Electronic Supplementary Material (ESI) for Chemical Communications
This journal is © The Royal Society of Chemistry 2012

Electronic Supplementary Information for

Chemoenzymatic synthesis of the alarm pheromone (+)-verbenone from geranyl diphosphate

Zulfa Yoosuf-Aly, Juan A. Faraldos, David J. Miller and Rudolf K. Allemann*

School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff CF10 3AT, UK

Materials & General Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Oligonucleotides for PCR reactions were purchased from Eurofins mwg-operon and synthetic genes were purchased from Epoch Biolabs.

Geranyl diphosphate (GDP) was prepared from geranyl bromide using the method of Davisson *et al.*[1]

^{1}H-NMR spectra were measured on a Bruker Avance DPX400 NMR spectrometer and are reported as chemical shifts in parts per million downfield from tetramethylsilane, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (to the nearest 0.5 Hz) and assignment, respectively. IR spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer and samples were prepared as thin films of neat liquid on sodium chloride discs. GC-MS analyses were performed on a Hewlett Packard 6890 GC fitted with a J&W scientific DB-5MS column (30 m x 0.25 mm internal diameter) and a Micromass GCT Premiere mass spectrometer detecting in the range *m/z* 50-800 in EI^+ mode with scanning once a second with a scan time of 0.9 s. Injections (10 μL) were performed in split mode (split ratio 5:1) at 50 °C. Chromatograms were begun with an oven temperature of 49 °C, which was held for 1 min and then increased at 4 °C min^{-1} for 25.25 min up to 150 °C. Chiral GC analyses were performed on a Perkin Elmer 8700 Gas Chromatograph using a Supelco 23404 beta-Dex L20 column (30 m x 0.25 mm internal diameter) with an FID detector. Chromatograms were performed isothermally at 80 °C for α-pinene and 110 °C for verbenone with the injector at 200 °C.

Expression and purification of (+)-APS

A synthetic gene for (+) α-pinene synthase from loblolly pine (*Pinus taeda*)[2] was purchased from Epoch Biolabs (http://www.epochlifescience.com). The synthetic gene, codon optimised for *E.coli* expression, was cloned into a pET21d vector between *NcoI* and *BamHI* restriction sites to produce pET21d pinus.

Gene and protein sequences for the synthetic gene were as shown below:

```
atggctctggttagcgccgttccgctgaacagtaaactgtgcctgcgccgtactctgttt
M  A  L  V  S  A  V  P  L  N  S  K  L  C  L  R  R  T  L  F
ggcttttctcatgagctgaaagcgattcacagcacggtccctaatctgggtatgtgtcgt
G  F  S  H  E  L  K  A  I  H  S  T  V  P  N  L  G  M  C  R
```

