Supporting Information

Cell-Free Total Biosynthesis of Plant Terpene Natural Products using an Orthogonal Cofactor Regeneration System

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Materials and Methods

1.1 Strains and general culture conditions

E. coli TOP10 and *E. coli* BL21(DE3) strains were used for cloning and protein expression, respectively. All *E. coli* strains were cultured in LB media at 37°C for cloning and 16°C for protein expression.

1.2 General synthesis conditions

Unless stated otherwise, reactions were conducted in flame-dried glassware under an atmosphere of nitrogen using anhydrous solvents (passed through activated alumina columns). All commercially obtained reagents were used as received. Geranyl acetate and potassium carbonate were purchased from Alfa Aesar. Selenium dioxide and *tert*-Butyl hydroperoxide solution (~5.5 M in decane, over 4Å molecular sieves) were obtained from Sigma Aldrich. Dess-Martin periodinane (DMP) was purchased from Combi-Blocks. Reaction temperatures were controlled using an IKAmag temperature modulator, and unless stated otherwise, reactions were performed at 23 °C. Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 pre-coated plates (0.25 mm) and visualized using anisaldehyde staining. Silicycle Siliaflash P60 (particle size 0.040–0.063 mm) was used for flash column chromatography.

1.3 DNA manipulations and cloning

All DNA manipulations in this study were conducted according to manufacturers' protocols. DNA restriction enzymes were used as recommended by the manufacturer (New England Biolabs, NEB). PCR was performed according to recommended protocol using Q5® High-Fidelity DNA polymerase (NEB). The primers used in this study are listed in Table S1. Plasmid DNAs were confirmed by restriction enzyme digestion checks and sequencing.

The genes for TfG8H, YkuN, and MLPL were synthesized by IDT technologies. They were then cloned into pCR-blunt vector by ligation and transformed into *E. coli TOP10* strain for storage. The genes were then amplified by using their respective primers listed in Table S1, digested with restriction enzymes NdeI and XhoI and ligated into pET28a vector linearized by NdeI and XhoI with T4 ligase (Invitrogen). The genes for GOR, ISY and NEPS1 were amplified from plasmids pJB042, pJB043 and pJB193, respectively, which were built in a previous study[1] with their respective primers listed in Table S1, and inserted into pET28a vector with digestion/ligation. The genes for FpR, FumC and MaeB were amplified from *E. coli* genomic DNA by PCR with their respective primers listed in Table S1 and inserted into pET28a vector as previously described, with HindIII being used in place of XhoI in FumC and NheI being used in place of NdeI in MaeB cloning. The primers were designed to give the proteins a hexa-His tag on their *N*-termini. The resultant plasmids were sequenced for verification and listed in Table S2. The plasmid encoding *C*-terminally hexa-His-tagged NoxE (pEUB50010) was obtained from Prof. James Bowie lab at UCLA Chemistry and Biochemistry. The full-length TfG8H plasmid (pEUB50001) was built by complementing the missing amino acids with a primer encoding appropriate overhang sequence. The resulting PCR product was inserted into pET28a as described above.

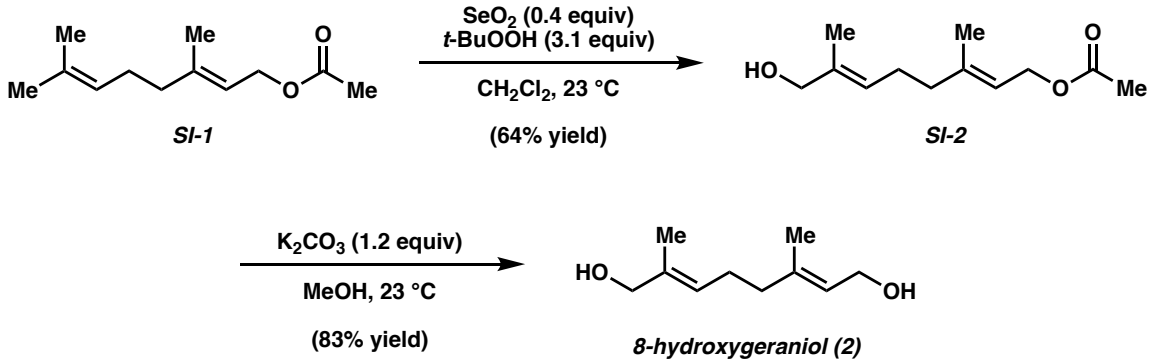
1.4 Protein expression and purification from *E. coli* BL21(DE3)

The plasmids listed in Table S3 were transformed into *E. coli* BL21(DE3) individually and the transformants were grown overnight in 5 mL of LB medium with 50 μg/mL kanamycin at 37°C. The overnight cultures were used as seed cultures for 1 L fresh LB media containing 50 μg/mL

kanamycin and incubated at 37°C until the OD_{600} reached 0.6. The cultures were cooled on ice, before the protein overexpression was induced with 0.1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG, GoldBio, USA) at 16°C overnight. Cultures were supplemented with 125 μM $FeSO_4$ for iron-containing enzymes and 80 mg/L 5'-aminolevulinic acid for P450 expression. The *E. coli* cells were harvested by centrifugation at 5300 rpm for 15 min and the cell pellets were stored at -80°C for later use. All transformants except for transformant harboring pEUB50006 were resuspended in 30 mL A10 buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, pH 8.0) containing 1 tablet of Pierce™ protease inhibitor (Thermo Scientific). The cell suspension was lysed on ice by sonication and the lysate was centrifuged at 17,000 *g* for 15 min at 4°C to remove the insoluble cellular debris. The recombinant *C*- or *N*- terminally hexa-His-tagged proteins were purified individually from corresponding soluble fractions by affinity chromatography with Ni-NTA agarose resin (Qiagen) according to the manufacturer's instructions. The purified proteins were concentrated and exchanged into storage buffer (50 mM Phosphate buffer, 100 mM NaCl, 10% glycerol, pH 8.0) with Centriprep filters (Amicon). SDS-PAGE was performed to check the protein purity and Bradford Protein Assay (Bio-Rad) was used to calculate protein concentration with bovine serum albumin (BSA, Sigma-Aldrich) as standard. The proteins were aliquoted and stored at -80°C until used in *in vitro* assays.

The cell pellet harboring pEUB50006 was resuspended in 30 mL MOPS10 buffer (50 mM MOPS, 100 mM NaCl, 10 mM imidazole, 1% Triton-X100, pH 7.5) containing 1 tablet of Pierce™ protease inhibitor (Thermo Scientific). The cell suspension was lysed on ice by sonication and the lysate was centrifuged at 17,000 *g* for 15 min at 4°C to remove the insoluble cellular debris. The recombinant *C*- terminally hexa-His-tagged protein was purified from corresponding soluble fractions by affinity chromatography with Ni-NTA agarose resin (Qiagen) according to the manufacturer's instructions. The purified protein was buffer exchanged into MOPS10 buffer with 10% glycerol with cellulose membrane. SDS-PAGE was performed to check the protein purity and Bradford Protein Assay (Bio-Rad) was used to calculate protein concentration with bovine serum albumin (BSA, Sigma-Aldrich) as standard. The protein was aliquoted and stored at -80°C until used in *in vitro* assays.

1.5 Synthesis of 8-hydroxygeraniol (2)

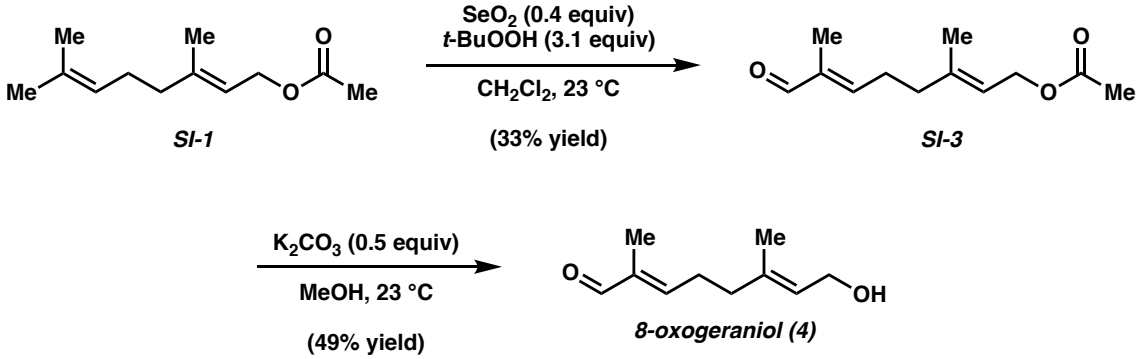


8-Hydroxygeranyl acetate (SI-2): To a flame-dried 100-mL round bottom flask equipped with a magnetic stir bar and selenium dioxide (226 mg, 2.04 mmol, 0.400 equiv) under N_2 was added CH_2Cl_2 (25 mL, 0.20 M), *tert*-butyl hydroperoxide (5.5 M in decane, 2.9 mL, 16 mmol, 3.1 equiv), and geranyl acetate (SI-1,1.09 mL, 5.09 mmol, 1.00 equiv). After stirring for 1.5 h at 23 °C, the reaction mixture was concentrated under reduced pressure. The crude oil was transferred to a separatory funnel with EtOAc (50 mL). The organic layer was washed successively with deionized water (2 x 20 mL), saturated aqueous $NaHCO_3$ (1 x 20 mL), deionized water (1 x 10 mL),

and brine (1 x 10 mL). The combined aqueous layers were extracted with EtOAc (1 x 80 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting crude oil was purified via flash chromatography (6:1 → 2:1 hexanes:EtOAc) to afford 8-hydroxygeranyl acetate (SI-2, 688 mg, 64% yield) as a colorless oil. ^{1}H-NMR spectral data match those previously reported.[2]

8-Hydroxygeraniol (2): A flame-dried 50-mL round bottom flask equipped with a magnetic stir bar was charged with 8-hydroxygeranyl acetate (SI-2, 633 mg, 2.98 mmol, 1.00 equiv) and methanol (19 mL, 0.16 M). Potassium carbonate (495 mg, 3.58 mmol, 1.20 equiv) was added in one portion. After stirring at 23 °C for 2.5 h, the solvent was removed under reduced pressure, and the reaction mixture was transferred to a separatory funnel with deionized water (10 mL). The aqueous layer was extracted with diethyl ether (3 x 20 mL). The combined organic layers were washed successively with 0.5 M aqueous HCl (1 x 10 mL), saturated aqueous $NaHCO_3$ (1 x 10 mL), brine (1 x 10 mL) and deionized water (1 x 10 mL). Next, the organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting crude oil was purified via flash chromatography (1:1 hexanes:EtOAc) to afford 8-hydroxygeraniol (2, 490 mg, 83% yield) as a light yellow oil. ^{1}H-NMR spectral data match those previously reported.[2]

1.6 Synthesis of 8-oxogeraniol (4)

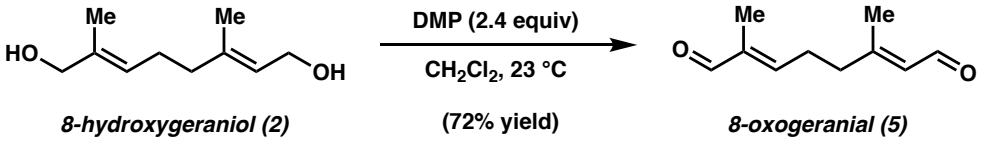


8-Oxogeranyl acetate (SI-3): A 500 mL round bottom flask was equipped with a magnetic stir bar and flame-dried then cooled under N_2. Selenium dioxide (1.36 g, 12.2 mmol, 0.400 equiv), CH_2Cl_2 (150 mL, 0.20 M), *tert*-butyl hydroperoxide (5.5 M in decane, 17 mL, 95 mmol, 3.1 equiv), and geranyl acetate (**SI-1**, 6.55 mL, 30.6 mmol, 1.00 equiv) were added to the flask. After stirring for 21 h at 23 °C, the reaction mixture was concentrated to an oil under reduced pressure. To the crude oil, EtOAc (100 mL) was added and the reaction was transferred to a separatory funnel. The organic layer was washed successively with deionized water (2 x 20 mL), saturated aqueous $NaHCO_3$ (1 x 20 mL), deionized water (1 x 20 mL), and brine (1 x 20 mL). The combined aqueous layers were extracted with EtOAc (100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude oil was purified via flash chromatography (6:1 hexanes:EtOAc) to afford 8-oxogeranyl acetate (**SI-3**, 2.14 g, 33% yield) as a colorless oil. ^{1}H-NMR spectral data match those previously reported.[3]

8-Oxogeraniol (4): To a flame-dried 25 mL round bottom flask charged with a stir bar and cooled under N_2, 8-oxogeranyl acetate (**SI-3**, 309 mg, 1.47 mmol, 1.00 equiv) was dissolved in methanol (9.2 mL, 0.16 M). To the stirring solution was added potassium carbonate (102 mg, 0.736 mmol, 0.500 equiv) and the solution was stirred at 23 °C for 2.5 h. After 2.5 h, the solvent was removed under reduced pressure, deionized water (10 mL) and diethyl ether (10 mL) were

added to the crude oil, and the reaction was transferred to a separatory funnel. The layers were separated and the aqueous layer was extracted with diethyl ether (3 x 10 mL). The combined organic layers were washed with brine (1 x 10 mL). The organic layers were then dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude oil was purified by flash chromatography (2:1 hexanes:EtOAc) to afford 8-oxogeraniol (**4**, 122 mg, 49% yield) as a light yellow oil. ^{1}H-NMR spectral data match those previously reported.[3]

