Synthesis and Enantioselective Baeyer–Villiger Oxidation of Prochiral Perhydro-pyranones with Recombinant *E. coli* Producing *Cyclohexanone Monooxygenase*

Marko D. Mihovilovic,*[a] Florian Rudroff,[a] Wolfgang Kandioller,[a] Birgit Grötzl,[a] Peter Stanetty,[a] Helmut Spreitzer[b]

[a] Vienna University of Technology, Institute for Applied Synthetic Chemistry, Getreidemarkt 9/163, 1060 Vienna, Austria
Fax +43(1)5880115499; E-mail: mmihovil@pop.tuwien.ac.at

[b] Vienna University, Institute of Pharmaceutical Chemistry, Vienna, Austria

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Abstract: Recombinant whole cells of *Escherichia coli* overexpressing *Acinetobacter* sp. NCIMB 9871 cyclohexanone monooxygenase (E.C. 1.14.13.22) have been utilized for the Baeyer–Villiger oxidation of prochiral perhydro-pyranones. The spatial limitations of the enzyme's active site have been estimated by increasing the chain length of *cis*-substituents in positions 2 and 6. A diastereoselective synthetic sequence to the required substrate ketones has been developed utilizing high pressure hydrogenation.

Key words: biocatalysis, recombinant whole-cell biotransformation, cyclohexanone monooxygenase, Baeyer–Villiger oxidation, enantioselectivity

Enzyme mediated Baeyer–Villiger reactions offer an environmentally benign entry to chiral lactones as important building blocks in synthetic chemistry.[1–6] To date, a significant number of organisms has been identified to utilize such an oxygen insertion process in their metabolic pathways.[7–9] Several genes encoding such flavin dependent Baeyer–Villiger monooxygenases (BVMOs) have been characterized.[10–12] Since most of these biocatalysts accept a multitude of non-natural substrates, this offers the option, to selectively overexpress the enzymes for protein isolation purposes or direct whole-cell mediated biotransformations.

Our approach towards the production of chiral lactones utilizes such engineered cells. This offers the advantage to circumvent the sometimes tedious process of protein purification and also simplifies the required cofactor recycling. BVMOs catalyze the Baeyer–Villiger oxidation by utilizing molecular oxygen as oxidant, whereas one O atom is incorporated into the substrate while the second is reduced to water at the expense of a reducing cofactor. As a consequence, this enzyme family is dependent on NAD(P)H, which has to be recycled to facilitate an efficient and economical biotransformation. The simplest way to achieve this goal is the utilization of living whole cells, in which all natural cofactor recycling systems are still intact.[13]

A disadvantage of using native cells is the low concentration of the required biocatalyst in the total proteome of the organism. Hence, undesired side reactions can compromise both yield and enantiomeric excess of the required product. To overcome this problem, we have recently developed an *E. coli* based overexpression system[14,15] for cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 (E.C. 1.14.13.22)[16] as the best studied BVMO so far.[17] In this engineered strain the production of CHMO is significantly increased and such unwanted side reactions are suppressed. We have demonstrated the efficiency of this CHMO producer for the production of chiral compounds[18] and contributed to the active site model, previously.[19] More recently we have proven the power of recombinant whole cell mediated Baeyer–Villiger oxidations by identifying pairs of enantiodivergent BVMOs originating from different microorganisms to allow access to both antipodal lactones.[20–22]

We demonstrated that CHMO can convert heterocyclic cores such as 4-piperidones, perhydro-4-pyranones, and perhydro-4-thiopyranones in addition to carbocyclic substrates, which have been studied extensively, in high chemoselectivity and good isolated yields.[23] In this contribution we expand these substrate acceptance studies on prochiral heterocyclic ketones and present the first enantioselective Baeyer–Villiger oxidation of heterocyclic substrate ketones by recombinant cells. By this strategy, it is possible to generate a set of new chiral centers in an asymmetrization step with a theoretical yield of 100% in contrast to kinetic resolutions, which are limited to 50% yield of the required antipode.[24] Based on our previous results, we selected the perhydro-4-pyranone system for a first model study, since this system gave the best conversion rates and isolated yields when studying unsubstituted hetero-ketones. A series of *cis*-2,6-dialkyl-perhydropyrans **1** with increasing chain length for the substituents was oxidized to the corresponding lactones **2** to investigate spatial requirements of the active site of CHMO to accommodate such substrates (Scheme 1). The refinement of active site models for BVMOs[25–30] exclusively depends on such substrate acceptance profiles, since crystallographic data for the structure of this enzyme family is not available, to date.