This journal is © The Royal Society of Chemistry 2012

```
ggcggtaaaagcattgccccttctatgagcatgagtagcacaacaagcgttagcaatgaa
G  G  K  S  I  A  P  S  M  S  M  S  S  T  T  S  V  S  N  E
gatggggtccctcgtcgtattgcagggcatcattctaatctgtgggacgatgatagtatt
D  G  V  P  R  R  I  A  G  H  H  S  N  L  W  D  D  D  S  I
gcgagtctgtctacgagctacgaagcaccgtcttatcgtaaacgcgccgataaactgatt
A  S  L  S  T  S  Y  E  A  P  S  Y  R  K  R  A  D  K  L  I
ggtgaagtaaagaatatctttgacctgatgagcgtggaagacggtgttttcacgtctccg
G  E  V  K  N  I  F  D  L  M  S  V  E  D  G  V  F  T  S  P
ctgtctgatctgcatcatcgcctgtggatggtggatagcgtcgagcgtctgggtatcgat
L  S  D  L  H  H  R  L  W  M  V  D  S  V  E  R  L  G  I  D
cgtcactttaaagatgagatcaactctgcactggatcatgtttatagctattggaccgag
R  H  F  K  D  E  I  N  S  A  L  D  H  V  Y  S  Y  W  T  E
aaaggtatcggtcgcggtcgcgagtctggtgttaccgatctgaactctaccgcactgggg
K  G  I  G  R  G  R  E  S  G  V  T  D  L  N  S  T  A  L  G
ctgcgtactctgcgtctgcacgggtatacggtaagtagtcatgttctggatcattttaag
L  R  T  L  R  L  H  G  Y  T  V  S  S  H  V  L  D  H  F  K
aatgagaaaggccagtttacatgtagcgctattcaaaccgaaggtgaaattcgtgacgtg
N  E  K  G  Q  F  T  C  S  A  I  Q  T  E  G  E  I  R  D  V
ctgaatctgttccgcgcgagcctgattgcgttcccaggcgaaaagattatggaggctgca
L  N  L  F  R  A  S  L  I  A  F  P  G  E  K  I  M  E  A  A
gaaattttcagcacgatgtatctgaaagatgcgctgcagaagattccaccgagtgggctg
E  I  F  S  T  M  Y  L  K  D  A  L  Q  K  I  P  P  S  G  L
tctcaagaaattgaatatctgctggagttcggctggcacacgaatctgccgcgcatggaa
S  Q  E  I  E  Y  L  L  E  F  G  W  H  T  N  L  P  R  M  E
actcgtatgtatattgacgtgttcggcgaggacaccacgttcgaaaccccgtacctgatc
T  R  M  Y  I  D  V  F  G  E  D  T  T  F  E  T  P  Y  L  I
cgcgaaaagctgctggaactggcaaagctggagttcaatatcttccattctctggtgaaa
R  E  K  L  L  E  L  A  K  L  E  F  N  I  F  H  S  L  V  K
cgcgagctgcagagcctgagccgttggtggaaagattatggtttcccagaaatcacgttc
R  E  L  Q  S  L  S  R  W  W  K  D  Y  G  F  P  E  I  T  F
tctcgtcaccgtcacgtggagtattacacactggctgcctgcattgcgaatgatccgaaa
S  R  H  R  H  V  E  Y  Y  T  L  A  A  C  I  A  N  D  P  K
cacagcgcttttcgcctgggttttggtaaaatctctcacatgatcacgatcctggatgac
H  S  A  F  R  L  G  F  G  K  I  S  H  M  I  T  I  L  D  D
atctacgataccttcggcacgatggaagagctgaagctgctgacagcggccttcaagcgc
I  Y  D  T  F  G  T  M  E  E  L  K  L  L  T  A  A  F  K  R
tgggacccgagttctatcgaatgcctgccggactatatgaaaggtgtatatatggcggtt
W  D  P  S  S  I  E  C  L  P  D  Y  M  K  G  V  Y  M  A  V
tatgataatatcaacgaaatggcccgtgaagcgcagaaaattcagggttgggatacagtg
Y  D  N  I  N  E  M  A  R  E  A  Q  K  I  Q  G  W  D  T  V
agctacgcccgtaaaagttgggaagcatttatcggcgcgtacatccaggaggcgaagtgg
S  Y  A  R  K  S  W  E  A  F  I  G  A  Y  I  Q  E  A  K  W
atctctagcgggtatctgccgacttttgacgaatatctggagaacgggaaagtcagcttt
I  S  S  G  Y  L  P  T  F  D  E  Y  L  E  N  G  K  V  S  F
ggcagccgcatcactactctggaaccaatgctgactctgggctttccgctgccgccgcgc
G  S  R  I  T  T  L  E  P  M  L  T  L  G  F  P  L  P  P  R
attctgcaagagattgactttcctagtaagtttaacgatctgatctgtgcgattctgcgt
I  L  Q  E  I  D  F  P  S  K  F  N  D  L  I  C  A  I  L  R
ctgaaaggcgatacccaatgttataaagcggatcgcgctcgcggcgaagaagcaagcgct
L  K  G  D  T  Q  C  Y  K  A  D  R  A  R  G  E  E  A  S  A
gtgagttgctacatgaaagaccatccaggcattaccgaagaggatgcagttaaccaagta
V  S  C  Y  M  K  D  H  P  G  I  T  E  E  D  A  V  N  Q  V
```

This journal is © The Royal Society of Chemistry 2012

```
aacgcgatggtagataacctgaccaaagagctgaactgggagctgctgcgccctgatagt
N  A  M  V  D  N  L  T  K  E  L  N  W  E  L  L  R  P  D  S
ggcgttccaattagttacaagaaagtggcctttgacatttgccgcgtgtttcattacggc
G  V  P  I  S  Y  K  K  V  A  F  D  I  C  R  V  F  H  Y  G
tacaaataccgtgacggctttagtgtggccagtattgaaattaagaacctggtgacccgc
Y  K  Y  R  D  G  F  S  V  A  S  I  E  I  K  N  L  V  T  R
accgtcgtcgaaaccgtcccactgtaatag
T  V  V  E  T  V  P  L  *  *
```

Introduction of C-terminal His-tag into APS

Oligonucleotides for PCR reactions were purchased from Eurofins mwg-operon. The primers were as follows (5' to 3'):

A- Forward: CACCACCACCACCACCAC
B- Reverse: GCCCTGAAAATACAGGTTTTCCAGTGGGACGGTTTCGAC

Both primers were phosphorylated at the 5' position. Insertion of the desired primer in to the APS gene was performed using *Phusion Site Directed Mutagenesis Kit* following the manufacturer's protocol.

