SUPPORTING INFORMATION

Mechanism-based post-translational modification and inactivation in terpene synthases

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Supporting information - Text

Protein expression and purification

A 10 mL Luria broth starter culture (25 g LB (Novagen), ad 1 L milli-Q water) with 50 µg/mL kanamycin was inoculated from a glycerol stock of *Escherichia coli* BL21(DE3) (Novagen) with a TPS gene-pET28a construct and incubated at 37 °C and 250 rpm for 8 h. A 1 L terrific broth culture (47.6 g terrific broth (Novagen), 4 mL glycerol, ad 1 L milli-Q water) with 50 µg/mL kanamycin was inoculated with the starter culture and incubated at 37 °C and 250 rpm. At an OD_{600} of ~1.0, IPTG was added to the culture to a final concentration of 0.5 mg/L and the culture was incubated for 16-20 h at 18 °C and 250 rpm. Cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, 500 mM sodium chloride, 20 mM imidazole, 1 % Tween 20, 10 % glycerol, 10 mM β-mercaptoethanol, pH 8). After adding 0.5 mg/mL lysozyme, resuspended cells were stirred at 4 °C for 30 min. Subsequently, the suspension was sonicated (8-12x 30s with 1s on/1s off intervals at 80% amplitude) and centrifugated (38465 g, 45 min, 4 °C). The supernatant was loaded once on a His Pur™ Ni-NTA resin column (Thermo Fisher Scientific) by gravity flow. The protein-loaded Ni-NTA column was washed with 20 column volumes of wash buffer (lysis buffer without Tween 20). His-tag protein was eluted with 5 column volumes of elution buffer (wash buffer with 250 mM imidazole, pH 8). Thrombin was added to the eluted protein (1 µg thrombin/mg eluted protein) and the eluted protein was dialyzed for 16 h at 4 °C with a 12-14 kD Spectra/Por 2 Dialysis membrane (Spectrumlabs) in dialysis buffer (50 mM Tris-HCl, 100 mM sodium chloride, 10 mM β-mercaptoethanol, pH 8). The dialyzed protein fraction was separated from cleaved His-tags by Ni-NTA-liquid chromatography and from thrombin by Benzamidine Sepharose™ 4 Fast Flow (high sub) (GE Healthcare) filtration and purified by fast protein liquid chromatography (Äkta FPLC system, Amersham Biosciences) with a Hi Load™ 16/60 Superdex™ 200 prep grad column (GE Healthcare) as a solid phase and the following mobile phase (50 mM Tris-HCl, 100 mM sodium chloride, 2 mM DTT, pH 8). Target protein fractions were combined, concentrated by 30 kD protein concentrators (Millipore), used for protein assays or crystallization or stored at -80 °C.

Table S1. Crystallographic data collection and refinement statistics[a]

Crystal	TEAS W273E alkylated	TEAS W273E nonalkylated
pdb code	5DHK	5DHI
Data collection		
Wavelength (Å)	1.12949	1.00004
Resolution range (Å)	50.42 - 2.43 (2.52 - 2.43)	56.48 - 2.25 (2.33 - 2.25)
Space group	$P4_12_12$	$P4_12_12$
Unit-cell parameters:		
a (Å)	126.7	126.3
b (Å)	126.7	126.3
c (Å)	122	123.4
Unique reflections	36829 (3142)	46034 (3914)
Multiplicity	8.5 (3.2)	8.7 (4.4)
Completeness (%)	96.88 (84.19)	96.10 (83.06)
$\langle I/\sigma I \rangle$	32.44 (1.26)	12.84 (1.78)
R_{merge}	0.1367 (1.018)	0.1282 (0.7686)
Refinement		
R_{work}	0.1942 (0.3157)	0.1907 (0.2981)
R_{free}	0.2327 (0.3402)	0.2271 (0.3659)
Resolution range (Å)	50.42 - 2.43 (2.52 - 2.43)	56.48 - 2.25 (2.33 - 2.25)
Number of non-hydrogen atoms	4479	4621
proteins	4344	4344
ligands	FAR: 15, MG: 2	MG: 3
water	118	274
Protein residues	536	536
RMS (bonds) (Å)	0.007	0.007
RMS (angles) (°)	0.84	0.81
Ramachandran favored (%)	95	98
Ramachandran outliers (%)	0.56	0.19
Average B-factor	53.6	45.6
protein	53.7	45.6
ligands	62.1	62.5
solvent	47.6	45.6

[a]Values in parentheses represent highest resolution shell.

Table S2. Primer sequences for site-specific mutagenesis

Protein	Mutant	Primer	Primer sequence
TEAS	W273C	fwd	GTTGAATGCTACTTTTCGGCATTAGGAGTTTATTTTGAGCCTC
		rev	GAGGCTCAAAATAAACTCCTAATGCCGAAAAGTAGCATTCAAC
	W273E	fwd	GTTGAATGCTACTTTGAGGCATTAGGAGTTTATTTTGAGCCTC
		rev	GAGGCTCAAAATAAACTCCTAATGCCTCAAAGTAGCATTCAAC
	W273F	fwd	GTTGAATGCTACTTTTTCGCATTAGGAGTTTATTTTGAGCCTC
		rev	GAGGCTCAAAATAAACTCCTAATGCGAAAAAGTAGCATTCAAC
	V277L	fwd	CTTTTGGGCATTAGGACTTTATTTTGAGCCTCAATAC
		rev	GTATTGAGGCTCAAAATAAAGTCCTAATGCCCAAAAG
	I294L	fwd	CATGCTCGTTAAGACCCTATCAATGATTTCGATTG
		rev	CAATCGAAATCATTGATAGGGTCTTAACGAGCATG
	Y404C	fwd	CTAGCAACTACCACATGTTACTACCTCGCGACAAC
		rev	GTTGTCGCGAGGTAGTAACATGTGGTAGTTGCTAG
	Y404F	fwd	CACTAGCAACTACCACATTTTACTACCTCGCGACAAC
		rev	GTTGTCGCGAGGTAGTAAAATGTGGTAGTTGCTAGTG
	L407I	fwd	CTACCACATATTACTACATCGCGACAACATCGTATTTGG
		rev	CCAAATACGATGTTGTCGCGATGTAGTAATATGTGGTAG
	L407P	fwd	CTACCACATATTACTACCCCGCGACAACATCGTATTTGG
		rev	CCAAATACGATGTTGTCGCGGGGTAGTAATATGTGGTAG
	L512I	fwd	CTCCTATTCTCAATATTGCTCGTATTGTTGAGGTTAC
		rev	GTAACCTCAACAATACGAGCAATATTGAGAATAGGAG
HPS	W280E	fwd	GCAGTTGAGTGCTACTTTGAGACGATGGGGGTGTATGC
		rev	GCATACACCCCCATCGTCTCAAAGTAGCACTCAACTGC
CVS	W273E	fwd	GTGGAGTTATATTTTGAGGATTTAGGGACATACTTCG
		rev	CGAAGTATGTCCCTAAATCCTCAAAATATAACTCCAC
ATAS	Y61C	fwd	GGTAACGTGCCTGTGCTTTCCGAAGGCGCTGG
		rev	CCAGCGCCTTCGGAAAGCACAGGCACGTTACC
PAS	W276E	fwd	GTGGAAAGCTATTTTGAGGCGAGCGGCAGC
		rev	GCTGCCGCTCGCCTCAAAATAGCTTTCCAC
Sspissy	W293E	fwd	GCTGCAGAGTTACATGGAGTCCTGCGCCATCGCAAGC
		rev	GCTTGCGATGGCGCAGGACTCCATGTAACTCTGCAGC
γHS	W315P	fwd	CGTGGAATTTTACTTCccGATGGCGGCGGCAATTAGC
		rev	GCTAATTGCCGCCGCCATCggGAAGTAAAATTCCACG

Abbreviations: TEAS – tobacco 5-*epi*-aristolochene synthase, HPS – *Hyoscyamus muticus* premnaspirodiene synthase, ATAS – *Aspergillus terreus* aristolochene synthase, CVS – citrus valencene synthase, SspiSSy – *Santalum spicatum* santalene synthase, γHS – γ-humulene synthase, PAS – patchoulol synthase, fwd – forward, rev – reverse.

