Macrocyclic Baeyer-Villiger monooxygenase oxidation of cyclopentadecanone on 1 L scale

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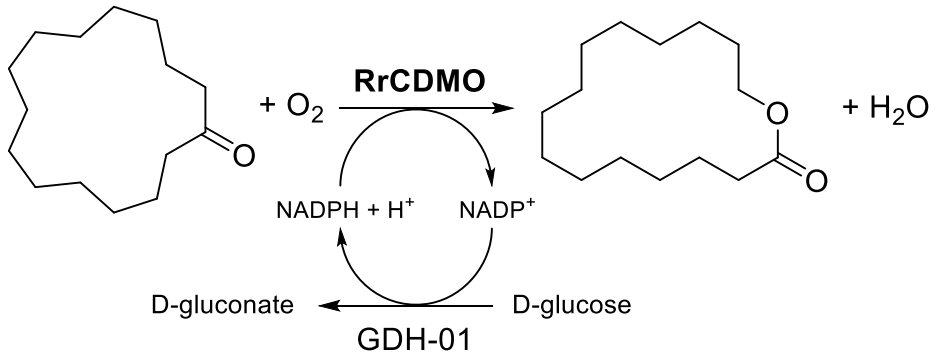
The enzyme catalysed Baeyer-Villiger oxidation of (cyclic) ketones into the corresponding esters and lactones is of interest because of the usually very high enantio- and regio-selectivity of Baeyer-Villiger Monooxygenases (BVMOs).[1] Even for unsubstituted cyclic ketones oxidation to the corresponding lactones by BVMOs is attractive because the use of harmful chemical oxidants as in chemical BVO can be avoided and no undesired regio-isomers as in the hydroxylation of fatty acids with cytochrome P450s can be formed.
In this contribution we describe the Baeyer-Villiger oxidation of cyclopentadecanone (CPD) to pentadecanolide (PDL) catalysed by Cyclododecanone Monooxygenase from *Rhodococcus ruber* SC1 (RrCDMO)[2] on 1 L scale with pure oxygen as oxidant and glucose dehydrogenease as auxiliary enzyme for regeneration of NADPH using glucose as sacrificial substrate (**Scheme 1**).



Scheme 1: Baeyer-Villiger Monooxygenase (BVMO) catalysed oxidation of cyclopentadecanone to pentadecanolide using O_2 as oxidant and NADPH as reduction equivlant producing H_2O as byproduct. NADPH was recycled using Glucose Dehydrogenase (GDH) and D-glucose as sacrificial substrate.

Key to the reaction optimisation towards synthetically relevant substrate and product concentrations and productivities was the application of methanol as cosolvent of the almost water insoluble cyclopentadecanone (5.3 $mg.L^{-1}$ or 0.024 $mmol.L^{-1}$ at 20 °C in 50 mM potassium phosphate buffer, pH 8.0). Up to 25 % (v/v) of methanol could be applied in the target reaction to solubilse the substrate (24.9 $mg.L^{-1}$ or 0.111 $mmol.L^{-1}$), because the employed RrCDMO was highly stable in a 25 % methanol/75 % aqueous potassium phosphate buffer and retained 95 % residual activity when stored for 7 days at 20 °C. Both enzymes, RrCDMO and GDH-01, were produced in fed-batch fermentations of recombinant *Escherichia coli* strains (data not shown, commercially available from InnoSyn) and applied as liquid enzyme formulations.

For the process development from 1 L scale onwards a controlled oxygen supply (eventually as compressed pure O_2 from a gas bottle) and the determination of oxygen dissolved in the reaction mix and in the off-gas were essential. The reactor (Figure 1) contained a frit to introduce the pressurized oxygen. The oxygen concentration was continuously monitored both in the liquid phase and in the gas outlet and nitrogen (100 $mL.min^{-1}$) was blown in the headspace to keep the oxygen concentration below 8 % (v/v). The oxygen measurements enabled certain oxygen control from the operator assuring that oxygen was always available in the reactor. Auto titration with 5 M NaOH was applied to compensate for the gluconate formed by the NADPH regeneration system and to keep the pH stable at 7.5.

