# Probing eudesmane cation- $\pi$ interactions in catalysis by aristolochene synthase with non-canonical amino acids 

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Materials. Competent cells of $E$. coli XL1 Blue and $E$ coli BL21(DE3) were purchased from Stratagene and E. coli TOP10 from Invitrogen. The QIAprep spin Miniprep plasmid purification kit was obtained from Qiagen. Isopropyl-thio-D-galactopyranoside (IPTG) and Ampicillin Na salt were purchased from Melford and $\beta$-mercaptoethanol, chloramphenicol and tetracycline were obtained from Aldrich. The pre-packed DEAE Sepharose Fast Flow anion exchange resin was obtained from GE Healthcare. Ni-NTA agarose was obtained from Fisher. Protein size marker was from BioLabs. The Amicon-YM30 membranes were from Millipore. $[1-3 \mathrm{H}] \mathrm{FPP}(20 \mathrm{Ci} / \mathrm{mmol})$ was purchased from American Radiolabeled Chemicals. Unlabelled FPP was synthesized following Poulter's two steps (chlorination/diphosphorylation) protocol ${ }^{1}$ with modifications ${ }^{2}$ from commercially available all trans-farnesol (Aldrich). Commercial $[1-3 \mathrm{H}]$ FPP was diluted by adding cold FPP to give a final specific activity of 75 $\mathrm{mCi} / \mathrm{mmol}$. All amino acids used were purchased from Bachem, Fluorochem or Sigma Aldrich.

General Methods. Protein concentrations were determined by the Bradford method ${ }^{3}$ using commercial reagents and bovine serum albumin (Sigma) as the calibration standard. FPLC (Fast Protein Liquid Chromatography) was performed on ÅKTA-FPLC P-920 or UPC-900 units from GE Healthcare. Liquid scintillation (LS) was performed on a Packard Tri-Carb LS analyzer model 2700TR using Ecoscint O (National Diagnostics) as scintillation cocktail. GC-MS analysis of incubation products was performed using a HP 6890 GC-MS system fitted with J \& W scientific HP-5MS column 30 m long with an internal diameter of 0.25 mm and a Micromass GCT Premiere mass spectrometer. The program uses an initial oven temperature of $50^{\circ} \mathrm{C}$ with a ramp of $4{ }^{\circ} \mathrm{C} \mathrm{min}^{-1}(25 \mathrm{~min})$ to $150{ }^{\circ} \mathrm{C}$ and a second ramp of $20^{\circ} \mathrm{C}(5 \mathrm{~min})$ to a final temperature of $250^{\circ} \mathrm{C}$.

Site directed mutagenesis of recombinant aristolochene synthase cDNA. Quickchange site-directed mutagenesis kit (Stratagene) was used to introduce the desired mutations according to the manufacturer instructions. The primers used for mutagenesis were as follows:

5'-CGGCAATGAGCAGTTTAGCAAGACCACGCG-3' and 5'-CGCGTGGTCTTGCTAAACTGCTCATTGCCG-3' for W334F;
5'-CGGCAATGAGCAGTATAGCAAGACCACGCG-3' and 5'-CGCGTGGTCTTGCTATACTGCTCATTGCCG-3' for W334Y;
5'-GCGGCAATGAGCAGCATAGCAAGACCACGCGTC-3' and 5'-GACGCGTGGTCTTGCTATGCTGCTCATTGCCGCTC-3' for W334H
5’-CGGCAATGAGCAGTAGAGCAAGACCACG-3' and 5'- CGTGGTCTTGCTCTACTGCTCATTGCCG-3' for W334TAG
Plasmids were purified from overnight cultures ( 10 mL LB medium containing ampicillin $50 \mu \mathrm{M} / \mathrm{mL}$ ) using the QIAprep Spin Miniprep Kit as described by the manufacturer. Mutations were confirmed by DNA sequence analysis using Walesbiogrid facilities (School of Bioscience, Cardiff University, UK)