Table S3. Estimation of alkylation rates of TEAS mutants W273E and Y404F based on kinetic parameters and alkylation level estimation from proteomic analysis.

Mutant	k_{cat} [s^{-1}]	K_m [μM]	Alkylation level estimate (12h)*	Alkylation rate estimate (per substrate turnover)
TEAS W273E	0.00039 ± 0.00001	4.3 ± 0.4	50%	3%
TEAS Y404F	1.16 ± 0.04	10.6 ± 1.1	5%	0.04%

* - based on proteomic analysis

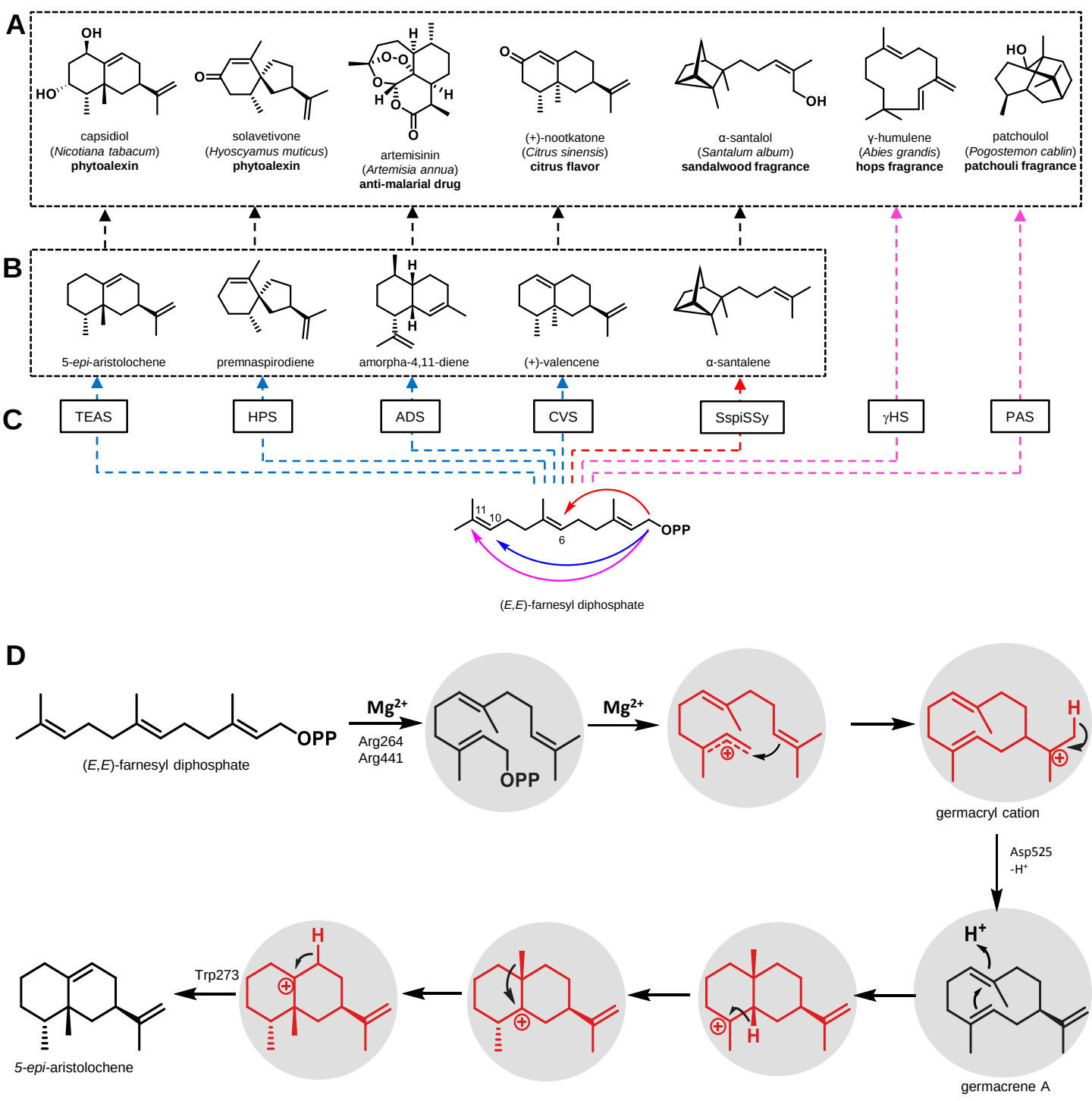
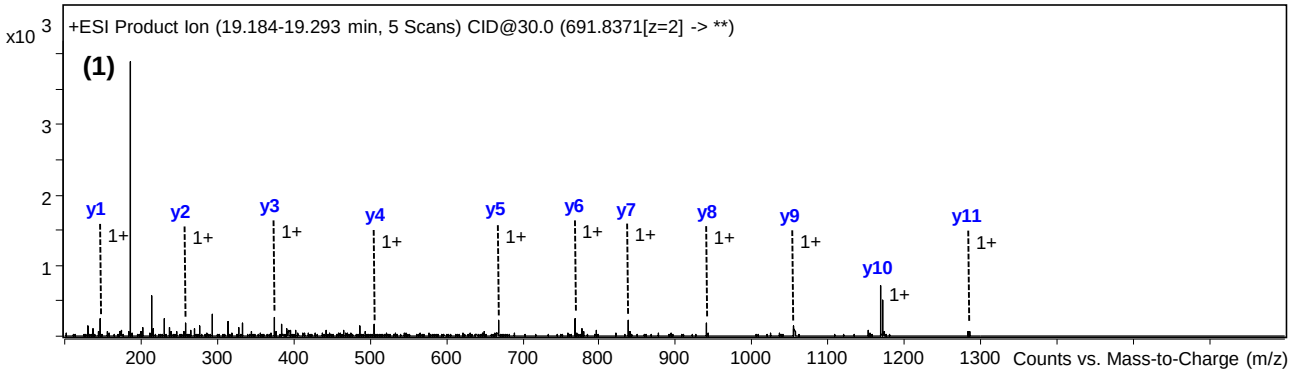


