Supporting Information

for

Targeting active site residues and structural anchoring positions in terpene synthases

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Amino acid sequence alignment, details about the mutagenesis, purification and analytical data
Consensus
Selina-4(15),7(11)-diene (S. pristinaespiralis)
Polytrichastrene A (C. polytrichastri)
Spirovioleone (S. violens)
a-Amorphene (S. viridochromogenes)
Germacrene A (M. marina)
Caryolan-1-ol (S. griseus subsp. griseus)
7-epi-Cubenol (S. griseus subsp. griseus)
Selina (S. avermitilis)
Pentalenene (S. exfoliatius)

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Avermitilol (S. avermitilis)  
Pentalenene (S. exfoliatus)
| Consensus                                         | ADXVAAXX------------------------XRGEXDXXAXXXRSVRRQVPAQRSA 352 |
| Selina-4(15),7(11)-dien (S. pristinaespiralis)   | PDDPANMSVFVTDVPTD---------------DSTEPLDPAVSVWWDLAEDARSVRRQVPAQRSA 365 |
| Polytrichastrene A (C. polytrichastri)           | GGYVDAEFD----------------------TQGE-----------------------------338 |
| Spiroviolene (S. violens)                        | LGRVFDPMEAPEWAESPADDARSAPRGLPTVAWWDDLALLGV---------------------359 |
| a-Amorphene (S. viridochromogenes)               | TLTPAPG-------------------------------335 |
| Germacrene A (M. marina)                         | ---------------------------------------------------------------316 |
| Caryolan-1-ol (S. griseus subsp. griseus)        | PDLVELD------------------------ERDSLSRHFAA-----------------------335 |
| epi-Cubenol (S. griseus subsp. griseus)          | ADVQYEEPEEYLETVGLVFPRASETAPACAGAEPFAR------------------------339 |
| 7-epi-a-Eudesmol (S. viridochromogenes)          | AKEGAVATAELA-------------------GGRGSVDDLLTV--------------------343 |
| Avermitilol (S. avermitilis)                     | APHVLPST----------------------GPYFDEVLPT-----------------------335 |
| Pentalenene (S. exfoliatus)                      | EFALAAG------------------------AQGYLEELGSSAH-------------------337 |

**Figure S1.** Amino acid sequence alignment some characterised sesqui- and diterpene synthases. Highly conserved residues and motifs are marked in yellow.
Site-directed mutagenesis

The site mutations were performed by the overlap extension PCR (OE-PCR) method.\(^1\) The template was the wildtype gene cloned into the pYE-Express expression vector.\(^2\) The mutational primers are listed in Table S1, the polymerase was purchased from NEB (Q5® High-Fidelity DNA polymerase, Ipswich, Massachusetts, USA). The first-round PCRs for individual amplification of the left and the right part of the mutated gene followed the program: 1) 98 °C for 30 s; 2) 98 °C for 10 s, 67 °C for 30 s, 72 °C for 40 s; repeated 32 times; 3) 72 °C for 2 min. The PCR products were analyzed by gel electrophoresis and purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). The obtained overlapping fragments were then mixed and were used for the next round PCRs over two steps. Step 1: 1) 98 °C for 30 s; 2) 98 °C for 10 s, 64 °C for 30 s, 72 °C for 45 s; repeated 5 times; 3) 72 °C for 2 min. Step 2: addition of primers 28B8 and 28B9 for amplification of the whole SmTS1 gene carrying additional homology arms for cloning into the pYE-Express expression vector by homologous recombination in yeast. The PCR was then continued: 1) 98 °C for 30 s; 2) 98 °C for 10 s, 67 °C for 30 s, 72 °C for 45 s; repeated 34 times; 3) 72 °C for 2 min. The mutated genes were then purified by the Wizard® SV Gel and PCR Clean-Up System.

