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Microbial *Baeyer-Villiger* Oxidation of Ketones by Cyclohexanone and Cyclopentanone Monooxygenase – A Computational Rational for Biocatalyst Performance

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Summary. Recombinant *Escherichia coli* overexpressing *Pseudomonas* sp. NCIMB 9872 cyclopentanone monooxygenase (CPMO, EC 1.14.13.16) and *Acinetobacter* sp. NCIMB 9871 cyclohexanone monooxygenase (CHMO, EC 1.14.13.22) have been utilized in whole-cell *Baeyer-Villiger* biotransformations of prochiral bicycloketones. A significant difference in substrate acceptance and stereoselectivity was observed for bicyclo[3.3.0] and bicyclo[4.3.0] substrates. A plausible mechanism of these transformations was established by means of high level DFT/B3LYP calculations suggesting an essential difference in electronic requirements for a successful enzymatic conversion, which was similarly encountered in recombinant whole-cell mediated biooxidations. Some of the lactones produced in the biocatalytic *Baeyer-Villiger* oxidation represent key intermediates for the synthesis of indole alkaloids.

Keywords. Biocatalysis; Recombinant whole-cell biotransformation; *Baeyer-Villiger* oxidation; DFT calculations; Transition states.

Introduction

Based on the successful application of enzymes in laboratory scale, biocatalytic methods have been introduced into the chemical and pharmaceutical industry due to an increasing demand for optically pure building blocks [1]. Out of more than 300 such processes [2], the microbial *Baeyer-Villiger* oxidation represents a particularly useful reaction for biotransformations in asymmetric mode [3–5].

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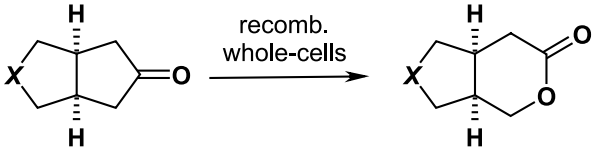
Recent advances in molecular biology enabled access to an increasing number of novel *Baeyer-Villiger* monooxygenases (*BV*MOs) originating from various natural sources [6–8]. Nature's diversity is additionally complemented by previous attempts to modify the stereospecificity of such enzymes [9], ultimately generating a toolbox of such biooxidation catalysts for the transformation of a large variety of structurally diverse substrates into high-value chiral lactones. In this context, the characterization of stereopreference and substrate acceptance of these enzymes is becoming a key aspect in order to understand and control the biocatalytic process on molecular level and to evaluate and tune their potential as biocatalysts in organic synthesis [10].

Utilization of engineered strains overexpressing particular *BV*MOs in place of wild-type organisms allows production of the required enzyme at high level by the use of strong and highly controlled promoters and simultaneously minimizing problems with unwanted side reactions and low monooxygenase activity. In addition, whole-cell mediated biotransformations offer a solution to obstacles associated with cofactor regeneration, as *BV*MOs are *NADP*H-dependent flavoenzymes. Finally, overexpression of enzymes in suitable non-pathogenic strains such as *E.coli* provides ''easy-to-use'' catalytic systems even for proteins originating from pathogenic organisms and require a minimum of special laboratory equipment and microbiological expertise. Consequently, such systems can be provided to preparative chemists for subsequent applications in asymmetric organic synthesis [11–13].

Results and Discussion

Recently, we have introduced a platform of *BV*MOs with enantiocomplementary stereopreference and overlapping substrate profiles [10, 14, 15]. The most prominent representatives were cycloehexanone monooxygenase (CHMO, EC 1.14.13.22 [16]) and cyclopentanone monooxygenase (CPMO, EC 1.14.13.16 [17]), which possess highly relaxed substrate acceptance for a variety of non-natural substrates. However, we were puzzled by the significant difference in substrate specificity and stereoselectivity for the biooxidation of fused bicycloketone **1b** (Scheme 1) [15], which we were studying in more detail in the current contribution. Compounds of this type are intermediates for several indole alkaloids [18].

