Synthesis of Natural Product Precursors by Baeyer–Villiger Oxidation with Cyclohexanone Monoxygenase from Acinetobacter

Ulrich Schwarz-Linek, Andreas Krödel, Friedrich-Alexander Ludwig, Alexander Schulze, Sebastian Risom, Udo Kragl, Vladimir I. Tishkov, Marina Vogel

Abstract: The Baeyer–Villiger oxidation of the 2-substituted ketones 1 and 3 with the coupled system cyclohexanone monoxygenase from Acinetobacter NCIMB 9871 / formate dehydrogenase from Pseudomonas sp. 101 provides the lactones (R)-2 and (R)-4 with high enantiomeric excess which are precursors in the synthesis of lipoic acid. The symmetrically trisubstituted ketone 5 was oxidised to the lactones 6a and 6b in a ratio of approx. 3:1. The absolute configuration of 6a and 6b was determined by hydrolysis of the racemic lactone with PLE yielding the hydroxycarboxylic acid (–)-7 with known absolute configuration.

Key words: oxidation, enzymes, kinetic resolution, hydrolysis, natural products

In a recent review, Hoberg pointed out the biological and chemical relevance of seven-membered oxacycles. One of the most powerful and elegant methods for expanding six-membered rings is the well-known Baeyer–Villiger oxidation. Unfortunately, there are only very few examples for achieving at least some enantioselectivity when oxidising prochiral or racemic ketones using chiral metal complexes, chiral auxiliaries, or the Sharpless epoxidation system. However, to our knowledge there is no method that could rival with the enzymatic Baeyer–Villiger oxidation concerning the stereoselectivities as well as the spectrum of substrates. Among several Baeyer–Villiger monoxygenases, cyclohexanone monoxygenase from Acinetobacter NCIMB 9871 (CHMO; EC 1.14.13.22) has been studied most intensively. Some interesting applications for the synthesis of precursors of natural products like azadirachtin and ionomycin were published.

In principle, there are two possibilities to carry out CHMO-catalysed Baeyer–Villiger oxidations: with whole cells or with isolated and enriched enzymes, respectively. The first method often suffers from over-metabolism and side reactions. This is also true if recombinant strains like a cyclohexanone monoxygenase expressing baker’s yeast or Escherichia coli are used. The second approach has some advantages. The reaction process can be executed in a simpler manner using well-defined concentrations of enzyme and substrate. However, this method has a considerable disadvantage; it requires an effective NADPH regeneration. The latter problem has probably been the reason for the fact that, barring one example, no Baeyer–Villiger oxidations using CHMO on a preparative scale have been reported. In contrast to NADH, regeneration of NADPH by enzymatic methods is much more difficult. Tishkov et al. developed a protein-engineered formate dehydrogenase (FDH) from Pseudomonas sp. 101 by multiple site-directed mutagenesis which accepts NADP+ to an extent making it suitable for NADPH regeneration.

Until now, three examples of application of this new promising enzyme are known. FDH was first used for cofactor regeneration in a dehydrogenase-catalysed reduction of acetophenone. The coupling of CHMO with the NADP+-dependent FDH in the Baeyer–Villiger oxidation of 4-methylcyclohexanone was the first application of this recombinant enzyme together with a monoxygenase. The corresponding chiral ε-lactone had been synthesised with high chemical and enantiomeric purity (ee >99%) in repetitive mode on a preparative scale. Oxygen delivery turned out to be the rate-limiting factor of the conversion. In order to avoid problems connected with oxygen feeding by bubbling or intensive stirring on such a scale, bubble-free aeration through a thin-walled silicon rubber tube was used. In addition to this first example, the Baeyer–Villiger oxidation of the ketones 1, 3 and 5 with the coupled enzyme system was investigated to yield lactones, which are interesting precursors of natural products.

The Baeyer–Villiger oxidation of ketone 1 was described for the first time using a monoxygenase MO2 from Pseudomonas putida NCIMB 10007. In a kinetic resolution, lactone (R)-2 was formed with considerable enantiomeric excess (93%) in good yield (30%). Starting from (R)-2, a useful intermediate for a synthesis of lipoic acid could be produced by ring-opening.