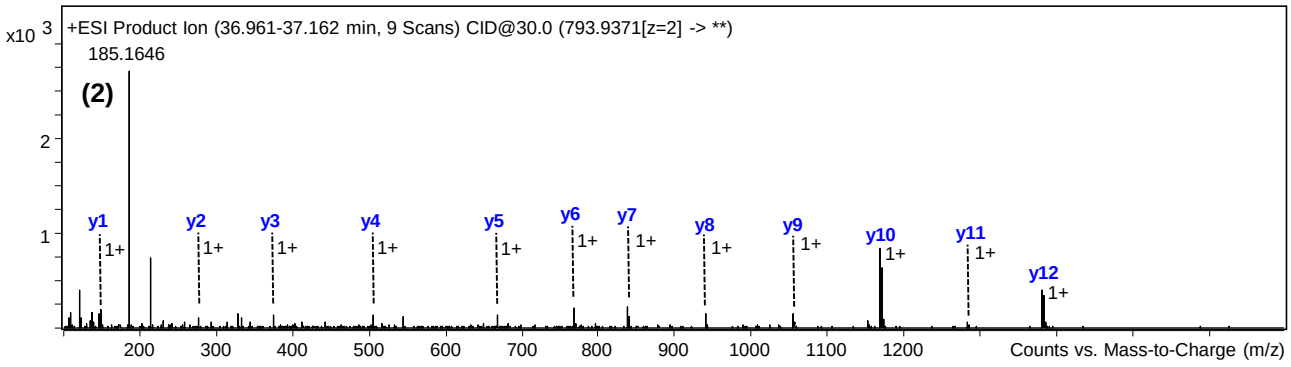
Figure S1. Structures, functions and biosynthesis of sesquiterpene natural products. (**A**) Structures of plant sesquiterpene natural products and corresponding cyclic sesquiterpene scaffolds (**B**). (**C**) Biosynthesis of cyclic sesquiterpene structures by plant sesquiterpene synthases via three different cyclization pathways from conventional sesquiterpene substrate (*E*,*E*)-farnesyl diphosphate. Abbreviations: TEAS – tobacco 5-*epi*-aristolochene synthase, HPS – *Hyoscyamus muticus* premnaspirodiene synthase, ADS – amorpha-4,11-diene synthase, CVS – citrus valencene synthase, SspiSSy – *Santalum spicatum* santalene synthase, γHS – γ-humulene synthase, PAS – patchoulol synthase. (**D**) Proposed biosynthetic mechanism of TEAS with cationic intermediates highlighted in red and active site intermediates highlighted in grey.

A



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y1	147.111	147.113	-0.002	14.0	y7	839.411	839.415	-0.004	4.8
y2	276.153	276.156	-0.003	11.0	y8	940.459	940.463	-0.004	4.3
y3	375.220	375.224	-0.004	11.0	y9	1055.496	1055.490	0.006	5.7
y4	504.266	504.267	-0.001	2.0	y10	1170.515	1170.517	-0.002	1.7
y5	667.331	667.330	0.001	1.5	y11	1283.606	1283.601	0.005	3.9
y6	768.375	768.378	-0.003	3.9					

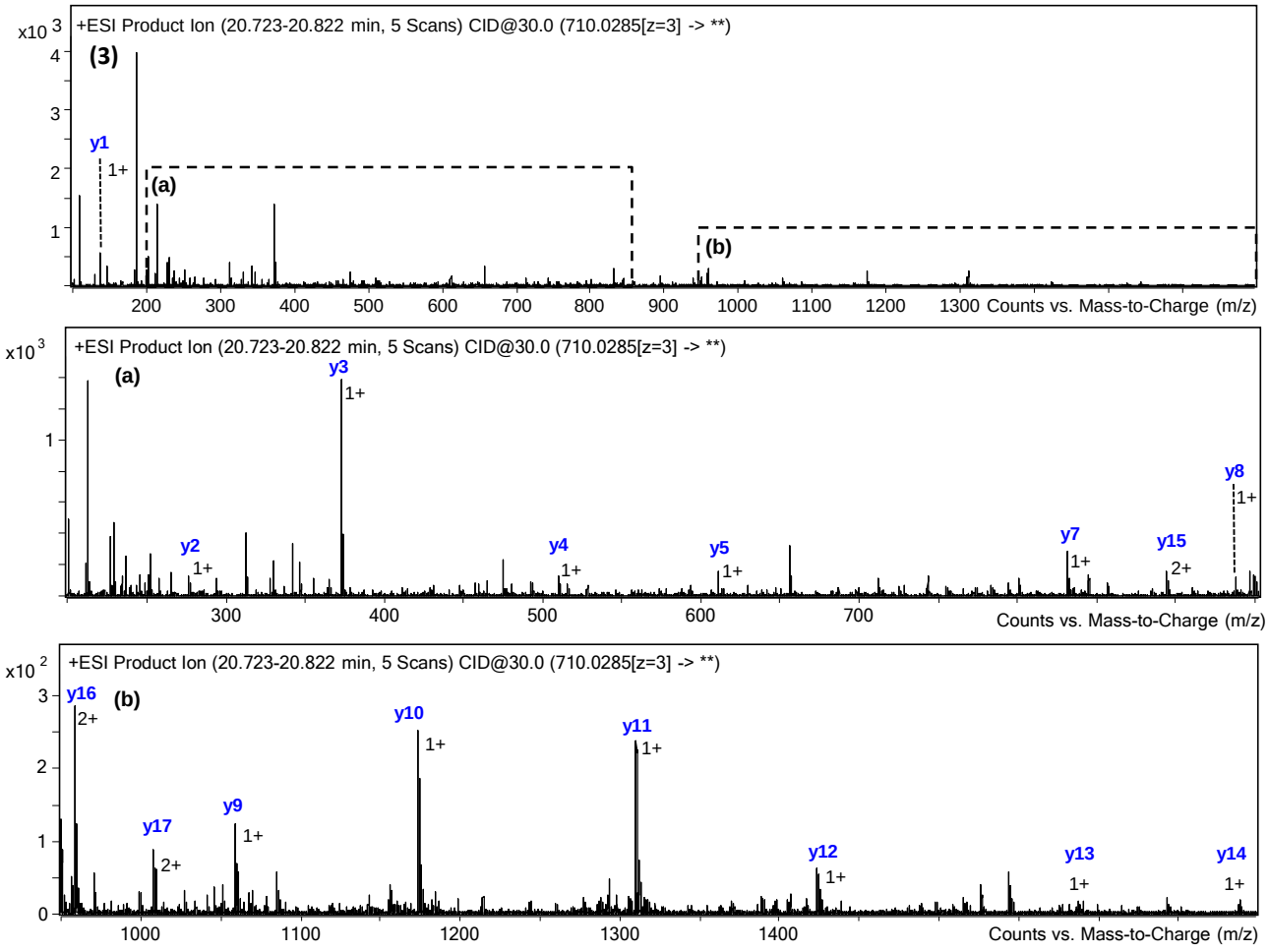
B



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y1	147.111	147.113	-0.002	13.6	y7	839.415	839.415	0.000	0.0
y2	276.156	276.156	0.000	0.0	y8	940.467	940.463	0.004	4.3
y3	375.222	375.224	-0.002	5.3	y9	1055.487	1055.490	-0.003	2.8
y4	504.268	504.267	0.001	2.0	y10	1170.519	1170.517	0.002	1.7
y5	667.334	667.330	0.004	6.0	y11	1283.598	1283.601	-0.003	2.3
y6	768.380	768.378	0.002	2.6	y12	1382.671	1382.669	0.002	1.5

Figure S2. Alkylation analysis of TEAS active site mutant W273E by bottom-up proteomic LC-MS. (**A**) MS/MS analysis of nonalkylated tryptic peptide V[442-453]K of TEAS W273E after reaction with (*E*,*E*)-FPP (**1**, Figure 2B). (**B**) MS/MS analysis of alkylated tryptic peptide I[515-532]K of TEAS W273E after reaction with (*E*,*E*)-FPP (**2**, Figure 2B).