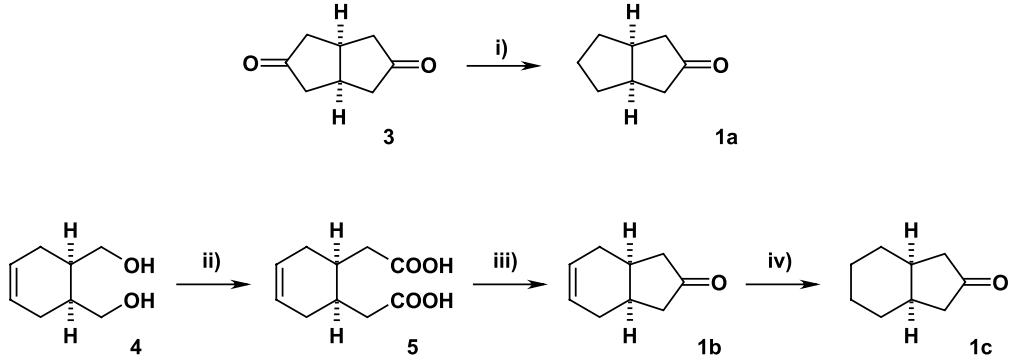
Substrate **1a** as a representative of the bicyclo[3.3.0] scaffold was accessed *via* literature methods [19] from diketone **3** (Scheme 2) [20]. Using diol **4** as entry into



1a (*X*: CH_2)
1b (*X*: CH=CH)
1c (*X*: CH_2CH_2)

2a (*X*: CH_2)
2b (*X*: CH=CH)
2c (*X*: CH_2CH_2)

Scheme 1



Scheme 2. (i) Ref. [19]; (ii) Ref. [15]; (iii) Ac_2O, pyridine, then 0.5 *N* HCl, 61%; (iv) Pd/C, H_2 81psi, *THF*, 77%

the bicyclo[4.3.0] series, we adapted [21] and optimized protocols [22] for tosylation, cyanation, and hydrolysis to the diacid **5** [15]. One-pot cyclization in acetic anhydride/pyridine and concomitant decarboxylation represents a shortcut to ketone **1b** compared to the usual *Dieckmann* condensation route [23]. Catalytic hydrogenation of olefin **1b** afforded **1c**.

Biocatalytic desymmetrizations were carried out using recombinant *E.coli* under growing conditions in baffled *Erlenmeyer* flasks according to our recently introduced standard protocol [24]. Protein production was induced by addition of isopropyl-β-*D*-galactopyranoside and biotransformations were usually carried out in the presence of β-cyclodextrin to improve solubility of ketones and in order to limit possible toxicity *vis-à-vis* whole-cells. Results of the biooxidation are summarized in Table 1.

Table 1. Biotransformation of fused bicyclo substrates with CHMO and CPMO expressing recombinant cells

Ketone	*X*	Lactone	Strain	Yield[a]/%	*e.e.*[b]/%	$[\alpha]_D^{20}/10^{-1}$ deg cm^2 g^{-1}
1a	CH_2	**2a**	CHMO	50	89	−4.7 *c* = 0.34, $CDCl_3$
1a	CH_2	**2a**	CPMO	85	9	+0.4 *c* = 1.0, $CHCl_3$
1b	CH=CH	**2b**	CHMO	33 (85)	∼5	−0.7 *c* = 0.36, CH_2Cl_2
1b	CH=CH	**2b**	CPMO	76	>99	+24.5 *c* = 1.0, $CHCl_3$
1c	CH_2CH_2	**2c**	CHMO	21 (65)	∼3	−1.2 *c* = 0.46, *Et*OH
1c	CH_2CH_2	**2c**	CPMO	83	99	+39.1 *c* = 1.0, $CHCl_3$

[a] Isolated yield after chromatographic purification; yield in parenthesis is based on consumed starting material; [b] *e.e.* determined by chiral phase gas chromatography; racemic reference material prepared by *m-CPBA* oxidation of ketones **1a**/**1c**

We observed an interesting trend for both enzymes: while CHMO converted the bicyclo[3.3.0] ketone **1a** readily and in good stereoselectivity, CPMO gave **2a** in poor optical purity with opposite specific rotation. In the case of bicyclo[4.3.0] substrates **1b**/**1c** also enantiocomplementary biooxidations were observed, however, now CHMO displayed poor stereopreference, while CPMO provided lactones **2b**/**2c** in excellent selectivity. The general behavior with respect to stereocomplementary oxidations was in agreement with our recently introduced model of two clusters for *BV*MO enzymes [10].

