Supporting Information

Tunable production of (*R*)- or (*S*)-citronellal from geraniol *via* a bi-enzymatic cascade using a Copper Radical Alcohol Oxidase and Old Yellow Enzyme

David Ribeaucourt[1,2,3 ‡], Georg T. Höfler[4 ‡], Mehdi Yemloul[2], Bastien Bissaro[1], Fanny Lambert[3], Jean-Guy Berrin[1], Mickael Lafond[2*], and Caroline E. Paul[4*]

[1] *INRAE, Aix Marseille Univ, UMR1163 Biodiversité et Biotechnologie Fongiques, 13009 Marseille, France*
[2] *Aix Marseille Univ, CNRS, Centrale Marseille, iSm2, 13013 Marseille, France*
[3] *V. Mane Fils, 620 route de Grasse, 06620 Le Bar sur Loup, France*
[4] *Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands*

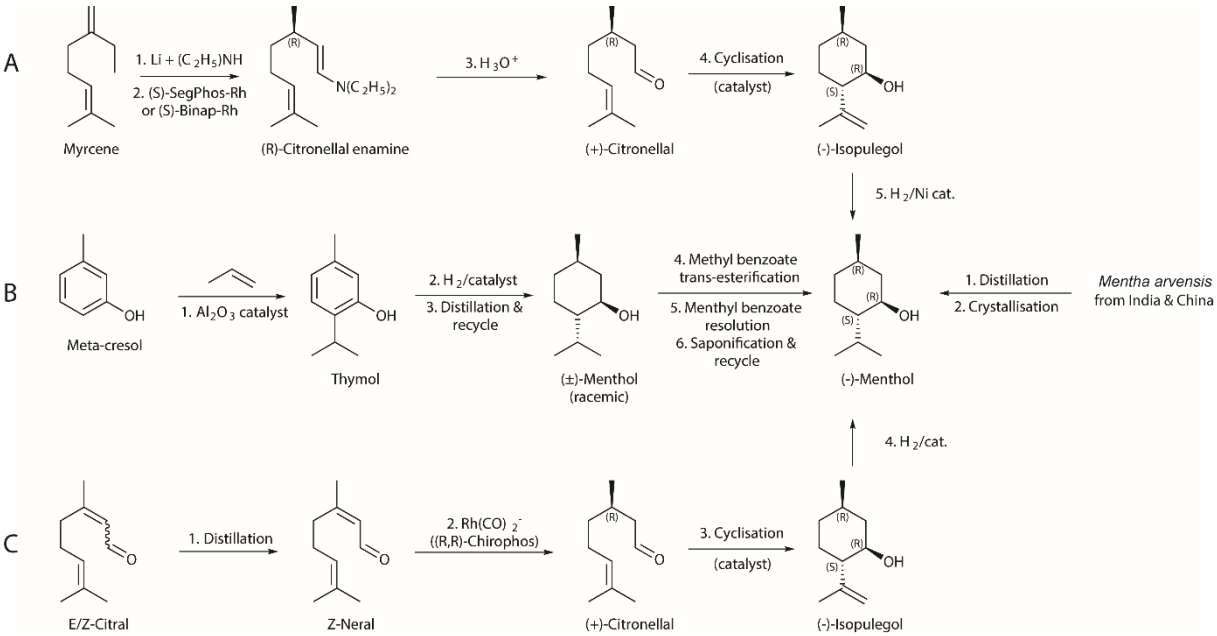
‡ These authors contributed equally
* corresponding authors: Dr. Caroline E. Paul (c.e.paul@tudelft.nl) and Dr. Mickael Lafond (mickael.lafond@univ-amu.fr)

Contents

1. General information

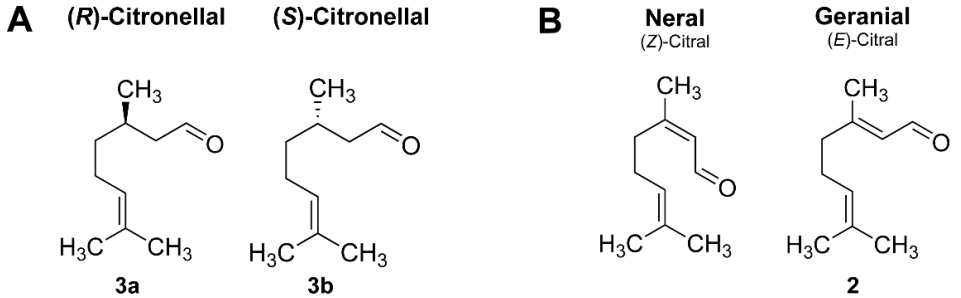
All chemicals (≥95% purity), HRP type II and catalase from bovine liver were purchased from Sigma-Aldrich (Germany). Molar concentrations of HRP and catalase were estimated by Bradford assay. DNA cloning work and production of *Pichia pastoris* X33 strains transformed with gene encoding *Cgr*AlcOx (GenBank ID EFQ30446.1) was carried out in previous studies.[1]

2. Synthetic routes to (–)-menthol



Scheme S1. Main industrial synthesis routes to (–)-menthol. (A) Takasago's process; **(B)** Symrise process; **(C)** BASF process. Adapted from Leffingwell *et al.*.[5]

3. Chemical structure of citral isomers and citronellal enantiomers



Scheme S2: (A) Chemical structure of the (*R*)- and (*S*)-enantiomers of citronellal. (B) Chemical structure of geranial and neral, the two isomers of citral.

4. Enzyme production and purification

2.1. Alcohol oxidase

*Cgr*AlcOx was produced using recombinant *Pichia pastoris* X33 strain in a 1.3 L New Brunswick BioFlo 115 fermenter (Eppendorf, Germany), and purified on a Resource Q-6 mL anion exchange chromatography column (GE Healthcare, USA), connected to an Äkta Xpress system (GE Healthcare)

as previously described.[2] Two runs of purification were necessary to purify the enzyme as the first run resulted in no binding of the enzyme on the column likely due to undesirable binding of impurities from the medium, which were removed after the first run. The *Cgr*AlcOx (ε_{280} = 101,215 $M^{-1}cm^{-1}$) concentration was determined by UV absorption at 280 nm using a Nanodrop ND-200 spectrophotometer (Thermo Fisher Scientific, USA). Final solutions (5.2 mg/mL, SDS-PAGE Figure S1) were flash-frozen using liquid nitrogen and stored at -80 °C for long-term storage or stored at 4 °C for immediate use.

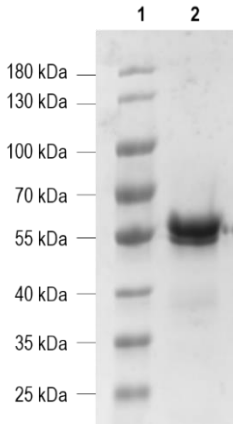


Figure S1. SDS-PAGE of purified recombinant *Cgr*AlcOx. Lane 1: molecular weight marker (PageRuler – Thermo Scientific; size expressed in kDa), 2: *Cgr*AlcOx. 3 µg of enzyme were loaded on a 10 % polyacrylamide gel. Gel was stained by Coomassie blue and displayed as grey shades.

2.2. Old Yellow Enzymes

OYE2 (from *Saccharomyces cerevisiae*, accession number Q03558) was recombinantly produced in *E. coli* BL21 Gold(DE3) cells with the plasmid pET-28b(+)-*oye2* (provided by Prof. D.J. Opperman from the University of the Free State, South Africa). A 100 mL pre-culture of LB medium containing 30 µg/mL of kanamycin was inoculated with 10 µL glycerol stock of *E. coli* BL21 Gold(DE3) pET-28b(+)-*oye2* cells and incubated overnight at 37 °C and 180 rpm. Overexpression was carried out in 2 L shake flasks containing 500 mL of TB medium supplemented with 30 µg/mL of kanamycin. The main cultures were inoculated to an optical density at 600 nm (OD_{600}) of approximately 0.05 using the required volume of pre-culture and grown at 37 °C and 180 rpm. When an OD_{600} of ~0.6 was reached (approximately 2 h 30 min), 0.1 mM of IPTG was added. After induction, cultures were incubated for 20 h at 25 °C and 180 rpm.