A number of methods has been reported previously to access 2,6-disubstituted γ-pyrones **5** as key precursors for the required ketones **1**.[31,32] We expanded a strategy

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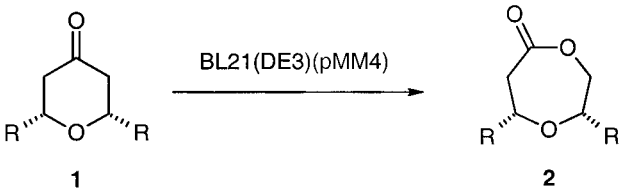


Scheme 1 Whole-cell biotransformations of prochiral perhydro-4-pyranones.

utilizing the magnesium-diacetonedicarboxylate complex **3** for an array of different substituents in the 2 and 6-positions (Scheme 2).[33] Initial cyclization leads to the corresponding 3,5-dicarboxylic acid esters **4** followed by hydrolytic decarboxylation.[34] Diastereoselective reduction was accomplished by high pressure hydrogenation with Pd/C in MeOH. High selectivity for the direct formation of thermodynamically favored *cis*-products is only ensured at 20 bar or above, while hydrogenation in a Parr-apparatus at approximately 5bar gave mixtures of partially reduced compounds in contrast to previous reports in the literature.[35]

It is interesting to note that in several cases ketals **6** were formed upon high-pressure hydrogenation. These products were only isolated as crude material and directly cleaved to give the required ketones **1**. The overall sequence allowed efficient access to an array of C_1 to C_4 substituted straight chain and branched perhydro-4-pyranones **1a**–**e** as probes to investigate the tolerance of the enzyme's active site for sterically demanding residues.[36]

Protein expression of CHMO in growing cultures of recombinant strain *BL21*(*DE3*)(*pMM4*) was triggered by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Fermentations were carried out according to our previously reported procedure on 100 mg scale in Erlenmeyer flasks[21,37] and β-cyclodextrin was added to facilitate membrane penetration.[38] For reference purposes, racemic lactones **2** were prepared by chemical oxidation of ketones **1** with MCPBA.

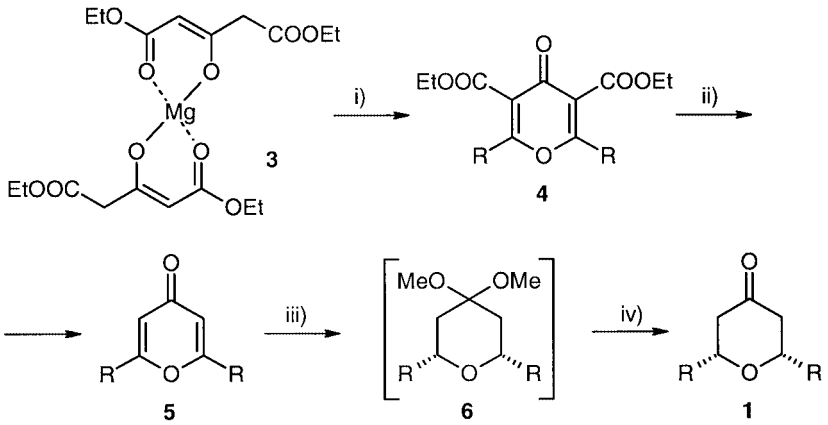
Results of the biotransformations of substrates **1a**–**e** with whole-cells of *BL21*(*DE3*)(*pMM4*) are summarized in Table 1. The size of substituents R had a substantial influence on the conversion of the substrates: While small straight chain groups such as methyl and ethyl were oxidized in excellent yield by the whole-cells, we observed a significantly decreased conversion for the *n*-propyl ketone **1c**. A straight chain with 3 carbons seems to represent the borderline with respect to steric demands of the substrate in the active site. Both branched substituents (*i*-propyl, **1d**) or longer chains (butyl, **1e**) seem to exceed the spatial limits imposed by the enzyme. These compounds are not (or only very poor in the case of **1d**) substrates for CHMO in preparative whole-cell biotransformations.