A most intriguing aspect was the very sluggish biotransformation of ketones **1b**/**1c** by CHMO, which may be attributed to spatial limitations within the active site of the enzyme. On the other hand, this enzyme had been demonstrated to accommodate also sterically demanding substrates [25], which prompted us to closer investigate the electronic situation in intermediates of the *Baeyer-Villiger* process.

For this purpose the transformation of ketones **1a** ($\mathbf{A^5}$ in the model) and **1c** ($\mathbf{A^6}$ in the model) to the corresponding lactones **2a** ($\mathbf{C^5}$ in the model) and **2c** ($\mathbf{C^6}$ in the model) was studied by means of high level DFT/B3LYP calculations. The energy profiles for these conversions utilizing H_2O_2 as oxidizing agent (representing a simple approximation for peroxy-*FAD*) are shown in Figs. 1 and 2.

A remarkable difference in the two transformations was observed for the initial attack by the peroxy-species at the ketone carbonyl center. In the case of $\mathbf{A^6}$, the energy barrier to be overcome is about 42 kJ/mol higher than for $\mathbf{A^5}$ and is approaching values for the transformation of aromatic ketones (Fig. 3). It has been established in early mechanistic studies, that aryl substituents have a retarding effect on the rate-determining step of *Criegee* intermediate formation [26]. It is

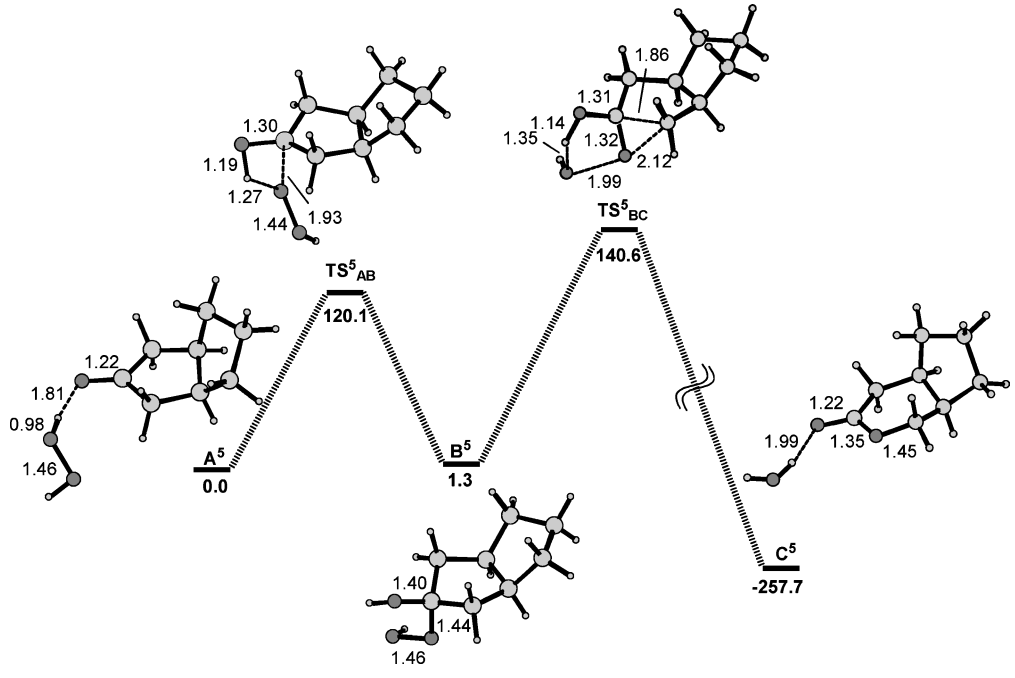


Fig. 1. Energy profile (in kJ/mol) for the transformation of ketone $\mathbf{A^5}$ to lactone $\mathbf{C^5}$ (distances in Å)