Cells were harvested by centrifugation (17,500 × *g* for 30 min at 4 °C). The obtained cell pellets were washed and re-suspended in approx. 150 mL MOPS-NaOH (20 mM, 300 mM NaCl, pH 7.0) buffer, supplemented with a spatula tip of DNaseI, $MgCl_2$, and one pill of EDTA-free cOmplete™ protease inhibitor cocktail. Cells were disrupted using a Multi Shot Cell Disruption System (Constant Systems Ltd, Daventry, UK) (two cycles) and the cell debris was separated from the crude extract by centrifugation (17,500 × *g* for 30 min at 4 °C).

For affinity chromatography purification, the obtained supernatant was loaded on a 5 mL GE Healthcare HisTrap FF Crude column. Buffer A: 20 mM MOPS-NaOH pH 7.0, 300 mM NaCl, 25 mM imidazole. Buffer B: 20 mM MOPS-NaOH pH 7.0, 300 mM NaCl, 500 mM imidazole. OYE2 eluted at 21% buffer B and fractions were gathered accordingly. The UV-vis spectrum was obtained to estimate flavin content. Purified enzyme was incubated with FMN 1:1 ratio for 30 min on ice. An AMICON with 30 kDa molecular cut-off was used to wash the excess FMN and concentrate the protein with 20 mM MOPS-NaOH (20 mM, 300 mM NaCl, pH 7.0) buffer, until the flow-through was colorless. The OYE2 was stored at -80 °C until later use. Protein concentration was measured by BCA assay and the purity of the preparation was accessed by SDS-PAGE comparing the band intensities (Figure S2).

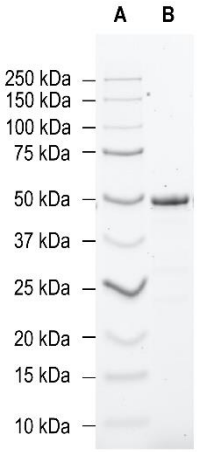


Figure S2. SDS-PAGE of OYE2 recombinant production in *E. coli* BL21 Gold(DE3) cells (OYE2: 47.3 kDa); A: Molecular Weight Marker OYE2; **B:** Purified OYE2.

*Ts*OYE (from *Thermus scotoductus* SA-01, accession number B0JDW3) was recombinantly produced in *E. coli* BL21(DE3) cells with the plasmid pET-22b(+)-*tsoye*. A 100 mL pre-culture of LB medium containing 100 µg/mL of ampicillin was inoculated with 10 µL glycerol stock of *E. coli* BL21(DE3)-pET-22b(+)-*tsoye* cells and incubated overnight at 37 °C and 180 rpm. Overexpression was carried out in 2 L shake flasks containing 500 mL of TB medium supplemented with 100 µg/mL of ampicillin. The main cultures were inoculated to an OD_{600} of approximately 0.04 using the required volume of pre-culture and grown at 37 °C and 180 rpm. When an OD_{600} of ~0.6 was reached (approximately 2 h 30 min), 0.1 mM of IPTG was added. After induction, cultures were incubated overnight at 30 °C and 180 rpm.
Cells were harvested by centrifugation (17,500 × *g* for 30 min at 4 °C). The obtained cell pellets were washed and suspended in approximately 150 mL MOPS-NaOH (20 mM, pH 7.0) buffer, supplemented with a spatula tip of DNase I, $MgCl_2$, and one pill of EDTA-free cOmplete™ protease inhibitor cocktail. Cells were disrupted using a Multi Shot Cell Disruption System (two cycles) and the cell debris was separated from the crude extract by centrifugation (17,500 × *g* for 30 min at 4 °C).
For heat purification, the supernatant was incubated in 50 mL Greiner tubes in a water bath at 70 °C for 1 h 30 min. Precipitated proteins were separated by centrifugation at 38,500 × *g* for 30 min at 4 °C (2 times). After centrifugation, the supernatant was a bright yellow solution. The UV-vis spectrum was obtained to estimate flavin content. FMN was supplemented to the supernatant as a 1:1 ratio, and left to incubate on ice for 30 min. An AMICON with 30 kDa molecular cut-off was used to wash the excess FMN and concentrate the protein with 20 mM MOPS-NaOH pH 7.0 buffer, until the flow-through was colorless, The *Ts*OYE was stored at -80 °C until later use. Protein concentration was measured by BCA assay and the purity of the preparation was accessed by SDS-PAGE comparing the band intensities (Figure S3). The *Ts*OYE double mutant C25D/I67T was produced with the same protocol.

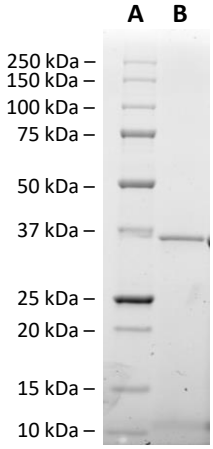


Figure S3. SDS-PAGE of *Ts*OYE recombinant production in *E. coli* BL21 Gold(DE3) cells (*Ts*OYE: 36 kDa); A: Molecular Weight Marker **B:** Heat purified *Ts*OYE.

GluER[3] (from *Gluconobacter oxydans*, accession number A1E8I9) was recombinantly produced in *E. coli* BL21(DE3) cells with the plasmid pET-28b(+)-*gluer* (purchased from BaseClear B.V. (Leiden, The Netherlands)). A 3 mL pre-culture of LB medium containing 50 µg/mL kanamycin was grown overnight at 37 °C and 180 rpm. 1 L TB media in 5 L a flask was inoculated with 3 mL of pre-culture and expression was induced at an OD_{600} of approximately 0.7 with 0.5 mM IPTG, and the cultures were left overnight (30 °C and 180 rpm).
Cells were harvested by centrifugation (10,000 rpm for 20 min at 4 °C). The obtained cell pellets were re-suspended and washed in MOPS-NaOH (20 mM, pH 7.5) buffer and transferred to a 50 mL plastic tube. After centrifugation (5,000 rpm for 15 min at 4 °C) the cell pellets were frozen at -80 °C. The obtained cell pellets were washed and suspended in approximately 2 mL per gram cell in 20 mM MOPS-NaOH (pH 7.5), supplemented with a spatula tip of DNase I, $MgCl_2$, and one pill of EDTA-free cOmplete™ protease inhibitor cocktail. Cells were disrupted using Multi Shot Cell Disruption System (two cycles) and the cell debris was separated from the crude extract by centrifugation (10,000 rpm for 20 min at 4 °C).
For affinity chromatography purification, the supernatant was loaded on a 5 mL GE Healthcare HisTrap FF Crude column. Buffer A: 50 mM Tris-HCl (pH 8.0), 10% glycerol, 10 mM imidazole and 300 mM NaCl. Buffer B: 50 mM Tris-HCl buffer (pH 8.0) with 10% glycerol, 500 mM imidazole and 300 mM NaCl. The fractions with His-tag containing proteins were collected. The UV-vis spectrum (600-300 nm) was obtained to estimate flavin content. Purified enzyme was incubated with FMN 1:1 ratio for 30 min on ice. An AMICON with 30 kDa molecular cut-off was used to wash the excess FMN and concentrate the protein with 20 mM MOPS-NaOH (20 mM, 300 mM NaCl, pH 7.0) buffer, until the flow-through was colorless. The GluER was stored at -80 °C until later use. Protein concentration was measured by BCA assay and the purity of the preparation was accessed by SDS-PAGE comparing the band intensities.