## Expression and purification of wild-type aristolochene synthase and protein mutants.

E. coli BL21(DE3) cells were transformed using $1 \mu \mathrm{~L}$ of plasmid. One colony was added to 100 mL of LB medium containing ampillicin ( $50 \mu \mathrm{~g}$ / mL ) and the culture was grown at $37^{\circ} \mathrm{C}$ with shaking ( 150 rpm ) overnight. Overnight cultures ( 5 mL ) were transferred to 3 X 500 mL of LB medium containing the same concentration of ampillicin as before. Cells were incubated at $37{ }^{\circ} \mathrm{C}$ with shaking, when the $\mathrm{O}^{\mathrm{D}} \mathrm{D}_{600}$ was between $0.6-0.8$, IPTG was added ( 0.5 mM ) and the cultures were incubated for 4 hours at $37^{\circ} \mathrm{C}$. Cells were harvested by centrifugation ( $5000 \mathrm{rpm}, 10$ minutes). The supernatant was discarded and the pellets were frozen at $-20^{\circ} \mathrm{C}$ until needed.

Pellets were thawed and re-suspended in 25 mL of cell lysis buffer ( 20 mM Tris ( pH 8.0 ), 5 mM EDTA, $5 \mathrm{mM} \beta$-mercaptoethanol (BME)). Samples were sonicated for $3 \mathrm{~min}(40 \%$ amplitude with 5 s on $/ 10 \mathrm{~s}$ off cycles). The lysed cells were centrifuged at $16,000 \mathrm{rpm}$ for 30 minutes and the supernatant was discarded (the proteins remained solely in the insoluble portion). The pellets were resuspended in 50 mL of lysis buffer by stirring at $4{ }^{\circ} \mathrm{C}$ for 30 min , then the pH of the solution was raised to 12 with 0.1 M NaOH and the solution was stirred for 30 min . The pH was then lowered to pH 7.5 with 0.1 M HCl and BME was added to a final concentration of 5 mM . The solution was stirred for 30 min at $4{ }^{\circ} \mathrm{C}$ and centrifuged at $17,000 \mathrm{rpm}$ for another 30 min . Proteins remained in the soluble supernatant.

For further purification a 65 mL Q Sepharose Fast Flow anion exchange column was used. The column was washed with 5 column volumes of lysis buffer. Then the protein was loaded onto the column and washed with another 5 column volumes of lysis buffer. A linear gradient ranging from $0-500 \mathrm{mM} \mathrm{NaCl}$ was applied to the column in 6 column volumes and the proteins eluted with $\sim 240 \mathrm{mM} \mathrm{NaCl}$. Fractions containing protein were analyzed on an SDS-PAGE gel and pooled together and dialysed overnight (MWCO 30000 Da ) against 20 mM Tris ( pH 8.0 ) and 5 mM BME. The volume of protein was reduced to $\sim 3 \mathrm{~mL}$ (AMICON system, YM 30). The concentration of protein was estimated using the Bradford assay. ${ }^{3}$

Introduction of C-terminal 6xHis tag into aristolochene synthase and PR-AS-W334TAG. Phusion® Site-Directed Mutagenesis Kit (Finnzyme) was used to introduce the C-terminal hexahistidine affinity tag ( $6 \times H i s$ tag). The reaction was performed according to the manufacturers recommendations using the following phosphorylated primers: 5'-CCACCATTAAGGATCCTCTAGAGTCGACCTGC-3' and 5'-TGATGATGGTGGCCGCTACCGCCGTTG-3' and plasmids PR-AS and AS-W334TAG as a templates. The PCR product (linear plasmid plus the $6 x$ His sequence) was purified using the QIAquick PCR purification Kit (Qiagen) and further recirculized using the Quick Ligation Kit (NEB). The ligation product was transformed into TOP10 cells plated on LB agar plates containing $100 \mu \mathrm{~g} / \mathrm{mL}$ of ampicillin. Plasmids were isolated from overnight cultures ( 5 mL LB medium containing ampicillin $100 \mu \mathrm{~g} / \mathrm{mL}$ ) using the QIAprep Spin Miniprep Kit (as described by the manufacturer). The insertion of the $6 \times$ His tag sequence was confirmed as mention above.