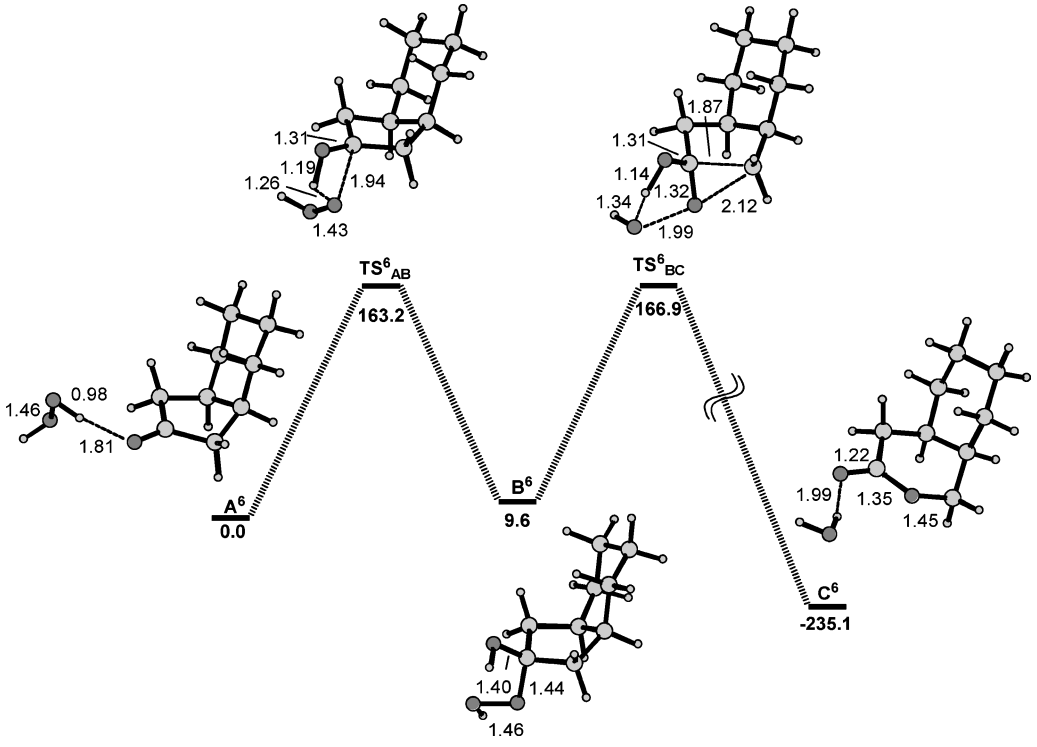


Fig. 2. Energy profile (in kJ/mol) for the transformation of ketone $\mathbf{A^6}$ to lactone $\mathbf{C^6}$ (distances in Å)