2.3. Glucose dehydrogenase

The *Bs*GDH (*Bacillus subtilis* glucose dehydrogenase double mutant E170K/Q252L) used for cofactor recycling was recombinantly produced in *E. coli* BL21 Gold(DE3) cells with the plasmid pACYC-*bsgdh*. Overexpression was carried out in 2 L shake flasks containing 350 mL of TB medium supplemented with 35 mg/mL of chloramphenicol. The main cultures were inoculated with 3.5 mL of pre-culture and grown at 37 °C and 180 rpm. When an OD_{600} of 0.4-0.6 was reached, 1 mM of IPTG was added for induction and cultures were incubated overnight at 30 °C and 180 rpm.
Cells were harvested by centrifugation (5,000 rpm for 15 min at 4 °C). The obtained cell pellets were re-suspended in potassium phosphate (KPi) buffer (100 mM, pH 7.4). Cells were lysed by sonication (5 × 30 s, 30 s intervals, 5 × 10 cycles, 40% power. The supernatant was recovered by centrifugation (16,000 rpm for 45 min at 4 °C).
For heat purification, the obtained supernatant was incubated at 60 °C for 1 h. Precipitated proteins were separated by centrifugation at 16,000 rpm for 45 min at 4 °C and the supernatant was stored at 80 °C until later use.
The *Bs*GDH activity was measured on a Cary 60 spectrophotometer at 340 nm with 1 mM $NADP^+$ and 100 mM glucose in 50 mM Tris-HCl pH 8.0 at 25 °C.

5. Biocatalytic reactions

3.1. Alcohol oxidase-catalyzed oxidation

Reactions were carried out in 4 mL-clear borosilicate glass vials closed by screw caps with a PTFE septum for a total reaction volume of 500 µL. Reactions contained 10 mM substrate (pre-diluted in acetone), 0.01-1 µM *Cgr*AlcOx (corresponding to 0.5-50 µg/mL *Cgr*AlcOx), 0-0.5 µM of catalase and/or 0-12 µM HRP, in 50 mM sodium phosphate (NaPi) buffer pH 8.0. Final concentration of acetone in the reaction mixture was 1 % (*v/v*). Reactions were incubated at 23 °C for 15 min, under shaking at 200 rpm in an Innova 42R incubator (New Brunswick, USA). Vials were placed lying down in the incubator.

Products and possible remaining substrate were extracted with 500 µL of ethyl acetate (EtOAc), followed by vortexing and centrifugation for 5 min at 1000 x *g.* The organic layer was transferred into a new vial by pipetting for further analyses.

3.2. Old Yellow Enzyme-catalyzed reduction

Reactions were carried out in 1.5 mL-clear borosilicate glass vials closed by screw caps with a septum for a total reaction volume of 400 µL. Reactions contained 20 mM citrate (2 M stock solution in acetone), 1.33–10.67 µM OYE2, 0.1-2 mM $NADP^+$, 6 U/mL *Bs*GDH, 40 mM glucose in 100 mM KPi buffer pH 8.0. Final concentration of acetone in the reaction mixture was 1% (v/v). Reactions were incubated at 25 °C for 5 h under shaking at 300 rpm in a thermoshaker. Products and possible remaining substrate were extracted by adding 400 µL of EtOAc through the septum with a syringe, followed by vortexing and centrifugation for 1 min at 12,000 × *g.* The organic layer was dried with $MgSO_4$ and analyzed by chiral GC-FID.

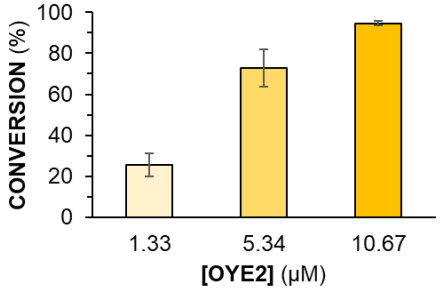


Figure S4. OYE2-catalyzed reduction of 20 mM citral varying the OYE2 concentration. Conditions: 20 mM citral 1.33-10.67 µM OYE2, 1 mM $NADP^+$, 40 mM glucose, 6 U/mL *Bs*GDH, KPi buffer (100 mM, pH 8.0), 1% v/v acetone, for 5 h at 25 °C and 300 rpm; 0.4 mL reaction volume. Analyses were done on a GC-FID with a chiral column. Error bars represent standard deviation (s.d., independent experiments, n = 2).

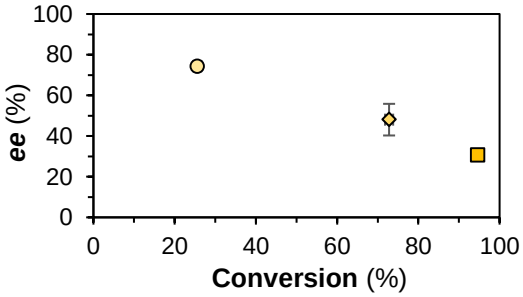


Figure S5. Enantiomeric excess of (*R*)-citronellal over conversion yield for the OYE2-catalyzed reduction of 20 mM citral using 1.33 (○), 5.34 (◇) or 10.67 (□) µM of OYE2. Conditions: 20 mM citral, 1.33-10.67 µM OYE2, 1 mM $NADP^+$, 40 mM glucose, 6 U/mL *Bs*GDH, KPi buffer (100 mM, pH 8.0), 1% v/v acetone, for 5 h at 25 °C and 300 rpm; 0.4 mL reaction volume. Analyses were done on a GC-FID with a chiral column. Error bars represent standard deviation (s.d., independent experiments, n = 2).

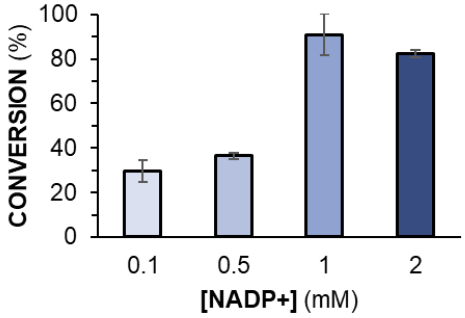


Figure S6. Influence of $NADP^+$ concentration on OYE2-catalyzed citral conversion. Conditions: 20 mM citral, 10.67 µM OYE2, 0.1-2.0 mM $NADP^+$, 40 mM glucose, 6 U/mL *Bs*GDH, KPi buffer (100 mM, pH 8.0), 1% v/v acetone, for 5 h at 25 °C and 300

rpm; 0.4 mL reaction volume. Analyses were done on a GC-FID with a chiral column. Error bars represent standard deviation (s.d., independent experiments, n = 2).

Table S1. OYE2-catalyzed reduction of citral, ratio of neral, geranial and citronellal with *ee* over a 5 h reaction

time (h)	neral (mM)	geranial (mM)	citronellal (mM)	*ee* (%)	ratio neral:geranial
1	7.3	6.7	6.0	70.4	1.1
2	4.4	4.7	10.8	58.5	0.9
3	2.1	3.3	14.6	53.6	0.6
4	1.9	2.2	15.9	48.0	0.8
5	0.5	0.5	19.0	44.4	1.0

Conditions: 20 mM citral, 10.67 µM OYE2, 1 mM $NADP^+$, 40 mM glucose, 6 U/mL *Bs*GDH, KPi buffer (100 mM, pH 8.0), 1% v/v acetone, for 6 h at 25 °C and 300 rpm; 0.4 mL reaction volume. Analyses were done on a GC-FID with a chiral column. Data are duplicate experiments.