Firstly, the oxidation of ketone 1 with the coupled system CHMO/FDH (Scheme 1) was carried out on analytical scale. The conversion was followed using chiral GC. Different ketone concentrations were tested (10, 20, 40, and
The formation of only one lactone isomer was obtained during the whole reaction period in all cases. The best result was obtained using a 20 mM substrate concentration (see experimental, Table 2). Hence, this concentration was used for the conversion of 1 on a preparative scale in the reactor with integrated bubble-free aeration. Ketone 1 was oxidised in two consecutive batches successfully (Figure 1). Kinetic resolution of 1 proceeded nearly completely yielding only one lactone with high enantiomeric purity (>99% GC). The specific rotation was determined to be \([\alpha]_D^{25} -27.2^\circ\) \((c 2.29, \text{CHCl}_3)\). A third batch was not successful, probably due to the irreversible inhibition of the monooxygenase by the lactone. The absolute configuration of the \((-\))-lactone was proposed to be \((R)\)-2 (Scheme 1).

In their investigations on a new synthesis of \((+)\)-lipoic acid, Willetts et al. oxidised ketone 3 using several monoxygenases, but not the CHMO from *Acinetobacter*. All enzymes produced the lactone \((R)\)-4 with good to satisfactory yields. Only a monoxygenase from *Pseudomonas putida* NCIMB 9872 formed the \((S)\)-lactone with low enantiomeric excess (42%) which is a direct precursor of \((+)\)-lipoic acid.

In the oxidation of ketone 3 with CHMO/FDH only one lactone was preferentially formed and very fast. In contrast to 1, the other ketone enantiomer was also accepted, but it was oxidised quite slowly (Figure 2). The conversion can be followed easily and quickly by chiral GC. Thus, if the reaction is stopped after 30 minutes, a lactone with high enantiomeric purity can be isolated (Table 1). The absolute configuration of the preferentially formed lactone was proposed to be \((R)\)-4 (Scheme 1) by comparison with the elution sequence of the chemically synthesised racemic compound \((\pm)\)-4 using the same GC conditions.

The racemic seven-membered lactone 6 is an interesting precursor in the synthesis of \((-\))-lardolure, the aggregation pheromone of the acarid mite *Lardoglyphus konoi*. \((-\))-Lardolure was prepared in a thirteen-step synthesis.

### Table 1 Conversion of the Ketones 1, 3 and 5 on an Analytical Scale

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Ketone</th>
<th>Yield(^a) (%)</th>
<th>ee(^b) (%)</th>
<th>Configuration(^c)</th>
<th>Lactone</th>
<th>Yield(^d) (%)</th>
<th>ee(^e) (%)</th>
<th>Configuration(^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60(^a)</td>
<td>1</td>
<td>60</td>
<td>64</td>
<td>S(^d)</td>
<td>2</td>
<td>39</td>
<td>&gt;99</td>
<td>R(^d)</td>
</tr>
<tr>
<td>30(^a)</td>
<td>3</td>
<td>66</td>
<td>76</td>
<td>S(^e)</td>
<td>4</td>
<td>34</td>
<td>&gt;99</td>
<td>R(^e)</td>
</tr>
<tr>
<td>1440</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>100</td>
<td>48</td>
<td>7S</td>
</tr>
</tbody>
</table>

\(^a\) At this time the reaction should be stopped to obtain a high enantiomeric excess.  
\(^b\) Determination by chiral GC (Lipodex E).  
\(^c\) Absolute configuration.  
\(^d\) The configuration was proposed only by comparison with the results published in Lit.\(^28\) The determination of the configuration is under investigation.  
\(^e\) The absolute configuration of lactone 4 was dedicated by comparison with the elution sequence of the racemic lactone in GC [(R)-4: \(t_R = 15.6 \text{ min}\), (S)-4: \(t_R = 16.3 \text{ min}\), column temp 150°C] with published data [(R)-4: \(t_R = 14.8 \text{ min}\), (S)-4: \(t_R = 15.3 \text{ min}\)] which were obtained under similar conditions (Lipodex E, 25 m, column temp 160°C).\(^31\)
A key step was the preparation of the enantiomerically pure 2,4-dimethyl-6-hydroxyheptanoic acids (+)-7 and (−)-7 by ring-opening of the racemic lactone (±)-6 with (S)-prolinol and separation of the formed diastereomers. An enzymatic Baeyer–Villiger oxidation of the meso-ketone 5 using the enzyme system CHMO/FDH could be an alternative way (Scheme 2).