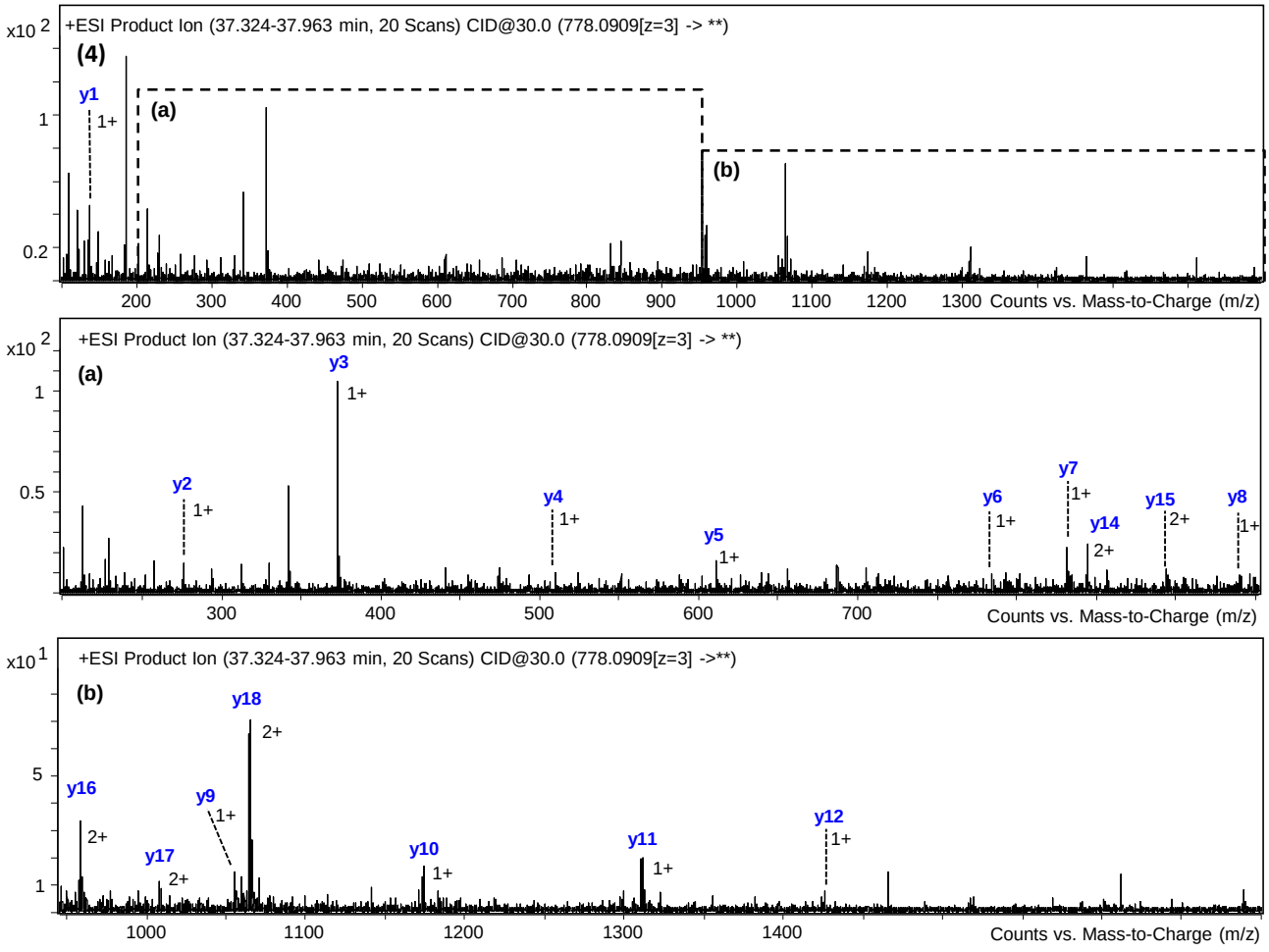
C



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y1	147.113	147.113	0.000	0.0	y10	1173.553	1173.554	0.001	0.1
y2	276.154	276.156	-0.002	7.2	y11	1310.609	1310.613	-0.004	3.1
y3	373.209	373.209	0.000	0.0	y12	1423.692	1423.697	-0.005	3.5
y4	510.262	510.268	-0.006	12.0	y13	1586.767	1586.760	0.007	4.4
y5	611.311	611.315	-0.004	6.5	y14	1687.802	1687.808	-0.006	3.6
y7	831.402	831.400	0.002	2.4	y15	1786.887	1786.877	0.010	5.6
y8	946.429	946.427	0.002	2.1	y16	1915.917	1915.919	-0.002	1.0
y9	1059.516	1059.511	0.005	4.7	y17	2014.970	2014.988	-0.018	8.9

Figure S2. Alkylation analysis of TEAS active site mutants by bottom-up proteomic LC-MS. (**C**) MS/MS analysis of non-alkylated tryptic peptide I[515-532]K of TEAS W273E after reaction with (*E,E*)-FPP (**3**, Figure 2B).

D



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y1	147.111	147.113	-0.002	13.6
y2	276.160	276.156	0.004	14.5
y3	373.208	373.209	-0.001	2.7
y4	510.274	510.268	0.006	11.8
y5	611.311	611.315	-0.004	6.5
y6	774.380	774.379	0.001	1.3
y7	831.403	831.400	0.003	3.6
y8	946.418	946.427	-0.009	9.5
y9	1059.524	1059.511	0.013	12.3

Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y10	1173.569	1173.554	0.015	12.8
y11	1310.617	1310.613	0.004	3.1
y12	1423.681	1423.697	-0.016	11.2
y14	1687.808	1687.808	0.000	0.0
y15	1786.855	1786.877	-0.022	12.3
y16	1915.897	1915.919	-0.022	11.5
y17	2014.969	2014.988	-0.019	9.4
y18	2128.074	2128.072	0.002	0.1

Figure S2. Alkylation analysis of TEAS active site mutants by bottom-up proteomic LCMS. (**D**) MS/MS analysis of alkylated tryptic peptide I[515-532]K of TEAS W273E after reaction with (*E,E*)-FPP (**4**, Figure 2B).