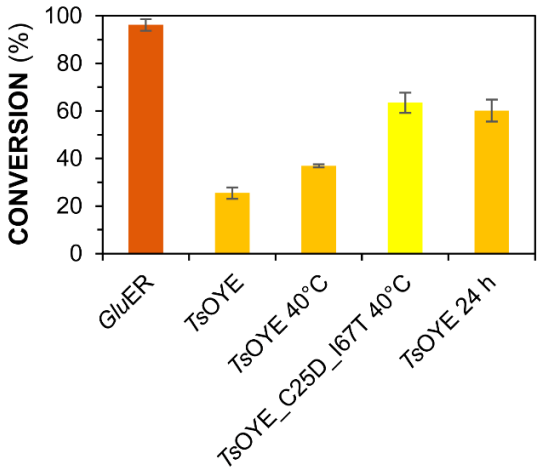


Figure S7. Screening of OYEs to catalyze the reduction of citral to form *(S)*-citronellal Conditions: 20 mM citral, 9.33 µM *Ts*OYE or all other OYE 8 µM, 1 mM $NADP^+$, 40 mM glucose, 6 U/mL *Bs*GDH, KPi buffer (100 mM, pH 8.0), 1% v/v acetone, for 5 h (or 24 h when indicated), at 25 °C (or 40 °C when indicated) and 300 rpm; 0.4 mL reaction volume. Analyses were done on a GC-FID with a chiral column. All enzymes showed high enantioselectivity with 99.9% *ee*. Error bars represent standard deviation (s.d., independent experiments, n = 2).

3.3. Bi-enzymatic cascade

Reactions were carried out in 4 mL-clear borosilicate glass vials closed by screw caps with a PTFE septum for a total reaction volume of 500 µL, with 10 mM geraniol (pre-diluted in acetone), 1 µM *Cgr*AlcOx, 0.5 µM catalase, 0.5 µM HRP, 10.67 µM OYE2 or 9.33 µM *Ts*OYE or 8 µM GluER, 6 U/mL *Bs*GDH, 40 mM glucose, 1 mM $NADP^+$, at pH 8 in 50 mM NaPi buffer. Final reaction contained 1% v/v acetone. Reactions were incubated at 23 °C for 2.5 h, under shaking at 200 rpm in an Innova 42R incubator (New Brunswick, USA). Vials were placed lying down in the incubator. For the "two steps, one pot" experiment, all conditions were the same except that *Bs*GDH was added only after the first 15 min of reaction. After addition of *Bs*GDH the reaction was incubated 2.5 h more in the same conditions as described above. Products and possible remaining substrate were extracted with 500 µL of EtOAc, followed by vortexing and centrifugation for 5 min at 1,000 × *g*. The organic layer was transferred into a new vial by pipetting for further analyses.

The scale-up was performed as a one-pot two-step reaction, with 20 mM geraniol in 20 mL volume in an Erlenmeyer flask. The reaction times were increased to one hour for the alcohol oxidation, and five hours for the conjugated alkene reduction. Prior to starting the reaction, the headspace and reaction media were saturated with pure oxygen. The reaction was quenched by extraction with EtOAc, which was further dried over $MgSO_4$ and evaporated. 44.3 mg (71.8% isolated yield) of (*R*)-citronellal was obtained with 95.14% *ee*. The final GC peaks observed were citronellal (90%), acetone (3%, used as co-solvent), geraniol (2%) and geranial (0.6%). Two new peaks (1.5% each) were also observed, possibly

corresponding to the two enantiomers of the overoxidation product (citronellic acid) that could have appeared due to prolonged reaction times. The purity of the isolated citronellal product was confirmed by NMR (Section 7) and the enantiomeric excess was confirmed by chiral GC-FID (Section 5).

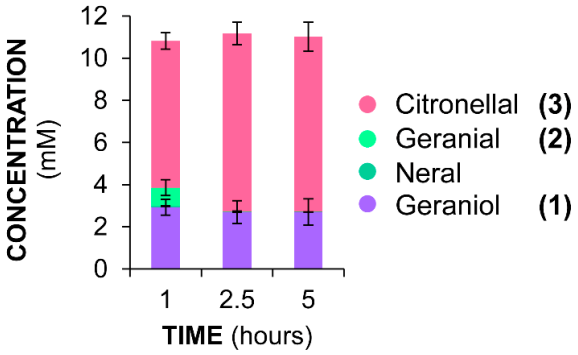


Figure S8. Time-course analysis of the concurrent one-pot, bi-enzymatic conversion of geraniol by *Cgr*AlcOx and OYE2. Conditions: 10 mM geraniol, 1 µM *Cgr*AlcOx, 10.67 µM OYE2, 0.5 µM CAT, 0.5 µM HRP, in NaPi buffer pH 8.0 for 1 to 5 h at 23 °C and 200 rpm. All reactions contained 1% v/v acetone. Analyses were done on a GC-FID. Error bars represent standard deviation (s.d., independent experiments, n = 3).

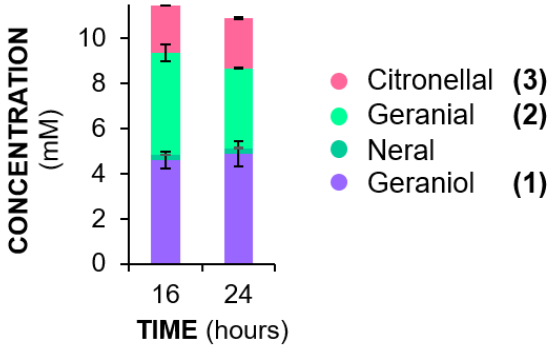


Figure S9. Time-course analysis of the concurrent one-pot, bi-enzymatic conversion of geraniol by *Cgr*AlcOx and *Ts*OYE. Conditions: 10 mM geraniol, 1 µM *Cgr*AlcOx, 9.33 µM *Ts*OYE, 0.5 µM CAT, 0.5 µM HRP, in NaPi buffer pH 8.0 for 16 to 24 h at 23 °C and 200 rpm. All reactions contained 1% v/v acetone. Analyses were done on a GC-FID. Error bars represent standard deviation (s.d., independent experiments, n = 3).

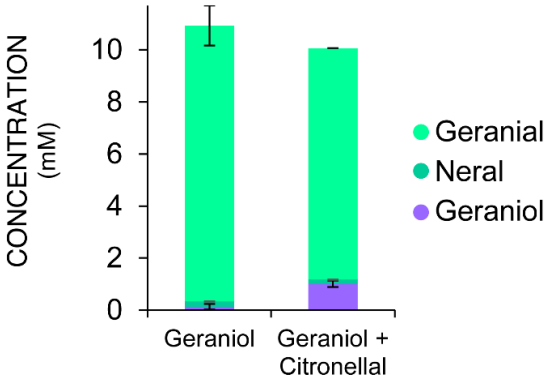


Figure S10. *Cgr*AlcOx incomplete conversion upon addition of citronellal (10 mM). Conditions: 10 mM geraniol (± 10 mM citronellal), 1 µM *Cgr*AlcOx, 1 µM CAT, 1 µM HRP, in NaPi buffer pH 8.0 for 2.5 h at 23 °C and 200 rpm. All reactions contained 2% v/v acetone. Analyses were done on a GC-FID. Error bars represent standard deviation (s.d., independent experiments, n = 3).

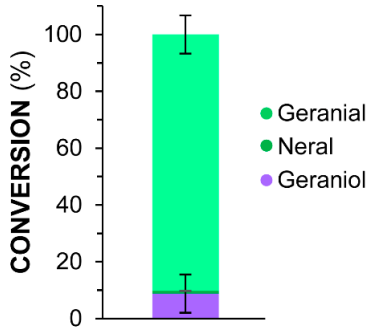


Figure S11. *Cgr*AlcOx conversion of 50 mM geraniol. Conditions: 50 mM geraniol, 1 µM *Cgr*AlcOx, 1 µM CAT, 1 µM HRP, in 50 mM KPi buffer pH 8.0 for 2.5 h at 23 °C and 200 rpm. Analyses were done on a GC-FID. Error bars represent standard deviation (s.d., independent experiments, n = 2).