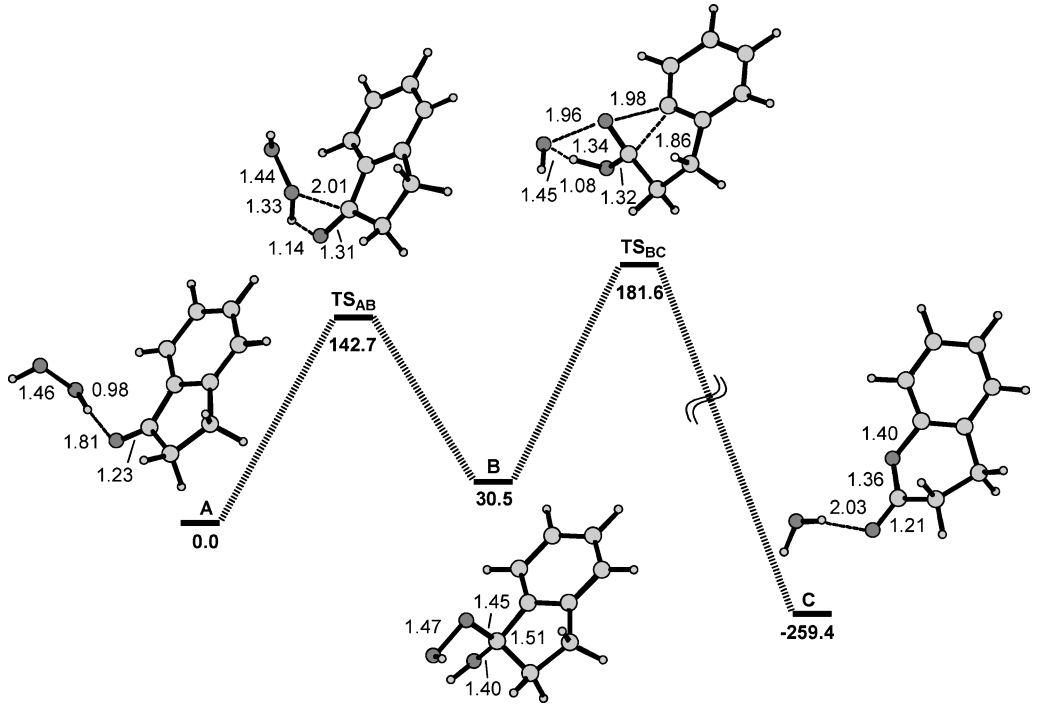


Fig. 3. Energy profile (in kJ/mol) for the transformation of 2,3-dihydroinden-1-one **A** to 3,4-dihydrochromen-2-one **C** (distances in Å)

interesting to note that the five-membered cyclopentanone moiety both in $\mathbf{A^5}$ and $\mathbf{A^6}$ exhibits a significant ring distortion which is changed during the conversion to intermediate $\mathbf{B^5}$ but maintained in the case of $\mathbf{B^6}$. Such structural differences of intermediates might be interpreted to be responsible for the complementary stereochemistry imposed by the two enzymes. However, more elaborate calculations involving the 3-dimensional structure of the proteins are required to further investigate this hypothesis.

Despite these changes in geometry the thermodynamics is very similar in both reactions being slightly endothermic (1.3 and 9.6 kJ/mol). On the other hand, the activation energy for the first step differs markedly suggesting that electronic rather than steric effects may be responsible for the different kinetic behavior. It may be speculated that this reflects a disturbance of the π-conjugation within the cyclopentanone ring.

Previously, CPMO has been demonstrated to convert several indanones to the corresponding lactones [27], while CHMO seems not active enough to facilitate this transformation. In a single case study, kinetic resolution of an α,β-unsaturated cyclopentanone has also been reported for CPMO [28]. The above presented data can also be interpreted in such a way. Again, CHMO requires sufficiently activated ketone substrates, in order to develop its biocatalytic potential. In contrast, CPMO is able to also attack less reactive carbonyl centers as in aromatic or α,β-unsaturated ketones.

Conclusions

Based on the computational model, substantially different electronic requirements within the substrate ketones seem relevant in CHMO and CPMO mediated *Baeyer-Villiger* biooxidations. While this was already indicated by selective acceptance of aromatic and unsaturated substrates, in this work we have also found a class of aliphatic ketones, where a similar effect seems to predominate. Molecular modeling of the *Baeyer-Villiger* oxidation of ketones **1a** and **1c** indicates a substantial difference in activation energy of the initial nucleophilic attack of a peroxo-species, which was not expected in such a magnitude. However, based on the theoretical study, the highly different efficiency of the two enzymes can be better rationalized.

Currently, our model only includes a non-chiral environment and is limited to explain electronic differences for the enzymatic transformation. We are currently trying to develop a more elaborate system, which should ultimately incorporate sterical aspects based on the first 3-dimentional structure of a BVMO reported recently [29].

Experimental

All chemicals and microbial growth media were purchased from commercial suppliers. 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. NMR-spectra were recorded from $CDCl_3$ or *DMSO*-d_6 solutions on a Bruker AC 200 (200 MHz) or Bruker Advance UltraShield 400 (400 MHz) spectrometer and chemical shifts are reported in ppm using *TMS* as internal standard. Enantiomeric excess was determined *via* GC

using a BGB 175 column (30 m × 0.25 mm ID, 0.25 μm film) on a HP 6890 Series chromatograph. Specific rotation $[\alpha]_D^{20}$ was determined using a Perkin Elmer Polarimeter 241 by the following equation: $[\alpha]_D^{20} = 100^*\alpha/(c^*l)$; c/g/100 cm^3, l/dm.

