

Synthesis of chiral ϵ -lactones in a two-enzyme system of cyclohexanone mono-oxygenase and formate dehydrogenase with integrated bubble-free aeration[†]

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Abstract: A two-enzyme system consisting of a cyclohexanone mono-oxygenase from *Acinetobacter* NCIMB 9871 and a protein-engineered formate dehydrogenase for the regeneration of the cofactor NADPH was used for the synthesis of chiral ϵ -lactones. 4-Methylcyclohexanone was used as the model substrate yielding (*S*)-(-)-5-methyl-oxepane-2-one with high chemical and enantiomeric purity. Syntheses were carried out in a repetitive-batch reactor with integrated bubble-free aeration by means of a thin-walled silicon tube. © 1997 Elsevier Science Ltd

Asymmetric Baeyer–Villiger oxidations of prochiral ketones yield chiral lactones which are interesting intermediates for the synthesis of natural products (see for example, Adger *et al.*¹). Several microbiological and enzymatic methods² have been described as well as chemical syntheses.^{3,4} *Acinetobacter* NCIMB 9871 and the cyclohexanone mono-oxygenase (CHMO) [EC 1.14.13.22]⁵ isolated from this strain have received most attention and have been successfully employed for the Baeyer–Villiger oxidation of substituted cyclohexanones.^{6–10} Cyclohexanone mono-oxygenase can easily be isolated and possesses a relatively high stability. It requires molecular oxygen and NADPH as a cofactor. Until now, cofactor regeneration for NADPH-dependent mono-oxygenases has mostly been realised using glucose-6-phosphate/glucose-6-phosphate dehydrogenase.¹¹ However, this method suffers from the application of stoichiometric amounts of the expensive substrate glucose-6-phosphate and, therefore, it is not applicable for syntheses of chiral lactones on a large scale.

The recently described use of a NADP⁺-dependent formate dehydrogenase allows an elegant, efficient and cheap method for the regeneration of the expensive cofactor NADPH.^{12,13} The enzyme was obtained by multiple site-directed mutagenesis of the relevant gene from *Pseudomonas* sp. 101 and uses formate as substrate.

For process development, 4-methylcyclohexanone **1** was chosen as the model substrate. It was oxidised by CHMO yielding 5-methyl-oxepane-2-one **2**, which contains a new stereocentre at C-5 (Figure 1).⁷ Initial experiments with the two-enzyme system described above yielded only moderate conversions in gently stirred solutions due to oxygen delivery being the limiting factor. Only 1.18 mmol L⁻¹ of molecular oxygen is soluble under the conditions used.¹⁴ Standard methods of oxygen feeding by bubbling of air or intensive stirring are not applicable due to the deactivation of the enzymes caused by gas–liquid interfaces. In order to avoid this problem a thin-walled silicon tube was used for aeration. This technique of bubble-free aeration has been successfully used, for example in cell

[†] Dedicated to Professor Dr. P. Welzel on the occasion of his 60th birthday.

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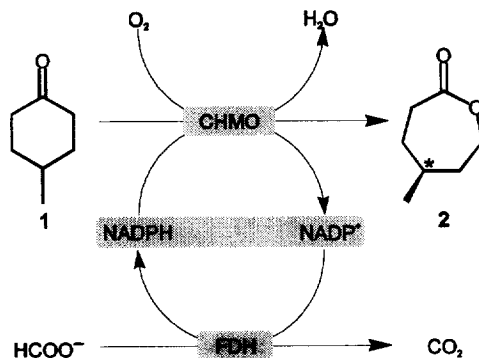


Figure 1. Enzymatic Baeyer-Villiger oxidation with enzyme-coupled cofactor regeneration.

culture technology.^{15,16} A scheme of the reactor used is shown in Figure 2. Conversions were carried out typically on a 30 mL scale under conditions guaranteeing the maximum activity of both CHMO and FDH. The stability of CHMO is drastically enhanced by the presence of NADPH. Therefore a twofold excess of FDH was used in order to ensure a sufficiently high NADPH concentration. Ketone concentrations of more than 40 mmol L⁻¹ significantly reduce the stability of cyclohexanone mono-oxygenase, so this concentration was used in all experiments. At a cofactor concentration of 0.25 mmol L⁻¹ a maximum total turnover number (ttn) of 160 could be reached. Further decrease of the cofactor concentration far below the K_M value of FDH at 0.4 mmol L⁻¹ is not reasonable even if the amount of FDH is not the limiting factor in our reaction. The reaction temperature was set to 30°C as a compromise between enzyme activity and stability of CHMO. A pH of 8.0 permits good activity of CHMO combined with still acceptable activity of FDH.¹⁷

Due to the sufficiently high stability of the enzymes it was possible to carry out the reaction in a repetitive batch mode using ultrafiltration to separate the enzymes after complete conversion of 1 and reusing them by adding fresh reaction solution.¹⁸ Figure 3 shows the conversion-time curve of a typical experiment. Even for the third batch no decrease of activity was observed. Product 2 could be extracted with high purity (>99%, GC) and with high enantiomeric excess (>99%, GC).