Production and purification of APS-His_6

E. coli BL21 codon plus (DE3)-RIL cells (Stratagene) were transformed with pET21d pinus-His_6. Transformed cells were grown at 37 °C in LB medium (6 x 500 mL) supplemented with ampicillin (0.1 mg/mL). When OD_{600} of the medium reached 0.6-0.7, the cultures were induced with IPTG (0.5 mM final concentration) and grown for another 4 h at 37 °C. Cells were harvested by centrifugation (6000*g*, 15 min) the supernatant solution was discarded and the pellets were stored at -80 °C.

For purification, frozen pellets were thawed and resuspended in cell lysis buffer (50 mM Tris, 5 mM EDTA, 5 mM βME, 10% Glycerol, pH 8.0). The resuspended cells were lysed by sonication on an ice bath. The cell lysate was clarified by centrifugation (17000*g*, 30 min) and the pellet resuspended in cell lysis buffer (50 mL). The pH of the well-stirred suspension was slowly changed to 12 by dropwise addition of 5 M NaOH and the basic solution stirred for 30 min at 4°C. βME (5 mM) was added and the pH reduced pH 8.0 by dropwise addition of 1 M HCl. This mixture was stirred for another 30 min at 4 °C and the suspension centrifuged (17000*g*, 30 min). The supernatant was dialyzed overnight against binding buffer (50 mM Tris-Base, 150 mM NaCl, 20 mM βME, 2 mM imidazole, pH 8.0). The solution was loaded onto a Ni^{2+}-Sepharose™ 6 Fast Flow drip column (GE Healthcare, 5 mL). After 10 min, the flow-through was collected and the column was washed with 10 column volumes (CV) of binding buffer. The protein was eluted with a gradient ranging from 50 to 500 mM imidazole (20 CV) followed by a wash at 500 mM imidazole (10 CV). APS-His_6 eluted at approximately 200-250 mM imidazole.

Fractions were analysed by SDS-PAGE and those corresponding to the correct molecular weight were pooled and dialysed overnight against 10 mM Tris-Base, 5 mM βME, pH 7.5. The protein was then concentrated to a final volume of ~ 10 mL (AMICON system, YM 30). Glycerol was added to the solution of purified enzyme

(10 %) and the solution stored at -20°C. The concentration of protein was measured using the Bradford method.[3]

Analytical incubation of APS-His_6 with GDP

To test the activity of the purified APS-His_6, the enzyme was incubated with GDP using 1.6 mM GDP (20 μL), buffer (Bis-Tris propane (100 mM), KCl (50 mM) and $MgCl_2$ (20 mM), pH 7.5) in a final volume of 1.0 mL. The assay solution was gently mixed with enzyme (150 μL, 18 μM), the reaction was overlaid with 1 mL of pentane and gently shaken overnight at room temperature. The organic layer was removed and the aqueous layer extracted with additional pentane (500 mL). The pooled pentane extracts were analyzed by GC-MS. The total ion chromatogram showed the presence of a major compound (*m/z* 136) with a retention time of 7.32 min. This enzymatically generated hydrocarbon was identified as α-pinene by co-elution with an authentic sample of α-pinene and form the mass-spectrum. All incubations were repeated without enzyme and also without filtration through silica to check for enzyme derived alcohol products.

