Synthesis of methyl propanoate by Baeyer-Villiger monooxygenases

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Abstract: Methyl propanoate is an important precursor for polymethyl methacrylates and the use of a Baeyer-Villiger monooxygenase (BVMO) to produce this compound was investigated. A suitable BVMO would allow for the fermentative production of this compound and thus the use of renewable feedstock. A collection of 55 different enzymes was screened for variants able to produce this compound, using growing cells and headspace GC. Several BVMOs were found to facilitate the desired conversion, producing yields up to 40% of the abnormal Baeyer-Villiger product methyl propanoate. This finding shows that an enzyme can force production of the chemically unexpected product on even the smallest asymmetric ketone.
Baeyer-Villiger monooxygenases (BVMOs) are versatile enzymes belonging to the class of oxidoreductases, which are able to convert ketones to esters [1,2]. Additionally, they are capable of performing heteroatom oxidations such as enantioselective sulfoxidations. BVMOs show exquisite regio- and stereoselective behavior and are often superior to metal-based catalysts [3]. The use of molecular oxygen instead of peroxides or peracids for Baeyer-Villiger (BV) oxidations also makes enzymatic approaches more attractive. BVMOs contain FAD which is reduced by the cosubstrate NADPH, allowing it to react with oxygen to form a peroxylavin intermediate. This forms a tetrahedral (Criegee) intermediate with the substrate homologous to the chemical BV oxidation [4], leading to the insertion of an oxygen adjacent to the carbonyl moiety.

Insertion next to the most substituted carbon creates the so-called normal product. Until now, a preference for the formation of the abnormal product has only been described for benzo-fused compounds and several chiral aliphatic compounds [1,5]. These conversions are generally stereo-electronically controlled; one enantiomer forms the normal product, while the other forms the abnormal product. This shows the excellent regioselectivity of these enzymes, which could potentially be used to synthesize exclusively the abnormal product.

Here we report for the first time the formation of the abnormal product from an achiral aliphatic ketone substrate by a BVMO. In fact, substrate 1 is the smallest ketone that can be converted into two different ester products (the normal product 2 and abnormal product 3) by BV oxidation (Figure 1). In contrast to the previously described abnormal products, the difference between the two sides of the ketone is in this case only one carbon. Product 3 is of industrial interest because it is used on a large scale as an intermediate in the production of acrylic plastics [6]. Therefore the enzymatic conversion of 1 to 3 could be used in a biocatalytic or fermentative process, allowing the use of a renewable feedstock instead of fossil fuels.

![Figure 1: BV oxidation of the aliphatic ketone 2-butanone (1) to the normal product ethyl acetate (2) and the abnormal product methyl propanoate (3). NADP = nicotinamide adenine dinucleotide phosphate.](image)

To find enzymes with selectivity for the abnormal product, we screened a large collection of cloned BVMOs showing different selectivities, using growing cells and headspace GC. Whole cells were used instead of isolated enzyme, because this greatly facilitates the screening of a large collection of enzymes and the *Escherichia coli* TOP10 cells did not show any interfering esterase activity. Phosphite-dehydrogenase (PTDH) fused versions of the BVMOs allow soluble expression or significantly improve expression for many BVMOs [7,8]. The fusion enzyme also facilitates recycling of the expensive NADPH cofactor, which was used for the experiments with isolated enzyme.
The tested BVMO collection contained 40 unique enzymes, 12 extensively investigated mutants with altered substrate specificities and 3 disulfide mutants of cyclohexanone monooxygenase from *Acinetobacter* NCIMB 9871 (CHMO<sub>Aci</sub>) with increased thermostability [9]. Of the 40 wild-type BVMOs tested, 22 produce at least trace amounts (>25 mM) of the normal product 2, highlighting the substrate promiscuity of BVMOs. Several BVMOs converted all of the substrate 1, but produced only the normal product 2 and only five of the investigated BVMOs produced both 2 and 3 (Table 1). These 5 BVMOs include the well-characterized CHMO<sub>Aci</sub> [7,10] and the crystallized CHMO from *Rhodococcus* HI-31 [11]. Both of these are closely related to the two other CHMOs producing 3, with sequence identities of at least 56%. Cyclopentadecanone monooxygenase (CPDMO) from *Pseudomonas* sp. strain HI-70 [12] also produced the abnormal product, but is only distantly related to the CHMOs with 20 to 24% identity to the CHMOs (Figure 2).

