Supplementary Information

Structure-guided product determination of the bacterial type II diterpene synthase Tpn2

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Supplementary Table 1. Strains used in this study

Strain	Description	Source (Reference)
E. coli NEB Turbo	Host for general cloning	New England Biolabs
E. coli NEB NiCo21	Host for high-level protein production	New England Biolabs
E. coli BL21 Star (DE3)	Host for high-level protein production	Invitrogen
Kitasatospora sp. CB02891	Putative terpentecin producer, used for isolation of genomic DNA for *tpn2* and *tpn3* amplification	Gift from Shen Lab[1]

Supplementary Table 2. Plasmids used in this study

Plasmid	Description	Source (Reference)
pRSF-Duet	General plasmid for cloning	Novagen
pCDF-Duet	General plasmid for cloning	Novagen
pET-Duet	General plasmid for cloning	Novagen
pUC19	General plasmid for cloning	Novagen
pET28a	Plasmid for heterologous expression in E. coli	Novagen
pBS3080	Plasmid created from pRSF-duet by cloning the TEV/LIC sites; used for cloning and protein expression	(2)
pJR1064	pET28a-MKI4: pET28a harboring kinases *Ec-Th*iM and *At-IPK*, *Ec-idi*, and GGPP synthase (*bnd3*). Ribosome binding sites were inserted before each gene.	(3)
pJR2001	pRSF/TEV/LIC harboring *tpn2*	This study
pJR2002	pRSF/TEV/LIC harboring *tpn2* (H180F)	This study
pJR2003	pRSF/TEV/LIC harboring *tpn2* (H180V)	This study
pJR2004	pRSF/TEV/LIC harboring *tpn2* (D296A)	This study
pJR2005	pRSF/TEV/LIC harboring *tpn2* (Y329F)	This study
pJR2006	pRSF/TEV/LIC harboring *tpn2* (H342A)	This study
pJR2007	pRSF/TEV/LIC harboring *tpn2* (H342F)	This study
pJR2008	pRSF/TEV/LIC harboring *tpn2* (H342A-D296A)	This study
pJR2009	pRSF/TEV/LIC harboring *tpn2* (H342F-D296A)	This study
pJR2010	pRSF/TEV/LIC harboring *tpn2* (K383A)	This study
pJR2011	pRSF/TEV/LIC harboring *tpn2* (G485D)	This study
pJR2012	pRSF/TEV/LIC harboring *tpn2* (Y489A)	This study
pJR2013	pET-Duet harboring *tpn2*, *tpn3*, and *tpn6* for heterologous expression in *E. coli*	This study
pJR2014	pCDF duet harboring *tpn2* for heterologous expression in *E. coli*	This study

pJR2015	pCDF duet harboring *tpn2* (G485D) for heterologous expression in *E. coli*	This study
pJR2016	pCDF duet harboring *tpn2* and *tpn3* with an additional RBS for heterologous expression in *E. coli*	This study
pJR2017	pCDF duet harboring *tpn2* and *tpn3* linked with a glycine-serine linker for heterologous expression in *E. coli*	This study
pJR2018	pCDF duet harboring *tpn2* (G485D) and *tpn3* for heterologous expression in *E. coli*	This study
pJR2019	pCDF duet harboring *tpn2* (G485E) for heterologous expression in *E. coli*	This study
pJR2020	pCDF duet harboring *tpn2* (G485E) and *tpn3* for heterologous expression in *E. coli*	This study

Supplementary Table 3. Primers used in this study

Name	Sequence	Purpose
pRSFtpn2_F	aaaacctctatttccagtcgatgagtgacgccgatcggatagc	*tpn2* or mutant amplification for protein expression in *E. coli*
pRSFtpn2_R	ctcgatcgcgtcgggcatcagtacctgctcacgacgg	
H180F_F	agacggcatggttcacactcgaggc	Tpn2 mutagenesis for H180F
H180F_R	gcctcgagtgtgaaccatgccgtct	
H180V_F	agacggcatgggtcacactcgaggc	Tpn2 mutagenesis for H180V
H180V_R	gcctcgagtgtgacccatgccgtct	
D296A_F	ccgcccgacggggccgacacggcctac	Tpn2 mutagenesis for D296A
D296A_R	gtaggccgtgtcggccccgtcgggcgg	
Y329F_F	cacttcgtttcgtttccgggggagcag	Tpn2 mutagenesis for Y329F
Y329F_R	ctgctcccccggaaacgaaacgaagtg	
H342A_F	ccgtgaacgcggccgccctcgagtac	Tpn2 mutagenesis for H342A
H342A_R	gtactcgagggcggccgcgttcacgg	
H342F_F	ccgtgaacgcgttcgccctcgagtac	Tpn2 mutagenesis for H342F
H342F_R	gtactcgagggcgaacgcgttcacgg	
K383A_F	ggctgctggtacgacgcatggaatgtctcgccg	Tpn2 mutagenesis for K383A
K383A_R	cggcgagacattccatgcgtcgtaccagcagcc	
G485D_F	ccgctgtggatggacaaggacctgtac	Tpn2 mutagenesis for G485D
G485D_R	gtacaggtccttgtccatccacagcgg	
G485E_F	ccgctgtggatggaaaaggacctgtac	Tpn2 mutagenesis for G485E
G485E_R	gtacaggtcctttccatccacagcgg	
Y489A_F	caaggacctggccacgcctttccgga	Tpn2 mutagenesis for Y489A
Y489A_R	tccggaaaggcgtggccaggtccttg	

NcoI_ala_Tpn2_F	aataaggagatataccatggcgagtgacgccgatcggatagc	*tpn2* WT amplification for in vivo expression
Tpn3_Hind_R	cattatgcggccgcaagctttcagcggtagcggtctgtct	*tpn3* amplification for in vivo expression
Tpn2-3_F	gcaggcgcgccatgagtgacgccgatcggatagc	*tpn2* or mutant amplification for in vivo expression
Tpn2_HindIII_R	tagcaagctttcagtacctgctcacgacgg	
Tpn1-3_R	cccaagctttcagcggtagcggtctgtct	
Tpn2_RBSOP_R	tatacgccccctatgccttaaataca tcagtacctgctcacgacgg	*tpn2* or mutant amplification for in vivo expression and addition of RBS sequence between *tpn2* and *tpn3*
RBSOP_Tpn3_F	tgtatttaaggcatagggggcgtata atgcccgacgcgatcgagtt	
Tpn2_GSlink_R	agaaccgccagaaccagaaccgtacctgctcacgacggcgc	*tpn2* or mutant amplification for in vivo expression and addition of linker sequence between *tpn2* and *tpn3*
GSlink_Tpn3_F	ggttctggttctggcggttctatgcccgacgcgatcgagtt	
Tpn6_F	gcacatatgtacaccgataccgccgaaac	*tpn6* amplification for in vivo expression
Tpn6_R	ggttaattaatcagtggttcctgagagcgac	
GSlink_tpn3_teda_F	ccgcttccggaggtgctggttctggttctggcggttct	*tpn3* amplification for in vivo expression
Tpn3_teda_R	gactctagaggatccccgggtcagcggtagcggtctgtct	

Supplementary Table 4. ^{1}H NMR (600 MHz) and ^{13}C NMR (151 MHz) spectroscopic data for TPP (**2**) and terpentetriene (**3**).[a]

No.	**2**[b]		**3**[c]	
	δ_H		δ_C	δ_H
1, CH_2	1.62 (dd, *J* = 12.7, 6.4); 1.33 (m)		17.8	1.43 (m); 1.67 (m)
2, CH_2	1.89 (m); 1.95 (m)		26.8	2.00 (m); 2.15 (m)
3, CH	5.21 (br s)		120.2	5.17 (ddd, *J* = 4.5, 2.7, 1.3)
4, qC			144.5	
5, qC			38.3	
6, CH_2	1.35 (m)		30.2	1.46 (m); 1.50 (m)
7, CH_2	1.22 (m), 1.89 (m)		25.6	1.32 (dq, *J* = 13.9, 3.3); 1.96 (m)
8, CH	1.47 (m)		35.2	1.69 (m)
9, qC			37.7	
10, CH	1.35 (m)		45.1	1.45 (m)
11, CH_2	1.08 (m), 1.47 (m)		38.3	1.20 (td, *J* = 12.8, 4.6); 1.64 (m)
12, CH_2	1.95 (m)		24.6	2.22 (m); 2.18 (m)
13, qC			147.8	
14, CH	5.41 (m)		139.2	6.40 (dd, *J* = 17.6, 10.9)
15, CH_2	4.38 (t, *J* = 6.4)		113.0	5.07 (dq, *J* = 10.8, 1.0); 5.27 (dd, *J* = 17.6, 1.2)
16, CH_3	1.65 (d, *J* = 1.5)		115.2	5.02 (s)
17, CH_3	0.85 (d, *J* = 6.7)		14.9	0.98 (s)
18, CH_3	0.98 (s)		20.6	1.08 (s)
19, CH_3	1.53 (s)		18.0	1.61 (m)
20, CH_3	0.86 (s)		20.4	0.99 (br s)

[a] δ in ppm, *J* in Hz

[b] NMR taken in D_2O

[c] NMR taken in $CDCl_3$

Supplementary Table 5. ^{1}H NMR (600 MHz) and ^{13}C NMR (151 MHz) spectroscopic data for *syn*-sclarene (**5**).[a]

No.	**5**[b]	
	δ_C	δ_H
1, CH_2	36.7	1.08 (m); 1.58 (m)
2, CH_2	19.2	1.46 (m); 1.65 (m)
3, CH	42.7	1.18 (td, *J* = 13.2, 3.6); 1.42 (m)
4, qC	33.2	
5, qC	45.8	1.32 (m)
6, CH_2	23.7	1.33 (m); 1.64 (m)
7, CH_2	31.62	2.13 (m); 2.22 (m)
8, CH	149.3	
9, qC	58.4	1.61 (m)
10, CH	38.0	
11, CH_2	25.2	1.53 (m); 1.75 (m)
12, CH_2	30.3	1.95 (m); 2.16 (m)
13, qC	147.2	
14, CH	139.0	6.38 (dd, *J* = 17.6, 10.8)
15, CH_2	113.1	5.06 (dq, *J* = 10.9, 1.2); 5.22 (dd, *J* = 17.6, 1.1)
16, CH_3	115.5	5.02 (s)
17, CH_3	109.6	4.59 (dd, *J* = 2.8, 1.7); 4.75 (t, *J* = 2.4)
18, CH_3	22.2	0.88 (s)
19, CH_3	33.5	0.89 (s)
20, CH_3	22.4	0.95 (s)