Expression and purification of His-tag aristolochene synthase. Plasmids were transformed into electrocompetent BL21(DE3) cells using 1 $\mu \mathrm{L}$ of plasmid. After transformation cells were plated on agar plates containing $100 \mu \mathrm{~g} / \mathrm{mL}$ ampicillin and incubated at $37^{\circ} \mathrm{C}$ over night. LB media ( 5 mL , containing $50 \mu \mathrm{~g} / \mathrm{mL}$ ampicillin) was inoculated with a single colony and grown overnight $37^{\circ} \mathrm{C}$ with shaking ( 250 rpm ). LB media ( 1 L containing $50 \mu \mathrm{~g} / \mathrm{ml}$ ampicillin) was inoculated with 2 mL of the saturated overnight culture and grown with shaking ( 200 rpm ) at $37^{\circ} \mathrm{C}$. The protein expression was induced with 0.5 mM IPTG at $\mathrm{OD}_{600 \mathrm{~nm}}=0.5-0.7$. After 4 h , the cultures were centrifuged ( $4400 \mathrm{rpm}, 25 \mathrm{~min}, 4^{\circ} \mathrm{C}$ ). Frozen cell pellets were thawed on ice and resuspended in 5 mL of buffer A ( 20 mM TrisHCl, $5 \mathrm{mM} \beta$-mercaptoethanol, pH 8 ) per 1 g cell pellet. Samples were sonicated on ice ( 5 min , amp $40 \%, 10 \mathrm{sec}$ on $/ 15 \mathrm{sec}$ off cycle) and centrifuged ( $45 \mathrm{~min}, 10000 \mathrm{rpm}, 4^{\circ} \mathrm{C}$ ). The pellet waw resuspended in buffer A ( 10 mL ) and stirred at $4^{\circ} \mathrm{C}$ for 1 h . The pH of the sample was raised to 12 followed by 30 min of stirring on ice. After addition of $50 \mu \mathrm{~L}$ of BME the pH was lowered to 8 . After additional 30 min of stirring the solution was centrifuged ( $45 \mathrm{~min}, 10000 \mathrm{rpm}, 4^{\circ} \mathrm{C}$ ) and the resulted supernatant was used for Ni-NTA purification. Samples from each step were collected for analysis. 1 ml of Ni-NTA agarose (ThermoScientific) was added to the supernatant and the solution was mixed for 1 hour at $4^{\circ} \mathrm{C}$ (rotator, 200 rpm ). Resin was then loaded on a reusable column and flow through was collected. Resin was washed with 10 column volumes of buffer $\mathrm{B}\left(50 \mathrm{mM} \mathrm{Na} 2 \mathrm{HPO}_{4}, 300 \mathrm{mM} \mathrm{NaCl} \mathrm{pH} 8\right)$ containing 20 mM imidazole and 5 volumes of buffer B containing 50 mM imidazole. The wash samples were collected. Protein was eluted with 10 mL of buffer B supplemented with 200 mM imidazole. All samples were analysed by SDS-PAGE and the obtained protein was dialysed into buffer A.