The mutated genes were cloned into the pYE-Express expression vector by homologous recombination in yeast following the standard PEG/LiOAc/salmon sperm protocol.\(^3\),\(^4\) S. cerevisiae cultures were grown on SM-URA plates for 3 days. Single colonies were then collected, and the plasmid DNA was isolated by using the Zymoprep™ Yeast Plasmid Miniprep II kit (Zymo Research, Irvine, CA, USA). The isolated plasmids were transformed into *E. coli* BL21(DE3) electrocompetent cells by electroporation. Cells were plated on LB medium (kanamycin) and grown at 37 °C over night. Single colonies were picked and used to inoculate 3 mL LB cultures (kanamycin). After incubation at 37 °C for 12 h, the cells were collected by centrifugation (5000 rpm × 5 min), and the plasmids were isolated by the PureYield™ Plasmid Miniprep System (Promega) and checked by sequencing. The transformants containing the correct mutations were stored in 20% glycerol at −78 °C and used for protein expression.
### Table S1. Primers used for site-directed mutagenesis of SmTS1.

| enzyme variant | primer | nucleotide sequence (5’ → 3’)[b] |
|----------------|--------|---------------------------------|
| SmTS1 wildtype| 28B8   | GGCAGGCTCAACCGGTC               |
|                | 28B9   | TCTCAGTGGTGTTGGTGTTGCTCTAGTCAGTCATATGGCTAGCATGACTGGTGGA                |
|                |        | CTATCCCAGGCTCTCGTTGTCG            |
| N86D           | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 32H8  | CGGGCGGAGGGTGAGGTGTCGAGGAGGGTGAGGTGTCGAGG |
|                | fragment 2 | 32H7  | CTGGGCTTCTCTGCGCACAGCTCACCCGCTCCCG |
|                |        | 28B7  | CTATCCCAGGCTCTCGTTGTCG |
| G180R          | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 32I1  | GCCCTCGGAGGCGTAAACCGAGGAGGTGAGGTGTCGAGG |
|                | fragment 2 | 32H9  | GTCAAGAACCACCTCAACCTCCTGCTCAGGCTGAGGAGG |
|                |        | 28B7  | CTATCCCAGGCTCTCGTTGTCG |
| Q227D          | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 32I3  | CGGATTTTGAATAGAGATAGGATACAGGTTCGAGGTGTCGAGG |
|                | fragment 2 | 32I2  | CCACCSCGGAATTCGACAGGCTGACTCTCTATCTGAAGATCGCG |
|                |        | 28B7  | CTATCCCAGGCTCTCGTTGTCG |
| R228L          | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 32I5  | GTGCGGATTTTGAAGATAGAGATACAGGTTCGAGGTGTCGAGG |
|                | fragment 2 | 32I4  | CGCGGATTTTGAAGATACAGGTTCGAGGTGTCGAGG |
|                |        | 28B7  | CTATCCCAGGCTCTCGTTGTCG |
| R242N          | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 32I7  | GAGSGATTTTGAAGATAGAGATACAGGTTCGAGGTGTCGAGG |
|                | fragment 2 | 32I6  | CGCGGATTTTGAAGATACAGGTTCGAGGTGTCGAGG |
|                |        | 28B7  | CTATCCCAGGCTCTCGTTGTCG |
| F307W          | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 32I9  | GAGSGATTTTGAAGATAGAGATACAGGTTCGAGGTGTCGAGG |
|                | fragment 2 | 32I8  | CTACGCTGCGCATCTACCTGCGCAAGGGCCTC |
|                |        | 28B7  | CTATCCCAGGCTCTCGTTGTCG |
| G184L          | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 33A4  | GGTGGCTTCTCGGCGCTAAAGACGGCGTAAAGG |
|                | fragment 2 | 33A3  | CCCCTCGGGCATCTACCTGCGCAAGGGCCTC |
|                |        | 28B7  | CTATCCCAGGCTCTCGTTGTCG |
| A222V          | fragment 1 | 28B6  | GTGACGCTCAACCGGTC |
|                |        | 33A2  | GTACGCTGCGCATCTACCTGCGCAAGGGCCTC |
|                | fragment 2 | 33A1  | CCACGCTGCGCATCTACCTGCGCAAGGGCCTC |