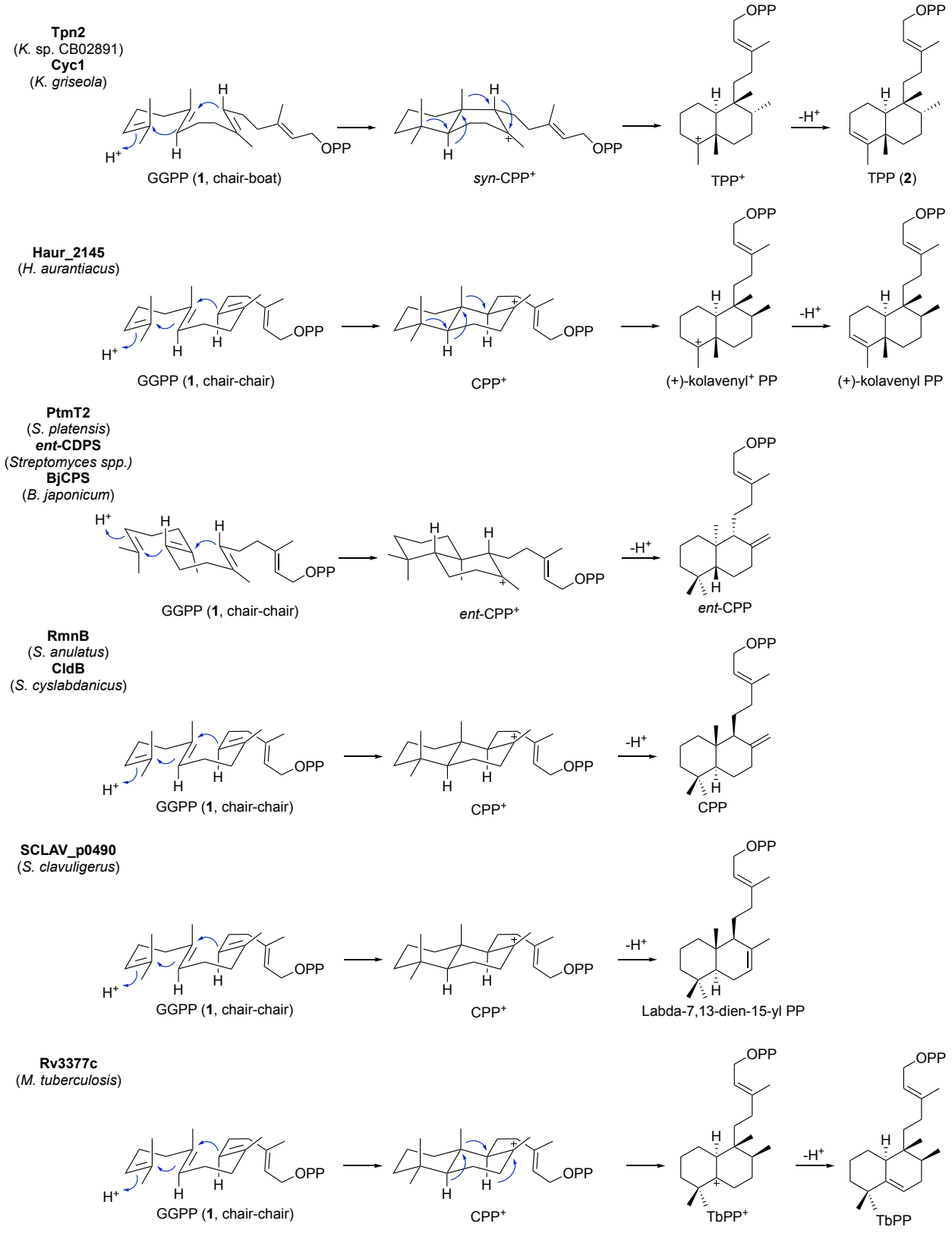
[a] δ in ppm, *J* in Hz

[b] NMR taken in $CDCl_3$

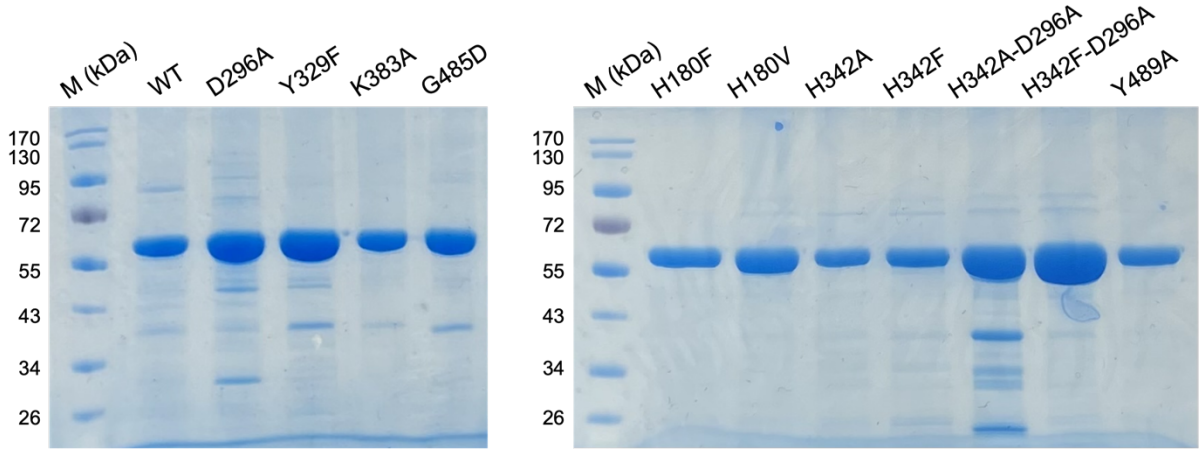
Supplementary Table 6. Data collection and refinement statistics for Tpn2 (molecular replacement).

	Tpn2
Data collection	
Space group	$P3_1$
Cell dimensions	
a, *b*, *c* (Å)	92.54, 92.54, 127.91
α, β, γ (°)	90.00, 90.00, 120.00
Resolution (Å)	30.00–2.57 (2.67–2.57)
R_{sym} or R_{merge}	12.0 (60.3)
$I / \sigma I$	15.7 (3.8)
Completeness (%)	99.3 (100.0)
Redundancy	4.7 (5.6)
Refinement	
Resolution (Å)	30.00–2.57
No. reflections	38716
R_{work} / R_{free}	0.256/0.288
No. atoms	
Protein	7443
Water	159
B-factors	
Protein	57.8
Water	50.2
R.m.s. deviations	
Bond lengths (Å)	0.0047
Bond angles (°)	1.36

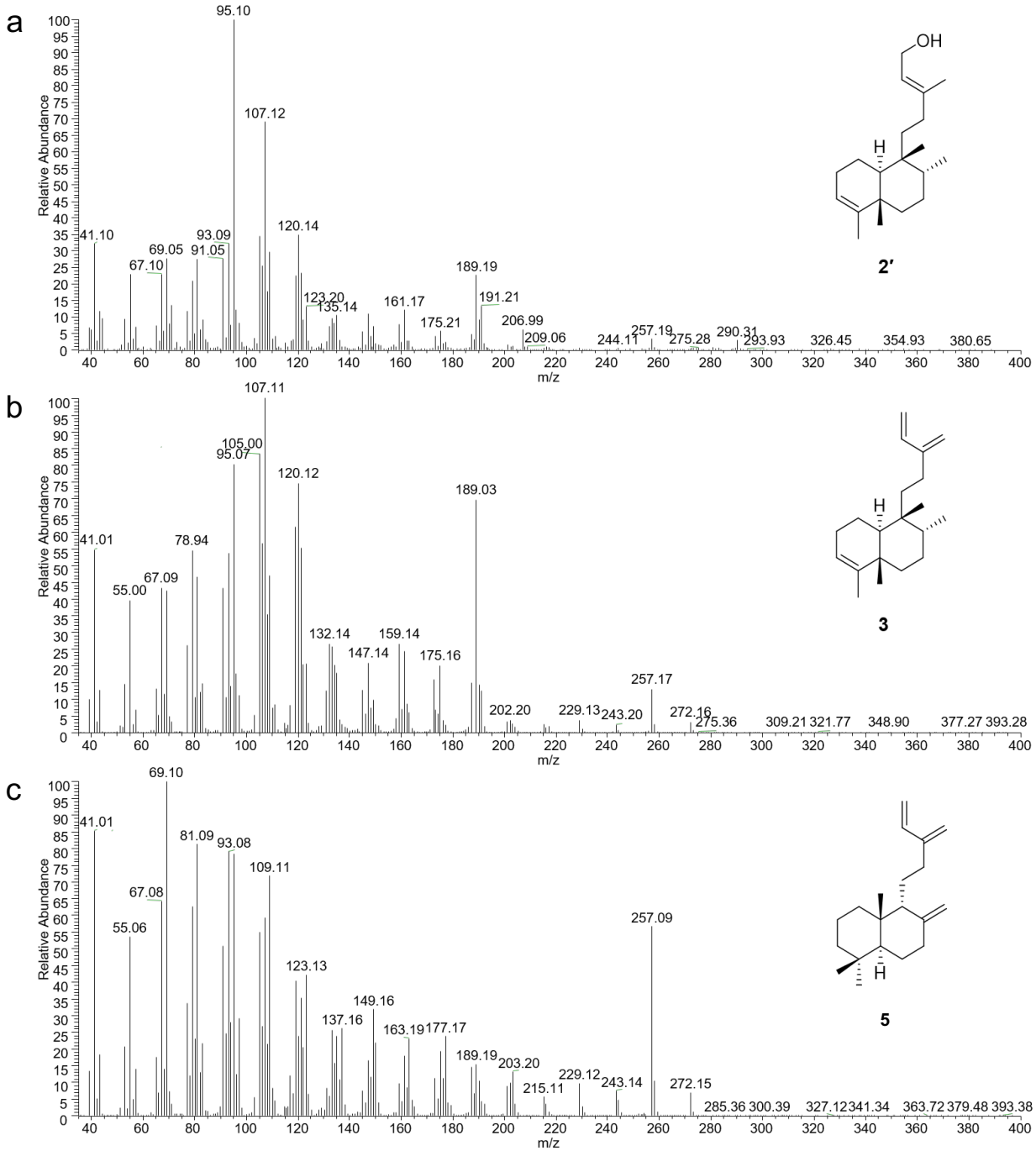
Supplementary Fig. 1. Bacterial type II TSs.



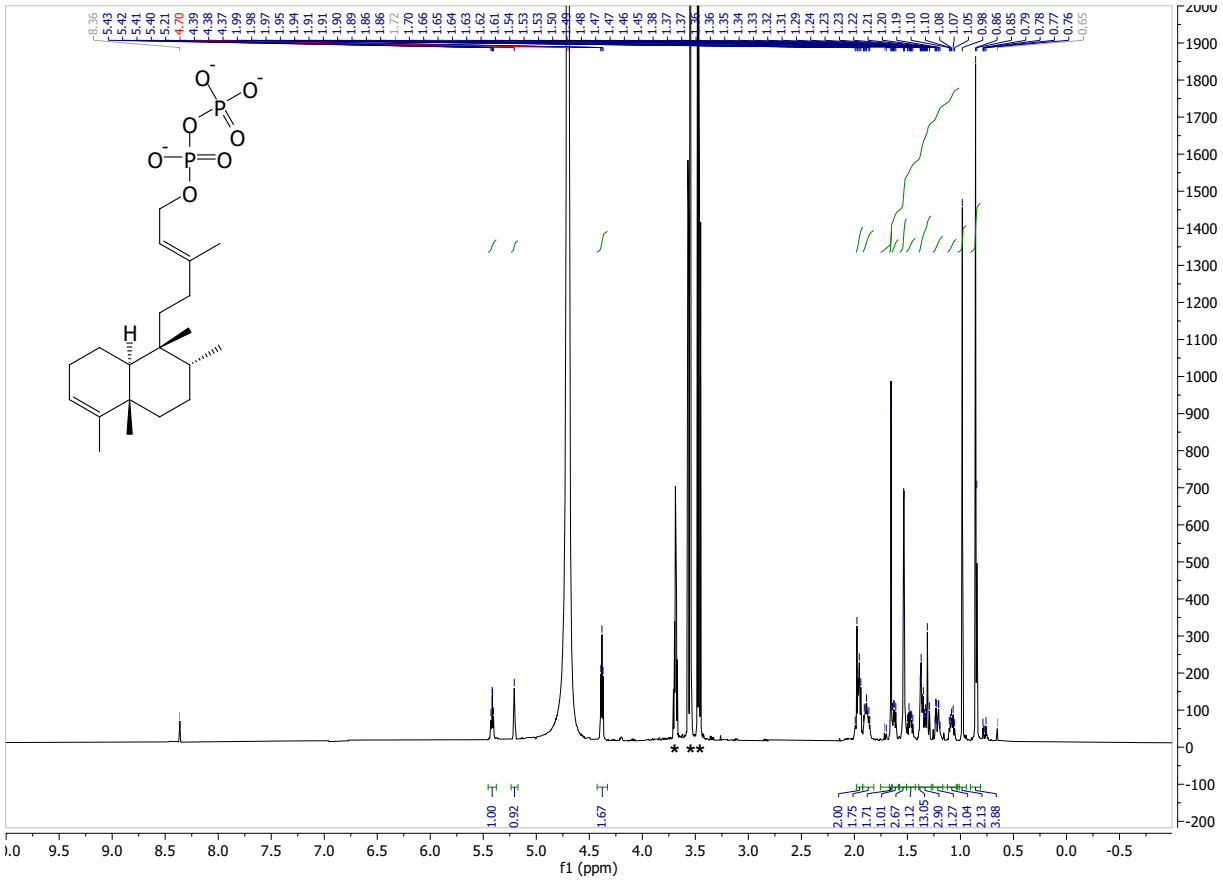
Supplementary Fig. 2. SDS-PAGE analysis of purified proteins. Expected size of Tpn2 is 56 kDa. Protein size analyzed using Fisher BioReagents™ EZ-Run™ Prestained Rec Protein Ladder.



