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Stereoselective Desymmetrizations by Recombinant Whole Cells Expressing the Baeyer–Villiger Monooxygenase from *Xanthobacter* sp. ZL5: A New Biocatalyst Accepting Structurally Demanding Substrates

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In this work the substrate profile and stereoselectivity of engineered whole cells overexpressing the Baeyer–Villiger monooxygenase from *Xanthobacter* sp. ZL5 with respect to biotransformations of prochiral substrates is characterized. This enzyme catalyzes the desymmetrization of cyclic ketones bearing different chemical features with stereoselectivity similar to that obtained with a related protein from *Acinetobacter* as a prototype representative of the cyclohexanone monooxygenase enzyme cluster. Moreover, this biocatalyst is able to convert sterically demanding substrates previously not transformed by other enzymes with excellent enantioselectivities. These results expand the repertoire of optically pure lactones accessible by whole-cell biotransformation processes, which are useful intermediates for the synthesis of natural and bioactive products. In addition, we observed a remarkable epoxidation reaction of a non-activated C=C bond catalyzed by this monooxygenase.

Introduction

Asymmetric catalysis in general is an extremely powerful tool to access optically pure intermediates efficiently.[1] Biocatalytic approaches offer great advantages over traditional methods since they are environmentally friendly and very efficient. Research in the area of enzyme-mediated Baeyer–Villiger oxidations has provided an outline of the great potential of these biocatalysts with respect to their high chemo-, regio-, and enantioselectivities.[2–6] While these studies have in the past been limited to a comparably small number of Baeyer–Villiger monooxygenases (BVMOs), a constantly growing number of such enzymes is becoming available by genome mining,[7] providing a potent platform for utilization of this biotransformation for the oxidation of diverse cyclic ketones to optically pure lactones. In particular, the identification of enzymes displaying enantiodivergent behavior was an important discovery, as it allows access to both antipodal lactones in excellent purities.[6]

Examples of such BVMOs producing enantiocomplementary lactones are cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 (CHMO) and cyclopentanone monooxygenase from *Comamonas* sp. NCIMB 9872 (CPMO). These enzymes are prototypes of the two main groups of cycloketone-converting Baeyer–Villiger monooxygenases: CHMO- and CPMO-type clusters. The enzymes belonging to each group display a high primary sequence identity and show similar trends both in regio- and enantiopreferences. The corresponding classification into two groups was initially proposed by our group on the basis of phylogenetic clustering, substrate preferences, and biocatalyst stereoselectivity.[8–12]

The BVMO from *Xanthobacter* sp. ZL5 was first cloned in 2003,[13] and in a previous study we determined that this new enzyme belongs to the CHMO-type branch of a phylogenetic tree comprising 18 BVMOs recombinantly expressed in a suitable host and with demonstrated activity for Baeyer–Villiger oxidations. The amino acid sequences of the CHMO from *Xanthobacter* sp. ZL5 and the one from *Acinetobacter* show 58% identity calculated using BLAST 2 SEQUENCES,[14] a BLAST-based tool at the NCBI WWW site (default parameters). We tested this biocatalyst both in kinetic resolutions of several 2-substituted cyclohexanones and in regioselective transformations of a representative set of racemic ketones and pure terpenone precursors. We observed that recombinant cells overexpressing the CHMO from *Xanthobacter* sp. ZL5 behaved like the CHMO from *Acinetobacter* both in regio- and enantioselectivities, again correlating phylogenetic data with substrate preference and

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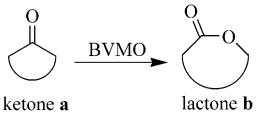
further supporting our classification into the clusters outlined above.[15]

Here we report on desymmetrization reactions of cyclic ketones as a highly attractive method for the generation of chirality in a de novo fashion from symmetrical precursors.[16] During screening experiments of the substrate scope of this BVMO we discovered that the biocatalyst is able to convert ketones bearing functionalized substituents with enantiomeric excesses comparable to or even better than those obtained with previous reference biocatalysts. Surprisingly, the CHMO from *Xanthobacter* sp. ZL5 in particular was able to accept and oxidize sterically demanding substrates with excellent enantioselectivities, offering a starting point for the synthesis of natural products or analogues. We also observed that the recombinant whole-cell system overexpressing this enzyme is able to catalyze two further reactions other than Baeyer–Villiger oxidation: the epoxidation of an oxo-bridged bicyclic ketone and the sequential two-step bioconversion of a cyclic alkenone.

Results and Discussion

1. Substrate Acceptance Profile of the CHMO from *Xanthobacter* sp. ZL5

In this work we challenged the ability of CHMO from *Xanthobacter* sp. ZL5 to catalyze desymmetrizations of prochiral ketones containing diverse structural features (Scheme 1). A large collection of mono-, bi-, and tricyclic ketones carrying different substituents were tested as substrates in screening scale experiments as described in the Experimental Section, and results are compiled in Table 1; reference data for prototype biocatalysts of the CHMO- and CPMO-type clusters are also given. All experiments were conducted with a recombinant expression strain of *E. coli* BL21 (DE3) transformed with the plasmid p11X5.1 carrying the structural gene for the BVMO from *Xanthobacter* sp. ZL5.[13]



Scheme 1. Typical Baeyer–Villiger biooxidation reaction.

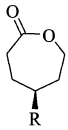
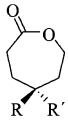
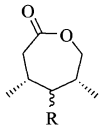
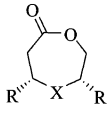
The desymmetrization of prochiral 4-monosubstituted cyclohexanones **1a**–**8a** to lactones **1b**–**8b** proceeded with excellent enantiomeric excesses, except in the case of 4-hydroxycyclohexanone (**2a**) which was converted moderately into racemic lactone (Table 1). As observed in previous biotransformation studies on hydroxy-functionalized ketones,[8,17,18] rearrangement of the initial lactone into a five-membered ring system was observed. With this exception, all the monosubstituted cyclohexanones were accepted by the enzyme with excellent stereoselectivities, and the configurations of the lactones were in agreement with our expectations for a CHMO-type BVMO (prototype reference: CHMO from *Acinetobacter*). Interestingly, sterically demanding 4-*tert*-butylcyclohexanone (**7a**) was an excellent substrate for the CHMO from *Xanthobacter* sp. ZL5 as a whole-cell biocatalyst, and a preparative-scale biotransformation was performed to confirm and characterize the product. The conversion of this ketone by the CHMO isolated from *Acinetobacter* sp. NCIMB 9871 was first reported in 1993.[18] Even though the yield was poor (17%), the lactones were obtained with an excellent enantiomeric excess (>98%) in favor of the (–)-enantiomer.[18] Most recently, the enzyme cyclopentadecanone monooxygenase from *Pseudomonas* has been reported to transform this ketone in 72% conversion and with 99% *ee*.[19] Another outstanding example is illustrated by the ability of the biocatalyst fully to convert bulky 4-phenylcyclohexanone (**8a**), producing lactone **8b** with a 98% *ee* in favor of the (–)-enantiomer. This result represents the first report of a whole cell-mediated Baeyer–Villiger biooxidation of 4-phenylcyclohexanone. To the best of our knowledge the only description of a Baeyer–Villiger biooxidation of this ketone was reported in 1999, using the purified CHMO from *Acinetobacter* sp. NCIMB 9871 and different co-solvents for the ketone.[20] The highest conversion (80%) was obtained by solubilizing the ketone in 5% ethylene glycol, but only a moderate enantiomeric excess (60%) was achieved.[20]

