ChemComm

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/c4cc08734h

Received 7th November 2014,
Accepted 5th January 2015

DOI: 10.1039/c4cc08734h

www.rsc.org/chemcomm

Non-hazardous Baeyer–Villiger oxidation of levulinic acid derivatives: alternative renewable access to 3-hydroxypropionates†

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Baeyer–Villiger monooxygenases catalyze the energetically challenging oxidation of levulinates (4-oxopentanoates) to 3-hydroxypropionic acid (3-HPA) derivates under ambient conditions, replacing propellant-grade H_2O_2 with aerial oxygen as the oxidant. This reaction enables a new pathway to a platform for chemical 3-HPA, an important intermediate in the non-petrol based production of a variety of bulk chemicals (acrylates, malonates, 1,3-propanediol).

Levulinic acid (LevOH) is generated as a by-product from carbohydrate dehydration in a significant percentage, and yearly tonnage is therefore available at a competitively low price.[1] Baeyer–Villiger oxidation (BVOx) of levulinates possibly leads to succinates upon unfavored methyl migration, or to 3-acetoxypropionates, which readily hydrolyze to give esters of 3-HPA. This compound is a versatile C_3 intermediate in the production of acrylates, malonates, 1,3-propanediol and other similar bulk chemicals.[2] It is currently being used as a crosslinking agent for polymer coatings and metal lubricants, and as an antistatic agent for textiles. More importantly, it was mentioned in third place in the current Top Value Added Chemicals from Biomass list of the US Department of Energy as a sugar-derived building block.[3] Bio-based access from levulinates to this key intermediate may thus create an alternative to direct fermentative approaches towards the formation of 3-HPA (from glucose or glycerol), while valorizing LevOH waste; so far, no fermentative process has achieved a level of economic viability on an industrial scale.[4]

In this work we present the biocatalytic BVOx of alkyl levulinates (LevOR, R = alkyl) to 3-acetoxypropionates and 3-hydroxypropionates on the gram scale under ambient conditions using aerial oxygen as the terminal oxidant. In the preceding literature the uncatalyzed BVOx of benzyl levulinate was the only example available, carried out using 90% H_2O_2 and trifluoroacetic anhydride in refluxing DCM and yielding the expected acetoxy product **5a** in 63% yield (Fig. 1).[5]

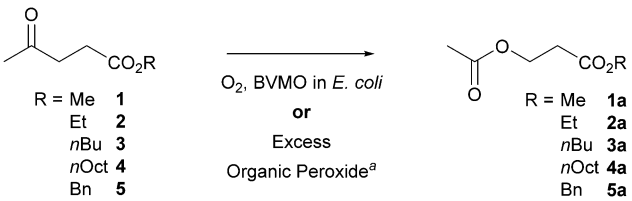

Fig. 1 BVOx of alkyl levulinates **1–5**. [a] *m*-Chloroperbenzoic acid or *t*-butyl hydroperoxide in DCM or toluene; peracetic acid or trifluoroperacetic acid generated *in situ* from 50% H_2O_2 and the corresponding carboxylic acid as solvent; for **5**: 90% H_2O_2, trifluoroacetic anhydride, DCM, 63% (ref. 5).

Since H_2O_2 at this concentration is mainly used as a rocket propellant[6] and the reaction was carried out on the 0.44 mol scale, we chose not to reproduce this experiment for safety reasons.

The first attempts to convert the initial test substrates LevOMe, LevOEt and LevO*n*Bu (**1–3**) using more common and comparably safe peroxide sources and activating agents were poorly successful (Fig. 1): only trifluoroperacetic acid at room temperature gave 24–29% conversion to a mixture of defined products (including **1a–3a**) after 48 h. All other reagents and conditions were either unreactive or led to complete decomposition of the starting materials (see ESI†).

LevOH is known to form α-angelica lactone *via* intramolecular enol lactonization under strongly acidic and dehydrating conditions.[7] The intermediate enol tautomer would be impervious to a nucleophilic peroxide attack, but no evidence for the enol form and/or intramolecular transesterification was found in comparative NMR analyses of $CDCl_3$ and CF_3CO_2D solutions of **1–3** (data not shown). These findings led us to believe that BVOx of levulinates are energetically demanding due to the stereoelectronic requirements[8] for a successful rearrangement of the Criegée intermediate, which obviously cannot be overcome by increased redox potential, nucleophile quality or concentration-kinetic effects (activation barriers for unreactive conformers have been shown to be higher by as much as 200 kJ mol^{-1} *via* DFT calculations).[9]

Baeyer–Villiger monooxygenases (BVMOs), a subclass of flavin- and NADPH-dependent monooxygenases, are well-studied biocatalysts for BVOx.[10] Their ability to induce chirality in this

