy}$	45%	93%	(*R*)[12a]	−45.0 (*c* 1.00, MeOH)
		$CHMO_{Brevi1}$	73%	98%	(*R*)	−47.3 (*c* 1.80, MeOH)
		$CPMO_{Coma}$	66%	37%	(*S*)	+16.9 (*c* 1.50, MeOH)
p-Cl-C_6H_4	**2e**	$CHMO_{Rhodo2}$	63%	95%	(S)[12b]	+44.2 (*c* 1.02, $CHCl_3$)
		$CHMO_{Brevi1}$	47%	87%	(*R*)	−37.6 (*c* 0.54, $CHCl_3$)
		$CHMO_{Brevi2}$	50%	42%	(*S*)	+16.8 (*c* 1.00, $CHCl_3$)
m-MeO-$C_6H_4CH_2$	**2f**	$CHMO_{Rhodo1}$	60%	98%	(*S*)[12a]	−6.50 (*c* 1.42, $CHCl_3$)
		$CHMO_{Brevi1}$	74%	35%	(*S*)	−2.10 (*c* 1.60, $CHCl_3$)
		$CHMO_{Brevi2}$	50%	45%	(*S*)	−1.85 (*c* 0.27, $CHCl_3$)
p-MeO-$C_6H_4CH_2$	**2g**	$CHMO_{Arthro}$	89%	97%	(*S*)[12c]	−6.11 (*c* 0.95, $CHCl_3$)
		$CHMO_{Brevi1}$	73%	26%	(*S*)	−2.10 (*c* 1.60, $CHCl_3$)
		$CHMO_{Brevi2}$	64%	24%	(*R*)	+1.08 (*c* 0.74, $CHCl_3$)
piperonyl	**2h**	$CHMO_{Rhodo2}$	52%	98%	(*S*)[12a]	−4.24 (*c* 0.75, $CHCl_3$)
		$CHMO_{Brevi1}$	61%	75%	(*R*)	+2.38 (*c* 1.51, $CHCl_3$)
		$CHMO_{Brevi2}$	53%	37%	(*S*)	−2.15 (*c* 1.30, $CHCl_3$)
3,4,5-$(MeO)_3$-$C_6H_2CH_2$	**2i**	$CHMO_{Arthro}$	72%	94%	(*S*)[34]	−6.10 (*c* 1.00, $CHCl_3$)
		$CHMO_{Brevi1}$	72%	79%	(*R*)	+4.38 (*c* 1.00, $CHCl_3$)
		$CHMO_{Brevi2}$	n.c.	n.a.	-	n.a.
$C_6H_4CH_2OCH_2$	**2j**	$CHMO_{Arthro}$	18%	58%	(*R*)[12a]	−19.1 (*c* 0.40, $CHCl_3$)
		$CHMO_{Brevi1}$	26%	55%	(*S*)	+17.1 (*c* 0.66, $CHCl_3$)
		$CPMO_{Coma}$	53%	63%	(*R*)	−18.9 (*c* 1.34, $CHCl_3$)

n. d. = not determined; n. a. = not applicable.

outline major trends. Full details for selected biotransformations of prochiral cyclobutanones **1a–j** to the corresponding lactones **2a–j** are given in Table 2. Comprehensive representation of all data for all enzymes is provided in the Supporting Information. The assignment of absolute configuration for lactones **2a–j** is based on previous literature. Purification of lactones **2a–j** was generally performed by preparative column chromatography and was analyzed by chiral GC and NMR. Complete conversion of cyclobutanones **1a–j** with BVMO producing cells gave good to acceptable yields of the desired butyrolactones **2a–j**.