The conversion of 5 is the first example for the enzymatic Baeyer–Villiger oxidation of a symmetrically trisubstituted ketone. Lactone 6a was found to be the main product. This was an expected result because the CHMO preferentially oxidises 2- and 4-alkyl substituted cyclohexanones and also 2,6-disubstituted cyclohexanones, respectively, to lactones with (S)-configuration. 13 Hence, the high amount of the enantiomeric lactone 6b was an unexpected result.

In summary, with the coupled enzyme system CHMO/FDH, a system is available to carry out enzymatic Baeyer–Villiger oxidations also on a laboratory scale. Using a reactor with bubble-free aeration, CHMO/FDH catalysed the formation of a precursor of the natural product (+)-lipoic acid on this scale. For the first time, a symmetrically trisubstituted ketone was oxidised with CHMO successfully. The determination of the absolute configurations of the lactones however, is one of the main problems. In the case of the trisubstituted lactone, a PLE-catalysed hydrolysis was used to form a hydroxycarboxylic acid with known absolute configuration.

Optical rotations were measured using a Perkin–Elmer 241 polarimeter, and a Polartronic D polarimeter (Schmidt & Haensch). Mass spectra were obtained using a HP 5890 Series II with MSD 5972 (70 eV) and a HP-5 column (30 m). IR spectra were recorded using a Genesis FT-IR-Spectrometer, ATI Mattson. 1H and 13C NMR spectra were recorded using a Varian Gemini 200 spectrometer. Gas chromatograms were recorded using a HP 5890 Series II with a CP-Chirasil-Dex column (25 m, Chrompack) or a Lipodex E
The mixture was warmed up to 30°C to facilitate homogeneity. 38 Two enzyme fractions CHMO 1 (6.6 U mL⁻¹) and CHMO 2 (46 U mL⁻¹) in SH-buffer (21 mM phosphate buffer with 10 mM mercaptoethanol, pH 7.2) were used. The FDH (10 U mL⁻¹) in phosphate buffer, 100 mM, pH 7.0, which contained 10 mM EDTA and (NH₂)₂SO₄ of approx. 5% of saturation.) was added, and the pH value decreased. It was stirred for 2 h under ice-cooling and then for 6 h at r.t. Then NaHSO₃ (H₂O-sat. NaHSO₃, 2:1; 45 mL) was added, and the reaction was stirred for another 20 min. After stirring for 10 min, the organic layer was separated. The aqueous layer was extracted with CH₂Cl₂ (2 × 10 mL). The combined organic layers were washed with H₂O (2 × 10 mL), sat. NaHCO₃ (2 × 10 mL), and concentrated in vacuo. Distillation of the crude product (1.05 g) in a Kugelrohr ball-tube distillation apparatus (Büchi GKR-51) yielded (±)-2 (266 mg, 24%), the diastereoisomers were separated by MPLC (PE, acetone powder) purchased from Sigma. Ketone 1 was purchased from Aldrich. Ketone 3 and the racemic lactone 4 were prepared, just as ketone 5 and the racemic lactone 6. 35