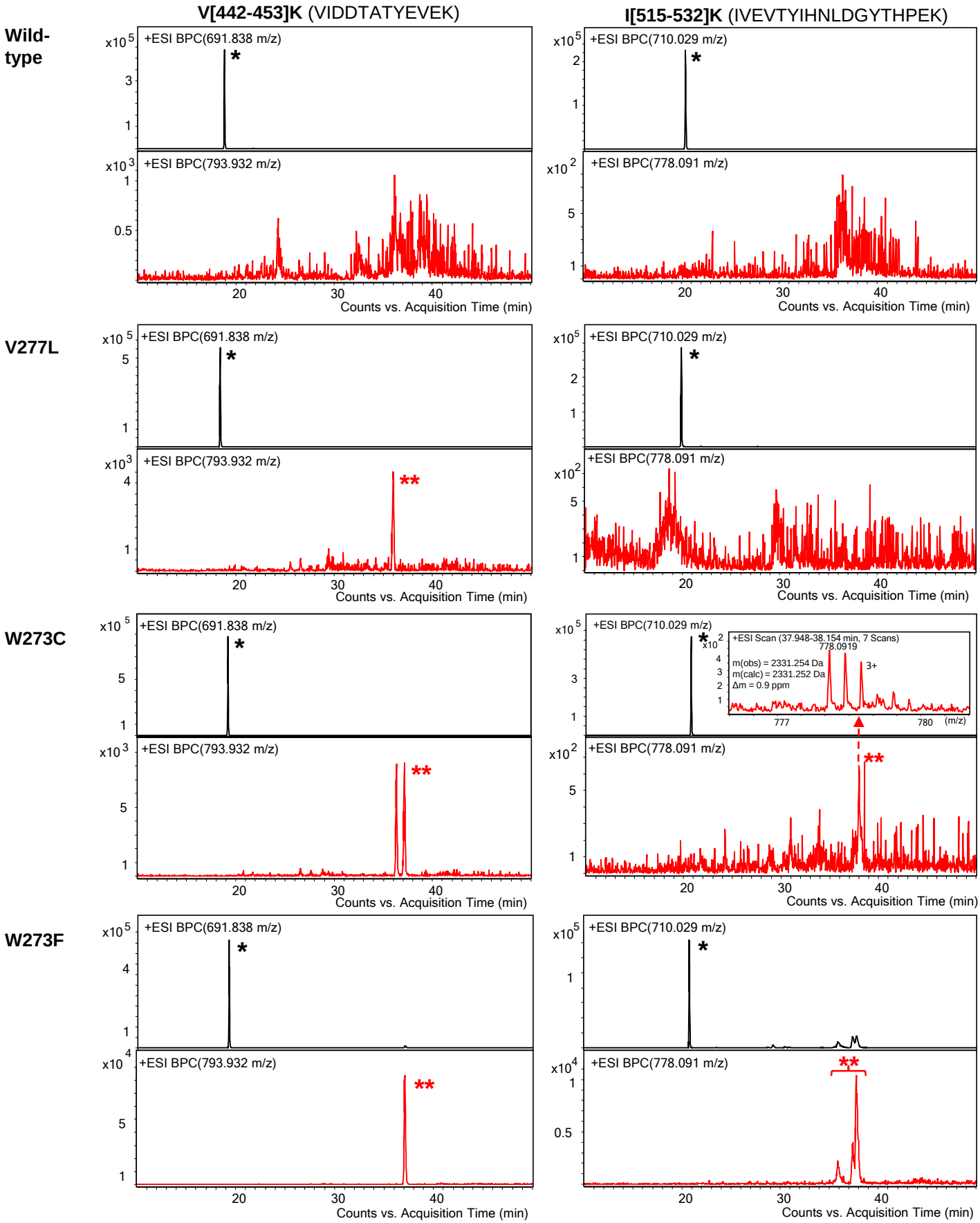


Figure S3. Alkylation analysis of TEAS active site mutants by bottom-up proteomic LC-MS. LC-MS analysis of nonalkylated and alkylated tryptic peptides V[442-453]K and I[515-532]K of TEAS active site mutants after reaction with (*E,E*)-FPP. Nonalkylated peptide BPCs are highlighted in black, monoalkylated peptide BPCs are highlighted in red. Single asteriks mark BPC peaks of non-alkylated peptides, double asteriks mark BPC peaks of alkylated peptides.

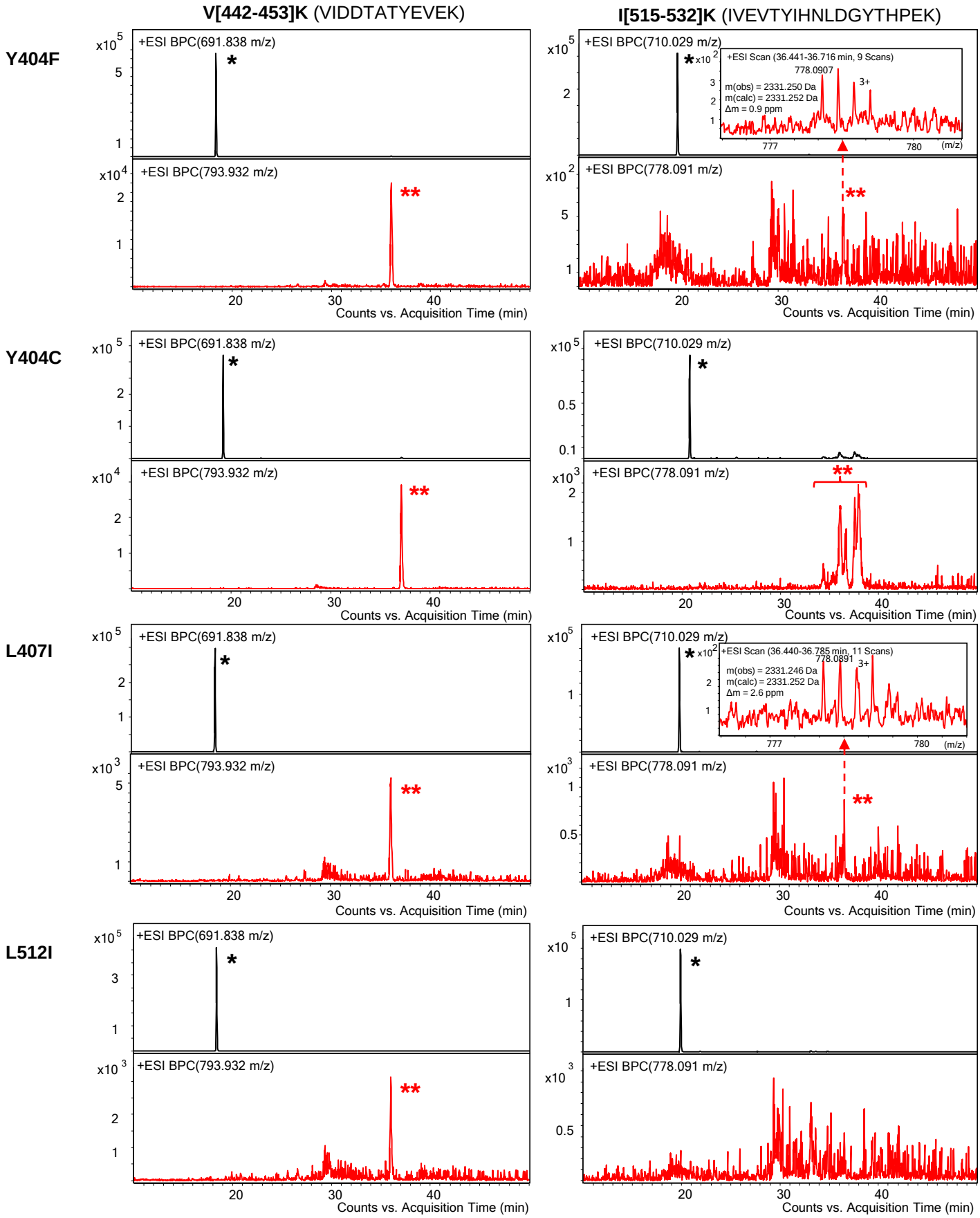


Figure S3. Alkylation analysis of TEAS active site mutants by bottom-up proteomic LC-MS. LC-MS analysis of nonalkylated and alkylated tryptic peptides V[442-453]K and I[515-532]K of TEAS active site mutants after reaction with (*E,E*)-FPP. Nonalkylated peptide BPCs are highlighted in black, monoalkylated peptide BPCs are highlighted in red. Single asteriks mark BPC peaks of non-alkylated peptides, double asteriks mark BPC peaks of alkylated peptides.