1,3,3a,4,7,7a-Hexahydro-2H-inden-2-one (**1b**)
Diacid **5** (8.25 g, 41.6 mmol [15]) was dissolved in 80 cm^3 Ac_2O and 6.4 cm^3 pyridine were added. The mixture became dark brown upon refluxing for 62 h. The mixture was evaporated *in vacuo* to remove pyridine. After addition of 55 cm^3 0.5 *N* HCl the mixture was refluxed for 1.5 h, cooled to rt, and treated with saturated K_2CO_3 solution until no further CO_2 evolution was observed. The aqueous layer was extracted with 3 × 100 cm^3 Et_2O. The combined organic layers were washed with 2 × 80 cm^3 2 *N* HCl, saturated $NaHCO_3$ solution, brine, dried (Na_2SO_4), and evaporated. The remaining yellow-orange oil was purified by *Kugelrohr* distillation to give 3.44 g **1b** (61%) as colorless oil. Bp 105–108°C/15 mbar; spectral properties agreed to Ref. [23].

cis-Octahydro-2H-inden-2-one (**1c**)
Ketone **1b** (1.00 g, 5.87 mmol) and 0.08 g 10% Pd/C were suspended in 15 cm^3 dry *THF*. The mixture was hydrogenated with H_2 (81 psi) using a *Parr*-apparatus for 24 h. After filtration (Celite®-bed) and evaporation, the slightly yellowish oil was purified by flash column chromatography (20 g SiO_2, LP:*Et*OA*c* = 15:1) to give 0.78 g **1c** (77%) as colorless oil with spectral properties in agreement with Ref. [22].

General Procedure for m-CPBA Oxidation (GP I)
The corresponding ketone was dissolved in dry CH_2Cl_2 (10% solution), *m-CPBA* (1.1–1.3 equiv, 50% chemical grade) was added, and the mixture was stirred overnight at rt until TLC indicated complete conversion. A white precipitate was formed. Excess triethylamine was added and the mixture was stirred for 0.5 h. Then the reaction was hydrolyzed upon addition of H_2O and extracted with CH_2Cl_2. The combined organic layers were washed with 2 *N* HCl, saturated $NaHCO_3$ solution, dried (Na_2SO_4), and the solvent was removed *in vacuo*.

General Procedure for Whole-Cell Biooxidation (GP II)
Fresh LB-ampicillin medium (250 cm^3; LB medium: 1% Bacto-Peptone, 0.5% Bacto-Yeast Extract, 1% NaCl in deion H_2O, supplemented by 200 μg/cm^3 ampicillin) was inoculated with a 2.5 cm^3 aliquot of an overnight preculture of the corresponding expression strain for CHMO or CPMO in a 1000 cm^3 baffled *Erlenmeyer* flask. The culture was shaken at 120 rpm at 37°C until it reached an optical density at 600 nm (OD_{600}) between 0.2 and 0.4, then isopropylthio-β-*D*-galactoside (*IPTG*) was added to a final concentration of 0.025 m*M*. The substrate was added neat and 1 equiv β-cyclodextrin was supplemented if required. The culture was shaken at 150 rpm at rt. Conversion was monitored by GC and reached completion between 18 and 36 h. Then, the biomass was separated by centrifugation (3500 rpm, 10 min) and the supernatant was filtered through a pad of Celite®. The clear solution was extracted with *Et*OA*c*, the combined organic layers were dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography.

cis-Hexahydrocyclopenta[c]pyran-3(1H)-one (**2a**)
Conversion of **1a** (0.50 g, 4.00 mmol) with *m-CPBA* according to GP I gave 0.28 g racemic **2a** (50%) as colorless liquid after flash column chromatography (LP:*Et*OA*c* = 2:1).

Biotransformation of 0.53 g **1a** (4.27 mmol) according to GP II with CHMO expressing recombinant *E.coli* gave 0.30 g (−)-**2a** (50%) after flash column chromatography (LP:*Et*OA*c* = 2:1) as colorless liquid with physical properties according to Table 1.

Biotransformation of 106 mg **1a** (0.84 mmol) according to GP II with CPMO expressing recombinant *E.coli* gave 106 mg (+)-**2a** (89%) after flash column chromatography (LP:*Et*OA*c* = 2:1) as colorless liquid with physical properties according to Table 1.