In a similar way, several 4,4-disubstituted cyclohexanones **9a**–**12a** were oxidized to completion by the *Xanthobacter* biocatalyst under standard screening conditions, though with only moderate enantioselectivity in the case of the 4-ethyl-4-methyl-disubstituted cyclohexanone **10a**. This may reflect the moderate energy differences for the methyl and ethyl groups adopting equatorial and axial positions, as proposed by us previously.[21] Surprisingly, 4-methyl-4-phenylcyclohexanone (**12a**) was completely oxidized in whole-cell mediated biotransformations with excellent enantiomeric excesses (95%), although the substrate is very bulky. In a previous survey by our laboratory this ketone was converted by none of eight recombinant whole-cell systems producing bacterial BVMOs of diverse origin.[22] Therefore, BL21 (DE3) expressing the CHMO from *Xanthobacter* sp. ZL5 is the first biocatalyst reported to date that is capable of accommodating and oxidizing sterically constrained 4-methyl-4-phenylcyclohexanone.

The efficient conversions and excellent enantioselectivities obtained for lactones **13b**–**17b** are in agreement with previous observations for CHMO-type enzymes[10,17,22,23] and indicate that at least the tested substituents in the 4-positions of 3,5-dimethylcyclohexanones impose no spatial limitations on this BVMO.

To investigate the ability of the recombinant BVMO from *Xanthobacter* sp. to catalyze the Baeyer–Villiger oxidation of heterocyclic ketones, six members of this series (**18a**–**23a**) were tested as potential substrates in whole-cell biotransformation reactions. It has been demonstrated previously that the CHMO family oxidizes these ketones in good yields.[24–26] As expected, all six ketones were good substrates for CHMO from *Xanthobacter* as well. In the

Table 1. Biooxidation of functionalized prochiral (hetero)cyclohexanones.

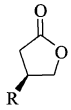
Lactone	R, R'		BVMO *Xanthobacter* sp. ZL5		Reference biotransformations		
			Cons.[a]	% *ee*[b]	% Yield	% *ee*[b]	Biocatalyst
	Me	**1**	+++	>99 (–)	83	>98 (–)	$CHMO_{Acineto}$[27,28,29,30]
					68	46 (+)	$CPMO_{Coma}$[31]
	OH	**2**	++	rac.	73	10 (–)	$CHMO_{Acineto}$[c][18]
					73	85 (+)	$CPMO_{Coma}$[12]
	Cl	**3**	+++	97 (–)	56	95 (–)	$CHMO_{Acineto}$[12]
					64	34 (+)	$CPMO_{Coma}$[12]
	Br	**4**	+++	99 (–)	63	97 (–)	$CHMO_{Acineto}$[21]
					70	64 (+)	$CPMO_{Coma}$[12]
	I	**5**	+++	>99 (–)	60	97 (–)	$CHMO_{Acineto}$[21]
					65	82 (+)	$CPMO_{Coma}$[12]
	COOEt	**6**	+++	98 (–)	15	93 (–)	$CHMO_{Acineto}$[12]
					83	64 (+)	$CPMO_{Coma}$[12]
	*t*Bu	**7**	+++	99 (–)	17	>98 (–)	$CHMO_{Acineto}$[18]
	Ph	**8**	+++	98 (–)	80[d]	60[d]	$CHMO_{Acineto}$[20]
	Me, Me'	**9**	+++	n.a.	61	n.a.	$CHMO_{Acineto}$[21]
					45	n.a.	$CPMO_{Coma}$[22]
	Me, Et'	**10**	+++	66 (–)	91	75 (–)	$CHMO_{Acineto}$[21]
					56	21 (–)	$CPMO_{Coma}$[22]
	Me, OH'	**11**	+++	>99 (–)	48	97 (–)	$CHMO_{Brachy}$[c][8]
					54	76 (+)	$CPMO_{Coma}$[8]
	Ph, Me'	**12**	+++	95 (–)	n.c.	n.a.	$CHMO_{Acineto}$[22]
					n.c.	n.a.	$CPMO_{Coma}$[22]
	H	**13**	+++	97 (–)	61	97 (–)	$CHMO_{Brevi1}$[10]
					56	99 (+)	$CHMO_{Brevi2}$[10]
	$=CH_2$	**14**	+++	>99 (+)	70	>99 (+)	$CHMO_{Brevi1}$[10]
					63	99 (–)	$CPMO_{Coma}$[8]
	cyclopropyl	**15**	+++	>99 (+)	57	>99 (+)	$CHMO_{Acineto}$[23]
					n.c.	n.a.	$CPMO_{Coma}$[23]
	cis-OH	**16**	+++	>95 (–)[e]	88	>98 (–)	$CHMO_{Acineto}$[17]
					n.c.	n.a.	$CPMO_{Coma}$[23]
	trans-OH	**17**	+++	>95 (+)[e]	80	96 (+)	$CHMO_{Acineto}$[23]
					n.c.	n.a.	$CPMO_{Coma}$[23]
	H (X = O)	**18**	+++	n.a.	79	n.a.	$CHMO_{Acineto}$[24]
					75	n.a.	$CPMO_{Coma}$[26]
	Me (X = O)	**19**	+++	>99 (–)	80	>99 (–)	$CHMO_{Acineto}$[25,26]
					n.c.	n.a.	$CPMO_{Coma}$[26]
	$CH=CH_2$ (X = O)	**20**	+++	>99 (–)	40	>99 (–)	$CHMO_{Acineto}$[26]
					64	98 (–)	$CHMO_{Brevi1}$[26]
	H (X = S)	**21**	+++	n.a.	48	n.a.	$CHMO_{Acineto}$[24]
	H (X = NMe)[f]	**22**	+++	n.a.	50	n.a.	$CHMO_{Acineto}$[24]
	H ($X = NCO_2Me$)	**23**	+++	n.a.	40	n.a.	$CHMO_{Acineto}$[24]