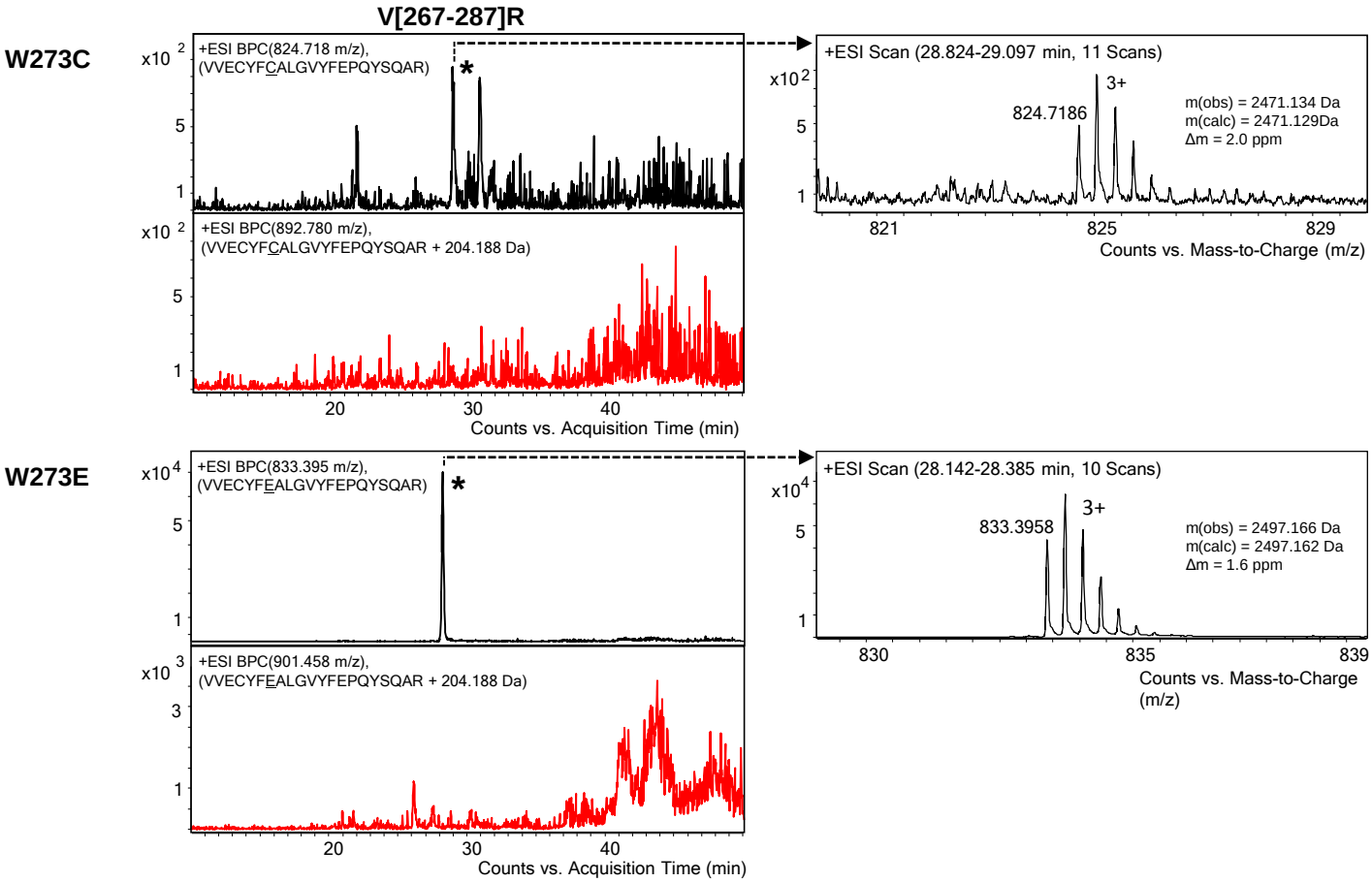


Figure S3. Alkylation analysis of TEAS active site mutants by bottom-up proteomic LC-MS. LC-MS and MS analysis of non-alkylated and alkylated tryptic peptide V[267-287]R of TEAS active site mutants W273C and W273E after reaction with (*E*,*E*)-FPP. Nonalkylated peptide BPCs are highlighted in black, monoalkylated peptide BPCs are highlighted in red. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. No mass signals of nonalkylated or alkylated tryptic peptide N[375-416]K of TEAS Y404C were detected by LC-MS (data not shown).

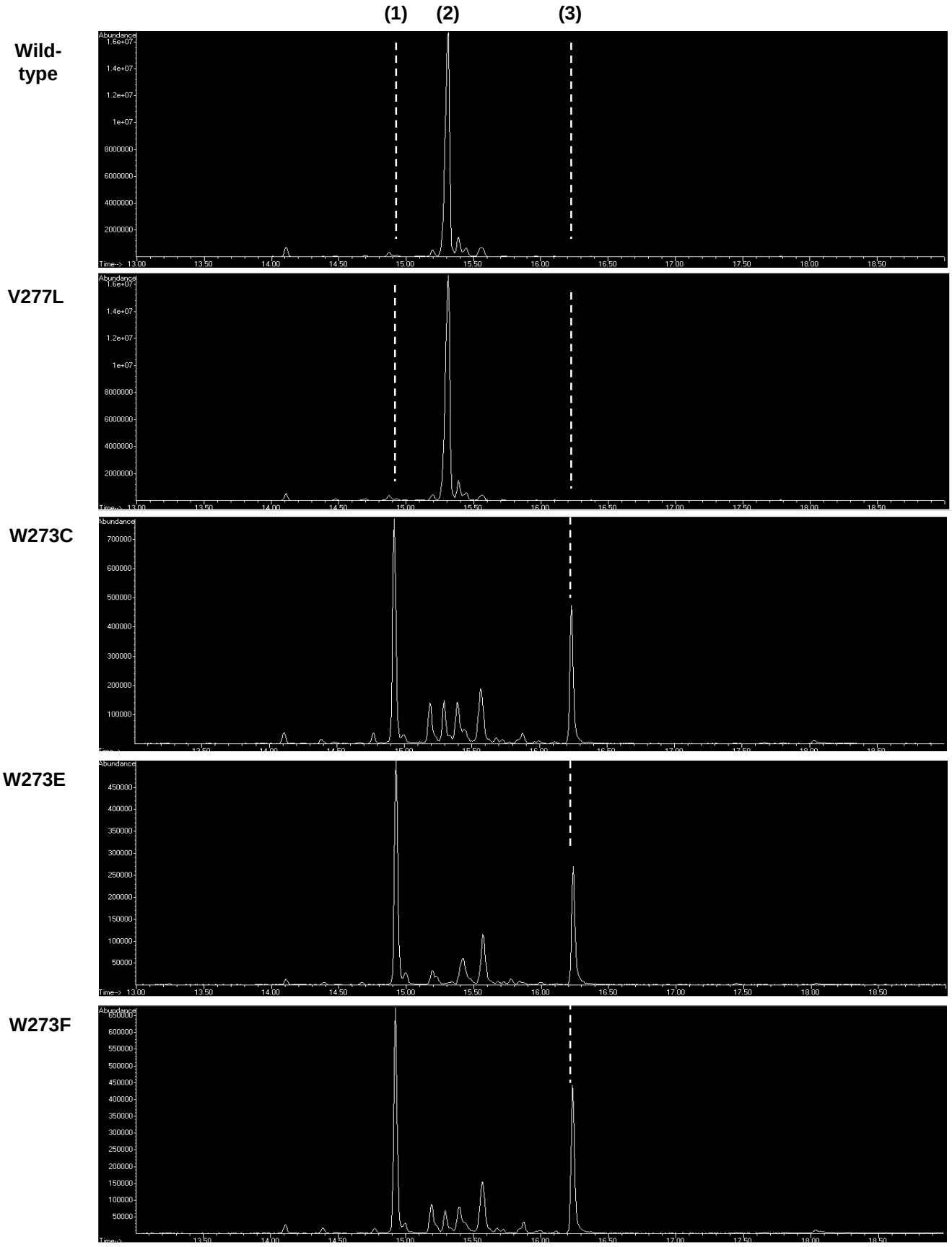


Figure S4. GCMS analysis of product profiles of TEAS active site mutants after reaction with (*E,E*)-FPP. Total ion chromatograms of hexane extracts of corresponding enzyme reactions are shown. Highlighted sesquiterpene products are β-farnesene (1), 5-*epi*-aristolochene (2) and farnesol (3).

