Supporting Information for

The amino-terminal segment in the β-domain of δ-cadinene synthase is essential for catalysis

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TABLE OF CONTENTS

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Materials and general procedures</td>
</tr>
<tr>
<td>2.</td>
<td>Construction of M8, M20 and M30</td>
</tr>
<tr>
<td>3.</td>
<td>Construction of CH-DCS-S24W and CH-DCS</td>
</tr>
<tr>
<td>4.</td>
<td>Construction of DCS-S30W</td>
</tr>
<tr>
<td>5.</td>
<td>Protein expression and purification</td>
</tr>
<tr>
<td>6.</td>
<td>Enzyme assay</td>
</tr>
<tr>
<td>7.</td>
<td>Identification of germacradien-4-ol</td>
</tr>
<tr>
<td>8.</td>
<td>GC-MS &amp; GC-FID analysis</td>
</tr>
<tr>
<td>9.</td>
<td>Michaelis-Menten plots</td>
</tr>
<tr>
<td>10.</td>
<td>Protein alignments of class I sesquiterpene synthases</td>
</tr>
<tr>
<td>11.</td>
<td>References</td>
</tr>
</tbody>
</table>

1. Materials and general procedures

Farnesyl diphosphate (FDP) was available from previous studies, and synthesized according to ref. 1. Oligonucleotides and primers for site directed mutagenesis were synthesized by Operon (UK). Pfu DNA polymerase was purchased from Promega (UK). Restriction enzymes were from New England Biolabs (UK). Site directed mutagenesis was carried out using the Quickchange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. QIAGEN miniprep kit and QIAquick gel extraction kit (QIAGEN, UK) were used for the purification of plasmids and DNA fragments from agarose gel respectively, according to the manufacturer’s instructions. All the mutated and ligated constructs were confirmed by DNA sequence analysis using the internal Walesbiogrid facilities (School of Bioscience, Cardiff University, UK). All other chemicals were from Sigma-Aldrich, Fisher or Melford. Product analysis by GC-MS was carried out as previously described.\(^2\) Multiple amino acid sequence alignment of sesquiterpene synthases was carried out with ClustalX using the default parameters.\(^3\)

S1
2. Construction of M8, M20 and M30

The gene for DCS already contains a NcoI restriction site at position 1, truncated proteins were all created by introducing a second NcoI restriction site in the gene at the desired position to produce the shorter versions of the protein. To create the second restriction site, a site-directed mutagenesis approach was adopted. The primers used for these mutagenesis transformations were as follows:

DCS-M8  
5'-CTTCACAAAGTTTCTCCATGGCTCTTCATCAACC-3'  
5'-GGGTGATGAAGAAGCCATGGGAGAAAATTTGTGAAG-3'

DCS-M20  
5'-CCATAAGGATGCATGCTCCAAAAGGCGATTTTC-3'  
5'-GAAAATCGGCTTTGGGACCCATGCGATCCTTATG-3'

DCS-M30  
5'-CAAGGCGATTTCAGCCATGCTTTGGGAGATCTCTTCTCC-3'  
5'-GGAAGGATCTCCAAAATCCATGGCTGAAAATCGGCTTTG-3'

DCS-M30 frame shift correction  
5'-GGGATATACCATGATTTGGGGAGATC-3'  
5'-GATCTCCCCAATCAGGTATATCTCCTCC-3'

The modified DNAs containing two NcoI restriction sites were digested with NcoI and the fragments corresponding to an open vector were purified by agarose gel. The open vector was then closed intra-molecularly by ligating the large fragment with T4 DNA ligase.

3. Construction of CH-DCS-S24W and CH-DCS

The gene encoding DCS-M30 was digested with the NcoI restriction enzyme. The correct size DNA bands were isolated from agarose gel and ligated to DNA fragments (formed by phosphorylation and annealing of a synthetic oligonucleotide pair, i.e. CH-DCS ligation fragment, encoding the 24 first amino acids of E-β-farnesene synthase (EBFS). The mutation (CH-DCS-S24W) created upon introducing the NcoI restriction site was subsequently corrected by site-directed mutagenesis using the synthetic oligonucleotide pair CH-DCS-W24S.