1.7 Synthesis of 8-oxogeranial (5)



8-hydroxygeraniol (2) (72% yield) *8-oxogeranial (5)*

8-Oxogeranial (5): To a flame-dried 250 mL round bottom flask equipped with a stir bar and cooled under N_2, 8-hydroxygeraniol (2, 1.00 g, 5.87 mmol, 1.00 equiv), DMP (5.98 g, 14.1 mmol, 2.40 equiv), and CH_2Cl_2 (59 mL, 0.10 M) were added. The flask was purged with N_2 and stirred at 23 °C for 1 h. After 1 h, the reaction was quenched with 1:1:1 sat. aq. $NaHCO_3$:sat. aq. $Na_2S_2O_3$:H_2O (60 mL) and was transferred to a separatory funnel. The aqueous layer was extracted with CH_2Cl_2 (3 x 60 mL). The combined organic layers were dried over Mg_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified via flash chromatography (4:1 Hex:EtOAc) to afford 8-oxogeranial (5, 705 mg, 72% yield) as a light yellow oil. ^{1}H-NMR spectral data match those previously reported.[3]

1.8 Small-scale *in vitro* enzymatic reactions

1.8.1 TfG8H *in vitro* assays

200 µL-scale *in vitro* assays were performed at 30°C for 2 hours in 100 mM Bis-Tris-Propane buffer (pH 9.0, unless otherwise indicated) generally containing 2 mM geraniol, 5 µM TfG8H, 10 µM FpR, 10 µM YkuN and 4 mM NADPH, unless otherwise indicated. Reactions with NADPH regeneration contain an additional 6 mM fumarate, 1 µM FumC and 10 µM MaeB. The reaction was stopped by extracting directly with 100 µL ethyl acetate (EtoAc) and centrifuged at 17,000 *g* for 5 min before the organic layer was subjected to GC/MS analysis on an Agilent Technologies GC-MS 6890/5973 equipped with a DB-FFAP column. An inlet temperature of 220 °C and constant pressure of 4.2 psi were used. The oven temperature was held at 60 °C for 5 min and then ramped at 60°C/min for 1.5 min, followed by a ramp of 15 °C/min for 16 min and a hold for 10 min.

1.8.2 GOR *in vitro* assays

200 µL-scale *in vitro* assays were performed at 30°C for 2 hours in 100 mM Bis-Tris-Propane buffer (pH 9.0) containing 2 mM 8-hydroxygeraniol, 10 µM GOR and 4 mM nicotinamide dinucleotide (NAD^+, Cayman Chemicals) unless otherwise specified. Reactions with NAD^+ regeneration contain 5 µM NoxE. The reactions were stopped by extracting directly with 100 µL ethyl acetate (EtoAc) and centrifuged at 17,000 *g* for 5 min before the organic layer was subjected to GC/MS analysis.

1.8.3 ISY and NmMLPL *in vitro* assays

200 µL-scale *in vitro* assays were performed at 30°C for 2 hours in 100 mM Bis-Tris-Propane buffer (pH 9.0) containing 2 mM 8-oxogeranial, 1 µM ISY, 10 µM NmMLPL and 4 mM NADPH unless otherwise specified. Reactions with NADPH regeneration contain an additional 6 mM fumarate, 1 µM FumC and 10 µM MaeB. The reactions were stopped by extracting directly with 100 µL ethyl acetate (EtoAc) and centrifuged at 17,000 *g* for 5 min before the organic layer was subjected to GC/MS analysis as described previously.

1.8.4 NoxE and FumC/MaeB cofactor selectivity assay

For NoxE cofactor selectivity, 200 µL-scale *in vitro* assays were performed in a 96-well plate (Corning® 96 Well plate, Sigma-Aldrich) at 30°C in 100 mM Phosphate buffer (pH 7.5) containing 1 µM NoxE, 400 µM NAD^+ or $NADP^+$. The absorbance at 340 nm was measured continuously for 2 hours.

For MaeB cofactor selectivity, 200 µL-scale *in vitro* assays were performed in a 96-well plate (Corning® 96 Well plate, Sigma-Aldrich) at 30°C in 100 mM Phosphate buffer (pH 7.5) containing 4 mM fumarate, 1 µM FumC, 10 µM MaeB, 4 mM NADH or NADPH. The absorbance at 340 nm was measured continuously for 2 hours.

1.8.5 Small-scale one-step or two-step one-pot reactions

200 µL-scale one-pot one-step *in vitro* assays were performed at 30°C for 2 hours in 100 mM Bis-Tris-Propane buffer (pH 9.0, unless otherwise indicated) containing 2 mM geraniol, 12 mM fumarate, 5 µM TfG8H, 10 µM FpR, 10 µM YkuN, 10 µM GOR, 0.5 µM ISY, 5 µM NmMLPL, 1 µM FumC, 10 µM MaeB, 5 µM NoxE, 100 µM NADPH and 100 µM NAD^+. The one-pot two-step *in vitro* assays were performed by directly adding 0.5 µM ISY, 5 µM NmMLPL and 6 mM fumarate after the conclusion of 2 hours reaction with 2 mM geraniol, 6 mM fumarate, 5 µM TfG8H-full, 10 µM FpR, 10 µM YkuN, 10 µM GOR, 1 µM FumC, 10 µM MaeB, 5 µM NoxE, 100 µM NADPH and 100 µM NAD^+. The reactions were then stopped by extracting directly with 100 µL EtoAc and centrifuged at 17,000 *g* for 5 min before the organic layer was subjected to GC/MS analysis as described above.

1.9. 10 mL-scale nepetalactol and nepetalactone production and purification

10 mL-scale one-pot two-step *in vitro* assays were performed at 28°C, 250 rpm in a 50 mL falcon tube by directly adding 0.5 µM ISY, 5 µM MLPL and 6 mM fumarate after the conclusion of 2 hours reaction with 2 mM geraniol, 6 mM fumarate, 5 µM TfG8H, 10 µM FpR, 10 µM YkuN, 10 µM GOR, 1 µM FumC, 10 µM MaeB, 100 µM NADPH and 100 µM NAD^+.

The 10 mL-scale one-pot drop-in *in vitro* assays were performed by first directly adding 10 µM GOR, 5 µM NoxE and 100 µM NAD^+ after the conclusion of 2 hours reaction with 2 mM geraniol, 6 mM fumarate, 5 µM TfG8H, 10 µM FpR, 10 µM YkuN, 1 µM FumC, 10 µM MaeB and 100 µM NADPH. Then 0.5 µM ISY, 5 µM NmMLPL and 6 mM fumarate was added after the conclusion of 2 hours reaction with GOR. Aliquots of 2 mM geraniol and 6 mM fumarate was added to 5 µM TfG8H, 10 µM FpR, 10 µM YkuN, 1 µM FumC, 10 µM MaeB and 100 µM NADPH and the reactions were run for 90 minutes each before GC/MS analysis in order to assess the *in-vitro* system's efficiency and robustness. For nepetalactone production 10 µM NEPS1 was added to the system at the same time as ISY and MLPL is added. All reactions were stopped by extracting directly with 5 mL EtoAc and centrifuged at 17,000 *g* for 5 min

before the organic layer was subjected to GC/MS analysis as described above. The organic layer was dried, leaving nepetalactol oil.

Supporting Tables

Table S1. Primers used in this study

Name	Sequence (5' – 3')	Used in construction of plasmid
pEUB50001 F	ttatcatatgggacagtcccgccgaccccacaccgtctac-ctcgatccggccaaaggatcgacatccccgcgcagcgacgcga actcctcgacaaaggcccggtggtacgcgtggctttccccggc	pEUB50001
pEUB50001 R	aatactcgagttacggcttcgggcgtaacag	pEUB50001
pEUB50002 F	aatagctagcatggctgattgggtaacaggcaaag	pEUB50002
pEUB50002 R	aatactcgagttaccagtaatgctccgctgtcatatg	pEUB50002
pEUB50003 F	aatacatatggcgaaggcattgatcacatac	pEUB50003
pEUB50003 R	aatactcgagttaactgacgtggatcttctctttgttcatg	pEUB50003
pEUB50004 F	cagccatatgactaaaactaattctccagccccatc	pEUB50004
pEUB50004 R	ggtgctcgagtgaacttaataacaactttgacacagtctggg	pEUB50004
pEUB50005 F	cagccatatgtcctggtggtggaaaaggtc	pEUB50005
pEUB50005 R	ggtgctcgagttggaatgaatctgtagtctctcatcttgtcg	pEUB50005
pEUB50006 F	ttatcatatgatggcgtcgaaactgg	pEUB50006
pEUB50006 R	ttatctcgagctaattttgacaagtgtgattcataccctt	pEUB50006
pEUB50007 F	tggacccatatgaatacagtacgcagcgaaaaagattcg	pEUB50007
pEUB50007 R	tggaccaagcttttaacgcccggctttcatactgc	pEUB50007
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pEUB50008 R	tggcaactcgagttacagcggttgggtttgcg	pEUB50008
pEUB50009 F	taatgctagcatggcatccacagcgaacc	pEUB50009
pEUB50009 R	taatgagctcctatgacggtgcgaagaaaggtaaac	pEUB50009

Table S2. Gene sequences used in this study

>TfG8H (pEUB50001)
ATGGGACAGTCCCGCCGACCCCACACCGTCTACCTCGATCCGGCCAAAGGAGTCGACATCCCCGCGCAG
CGACGCGAACTCCTCGACAAAGGCCCGGTGGTACGCGTGGCTTTCCCCGGCAATTTGGAGGTCTGGGCA
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>FpR (pEUB50002)
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>YkuN (pEUB50003)
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GTATATCAGGAAACTTTGAAAATCGAGTTGGCACCTGAGACCGACGAAGATGTTGAATCATGTCGTGCC
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>GOR (pEUB50004)
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>ISY (pEUB50005)
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TAA

Table S3. Plasmids used in this study

Plasmid	Vector	Encoded protein
pET-28a	pET-28a	vector only
pET-22a	pET-22a	vector only
pEUB50001	pET-28a	T7-*N*-his-TfG8H
pEUB50002	pET-28a	T7-*N*-his-FpR
pEUB50003	pET-28a	T7-*N*-his-YkuN
pEUB50004	pET-28a	T7-*N*-his-GOR
pEUB50005	pET-28a	T7-*N*-his-ISY
pEUB50006	pET-28a	T7-*N*-his-MLPL
pEUB50007	pET-28a	T7-*N*-his-FumC
pEUB50008	pET-28a	T7-*N*-his-MaeB
pEUB50009	pET-28a	T7-*N*-his-NEPS1
pEUB50010	pET-22a	T7-NoxE-*C*-his

Table S4. Cost analysis for 1 g nepetalactol production

component	mass per 1 g nepetalactol (g)	component cost ($/g)	cost per 1 g nepetalactol ($)	commercial source
geraniol	0.92	$0.54	$0.53	Fisher Scientific
enzyme	2.28	$2.50 (low) $25.00 (high)	$6.13 (low) $61.29 (high)	*
NADPH	0.076	$450.00	$36.77	Calzyme
NAD^+	0.068	$14.00	$1.02	Calzyme
fumarate	0.68	$0.04	$0.03	Fisher Scientific
BSA	0.123	$31.70	$4.19	Fisher Scientific
bis-tris propane	28.4	$0.60	$18.32	Fisher Scientific
Total material cost to make 1 g nepetalactol			**$67.01 (low) $122.17 (high)**	

* Enzyme cost at scale is estimated to be between $250 and $2500 per kilogram based on communication with industry. For our material cost estimate, we have taken the upper bound ($2.50/g) as our low price point and a 10-fold increase ($25.00/g) as our high price point to account for the absence of economies of scale.