General method for incorporation of non-canonical amino acids (NAAs). A plasmid harbouring the $6 \times$ His PR-AS-W334TAG gene in combination with the appropriate pDULE-NAA-RS plasmid ${ }^{4,5}$ (pDULE plasmids were kindly supplied by Professor Ryan Mehl, Franklin \& Marshal College, Lancaster, PA, USA) were co-transformed into electrocompetent BL21(DE3). The pDULE-NAA-RS plasmids contain an aminoacyl-tRNA synthetase gene specific for each NAA as well as the suppressor $\mathrm{tRNA}_{\text {CUA }}$ gene. After transformation, cells were plated on agar plates containing $100 \mu \mathrm{~g} / \mathrm{ml}$ ampicillin and $9 \mu \mathrm{~g} / \mathrm{ml}$ tetracycline and incubated for 12 h at $37^{\circ} \mathrm{C}$. Each expression of His-tag W334TAG mutant in the presence of the required unnatural amino acid was carried out as described. ${ }^{4,5}$ A single colony was used to inoculated 5 mL of LB broth media containing the appropriate antibiotics and the culture was grown at $37^{\circ} \mathrm{C}$ overnight with shaking ( 250 rpm ). Afterwards, 1 L of LB media containing the appropriate antibiotics was inoculated with 4 mL of the saturated overnight culture and grown with shaking ( 200 rpm ) at $37^{\circ} \mathrm{C}$. When $\mathrm{OD}_{600}$ reached 0.3 the cultures were supplemented with the appropriate unnatural amino acid (typically $2-5 \mathrm{mM}$ ). As a negative control a duplicate culture was grown without unnatural amino acid to generate the inactive truncated W334TAG protein. The protein over-expression was induced by addition of 0.5 mM IPTG at O.D. $600 \mathrm{~nm}=0.5-0.7$. After 4 h at $37^{\circ} \mathrm{C}$, the cultures were centrifuged at $4{ }^{\circ} \mathrm{C}(25 \mathrm{~min}$ at 4400 rpm$)$. The resulting pellet was stored at $-80^{\circ} \mathrm{C}$ overnight.

Protein purification. The purification of $6 \times$ His PR-AS mutants harbouring non-canonical amino acids follows the protocol mention above for wild-type and His-tag PR-AS (see page S3).

GC/MS Analysis. Proteins ( 50 mM ) were incubated with FPP ( 1 mM ) in incubation buffer [25 mM Tris ( pH 7.5 ), 5 mM BME, $5 \mathrm{mM} \mathrm{MgCl}_{2}$ and $15 \%$ glycerol] in a total volume of $250 \mu \mathrm{~L}$. The reaction was overlaid with pentane and left overnight at room temperature. Hydrocarbon products were extracted using pentane ( $2 \times 1 \mathrm{~mL}$ ), and purified by passing the pentane solutions through a short column containing alumina. GC-MS analysis was performed on a Waters 6890 N GC fitted with HP-5MS column (Agilent Technologies) and a GCT Premier mass spectrometer detecting in the range $m / z 50-800$ in $\mathrm{EI}^{+}$mode. Splitless injections of $10 \mu \mathrm{~L}$ were made at $50^{\circ} \mathrm{C}$.

Enzyme Kinetics. Kinetics assays were carried out according to the standard, linear range, micro-assay procedure developed for limonene and bornyl diphosphate synthases ${ }^{6}$ with modifications. ${ }^{7}$ This protocol involved the incubation of varying amounts of $\left[1-{ }^{3} \mathrm{H}\right]$ FPP (specific activity 75 $\mathrm{mCi} / \mathrm{mmol}$ ) with fix concentration of purified enzymes ( 100 nM ) in 20 mM Tris buffer containing $5 \mathrm{mM} \mathrm{MgCl} 2,5 \mathrm{mM} \beta$-mercaptoethanol and $15 \%$ glycerol at pH 7.5 . The reaction mixtures containing buffer, FPP and protein were prepared on ice in a total volume of $250 \mu \mathrm{~L}$ and were overlaid with 1 mL of HPLC-grade hexane prior to incubation. The assay mixtures were incubated at $31^{\circ} \mathrm{C}$ for a fixed period of time, usually 10 or 15 min . The reactions were immediately ice-cooled and quenched by addition of $200 \mu \mathrm{~L}$ of 100 mM EDTA ( pH 8.5 ) and brief vortexing. The hexane overlay and two additional 1 mL hexane extracts were passed through a short pipette column containing alumina. The column was washed with additional hexane ( 1 mL ) and the combined filtrates were analyzed by liquid scintillation counting using 15 mL of scintillation cocktail. Steady-State kinetic parameters for wild-type $6 \times$ His PR-AS and mutants were obtained by direct fitting of the data to the Michaelis-Menten equation by nonlinear least squares regression or by the graphical procedure developed by Lineweaver-Burk ${ }^{8}$ using the commercial SigmaPlot package (Systat Software).