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† Electronic supplementary information (ESI) available: Experimental details, copies of NMR spectra. See DOI: 10.1039/c4cc08734h

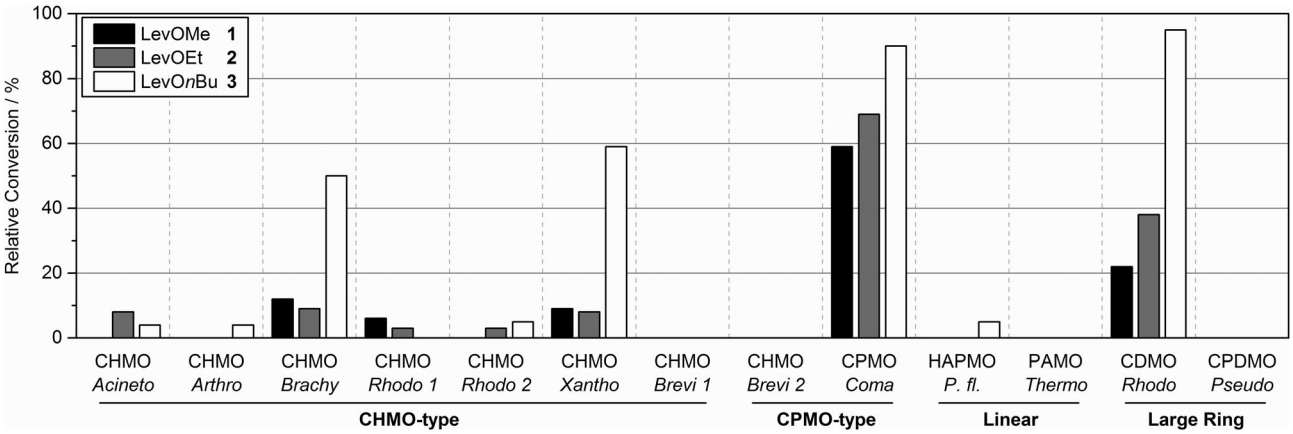

Fig. 2 Analytical scale screening of wild-type BVMOs for the oxidation of levulinates **1–3**; relative conversion as determined by GC-MS(EI); reaction conditions: BVMO in recombinant *E. coli* BL21(DE3), air, LB medium, 4 mM substrate, 0.5% v/v 1,4-dioxane, 1 mL reaction volume, 24 °C, 24 h.

reaction class at room temperature is so far unsurpassed by any other catalytic system, which in turn highlights their excellent ability to (specifically) lower activation energy. Analytical scale whole-cell enzyme screening was performed on esters **1–3** with a selection of 13 BVMOs: seven cyclohexanone monooxygenase-type (CHMO), two cyclopentanone monooxygenase-type (CPMO), two linear ketone-converting and two large ring-converting BVMOs. This demonstrated the excellent stereoelectronic control of these catalysts: the substrates were partly converted to the acetoxy products **1a–3a** using aerial oxygen under ambient conditions by nine biocatalysts (Fig. 2). Interestingly, the best results were achieved with $CPMO_{Coma}$ (ref. 11) and cyclododecanone monooxygenase $CDMO_{Rhodo}$.[12] On the other hand, both linear ketone-converting BVMOs (*p*-hydroxyacetophenone monooxygenase $HAPMO_{P.fl.}$ (ref. 13) and phenylacetone monooxygenase $PAMO_{Thermo}$)[14] failed to convert this open-chain ketone (**3a** was detected in trace amounts using $HAPMO_{P.fl.}$). These findings fit into the general substrate profiles of the two best-performing wild-type catalysts: $CPMO_{Coma}$ is known to preferentially stabilize only one conformer in the Criegée intermediate, resulting in regioisomerically homogeneous products.[15] $CDMO_{Rhodo}$ was found to be an excellent catalyst for the oxidation of linear β-amino ketones, among other substrate classes.[16] In addition to the enzyme-specific interpretation, a clear positive correlation between observed reaction rate and ester chain length was observed. Esters of longer chain alcohols are converted faster and are tolerated at higher substrate concentrations (Fig. 3). So far, we have not been able to determine to what extent this effect is based on enhanced lipophilicity and thus faster transmembrane transport of substrates in whole-cell biocatalysis.