[a] Consumption of starting material according to gas chromatography: +++ >90%, ++ 50–90%, + <50%. [b] *ee* values determined by chiral-phase gas chromatography; sign of specific rotation is given in parentheses. [c] Isolated as rearranged product (see corresponding references). [d] Reported conversion and *ee* for a biotransformation using isolated CHMO from *Acinetobacter* sp. NCIMB 9871; specific rotation was not reported in the literature. [e] No clear baseline separation. [f] After 6 h of biotransformation; n.a.: not applicable, n.c.: no conversion, rac.: racemic.

cases of lactones **19b** and **20b** the (–)-enantiomers were formed, again showing a behavior similar to that of CHMO from *Acinetobacter*.[25,26] Like other BVMOs tested previously,[26] the CHMO from *Xanthobacter* displayed great chemoselectivity for the conversion of divinyl ketone **20a**, since no evidence of epoxidation at the two double bonds was detected and only lactone was obtained. All reactions were stopped after 24 h except in the case of ketone **22a**; this substrate had been fully consumed after 24 h, although only a small amount of lactone was observed. To analyze this reaction in detail, a time-course study of the bioconversion was carried out, with samples being taken at 0, 1, 6, 18, and 24 h after addition of the substrate, extracted, and analyzed by GC-MS. An evident peak corresponding to lactone was already identifiable after 6 h of biotransformation and, in the course of time, most of this product was obviously further metabolized by the recombinant organism and disappeared, leaving only a small amount of lactone at 24 h reaction time.

Prochiral 3-substituted cyclobutanones (Table 2) are amongst the most readily accepted substrates within the BVMO family. Even BVMOs with narrow substrate accept-

Table 2. Biooxidation of monosubstituted cyclobutanones.

Lactone	R		BVMO *Xanthobacter* sp. ZL5		Reference biotransformations		
			Cons.[a]	% *ee*[b]	% Yield	% *ee*[b]	Biocatalyst
	Ph	**24**	+++	83 (–)	53	62 (–)	$CHMO_{Acineto}$[37]
					66	37 (+)	$CPMO_{Coma}$[37]
	4-Cl-Ph	**25**	+++	81 (+)	88	85 (+)	$CHMO_{Acineto}$[35]
					78	44 (+)	$CPMO_{Coma}$[37]
	Bn	**26**	+++	88 (–)	57	82 (–)	$CHMO_{Acineto}$[38]
					37	31 (–)	$CPMO_{Coma}$[37]
	3-MeO-Bn	**27**	+++	97 (–)	83	96 (–)	$CHMO_{Acineto}$[38]
					70	45 (–)	$CPMO_{Coma}$[37]
	3,4-(OCH_2O)-Bn	**28**	+++	99 (–)	83	95 (–)	$CHMO_{Acineto}$[38]
					56	40 (–)	$CPMO_{Coma}$[37]
	*i*Bu	**29**	+++	76 (–)	53	rac.	$CHMO_{Acineto}$[37]
					63	76 (–)	$CPMO_{Coma}$[37]
	3,4,5-triMeO-Bn	**30**	+++	95 (–)	90	90 (–)	$CHMO_{Acineto}$[37]
					n.c.	n.a.	$CPMO_{Coma}$[37]

[a] Consumption of starting material according to gas chromatography: +++ >90%, ++ 50–90%, + <50%. [b] *ee* values determined by chiral-phase gas chromatography; sign of specific rotation is given in parentheses; n.a.: not applicable, n.c.: no conversion, rac.: racemic.

ance profiles such as PAMO[32,33] are able to convert ketones of this class when they are substituted with aromatic groups. As the corresponding butyrolactones are interesting precursors for the synthesis of bioactive compounds and natural products,[34–37] we decided to include a systematic investigation of representative cyclobutanones carrying aromatic and aliphatic substituents in position 3 (see **24a**–**30a**).

As shown in Table 2, the *Xanthobacter* sp. ZL5 BVMO converted these substrates with the same stereopreference, with enantiomeric excess of lactones comparable to or even better than those obtained with the CHMO from *Acinetobacter*. Surprisingly, 3-isobutylcyclobutanone (**29a**) was accepted by this biocatalyst, giving the corresponding (–)-lactone **29b** with a moderately good enantiomeric excess (76%), in contrast to the racemic product obtained with CHMO from *Acinetobacter* but in agreement with biooxidations catalyzed by other BVMOs in whole-cell systems.[37]

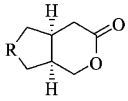
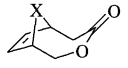
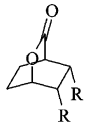
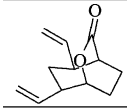
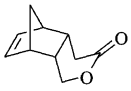
Table 3 summarizes the performance of the novel biocatalyst on polycyclic ketones. Fused bicyclic ketones **31a** and **32a** were completely biooxidized by the CHMO from *Xanthobacter* sp. with the same stereopreference as the members of the CHMO family tested previously.[8–10,39] The result obtained for lactone **32b** represents a slight improvement in the access to the corresponding (–)-enantiomer as an interesting intermediate for indole alkaloids.[9,40,41,42]

In line with the results obtained for the biooxidation of bridged oxo-ketones by the CHMO family,[8] the CHMO from *Xanthobacter* was unable to convert the oxo-bridged compound **34a** into the corresponding lactone, but a major biotransformation product was identified as an epoxide. Only CPMO from *Comamonas* and CHMO 2 from *Brevibacterium* – both members of the CPMO family – convert this ketone in moderate yields but with very good enantiomeric excesses in favor of the (+)-lactone **34b**.[8]

Different and more appealing behavior was found in the case of the carbo-bridged ketone **33a**. In agreement with the proposed stereodivergence between biooxidations by CHMO- and by CPMO-type enzymes, this ketone is converted into antipodal lactones **33b** by the two enzyme groups. While CPMO-type enzymes give full conversion to the (+)-lactone with good *ee*s, previous members of the CHMO family only displayed fair formation of the (–)-lactone at best (unpublished results). So far, the BVMO from *Xanthobacter* sp. is the first BVMO reported to catalyze the desymmetrization of ketone **33a** in whole-cell systems, providing excellent access to the resultant carbo-bridged bicyclic lactone (–)-**33b**. In contrast with the biooxidation of the oxo-bridged ketone, no indication of epoxidation at the C=C double bond was detected. Thus, both (+)- and (–)-lactones are now efficiently accessible from ketone **33a** in either CPMO- or CHMO-mediated Baeyer–Villiger reactions. However, the *N*-substituted bridged bicyclic ketone **35a** was not a substrate for this biocatalyst. These results may indicate a limitation to the accommodation and conversion of bridged bicyclic ketones of higher polarity. This trend in chemoselectivity is compatible with a prior proposal suggesting a polar constraint on the interaction between the active site of *Acinetobacter* CHMO and a set of heterocyclic cyclohexanones.[24] In principle, the same reasoning could be applied in the case of *Xanthobacter* sp., although further studies should be performed to confirm this hypothesis.