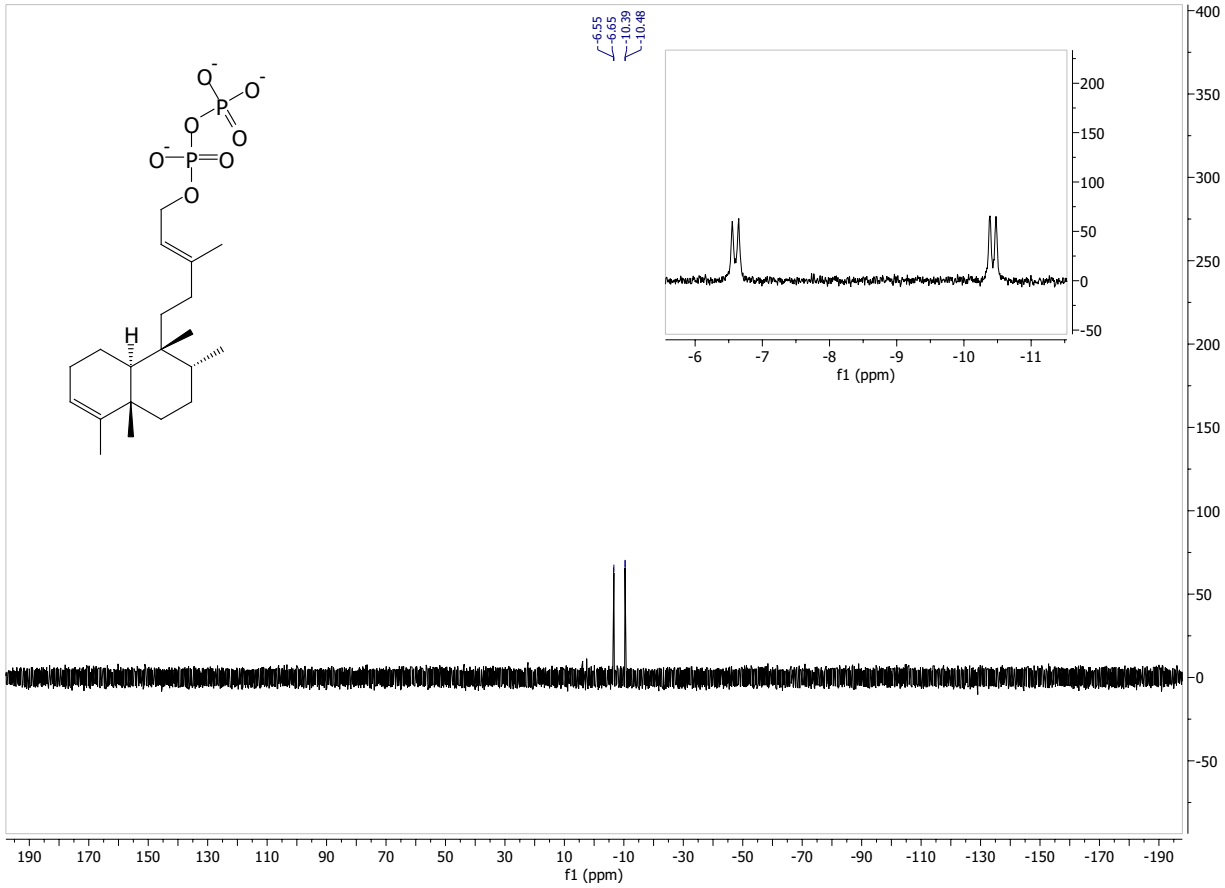
Supplementary Fig. 3. EI mass spectra of **2′**, **3**, and **5**. (a) EIMS **2′**: *m*/*z* (%): 95 (100), 107 (69), 120 (35), 41 (32), 69 (28), 189 (23), 257 (3), 290 (M^+, 3). (b) EIMS **3**: *m*/*z* (%): 107 (100), 95 (80), 120 (75), 189 (69), 41 (55), 79 (54), 67 (43), 55 (40), 132 (26), 159 (26), 257 (13), 272 (M^+, 3). (c) EIMS **5**: *m*/*z* (%): 69 (100), 41 (85), 81 (81), 93 (79), 109 (72), 257 (57), 55 (54), 123 (42), 149 (32), 272 (M^+, 7).



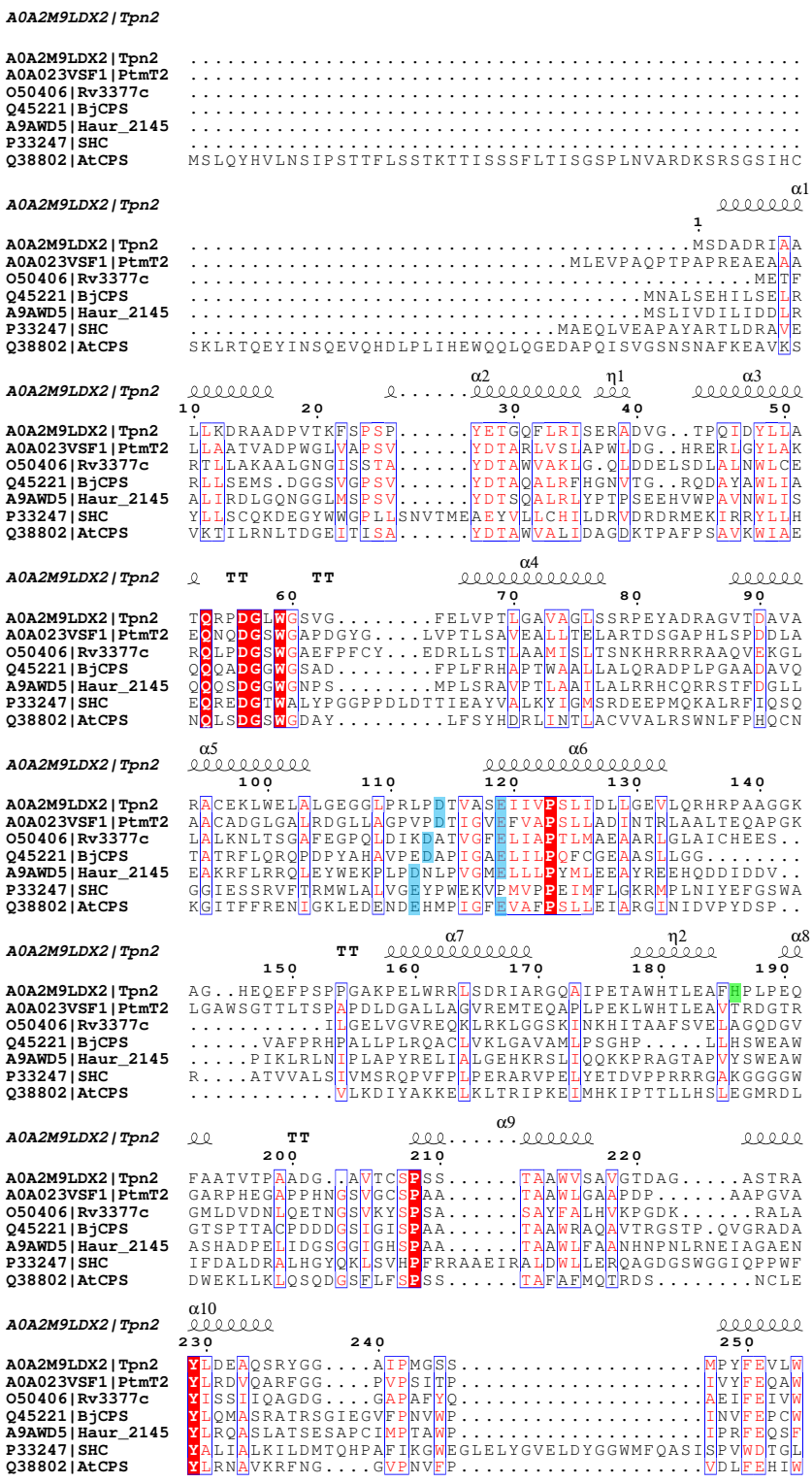
Supplementary Fig. 4. ^{1}H NMR spectrum of **2** in D_2O (600 MHz). TPP (**2**) was isolated in the presence of glycerol; peaks marked with asterisks.