Supporting Figures

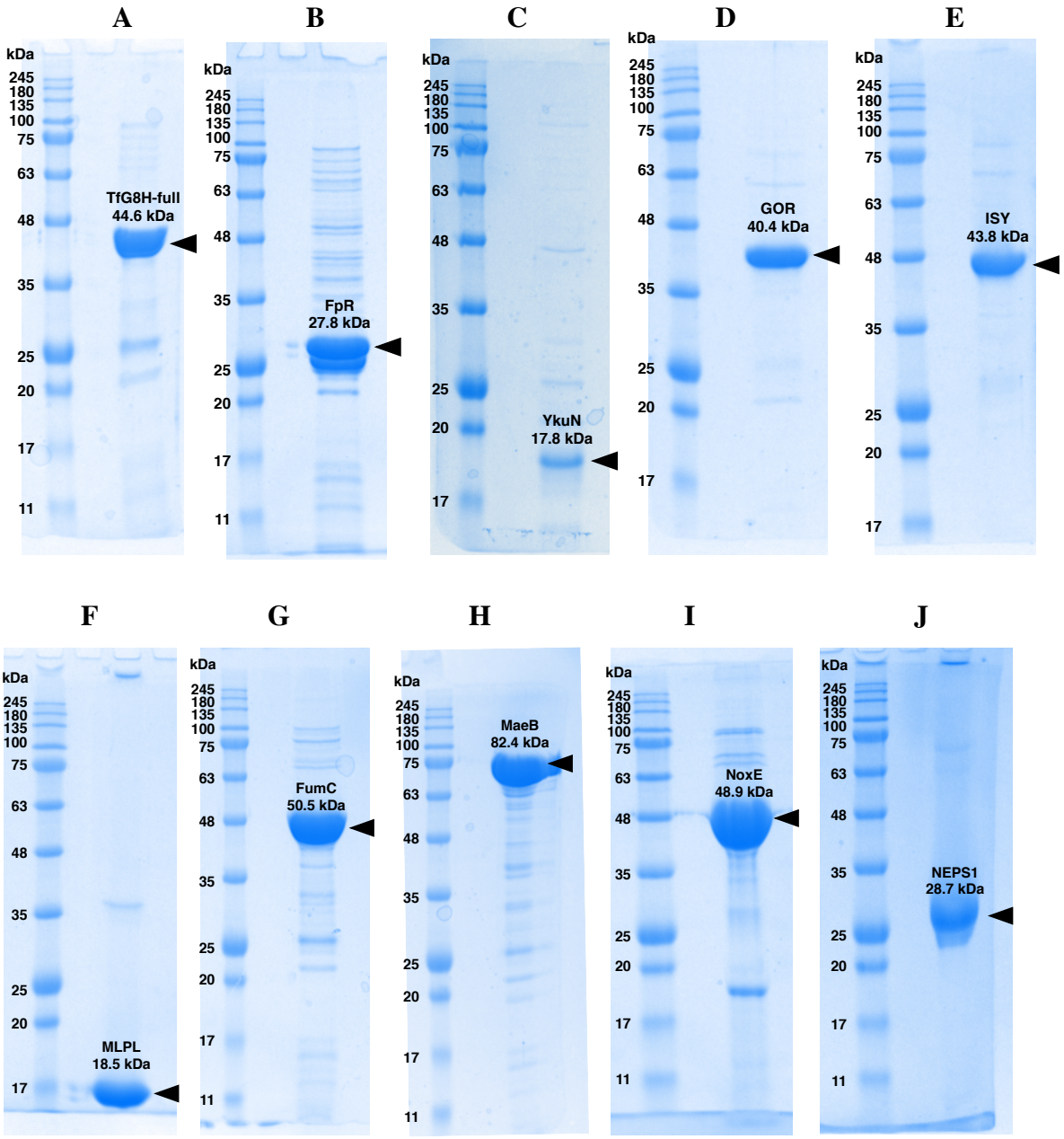

Figure S1. SDS-PAGE of purified proteins A. TfG8H, B. FpR, C. YkuN, D. GOR, E. ISY, F. MLPL, G. FumC, H. MaeB, I. NoxE. J. NEPS1

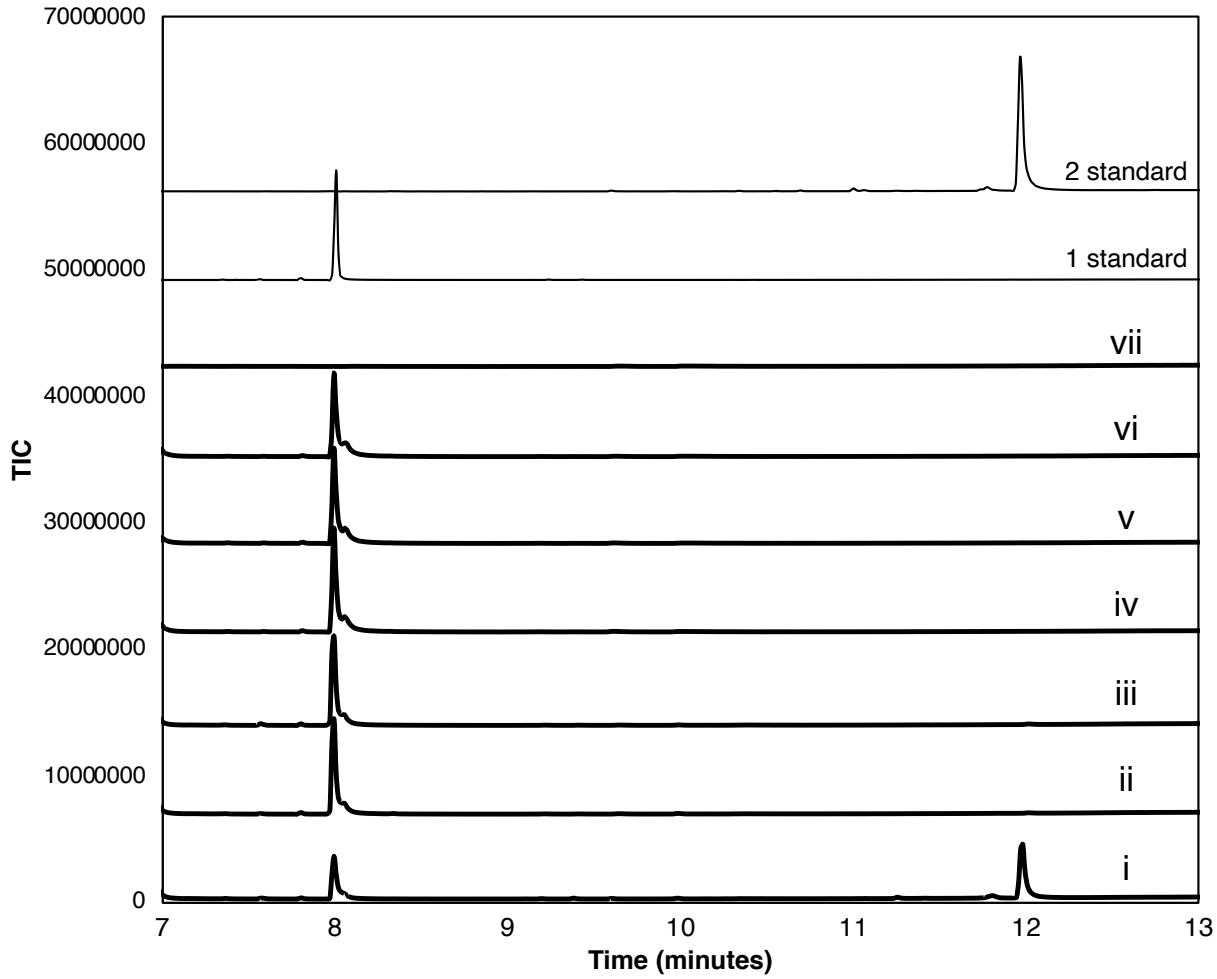

Figure S2. GC/MS analysis of geraniol hydroxylation activity of TfG8H. Each reaction contains 2 mM geraniol, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 4 mM NADPH unless otherwise specified, i. sample reaction, ii. only 100 μM NADPH, iii. no TfG8H, iv. no FpR, v. no YkuN, vi. no NADPH, vii. no geraniol.

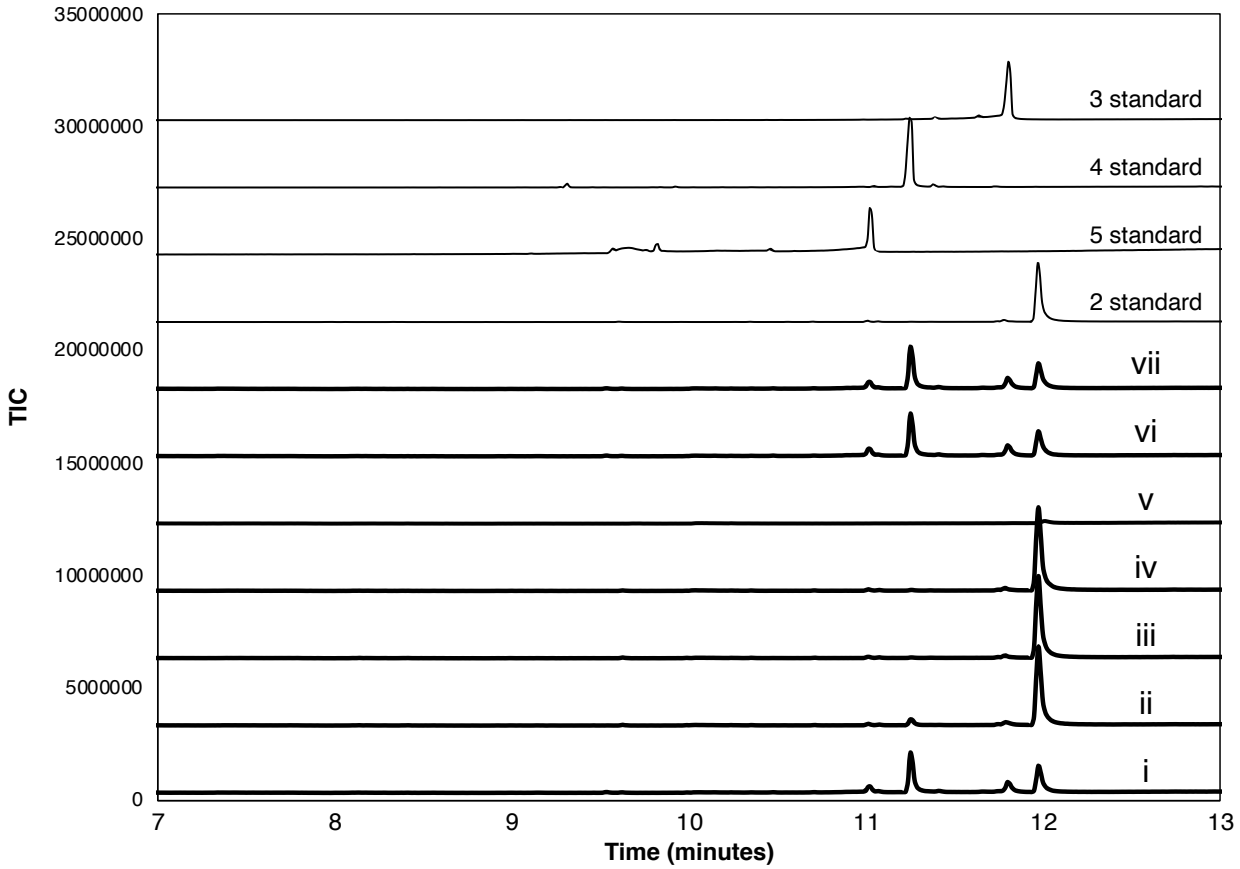

Figure S3. GC/MS analysis of 8-hydroxygeraniol oxidation by GOR. Each reaction contains 2 mM 8-hydroxygeraniol, 10 μM GOR and 4 mM NAD^+ in BTP buffer (pH 9.0) unless otherwise specified, i. sample reaction, ii. only 100 μM NAD^+, iii. no GOR, iv. no NAD^+, v. no 8-hydroxygeraniol, vi. Phosphate buffer pH 7.5, vii. Tris-HCl buffer pH 8.0.

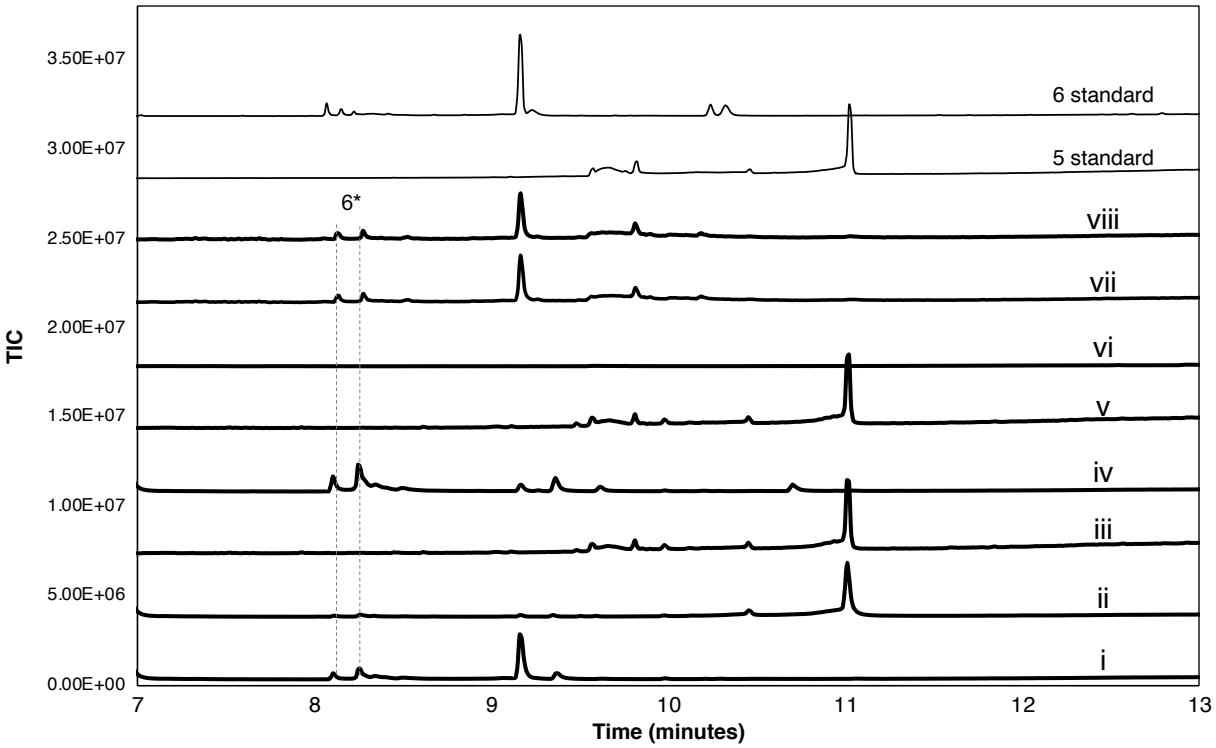

Figure S4. GC/MS analysis of 8-oxogeranial reduction and cyclization by ISY and MLPL. Each reaction contains 2 mM 8-oxogeranial, 1 μM ISY, 10 μM MLPL and 4 mM NADPH in BTP buffer (pH 9.0) unless otherwise specified, i. sample reaction, ii. only 100 μM NADPH, iii. no ISY, iv. no MLPL, v. no NADPH, vi. no 8-oxogeranial, vii. Phosphate buffer pH 7.5, viii. Tris-HCl buffer pH 8.0.