The product structure and integrity of the analytical results were confirmed with preparative biotransformations in shake flasks using $CPMO_{Coma}$ (esters **1–3**) and $CDMO_{Rhodo}$ (esters **2** and **3**) on the 100 mg scale. We obtained the expected 3-acetoxypropionates **1a–3a**, but missing pH regulation in the simple vessels partly led to the cleavage of the acetate moiety during the reaction and/or workup; only methyl ester **1a** remained intact and was obtained in 80% yield. This information proved to be valuable: hydrolysis of the BVOx products to 3-hydroxypropionates proceeds smoothly in

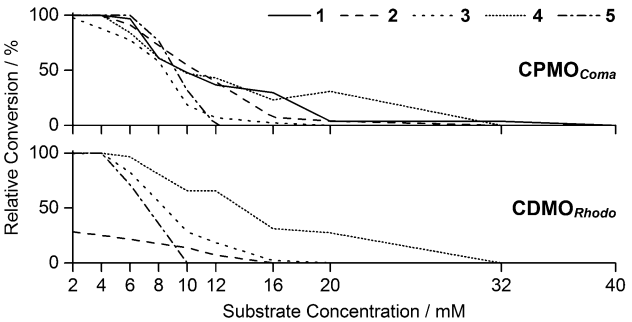

Fig. 3 Concentration screening of levulinates **1–5** using whole-cell BVMO catalysts; at all concentrations no visible formation of a second liquid phase was observed; relative conversion determined by GC-FID; LevOMe **1** not done with $CDMO_{Rhodo}$; *x*-axis ticks indicate tested initial substrate concentrations.

an aqueous environment at sub-physiological pH and room temperature. Nevertheless, both product compounds could easily be isolated from the medium by ethereal solvent extraction. In any case, no regioisomeric oxidation products (methyl alkyl succinates) were detected.

Next, we tested a CAST library of CPMO variants in order to further improve the productivity of this reaction. This library was already previously generated and described with the intent of improving the selectivity and/or extend the substrate acceptability of wild-type CPMO.[17] Four active site positions had been targeted (F156, G157, G449, F450), modulating available space and hydrogen bonding properties specifically without randomization. Four double mutants were also included. In order to screen for enhanced activity, further tests were performed at 8 mM substrate loading (Fig. S3, ESI†). Two mutants showed significantly increased activity with at least one of the substrates: $CPMO_{F156L}$ converted esters **1–3** almost completely within 24 h, and F450I exchange improved the reaction rate for LevO*n*Bu. We hypothesize that these two amino acids are close to the ester residue, since reduced steric bulk results in higher activity, and substitution of the native phenylalanine to non-aromatic amino acids significantly decreased conversion of LevOBn **5**, suggesting π–π interactions in the wild-type (Fig. S1 and S2, ESI†). Moreover, the previously observed activity trend seemed to be reversed or not present in

the mutant F156L. Within this mutant study, we also tested a CPMO-templated quadruple mutant of $PAMO_{Thermo}$,[18] with positive results. In contrast to its parent enzyme, $PAMO_{15\text{-}F5}$ was active on esters **2** and **3**, further substantiating this strategy in enzyme engineering (Fig. S3, ESI†).

Eventually, $CPMO_{F156L}$ was chosen as the best candidate for biotransformation up-scaling and process development. LevOEt **2** was used as the model substrate based on the compromise between favorable kinetics and atom economy. Moreover, ethyl 3-HPA was explicitly stated as a desired commodity chemical target.[3] Oxidation of **2** using whole-cells under monitored conditions in a bioreactor on a 2.6 g scale (1.8 L reaction volume, 10 mM initial substrate titer, 20 h reaction time) gave **2a** in 66% yield and high purity after simple extraction of the centrifuged culture medium. Uncontrolled hydrolysis of the acetate was avoided by maintaining a neutral pH during the reaction and workup. This results in a space-time yield (STY) of 1.9 g L^{-1} d^{-1} by conversion, or 1.3 g L^{-1} d^{-1} based on the isolated product.

We further investigated the limits of the whole-cell reaction mode and observed the significant toxicity at >10 mM of **2** (1.44 g L^{-1}, Fig. 4). This represented a serious obstacle for improvements along this line. Thus, using a solid polystyrene substrate and product reservoir (Lewatit VP OC 1163),[19] we were able to maintain a non-toxic substrate titer and increase the STY to 2.4 g L^{-1} d^{-1} in small scale experiments (100 mL; data not shown).

In order to circumvent the unexpectedly strong toxicity of **2** to the recombinant host organism, we opted for crude cell extract (CCE) reactions at the cost of xenobiotic enzyme denaturation and its intrinsic decay. For economic viability, *in situ* NADPH recycling was facilitated by a secondary enzymatic system (glucose-6-phosphate dehydrogenase). In comparison with whole-cell reactions, the productivity of CCE biotransformations was increased up to a concentration of 20 mM LevOEt (Fig. 5). Although no substrate inhibition and/or denaturation of the enzyme was observed up to a titer of 150 mM **2** (approx. 22 g L^{-1}), $CPMO_{F156L}$ seems to be strongly inhibited by the reaction product **2a** at >20 mM available aqueous concentration. This was cross-validated in a competition experiment: a CCE solution pre-saturated with 20 mM of **2a** could still fully oxidize 1 mM LevOEt (data not shown).