Biooxidation of *n*-butylcyclobutanone **1a** gave exclusively the (*S*)-enantiomer in good yields. Interestingly, recombinant cells producing $CHMO_{Brevi1}$ gave the product with the highest enantioselectivity [>99% *ee*, (*S*)-**2a**], whereas all other representatives like $CPMO_{Coma}$ [76% *ee*, (*S*)-**2a**] or $CHMO_{Acineto}$ [17% *ee*, (*S*)-**2a**] displayed significantly lower stereoselectivity. Similar results were obtained for the biotransformation of isobutylcyclobutanone **1b**. Isolated yields were moderate to acceptable and best stereoselectivity was again observed for $CHMO_{Brevi1}$ [>99% *ee*, (*S*)-**2b**], superceeding previously reported biooxidation results.

Aryl-containing cyclobutanones **1c** and **1d** usually gave (−)-lactones in good enantioselectivities [$CHMO_{Arthro}$: 93% *ee*, (*R*)-**2c**; $CHMO_{Brachy}$: 93% *ee*, (*R*)-**2d**]. Some biocatalysts like $CPMO_{Coma}$ displayed a significantly lower stereoselectivity for lactone **2c** [31% *ee*, (*R*)-**2c**] and $CHMO_{Brevi2}$ produced antipodal product **2d** [39% *ee*, (*S*)-**2d**]. Again, biooxidations with $CHMO_{Brevi1}$ gave very good results with both substrates.

p-Chlorophenylbutyrolactone **2e**, a precursor for the synthesis of a $GABA_B$ receptor agonist, was obtained in both antipodal stereoisomers. Whereas the majority of BVMOs produced the (*S*)-enantiomer [$CHMO_{Rhodo1}$: 95% *ee*, (*S*)-**2e**], $CHMO_{Brevi1}$ gave the opposite stereoisomer in good optical purity [87% *ee*, (*R*)-**2e**].

m-Methoxycyclobutanone **1f** as precursor for enterolactone was converted to lactone **2f** in excellent enantioselectivity [$CHMO_{Rhodo1}$: 98% *ee*, (*S*)-**2f**] with several enzymes, while $CPMO_{Coma}$, $CHMO_{Brevi1}$, and $CHMO_{Brevi2}$ gave moderate to poor enantioselectivities [35–45% *ee*, (*S*)-**2f**]. Interestingly, no antipodal

m-methoxybutyrolactone was obtained within this collection of BVMOs. Biooxidation of *p*-methoxyphenylcyclobutanone **1g** gave the expected lactone **2g** with all expression strains. Comparison of specific rotation with previously published literature data showed, that $CHMO_{Arthro}$ gave the (*S*)-enantiomer **2g** in excellent optical purity [97% *ee*, (*S*)-**2g**], while $CHMO_{Brevi2}$ generated the antipodal lactone (*R*)-**2g** in poor enantioselectivity (24% *ee*).

Two other interesting substrates for the biooxidation were piperonylcyclobutanone **1h** and the 3,4,5-trimethoxybenzyl analogue **1i**. The corresponding lactones (**2h**, **2i**), obtained in both antipodal forms, are valuable precursors for the synthesis of various lignans (Scheme 1). Several recombinant strains like the $CHMO_{Rhodo2}$ producer converted ketone **1h** to (−)-butyrolactone **2h** with excellent stereoselectivity [98% *ee*, (*S*)-**2h**]. However, $CPMO_{Coma}$ and $CHMO_{Brevi2}$ gave low enantiomeric excess [37–40% *ee*, (*S*)-**2h**]. Antipodal (+)-butyrolactone **2h** was obtained in acceptable enantioselectivity [75% *ee*, (*R*)-**2h**] and moderate yield only upon oxidation with $CHMO_{Brevi1}$.

The enzymatic Baeyer–Villiger oxidation of **1i** showed a similar trend as with other oxygen-functionalized benzyl ketones: The majority of BVMOs accepted the substrate readily and the corresponding lactone **2i** was obtained with excellent enantioselectivity [$CHMO_{Arthro}$: 95% *ee*, (*S*)-**2i**]. Compound **1i** was essentially no substrate for $CPMO_{Coma}$ and $CHMO_{Brevi2}$. Again, $CHMO_{Brevi1}$ provided access to the antipodal lactone **2i** in acceptable enantioselectivity [79% *ee*, (*R*)-**2i**] and yield.

