Towards Preparative-Scale, Biocatalytic Alkene Reductions

Supporting Information

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Reagents were obtained from commercial suppliers and used as received. LB medium contained Bacto-Tryptone (1%), Bacto-Yeast Extract (0.5%) and NaCl (1%). Agar (1.5%) was used to form solid medium when required. GC/MS analyses were performed on a Chirasil-Dex CB column (25 m × 0.25 mm) using a mass-selective detector (EI, 70 eV). The temperature program involved 60°C (2 min) followed by a 10°C / min increase to 180°C (10 min). Under these conditions, peaks eluted at 10.5 min (citronellal), 11.8 min (neral), 12.2 min (geranial) and 12.5 min (cintronellol). Chiral analyses were performed on a BetaDex column (30 m × 0.25 mm). The temperature program involved 95°C (35 min) followed by a 5°C / min increase to 160°C (2 min), then a 10°C / min increase to 200°C (5 min). Under these conditions, peaks eluted at 26.6 min ((S)-citronellal), 27.0 min ((R)-citronellal), 36.6 min ((S)-citronellol), 36.9 min ((R)-citronellol), 43.4 min (neral) and 46.2 min (geranial). Optical rotations were measured with a Perkin-Elmer 241 polarimeter operating a room temp. GST-OYE 2.6 was purified with High-Affinity GST resin (GenScript) as described earlier. Total protein concentrations were determined by Bradford assays using BSA as a standard. OYE catalytic activity was determined against 2-cyclohexenone.

Geranial 1. Geraniol (33.4 mmoles, 5.25 g) was stirred with MnO2 (125 mmoles, 10.85 g) in 30 mL of CH2Cl2 at room temp. After 24 hr, additional MnO2 was added (63 mmoles, 5.47 g) and stirring was continued overnight. Analysis by GC/MS showed that all starting material had been consumed. Solids were removed by filtration through a silica pad, then solvent was removed under vacuum to yield the title compound (4.72 g, 89% yield) as a bright yellow oil, which was stored under Ar at -20°C. GC analysis of the final product showed 96% geranial and 4% neral.
Neral 4. Nerol (136 mmoles, 21.0 g) was stirred with MnO₂ (500 mmoles, 43.40 g) in 150 mL of CH₂Cl₂ at room temp. After 22 hr, additional MnO₂ was added (125 mmoles, 10.85 g) and stirring was continued for an additional 4 hr. Analysis by GC/MS showed that all starting material had been consumed. Solids were removed by filtration through a silica pad, then solvent was removed under vacuum to yield the title compound (20.30 g, 98% yield) as a yellow oil, which was stored under Ar at -20°C. GC analysis of the final product showed 97% neral and 3% geranial.

Preparation of *P. stipitis* GST-OYE 2.6 crude extract. *E. coli* BL21(DE3) cells overexpressing OYE 2.6 were grown on LB plates supplemented with 200 μg/mL ampicillin. A single colony was used to inoculate 40 mL of LB medium containing 200 μg/mL ampicillin. After overnight growth at 37°C, this was added to 4 L of LB medium supplemented with 80 mL of sterile 20% glucose, 2 g/L ampicillin and 0.5 mL of Sigma AF 204 antifoam. Cells were grown in a New Brunswick M19 fermenter at 37°C with stirring and airflow maintained at 700 rpm and 4 L/min, respectively. After 2 hr, the O.D₆₀₀ value reached 0.6. The culture was cooled to 30°C and protein overexpression was induced by adding 0.48 mL of sterile 840 mM IPTG solution. The culture conditions were maintained for an additional 4 hr, when the O.D₆₀₀ value reached 4.2. Cells were harvested by centrifugation (6,500 × g at 4°C) to yield 28.2 g (wet cell weight) of biomass that could be stored at -20°C or used directly.