Bridged tri- and tetracyclic ketones **36a**, **37a**, and **38a**, with more sterically demanding structures, were accepted by the BVMO from *Xanthobacter* sp. and were successfully

Table 3. Biooxidation of polycyclic ketones.

Lactone	R		BVMO *Xanthobacter* sp. ZL5		Reference biotransformations		
			Cons.[a]	% *ee*[b]	% Yield	% *ee*[b]	Biocatalyst
	*exo*>CHCl	**31**	+++	>99 (–)	78	>99 (–)	$CHMO_{Acineto}$[8]
					59	60 (+)	$CHMO_{Brevi2}$[8]
	CH=CH	**32**	+++	88 (–)	56	85 (–)	$CHMO_{Brachy}$[8]
					76	>99 (+)	$CPMO_{Coma}$[8]
	X = CH_2	**33**	++	>99 (–)			
	X = O	**34**	[c]	n.a.	n.c.	n.a.	$CHMO_{Acineto}$[8]
					53	95 (+)	$CPMO_{Coma}$[8]
	X = NCO_2Me	**35**	n.c.	n.a.			
		**36**	+++	97 (–)	63	97 (–)	$CHMO_{Acineto}$[11]
					57	83 (+)	$CPMO_{Coma}$[11]
		**37**	+++	96 (–)	47	97 (–)	$CHMO_{Acineto}$[11]
					45	91 (+)	$CPMO_{Coma}$[11]
		**38**	+++	92 (–)	49	98 (–)	$CHMO_{Acineto}$[45]
					n.c.	n.a.	$CPMO_{Coma}$[45]
	CO_2Me	**39**	n.c.	n.a.	n.c.	n.a.	$CHMO_{Acineto}$[45]
					n.c.	n.a.	$CPMO_{Coma}$[45]
		**40**	+++	98:2[d]			
	*endo*	**41**	+	94 (+)	n.c.	n.a.	$CHMO_{Acineto}$[11]
					73	36 (–)	$CPMO_{Coma}$[11]
	exo	**42**	+++	43 (+)	++[e]	ca. 60 (+)	1-H7-F4[44]
					++[e]	ca. 90 (–)	1-K2-F5[44]

[a] Consumption of starting material according to gas chromatography: +++: >90%, ++: 50–90%, +: <50%. [b] *ee* values determined by chiral-phase gas chromatography; sign of specific rotation is given in parentheses. [c] Mainly epoxide was formed; n.a. not applicable; n.c. no conversion. [d] Diastereomeric ratio (*anti*/*syn*). [e] Reported as conversion in the literature stated.

converted into the lactones with excellent enantiomeric excesses. In these cases this allows control over four to six stereogenic centers in a single biotransformation step. Moreover, the location of the ketone functionalities in the bridges of these compounds did not hamper the performance of the enzyme, indicating either that its structure is flexible or that the active site is large enough to accommodate such substrates. The limits in substrate acceptance for the BVMO from *Xanthobacter* were obviously reached in the case of bifunctional ketone **39a** bearing two ester substituents; this compound could not be biooxidized enzymatically, so far. Excellent substrate acceptance was also observed for the related divinyl-substituted compound **40a**. A sample of the *anti*-ketone contaminated with traces of the corresponding *syn* isomer was efficiently oxidized to lactone **40b** (*anti*:*syn* = 98:2) in high optical purity as detected by GC-MS and confirmed by NMR analysis. Ketone **40a** was obtained from a corresponding bridged tricyclo-precursor by ring-opening metathesis, analogously with the previously reported protocol using ethylene.[43] This compound class was also tested for substrate acceptance; however, the enzyme was incapable of oxidizing such structures. So far, this represents the only spatial limitation for biotransformation of polycyclic precursors by this BVMO from *Xanthobacter* in our hands.

In contrast, the enzyme hardly afforded desymmetrization of *endo*-tricyclic ketone **41a** (though with excellent enantioselectivity), as was also observed for other CHMO-type enzymes. The conversion of **41a** with the CHMO and CPMO families has been studied before:[11] CPMO from *Comamonas* and CHMO 2 from *Brevibacterium* showed good conversion rates but moderate to poor stereoselectivities, while some members of the CHMO family only produced traces of the corresponding (+)-lactone, but with excellent selectivities. The unsaturated *exo*-tricyclic ketone **42a** was oxidized by the BVMO from *Xanthobacter* sp. ZL5, resulting in the formation of the corresponding lactone **42b** in 43% enantiomeric excess in favor of the (+)-enantiomer. Ketone **42a** is not converted by wild-type CHMO from *Acinetobacter*, although two random mutants of this enzyme (1-K2-F5 and 1-H7-F4) were found to display moderate conversions and enantiocomplementary behavior.[44] Hence, the CHMO from *Xanthobacter* sp. ZL5 is the first wild-type BVMO to show significant acceptance of *exo*-ketone **42a**.

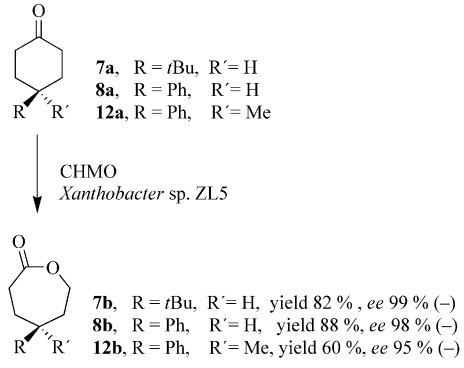
The fact that the only difference between these two tricyclic ketones is the steric arrangements of the ring systems makes these substrates very appealing candidates for further studies.

Summarizing our substrate profiling, we were clearly able to support our previous classification of the BVMO from *Xanthobacter* sp. ZL5 as belonging to the group of CHMO-type biocatalysts. Both the substrate acceptance and the stereoselectivity compare favorably to the behavior of the CHMO from *Acinetobacter* as a prototype CHMO-group enzyme, as opposed to the biocatalytic performance of CPMO-type proteins.