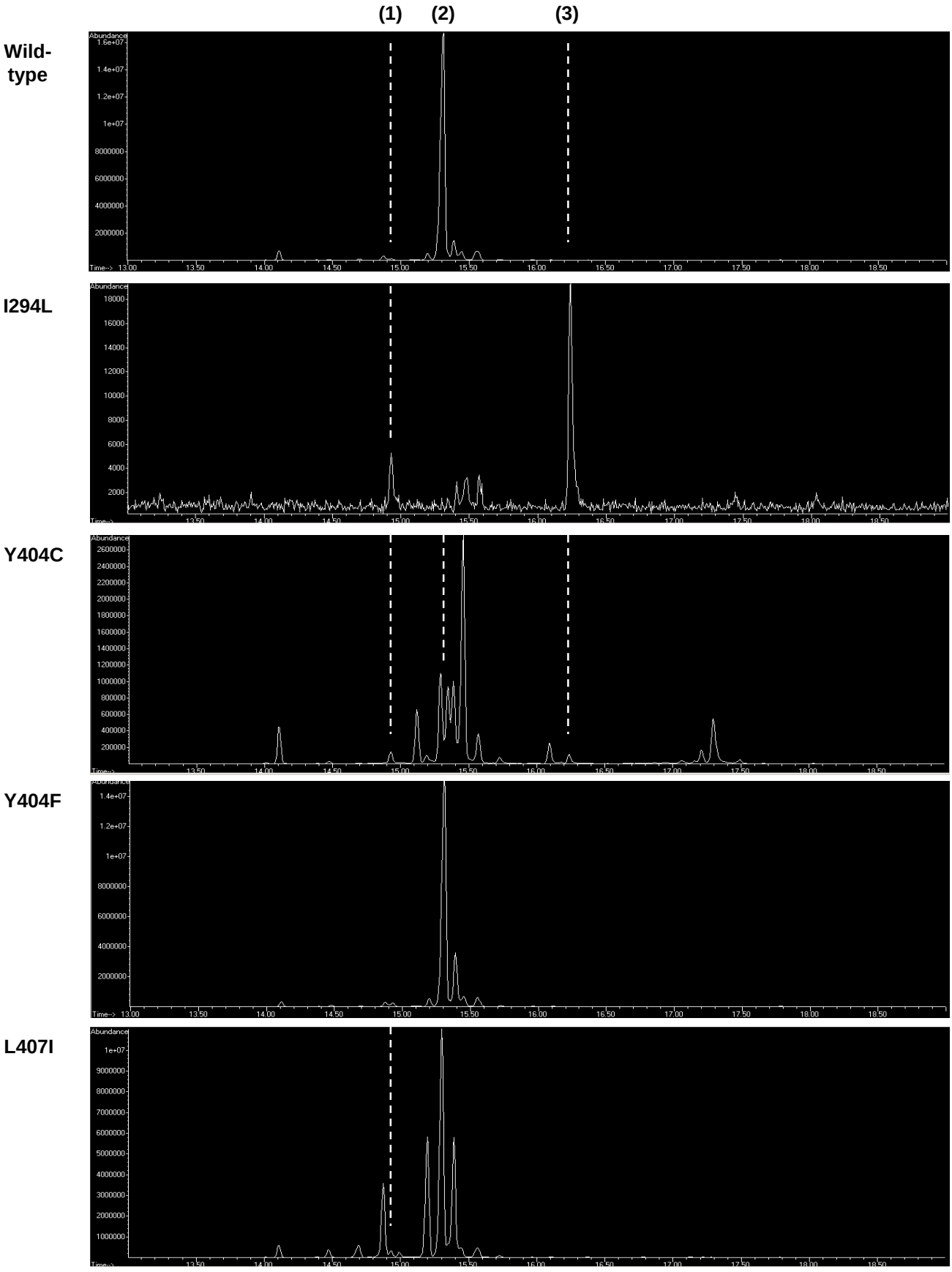


Figure S4. GCMS analysis of product profiles of TEAS active site mutants after reaction with (*E,E*)-FPP. Total ion chromatograms of hexane extracts of corresponding enzyme reactions are shown. Highlighted sesquiterpene products are β-farnesene (1), 5-*epi*-aristolochene (2) and farnesol (3).

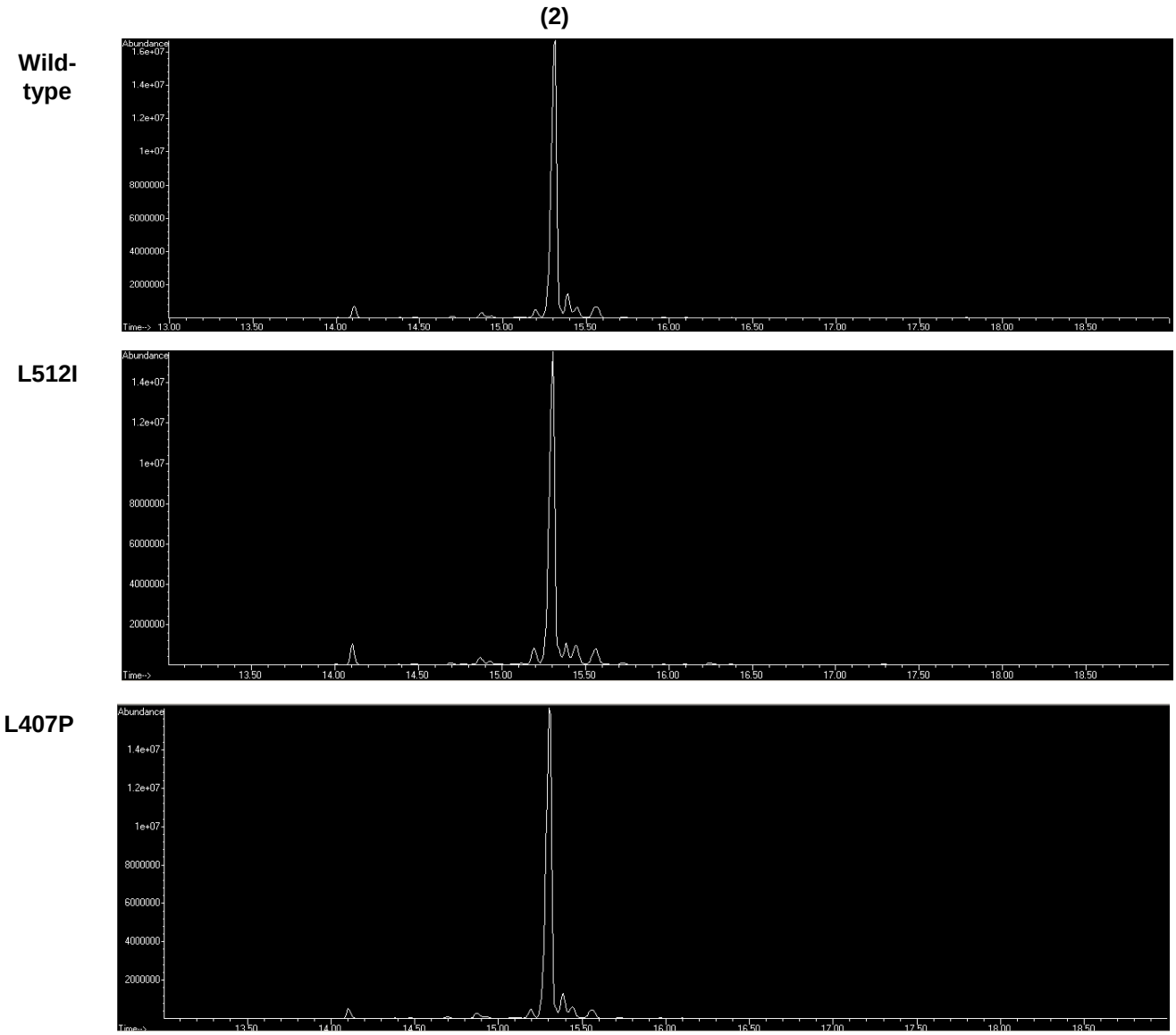
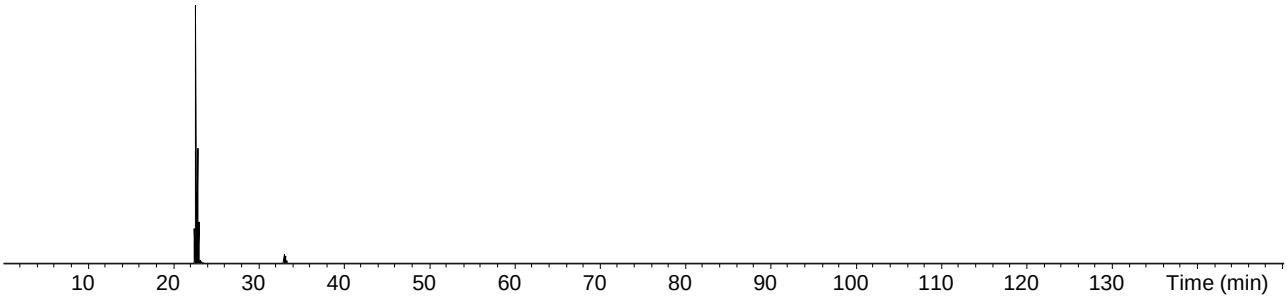
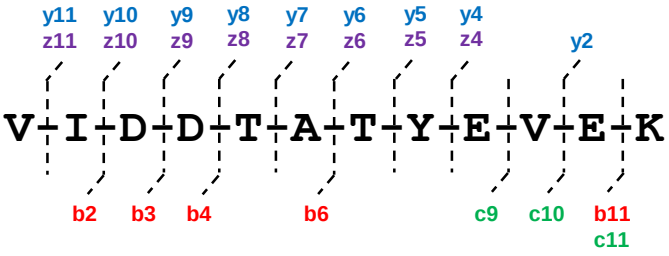