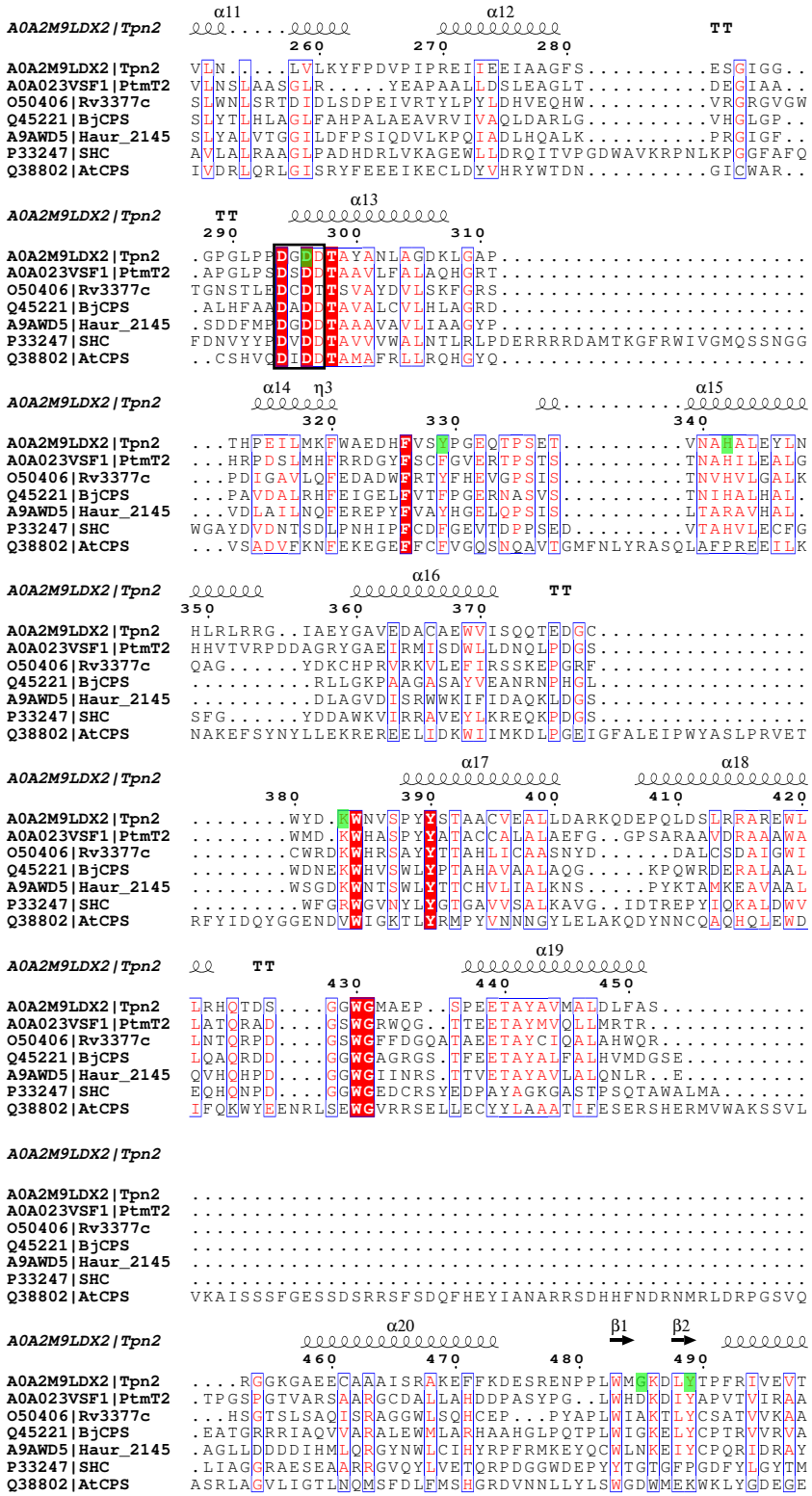
Supplementary Fig. 5. ^{31}P NMR spectrum of **2** in D_2O (243 MHz).



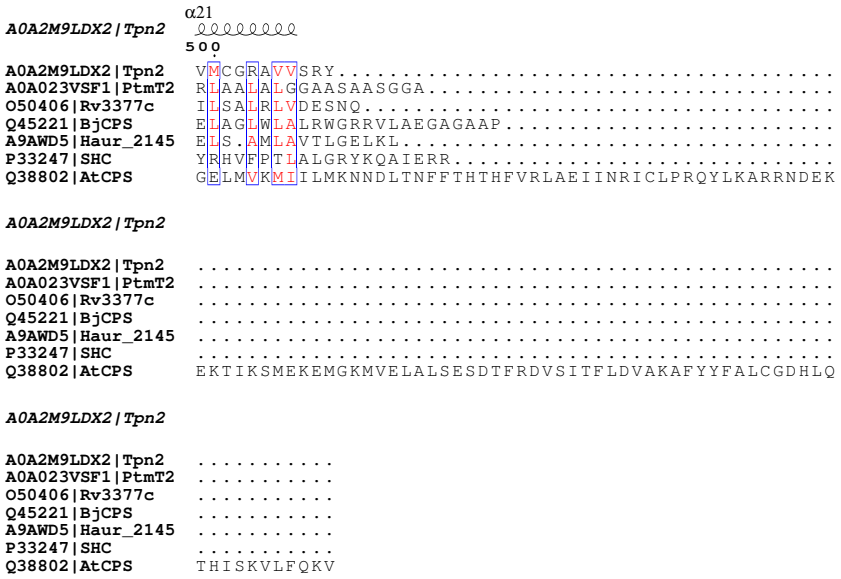
Supplementary Fig. 6. Sequence alignment of selected bacterial and plant type II TSs. Residues are colored based on conservation (identity: red box with white residue; similarity: red residue; similar across group: blue frame). The secondary structure of Tpn2 is shown above the alignment. The DxDD motif is in a black frame, residues mutated in this study are in green, and DxxxxE is in blue. The alignment was created with Clustal Omega[4] and rendered with ESPript.[5] The α domain of AtCPS was removed.



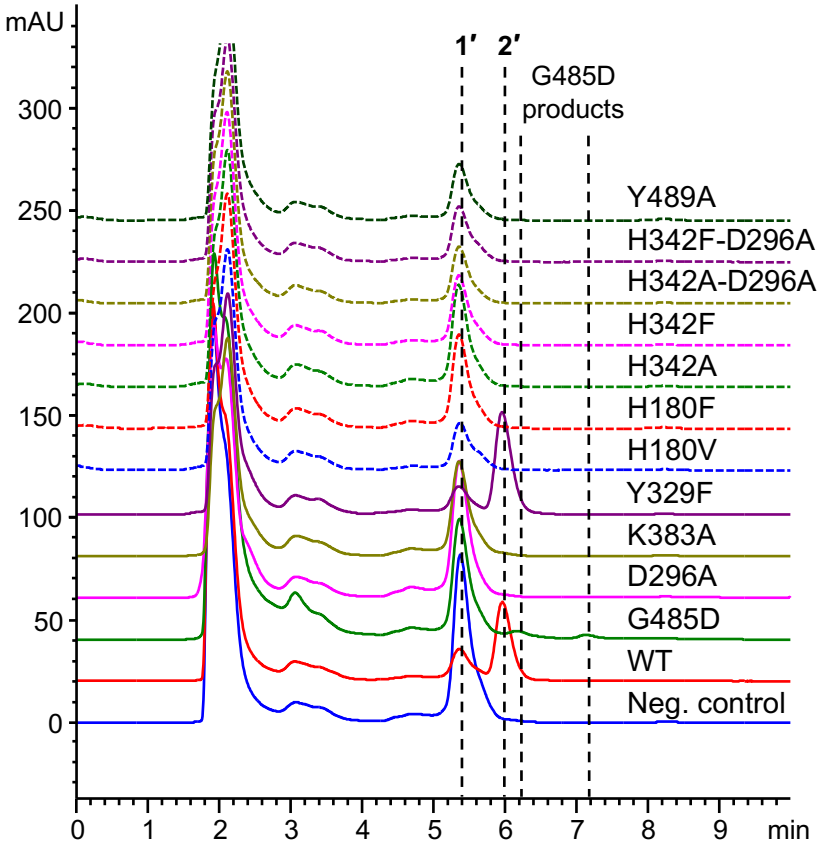
Supplementary Fig. 6 cont. Sequence alignment of selected bacterial and plant type II TSs.



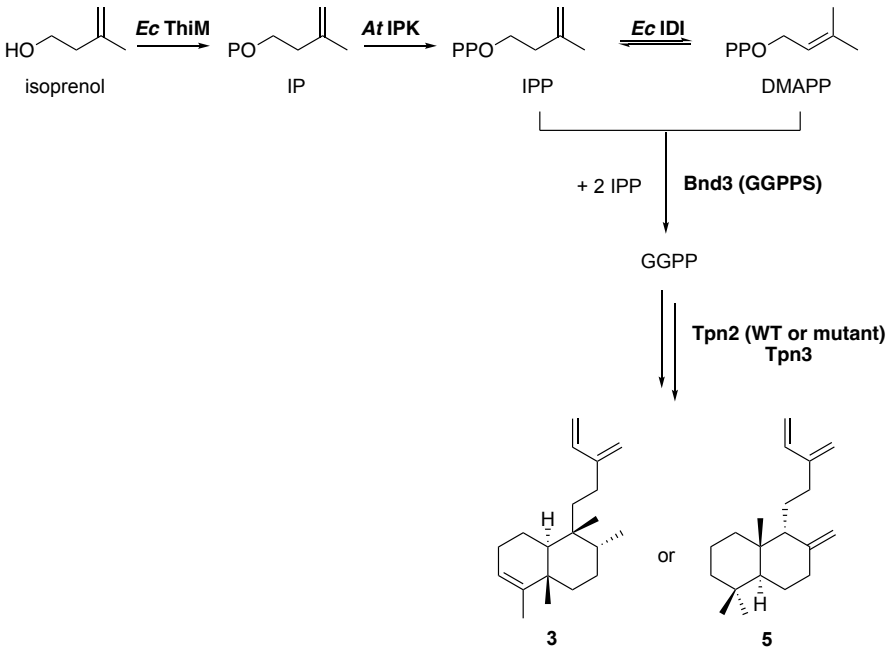
Supplementary Fig. 6 cont. Sequence alignment of selected bacterial and plant type II TSs.



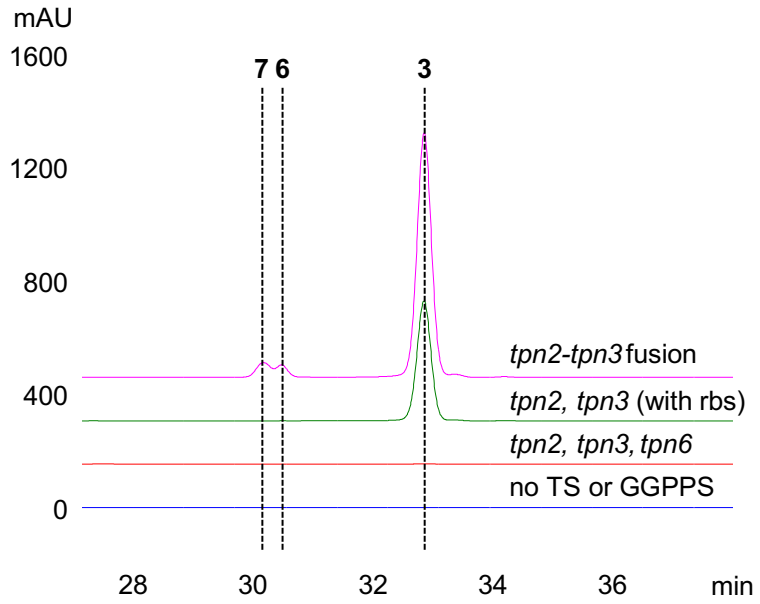
Supplementary Fig. 7. HPLC chromatograms showing in vitro enzyme reactions of Tpn2 and mutants after desphosphorylation.