[a] The third nucleotide of each primer is shown in lowercase.
[b] The lowercase letters represent the third nucleotide of each primer.
| Fragment | 28B7 | 28B6 | 34D4 | 34D3 | 34D2 | 34D1 | 34D7 | 34D6 | 34D5 |
|----------|------|------|------|------|------|------|------|------|------|
| A222M    |      |      |      |      |      |      |      |      |      |
| fragment 1 | 28B7 |      |      |      |      |      |      |      |      |
| fragment 2 | 28B7 |      |      |      |      |      |      |      |      |
| A222L    |      |      |      |      |      |      |      |      |      |
| fragment 1 | 28B7 |      |      |      |      |      |      |      |      |
| fragment 2 | 28B7 |      |      |      |      |      |      |      |      |
| A222I    |      |      |      |      |      |      |      |      |      |
| fragment 1 | 28B7 |      |      |      |      |      |      |      |      |
| fragment 2 | 28B7 |      |      |      |      |      |      |      |      |
| A222F    |      |      |      |      |      |      |      |      |      |
| fragment 1 | 28B7 |      |      |      |      |      |      |      |      |
| fragment 2 | 28B7 |      |      |      |      |      |      |      |      |
| A222Y    |      |      |      |      |      |      |      |      |      |
| fragment 1 | 28B7 |      |      |      |      |      |      |      |      |
| fragment 2 | 28B7 |      |      |      |      |      |      |      |      |
| A222W    |      |      |      |      |      |      |      |      |      |
| fragment 1 | 28B7 |      |      |      |      |      |      |      |      |
| fragment 2 | 28B7 |      |      |      |      |      |      |      |      |

[a] Homology arms for gene cloning are shown in bold. DNA base exchanges introduced for mutations are underlined.
Expression and purification of SmTS1 variants

The transformants containing the mutations were inoculated in LB broth (3 mL, kanamycin). The cultures were grown at 37 °C to form the precultures, the precultures (0.4 mL) were then used to inoculate the expression cultures (400 mL, kanamycin). The expression cultures were incubated at 37 °C until OD600 = 0.4–0.6 was reached. The expression cultures were cooled to 18 °C, and IPTG (0.4 mL; 400 mM in H2O) was added into the expression culture to induce protein expression. Incubation was continued at 18 °C for 16 h.

The cells were collected by centrifugation (3500 rpm × 30 min), and resuspended in binding buffer (7 mL; 50 mM Tris, 300 mM NaCl, 20 mM imidazole, 2 mM β-mercaptoethanol, 1 mM MgCl2, pH = 7.6). The suspension was then lyzed by ultra-sonification (on ice, 6 × 45 s), and the lysates were centrifuged (10000 rpm × 20 min) to remove the cell debris. The supernatant was filtered through a syringe filter (0.45 μm) and loaded onto a Ni2+-NTA affinity chromatography column (Protino™ Ni-NTA, Macherey-Nagel, Düren, Germany). The column was washed with binding buffer (1 mL), washing buffer (2 × 1 mL; 50 mM Tris, 300 mM NaCl, 60 mM imidazole, 2 mM β-mercaptoethanol, 1 mM MgCl2, pH = 7.6), and the protein was obtained by using elution buffer (1 mL; 50 mM Tris, 300 mM NaCl, 300 mM imidazole, 2 mM β-mercaptoethanol, 1 mM MgCl2, pH = 7.6).

Figure S2. SDS-PAGE analysis of the SmTS1 variants.