^{1}H NMR ($CDCl_3$): $\delta = 1.20–2.08$ (m, 6H), 2.25–2.69 (m, 4H), 4.00, 4.37 (2dd, $J = 7$, 20 Hz, 2H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 25.0$ (t), 28.9 (t), 34.2 (t), 34.3 (t), 34.4 (d), 36.3 (d), 67.4 (t), 173.4 (s) ppm.

1,4,4a,5,8,8a-Hexahydro-3H-2-benzopyran-3-one (**2b**)
Biotransformation of 106 mg **1b** (0.78 mmol) according to GP II with CHMO expressing recombinant *E.coli* followed by flash column chromatography (LP:*Et*OAc = 10:1) resulted in recovery of 38 mg **1b** (36%) and afforded 40 mg (4a*S*,8a*S*)-(−)-**2b** (33%; 85% based on consumed and unrecovered starting material) as colorless oil with physical properties according to Table 1.

Biotransformation of 100 mg **1b** (0.73 mmol) according to GP II with CPMO expressing recombinant *E.coli* gave 91 mg (4a*R*,8a*R*)-(+)-**2b** (76%) after flash column chromatography (LP:*Et*OAc = 10:1) as colorless oil with physical properties according to Table 1.

^{1}H NMR ($CDCl_3$): $\delta = 1.80–2.13$ (m, 2H), 2.16–2.44 (m, 4H), 2.50–2.60 (m, 2H), 4.22–4.40 (m, 2H), 5.67 (bs, 2H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 24.0$ (t), 28.4 (t), 28.5 (d), 29.6 (d), 33.7 (t), 72.1 (t), 124.1 (d), 124.6 (d), 170.6 (s) ppm.

cis-Octahydro-3H-2-benzopyran-3-one (**2c**, $C_9H_{14}O_2$)
Ketone **1c** (200 mg, 1.44 mmol) was oxidized according to GP I to give 109 mg **2c** (49%) as colorless oil after flash column chromatography (LP:*Et*OAc = 20:1).

Biooxidation of 106 mg **1c** (0.70 mmol) according to GP II with CHMO expressing recombinant *E.coli* followed by flash column chromatography (LP:*Et*OAc = 10:1) resulted in recovery of 40 mg **1c** (40%) and afforded 23 mg (4a*S*,8a*S*)-(−)-**2c** (21%; 65% based on consumed and unrecovered starting material) as colorless oil with physical properties according to Table 1.

Biotransformation of 106 mg **1c** (0.70 mmol) according to GP II with CPMO expressing recombinant *E.coli* gave 100 mg (4a*R*,8a*R*)-(+)-**2c** (83%) after flash column chromatography (LP:*Et*OAc = 10:1) as colorless oil with physical properties according to Table 1.

^{1}H NMR ($CDCl_3$): $\delta = 1.22–1.63$ (m, 8H), 1.83–2.01 (m, 1H), 2.05–2.25 (m, 1H), 2.40–2.55 (m, 2H), 4.25 (d, 2H, $J = 8$ Hz) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 21.5$ (t), 23.3 (t), 24.6 (t), 28.6 (t), 31.0 (d), 32.7 (d), 32.8 (t), 72.4 (t), 171.1 (s) ppm.

Computational Details
All calculations were performed using the Gaussian98 software package on the Silicon Graphics Origin 2000 of Vienna University of Technology [30]. The geometry and energy of the model compounds and the transition states were optimized at the B3LYP level [31]. For C, O, and H atoms the 6–31 g** basis set was employed [32]. A vibrational analysis was performed to confirm that the structures of the model compounds have no imaginary frequency. Transition state optimizations were performed with the Synchronous Transit-Guided Quasi-*Newton* Method (STQN) developed by *Schlegel et al.* [33]. Frequency calculations were performed to confirm the nature of the stationary points, yielding one imaginary frequency for the transition states and none for the minima. The vibrational eigenvectors corresponding to the reaction coordinate (with imaginary frequency) of all transition states were visually checked to confirm the connectivity of transition states with the reactants and the products. All geometries were optimized without symmetry constraints.

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