Finally, substrate **1j** was biooxidized with all protein expressing strains: while both antipodal lactones **2j** were obtained, the stereoselectivity of the biooxidation was generally modest.

In summary, the results demonstrate the potential of this library of bacterial BVMOs to produce antipodal butyrolactones and have significant consequences on our previous attempts to classify BVMOs. So far, we had outlined a hypothesis of two BVMO groups (CPMO group consisting of $CPMO_{Coma}$ and $CHMO_{Brevi2}$; CHMO group containing the remaining six enzymes),[10a] which is based on protein sequence alignment and biocatalytic performance. We have observed either enantiocomplementary oxidation or selective substrate acceptance by the two groups in a large number of biotransformations. This clustering into two groups providing access to antipodal lactones is particularly pronounced on substrates with large energy differences for various conformational forms. Adopting axial or equatorial positions of substitutents at the cyclic core of the substrate ketones seems to determine the final stereochemical outcome of the biotransformation.[22] Within six- and five-membered systems, the energy difference of these two conformations seems large enough to be affected by the particular nature of the substituent only to a minor degree. However, a much more pronounced effect by individual functional groups is observed in the cyclobutanone series. Consequently, already minor additional interactions between substrate and parts of the active site of the BVMO can affect the orientation of the ketone within the enzyme, leading to diverse migratory preferences and, ultimately, to different antipodal products.

In this cyclobutanone series, biotransformation of compounds **1a–d** with $CHMO_{Brevi1}$ gave the best enantioselectivities and this enzyme enabled access to antipodal products **2e**, **2h**, **2i**. In several cases, formation of enantiocomplementary lactones was not observed by the two enzyme clusters, but stereoselectivities of CPMO-type biocatalysts were often low (**2a–c**, **2f**, **2h**) or no conversion was observed (**1i**); only in the case of substrates **1d** and **1g** were the stereoisomeric lactones obtained.

It is interesting to observe that within this class of substrates $CHMO_{Brevi1}$ displays enantiocomplementary behavior compared to the other members of the BVMO collection studied. Already within our previous contributions on the clustering of cycloketone accepting BVMOs we noticed the borderline position of this enzyme between the CHMO and the CPMO group in the phylogenetic sequence analysis. This aspect will be investigated in more detail in upcoming studies.

Almost all whole-cell mediated biotransformations performed on the preparative scale gave lactones in acceptable to good yields. Recent advances in the fermentation "up-scaling" of microbial Baeyer–Villiger oxidations clearly outline the applicability of this methodology to access valuable chiral building blocks on multi-gram scale.[23]

Conclusions

Chiral butyrolactone precursors for several natural products were obtained on a preparative scale by BVMO-mediated biooxygenation using recombinant whole cells. The available enzyme collection enabled access to enantiocomplementary products in several cases in moderate to excellent optical purities. So far, only the natural diversity of BVMOs was exploited by using wild-type enzymes. Recent contributions in the field successfully outlined strategies to improve the stereoselectivity of such enzymes.[5] By discovering BVMOs to produce enantiocomplementary butyrolactones as highly valuable intermediates for subsequent elaboration in natural product syntheses, better entry points for optimization efforts to improve stereoselectivity and/or substrate acceptance can be provided, ultimately improving the success chance to fine-tune the catalytic performance of a mutant enzyme.

Experimental Section

Unless otherwise noted, chemicals and microbial growth media were purchased from commercial suppliers and used without further purification. All solvents were distilled prior to use. Flash column chromatography was performed on silica gel 60 from Merck (40–63 μm). Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected.