2. Preparative-Scale Biotransformations

Preparative-scale biotransformations and purification of products by flash column chromatography were carried out to characterize the most representative novel products that can be obtained with the CHMO from *Xanthobacter* sp. ZL5 as biocatalyst. The structures of pure lactones were confirmed by NMR, and their optical rotations were measured for correct assignment (see Exp. Sect. for details). All reactions proceeded with very good yields and high enantiomeric purities (consistent with screening experiments).

Scheme 2 summarizes the selected reactions with substituted cyclohexanones. In all three cases, (–)-lactones **7b**, **8b**, and **12b** were formed by *Xanthobacter* CHMO-mediated Baeyer–Villiger oxidation of the corresponding ketones. Lactones were usually isolated in good yields and with excellent enantiomeric excesses (Scheme 2). The biocatalytic formation of **7b** in synthetically useful quantities is particularly noteworthy, as only moderate conversion (72%) of the corresponding substrate had previously been reported, for



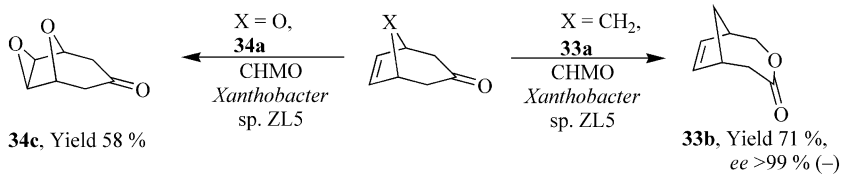
Scheme 2. Desymmetrizations of 4-substituted cyclohexanones.

CPDMO as the most effective BVMO.[19] The efficient production of lactone **8b** by a whole-cell biotransformation process is described here for the first time. Moreover, the recombinant *E. coli* overexpressing CHMO from *Xanthobacter* is the first biocatalyst able to produce lactone **12b**. The absolute configuration of lactone **7b** was assigned as in the literature.[18] For lactones **8b** and **12b**, assignment of absolute configuration is still pending.

Another interesting result was encountered with the conversion of bridged bicyclic ketones (Scheme 3). The CHMO from *Xanthobacter* sp. ZL5 proved to be an excellent catalyst for the conversion of the carbo-bridged bicyclic ketone **33a**, giving the enantiopure (–)-lactone **33b**.

However, the fact that the CHMO from *Xanthobacter* sp. ZL5 mainly catalyzes the epoxidation of ketone **34a** to compound **34c** instead of the Baeyer–Villiger oxidation to lactone is an interesting starting point for further studies. Baeyer–Villiger monooxygenases are known to catalyze oxidations at other atoms apart from the classical oxidations of ketones (reviewed in;[6] see also references therein and[33]). In 2002, the first example of the epoxidation of vinyl-phosphonates mediated by CHMO from *Acinetobacter* was reported.[46] Indeed, we have confirmed this observation in analytical-scale parallel experiments. Judging by chiral-phase GC analysis, control experiments with BL21 (DE3) cells not containing the expression plasmid p11X5.1 but in the presence of IPTG and un-induced BL21 (DE3) cells transformed with the expression plasmid coding for CHMO from *Xanthobacter* were unable to catalyze the epoxidation of ketone **34a** (only traces were detected) or partially converted it to other minor side products (not identified). In contrast, only BL21 (DE3) transformed with the plasmid p11X5.1 and induced with IPTG catalyzed the epoxidation reaction. Thus, we can exclude any interfering reaction from the host and we firmly attribute this behavior to the CHMO from *Xanthobacter* sp. ZL5. Future studies with the whole-cell biocatalyst as well as the pure enzyme are planned in order to investigate these findings more thoroughly.

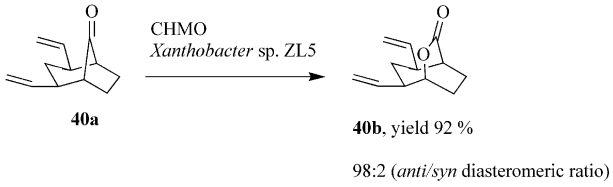
Nevertheless, we consider our findings to represent a very exceptional behavior for a BVMO. In the previously reported case of a BVMO-mediated epoxidation, a Michael acceptor-type alkene was oxygenated. This points to a nucleophilic reaction, as can be expected according to the generally accepted biocatalytic reaction cycle via an FAD-peroxyanion intermediate.[47] However, the C=C double bond in **34a** represents an electron-rich functionality, and an electrophilic oxygenation seems more likely to explain the formation of epoxide **34c**. Such a process is more similar to



Scheme 3. CHMO *Xanthobacter* sp. ZL5-mediated conversion of bridged ketones.

the BVMO-mediated oxidation of heteroatoms and, to the best of our knowledge, so far unprecedented at carbon centers.

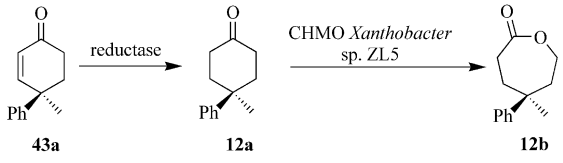
The capacity of the enzyme to accept large substrates is illustrated by the excellent isolated yield for lactone **40b** (Scheme 4). When we challenged nine BVMOs in screening experiments, poor isolated yields at best were observed in the CHMO family, while CPMO-type enzymes did not transform the substrate at all. Undoubtedly, the CHMO from *Xanthobacter* sp. gave the highest isolated yield.



Scheme 4. Example of the conversion of structurally-demanding ketones.

3. Sequential Two-Step Biotransformation Reaction

To characterize the biocatalytic behavior of this recombinant whole-cell system further, we studied the oxidation of the α,β-unsaturated cyclohexenone **43a** (Scheme 5). Few examples describing acceptance and oxidation of enones by Baeyer–Villiger monooxygenases have been reported. In an early contribution, it was shown that wild-type whole cells of *Pseudomonas* NCIMB 10007 transformed α,β-unsaturated cyclohexenones into mixtures of saturated and unsaturated ketones and lactones.[48] In 1996, Bes et al. reported the partial conversion of 5-hexylcyclopent-2-enone into the unsaturated lactone by CPMO from *Comamonas*, both as a whole-cell system and as pure enzyme.[49] Another BVMO able to accept unsaturated aryl ketones is HAPMO from *Pseudomonas fluorescens* ACB.[50] The chemoselective natures of biocatalysts give access to lactones otherwise almost impossible to obtain by classical chemical Baeyer–Villiger oxidation, given that the oxidation of this kind of ketones with *m*CPBA yields mainly epoxide products instead of lactones.



Scheme 5. Sequential two-step biotransformation of a 4-substituted cyclohexenone.