6. GC analyses

Analyses were carried out on a GC-2010 apparatus (Shimadzu, Japan) equipped with an AOC-20i auto injector and a flame ionization detector (FID). For AlcOx-catalyzed reactions, products were confirmed by reference standards, with oven method A (see below) and concentrations were obtained with a calibration curve equation using 1 mM dodecane as an internal standard. For OYE-catalyzed reactions and *ee* determination, products were confirmed by reference standards (geraniol, geranial, neral, (*R*)-citronellal, (*S*)-citronellal) with oven method B and concentrations were obtained with a calibration curve equation using 5 mM dodecane as an internal standard. Below are details on the column used with their methods, injection temperature, linear velocity, column flow, oven temperature program and retention times (Table S2):

A. **DB-5** (Agilent J&W, USA), 30 m × 0.25 mm × 0.25 µm (length, internal diameter, film thickness), injection at 250 °C, split ratio 10, linear velocity 34 cm/sec, column flow 1.3 mL/min, nitrogen as carrier gas.

B. **Hydrodex ß-TBDAc** (Macherey-Nagel, Germany), 50 m × 0.25 mm × 0.25 µm (length, internal diameter, film thickness), injection at 250 °C, split ratio 100, linear velocity 38 cm/sec, column flow 2.27 mL/min, helium as carrier gas.

Table S2. GC-FID retention times of identified compounds on the specified columns and methods.

Column	Column oven temperatures	Compound	Retention time (min)
A	110 °C 5°C/min to 160 °C 60 °C/min to 250 °C hold for 2.5 min.	Dodecane	4.81
		Geraniol	5.62
		Geranial	5.96
		Neral	5.47
		Citronellal	4.25
B	70 °C for 5 min. 5 °C/min to 80 °C hold for 5 min 5 °C/min to 90 °C hold for 5 min 5 °C/min to 110 °C hold for 5 min 5 °C/min to 130 °C hold for 5 min 5 °C/min to 150 °C hold for 5 min 10 °C/min to 220 °C hold for 1 min.	Acetone	14.87
		Dodecane	24.93
		Geraniol	39.07
		Geranial	40.7
		Neral	43.87
		(*R*)-Citronellal	33.41
		(*S*)-Citronellal	33.22

7. GC chromatograms

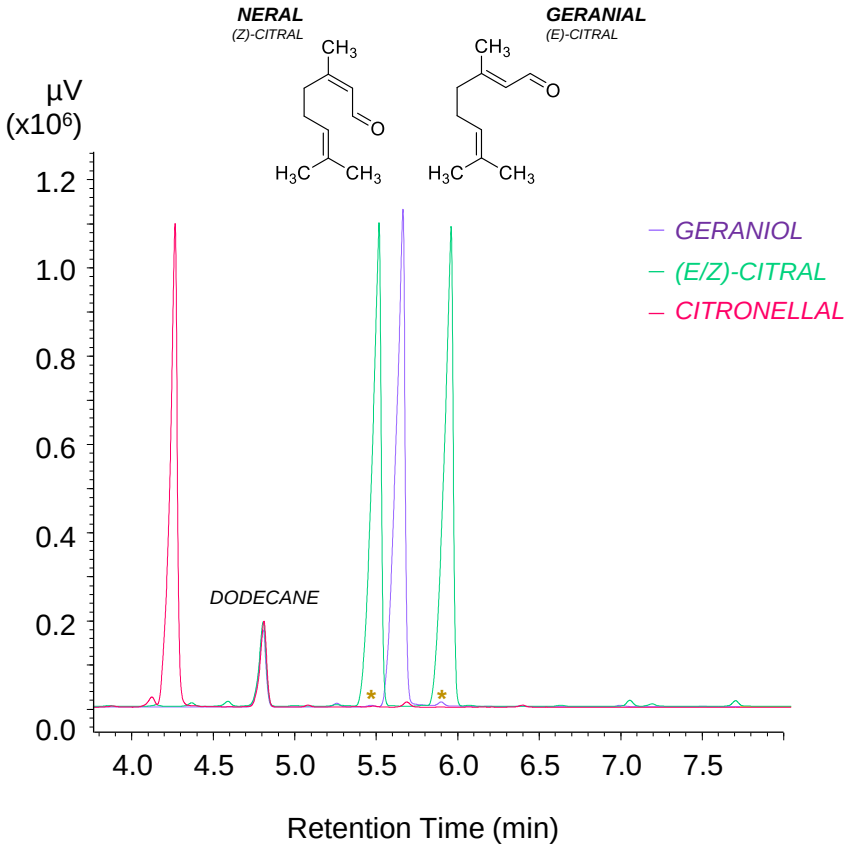


Figure S12. GC-FID chromatograms of chemical standards for AlcOx-catalyzed oxidation. Overlay of geraniol (10 mM), citral (20 mM – mixture (1:1) of geranial and neral), citronellal (10 mM). Standards were diluted in EtOAc. Dodecane (1 mM) was used as internal standard. Note*: The peaks at 5.47 and 5.96 min correspond to neral (0.2%) and geranial (1.1%) respectively (identified by ^{1}H NMR – see **Figure S17**), which were detected in geraniol stock solution.

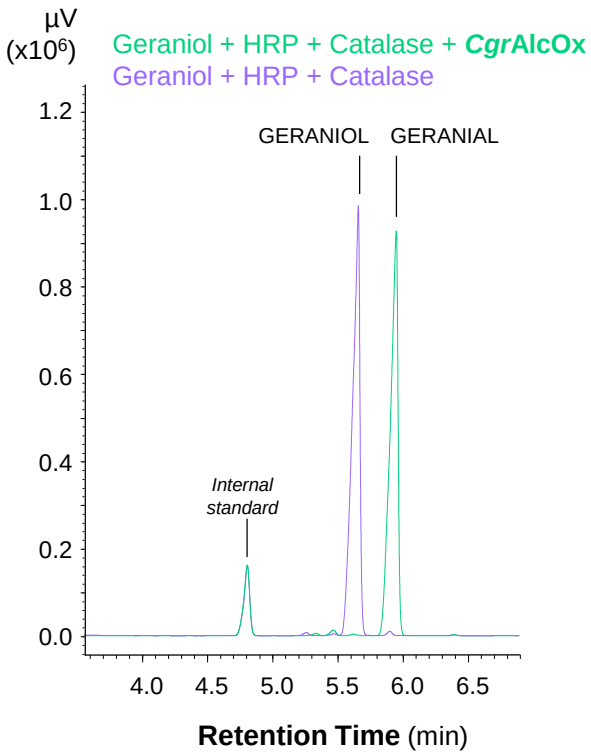


Figure S13: GC-FID chromatograms of *Cgr*AlcOx-catalyzed oxidation of geraniol (10 mM) in presence of HRP (12 µM) and CAT (0.5 µM). Dodecane was used as internal standard.