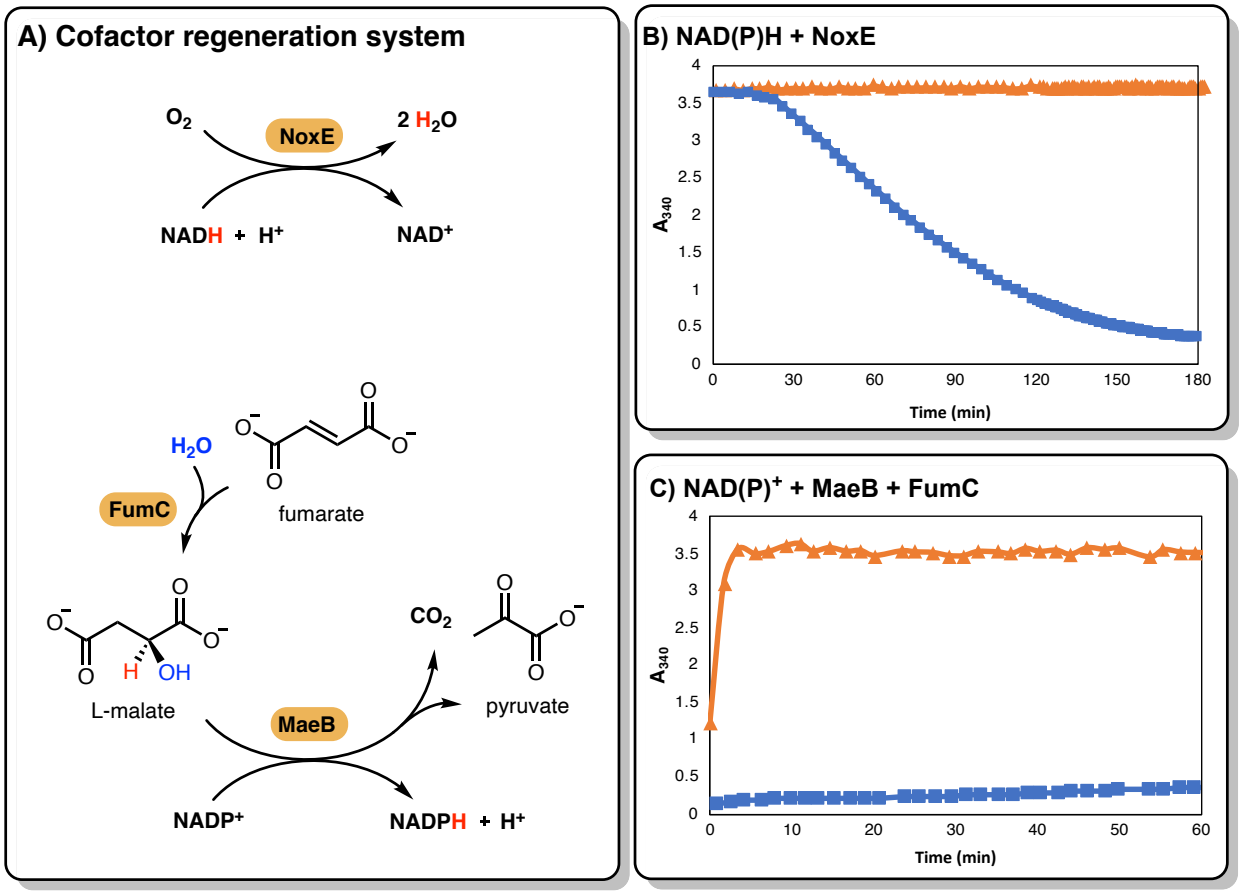

Figure S5. (A) Cofactor regeneration systems (B) NoxE is used as a NAD+ regeneration enzyme. Substrate specificity for NADH (blue) over NADPH (orange) is confirmed when 1 μM NoxE, 400 μM NAD^+ or $NADP^+$ was used; (C) FumC/MaeB pair is used as a NADPH regeneration system. Substrate specificity for $NADP^+$ over NAD^+ is confirmed when 4 mM fumarate, 1 μM FumC, 10 μM MaeB, 4 mM NADH or NADPH was used. Both assays in (B) and (C) were performed by spectroscopically at λ=340 nm.

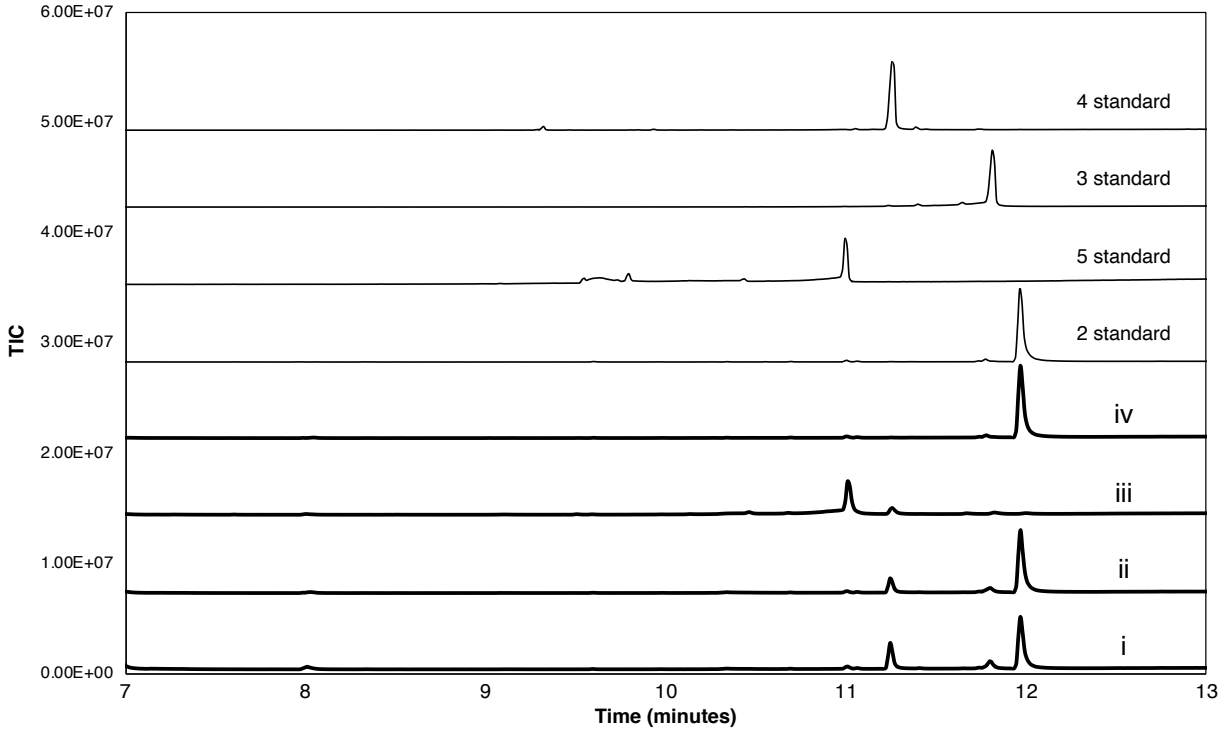

Figure S6. GC/MS analysis of 8-hydroxygeraniol oxidation with GOR with NAD^+ regeneration. Each reaction contains 2 mM 8-hydroxygeraniol, 10 μM GOR, 5 μM NoxE and 4 mM NAD^+ in BTP buffer (pH 9.0) unless otherwise specified, i. no NoxE, ii. no NoxE with only 100 μM NAD^+, iii. with NoxE and only 100 μM NAD^+, iv. no GOR.

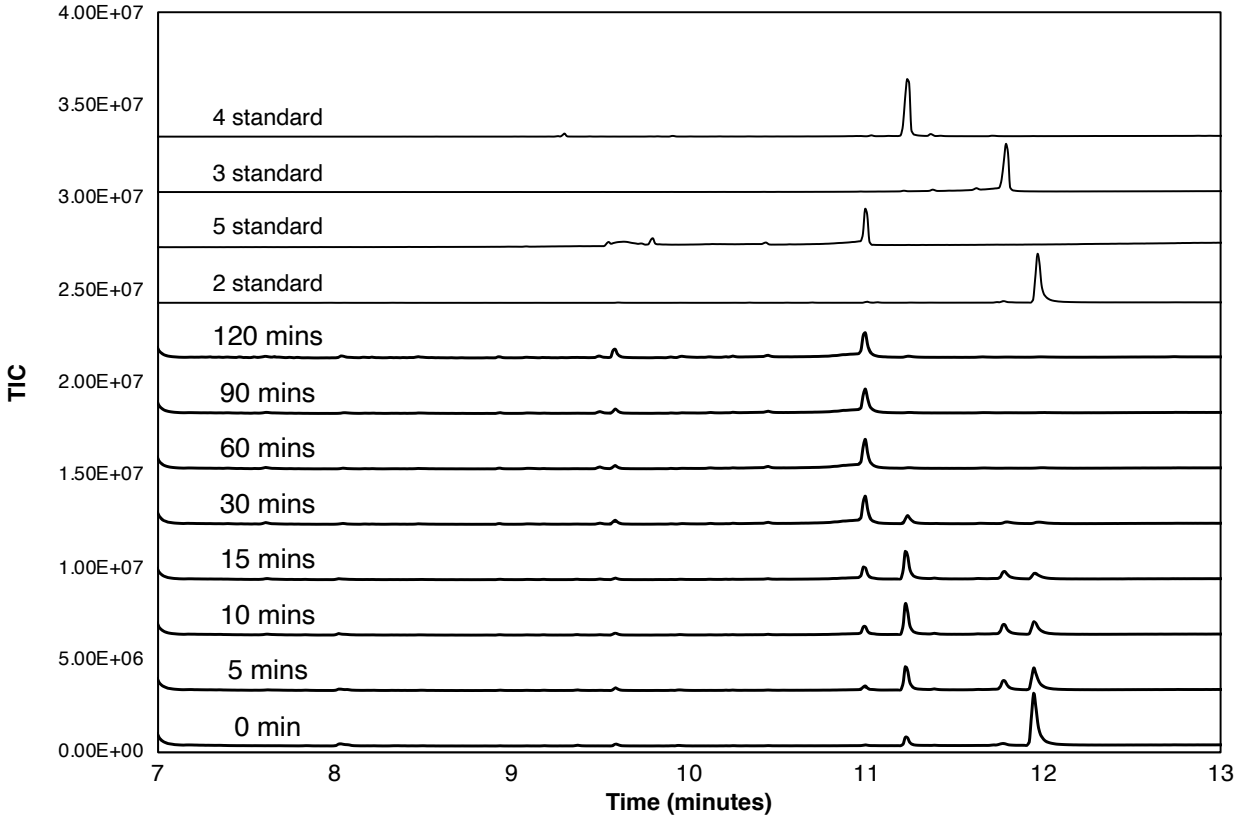

Figure S7. Time-course of 8-hydroxygeraniol oxidation with GOR with NAD^+ regeneration. Each reaction contains 2 mM 8-hydroxygeraniol, 10 μM GOR, 5 μM NoxE and 100 μM NAD^+ in BTP buffer (pH 9.0).