The biotransformation of a set of cyclic alkenones was recently studied for recombinant *E. coli*-based expression systems for CHMO from *Acinetobacter*, CHMO 2 from *Brevibacterium*, and CPMO from *Comamonas*.[22] On the basis of parallel experiments with and without induction of the BVMO, our group proposed that two sequential enzymatic reactions took place. Firstly, a (so far unknown) "reductase" would catalyze the reduction of the double bond, and then, in a second step, this saturated ketone would be oxidized by the BVMO.[22] However, in this context the presence of such reduced intermediates has never been demonstrated in situ for the biotransformation of a cyclic alkenone. To address this question, cyclohexenone **43a** was used as a model substrate for the reaction with the CHMO from *Xanthobacter* sp. ZL5 as a whole-cell biocatalyst. Control experiments were carried out with BL21 (DE3) cells not containing the expression plasmid. Here, a clear kinetic resolution of racemic unsaturated ketone **43a** was observed, yielding a mixture of prochiral **12a** and (almost) optically pure **43a**. In parallel experiments, BL21 (DE3) cells transformed with plasmid p11X5.1, in which protein expression was induced with IPTG, partially converted the α,β-unsaturated ketone **43a** into fully saturated lactone **12b** with the same enantioselectivity as achieved in the biooxidation starting from saturated ketone **12a**. Interestingly, it was possible to detect a small amount of saturated ketone **12a** by chiral-phase GC and GC-MS, confirming the sequential biotransformation process proposed previously (Scheme 5). When a similar experiment was conducted with transformed BL21 (DE3) cells containing the expression plasmid p11X5.1 but without addition of IPTG, we nevertheless observed formation of chiral lactone **12b**; small amounts of the intermediate **12a** of the redox cascade were found according to chiral-phase GC, and optically pure **43a** remained as result of the kinetic resolution catalyzed by the reductase. We attribute these findings to basal transcription from the promoter in the absence of inducer, especially after long fermentation periods.

Accordingly, no unsaturated lactone was detected in the fermentation medium. This finding has two major implications: on one hand, it confirms our previous hypothesis of a biocatalytic redox cascade reaction. On the other hand, it emphasizes the biotransformation of 4-methyl-4-phenylcyclohex-2-enone (**43a**) and its corresponding saturated form, a ketone until now exclusively described as substrate for CHMO *Xanthobacter* sp. As enone **43a** is also a precursor in the chemical synthesis of **12a**, this redox cascade biotransformation reaction represents an appealing short-cut to access optically pure **12b**, underscoring the efficiency of this recombinant whole-cell approach with respect to system sustainability.

Conclusions

In this work we have confirmed our previous classification of the BVMO from *Xanthobacter* sp. ZL5 as a CHMO-type enzyme according to its substrate selectivity and preference.[15] We have challenged the CHMO from *Xanthobacter* sp. ZL5 as a whole-cell biocatalyst with a wide variety of prochiral ketones containing diverse functional substituents in desymmetrization reactions. During this extensive profiling, conversions were in most cases complete or greater than 90%, and lactones were obtained with very good to excellent enantiomeric excesses. The biocatalyst be-

haved similarly to CHMO from *Acinetobacter* with all substrates reported in this work, with respect both to acceptance and to enantioselectivity.

While a great number of ketones were accepted and successfully converted, six substrates gave access to highly interesting enantiopure lactones containing sterically hindered structural motifs. These were comprehensively characterized after preparative biotransformations mediated by CHMO from *Xanthobacter* sp. ZL5. The products are of great interest as key intermediates for the first-time synthesis of natural products by BVMO-mediated biooxidations. A particularly promising example is the conversion of the carbo-bridged ketone **33a** in light of the great synthetic versatility of this scaffold, as previously outlined by us.[51] Another rare and attractive feature of this enzyme is the ability to catalyze the epoxidation of ketone **34a**. Whether this enzyme will accept substrates other than ketones will be explored in future investigations. Further expression studies will be also carried out to address the problem of the basal activity encountered for this system.

In conclusion, this versatile biocatalyst greatly expands the repertoire of optically pure lactones that can be obtained by whole-cell Baeyer–Villiger biooxidations. Its potential for applications in key chemoenzymatic steps in the synthesis of natural products and bioactive compounds is presently being addressed by our group.

Experimental Section

Abbreviations: BVMO = Baeyer–Villiger monooxygenase, CHMO = cyclohexanone monooxygenase, CPMO = cyclopentanone monooxygenase, PAMO = phenylacetone monooxygenase, HAPMO = 4-hydroxyacetophenone monooxygenase, CPDMO = cyclopentadecanone monooxygenase.

General: Chemicals and components of bacterial growth media were purchased from commercial suppliers. Substrates were either commercially available or synthesized in our laboratory (see corresponding references in Tables 1, 2, and 3). Solvents were distilled before use. Whenever possible, *m*-chloroperbenzoic acid was used to oxidize ketones to lactones in order to obtain racemic standards.[52]

Chiral-phase gas chromatography was performed with a Thermo-Finnigan Trace GC 2000 or a Focus GC instrument with a BGB 173 or a BGB 175 column, 30 m × 0.25 mm ID, 0.25 μm film. For GC-MS analysis a GC Top 8000/MS Voyager (quadrupole, EI+) instrument equipped with a standard BGB5 capillary column, 30 m × 0.32 mm ID was employed. Combustion analysis was carried out in the Microanalytic Laboratory, University of Vienna.

NMR spectra were recorded from $CDCl_3$ solutions on a Bruker AC 200 (200 MHz) or a Bruker Avance UltraShield 400 (400 MHz) spectrometer, and chemical shifts are reported in ppm from Me_4Si as internal standard. Peak assignment was based on correlation experiments. Specific rotations ($[\alpha]_D$) were determined with a Perkin–Elmer Polarimeter 241 by the following equation: $[\alpha]_D = 100 \times \alpha/(c \times l)$; c [g/100 mL], l [dm].