Activity of enzyme variants

The protein concentration was determined by the Bradford method,[5] and adjusted to 80 μg mL⁻¹ for all variants to test the enzyme activities. The prepared protein solution (200 μL), Tris buffer (200 μL; 50 mM Tris, 1 mM MgCl2, pH = 7.6), incubation buffer (0.5 mL; 50 mM Tris, 10 mM MgCl2, 20% glycerol, pH = 7.6) and GFPP (1 mL; 1 mg mL⁻¹ in 25 mM aqueous NH4CO3) were mixed and incubated at 28 °C overnight. The products were extracted with hexane (200 μL; containing 0.08 mg L⁻¹ octadecane as internal standard) for GC/MS analysis. For every enzyme variant reactions were performed in triplicates.

GC/MS

GC/MS analyses were carried out on a 7890B/5977A series gas chromatography/mass selective detector (Agilent, Santa Clara, CA, USA). The GC was equipped with an HP5-
MS fused silica capillary column (30 m, 0.25 mm i. d., 0.50 μm film; Agilent) and operated using the settings 1) inlet pressure: 77.1 kPa, He at 23.3 mL min⁻¹, 2) injection volume: 1–2 μL, 3) temperature program: 5 min at 50 °C then increasing 5 °C min⁻¹ to 320 °C, 4) 60 s valve time, and 5) carrier gas: He at 1.2 mL min⁻¹. The MS was operated with settings 1) source: 230 °C, 2) transfer line: 250 °C, 3) quadrupole: 150 °C and 4) electron energy: 70 eV.

**NMR spectroscopy**

NMR spectra were recorded at 298 K on a Bruker (Billerica, MA, USA) Avance III HD Cryo (700 MHz) NMR spectrometer. Spectra were measured in C₆D₆ and referenced against solvent signals (¹H NMR, residual proton signal: δ = 7.16 ppm; ¹³C-NMR: δ = 128.06 ppm).[^6]
Figure S3. Total ion chromatograms of extracts from incubations of GFPP with A) wildtype SmTS1, and the SmTS1 variants B) N86D, C) G180R, D) Q227D, E) R242N, F) F307W, G) G184L, and H) A222V.
Table S2. Production of compounds 1–6 and unknown a–c by wildtype SmTS1 and its enzyme variants, and relative enzyme activities.

| enzyme variant | 2     | 1+6[a] | a     | b+c   | 3+4   | 5     | activity[b] |
|----------------|-------|--------|-------|-------|-------|-------|-------------|
| wildtype       | 48±3  | 100±7  | 25±5  | 46±7  | 53±4  | 30±9  | 100±19      |
| N86D           | 15±2  | 148±9  | 15±1  | 68±5  | 32±3  | 70±7  | 104±9       |
| G180R          | 25±2  | 41±1   | 9±1   | 32±3  | 35±23 | 20±5  | 51±16       |
| Q227D          | 61±6  | 163±15 | 37±4  | 89±11 | 79±10 | 54±12 | 155±13      |
| R242N          | 54±6  | 105±9  | 24±3  | 51±7  | 60±14 | 25±4  | 94±17       |
| F307W          | 23±2  | 54±5   | 17±1  | 39±3  | 26±3  | 23±3  | 53±6        |
| A222V          | –     | –      | –     | –     | –     | –     | –           |
| G184L          | –     | –      | –     | –     | –     | –     | –           |

[a] Production of compounds 1 – 6 by peak integration of total ion chromatograms from triplicates. Production of 1 and co-eluting 6 by wildtype SmTS1 is set to 100%. [b] Enzyme activities were calculated from total production of all compounds by peak integrations from triplicates. Activity of wildtype SmTS1 is set to 100%.
Figure S4. Production of compounds 1–6 and a–c by peak integration of total ion chromatograms (mean ± standard deviation from triplicates). Production of 1 with co-eluting 6 by wildtype SmTS1 is set to 100%. The production of A) 2, B) 1 + 6, C) unknown a, D) unknown b + c, E) 3 + 4, F) 5 and G) total activities of the wildtype and each variant. For a comparison of enzyme activities, the peak integrals of all produced compounds were summarised. Activity of wildtype SmTS1 is set to 100%.
Figure S5. The A222V enzyme variant. A) Total ion chromatogram of an extract from an incubation with GGPP, and mass spectra of B) 8 and C) 9.
Table S3. Production of sesterterpenes and diterpenes by wildtype SmTS1 and its enzyme variants related to the position A222, and relative enzyme activities.