Cells (ca. 30 g) were resuspended in 30 mL of 100 mM KPᵢ, pH 7.5, then lysed by two passages through a chilled French pressure cell in the presence of 10 μM PMSF. Debris was removed by centrifugation (20,000 × g for 45 min at 4°C), then the supernatant (44.5 mL) was slowly mixed with 15 mL of saturated (NH₄)₂SO₄ to yield a final value of 25% saturation. After stirring gently for 10 min at 4°C, insoluble material was removed by centrifugation (20,000 × g
for 45 min at 4°C). Solid (NH₄)₂SO₄ (5.0 g) was slowly dissolved in the supernatant (55 mL) by stirring at 4°C. After an additional 10 min, insoluble proteins were removed by centrifugation (20,000 × g for 45 min at 4°C) and the supernatant was discarded. The pellet was resuspended in 25 mL of 100 mM KPi, pH 7.5, then insoluble material was removed by centrifugation as described above to yield 29 mL of protein solution that was frozen in aliquots at -20°C until needed. Activity assays showed 7.9 U/mL (2-cyclohexenone substrate).

**Preparation of *E. coli* GST-NemA crude extract.** *E. coli* BL21(DE3) cells overexpressing NemA were grown on LB plates supplemented with 200 μg/mL ampicillin. Using the procedure described above, cells were grown, induced at O.D₆₀₀ = 0.8 and harvested to yield 28.15 g of cells (wet weight). The biomass was suspended in 30 mL of 100 mM KPi, pH 7.5, lysed by two passages through a chilled French pressure cell and clarified by centrifugation (20,000 × g for 45 min at 4°C) to afford 45 mL of crude lysate that showed an activity of 44 U/mL (2-cyclohexenone substrate).

**SDS-PAGE analysis of purified *S. stipitis* OYE 2.6 and *E. coli* NemA fusion proteins.**

Purified proteins were separated on a 10% polyacrylamide gel, then proteins were stained with Coomassie blue. Theoretical values: GST-OYE 2.6, 71.3 kDal; GST-NemA, 65.5 kDal.
**CLEA preparation.** Purified GST-OYE 2.6 (17 mg, 0.50 mL) was mixed with purified GDH-102 (6 mg) and 0.50 mL of 100 mM KP _, pH 7.5_. A 0.50 mL aliquot was transferred to a microcentrifuge tube and 0.50 mL of saturated (NH₄)₂SO₄ solution was added. The tube was rotated gently at 4°C for 15 min, then 384 mg of solid (NH₄)₂SO₄ was added the tube was rotated gently at 4°C for an additional 15 min. Glutaraldehyde (15 μL, 50% aqueous solution) was added to a final concentration of 75 mM and the tube was rotated gently at 4°C for an additional 2 hr. The CLEA was collected by centrifugation and washed three times with cold 100 mM KP _, pH 7.5_.

**Acetylation of GST-OYE 2.6.** Four microcentrifuge tubes containing 4 mg of purified GST-OYE 2.6 in a volume of 0.50 mL were prepared (1 – 4). Sodium acetate (190 mg, 50% aqueous solution) was added to tubes 2 and 4. Neat acetic anhydride (1 μL) was added to tubes 3 and 4. All tubes were gently rotated at 4°C for 1 hr, then samples 2 – 4 were dialyzed against 100 mM KP _, pH 7.5_, 50 mM NaCl, 50% glycerol for 3 hr. Aliquots (250 μL) from samples 1 – 4 were transferred to microcentrifuge tubes and citronellal was added to a final concentration of 25 mM (28 μL of a 250 mM stock in EtOH) and gently rotated overnight at 4°C. Each sample, along with a control that was not treated with citronellal was diluted 1 : 10 with 100 mM KP _, pH 7.5_ and assayed for protein concentration and catalytic activity. The latter quantities were measured by incubating 2.5 mM 2-cyclohexenone (from a X M EtOH stock solution) with 100 mM KP _, pH 7.5 that contained 0.2 mM NADPH and an appropriate quantity of protein in a total volume of 1.0 mL. The change in A₃₄₀ was measured at 25°C. The small change in A₃₄₀ observed during the same time period in a control reaction lacking 2-cyclohexenone was subtracted from all of the determinations.
<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>NaOAc</th>
<th>Ac₂O</th>
<th>NaOAc, Ac₂O</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.75 ± 0.17</td>
<td>1.43 ± 0.08</td>
<td>0.12 ± 0.01</td>
<td>1.02 ± 0.04</td>
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<tr>
<td>25 mM citronellal</td>
<td>1.47 ± 0.09</td>
<td>1.46 ± 0.13</td>
<td>0.26 ± 0.02</td>
<td>0.84 ± 0.04</td>
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References