Table 1 Recombinant Whole-Cell Biotransformations of 2,6-Disubstitued Perhydro-4-pyranones

Substrate	R	Product	Yield[a]	ee[b]	$[\alpha]_D^{20}$
1a	Me	**2a**	80%	>99.5%	–50.6 (*c* 1.25, $CHCl_3$)
1b	Et	**2b**	90%	>99.5%[c]	–4.3 (*c* 1.32, $CHCl_3$)
1c	*n*-Pr	**2c**	19%	98%	–22.5 (*c* 1.0, $CHCl_3$)
1d	*i*-Pr	**2d**	traces	n.d.	n.d.
1e	Bu	**2e**	no conv.	n.a.	n.a.

[a] Isolated yield after chromatographic purification.
[b] Ee determined by chiral gas phase chromatography; racemic reference material prepared by MCPBA oxidation of ketones **1**.
[c] Ee determined by chiral phase gas chromatography after reduction of lactone **2b** with $LiAlH_4$ to the corresponding diol.

For those ketones, which can be accommodated by the active site of CHMO (**1a**–**c**), the biooxidation generates two new stereogenic centers and proceeds with excellent enantioselectivity. Assignment of the absolute configuration is based on previous results on similar carbocyclic systems by Taschner and coworkers.[39] Assuming the same configuration for lactones **2**, which is supported by



Scheme 2 (i) RCOCl/Et_3N or $(RCO)_2O$, 32–59%; (ii) AcOH, HCl, H_2O, heat, 53–87%; (iii) 20 bar H_2, Pd/C, MeOH; (iv) 2N HCl, THF, r.t., 35–57% (2 steps).

the same sign of specific rotation, and considering the change of priorities according to the Cahn–Ingold–Prelog rules, the absolute configuration of fermentation products **2a–c** can be assigned as 2*S*,7*R*.[40]

In summary, we have developed a diastereoselective route to substituted hetero-ketones **1** as substrates for whole-cell biotransformations. The compounds serve as probes to determine spatial requirements by the enzyme and we demonstrated, that straight chain substitution up to C_3 is accepted by the recombinant whole-cell expression system for the production of chiral lactones **2**.

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(36) Typical procedure for the high pressure hydrogenation: Precursor **5** dissolved in anhyd MeOH was hydrogenated with Pd/C (10%, 300 mg) in a Büchi steel autoclave under H_2 atmosphere (20 bar) for 2 d. The solution was filtered through a bed of Celite® and MeOH was evaporated. In the case of partial ketal formation (**6**), the crude material was treated with a 5:1 mixture of THF and 0.1 N HCl at r.t. overnight. The solution was washed with $NaHCO_3$, extracted with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated in vacuo. Pure **1** was obtained after Kugelrohr distillation or flash column chromatography.
***cis*-Tetrahydro-2,6-dimethyl-4*H*-pyran-4-one (1a):** 42% yield, colorless liquid, bp 50 °C/12mbar (Kugelrohr),
^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.35 (d, *J* = 6 Hz, 6 H), 2.10–2.45 (m, 4 H), 3.61–3.80 (m, 2 H),
^{13}C NMR(50 MHz, $CDCl_3$): δ = 21.9 (q), 48.8 (t), 72.9 (d), 207.2 (s).
***cis*-Tetrahydro-2,6-diethyl-4*H*-pyran-4-one (1b):** 57% yield, colorless liquid, bp 81–83 °C/11mbar (Kugelrohr).
^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.00 (t, *J* = 7 Hz, 6 H), 1.43–1.81 (m, 4 H), 2.11–2.45 (m, 4 H), 3.39–3.55 (m, 2 H).
^{13}C NMR (50 MHz, $CDCl_3$): δ = 9.6 (q), 29.3 (t), 47.5 (t), 78.2 (d), 207.7 (s).
***cis*-Tetrahydro-2,6-dipropyl-4*H*-pyran-4-one (1c):** 56% yield, beige oil.
^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.90 (t, *J* = 6 Hz, 6 H), 1.30–1.80 (m, 8 H), 2.15–2.40 (m, 4 H), 3.60–3.75 (m, 2 H).
^{13}C NMR (50M Hz, $CDCl_3$): δ = 14.3 (q), 19.0 (t), 38.9 (t), 48.4 (t), 77.1 (d), 207.9 (s).
***cis*-Tetrahydro-2,6-bis-(1-methylethyl)-4*H*-pyran-4-one (1d):** 71% yield, colorless oil, bp: 90–95 °C/0.1 mbar (Kugelrohr).
^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.90, 0.95 (2 × d, *J* = 6 Hz,