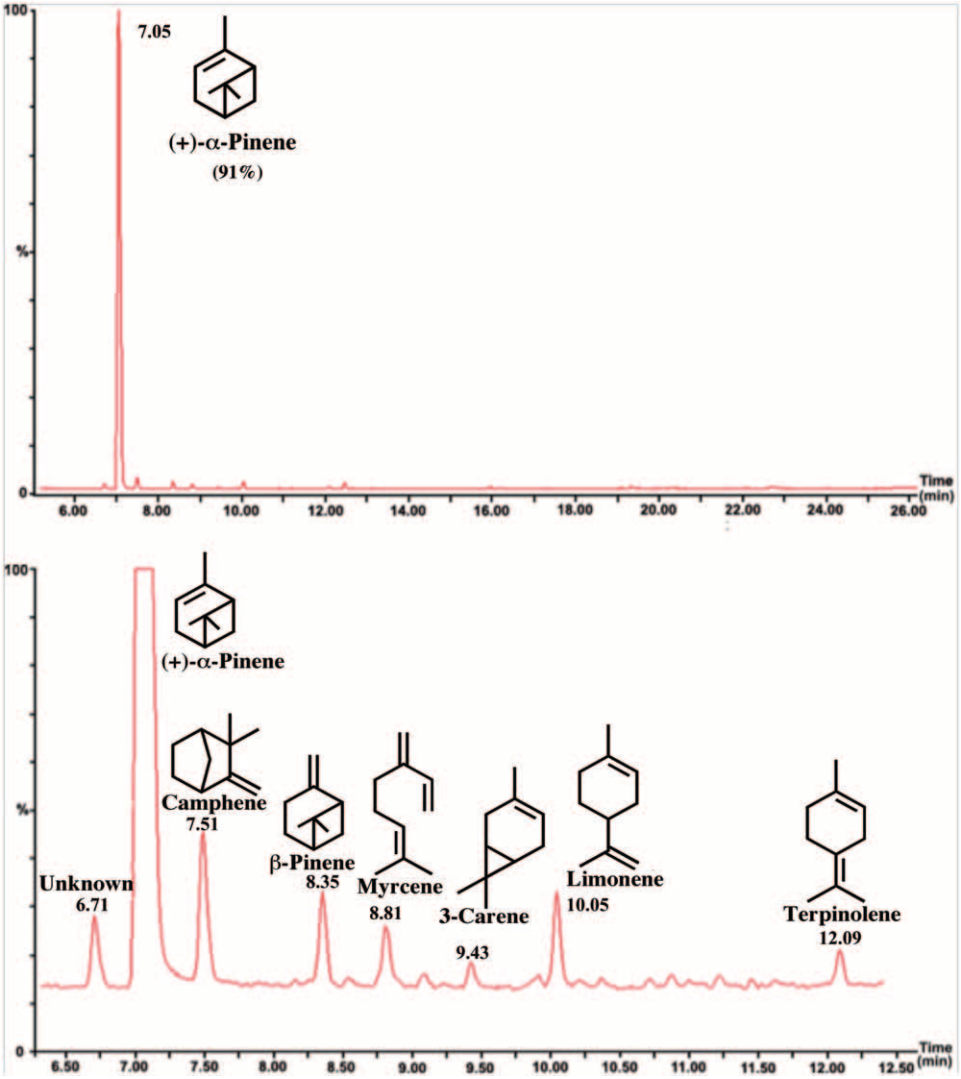


Figure S2: Total ion chromatograms of the pentane extractable products derived from incubation of GDP with APS-His_6 at two levels of sensitivity. For a tentative identification of the material eluting at 6.71 min see Figs. S3 and S4.

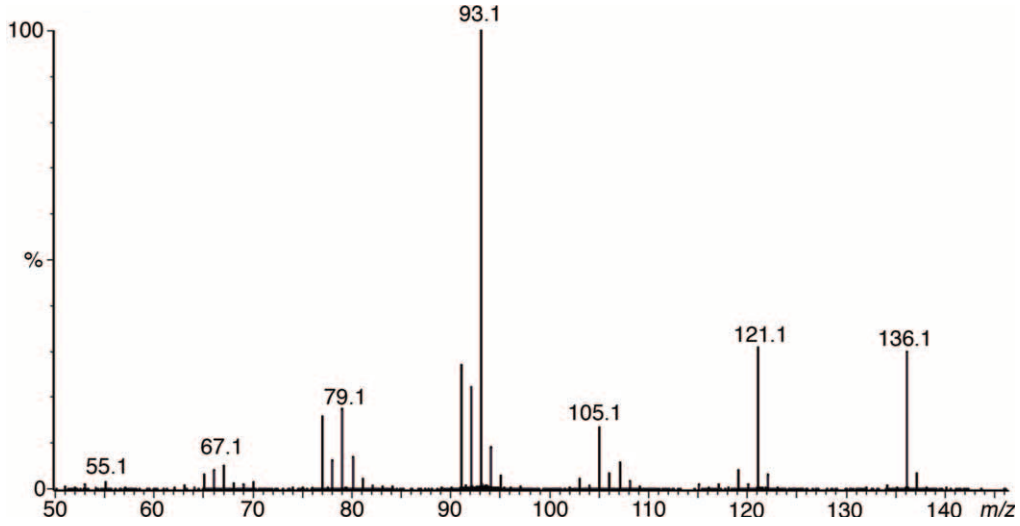


Figure S3. Mass spectrum compound eluting at 6.71 min in Fig. S2.