Further enhancement of total turnover number of the cofactor should be possible using an enzyme-membrane reactor with integrated bubble-free aeration. The use of a nanofiltration membrane would allow an at least partial retention of the cofactor.¹⁹

In conclusion, we describe here an efficient method for cofactor regeneration combining an NADPH-dependent mono-oxygenase and a formate dehydrogenase.²⁰ This permits the conversion of substituted

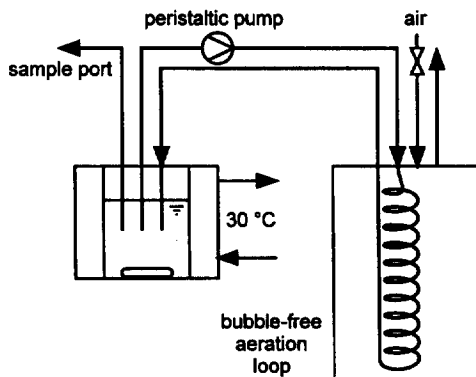


Figure 2. Reactor set-up with integrated bubble-free aeration.

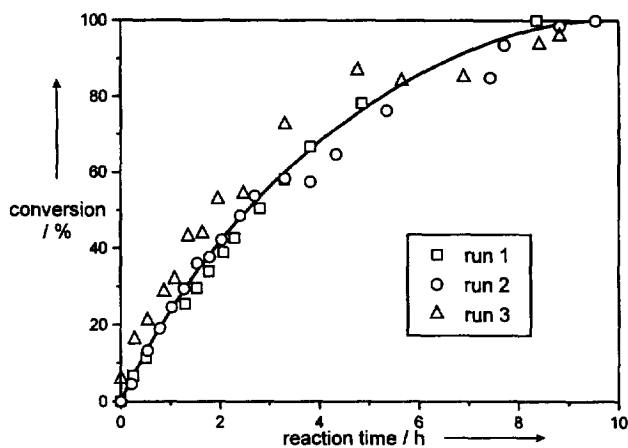


Figure 3. Conversion–time curve of the synthesis of **2** in three consecutive batches.

cyclohexanones to the corresponding ϵ -lactones on a preparative scale. (*S*)-(-)-5-Methyl-oxepane-2-one **2** could be obtained with high chemical and enantiomeric purity from the reaction mixture by simple extraction.

Representative procedure for the synthesis of **2** in a repetitive batch

4.7 U CHMO (0.16 U mL⁻¹) and 9.6 U FDH (0.32 U mL⁻¹) are added to 30 mL of a solution containing 40 mmol L⁻¹ **1** (1.2 mmol), 0.25 mmol L⁻¹ NADP⁺ (7.5 μ mol) and 200 mmol L⁻¹ sodium formate in 50 mmol L⁻¹ Tris/HCl-buffer, pH 8.0. The mixture is kept at 30°C and stirred gently. The solution is aerated by pumping it through a thin-walled silicon tube (5 mm i.d. \times 40 cm) at a rate of 450 mL h⁻¹ with an exterior air flow of about 60 L h⁻¹. After complete conversion the reaction mixture is concentrated to 2 mL through an Amicon YM 30 ultrafiltration membrane, the concentrate is washed twice with 4 mL of buffer, and finally added to fresh substrate solution. The product is recovered from the aqueous filtrate by fourfold extraction with 20 mL portions of ethyl acetate, subsequently dried with sodium sulfate, and the solvent evaporated *in vacuo*. The specific rotation of the product was determined to be $[\alpha]_D^{25} = -44$ (*c* 1.0, CHCl₃), which was consistent with the literature value.⁷ GC analysis (FS-Cyclodex β I/p (CS, Langerwehe) isothermal at 130°C, 1.8 bar H₂, FID) yielded an *ee* > 99%.

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