Lactone (±)-3

Peracetic acid (40%, 20 mL), d-water (10 mL), and NaHCO₃ (0.91 g, 10.8 mmol) in CH₂Cl₂ (40 mL) during 20 min. The mixture was stirred for 2 h under ice-cooling and then for 6 h at r.t. Then H₂O (H₂O-sat. NaHSO₃, 2:1; 45 mL) was added, and the solution was kept at 30°C and stirred gently. The reaction was carried out in a reactor with integrated bubble-free aeration. 24 To a solution of ketone 1 (110 mg, 0.66 mmol) in Tris/HCl buffer (pH 8, 20.3 mL) were added sodium formate (6 mL, 1 M in Tris/HCl buffer, pH 8, 6 mmol), NADP⁺ (1.5 mL, 8 mM in Tris/HCl buffer, 12 mM), FDH (18 U, 1.8 mL, 0.6 U mL⁻¹), and CHMO (6 U, 273 µL, 0.2 U mL⁻¹). The mixture was kept at 30°C and stirred gently. The solution was aerated by opening the reaction mixture through a thin-walled silicon tube (4 mm i.d. × 40 cm) at a rate of 5 mL min⁻¹ with an external air flow of about 14.5 L h⁻¹. The reaction was stopped after 150 min (48% conversion of 1) by ultrafiltration through an Amicon PM 10 membrane. The concentrate (the enzyme solution) was washed with Tris/HCl buffer (2 × 20 mL) and concentrated again. This enzyme solution (6.8 mL) was ready for use in the second batch.

The combined aqueous filtrates were extracted with EtOAc (4 × 20 mL), dried (Na₂SO₄), and concentrated in vacuo. After flash chromatography (PE–EtOAc, 2:1) of the crude product, 42.0 mg (35%) of the lactone (±)-2, [α]D²⁵ ±27.2˚ (c 2.9, CHCl₃), ee >99%, tₚ = 23.9 min; and 16.1 mg (15%) of the residual ketone 1, [α]D²⁵ ±23.6˚ (c 2.29, CHCl₃), ee = 80%, tₚ = 7.2 min (Lipodex E, 120°C), were isolated.

Enzymatic Hydrolysis of Lactone (±)-6

In a vial (3.5 mL) were placed the buffer NaH₂PO₄ (0.1 M adjusted to pH 7.2; 1 mL), the lactone (±)-6 (100 mg, 0.64 mmol), and the crude enzyme PLE (100 µL). The mixture was stirred gently at r.t. The reaction started immediately, and the pH value decreased. It was corrected by the addition of NaOH (2 M). After a consumption of NaOH (180 µL) corresponding to about 60% conversion, a sample was taken from the reaction mixture, extracted with EtOAc (60 µL), acidified with 2 N HCl (3 µL) and analysed in GC (programme: 120°C, 4 min, 10°C min⁻¹ to 180°C). A ratio of 6b (tₚ = 6.7 min) to 6a (tₚ = 6.2 min) of 15:1 was found after 3.5 h. NaOH (30 µL) was added and the mixture was stored in a refrigerator overnight. The solution was extracted with Et₂O (7 × 2 mL). The combined organic layers were washed with 10% NaHCO₃ (3 × 5 mL), dried (Na₂SO₄), and concentrated in vacuo to give 39 mg (39%) of lactone 6b (>99% GC; tₚ = 6.7 min) as a colourless liquid; ee >99%: [α]D²⁵ ±22.2˚ (c 5.7, CHCl₃).

The aqeous layers were treated with the washing solution, acidified with 2 N HCl (7 × 5 mL), dried (Na₂SO₄), and concentrated to give the optically active hydroxy acid 7; yield: 60 mg (54%); colourless needles from hexane–Et₂O (10:1), mp 81–83°C (Lit. 31 mp 83.5–85.5°C), ee = 50% (GC; tₚ = 11.5 min, shoulder detection; the enantiomers could not be separated; [α]D²⁵ ±2.5˚ (c 1.4, CHCl₃) [Lit. 31 [α]D²⁵ ±4.2˚ (c 8.69, CHCl₃)].

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Reaction Conditions of the Enzymatic Baeyer–Villiger Oxidation of Ketones 1, 3 and 5 on an Analytical Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketone</td>
<td>Amount (mg)</td>
</tr>
<tr>
<td>1</td>
<td>3.68</td>
</tr>
<tr>
<td>3</td>
<td>2.00(*)</td>
</tr>
<tr>
<td>5</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* CHMO 1.
* Ketone 3 was dissolved in EtOH (1% v/v).
* CHMO 2.

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References

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(30) The reaction was published without any experimental details.


(32) The lactone (R)-2 was described only in ref. 27 but without any physical data.


(38) (a) Schwarz-Linek, U. PhD Thesis; University of Leipzig: Germany, 1998. (b) Details of the enzyme preparation will be reported in due course.