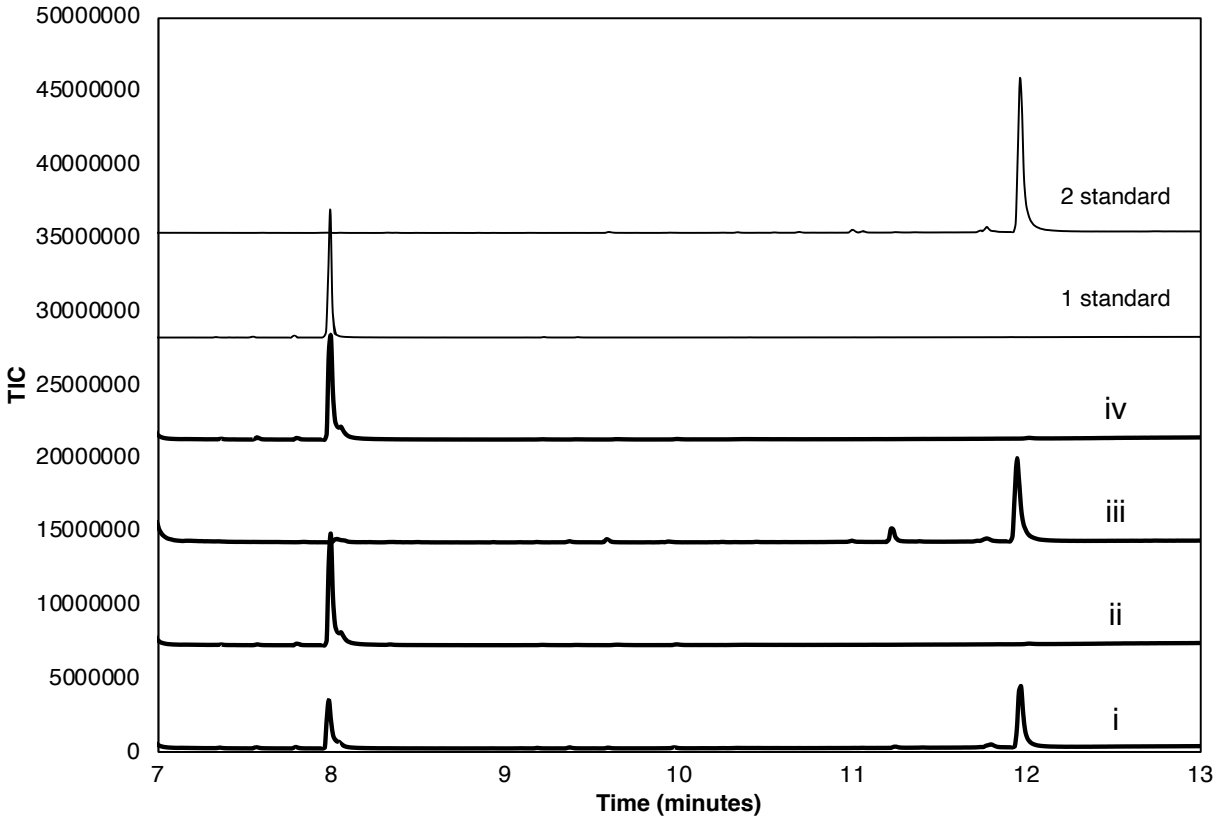

Figure S8. Geraniol hydroxylation by TfG8H with NADPH regeneration. Each reaction contains 2 mM geraniol, 6 mM fumarate, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 1 μM FumC, 10 μM MaeB and 4 mM NADPH in BTP buffer (pH 9.0) unless otherwise specified, i. no FumC/MaeB, ii. no FumC/MaeB with only 100 μM NADPH, iii. with FumC/MaeB and only 100 μM NADPH, iv. no TfG8H.

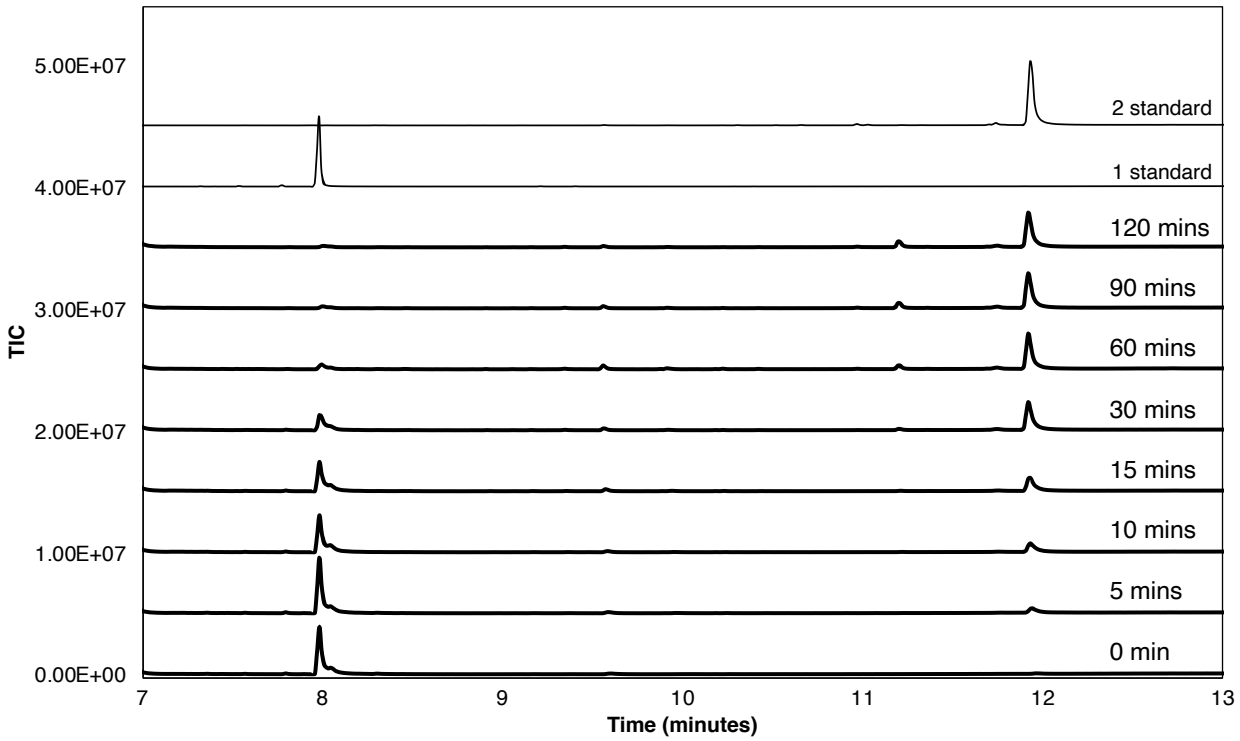

Figure S9. Time-course of geraniol hydroxylation by TfG8H with NADPH regeneration. Each reaction contains 2 mM geraniol, 6 mM fumarate, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 1 μM FumC, 10 μM MaeB and 100 μM NADPH in BTP buffer (pH 9.0).

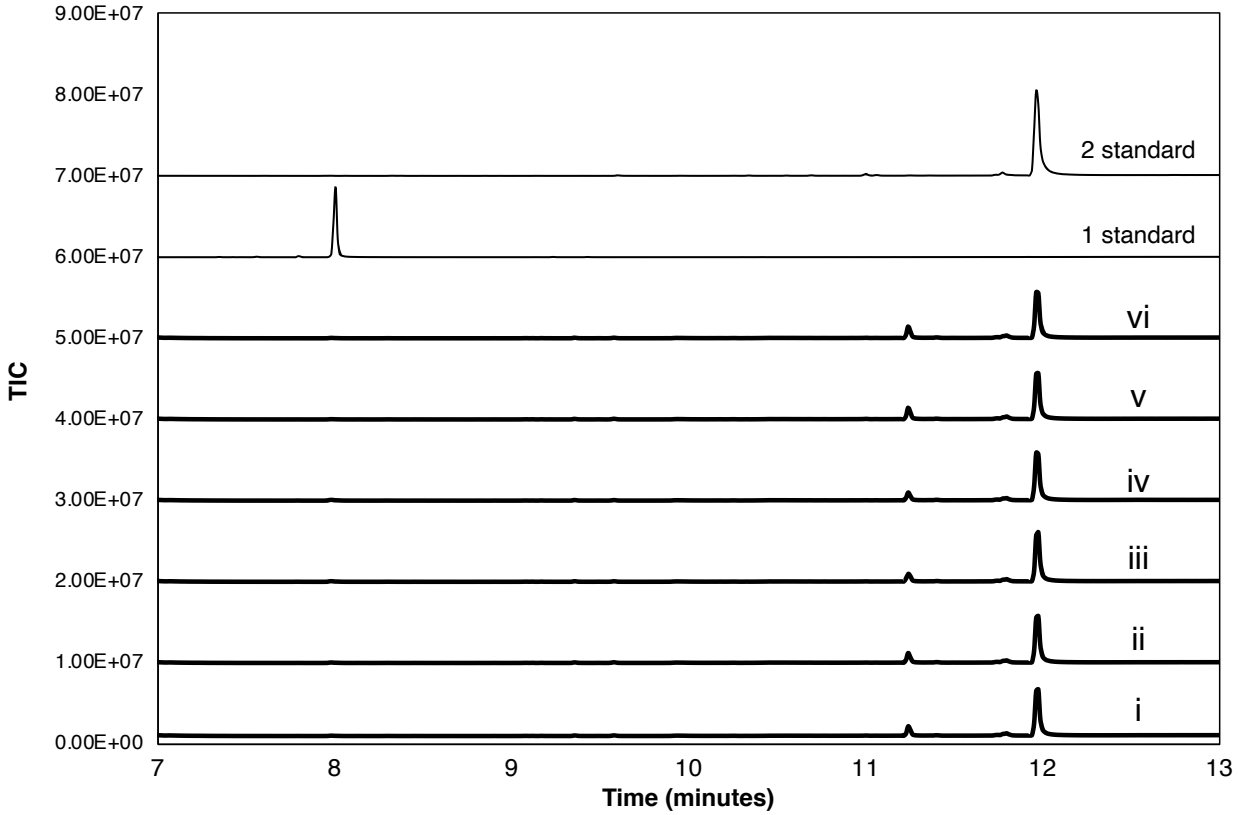

Figure S10. Geraniol hydroxylation by TfG8H with NADPH regeneration with different concentrations of pyruvate. Each reaction contains 2 mM geraniol, 6 mM fumarate, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 1 μM FumC, 10 μM MaeB, 100 μM NADPH and different concentrations of pyruvate in BTP buffer (pH 9.0). i. 0 mM pyruvate, ii. 2 mM pyruvate, iii. 4 mM pyruvate, iv. 6 mM pyruvate, v. 9 mM pyruvate, vi. 12 mM pyruvate.

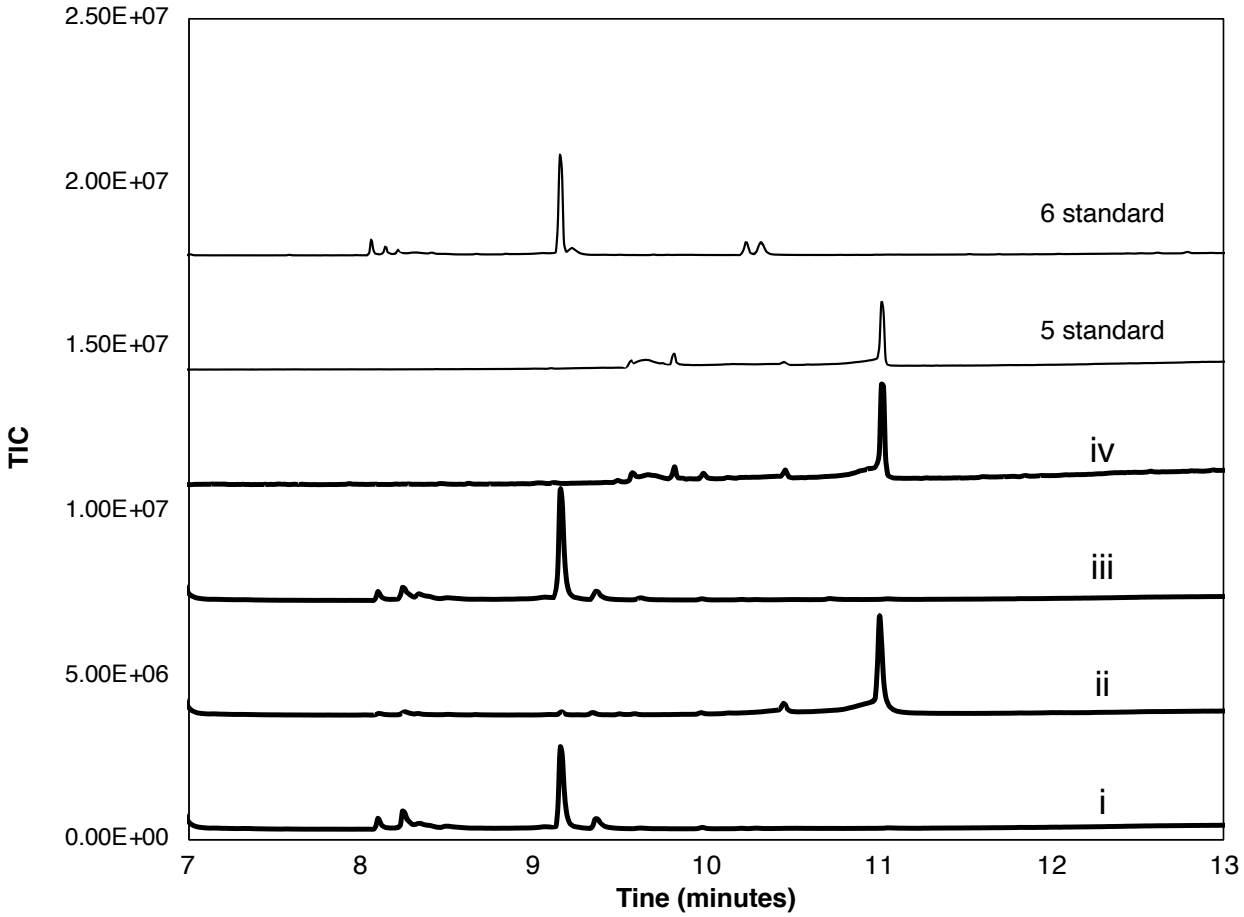

Figure S11. 8-oxogeranial reduction and cyclization by ISY/MLPL with NADPH regeneration. Each reaction contains 2 mM 8-oxogeranial, 6 mM fumarate, 0.5 μM ISY, 5 μM MLPL, 1 μM FumC, 10 μM MaeB and 4 mM NADPH in BTP buffer (pH 9.0) unless otherwise specified, i. no FumC/MaeB, ii. no FumC/MaeB with only 100 μM NADPH, iii. with FumC/MaeB and only 100 μM NADPH, iv. no ISY/MLPL.