DNA fragments for ligation and primers for site-directed mutagenesis were as follows:

CH-DCS ligation fragment  
5'-CATGGAACCTTCTTCATCACCCCTTTCTTCTCAATAAGGATGAAATGCCTCCAAAGCCGATTTTCAGCCTAC-3'  
5'-CATGGTAGGGCTGAAAATCGGCTTTGGGAGATCTCTTATCCTCCATCCTTATGGAAGAAGGGGAGATGAGAAGGTTCC-3'

CH-DCS-W24S  
5'-GAAGCATGCGCAAGCATTGTTGGGAGATC-3'  
5'-GATCTCCCCAAATGCTTGGGCATGCTTCC-3'

4. Construction of DCS-S30W

The primers used for mutagenesis were as follows:
5. Expression and purification

Parent and mutated constructs were expressed in *E. coli* BL21(DE3) host cells and produced following protocols previously described. Cells were harvested by centrifugation (3000 g, 10 minutes) and cell pellets stored at -20 °C until purification.

DCS proteins were solubilized by the base extraction method as described herein; defrosted cell pellets were resuspended in buffer (20 mM Tris, pH 8.0 containing 5 mM EDTA and 5 mM β-mercaptoethanol) and lysed by sonication using 3 × 2 min pulses. The lysate was centrifuged at 11,000 g for 30 min and the supernatant solution discarded. The pellets were resuspended in 50 mL of fresh buffer and titrated on ice with 5 M NaOH until the solution became clear (approximately pH 12). After stirring on ice for 30 min, the pH was lowered to 7.2 by slow addition of 1 M HCl (dropwise). β-Mercaptoethanol was added to a final concentration of 5 mM. The resulting solution was stirred for 30 min on ice and centrifuged at 11,000 g for 30 min, after which the protein was found in the supernatant solution.

The protein solution was then loaded onto a 75 mL DEAE-Sepharose Fast Flow column (GE Healthcare) and eluted with 0 - 1 M NaCl gradient. Fractions were analyzed by SDS-PAGE and those containing pure protein were collected and concentrated by ultrafiltration (AMICON system, YM 30). Protein concentrations were determined using the Bradford assay.

6. Enzyme assay

Steady-state kinetics assays were carried out using [1-3H]-*(E,E)*-FDP (240000 dpm/nmol) in buffer (20 mM Tris, pH 8.0 containing 5 mM β-mercaptoethanol and 5 mM MgCl₂) as previously described. Reactions (250 µl) were initiated by addition of enzyme (0.3-0.6 µM) to assay buffer solutions containing [1-3H]-*(E,E)*-FDP (concentrations ranging from 0-120 µM) at 0 °C overlaid with 0.75 mL of hexane. The resulting enzymatic preparations were incubated at 30 °C for 15-30 min (depending on the time to reach saturation in test trials). Reactions were quenched by adding 100 µL of 100 mM EDTA. Products were extracted with 3 × 750 µL portions of hexane. The organic solution was diluted with 15 mL EcoScint™ fluid (National Diagnostics) before analysis on a scintillation counter (Packard 2500 TR™) in 3H mode for 4 min per sample. The reaction time and enzyme concentration were optimised to ensure the reaction was in the initial linear phase and was not reaching saturation during the experiment. The *Kₘ* and *kₐₜ* were calculated by fitting the data to the equation $V = (kₐₜ[E_{total}][S])/(Kₘ + [S])$ using Systat SigmaPlot (Table S1).
Table S1: Summary of kinetic constants of DCS wild-type and N-terminal segment and related mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$s$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>DCS</td>
<td>0.010 ± 0.001</td>
<td>3.2 ± 0.3</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>M8</td>
<td>0.008 ± 0.001</td>
<td>2.9 ± 0.4</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>M20</td>
<td>0.0031 ± 0.001</td>
<td>2.5 ± 0.7</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>M30</td>
<td>Not measurable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH-DCS</td>
<td>0.006 ± 0.003</td>
<td>4.7 ± 0.3</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>CH-DCS-W24S</td>
<td>Inactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCS-S30W</td>
<td>Inactive</td>
<td></td>
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7. Identification of (-)-germacradien-4-ol