NMR-spectra were recorded from $CDCl_3$ or DMSO-d_6 solutions on a Bruker AC 200 (200 MHz) or Bruker Avance UltraShield 400 (400 MHz) spectrometer and chemical shifts are reported in ppm using TMS as internal standard. Combustion analysis was carried out in the Microanalytic Laboratory, University of Vienna.

General conversion control and analyses of purified products were performed on a GC Top 8000/MS Voyager (quadropol, EI+) using a standard capillary column BGB5 (30 m × 0.32 mm ID). Enantiomeric excesses were determined by chiral-phase GC using a BGB 175 column (30 m × 0.25 mm ID, 0.25 μm film) and a BGB 173 column (30 m × 0.25 mm ID, 0.25 μm film) on a ThermoQuest Trace GC 2000 and a Thermo Focus GC.

Specific rotations, $[\alpha]_D^{20}$ were determined using a Perkin–Elmer Polarimeter 241 by the following equation: $[\alpha]_D^{20} = 100 \times \alpha/[c] \times l$; c [g/100 mL], l [dm].

Bacterial Strains and Growth

E. coli containing the structural genes for particular BVMOs were routinely cultivated on LB-agar (1% Bacto-Peptone, 0.5% Bacto-Yeast Extract, 1% NaCl, 1.5% agar) plates supplemented by ampicillin (200 μg mL^{-1}) and stored as frozen stocks (addition of glycerol to a final concentration of 15%) at −80 °C.

Liquid cultures were grown in standard LB media (1% Bacto-Peptone, 0.5% Bacto-Yeast Extract, 1% NaCl) supplemented by ampicillin in baffled Erlenmeyer flasks on an orbital shaker (120 rpm at 37 °C). Protein expression was induced at 25 °C by addition of IPTG to a final concentration of 0.004 wt/v.

Cyclobutanone Substrates

Prochiral cyclobutanone substrates were prepared according to the literature either by Cu/Zn couple-mediated cyclization under thermal or ultrasound conditions from the corresponding alkenes and tricholoracetyl chloride. Subsequent dechlorination gave the required ketone substrates. Cyclobutanone **1i** was prepared according to the sequence below.

2-(Allyloxy)-1,3-dimethoxybenzene (4)

Sodium (1.79 g, 78.0 mmol, 1.2 equivs.) was suspended in dry EtOH (150 mL) and stirred under argon atmosphere until completely dissolved. Then a solution of 2,6-dimethoxyphenol (10.0 g, 65 mmol) in dry EtOH (120 mL) was added dropwise *via* syringe and the mixture was stirred for one hour. Freshly distilled allyl bromide (9.44 g, 78.0 mmol, 1.2 equivs.) was added dropwise and the reaction mixture was stirred overnight at room temperature to become a grey slurry. Additional allyl bromide (1.00 g, 8.3 mmol) was added and the mixture was stirred until GC/MS showed full conversion. The reaction mixture was concentrated at reduced pressure and the remaining suspension was then diluted with water (400 mL). After extraction at pH 12 with Et_2O (approx. 800 mL), the combined organic layers were dried over Na_2SO_4. Evaporation of the solvent provided the allyl ether **4** as golden yellow oil; yield: 10.5 g (54.1 mmol, 83% yield; purity: >99% according to GC/MS). Spectral data are according to the literature.[24] MS: $m/z = 194$ (61, M^+), 153 (100), 125 (48), 110 (56), 95 (38), 93 (40).

4-Allyl-2,6-dimethoxyphenol (5)

Pure 2-allyloxy-1,3-dimethoxybenzene (**4**; 2.0 g, 10.3 mmol) was sealed in a microwave tube and irradiated for 75 min (250 W, 180 °C) in a CEM Explorer PLS microwave unit. Reaction control by GC/MS indicated complete conversion. The desired compound **5** was obtained as brown oil and was used without purification in the following reaction step; yield: 2.00 g (100%). Spectral data according to the literature.[24] MS: $m/z = 194$ (100, M^+), 179 (11), 147 (10), 131 (14), 119 (20), 91 (25), 77 (13).