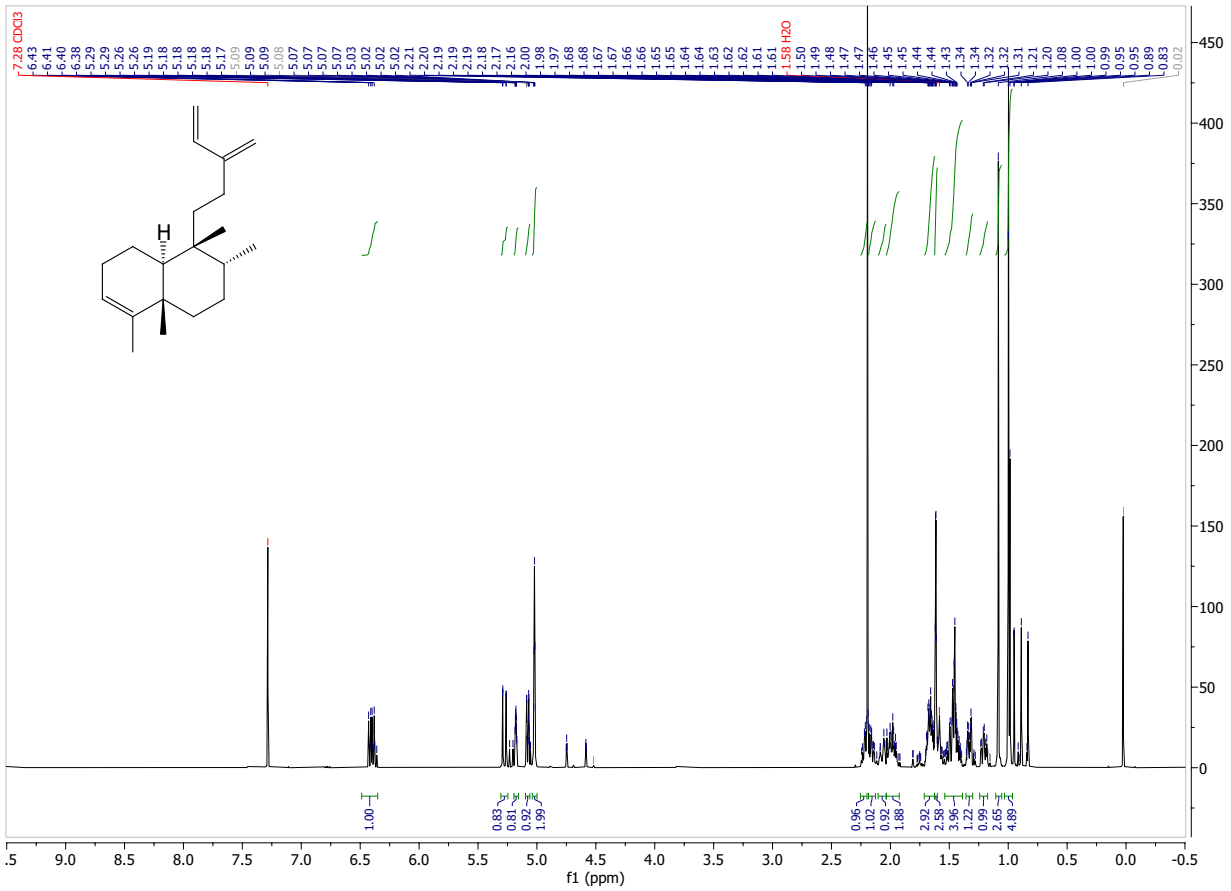
Supplementary Fig. 8. Scheme of the in vivo terpenoid upregulation system used in *E. coli* for heterologous production of Tpn2 products.[3]



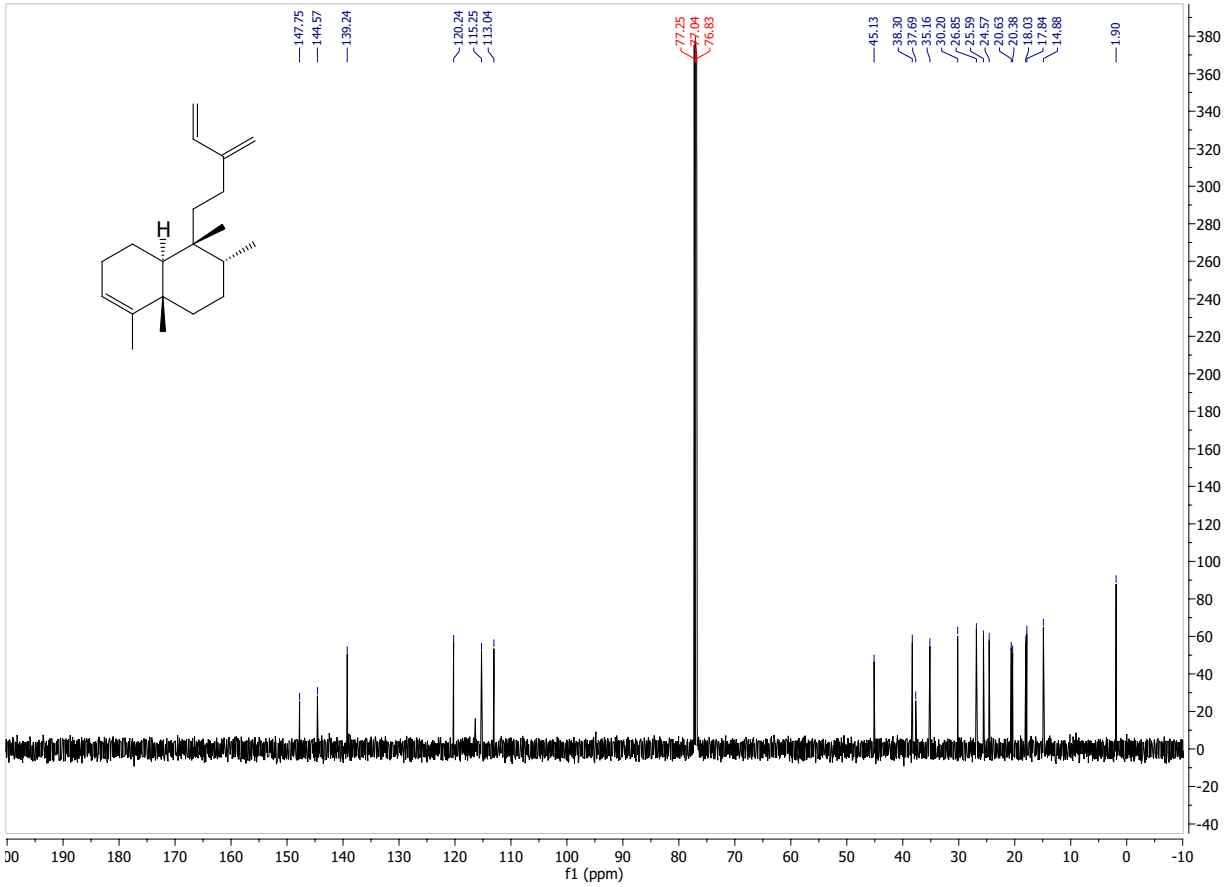
Supplementary Fig. 9. HPLC analysis showing production of terpentetriene (**3**) in vivo using the MKI4 system. The acyclic compounds **6** and **7** are produced via diphosphate elimination of GGPP by Tpn3.



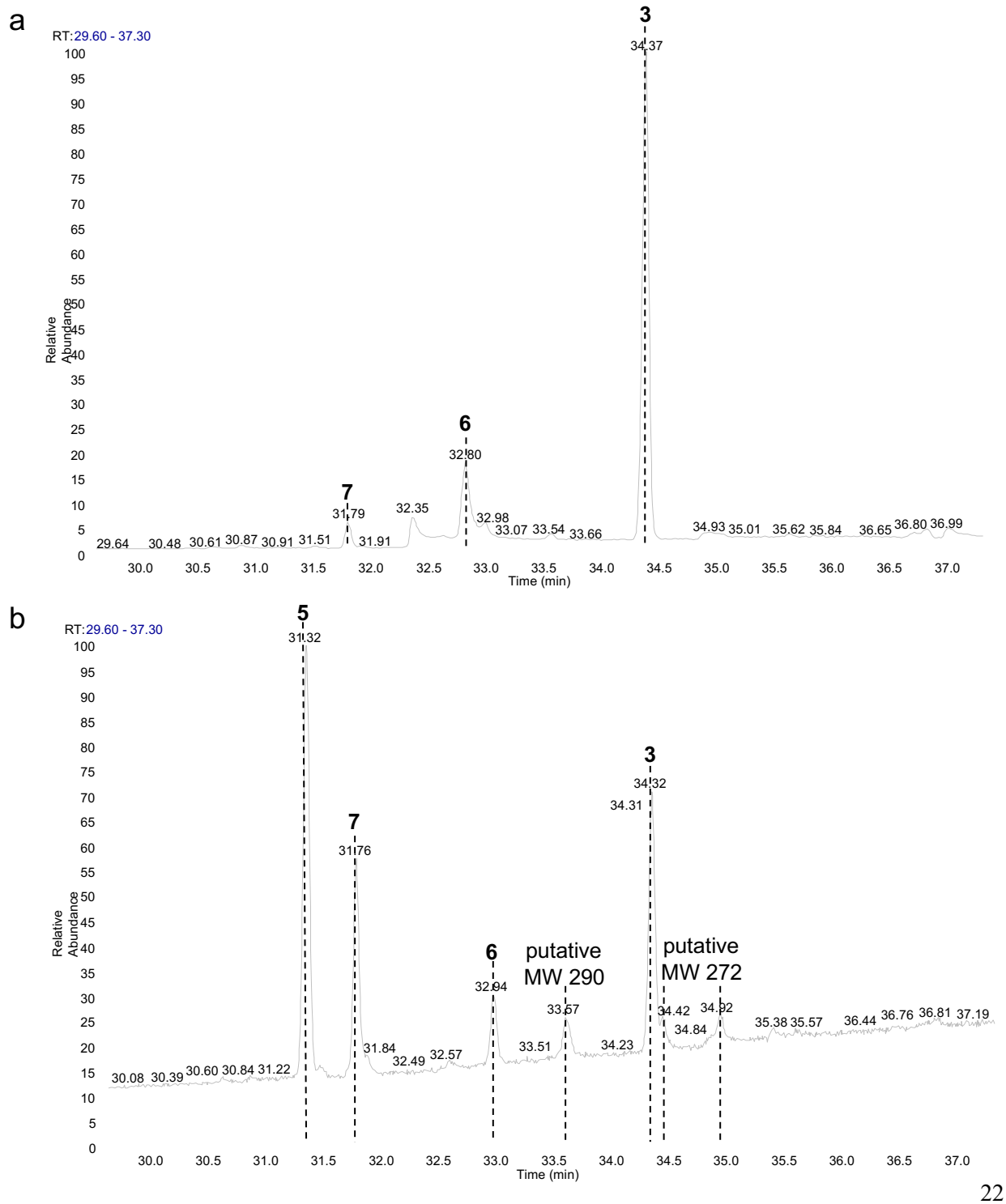
Supplementary Fig. 10. ^{1}H NMR spectrum of terpentetriene (**3**) in $CDCl_3$ (600 MHz).