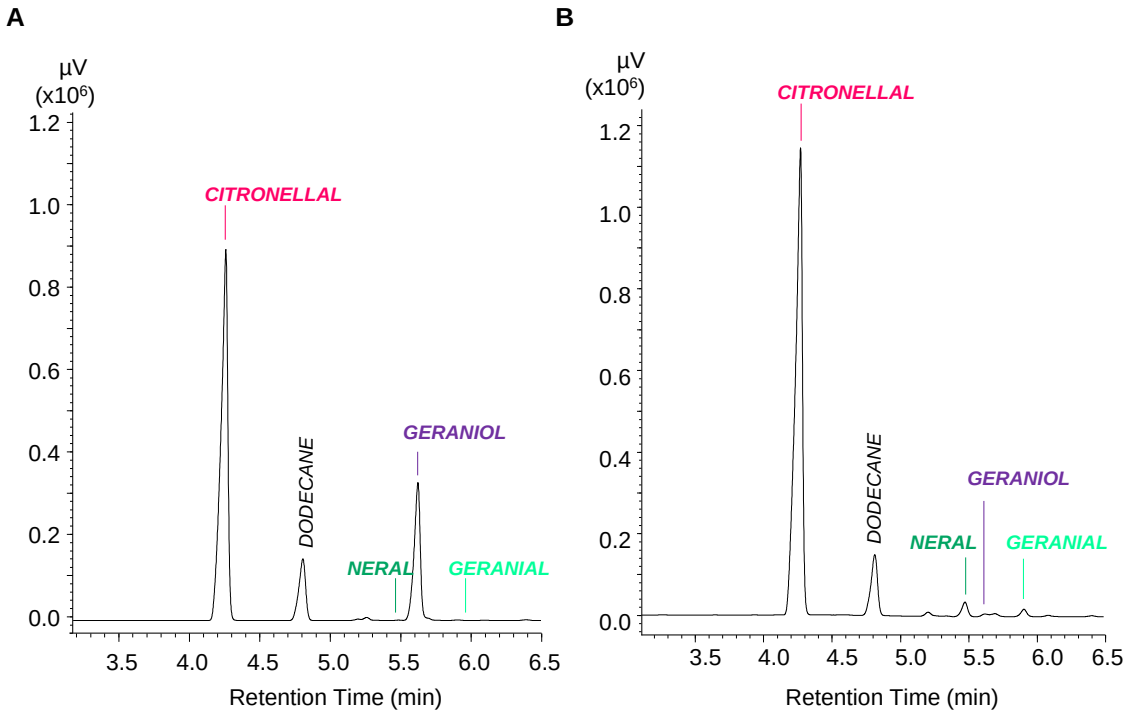
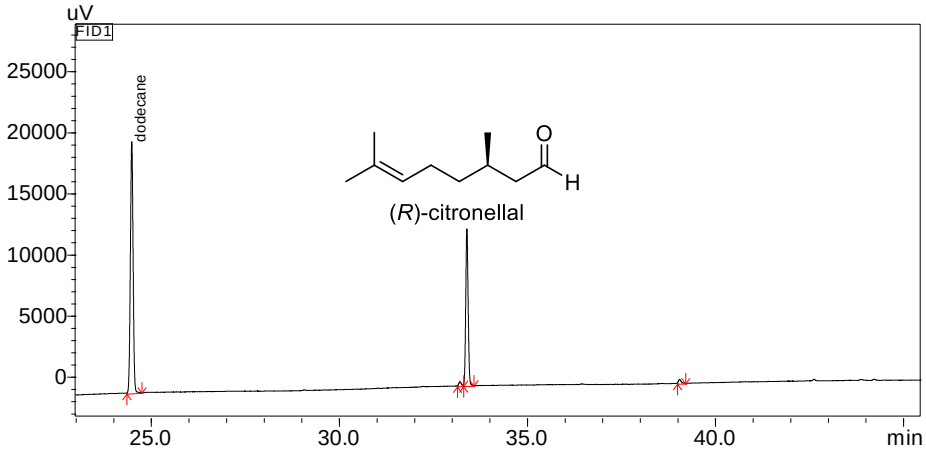


Figure S14. GC-FID chromatograms of *Cgr*AlcOx-OYE2 bi-enzymatic cascade. (A) Concurrent one-pot reaction performed in 2.5 h. **(B)** Sequential one-pot cascade reaction: first step (*Cgr*AlcOx conversion of geraniol to geranial) performed in 15 min (all reagents added except for *Bs*GDH) and subsequent second step (OYE conversion of geranial to citronellal) performed in 2.5 h (after addition of *Bs*GDH). Analysis by GC-FID. Reaction conditions: 1 µM *Cgr*AlcOx, 0.5 µM catalase, 0.5 µM HRP, 10.67 µM OYE2 or 8 µM GluER, 6 U/mL *Bs*GDH, 40 mM glucose, 1 mM $NADP^+$, pH 8 (50 mM NaPi buffer), 1% v/v acetone. Reactions were incubated at 23 °C, under shaking (200 rpm). Dodecane (1 mM) was used as internal standard.

A



B

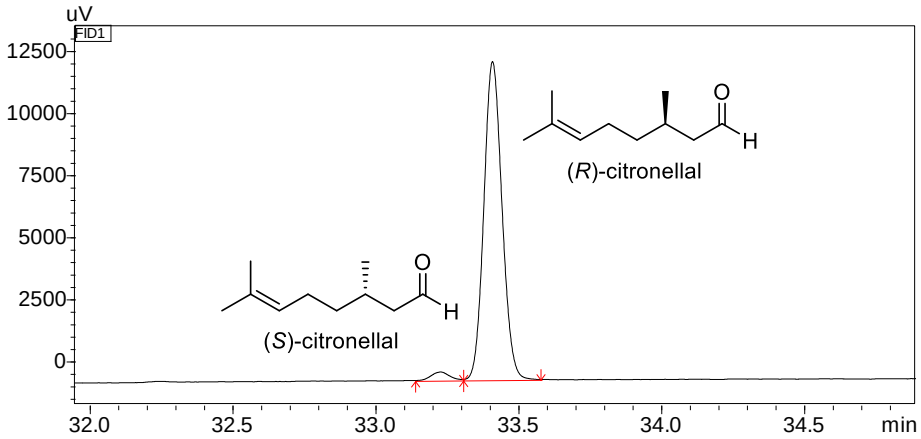
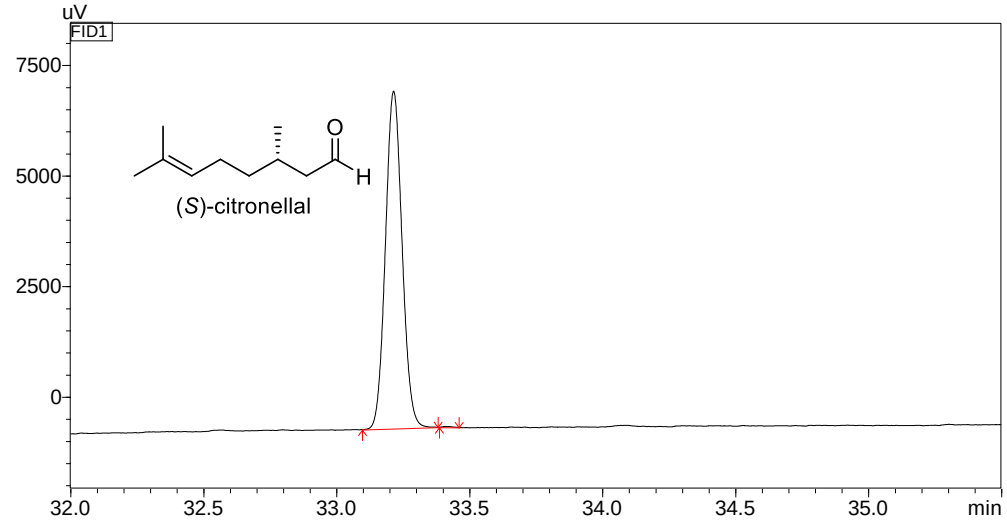


Figure S15. A: GC-FID chromatogram of the 20 mL scale *Cgr*AlcOx-OYE2 bi-enzymatic cascade. Sequential one-pot cascade reaction: first step (*Cgr*AlcOx conversion of geraniol to geranial) performed in 1 h (all reagents added except for *Bs*GDH) and subsequent second step (OYE conversion of geranial to citronellal) performed in 5 h (after addition of *Bs*GDH). Analysis by GC-FID. Reaction conditions: 20 mM geraniol (2 M in acetone), 1% v/v acetone, 40 mM glucose, 1 mM $NADP^+$, 10.67 µM OYE2, 0.5 µM catalase, 0.5 µM HPR, 6 U/mL *Bs*GDH, 1 µM *Cgr*AlcOx in 50 mM KPi pH 8, pure oxygen atmosphere in 250 mL Erlenmeyer flask, 23 °C, 200 rpm in incubator shaker, 20 mL reaction volume. **B:** zoom-in of the isolated citronellal, 95.1% *ee*. Left (*S*)-, right (*R*)-citronellal.