5-Allyl-1,2,3-trimethoxybenzene (6)

4-Allyl-2,6-dimethoxyphenol (**5**; 8.23 g, 42.4 mmol) was dissolved in 10% aqueous potassium hydroxide solution (2.97 g KOH, 53.0 mmol, 1.25 equivs.) under vigorous magnetic stirring. The reaction mixture turned dark blue and later to a greenish yellow and became a slurry. An ice bath was installed and freshly distilled dimethyl sulfate (5.88 g, 46.7 mmol, 1.1 equivs.) was added slowly; no temperature change was observed. The cooling bath was removed and the mixture was stirred for 4 h at room temperature. Additional dimethyl sulfate (0.54 g, 0.1 equiv.) was added to complete the conversion and the reaction mixture was heated to reflux. After 30 min reaction control by TLC and GC-MS showed complete conversion. The reaction solution was extracted with Et_2O (5 × 60 mL), the combined organic layers were dried over anhydrous sodium sulfate and concentrated under vacuum. Bulb-to-bulb distillation of the crude product afforded the desired compound as colorless oil; yield: 8.20 g (39.4 mmol, 93%); bp 106–108 °C/1 mbar, (Lit.[25] 106–107 °C/10 mbar). Spectral data are according to the literature.[26] MS: $m/z = 208$ (100, M^+), 193 (62), 177 (13), 133 (17), 118 (13), 91 (15), 77 (18).

3-(3,4,5-Trimethoxybenzyl)-cyclobutanone (1i)

2,2-Dichloro-3-(3,4,5-trimethoxybenzyl)cyclobutanone: A stirred suspension of zinc dust (10 g, 0.15 mol) in water (40 mL) was degassed by passage of N_2 for 15 min. Subsequently, $CuSO_4$ (750 mg, 4.7 mmol) was added at once and the black suspension was stirred while N_2 was passed through for an additional 45 min. The Cu/Zn-couple was collected on a sintered glass funnel and was washed successively with 100 mL degassed water and acetone. The Cu/Zn-couple was dried in vacuum and stored (maximum 2 days) under N_2. The freshly prepared Cu/Zn-couple (0.56 g, 8.64 mmol, 1.2 equivs.) was suspended in dry diethyl ether (20 mL). 5-Allyl-1,2,3-trimethoxybenzene (**6**) (1.50 g, 7.20 mmol) was added and the reaction mixture was set under a nitrogen atmosphere. A mixture of freshly distilled

Cl_3CCOCl (1.57 g, 8.64 mmol, 1.2 equivs.) and $POCl_3$ (1.32 g, 8.64 mmol, 1.2 equivs.) dissolved in dry diethyl ether (10 mL) was added subsequently over a period of one hour. The reaction mixture was refluxed for six hours, cooled to room temperature and a mixture of Cl_3CCOCl, $POCl_3$ (1.2 equivs.) and Cu/Zn-couple (1.2 equivs.) was added again. After 12 h complete conversion was observed by TLC. The crude reaction mixture was diluted with CH_2Cl_2 and washed with saturated sodium bicarbonate solution. The aqueous phase was re-extracted three times with ethyl acetate. The combined organic layers were dried over sodium sulfate. After evaporation of the solvent the desired compound was isolated as a crude material to be utilized in the next step without further purification; yield: 2.25 g (7.20 mmol, 100%, purity: >95% according to GC/MS).