![Phylogenetic tree of the BVMOs used in the screening. The size of the pie charts corresponds to the amount of 2 and 3 produced (small <20% conversion, large >80% conversion). In each pie chart the fraction of 2 is shown in white. Full names and accession numbers are listed in the appendix and together with exact conversions in the online supplementary information.](image)

**Table 1**: Conversion of 5 mM 2-butanone by whole cells expressing different BVMOs.[a]

<table>
<thead>
<tr>
<th>BVMO</th>
<th>Conv. [%]</th>
<th>3 [% of product]</th>
<th>2 [% of product]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHMO&lt;sub&gt;Aci&lt;/sub&gt;</td>
<td>45</td>
<td>26</td>
<td>74</td>
</tr>
<tr>
<td>CHMO&lt;sub&gt;Rho&lt;/sub&gt;</td>
<td>49</td>
<td>14</td>
<td>86</td>
</tr>
<tr>
<td>CHMO&lt;sub&gt;Xf&lt;/sub&gt;</td>
<td>41</td>
<td>26</td>
<td>74</td>
</tr>
<tr>
<td>CHMO&lt;sub&gt;Bp&lt;/sub&gt;</td>
<td>14</td>
<td>27</td>
<td>73</td>
</tr>
<tr>
<td>CPDMO</td>
<td>42</td>
<td>26</td>
<td>74</td>
</tr>
</tbody>
</table>

[a] Reaction in 1 mL LB, inoculated with 50 µL overnight culture and incubated at 17 °C or 24 °C for 24 h after starting the reaction by adding substrate and L-arabinose to induce expression at the same time.
Detailed information about the sequence relationship between the different BVMOs is shown in Figure 2. Several BVMOs that were in our test panel had previously been shown to be active on 1 [13-15], but until now no product analysis had been carried out. Here we show that these specific BVMOs only produce the normal product 2 (Figure 2, Table S1). Interestingly, most of the enzymes converting 1 efficiently to the normal product cluster together like the abnormal product producing CHMOs.

For exploring the catalytic potential of BVMOs to produce 3, we investigated CHMO_{Aci} as biocatalyst in more detail. Intriguingly, when testing different concentrations of substrate 1, decreasing amounts of the undesired product 2 were formed when higher concentrations of the substrate 1 were used (Figure 3). As has been reported in literature, the specificity of BVMOs can be adapted by the addition of cosolvent [16-19]. Here a similar effect was achieved by the addition of an excess of 1, which suppressed the formation of the normal product 2, leading to a better ratio of abnormal over normal product. At higher concentrations of 1 there are some signs of substrate inhibition, which is quite common for BVMOs and illustrated by the reaction of CHMO_{Aci} in combination with cyclohexanone as substrate [20].

Figure 3: Conversions using increasing concentrations of the ketone substrate 1. The experiment was performed using 4 µM purified PTDH-CHMO_{Aci}, 100 µM NADPH and 20 mM Na_{2}HPO_{4}. □: absolute amount of 3 (left axis). —: relative amount of 3 (% of total product, right axis).

Several chemical BV oxidations of 1 using atypical oxidants (e.g. immobilized 4-aminoperbenzoic acid) have been shown to produce small amounts (<20%) of 3 as a side product [21,22]. Clearly, the BVMOs identified in this study provide better candidates for the production of 3 from 1.

Even though the conversion of the substrate to the abnormal product 3 was incomplete, it is an interesting discovery that BVMOs are able to create this product. We have shown that the collapse of the Criegee intermediate, the reaction intermediate preceding ester formation, can be influenced by the enzyme microenvironment even for very small asymmetric ketones; previously this has only been shown for bulky cyclic ketones or chiral ketones containing substituents. This proof of principle is a
valuable starting point for possible enzyme engineering efforts to create an industrially relevant BVMO that only produces the abnormal product. We have also shown that better specificity for the desired product can be achieved by adapting the reaction conditions.

**Experimental Section**

All chemicals were purchased from Sigma-Aldrich, ABCR, Biosynth, Oriental Yeast Co. or Merck and used without further purification.

Biocatalysis using growing cells was performed by inoculating 1 mL of LB<sub>Amp</sub> with 50 µL preculture in a 20 mL headspace vial, growing the cells at room temperature for 2 h. Subsequently expression was induced by the addition of 0.002 or 0.02 % (w/v) L-arabinose and the reaction initiated by adding 10 mM 1 at the same time. After 24-30 h at the optimal temperature for expression (17 – 37 °C), the samples were analyzed by headspace GC.

PTDH-CHMO<sub>Ac</sub> was purified as described previously.[9] For the biocatalysis experiments using isolated enzyme, 4 mM PTDH-CHMO, increasing concentrations of 1, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 µM NADPH and 10 µM FAD in 1 mL 50 mM Tris-HCl pH 8.5 were incubated for 24 h at room temperature in a 20 mL headspace vial.

GC analyses were carried out on a Shimadzu GC-QP2010 gas chromatograph with a MS detector using a HP1 column. 250 µL samples were taken from a 20 µL headspace vial with a 45 °C syringe after vials were heated to 40 °C for 2:30. The injection temperature was 150 °C and the oven temperature was set at 35 °C (isothermal). Retention times of the compounds were 2.22 min for 1, 2.42 min for 2 and 2.60 min for 3. The compounds were accurately identified by MS and the retention times were consistent compared to the commercially available reference compounds.

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**Supporting information**

The supplementary material includes the sequences and raw data belonging to figure 2 and can be found linked to the online version of this chapter. A list of complete names and accession numbers can be found in the appendix.
References