A



B

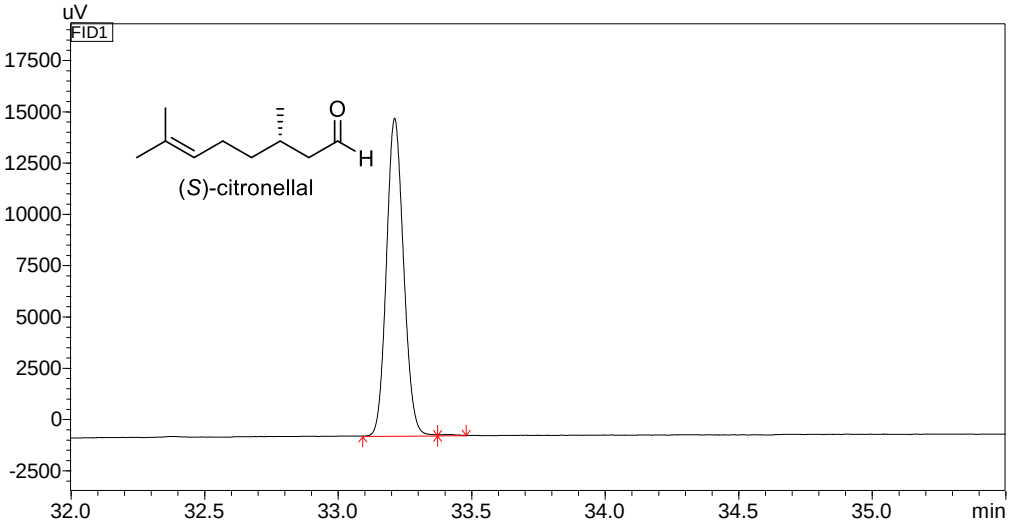


Figure S16. GC-FID chromatograms zoom-in of >99% *ee* (*S*)-citronellal obtained from geraniol catalyzed by *Cgr*AlcOx-OYE. A: with *Ts*OYE. **B:** with GluER.

8. NMR spectroscopy data

*Cgr*AlcOx-catalyzed reactions for ^{1}H NMR spectroscopy analyses were conducted as described in section 3.1 but in deuterated solvents: H_2O was replaced with D_2O; HRP (final concentration: 12 µM) and catalase (final concentration: 0.5 µM) were dissolved in D_2O; stock solution of geraniol and citral (final concentration 10 mM) were prepared in acetone-d_6 (final concentration 1% v/v). Final volume was 1 mL. After 15 min, the reactions were extracted with 1 mL of $CDCl_3$ and 500 µL of organic layer were pipetted in 5 mm NMR tubes. For standard analysis, citral (final concentration 20 mM) and geraniol (10 mM) were diluted and analyzed in acetone-d_6.^{1}H NMR spectra were recorded on Bruker Advance III-600 MHz spectrometer equipped with a triple resonance high-resolution probe producing pulse field gradients with a maximum strength of 60 G cm^{-1}. The experiment time of the standard one pulse sequence was approximately 3 min with 32 scans, an inter-scan delay of 2 sec and an acquisition time of 2.27 sec using 32k data points in the time domain. The integration value was used to calculate percent of each relevant species. The analyses were performed at 23 °C.

^{1}H and ^{13}C NMR spectra of the scale up isolated citronellal product were recorded on an Agilent 400 NMR spectrometer, internally referenced to residual proton signals in $CDCl_3$.

9. NMR spectra

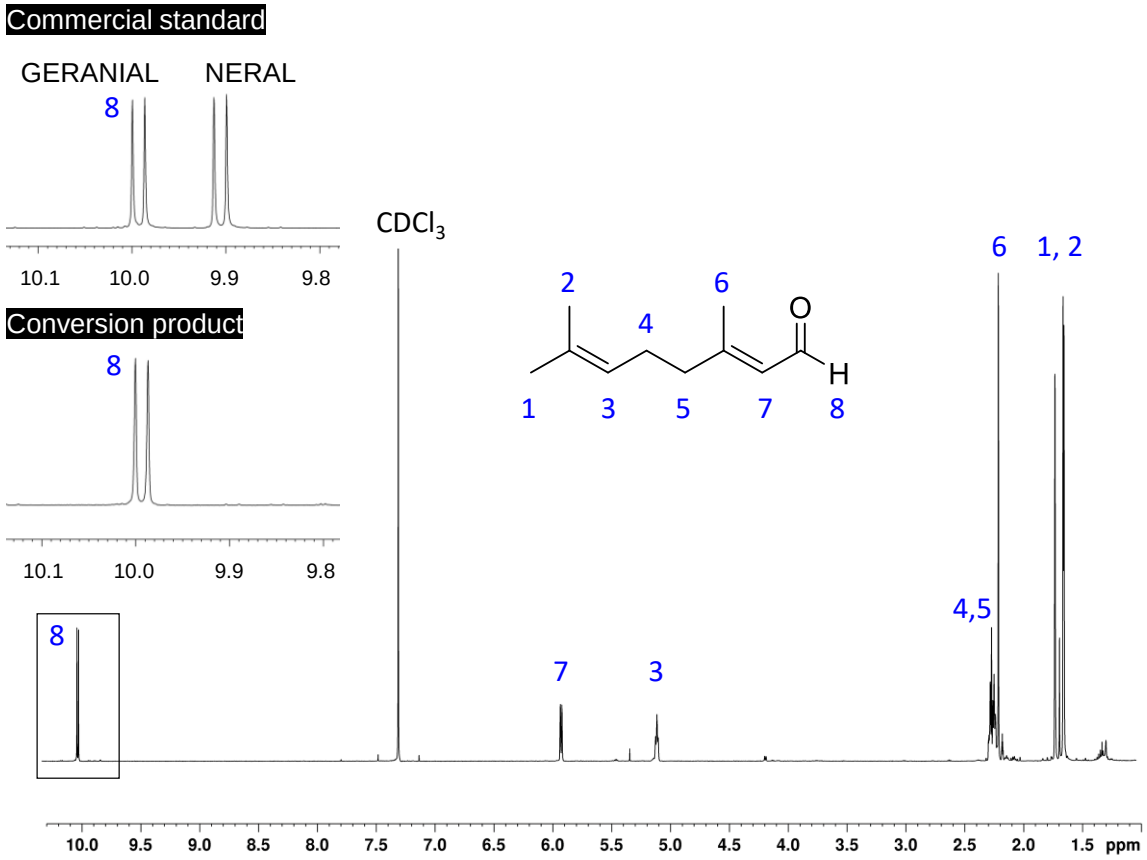


Figure S17. ^{1}H NMR spectrum of *Cgr*AlcOx-catalyzed oxidation of geraniol (10 mM) in presence of HRP (12 µM) and catalase (0.5 µM) for 15 min at 23 °C. On the left-hand side of the figure, we provide a zoom-in view of the aldehyde proton observed in the reaction sample *vs* in the citral commercial mixture (identification is based on Zeng *et al.* data[4]).