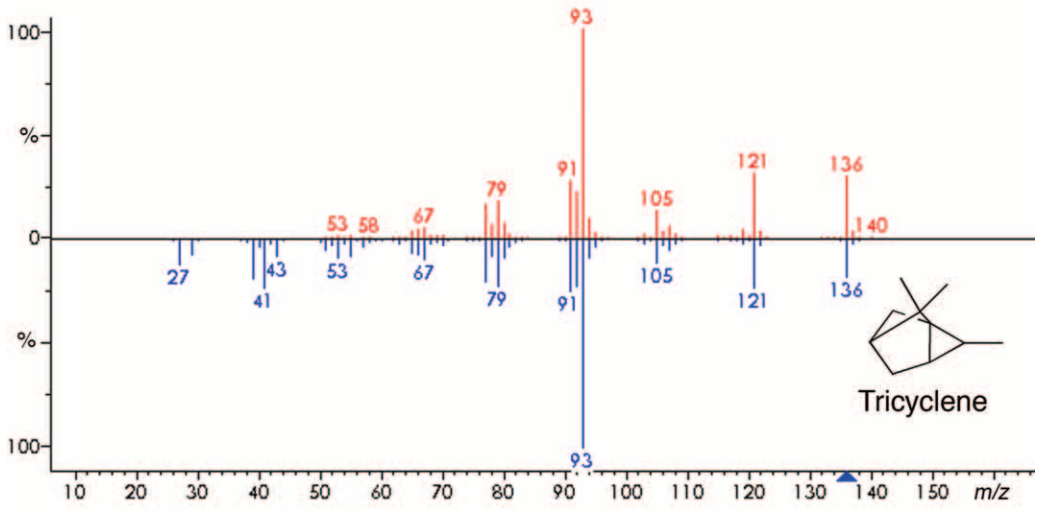


Figure S4. Head to tail comparison of the mass spectrum of the compound eluting at 6.71 min in Fig. S2 (red) with the mass spectrum of tricyclene from the NIST library (blue).

Assay of enzyme activity

Kinetic assays were performed in a manner similar to that previously described for aristolochene synthase.[4] Assays (final volume 250 μL) were initiated by addition of a purified enzyme solution (0.4 μM) to 20-120 μM [1-^{3}H]-geranyl diphosphate (6500 dpm/nmol) in Bis-Tris propane (100 mM), KCl (50 mM) and $MgCl_2$ (100 mM), pH 7.5). After incubation for 15 min, reactions were stopped by addition of 100 mM EDTA and overlaid with hexane (1 mL). The samples were mixed for 10 s on a vortex mixer, the hexane layer was removed and the sample extracted with further (2 x 1 mL) hexane. The pooled hexane extracts were filtered through 40 mg of silica gel, emulsified with Ecoscint O (15 mL) and analyzed by scintillation counting. The Michaelis constant K_M (40 ± 8 μM) and the turnover number k_{cat} (0.001 ± 0.0004 s^{-1}) are the average of three measurements:

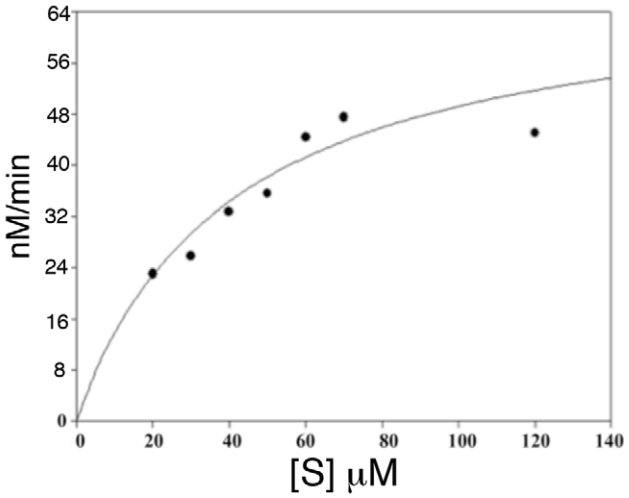


Figure S5: Typical Michaelis-Menten plot for turnover of [1-^{3}H]-GDP with APS-His_6.

Calibration of α-pinene concentration

In order to measure the concentration of (+)-α-pinene produced from GDP by APS-His_6, known concentrations (0.1 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.8 mM and 0.9 mM) of racemic α-pinene in pentane were prepared. These samples were analysed by GC-MS (conditions as above) and the total ion counts under the peak corresponding to α-pinene in the TIC were plotted against the concentration of α-pinene (Fig. S6).