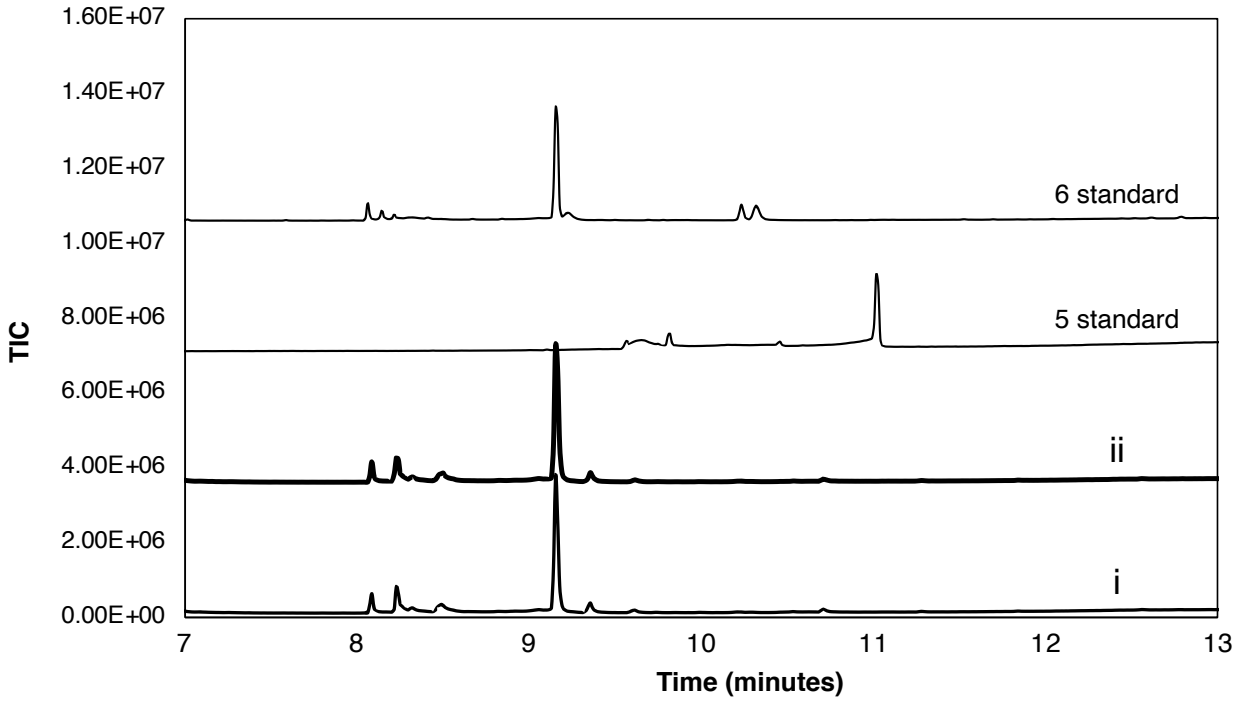

Figure S12. 8-oxogeranial reduction and cyclization by ISY/MLPL with NADPH regeneration with additional pyruvate. Each reaction contains 2 mM 8-oxogeranial, 6 mM fumarate, 0.5 μM ISY, 5 μM MLPL, 1 μM FumC, 10 μM MaeB and 100 μM NADPH in BTP buffer (pH 9.0), i. no pyruvate, ii. 12 mM pyruvate.

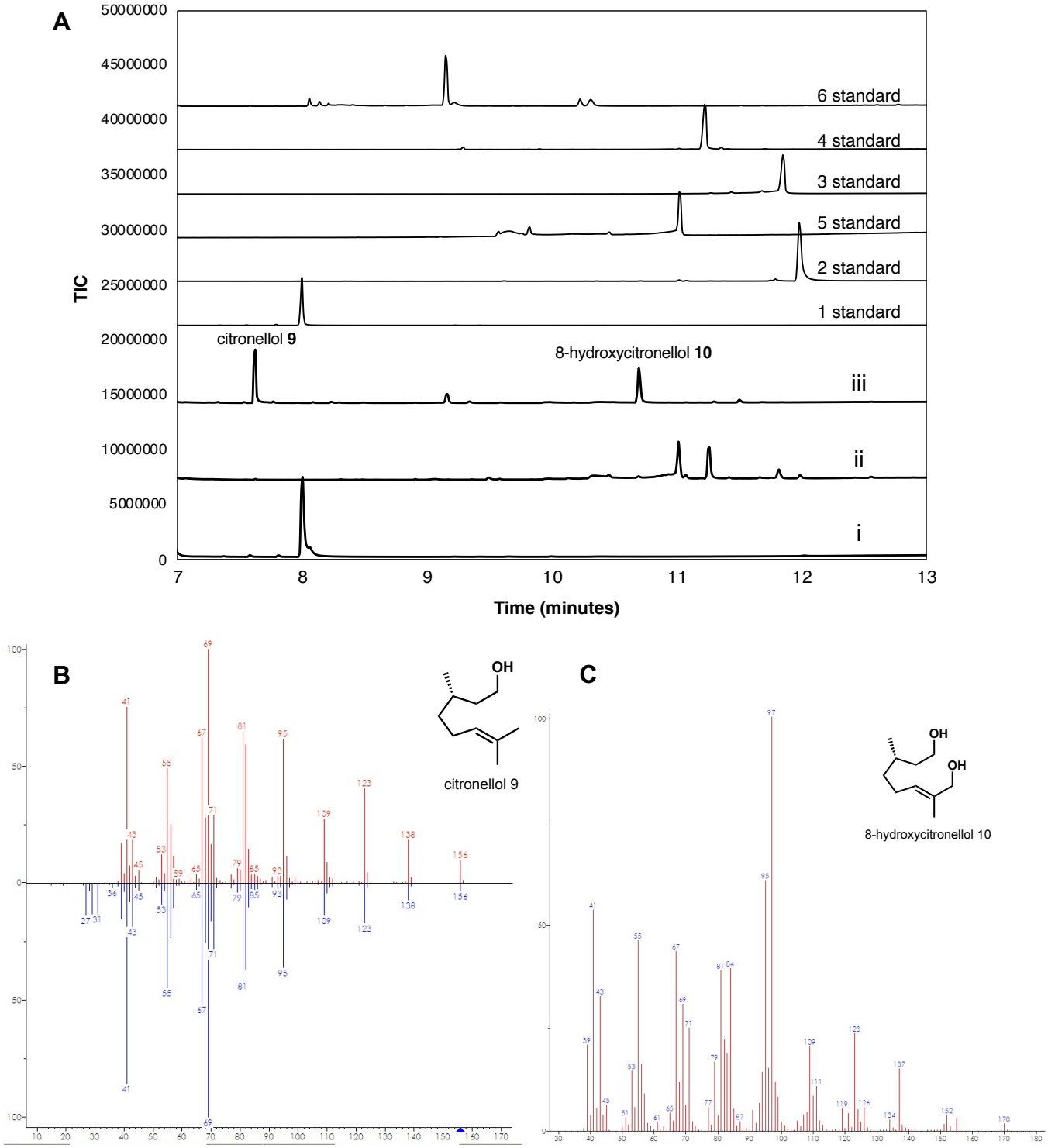

Figure S13. Small-scale one-pot one-step bioconversion of geraniol to nepetalactol. **A.** Each reaction contains 2 mM geraniol, 6 mM fumarate, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 10 μM GOR, 0.5 μM ISY, 5 μM MLPL, 1 μM FumC, 10 μM MaeB, 100 μM NADPH and 100 μM NAD^+ in BTP buffer (pH 9.0) unless otherwise specified, i.0 min, ii. no ISY, iii. with ISY**.** Mass fragmentation spectrum of citronellol 9 (**B**), and 8-hydroxycitronellol 10 (**C**). Red fragmentations depict patterns of compound produced in the reaction mixture from Figure S13, whereas blue fragmentations depict the compound fragmentation in GC-MS library search.

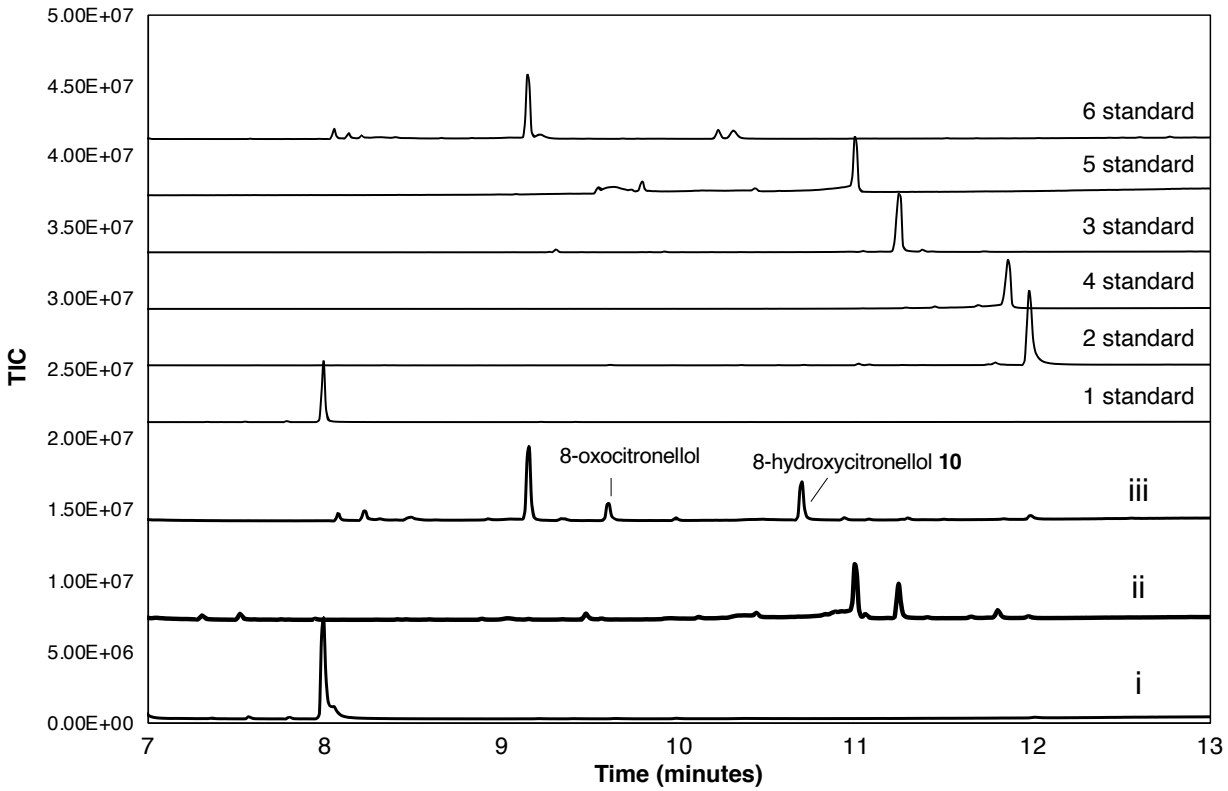

Figure S14. Small-scale one-pot two-step geraniol to nepetalactol conversion. Each reaction contains 2 mM geraniol, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 10 μM GOR, 0.5 μM ISY, 5 μM MLPL, 1 μM FumC, 10 μM MaeB, 100 μM NADPH, 100 μM NAD^+ and 6 mM fumarate in BTP buffer (pH 9.0) unless otherwise specified, i.0 min, ii. no ISY, iii. ISY/MLPL added after 2 hours of TfG8H/FpR/YkuN and GOR reaction.