Strains and Plasmid: The plasmid p11X5.1[13] encoding the cyclohexanone monooxygenase from *Xanthobacter* sp. ZL5 was isolated from recombinant *Escherichia coli* DH5α host cells by use of the Wizard Plus SV Minipreps DNA Purification System (Promega). Prior to activity assays, *E. coli* BL21(DE3) cells were chemically transformed with this plasmid by standard procedures.[53]

Typical Procedure for Screening in Multiwell Plates: An overnight preculture of *E. coli* BL21 (DE3) transformed with the plasmid p11X5.1 was prepared by inoculating a single colony of the strain into LB medium (10 mL) supplemented with ampicillin (200 μg mL^{-1}) and grown at 30 °C. Cultivation of recombinant bacteria for the expression of the CHMO from *Xanthobacter* sp. ZL5 was carried out in baffled Erlenmeyer flasks containing LB medium supplemented with ampicillin (200 μg mL^{-1}) and inoculated with the overnight-grown preculture (1% v/v). The culture was shaken at 120 rpm and at 30 °C until it reached an OD_{590} of about 0.7. At this point, IPTG was added to a final concentration of 0.1 mM, and the culture was mixed well and distributed into 24-well plate dishes. Aliquots of 1 mL were loaded in each well, the appropriate ketone substrate (0.3–0.5 mg, as a solution in dioxane or ethanol) was added, and the plate was shaken at 24 °C and at 120 rpm. After 24 h of reaction, samples (700 μL) were centrifuged, and the supernatant was extracted with ethyl acetate or methylene dichloride (700 μL) containing methyl benzoate (1 mM) as internal standard. The organic phase was dried with anhydrous Na_2SO_4 and analyzed by chiral-phase GC. Assignments were performed according to the literature, with comparison of results with data from CHMO *Acinetobacter* sp. NCIMB 9871 and/or results from other BVMO-mediated biotransformations as indicated in Table 1, Table 2, and Table 3. If available, experimental data from biotransformations catalyzed by CHMO-type and CPMO-type enzymes have been provided in Tables 1, 2, and 3 as references.

Preparative-Scale Biotransformations, Product Isolation, and Characterization: Luria Bertani [LB: peptone (1% w/v), yeast extract (0.5% w/v), and NaCl (1% w/v)] or Terrific Broth [TB: tryptone (1.2% w/v), yeast extract (2.4% w/v), glycerol (0.4% v/v), KH_2PO_4 (17 mM), and K_2HPO_4 (72 mM)] media supplemented with ampicillin (200 μg mL^{-1}) were used for bacterial biotransformations. Precultures of *E. coli* BL21 (DE3) transformed with plasmid p11X5.1 were prepared in LB/ampicillin starting from an isolated colony of fresh transformants and grown overnight at 30 °C with gentle shaking (120 rpm). As a general procedure, fresh medium containing ampicillin (200 μg mL^{-1}) in baffled Erlenmeyer flasks was inoculated with an overnight-grown preculture of the bacteria (1% v/v). Antifoam was used if required. Cultures were shaken at 120 rpm at 30 °C, and protein expression was induced with IPTG at the appropriate OD_{590}. Biotransformations were initiated upon addition of the corresponding substrate and β-cyclodextrin. Reaction mixtures were incubated at 120 rpm and at 24 °C until completion or until no further conversion was observed. This time-lapse was different for each substrate, but as a general rule biotransformations lasted between 14 and 20 h. Cells were then removed by centrifugation, and the supernatant was extracted repeatedly with ethyl acetate or methylene dichloride (if necessary after saturation with sodium chloride). The combined organic layers were dried with anhydrous Na_2SO_4, filtered, and concentrated. The crude products were purified by standard flash column chromatography.

Particular biotransformation procedures and physical properties of products are described in detail below. ^{1}H NMR, ^{13}C NMR, *m/z* and combustion analysis data are provided only for compounds not reported in the literature previously.

(–)-(5*S*)-5-*tert*-Butyloxepan-2-one (7b): Fresh LB-ampicillin medium (125 mL) was inoculated with an overnight preculture of the corresponding recombinant *E. coli* strain (1% v/v) in a baffled Er-

lenmeyer flask. The culture was incubated at 120 rpm and at 30 °C on an orbital shaker for 3–4 h. After the system had reached an optical density OD_{590} = 0.6–0.7, IPTG stock solution was added to provide a final concentration of 0.134 mM. Substrate **7a** (55 mg, 0.357 mmol) was added neat along with β-cyclodextrin (1 equiv.). The culture was incubated at 24 °C for 20 h. The biomass was removed by centrifugation, saturated with sodium chloride, and extracted repeatedly with ethyl acetate. The combined organic layers were dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed in vacuo.

The crude product was purified by flash column chromatography (silica gel, PE/EtOAc, 8:2), and (–)-(5*S*)-**7b** was obtained as colorless crystals (50 mg, 0.294 mmol, 82%), m.p. 76–78 °C (lit. 58.5 °C[54]). $[\alpha]_D^{20}$ = –38.5 (99% *ee*, *c* = 0.75, $CHCl_3$) (lit. $[\alpha]_D^{20}$ = –34.9 (98% *ee*, *c* = 0.78, $CHCl_3$[18]). Spectral properties were in accordance with previous literature reports.[55,19]

(–)-5-Phenyloxepan-2-one (8b): Fresh LB-ampicillin medium (125 mL) was inoculated with an overnight preculture of the corresponding recombinant *E. coli* strain (1% v/v) in a baffled Erlenmeyer flask. The culture was incubated at 120 rpm and at 30 °C on an orbital shaker for 3–4 h. After an optical density OD_{590} = 0.6–0.7 had been reached, IPTG stock solution was added to provide a final concentration of 0.134 mM. Compound **8a** (55 mg, 0.316 mmol) was added neat, along with β-cyclodextrin (1 equiv.). The culture was incubated at 24 °C for 20 h. The biomass was removed by centrifugation, saturated with sodium chloride, and extracted repeatedly with ethyl acetate. The combined organic layers were dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed in vacuo.

The crude product was purified by standard flash column chromatography (silica gel, PE/EtOAc, 8:2), and **8b** was obtained as colorless crystals (53 mg, 0.279 mmol, 88%), m.p. 90–92 °C (lit. 100–101 °C[56]). $[\alpha]_D^{20}$ = –63.5 (98% *ee*, *c* = 2.41, $CHCl_3$). Spectral properties were in accordance with previous literature reports.[57]

(–)-5-Methyl-5-phenyloxepan-2-one (12b): Fresh LB-ampicillin medium (230 mL) was inoculated with an overnight preculture of *E. coli* BL21 (DE3) transformed with p11X5.1 (1% v/v) in a baffled Erlenmeyer flask. Antifoam was added to prevent the formation of foam. The culture was incubated at 120 rpm and at 30 °C on an orbital shaker until an optical density of 0.7 at 590 nm was reached. Then, IPTG was added to provide a final concentration of 0.1 mM and, after an initial induction period of about 1 h, ketone **12a** (100 mg, 0.532 mmol, solution in dioxane) and β-cyclodextrin (0.1 equiv.) were further added to start the reaction. Biotransformation was carried out over 15 h at 24 °C. After this period, cells were removed by centrifugation and the supernatant was repeatedly extracted with ethyl acetate after saturation with sodium chloride. The combined organic layers were dried with anhydrous Na_2SO_4, filtered, and concentrated.