## Sequence alignment of Sesquiterpene Synthases using ClustalW2．${ }^{9}$



Alignment of several fungi sesquiterpene synthases amino acids：AS＿PR，Aristolochene Synthase from Penicillium roqueforti（UnitProtKB／ TrEMBL accession number Q03471）；AS－AT，Aristolochene Synthase from Aspergillus terreus（UnitProtKB／TrEMBL accession number Q9UR08）；PS＿Strep Pentalenene synthase from Streptomyces sp．（strain UC5319）（UnitProtKB／TrEMBL accession number Q55012）；TS＿FS Trichodiene synthase from Fusarium sporotrichioides（UnitProtKB／TrEMBL accession number P13513））
---------------------------------------------------------------------------------------VDNQVAEKYAQEIETLKEQTSTMLSAACGTT--LT 33 ---------------------------------------------MASAAVANYEEEIVRPVAD--FSPSLWGDQFL-SFSIDNQVAEKYAKEIEALKEQTRNML-LATGMK--LA 65
 ----------------------------------------MASQVSQMPSSSPLSSNKDEIRPKAD--FQPSIWGDFFL-NCPDKNIDA-GTEKRHQQLKEEVRKMIVAPMAN---ST 71 _-_------------------------MAASS-----ADKCRPLAN--FHPSVWGYHFL-SYT-HEITN-QEKVEVDEYKETIRKMLVETCDN---ST 58
 -------------------------------------------MSLTE-----EKPIRPIAN--FPPSIWGDQFL-IYEKQVEQ--GVEQIVNDLKKEVRQLLKEALDIPMKHA 61

KLNLIDIIERLGIAYHFEKOIEDMLDHIYRADPYFEAHEYNDLNTSSVOFRLLROHGYNVSPNIFSRFODANGKFKESLRSDIRGLLNLYEASHVRTHKEDILEEALVFSVGHLESAAP 153 DTLNLIDTIERLGISYHFEKEIDDILDQIYNQN-----SNCNDLCTSALQFRLLRQHGFNISPEIFSKFQDENGKFKESLASDVLGLLNLYEASHVRTHADDILEDALAFSTIHLESAAP 180 LKLAFIDSVQGLGVSYHFTKEIEDELENIYHNN----NDAENDLYTTSLRFRLLREHGFHVSCDVFNKFKDEQGNFKSSVTSDVRGLLELYQASYLRVHGEDILDEAISFTSNHLSLAVA 187 QKLAFIDSVQRLGVSYHFTKEIEDELENIYHNN----NDAENDLYTTSLRFRLLREHGYNVSCDVFNKFKDEQGNFKSSVTSDVQGLLELYQASYLRVHGEDILDEAISFTTNHLSLAVS 187 QKLVLIDAMQRLGVAYHFDNEIETSIQNIFDASSK-QNDNDNNLYVVSLRFRLVRQQGHYMSSDVFKQFTNQDGKFKETLTNDVQGLLSLYEASHLRVRNEEILEEALTFTTTHLESIVS 177 NLLKLIDVIQRLGIAYYFEHEITQALDHIYSVY-----GDEWNGGRTSLWFRLLRQQGFYVSCDIFNIYKLDNGSFKDSLTKDIECMLELYEAAYMRVQGEIILDEALEFTKTHLEHIAK 178 NLLKLIDVIQRLGIAYYFEHEITQALDHIYNVY-----GDEWNGGSTSLWFRLLRQQGFYVSCDIFNIYKLDNGSFKDSLTKDIECMLELYEAAYMRVQGEIILDEALEFTKTHLEQIAK 180 NLLKLIDEIQRLGIPYHFEREIDHALQCIYETY-----GDNWNGDRSSLWFRLMRKQGYYVTCDVFNNYKDKNGAFKQSLANDVEGLLELYEATSMRVPGEIILEDALGFTRSRLSIMTK 176 QQMTLIDTLERLGLSFHFETEIEYKIELINAAE-----DDGFDLFATALRFRLLRQHORHVSCDVFDKFIDKDGKFEESLSNNVEGLLSLYEAAHVGFREERILQEAVNFTRHHLEGAEL 18O