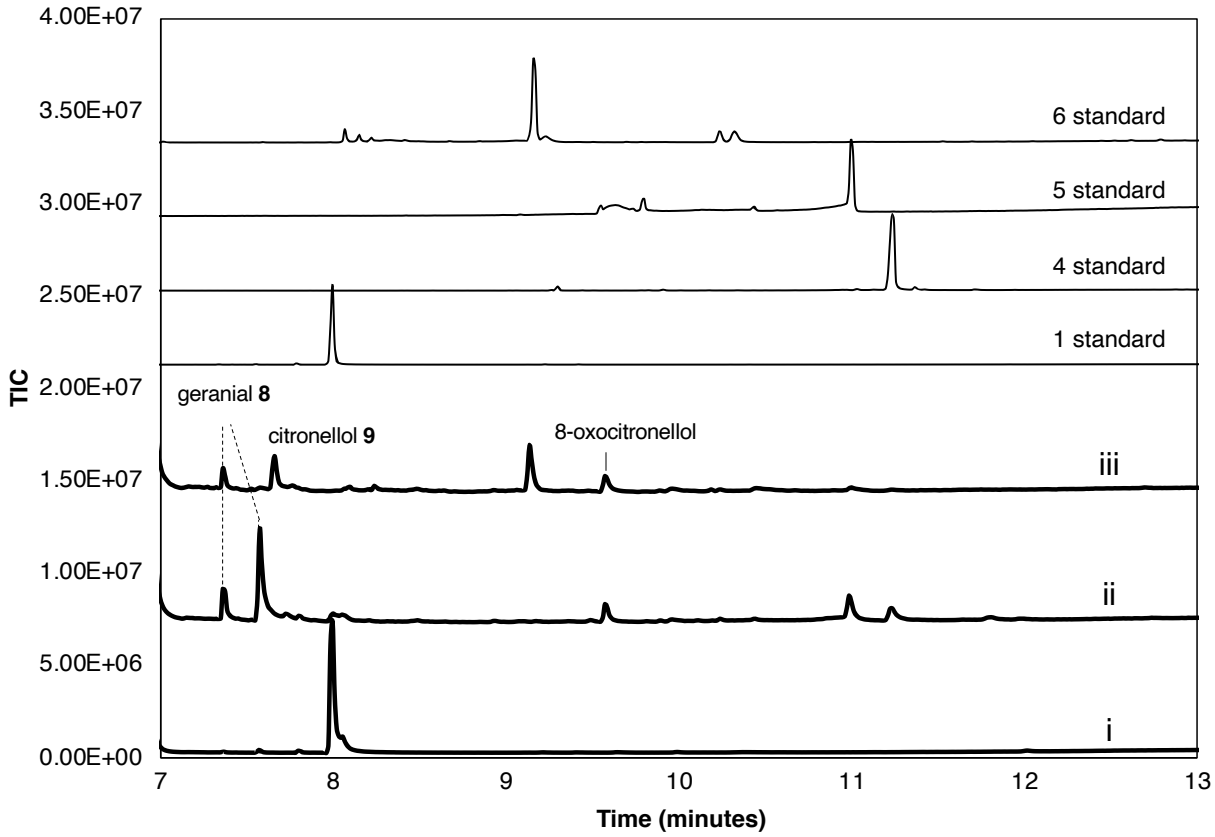

Figure S15. 10 mL-scale one-pot two-step geraniol to nepetalactol conversion. Each reaction contains 2 mM geraniol, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 10 μM GOR, 0.5 μM ISY, 5 μM MLPL, 1 μM FumC, 10 μM MaeB, 100 μM NADPH, 100 μM NAD^+ and 6 mM fumarate in BTP buffer (pH 9.0) unless otherwise specified, i.0 min, ii. no ISY/MLPL, iii. ISY/MLPL added after 2 hours of TfG8H/FpR/YkuN and GOR reaction.

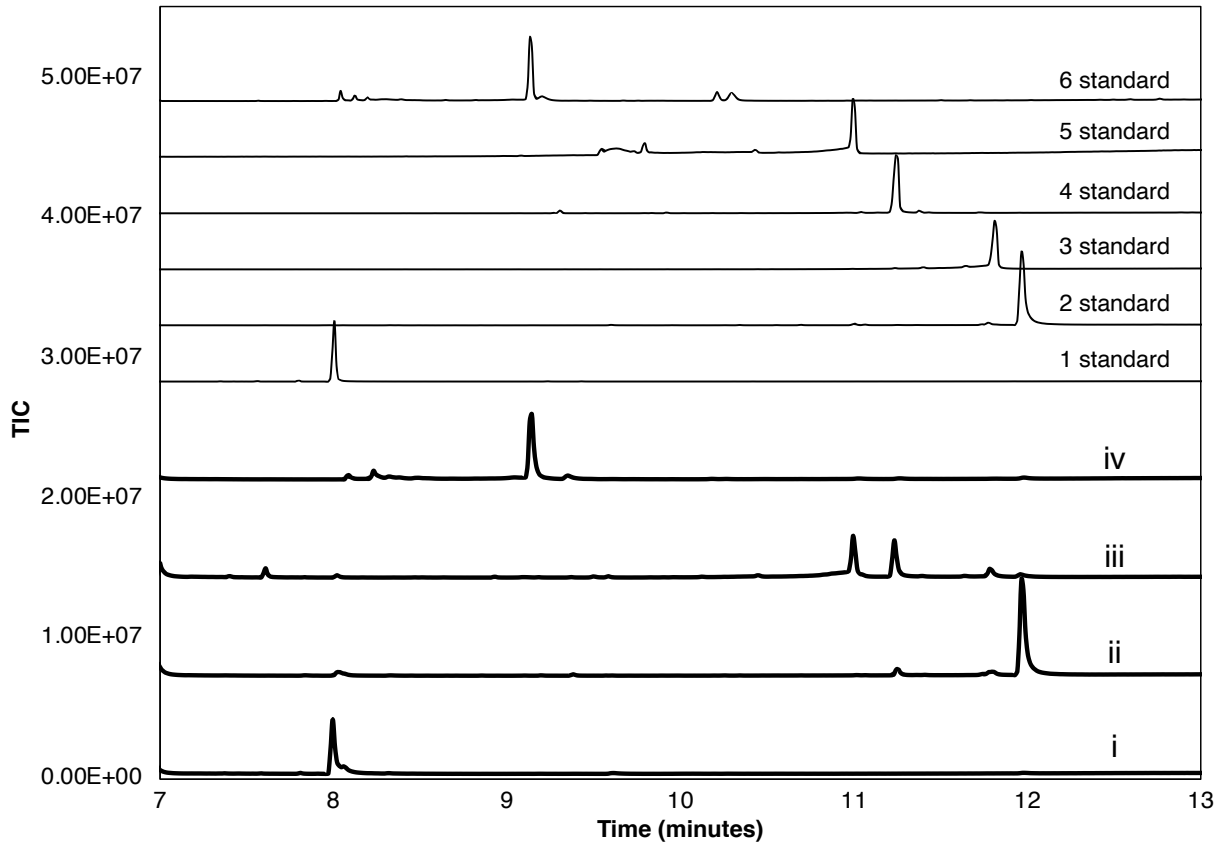

Figure S16. GC-MS chromatograms of 10 mL-scale one-pot drop-in geraniol to nepetalactol conversion. Reaction contains 2 mM geraniol, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 10 μM GOR, 0.5 μM ISY, 5 μM MLPL, 1 μM FumC, 10 μM MaeB, 100 μM NADPH, 100 μM NAD^+ and 6 mM fumarate in BTP buffer (pH 9.0) unless otherwise specified, i.0 min, ii. 2 hours reaction with TfG8H, iii. 2 hours reaction with GOR after TfG8H, iv. 2 hours reaction with ISY/MLPL after TfG8H and GOR.

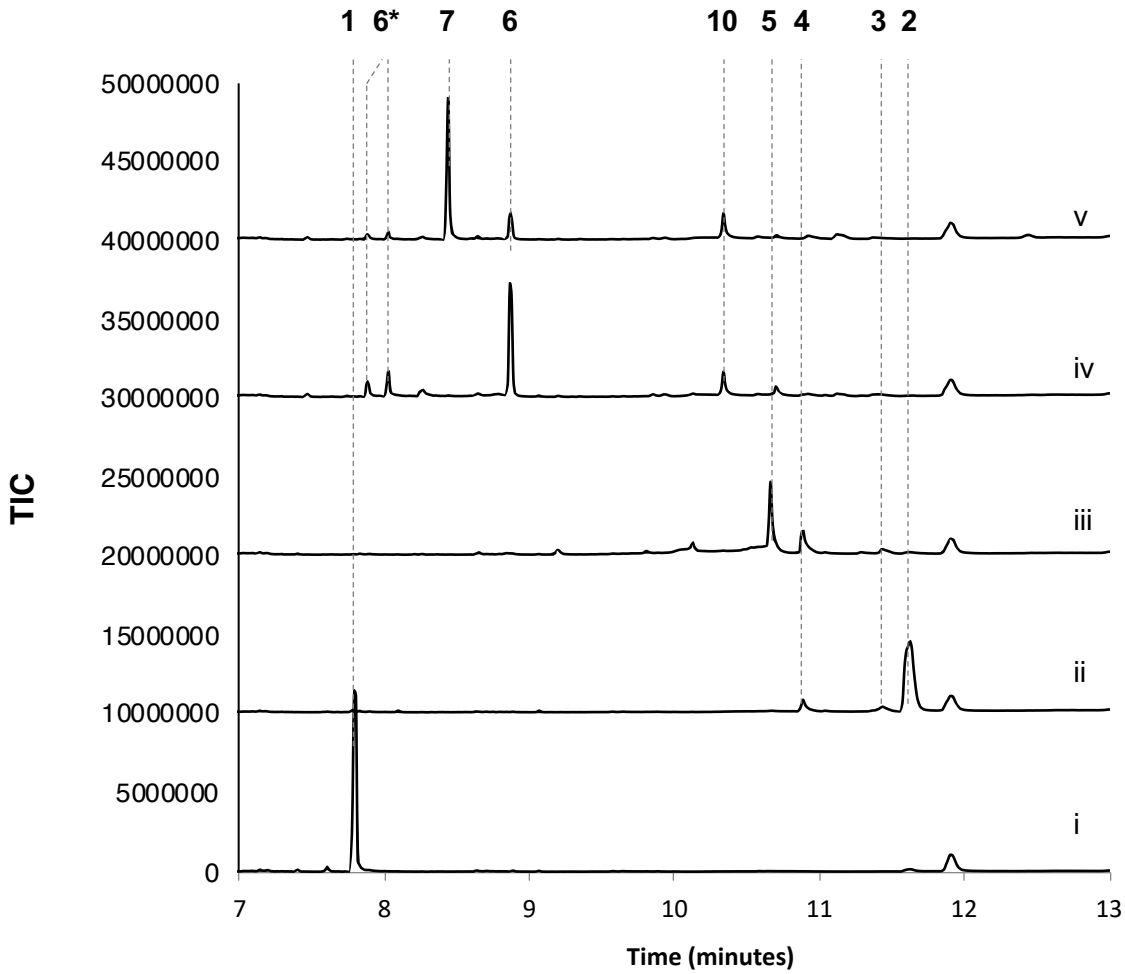

Figure S17. GC-MS chromatograms for 10 mL-scale one-pot conversion of 6.2 mM geraniol to nepetalactol and nepetalactone. Final reaction contained 957 mg/L geraniol **1**, 5 μM TfG8H, 10 μM FpR, 10 μM YkuN, 10 μM GOR, 0.5 μM ISY, 5 μM MLPL, 1 μM FumC, 10 μM MaeB, 100 μM NADPH, 100 μM NAD^+ and 18 mM fumarate in BTP buffer (pH 9.0) unless otherwise specified. (i) starting material, 6 mM **1**, (ii) 3-hour reaction with TfG8H, (iii) 2-hour reaction after GOR was added to (ii), (iv) 2-hour reaction after ISY/MLPL were added to (iii), (v) 2-hour reaction after ISY/MLPL and NEPS1 were added to (iii).

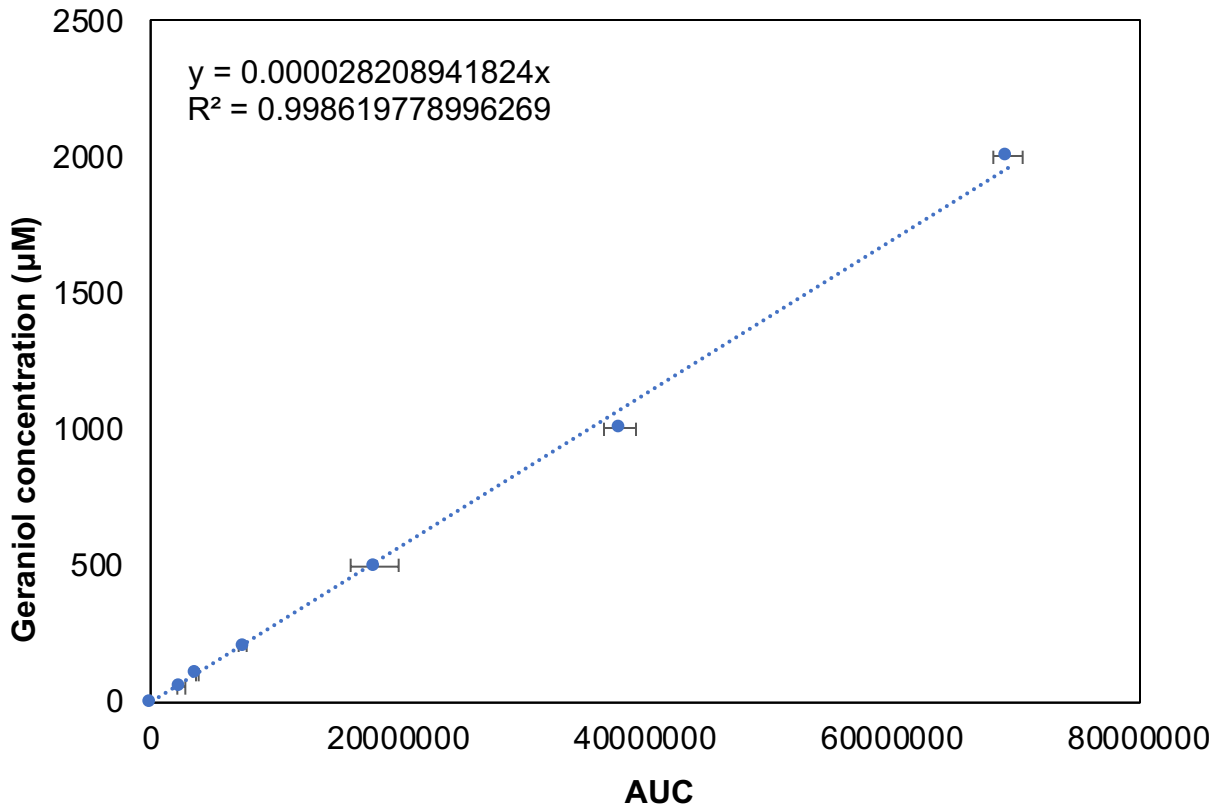

Figure S18. Geraniol calibration curve.