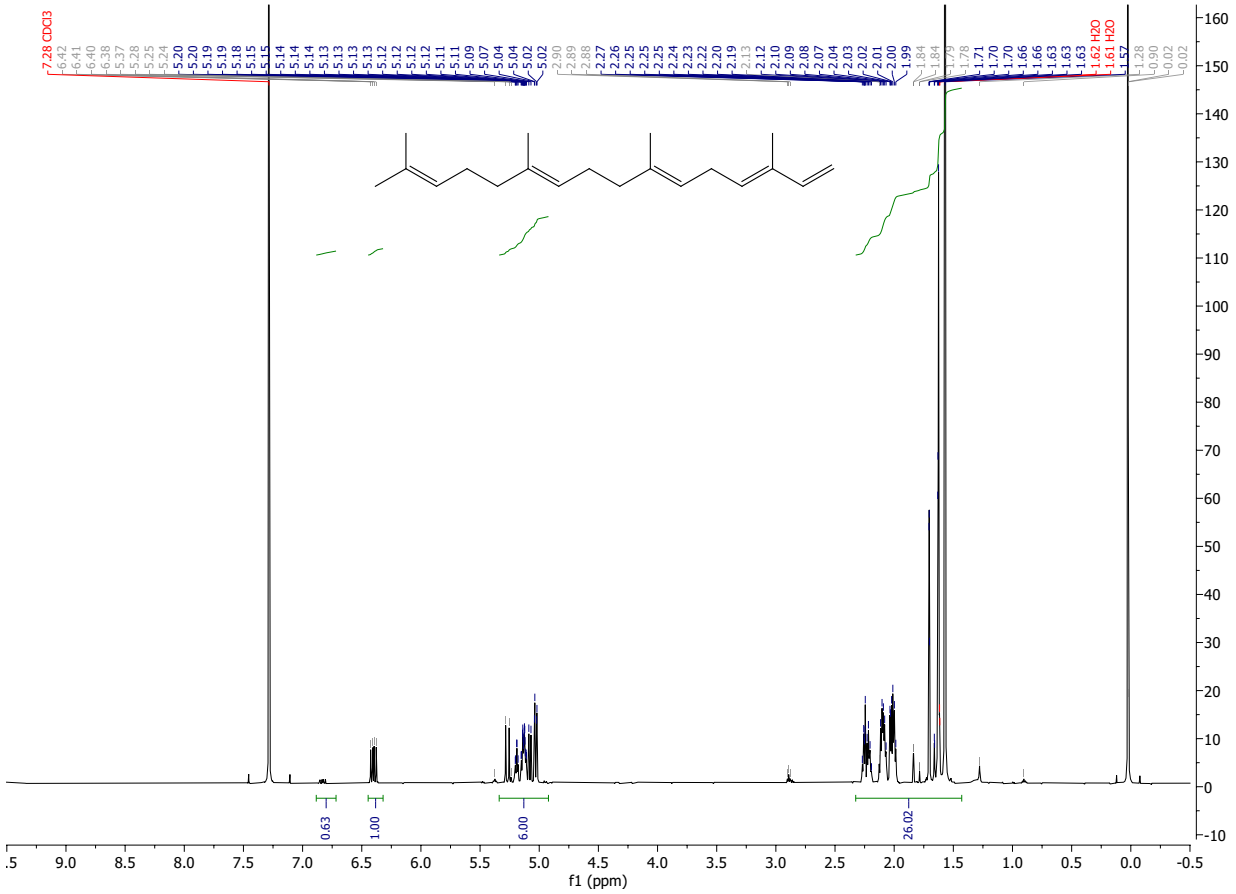
Supplementary Fig. 11. ^{13}C NMR spectrum of terpentetriene (**3**) in $CDCl_3$ (151 MHz).



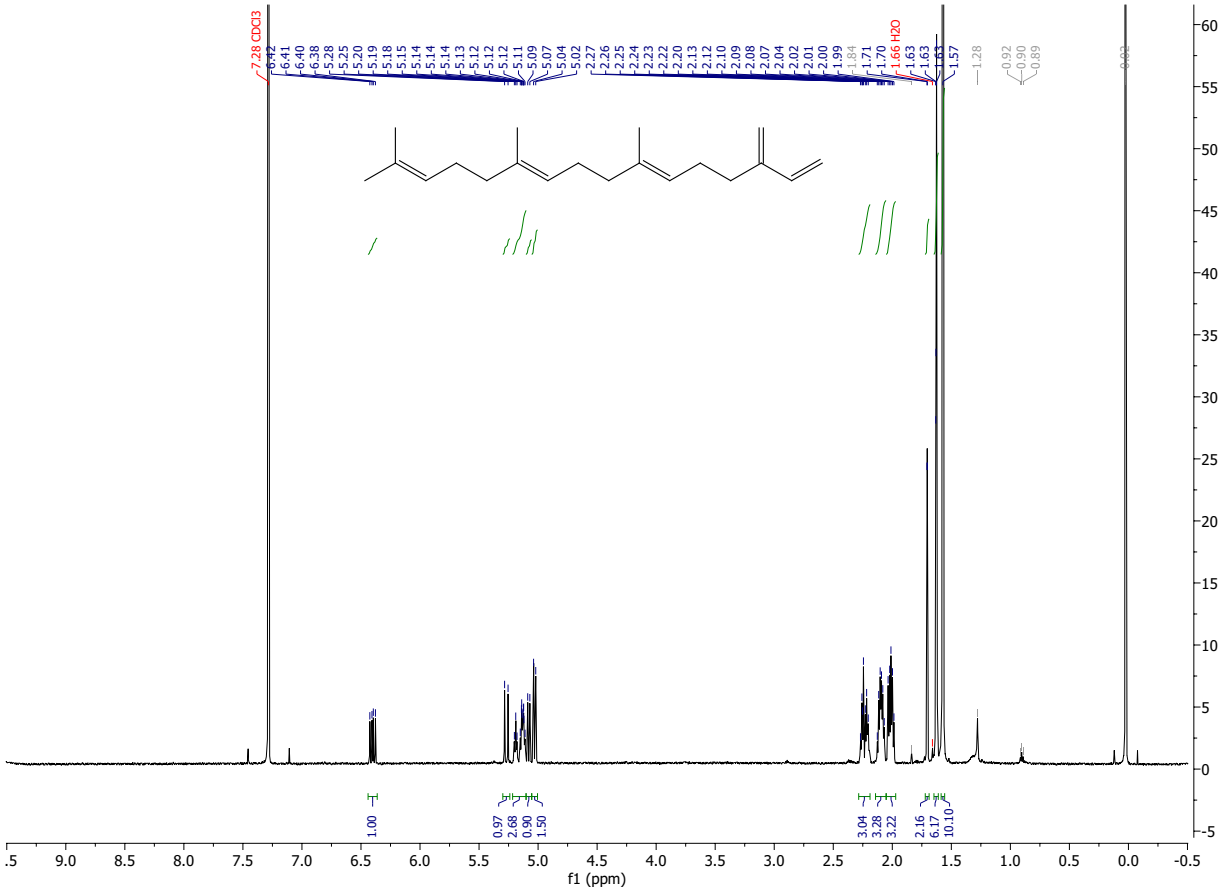
Supplementary Fig. 12. GC-MS analysis of in vivo products from Tpn2-Tpn3 and Tpn2^{G485D}-Tpn3 fusion proteins using the MKI4 system. Extracts of Tpn2-Tpn3 (a) and Tpn2^{G485D}-Tpn3 (b) revealed the production of major products **3** and **5**, respectively. The acyclic compounds **6** and **7** are produced via diphosphate elimination of GGPP by Tpn3. Two additional compounds produced by Tpn2^{G485D}-Tpn3 are proposed to be diterpene-related based on their *m/z* values of 272 and 290 and comparison to the NIST database (NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library, Version 2.3, build May 4, 2017); the titers of these compounds were too low for isolation.



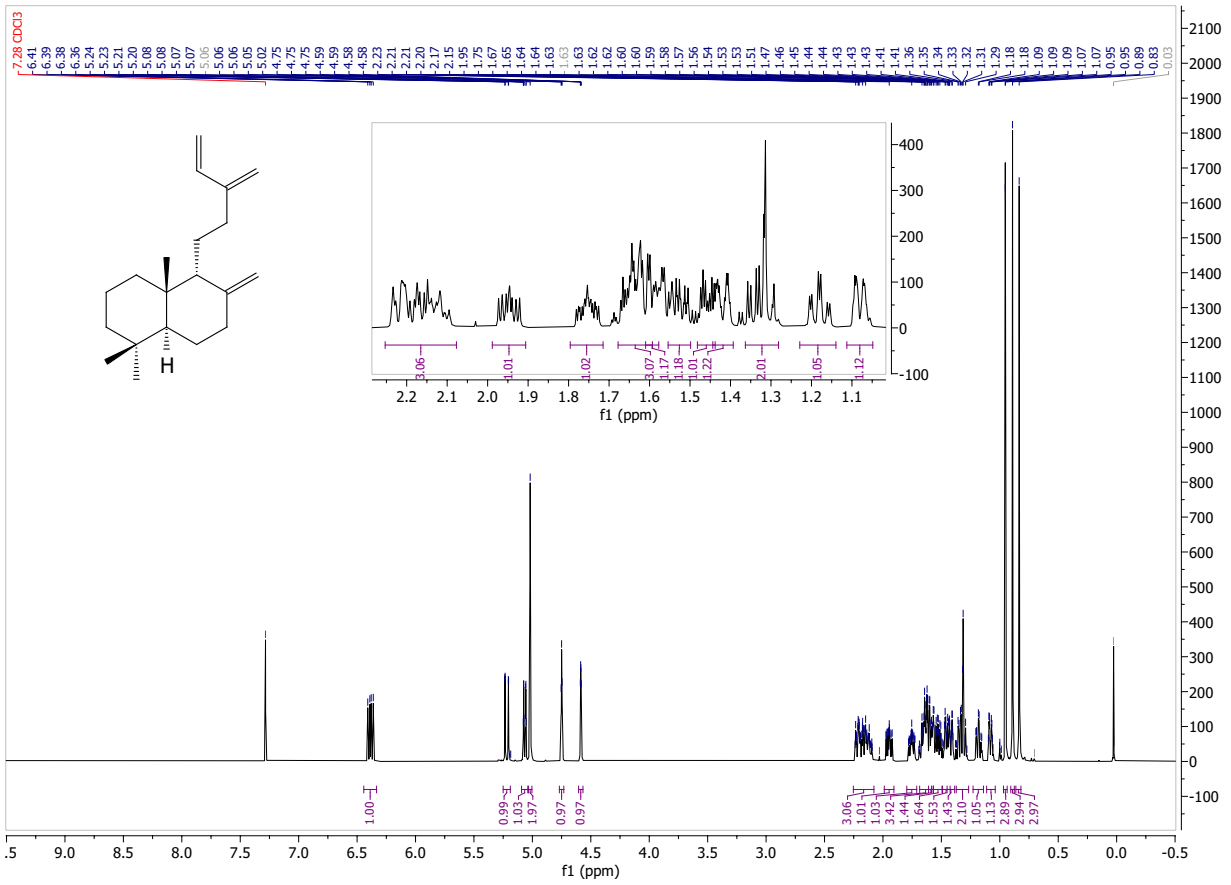
Supplementary Fig. 13. ^{1}H NMR spectrum of α-springene (**6**) in $CDCl_3$ (600 MHz).