Procedure 1: Biocatalytic conversion of cyclopentadecanone to pentadecanolide

Materials and Equipment

- Cyclopentadecanone was a generous gift from Givaudan Schweiz AG
- RrCDMO; Liquid enzyme formulation (commercially available from Innosyn)
- GDH-01; Liquid enzyme formulation (commercially available from Innosyn)
- Potassium phosphate buffer pH 7.5, 100 mM
- Nicotinamide dinucleotide phosphate disodium salt ($NADP^+$)
- Methanol
- 5 M Aqueous sodium hydroxide
- 1 L Reactor set-up
- Top stirrer
- pH Electrode
- Reflux condenser
- Oxygen sensor in reaction mixture
- Oxygen sensor in gas outlet
- Mass flow meters for controlled oxygen, air and nitrogen supply
- Gas inlet (frit)
- Oxygen
- Nitrogen
- Automatic titration device connected to pH electrode
- Thermostat for heating up the reaction
- Cryostat for cooling the condenser
- Dicalite 4208
- Glass filter (P3)
- Separation funnel (1 L)
- Agilent 7693 GC System with a PTV (Programmed Temperature Vaporization) and FID detection
- GC column: Rtx 5 Sil MS (30 m x 0.25 mm 0.50 µm)

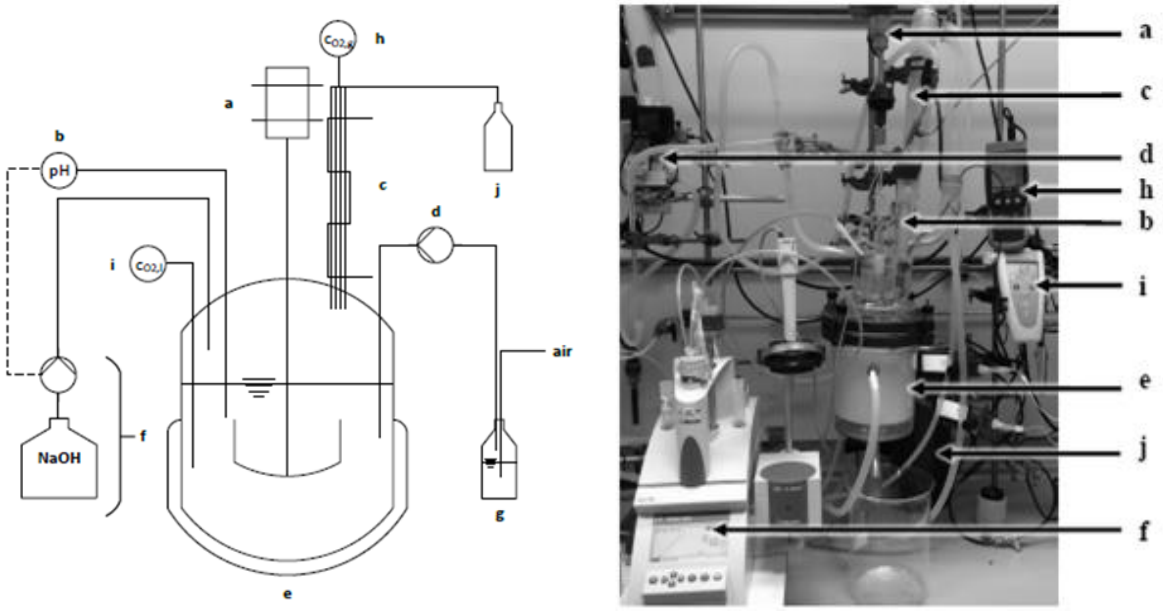


Figure 1: 1 L scale reactor set-up used for the Baeyer-Villiger oxidation of cyclopentadecanone to pentadecanolide by RrCDMO. Left scheme, right photo of the set-up. a) stirrer, b) pH electrode, c) reflux-condenser, d) controlled gas supply, e) reactor, f) automatic titration device connected to pH electrode, g) gas washing bottle filled with water, h) oxygen sensor in gas outlet, i) oxygen sensor in reaction mixture, j) cooling trap. Not in the picture: compressed O_2 bottle and gas flow controller.