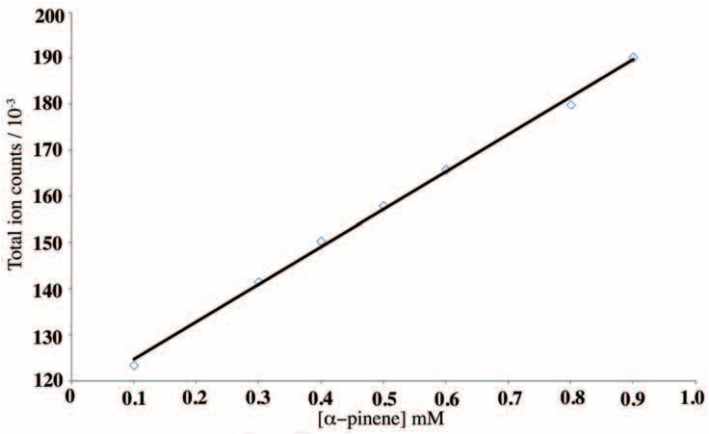


Figure S6: Graph of total ion counts versus α-pinene concentration used to determine α-pinene concentrations found in preparative incubations of GDP with APS-His_6.

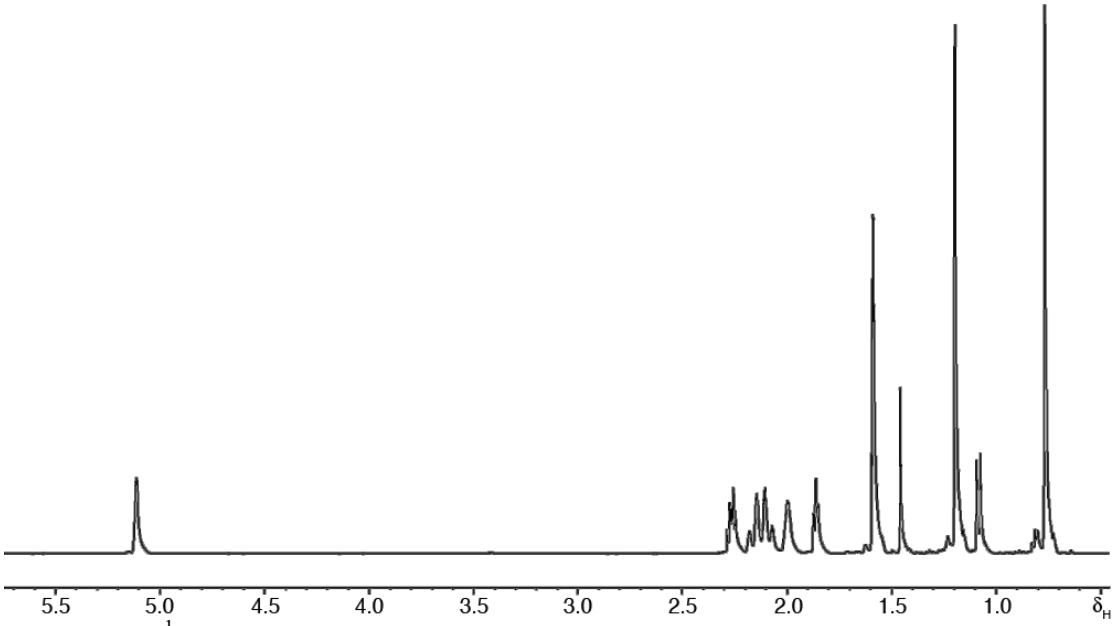
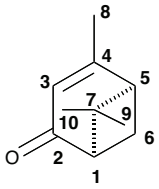


Figure S7: ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of enzymatically generated α-pinene. δ_H (500 MHz, $CDCl_3$) 0.75 (3 H, s, H-9), 1.09 (1 H, d, J = 7, H-6), 1.18 (3 H, d, H-10), 1.60 (3 H, s, H-8), 1.85 (2 H, m, H-5), 2.00 (1 H, m, H-2), 2.12 (3 H, m, H-2 and H-1), 2.25 (1 H, m, H-6), 5.12 (1 H, s, H-3), numbering as for verbenone (see below).

Optimised synthesis of (+)-verbenone from GDP



1.6 mM GDP (16 mg, 40 mmol) in assay buffer buffer (Bis-Tris propane (100 mM), KCl (50 mM) and $MgCl_2$ (20 mM), pH 7.5) in a final volume of 27.0 mL was gently mixed with APS-His_6 (4.0 mL, 4 mM). The resulting solution was overlaid with pentane (10 mL) and tightly sealed. The sealed vessel was then gently shaken overnight at room temperature. The aqueous layer was then carefully removed and then further extracted with pentane (5 × 4 mL).