Germacradien-4-ol synthase (GdolS) was expressed and purified as previously reported.$^6$ An incubation containing GdolS (4 µM) and FDP (0.4 mM, 89 mg, 0.205 mmol) in buffer ($V_{tot}$ 500 mL, 50 mM tris, pH 8.0, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol) was prepared and overlaid with pentane (50 mL). The incubation was slowly stirred at room temperature for 24 h, followed by approximately 60 h at 4 °C. The pentane layer was removed and the aqueous phase was gently extracted with pentane (10 x 20 mL), pentane extracts were combined along with any minor emulsion formed, washed with brine (1 x 100 mL), and the solvent gently removed under reduced pressure (25 °C, 550 mbar) to yield germacradien-4-ol in 80.5 % yield (36.8 mg, 0.165 mmol).

NMR spectra and specific rotation (-163.4°) analyses were in agreement with previous reports.$^7$ For further assessment of the optical purity (Figure S6), a small sample was analysed by GC-FID using a SUPELCO Aztec CHIRALDEX™ B-DM chiral silica capillary column.

8. GC-MS and GC-FID analysis

All product profiles were determined by incubating 10 µM of purified enzyme with 1 mM (E,E)-FDP in the same buffer as for kinetic assay. Reactions were overlaid with 700 µL HPLC grade pentane and, typically, left overnight. Sesquiterpene products were extracted using 2 x 500 µL pentane (HPLC grade), and the resulting solutions were analyzed by GC-MS using a Hewlett-Packard 6890 gas chromatograph fitted with a J&W Scientific DB-5MS column (30 m with an internal diameter of 0.25 mm) and a Micromass GCT Premiere detecting in the range of m/z 50-800 in EI$^+$ mode, as previously reported.$^2$ The program uses an initial oven temperature of 50 °C with a ramp of 4 °C min$^{-1}$ (25
min) to 150 °C and a second ramp of 20 °C min\(^{-1}\) (5 min) to 250 °C. The calculated errors for the product distributions were less than 2% in all cases. Optical purity was assessed using GC-FID. This was performed on an Agilent 7890A GC system fitted with a SUPELCO Aztec CHIRALDEX™ B-DM silica capillary column; The oven temperature was held at 90 °C for 2 minutes and then raised 5 °C/min to 170 °C and held for 40 minutes.

**Figure S1:** Total ion chromatogram (EI\(^{+}\)-TOF) of the pentane extractable product formed from incubation of FDP with wild-type DCS (left) and Ei-mass spectrum of the product at 26.15 minutes (right).

**Figure S2:** Total ion chromatogram (EI\(^{+}\)-TOF) of the pentane extractable product formed from incubation of FDP with M8
Figure S3: Total ion chromatogram (EI⁻-TOF) and GC peaks integration for of the pentane extractable product formed from incubation of FDP with M20

Figure S4: Total ion chromatogram (EI⁻-TOF) of the pentane extractable product formed from incubation of FDP with M30

Figure S5: Total ion chromatogram (EI⁻-TOF) of the pentane extractable product formed from incubation of FDP with CH-DCS
**Figure S6:** FID chromatogram of an authentic sample (enzymatically produced) of (-)-germacradien-4-ol (SUPELCO Aztec CHIRALDEX™ B-DM silica capillary column).

**Figure S7:** FID chromatogram of the pentane extractable product formed from incubation of FDP with M20. Peak integrations are shown for comparison with Figure S8, δ-cadinene, 32.1671; (-)-germacradien-4-ol, 87.8438, δ-cadinene/(-)-germacradien-4-ol, 0.37.

**Figure S8:** FID chromatogram of the co-injection of the pentane extracted products of the above incubation of FDP with M20 (Figure S7) with an authentic sample (enzymatically produced) of (-)-germacradien-4-ol (Figure S6). Peak integrations are shown for comparison with Figure S7, δ-cadinene, 173.253; (-)-germacradien-4-ol, 525.692, δ-cadinene/(-)-germacradien-4-ol, 0.33.
9. Collection of Michaelis-Menten Plots

**Figure S9:** Michaelis-Menten plots for DCS

**Figure S10:** Michaelis-Menten plots for M8 (left) and M20 (right).

**Figure S11:** Michaelis-Menten plot for CH-DCS