Procedure

1. The reactor jacket temperature was set to 30 °C
2. Potassium phosphate buffer pH 7.5, 100 mM (100 mL) was charged to the reactor
3. Water was added (666 mL)
4. $NADP^+$ disodium salt was added (400 mg)
5. The stirrer speed was set at 200 rpm
6. RrCDMO was added (180 mL liquid enzyme formulation)
7. GDH-01 was added (2 mL liquid enzyme formulation)
8. The stirrer speed was set at 400 rpm
9. The automatic titration device was set at pH 7.5 (titration via 5 M NaOH)
10. The oxygen flow rate was set at 25 $mL.min^{-1}$ (and was decreased to 10 $mL.min^{-1}$ over the course of the reaction)
11. Cyclopenatadecanone (52 g, 231 mmol) dissolved in methanol was added in 9 portions (initially 10 g and ca. 5 g portions over time) at regular intervals over 5.5 h
12. Samples were taken and analyzed by GC to follow the reaction progress
13. After 8 h the cyclopentadecanone conversion was > 95 %.
14. The stirrer speed was set at 200 rpm
15. *n*-Heptane (500 mL) was added
16. The reactor was heated up to 75 °C for 1 h
17. 25 g Dicalite 4208 and 25 g sodium sulfate were added as filter aid
18. After 30 min stirring, the mixture was filtered over a glass filter (P3) containing a pre-coat of Dicalite 4208

19. The filter cake was washed 2 times with *n*-heptane (250 mL) at 75 °C
20. The filtrate was separated into the organic product layer and a water layer
21. From the organic product layer *n*-heptane was evaporated under vacuum at ca. 60°C
22. The overall PDL yield was of 47 g or 90.7 % in the product oil of 98 % chemical purity.
23. The determined product losses in the extraction step (1.2%), the filtration step (1.8%) and the solvent evaporation step (<0.1%) were low.

Analysis methods

Samples were taken from the reactor while stirring. The sample amount was weighed (~200 mg) and diluted 10x with acetonitrile containing naphthalene as internal standard (1.0-1.5 $mg.mL^{-1}$) and weighed again. The mixture was shaken and centrifuged. The clear supernatant was analysed. The retention times of CPD and PDL were 13.2 and 13.4 min, respectively (naphthalene 5.4 min).

GC parameters

Temperature program:

Initial temperature:	150 °C
Hold 1:	1 min
Rate 1:	10 $°C.min^{-1}$
Temperature:	290 °C
Hold 2:	2 min
Injector temperature:	280 °C
Detector temperature:	280 °C
Analysis time:	17 min

Conclusion

Dosing the CPD substrate dissolved in methanol in portions to the reaction over time and keeping the maximum methanol concentration below 25 % (v/v) enabled an almost complete substrate conversion of 230 mM CPD within 8 h reaction time (**Figure 2**). The mass balance was ≥90 % throughout the whole reaction despite a challenging sampling of the reaction mixture. In the beginning of the reaction the base consumption (titrating the gluconic acid from the NADPH cofactor regeneration) was well in line with the substrate dosing and the product formation demonstrating a coupling efficiency of the BVMO reaction, while after about 6-7 h when the BVMO reaction stopped, titration continued at a lower rate indicating NADPH consuming or other acid producing background reactions (**Figure 2**).