2 × 6 H), 1.75 (oct, *J* = 6 Hz, 2 H), 2.10–2.45 (m, 4 H), 3.20–3.30 (m, 2 H).
^{13}C NMR (50 MHz, $CDCl_3$): δ = 18.2 (t), 33.4 (d), 45.3 (t), 81.7 (d), 208.9 (s).
***cis*-Tetrahydro-2,6-dibutyl-4*H*-pyran-4-one (1e):** 35% yield, beige oil.
^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.90–1.00 (m, 6 H), 1.20–1.80 (m, 12 H), 2.10–2.40 (m, 4 H), 3.40–3.55 (m, 2 H).
^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.4 (q), 22.9 (t), 27.9 (t), 36.5 (t), 48.8 (t), 77.4 (d), 208.4 (s).

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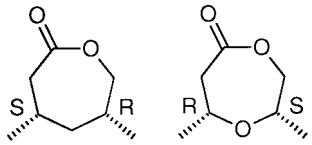

Figure 1

(40) Physical and spectroscopic data of lactones **2**:
***cis*-2,7-Dimethyl-1,4-dioxepan-5-one (2a):** colorless oil.
^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.18 (d, *J* = 6 Hz, 3 H), 1.29 (d, *J* = 6 Hz, 3 H), 2.67 (dd, *J* = 14 Hz, ca 1 Hz, 1 H), 2.92 (dd, *J* = 14 Hz, 5 Hz, 1 H), 3.79–3.99 (m, 2 H), 4.02 (dd, *J* = 13 Hz, ca 1 Hz, 1 H), 4.20 (dd, *J* = 14 Hz, 6 Hz, 1 H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.6 (q), 23.5 (q), 45.6 (t), 71.0 (d), 74.4 (t). 75.5 (d), 173.8 (s).
***cis*-2,7-Diethyl-1,4-dioxepan-5-one (2b):** colorless oil.
^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.99 (t, *J* = 9 Hz, 3 H), 1.00 (t, *J* = 9 Hz, 3 H), 1.40–1.75 (m, 4 H), 2.69 (dd, *J* = 16 Hz, ca 1 Hz, 1 H), 2.90 (dd, *J* = 16 Hz, 10 Hz, 1 H), 3.50–3.67 (m, 2 H), 4.08 (dd, *J* = 13 Hz, ca 1 Hz, 1 H), 4.21 (dd, *J* = 13 Hz, 6 Hz, 1 H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 9.8 (q), 9.9 (q), 25.5 (t), 30.0 (t), 43.8 (t), 73.4 (t), 75.5 (d), 80.3 (d), 173.6 (s).
***cis*-2,7-Dipropyl-1,4-dioxepan-5-one (2c):** beige colored oil.
^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.90 (t, *J* = 7 Hz, 6 H), 1.20–1.60 (m, 8 H), 2.65 (d, *J* = 13 Hz, 1 H), 2.90 (dd, *J* = 13 Hz, 8 Hz, 1 H), 3.55–3.70 (m, 4 H), 4.05 (d, *J* = 13 Hz, 1 H) 4.25 (dd, *J* = 13 Hz, 8 Hz, 1 H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.6 (q), 18.6 (t), 34.2 (t), 38.9 (t), 44.2 (t), 73.7 (t), 73.9 (d), 78.7 (d), 173.5 (s).