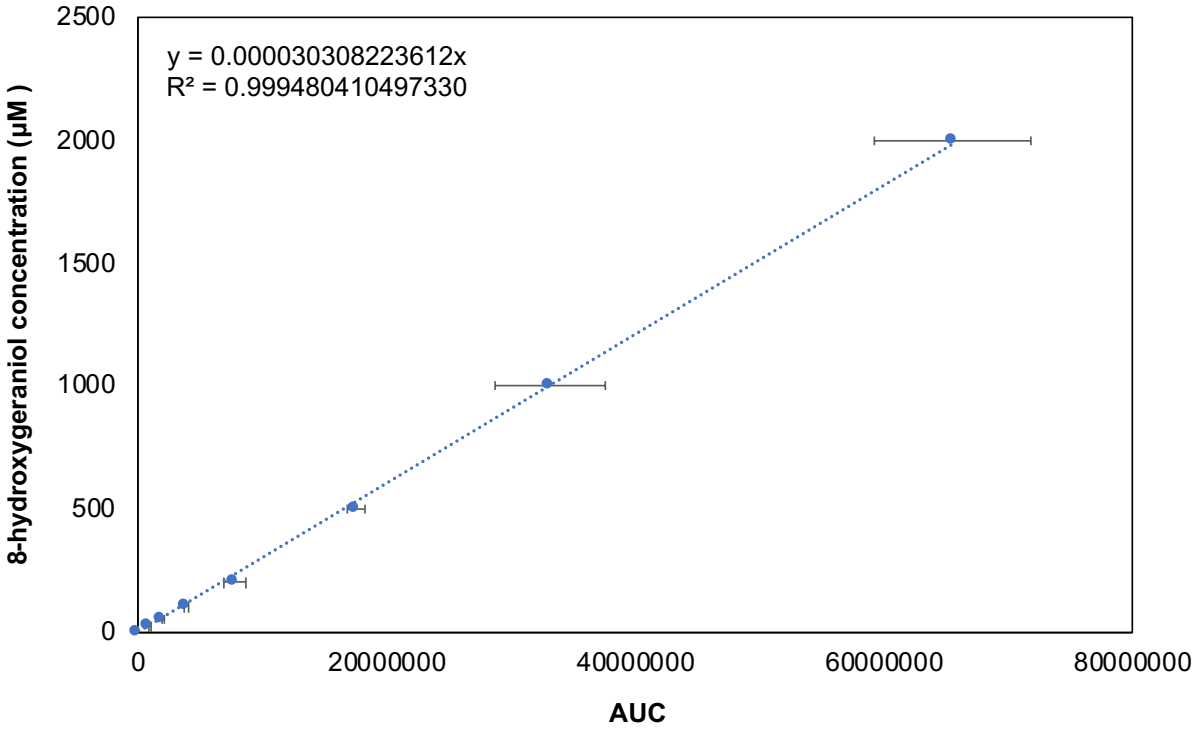

Figure S19. 8-hydroxygeraniol calibration curve.

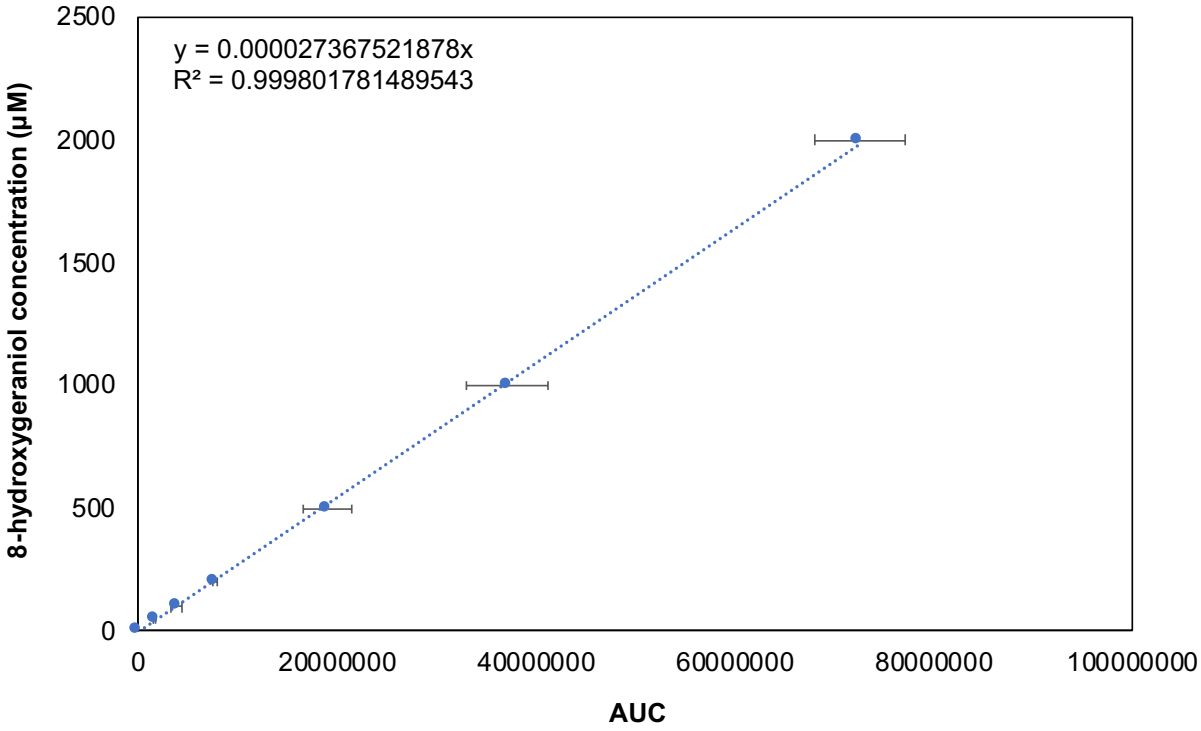

Figure S20. 8-oxogeranial calibration curve.

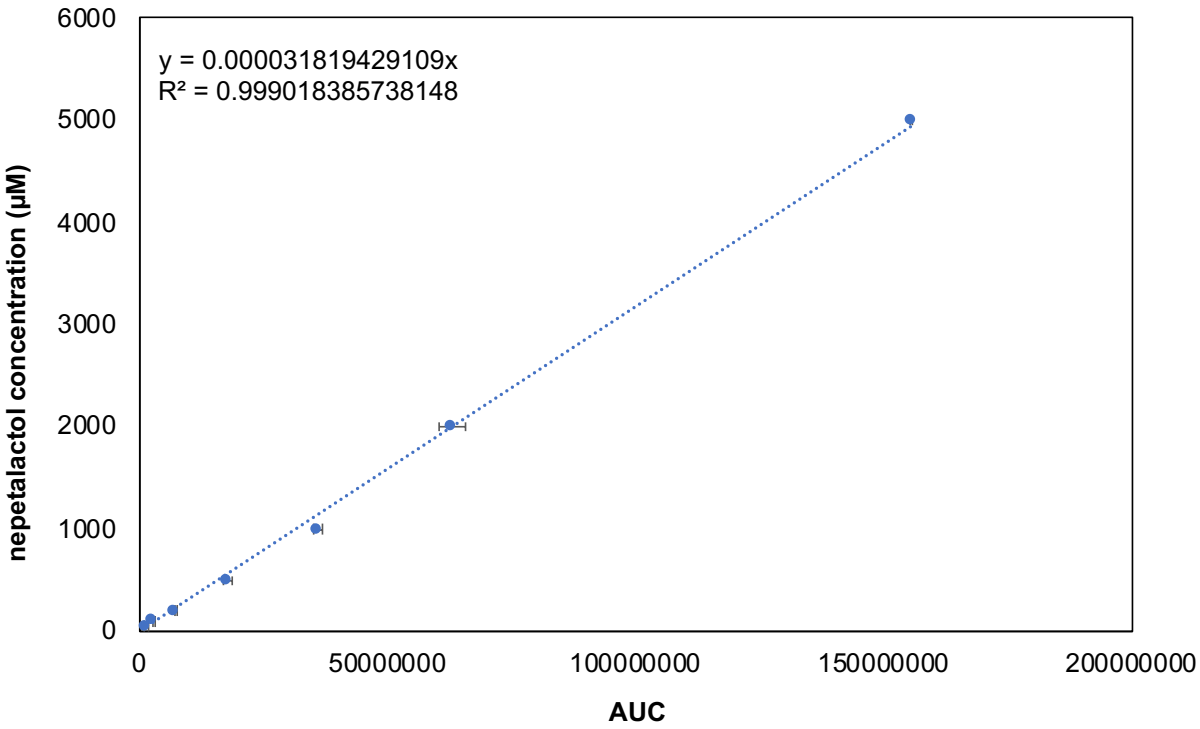

Figure S21. Nepetalactol calibration curve.

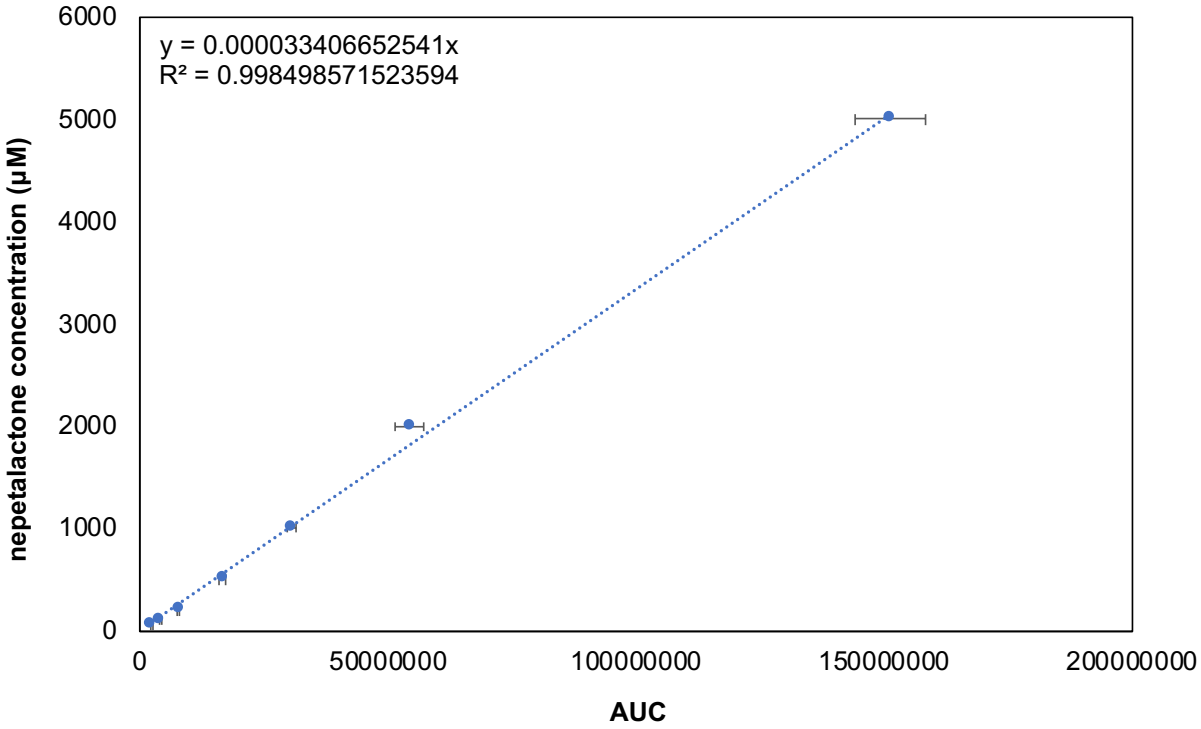

Figure S22. Nepetalactone calibration curve.

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