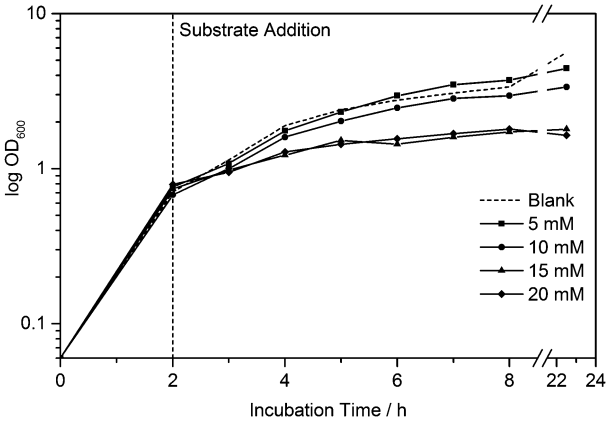

Fig. 4 Growth curves of recombinant *E. coli* BL21(DE3) expressing $CPMO_{F156L}$ at various conc. of LevOEt **2**; cultures were inoculated to OD_{600} = 0.05 from an overnight culture and grown at 37 °C; after 2 h substrate was added and cultivation was continued at 24 °C (expression temp.).

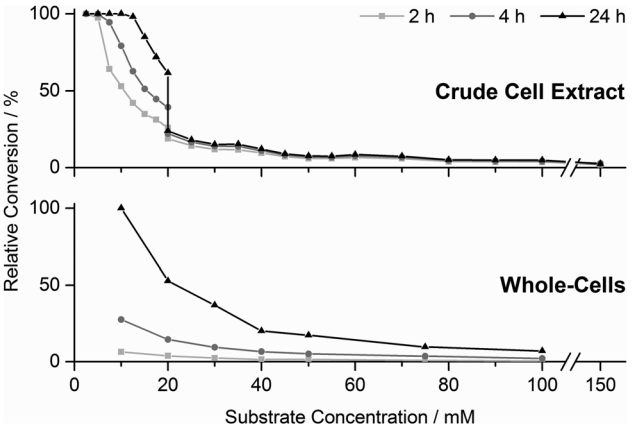

Fig. 5 Comparison of concentration effects of **2** on CCE and whole-cells of *E. coli* BL21(DE3) expressing $CPMO_{F156L}$; top: 1% w/v CCE, neat substrate addition, 2% v/v MeOH co-solvent for experiments >20 mM substrate; bottom: growing cells (24 °C), induction and substrate addition after a 2 h incubation time (37 °C), 0.5% v/v 1,4-dioxane as the co-solvent for all experiments; GC-FID analysis.

Nevertheless, this reaction setup leads to a calculated average productivity of 5 mM h^{-1} (STY approx. 17 g L^{-1} d^{-1}), as long as the resulting BVOx product can continuously be removed from the aqueous phase. The compatibility of such a substrate feed and product removal system (as described above with whole cells) was already reported for other redox biocatalysts.[20] We are currently investigating the feasibility of this concept with BVMOs.

In summary, we have demonstrated another instance of the potential of BVMOs as catalysts in achiral transformations: a propellant-grade stoichiometric oxidant in a corrosive organic solvent mixture could be replaced with aerial oxygen in water under ambient conditions. Levulinates, by-products from biomass degradation, could be converted to derivatives of 3-HPA, a key building block for many petrol-dependent bulk chemicals (acrylates, malonates, 1,3-propanediol) in a safe way. Using generically designed variants of $CPMO_{Coma}$ it was possible to convert the model substrate LevOEt on the gram scale. We are confident that tailored enzyme engineering is capable of further boosting the productivity of this transformation beyond the lab scale.

We thank Dr Martin Schürmann (DSM) and Dr Robert DiCosimo (DuPont) for suggestions and scientific advice, and Prof. Margaret M. Kayser (University of New Brunswick) and Prof. Manfred T. Reetz (Max Planck Institute for Coal Research, Mülheim an der Ruhr) for the kind donation of the CPMO variants. $PAMO_{15\text{-}F5}$ was provided by Prof. Marco W. Fraaije within the Oxygreen consortium (EU-FP7 grant # 212281). M.J.F. was also funded under this program.

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