| enzyme variant | sesterterpenes$^a$ | 8   | 9   | diterpenes$^b$ |
|----------------|---------------------|-----|-----|----------------|
| Wildtype       | 100±8%              | 0   | 0   | 0              |
| A222V          | 0                   | 98±11% | 31±2% | 129±8%         |
| A222M          | 39±9%               | 82±3% | 80±2% | 162±3%         |
| A222I          | 0                   | 110±2% | 47±2% | 157±1%         |
| A222L          | 13±4%               | 89±3% | 54±8% | 142±11%        |
| A222F          | 0                   | 21±2% | 15±8% | 37±8%          |

[a] Production of sesterterpenes by wildtype SmTS1 is set to 100%. [b] Enzyme activities were calculated from total production of 8 and 9 by peak integrations from triplicates.
Figure S6. Total ion chromatograms of extracts from incubations of GFPP with A) wildtype SmTS1, and the SmTS1 variants B) A222M, and C) A222L.
Figure S7. Total ion chromatograms of extracts from incubations of GGPP with the SmTS1 variants A) A222V, B) A222M, C) A222I, D) A222L, and E) A222F. Asterisks indicate degradation products from GGPP also observed without enzyme.
Figure S8. Production of sesterterpenes and diterpenes from SmTS1 and its variants in the position of A222. Production of A) sesterterpenes, B) compound 8, C) compound 9 and D) diterpenes (sum of 8 and 9) by the wildtype and its variants. The data were obtained by peak integration of total ion chromatograms (mean ± standard deviation from triplicates). Production of sesterterpenes by wildtype is set to 100%.
Figure S9. Structure elucidation of 8. Bold: $^1$H,$^1$H COSY, single headed arrows: key HMBC, and double headed arrows: key NOESY correlations. Carbon numbering follows GGPP numbering to indicate the origin of each carbon.

Table S4. NMR data of cembrene A (8) in C$_6$D$_6$ recorded at 298 K.

| C$^{[a]}$ | $^{13}$C$^{[b]}$ | $^1$H$^{[b]}$                      |
|--------|----------------|----------------------------------|
| 1      | 33.02          | 2.16 (m, 1H); 2.02 (m, 1H)       |
| 2      | 124.57         | 5.32 (ddq, J = 8.1, 6.7, 1.3 Hz, 1H) |
| 3      | 134.91         | –                                |
| 4      | 39.38          | 2.15 (m, 1H)                     |
|        |                | 2.11 (m, 1H)                     |
| 5      | 25.35          | 2.26 (m, 1H)                     |
|        |                | 2.13 (m, 1H)                     |
| 6      | 126.45         | 5.08 (dddd, J = 7.0, 5.5, 2.6, 1.3 Hz, 1H) |
| 7      | 133.48         | –                                |
| 8      | 39.85          | 2.10 (m, 2H)                     |
| 9      | 24.22          | 2.15 (m, 2H)                     |
| 10     | 122.33         | 5.25 (ddq, J = 6.5, 4.6, 1.5 Hz, 1H) |
| 11     | 133.86         | –                                |
| 12     | 34.37          | 2.04 (m, 1H)                     |
|        |                | 1.94 (m, 1H)                     |
| 13     | 28.59          | 1.80 (ddt, J = 13.9, 10.0, 3.9 Hz, 1H) |
|        |                | 1.41 (dddd, J = 13.8, 10.1, 6.7, 3.8 Hz, 1H) |
| 14     | 46.55          | 2.22 (m, 1H)                     |
| 15     | 149.25         | –                                |
| 16     | 19.37          | 1.63 (t, J = 1.2 Hz, 3H)         |
| 17     | 110.80         | 4.83 (m, 2H)                     |
| 18     | 18.33          | 1.60 (s, 3H)                     |
| 19     | 15.40          | 1.54 (s, 3H)                     |
| 20     | 15.64          | 1.55 (s, 3H)                     |