To this enzymatically produced solution of (+)-α-pinene (4.9 mg, 36 μmol) in pentane (40 mL) was added, drop-wise, *tert*-butyl hydroperoxide (70% wt. aqueous solution, 39.8 μL, 0.29 mmol) and $Cr(CO)_6$ (15.8 mg, 72 μmol). To this mixture was added acetonitrile (4 mL) and the mixture was heated under reflux for 18 h. After cooling in ice the solution was filtered and the residue was washed with additional pentane (2 mL). The filtrate was extracted with 5% NaOH (3 × 5 mL), and the pooled aqueous extracts were back extracted with pentane (5 mL). The pooled fractions were dried with $MgSO_4$, filtered and the solvent wascarefully removed under reduced pressure. The residue was dissolved in hexane (1 mL) and (+)-verbenone purified by normal phase HPLC on an Agilent 1100 series system using a Waters Nova-PAK silica HR (3.9 mm × 300 mm) column, eluting with 1% isopropanol in hexane at 1 mL/min. Verbenone eluted at 8.84 min (3.0 mg, 50%).

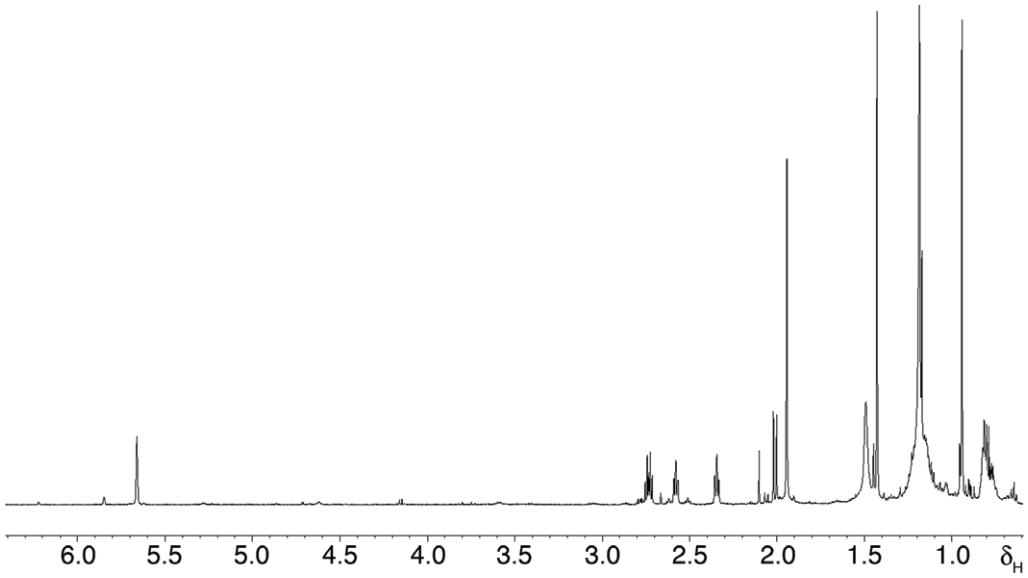


Figure S8. ^{1}H-NMR spectrum ($CDCl_3$) of chemoenzymatically produced (+)-verbenone (**3**). δ_H (400 MHz, $CDCl_3$) 1.02 (s, 3 H, H-9); 1.47 (3 H, s, H-10); 2.00 (3 H, d, J = 1.5 Hz, H-8); 2.10 - 2.08 (1 H, m, H-6); 2.42 (1 H, m, H-5); 2.70 - 2.62 (1 H, m, H-1); 2.91 - 2.80 (1 H, m , H-6); 5.71(1 H, m, H-3); υ_{max}/cm^{-1} (thin film) 3502, 3039, 2940, 2871, 2360, 2342, 1683 and 1617; *m/z* (EI$^+$) 150.1 (90 %, M$^+$), 135.1 (100, [M-CH_3]$^+$), 122.1 (22), 107.1 (75), 91.1 (40), 71.1 (20), 67.1 (5); $[\alpha]^{20}$ = + 233.3 °dm^{-1}cm^{3}g^{-1} (c = 0.06, $CHCl_3$). (lit. +258 (c = 1.0 in $CHCl_3$).[5]