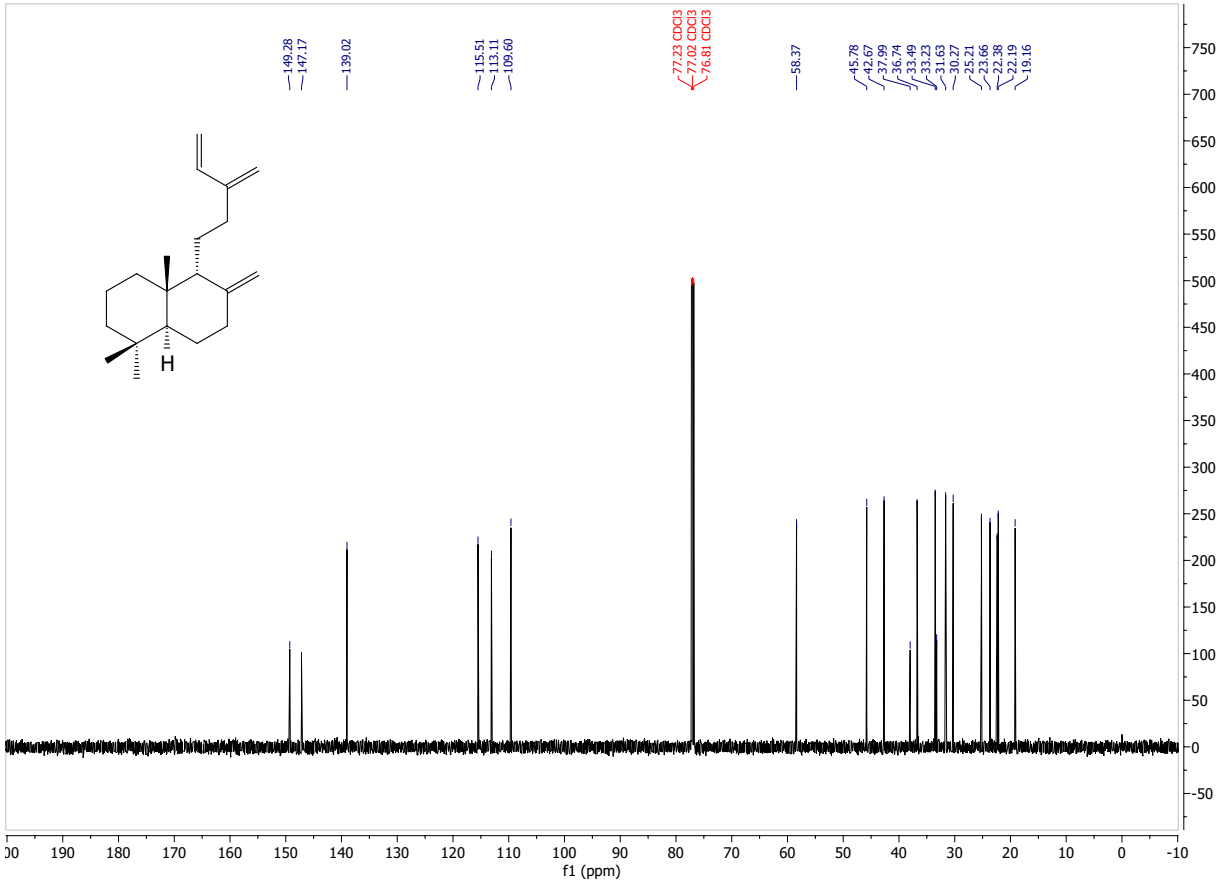
Supplementary Fig. 14. ^{1}H NMR spectrum of β-springene (**7**) in $CDCl_3$ (600 MHz).



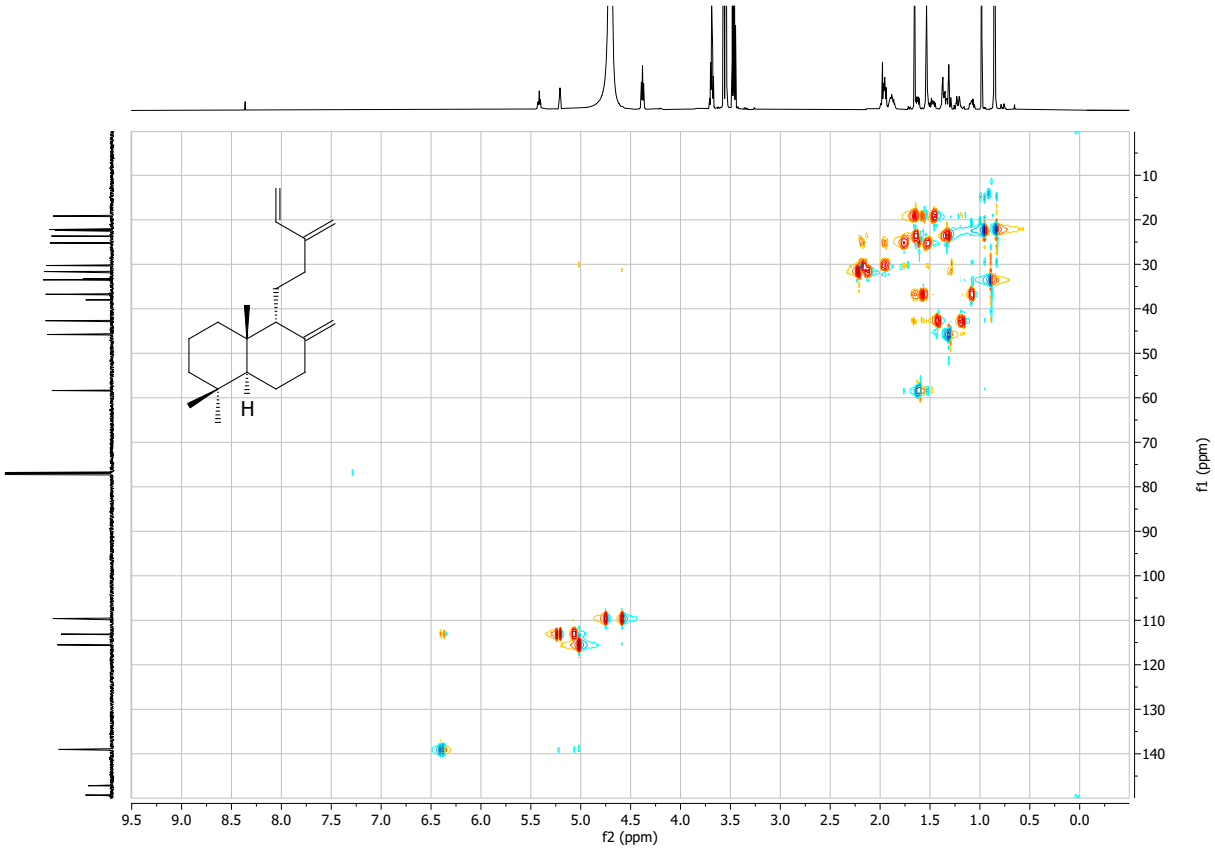
Supplementary Fig. 15. ^{1}H NMR spectrum of *syn*-sclarene (**5**) in $CDCl_3$ (600 MHz).



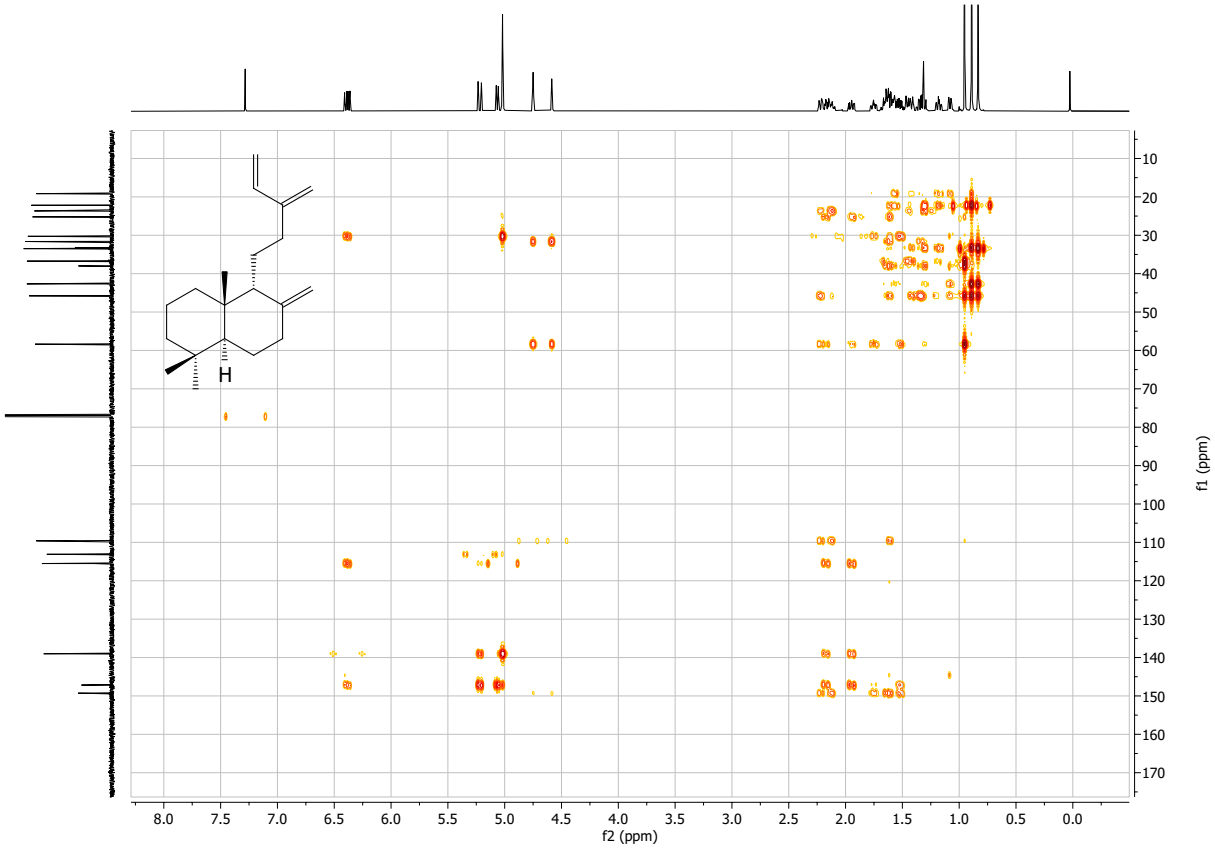
Supplementary Fig. 16. ^{13}C NMR spectrum of *syn*-sclarene (**5**) in $CDCl_3$ (151 MHz).



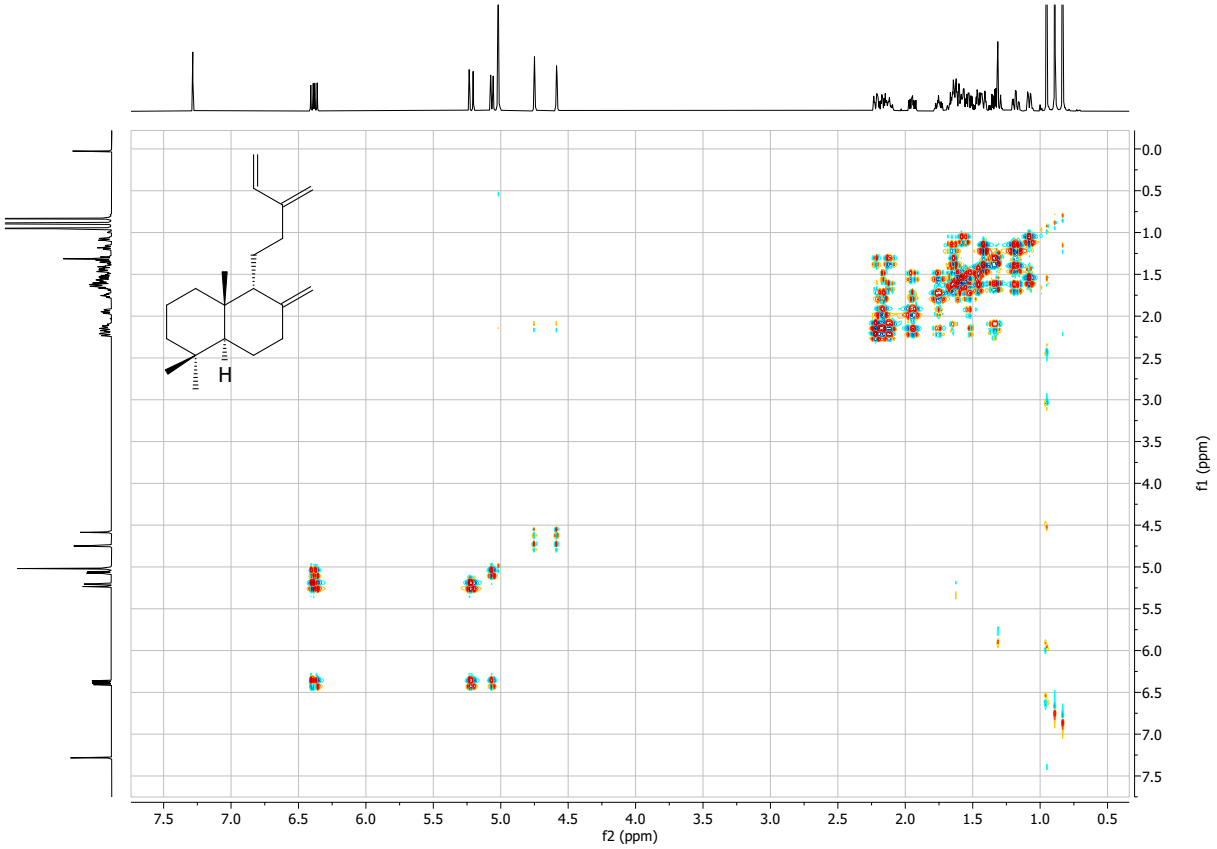
Supplementary Fig. 17. HSQC spectrum of *syn*-sclarene (**5**) in $CDCl_3$. Blue cross peaks represent –CH or $–CH_3$; orange represent $–CH_2$.