[a] Carbon numbering as shown in Figure S9. [b] Chemical shifts δ in ppm, multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.
Figure S10. $^1$H NMR spectrum of 8 (700 MHz, C$_6$D$_6$).
Figure S11. $^{13}$C NMR spectrum of 8 (176 MHz, C$_6$D$_6$).
Figure S12. $^{13}$C DEPT spectrum of 8 (176 MHz, $C_6D_6$).
Figure S13. $^1$H,$^1$H COSY spectrum of 8 (700 MHz, C$_6$D$_6$).
Figure S14. HSQC spectrum of 8 (C₆D₆).
Figure S15. HMBC spectrum of 8 (C₆D₆).
Figure S16. NOESY spectrum of 8 (C₆D₆).
**Figure S17.** Structure elucidation of 9. Bold: $^1$H,$^1$H-COSY, single headed arrows: key HMBC, and double headed arrows: key NOESY correlations. Carbon numbering follows GGPP numbering to indicate the origin of each carbon.

| C[a] | $^{13}$C[b] | $^1$H[b] |
|------|-------------|----------|
| 1    | 29.04       | 2.20 (m, 1H) |
|      |             | 1.91 (dt, $J = 15.1, 7.6$ Hz, 1H) |
| 2    | 126.93      | 5.26 (tq, $J = 7.2, 1.3$ Hz, 1H) |
| 3    | 133.04      | –        |
| 4    | 39.31       | 2.13 (m, 2H) |
| 5    | 25.16       | 2.19 (m, 2H) |
| 6    | 126.29      | 5.07 (t, $J = 6.7$ Hz, 1H) |
| 7    | 133.02      | –        |
| 8    | 39.88       | 2.10 (m, 1H) |
|      |             | 2.06 (m, 1H) |
| 9    | 24.49       | 2.14 (m, 2H) |
| 10   | 125.33      | 5.16 (t, $J = 6.8$ Hz, 1H) |
| 11   | 134.28      | –        |
| 12   | 38.21       | 2.16 (m, 1H) |
|      |             | 2.09 (m, 1H) |
| 13   | 28.77       | 1.66 (m, 1H) |
|      |             | 1.24 (m, 1H) |
| 14   | 48.8        | 1.34 (m, 1H) |
| 15   | 73.21       | –        |
| 16   | 27.85       | 1.07 (s, 3H) |
| 17   | 27.88       | 1.06 (s, 3H) |
| 18   | 15.79       | 1.59 (s, 3H) |
| 19   | 15.44       | 1.54 (d, $J = 1.3$ Hz, 3H) |
| 20   | 15.69       | 1.57 (s, 3H) |

[a] Carbon numbering as shown in Figure S17. [b] Chemical shifts $\delta$ in ppm, multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.
Figure S18. $^1$H NMR spectrum of 9 (700 MHz, C$_6$D$_6$).
Figure S19. $^{13}$C NMR spectrum of 9 (176 MHz, C$_6$D$_6$).
Figure S20. $^{13}$C DEPT spectrum of 9 (176 MHz, C$_6$D$_6$).
Figure S21. $^1$H,$^1$H COSY spectrum of 9 (700 MHz, C$_6$D$_6$).
Figure S22. HSQC spectrum of 9 (C₆D₆).
**Figure S23.** HMBC spectrum of 9 (C₆D₆).
Figure S24. NOESY spectrum of 9 (C₆D₆).
Figure S25. Determination of the absolute configuration of compounds 8 and 9 from SmTS1 variants. Partial HSQC spectra of labelled 8 and 9 obtained from (R)-(1\(^-^{13}\)C,1\(^-^{2}\)H)GGPP (left) and (S)-(1\(^-^{13}\)C,1\(^-^{2}\)H)GGPP (right) with variant A) A222V, B) A222L, C) A222I, and D) A222F.
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