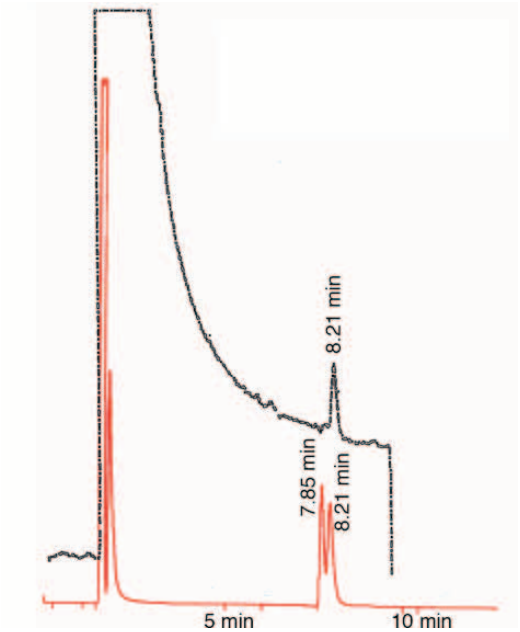


Figure S9. Red: FID gas chromatogram of commercial (±)-α-pinene passed over a β-cyclodextrin stationary phase at 80°C. Black: α-pinene generated from incubation of GDP with APS-His_6, only one enantiomer is detectable.

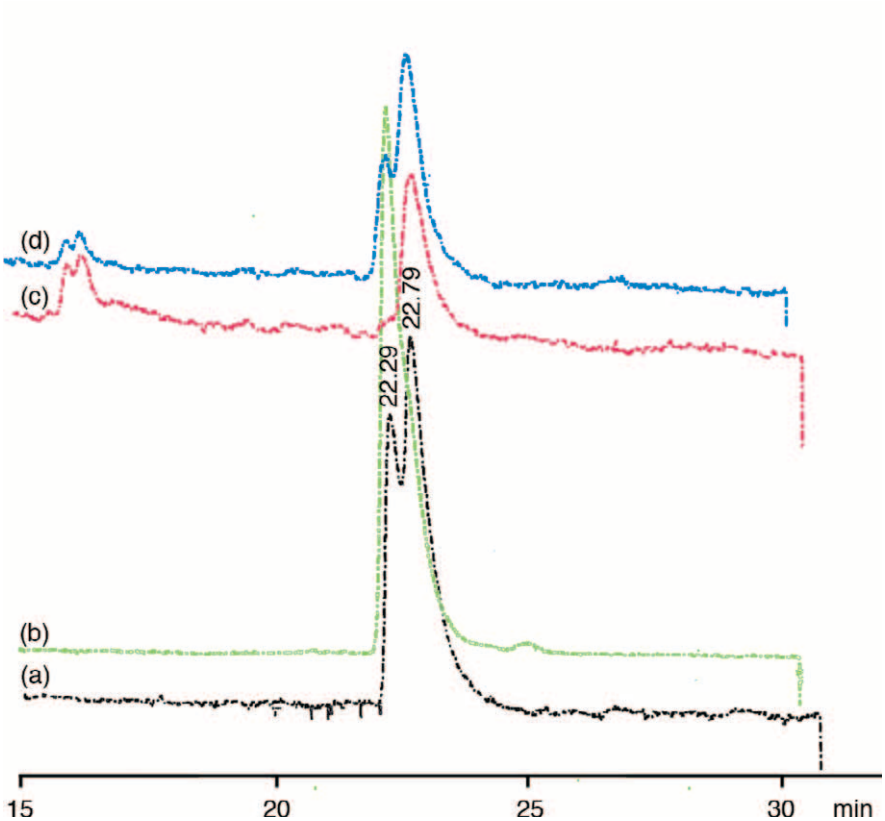


Figure S10. Chiral GC-analysis of chemoenzymatically generated verbenone. (a) Chromatogram of racemic verbenone generated from racemic pinene using ^{t}BuOOH and $Cr(CO)_6$; (b) chromatogram of (-)-verbenone obtained from Sigma-Aldrich; (c) chromatogram of chemoenzymatically generated (+)-verbenone; (d) chromatogram of racemic verbenone spiked with chemoenzymatically generated (+)-verbenone (the peak at t_R = 22.79 min is enhanced).

This journal is © The Royal Society of Chemistry 2012

References

1. V. J. Davisson, A. B. Woodside, T. R. Neal, K. E. Stremler, M. Muehlbacher and C. D. Poulter, *J. Org. Chem.*, 1986, **51**, 4768-4779.
2. M. A. Phillips, M. R. Wildung, D. C. Williams, D. C. Hyatt and R. Croteau, *Arch. Biochem. Biophys.*, 2003, **411**, 267-276.
3. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-254.
4. M. J. Calvert, P. R. Ashton and R. K. Allemann, *J. Am. Chem. Soc.*, 2002, **124**, 11636-11641.
5. R. P. Limberger, A. M. Aleixo, A. G. Fett-Neto and A. T. Henriques, *Electron. J. Biotechno.*, 2007, **10**, 500-507.