3-(3,4,5-Trimethoxybenzyl)cyclobutanone (1i): Crude 2,2-dichloro-3-(3,4,5-trimethoxybenzyl)cyclobutanone (2.25 g, 7.20 mmol) was dissolved in glacial acetic acid (20 mL). Then zinc dust (1.4 g, 21.6 mmol, 3 equivs.) was added. The reaction mixture was refluxed overnight. TLC control showed complete conversion and the reaction mixture was cooled to room temperature and diluted with ethyl acetate. After washing with saturated aqueous sodium bicarbonate solution the organic layer was dried and the solvent was evaporated. The crude compound was purified by flash column chromatography (LP/EtOAc = 4/1 to 2/1) and was obtained as colorless oil; yield: 864 mg (48% over 2 steps). ^{1}H NMR ($CDCl_3$): δ = 2.67–2.86 (m, 5H), 3.08–3.24 (m, 2H), 3.83 (s, 3H), 3.86 (s, 6H), 6.41 (s, 2H); ^{13}C NMR ($CDCl_3$): δ = 25.1 (d), 42.2 (t), 52.3 (2t), 56.1 (2q), 60.8 (q), 105.4 (2s), 135.7 (2s), 136.4 (s), 150.9 (s), 207.7 (s); MS: *m/z* = 250 (60, M^+), 208 (22), 193 (41), 181 (100), 91 (19), 77 (25); anal. calcd. for $C_{14}H_{18}O_4$ (250.3): C 67.18%, H 7.25%; found: C 67.05%, H 7.25%.

Biotransformations

Fresh LB_{amp} medium (250 mL) was inoculated with 1% (2.5 mL) of an overnight preculture of recombinant *E. coli* strains: $CHMO_{Acineto}$, $CPMO_{Coma}$, $CHMO_{Brevi1}$, $CHMO_{Brevi2}$, $CHMO_{Rhodo1}$, $CHMO_{Rhodo2}$, $CHMO_{Brachy}$, $CHMO_{Arthro}$, in a baffled Erlenmeyer flask. The culture was incubated at 120 rpm at 37 °C on an orbital shaker for 2 h, and then 50 μL IPTG stock solution were added to a final concentration of 0.004 wt/v. The substrate was added neat along with β-cyclodextrin (1 equiv.). The culture was incubated overnight at 120 rpm on an orbital shaker at room temperature until GC (sample of 700 μL) showed complete conversion of the ketone (12–24 h). After complete conversion the biomass was separated by centrifugation (15 min, 4000 rpm). The supernatant was filtered through a bed of Celite, which was subsequently washed with the extraction solvent (EtOAc or CH_2Cl_2).

The aqueous layer was extracted with the same solvent (2×200 mL). The combined organic layers were dried over sodium sulfate, filtered, and the solvent was removed under vacuum. The crude lactones were purified by flash column chromatography and subsequently analyzed by NMR and chiral phase GC.

4-Butyldihydrofuran-2(3*H*)-one (2a): Biotransformation of 3-butylcyclobutanone (**1a**; 150 mg, 1.18 mmol) with $CHMO_{Acineto}$ gave (*S*)-**2a** (yield: 105 mg, 0.73 mmol, 62%, 17% *ee*). Oxidation of **1a** (150 mg, 1.18 mmol) with $CHMO_{Brevi1}$ gave (*S*)-**2a** (yield: 110 mg, 0.77 mmol, 65%, >99% *ee*). Transformation of 3-butylcyclobutanone (**1a**; 150 mg, 1.18 mmol) with $CHMO_{Brevi1}$ gave (*S*)-**2a** (yield: 121 mg, 0.85 mmol, 72%, 76% *ee*). The crude product was purified in all experiments by column chromatography (silica gel, LP/EtOAc = 7/1) and was obtained as a yellow oil.[27]