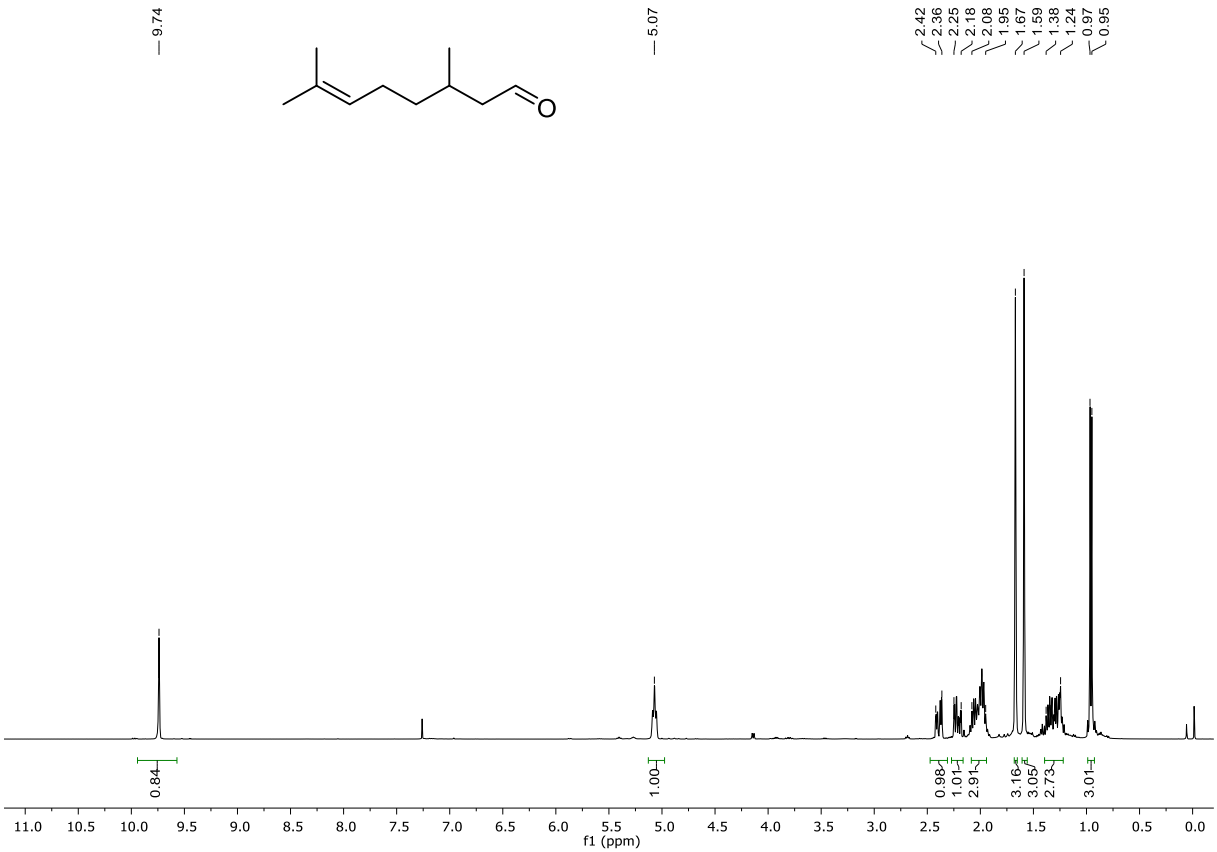


Figure S18. ^{1}H NMR spectrum of the isolated citronellal product, in $CDCl_3$ (referenced at 7.26 ppm) with tetramethylsilane (TMS), from the 20 mL scale reaction.

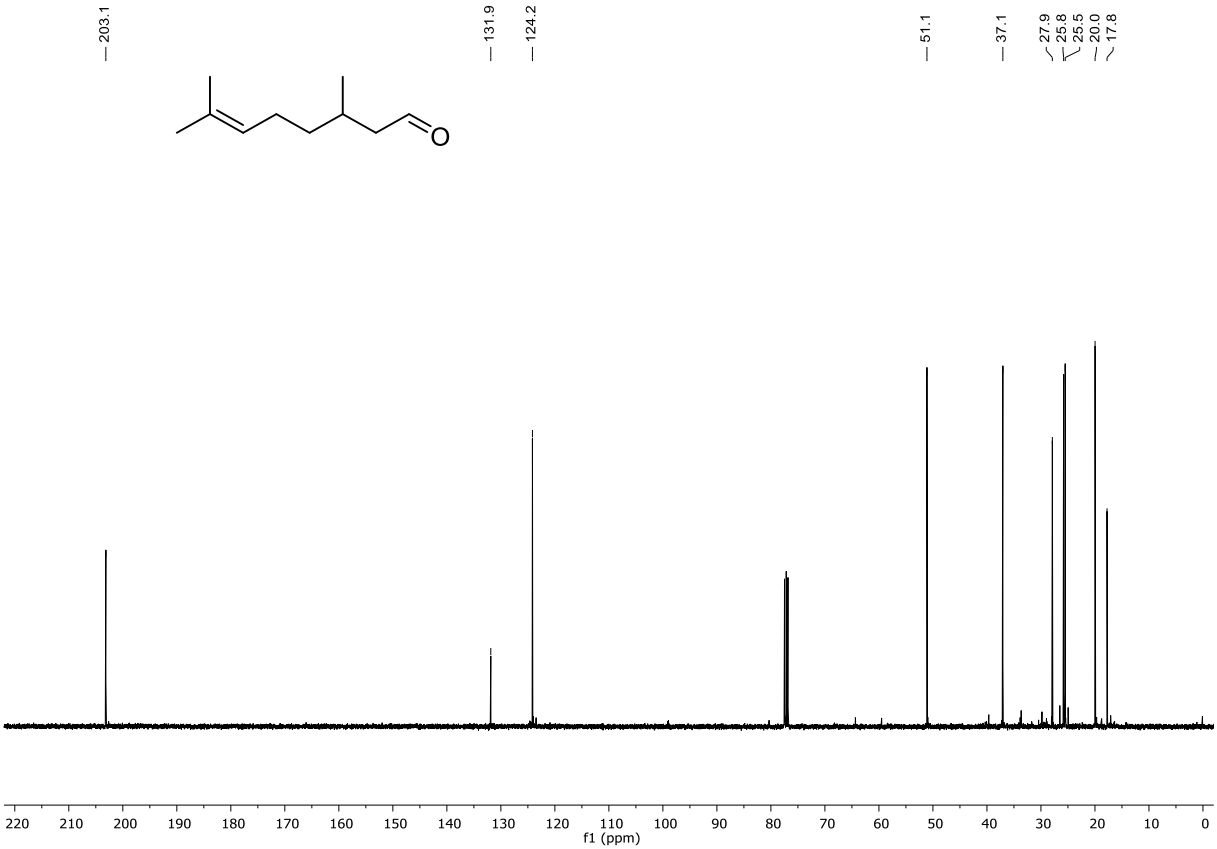


Figure S19. ^{13}C NMR spectrum of the isolated citronellal product, in $CDCl_3$ (referenced at 77.16 ppm) with TMS, from the 20 mL scale reaction.

10. References

(1) Yin, D.; Urresti, S.; Lafond, M.; Johnston, E. M.; Derikvand, F.; Ciano, L.; Berrin, J.-G.; Henrissat, B.; Walton, P. H.; Davies, G. J.; Brumer, H. Structure–Function Characterization Reveals New Catalytic Diversity in the Galactose Oxidase and Glyoxal Oxidase Family. *Nat. Commun.* **2015**, *6*, 10197.

(2) Ribeaucourt, D.; Bissaro, B.; Guallar, V.; Yemloul, M.; Haon, M.; Grisel, S.; Alphand, V.; Brumer, H.; Lambert, F.; Berrin, J.-G.; Lafond, M. Comprehensive Insights into the Production of Long Chain Aliphatic Aldehydes Using a Copper-Radical Alcohol Oxidase as Biocatalyst. *ACS Sustain. Chem. Eng.* **2021**, *9*, 4411–4421.

(3) Richter, N.; Gröger, H.; Hummel, W. Asymmetric Reduction of Activated Alkenes Using an Enoate Reductase from *Gluconobacter oxydans*. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 79–89.

(4) Zeng, S.; Kapur, A.; Patankar, M. S.; Xiong, M. P. Formulation, Characterization, and Antitumor Properties of *trans*- and c*is*-Citral in the 4T1 Breast Cancer Xenograft Mouse Model. *Pharm. Res.* **2015**, *32*, 2548–2558.

(5) Leffingwell, J.; Leffingwell, D. Chiral Chemistry in Flavours & Fragrances. *Spec. Chem. Mag.* **2011**, *2011*, 30–33.