HLKSP----LSKQVTHALEQSLHKSIPRVEIRYFIS-IYEEEEFKNDLLLRFAKLDYNLLQMLHKHELSEVSRWWKDLDFVTTLPYARDRAVE YF VTMGVYAEPQYSQARVMLAKTIAM 268 HLKSP----LREQVTHALEQCLHKGVPRVETRFFISSIYDKEQSKNNVLLRFAKLDFNLLQMLHKOELAQVSRWWKDLDFVTTLPYARDRVVEGY SLDHP----LSEEVSHALKQSIRRGLPRVEARHYLS-VYODIESHNKVLLEFAKIDFNMVQLLHRKELSEISRWWKDLDFORKLPYARDRVVE LDDHP----LSEEVSHALKQSIRRGLPRVEARHYLS-VYQDIESHNKALLEFAKIDFNMLQFLHRKELSEICRWWKDLDFQRKLPYARDRVVE NLSNNNN-SLKVEVGEALTQPIRMTLPRMGARKYIS-IYENNDAHHHLLLKFAKLDFNMLQKFHORELSDLTRWWKDLDFANKYPYARDRLVE DPLRCNN-TLSRHIHEALERPVQKRLPRLDAIRYIP-FYEQQDSHNKSLLRLAKLGFNRLQSLHKKELSQLSKWWKEFDAPKNLPYVRDRLVE DPLRCNN-TLSRHIYEALKRPIRKRLPRVDALOYMP-FYEOODSHNKSLLRLAKLGFNRLOSLHKKELSOLSKWWKEFDAPKNLRYVRDRLVE DAFSTNP-ALFTEIQRALKOPLWKRLPRIEAAQYIP-FYQQQDSHNKTLLKLAKLEFNLLQSLHKEELSHVCKWWKAFDIKKNAPCLRDRIVE DQSPLLI---REKVKRALEHPLHRDFPIVYARLFIS-IYEKDDSRDELLLKLSKVNFKFMONLYKEELSOLSRWWNTWNLKSKLPYARDRVVE促 SGYFEPQYSLGRKMLTKVIAM 302 SGYEPQYSLGRKMLTKVIAM 302 GVYY IGVYFRPQYSRRIFLTKTIKM 296 GLGSGYEPQYSRARVFFTKAVAV 294 GVGYHYEPQYSYVRMGLAKGVLI 296