4-*i*-Butyldihydrofuran-2(3*H*)-one (2b): Biotransformation of 3-(2-methylpropyl)cyclobutanone (**1b**; 172 mg, 1.37 mmol) with $CHMO_{Acineto}$ gave **2b** (yield: 103 mg, 0.73 mmol, 53%, *rac.*). Conversion of **1b** (172 mg, 1.37 mmol) with $CHMO_{Brevi1}$ gave (*S*)-**2b** (yield: 58 mg, 0.41 mmol, 30%, >99% *ee*). Transformation of 3-(2-methylpropyl)cyclobutanone (**1b**; 172 mg, 1.37 mmol) with $CPMO_{Coma}$ gave (*S*)-**2b** (yield: 123 mg, 0.86 mmol, 63%, 76% *ee*). The crude product was generally purified by flash column chromatography (LP/EtOAc = 35/1) and was obtained as a beige odorant oil.[28]

Dihydro-4-(phenylmethyl)-furan-2(3*H*)-one (2c): Biotransformation of 3-(phenylmethyl)cyclobutanone (**1c**; 106 mg, 0.66 mmol) with $CHMO_{Arthro}$ gave (*S*)-**2c** (yield: 65 mg, 0.37 mmol, 56%, 93% *ee*). Oxidation of **1c** (106 mg, 0.66 mmol) with $CHMO_{Brevi1}$ gave (*S*)-**2c** (yield: 35 mg, 0.20 mmol, 30%, 93% *ee*). Transformation of **1c** (106 mg, 0.66 mmol) with $CPMO_{Coma}$ gave (*S*)-**2c** (yield: 43 mg, 0.24 mmol, 37%, 31% *ee*). The crude product was purified by column chromatography (LP/EtOAc = 10/1) and was obtained as a pink odorous oil.[29]

Dihydro-4-phenylfuran-2(3*H*)-one (2d): Biotransformation of 3-phenylcyclobutanone (**1d**; 110 mg, 0.75 mmol) with $CHMO_{Brachy}$ gave (*R*)-**2d** (yield: 45 mg, 0.34 mmol, 45%, 93% *ee*). Oxidation of **1d** (110 mg, 0.75 mmol) with $CHMO_{Brevi1}$ gave (*R*)-**2d** (yield: 89 mg, 0.55 mmol, 73%, 98% *ee*). Transformation of **1d** (110 mg, 0.75 mmol) with $CPMO_{Coma}$ gave (*S*)-**2d** (yield: 81 mg, 0.50 mmol, 66%, 37% *ee*). After purification by column chromatography (silica gel, LP/EtOAc = 15/1) lactone **2d** was obtained as colorless crystals; mp 50–55 °C, (Lit.[30]: 47–48.5 °C).

4-(4-Chlorophenyl)-dihydrofuran-2(3*H*)-one (2e): Biotransformation of 3-(4-chlorophenyl)-cyclobutanone (**1e**; 100 mg, 0.55 mmol) with $CHMO_{Rhodo2}$ gave (*S*)-**2e** (yield: 69 mg, 0.35 mmol, 63%, 95% *ee*). Oxidation of **1e** (100 mg, 0.55 mmol) with $CHMO_{Brevi1}$ afforded (*R*)-**2e** (yield: 51 mg, 0.26 mmol, 47%, 87% *ee*). Transformation of **1e** (100 mg, 0.55 mmol) with $CHMO_{Brevi2}$ gave (*S*)-**2e** (yield: 55 mg, 0.28 mmol, 50%, 37% *ee*). The crude product was purified by column chromatography (silica gel, LP/EtOAc = 3/1) and was obtained as a yellow oil.[12b]

Dihydro-5-(3-methoxyphenyl)-furan-2(3*H*)-one (2f): 3-[(3-Methoxyphenyl)methyl]cyclobutanone (**1f**; 108 mg, 0.57 mmol) was oxidized with $CHMO_{Rhodo1}$ to give (*S*)-**2f** (yield: 72 mg, 0.34 mmol, 60%, 98% *ee*). Oxidation of **1f** (108 mg, 0.57 mmol) with $CHMO_{Brevi1}$ gave (*S*)-**2f** (yield: 80 mg, 0.42 mmol, 74%, 35% *ee*). Transformation of **1f** (108 mg, 0.57 mmol) with $CHMO_{Brevi2}$ gave (*S*)-**2f** (yield: 59 mg, 0.29 mmol, 50%, 45% *ee*). The crude product was purified *via* column chromatography (LP/EtOAc = 8/1) and the product was obtained as a yellow odorous oil.[31]