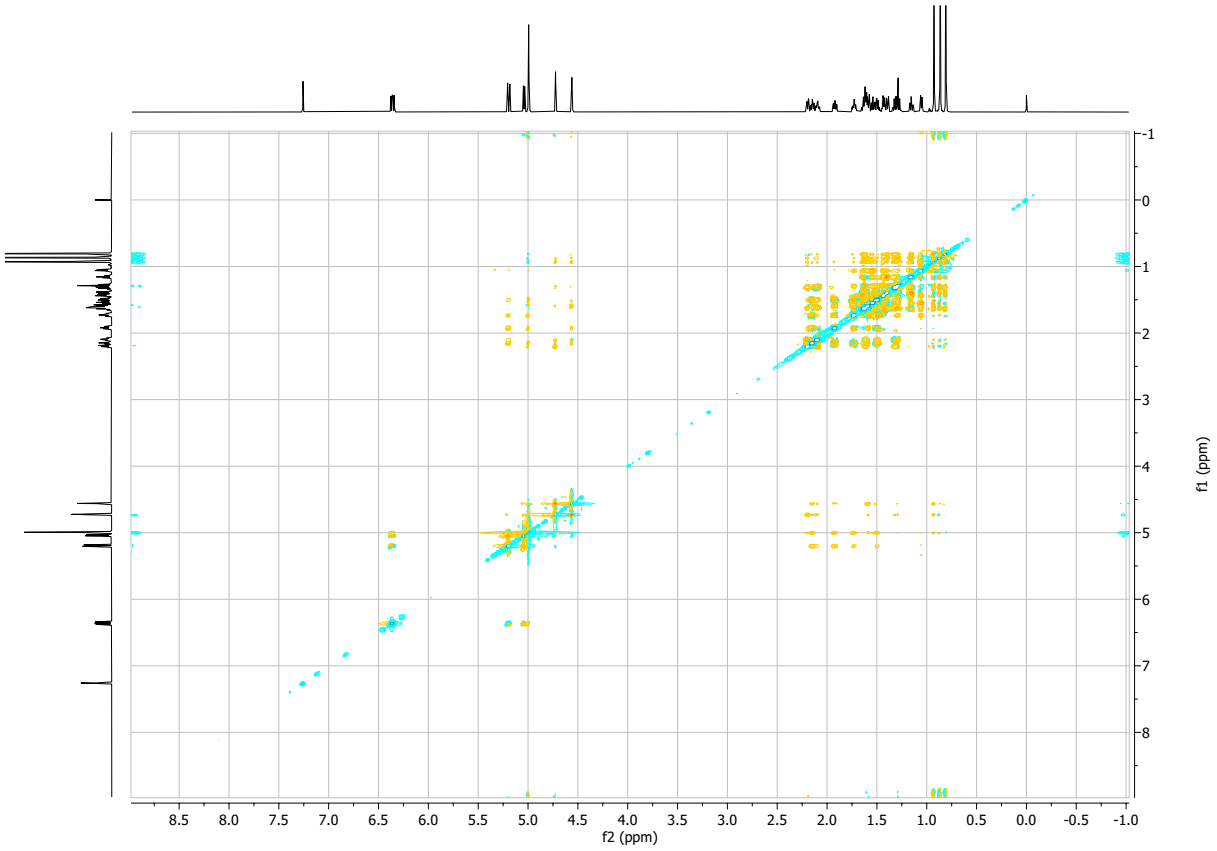
Supplementary Fig. 18. HMBC spectrum of *syn*-sclarene (**5**) in $CDCl_3$.



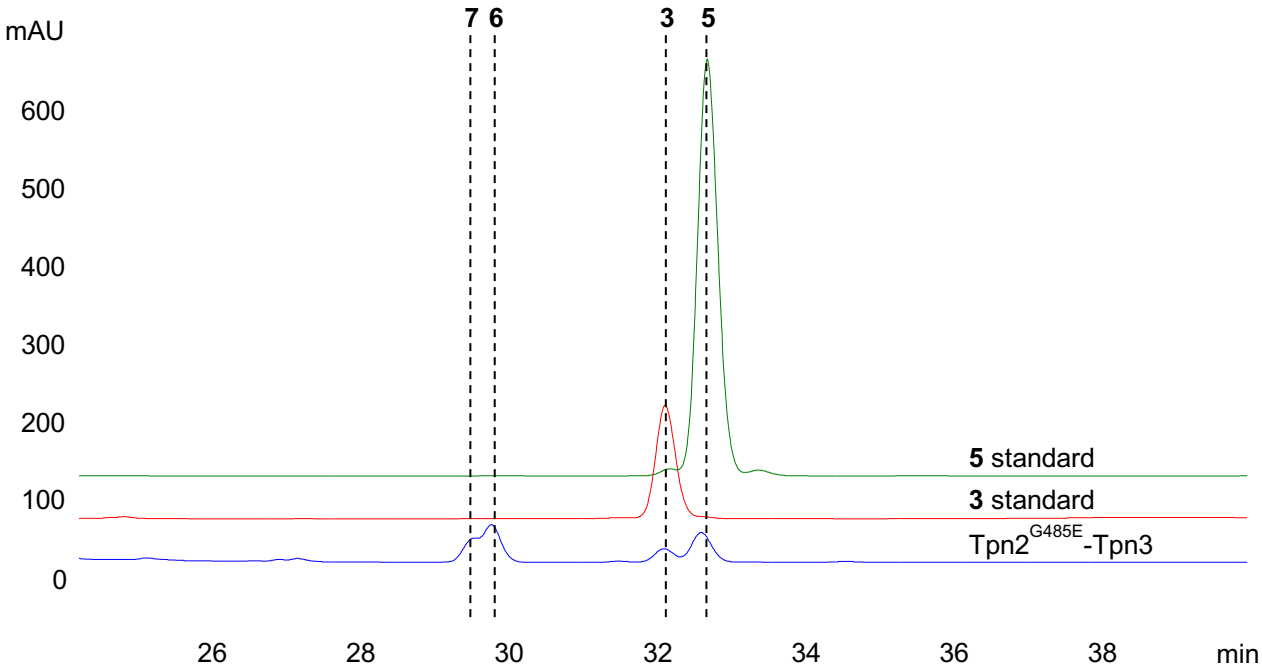
Supplementary Fig. 19. COSY spectrum of *syn*-sclarene (**5**) in $CDCl_3$.



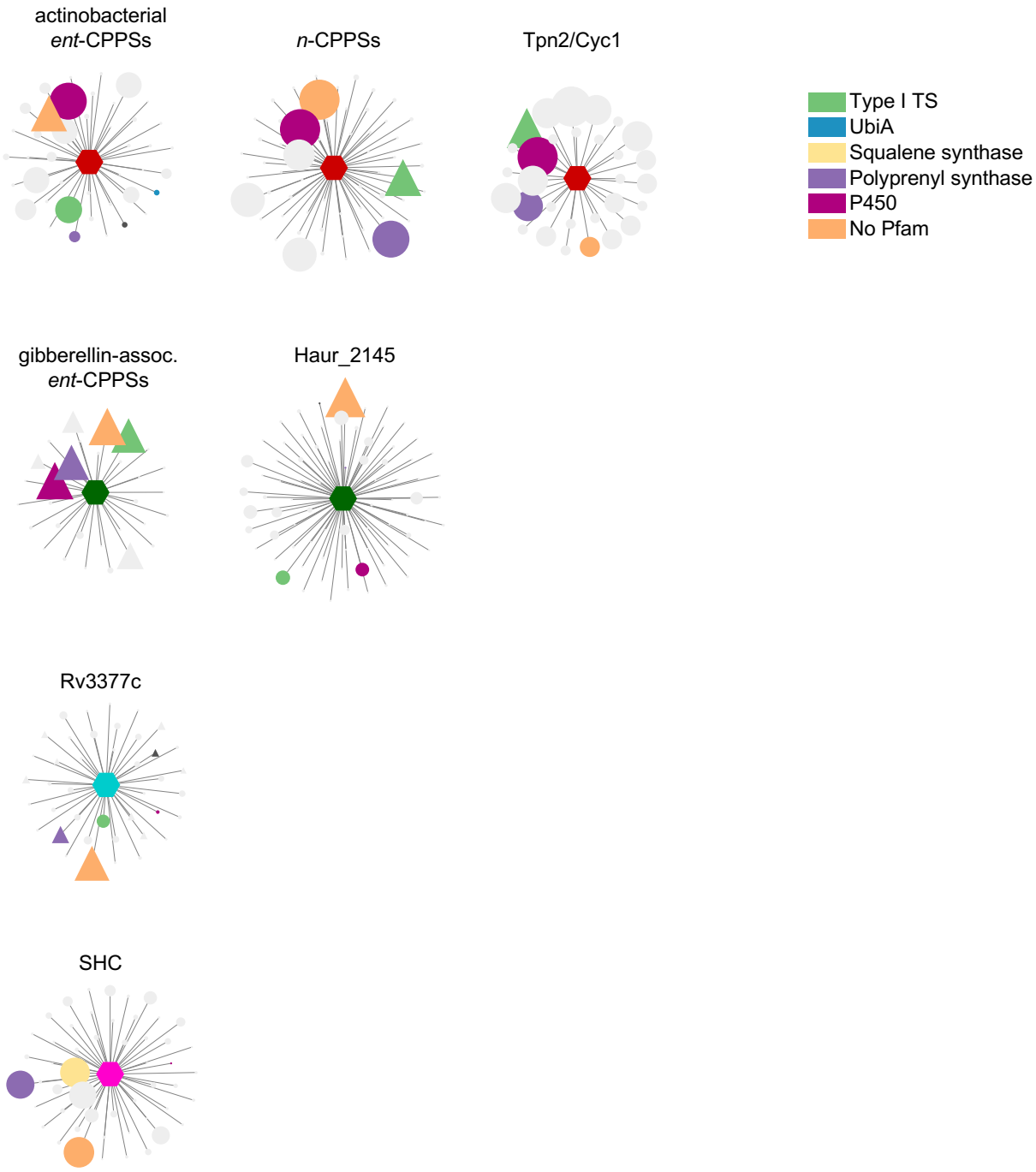
Supplementary Fig. 20. 2D NOESY spectrum of *syn*-sclarene (**5**) in $CDCl_3$ with key NOE correlations shown.



Supplementary Fig. 21. HPLC analysis of in vivo products from Tpn2^{G485E}-Tpn3 fusion protein using the MKI4 system.



Supplementary Fig. 22. Genome neighborhood network of bacterial type II TSs.



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