ISIVDDTFDAYGIVKELEVYTDAIQRWDISQIDRLPEYMKISYKALLDLYDDYEKELSKDGRSDVVHYAKERMKEIVGNYFIEGKWFIEG-YMPSVSEYLSNALATSTYYLLTTTSYLGM 387 ISIVDDTFDAYGTVKELEAYTDAIQRWDINEIDRLPDYMKISYKAILDLYKDYEKELSSAGRSHIVCHAIERMKEVVRNYNVESTWFIEG-YTPPVSEYLSNALATTTYYYLATTSYLGM 415 ASIVDDTYDSYATYEELIPYTNAIERWDIKCIDELPEYMKPSYKALLDVYEEMEQLVAEHGRQYRVEYAKNAMIRLAQSYLVEARWTLQN-YKPSFEEFKANALPTCGYAMLAITSFVGM 421 ASIVDDTYDSYATYEELIPYTNAIERWDIKCIDELPEYMKPSYKALLDVYKEMEQLVAEHGRQYRVEYAKNAMIRLAQSYLVEARWTLQN-YKPSFEEFKANALPTCGYAMLAITSFVGM 421 TSIIDDTFDAYATFDELVTFNDAIQRWDANAIDSIOPYMRPAYOALLDIYSEMEOVLSKEGKLDRVYYAKNEMKKLVRAYFKETQWLNDCDHIPKYEEQVENAIVSAGYMMISTTCLVGI 415 AAILDDTYDIYGTYEELEIFTKAVQRWSITCMDTLPDYMKVIYKSLLDVYEEMEEIIEKDGKAYQVHYAKESMIDLVTSYMTEAKWLHEG-HVPTFDEHNSVTNITGGYKMLTASSFVGM 415 ATILDDTYDIHGTYEELEIFTKAVQRWSITCMDTLPDYMKMIYKSLLDVYEEMEEIIEKDGKAYQVHYAKDSMIDLVTSYMTEAKWLHEG-HVPTFEEYNSITNLTGGYKMLTTSSFVDM 417 ITLIDDTYDAYGTYEELKIFTEAVERWSITCLDTLPEYMKPIYKLFMDTYTEMEEFLAKEGRTDLFNCGKEFVKEFVRNLMVEAKWANEG-HIPTTEEHDPVVIITGGANLLTTTCYLGM 413 CGIMDDTYDNYATLNEAQLFTQVLDKWDRDEAERLPEYMKIVYRFILSIYENYERDAAKLGKSFAAPYFKETVKQLARAFNEEQKWVMER-QLPSFQDYVKNSEKTSCIYTMFASIIPGL 415

| VEtI | K--SATKEHFEWLATNPKILEANATLCRVVDDIATYEVEKGRGQIATGIECYMRDYGVSTEVAMEKFQEMADIAWKDVNEEILRPT--PVSSEILTRILNLARIIDVTYKHNQDGYTHPE 503 |
| :---: | :---: |
| EPI | K--SATEQDFEWLSKNPKILEASVIICRVIDDTATYEVEKSRGQIATGIECCMRDYGISTKEAMAKFQNMAETAWKDINEGLLRPT--PVSTEFLTPILNLARIVEVTYIHNLDGYTHPE 531 |
| DCS_G | G-DIVTPETFKWAANDPKIIQASTIICRFMDDVTEHKFKHRREDDCSAIECYMEEYGVTAQEAYDVFNKHVESAWKDVNQGFLKPT--EMPTEVLNRSLNLARVMDVLYREG-DGYTYVG 537 |
| DCS-G | G-DIVTPETFKWAANDPKIIQASTIICRFMDDVAEHKFKHRREDDCSAIECYMEEYGVSAQEAYDVFNKHVESAWKDVNQEFQKPT--EMPTEVLNRSLNLARVMDVLYREG-DGYTYVG 537 |
| GCS | E-EFISHETFEWLMNESVIVRASALIARAMNDIVGHEDEQERGHVASLIECYMKDYGASKQETYIKFLKEVTNAWKDINKQFSRPT--EVPMFVLERVLNLTRVADTLYKEK-DTYSTAK 531 |
| GAS | HGDIVTQESFKWVLNNPPLIKASSDISRIMNDIVGHKEEQQRKHIASSVEMYMKEYNLAEEDVYDFLKERVEDAWKDINRETLTCK--DIHMALKMPPINLARVMDMLYKNG-DNLKNVG 532 |
| GDS | PGDIVTQESFRWALNNPPLIKASADVSRIMDDIVGHKEEQQRKHLPSRVEMYMKKYHLAEEDVYDLLKQRVEDAWKDLNRETLTCK--DIHMALKMRPINLARVIDMLYKND-DNLKNVG 534 |
| AMDS | S-DIFTKESVEWAVSAPPLFRYSGILGRRLNDLMTHKAEQERKHSSSSLESYMKEYNVNEEYAQTLIYKEVEDVWKDINREYLTTK--NIPRPLLMAVIYLCQFLEVQYAGK-DNFTRMG 529 |
| EBFS | K--SVTQETIDWIKSEPTLATSTAMIGRYWNDTSSQLRESKGGEMLTALDFHMKEYGITKEEAASKFEGLVEETWKDINKEFIATTNYNVGREIAITFLNYARICEASYSKTDGDAYLDP 533 |
| VETI | KVLKPHIIALVVDSIDI 520 |
| EPI | KVLKPHIINLLVDSIKI 548 |
| DCS_GH | KAAKGGITSLLIEPIAL 554 |
| DCS-GA | KAAKGGITSLLIEPIAL 554 |
| GCS | GKLKNMINPILIESVKI 548 |
| GAS | QEIQDYMKSCFINPMSV 549 |
| GDS | QEIQDYIKSCFINAISV 551 |
| AMDS | DEYKHLIKSLLVYPMSI 546 |
| EBFS | NVAKANVVALFVDAIVF 550 |