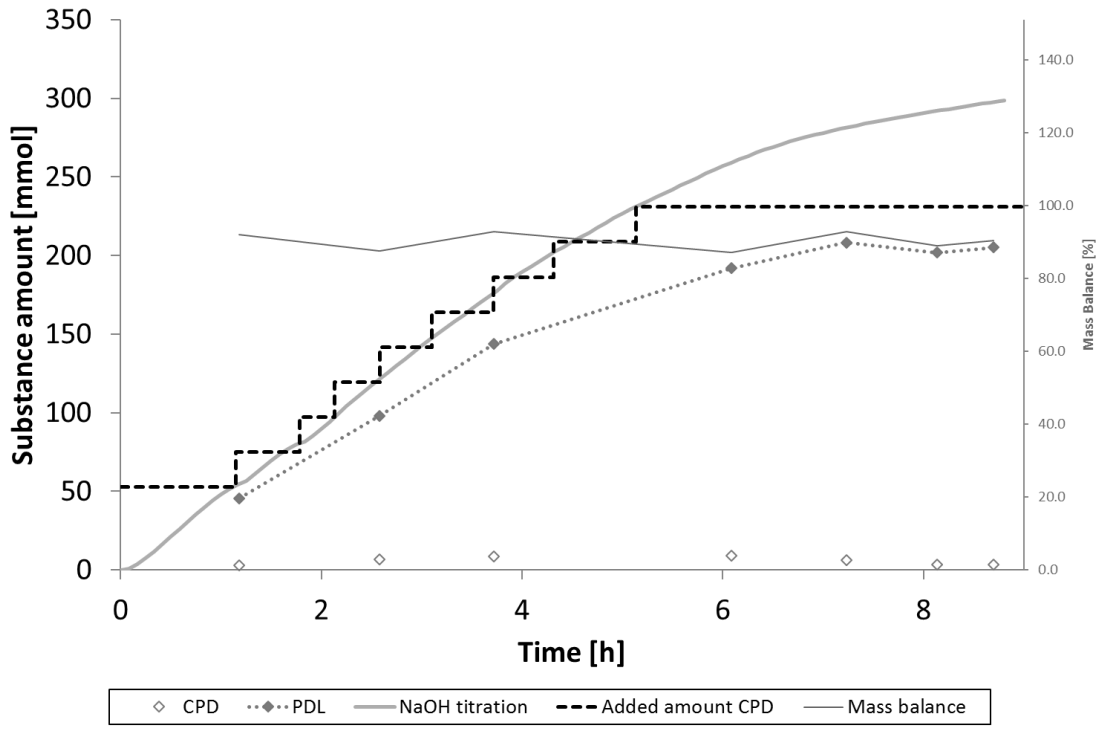


Figure 2: Progress curve of cyclopentadecaone (CPD) oxidation to pentadecalactone (PDL) with RrCDMO and GDH-01 on 1 L scale. Reaction conditions: V = 1 L, T = 30 °C, pH 7.5, 10 - 25 mL.min^{-1} O_2, stirring rate: 400 rpm; Addition of CPD portions in methanol, 1.5 eq. D-glucose, 0.5 mM $NADP^+$, finally 20 % (v/v) methanol, 4 mM Tween-80, 10 mM potassium phosphate pH 7.5, 0.2 % (v/v) GDH-01 liquid enzyme formulation, 18 % (v/v) RrCDMO liquid enzyme formulation. Open diamonds: CPD substrate; filled diamonds: PDL product; Bold line: NaOH titration; broken line: amount of CPD added; thin line: total mass balance of CPD and PDL based on GC analysis.

With the implementation of mentioned process conditions >95 % cyclopentadecanone conversion was achieved within a short reaction time. The down-stream procedure in combination with the applied reaction protocol resulted in an overall PDL yield of 90.7 % in the product oil of >98 % chemical purity with unreacted CPD being the main impurity. The determined product losses in the extraction step (1.2 %), the filtration step (1.8 %) and the solvent evaporation step (<0.1 %) were comparably low.

Finally, the developed process protocol was scaled up 100-fold for demonstration in our 200 L pilot plant reactor. In a 100 L reaction more than 4 kg pentadecalactone were produced and isolated according to the scaled up protocol.

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