The crude material was purified by flash column chromatography (silica gel, stepwise gradient system of PE/EtOAc from 100% PE to 100% EtOAc), and (–)-**12b** was obtained as colorless crystals (65 mg, 0.318 mmol, 60%), m.p. 94–96 °C. $[\alpha]_D^{21}$ = –23.2 (95% *ee*, *c* = 4.13, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.30 (s, 3 H), 1.75–2.05 (m, 2 H), 2.30–2.80 (m, 4 H), 4.05–4.35 (m, 2 H), 7.20–7.45 (m, 5 H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 30.6 (t), 31.9 (q), 33.9 (t), 40.3 (t), 40.4 (s), 65.3 (t), 125.9 (d), 126.4 (d), 128.9 (d), 145.6 (s), 175.9 (s) ppm. ^{1}H NMR and ^{13}C NMR results represent a correction to previously reported data.[22] *m*/*z*: 207 (2) $[M]^+$, 121 (100), 93 (60), 65 (68).

(–)-3-Oxabicyclo[4.2.1]non-7-en-4-one (33b): To improve the performance of CHMO from *Xanthobacter* sp. ZL5 on ketone **33a**, TB-ampicillin containing a drop of antifoam (200 mL) was used to grow the cells at 30 °C until an optical density of 11 at 590 nm was reached. Then, IPTG (0.1 mM) was added to initiate the induction of protein expression. After about 30 min at 24 °C, ketone **33a** (50 mg, 0.409 mmol; as a solution in dioxane) and β-cyclodextrin (1 equiv.) were added to start the reaction. Biotransformation was performed at 24 °C and 120 rpm over 14 h. After this period, cells were removed by centrifugation and the supernatant was extracted repeatedly with methylene dichloride. The combined organic layers were dried with anhydrous Na_2SO_4, filtered, and concentrated.

The crude product was purified by flash column chromatography (silica gel, stepwise gradient system of PE/EtOAc from 100% PE to 100% EtOAc), and lactone **33b** was obtained as colorless crystals (40 mg, 0.289 mmol, 71%), m.p. 50–52 °C. $[\alpha]_D^{21}$ = –83.3 (>99% *ee*, *c* = 0.51, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.64 (d, *J* = 11.5 Hz, 1 H), 2.20–2.45 (m, 1 H), 2.65–2.87 (m, 2 H), 2.89–3.07 (m, 1 H), 4.00–4.24 (m, 2 H), 5.83 (dd, *J* = 2.9, 5.7 Hz, 1 H), 6.10 (dd, *J* = 2.9, 5.7 Hz, 1 H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 36.7 (d), 42.6 (d), 44.4 (t), 44.7 (t), 70.7 (t), 131.0 (d), 137.0 (d), 174.1 (s) ppm. *m*/*z*: 138 (100) $[M]^+$, 109 (62), 79 (45), 66 (100). $C_8H_{10}O_2$ (138.17): calcd. C 69.55, H 7.30; found C 69.29, H 7.25.

3,9-Dioxatricyclo[3.3.1.0^{2,4}]nonan-7-one (34c): Fresh LB-ampicillin medium (125 mL) was inoculated with an overnight preculture of *E. coli* BL21 (DE3) transformed with p11X5.1 (1% v/v) in a baffled Erlenmeyer flask. A drop of antifoam was added to avoid the formation of foam. The culture was incubated at 120 rpm and at 30 °C on an orbital shaker until the optical density had reached 0.8 at 590 nm and IPTG was provided to give a final concentration of 0.1 mM. After an initial induction period of 1 h, ketone **34a** (50 mg, 0.403 mmol; as a solution in ethanol) and β-cyclodextrin (1 equiv.) were added to start the reaction. Biotransformation was carried out over 24 h at 24 °C. After this period, cells were removed by centrifugation, and the supernatant was extracted repeatedly with methylene dichloride. The combined organic layers were dried with anhydrous Na_2SO_4, filtered, and concentrated.

The crude product was purified by flash column chromatography (silica gel, stepwise gradient system of PE/EtOAc from 100% PE to 100% EtOAc), and epoxide **34c** was obtained as a colorless solid (33 mg, 0.235 mmol, 58%), m.p. 85–87 °C (pentane) {lit. 97–98.5 °C [ethyl acetate,[58] 93 °C (CCl_4)[59]]}. Spectral properties were in accordance with previous literature reports;[58,59] *m*/*z*: 140 (72) $[M]^+$, 111 (8), 98 (72), 69 (100).

2,4-Divinyl-6-oxabicyclo[3.2.2]nonan-7-one (40b): Fresh LB-ampicillin medium (250 mL) was inoculated with an overnight preculture of the corresponding recombinant *E. coli* strain (1% v/v) in a baffled Erlenmeyer flask. The culture was incubated at 120 rpm and at 30 °C on an orbital shaker. After 3 h, the culture had reached an optical density of 0.6 at 590 nm, and IPTG was added to give a final concentration of 0.168 mM. Substrate **40a** (70 mg, 0.400 mmol) was added, together with β-cyclodextrin (1 equiv.). The culture was incubated at 24 °C for 20 h. The biomass was removed by centrifugation; the supernatant was saturated with sodium chloride, and extracted repeatedly with ethyl acetate. The combined organic layers were dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed.

The crude material was purified by flash column chromatography (silica gel, PE/EtOAc, 8:1), and lactone **40b** was obtained as a colorless oil (70 mg, 0.360 mmol, 92%, diastereomeric ratio 98:2 *anti*/*syn*). $[\alpha]_D^{26}$ = +35.5 (*c* = 1.91, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 1.28–1.32 (m, 1 H), 1.72–1.77 (m, 1 H), 1.79–1.81 (m, 1 H), 1.91–1.96 (m, 1 H), 2.07–2.14 (m, 2 H), 2.25–2.30

(m, 1 H), 2.32–2.38 (m, 1 H), 2.77 (d, J = 5.8 Hz, 1 H), 4.56 (d, J = 5.9 Hz, 1 H), 4.95–5.07 (m, 4 H), 5.77–5.91 (m, 2 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 24.2 (t), 25.8 (t), 32.9 (t), 44.9 (d), 46.0 (d), 46.7 (d), 79.9 (d), 114.0 (t), 114.4 (t), 140.6 (d), 140.9 (d), 173.2 (s) ppm. *m/z*: 192 (4) $[M]^+$, 177 (8), 164 (14), 148 (22), 135 (28), 107 (26), 91 (45), 79 (100), 67 (40), 53 (35). $C_{12}H_{16}O_2$ (192.26): calcd. C 74.97, H 8.39; found C 75.17, H 8.42.

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