Alignment of several plants sesquiterpene synthases amino acids : VETI, Vetispiradiene synthase from Hyoscyamus muticus (UnitProtKB/ TrEMBL accession number Q40577); EPI, Aristolochene Synthase from Nicotiana tabacum (UnitProtKB/TrEMBL accession number Q40577); DCS_GH, (+)- $\delta$-cadinene synthase from Gossypium arboreum (UnitProtKB/TrEMBL accession number P93665); DCS_GH (+)- $\delta$-cadinene synthase from Gossypium hirsutum (UnitProtKB/TrEMBL accession number Q39761); GCS, Germacrene C synthase from Solanum lycopersicum (UnitProtKB/TrEMBL accession number O64962); GAS, Germacrene A synthase from Solidago canadensis (UnitProtKB/TrEMBL accession number Q9AR67); GDS, (+)-germacrene D synthase from Solidago canadensis (UnitProtKB/TrEMBL accession number Q6TH92); AMDS, Amorpha-4,11-diene synthase from Artemisia annua (UnitProtKB/TrEMBL accession number Q9AR04); EBFS, (E)- $\beta$-farnesene synthase from Mentha piperita (UnitProtKB/TrEMBL accession number Q48935)

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## GC-MS: Cyclization of FFP catalyzed by His-tag WT

(Table 2 , entry 1)


## GC-MS: Cyclization of FFP catalyzed by W334F mutant

(Table1, entry 2)


## GC-MS: Cyclization of FFP catalyzed by W334Y mutant

(Table 1 , entry 3)


GC-MS: Cyclization of FFP catalyzed by W334H mutant
(Table 1 , entry 4)


GC-MS: Cyclization of FFP catalyzed by W334L mutant
(Table 1 , entry 5)


GC-MS: Cyclization of FFP catalyzed by W334Naphthyl mutant
(Table 2 , entry 2)


## GC-MS: Cyclization of FFP catalyzed by W334p-(CI)F

(Table 2 , entry 3)


## GC-MS: Cyclization of FFP catalyzed by W334p-( $\left.\mathrm{CF}_{3}\right)$ F

(Table 2 , entry 4)


## GC-MS: Cyclization of FFP catalyzed by W334p-( $\mathrm{NO}_{2}$ )F

(Table 2 , entry 5)


Lineweaver-Burk plot for His-tag WT Aristolochene Synthase (Table 2 , entry 1)


Michaelis-Menten plot for W334F mutant
(Table 1, entry 2)


Michaelis-Menten plot for W334Y mutant
(Table 1 , entry 3)


## Lineweaver-Burk plot for W334H mutant

(Table 1 , entry 4)


## Lineweaver-Burk plot for W334L mutant

(Table 1 , entry 5)


Lineweaver-Burk plot for W334Naphtyl mutant
(Table 2 , entry 2)


Lineweaver-Burk plot for W334p-(CI)F mutant
(Table 2 , entry 3)


## Lineweaver-Burk plot for W334p-( $\left.\mathrm{CF}_{3}\right)$ F mutant

(Table 2 , entry 4)


Lineweaver-Burk plot for W334p-( $\mathrm{NO}_{2}$ )F mutant
(Table 2 , entry 5)