Dihydro-4-[(4-methoxyphenyl)methyl]-2(3*H*)-furanone (2g): 3-[(4-Methoxyphenyl)methyl]cyclobutanone (**1g**; 69 mg, 0.36 mmol) was oxidized with $CHMO_{Arthro}$ and gave

(*S*)-**2g** (yield: 69 mg, 0.32 mmol, 89%, 97% *ee*). Oxidation of **1g** (100 mg, 0.53 mmol) with $CHMO_{Brevi1}$ gave (*S*)-**2g** (yield: 80 mg, 0.39 mmol, 73%, 26% *ee*). Transformation of **1g** (69 mg, 0.36 mmol) with $CHMO_{Brevi2}$ gave (*R*)-**2g** (yield: 48 mg, 0.20 mmol, 64%, 24% *ee*). The crude product was purified by column chromatography (LP/EtOAc = 10/1) and was obtained as a yellow odorous oil.[32]

Dihydro-4-(1,3-benzodioxo-5-ylmethyl)-furan-2(3*H*)-one (2h): Biotransformation of 3-(1,3-benzodioxo-5-ylmethyl)-cyclobutanone **1h** (94 mg, 0.46 mmol) with $CHMO_{Rhodo2}$ gave (*S*)-**2h** (yield: 53 mg, 0.24 mmol, 52%, 98% *ee*). Oxidation of **1h** (94 mg, 0.46 mmol) with $CHMO_{Brevi1}$ gave (*R*)-**2h** (yield: 62 mg, 0.28 mmol, 61%, 75% *ee*). Conversion of **1h** (94 mg, 0.46 mmol) with $CHMO_{Brevi2}$ gave (*S*)-**2h** (yield: 53 mg, 0.24 mmol, 53%, 37% *ee*). The crude product was purified *via* column chromatography (LP/EtOAc = 6/1) and was obtained as brown odorant oil.[33]

Dihydro-4-(3,4,5-trimethoxybenzyl)-furan-2(3*H*)-one (2i): Biotransformation of 3-(3,4,5-trimethoxybenzyl)cyclobutanone (**1i**; 65 mg, 0.26 mmol) with $CHMO_{Arthro}$ gave (*S*)-**2i** (yield: 50 mg, 0.19 mmol, 72%, 94% *ee*). Oxidation of **1i** (65 mg, 0.26 mmol) with $CHMO_{Brevi1}$ gave (*R*)-**2i** (yield: 50 mg, 0.26 mmol, 72%, 79% *ee*). The crude product was purified *via* column chromatography (LP/EtOAc = 2/1) and isolated as colorless crystals;[34] mp 93–96 °C (Lit.[35] 98–99 °C).

4-Benzyloxymethyldihydrofuran-2(3*H*)-one (2j): Biotransformation of 3-benzyloxymethylcyclobutanone (**1j**; 116 mg, 0.61 mmol) with $CHMO_{Arthro}$ gave (*R*)-**2j** (yield: 23 mg, 0.11 mmol, 18%, 58% *ee*). Oxidation of **1j** (116 mg, 0.61 mmol) with $CHMO_{Brevi1}$ gave (*S*)-**2j** (yield: 33 mg, 0.16 mmol, 26%, 55% *ee*). Transformation of **1j** (116 mg, 0.61 mmol) with $CHMO_{Brevi2}$ gave (*R*)-**2j** (67 mg, 0.32 mmol, 53%, 63% *ee*). The crude product was purified *via* column chromatography (LP/EtOAc = 2/1) and was obtained as a yellow oil.[36]

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