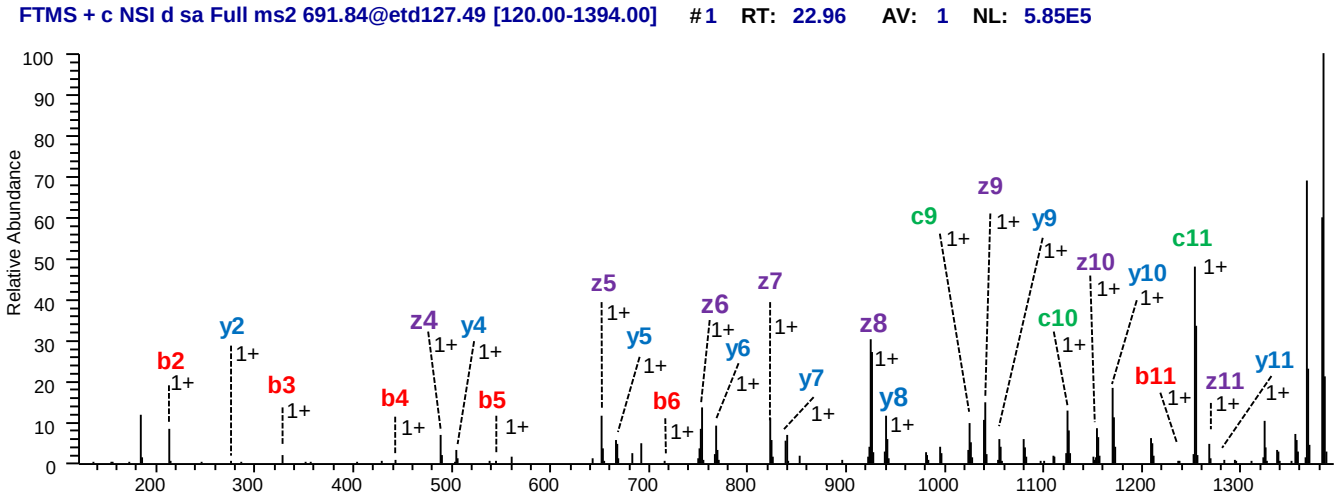
Figure S4. GCMS analysis of product profiles of TEAS active site mutants after reaction with (*E*,*E*)-FPP. Total ion chromatograms of hexane extracts of corresponding enzyme reactions are shown. Highlighted sesquiterpene products are β-farnesene (1), 5-*epi*-aristolochene (2) and farnesol (3).

A



B

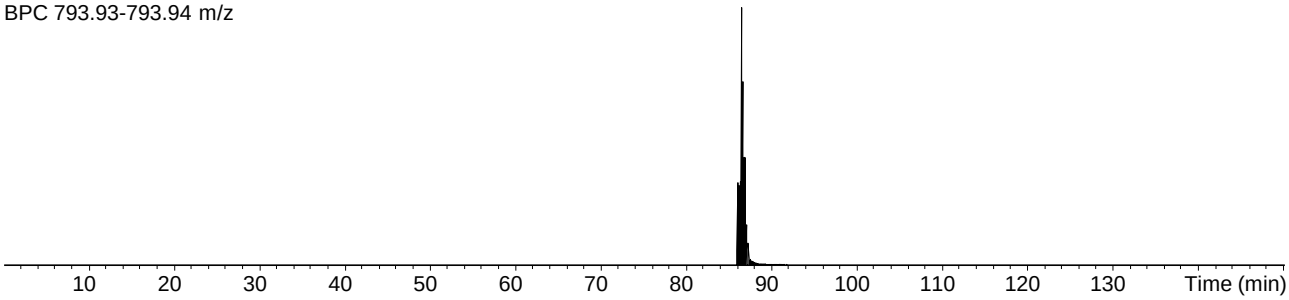




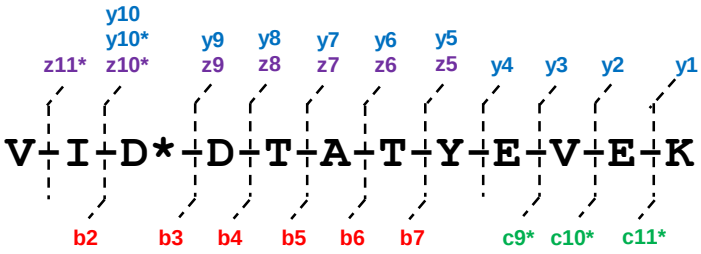
Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
b2	213.1580	213.1598	0.0018	8.4	y7	839.405	839.4145	0.0095	11.3
y2	276.1519	276.1554	0.0035	12.7	z8	924.437	924.4435	0.0065	7.0
b3	328.1833	328.1867	0.0034	10.4	y8	940.4545	940.4622	0.0077	8.2
b4	443.2116	443.2136	0.002	4.5	c9	1025.4695	1025.4786	0.0091	8.9
z4	488.2449	488.2477	0.0028	5.7	z9	1039.463	1039.4704	0.0074	7.1
y4	504.2642	504.2664	0.0022	4.4	y9	1055.4797	1055.4891	0.0094	8.9
b5	544.2599	544.2613	0.0014	2.6	c10	1124.5396	1124.547	0.0074	6.6
z5	651.3063	651.3110	0.0047	7.2	z10	1154.4873	1154.4974	0.0101	8.8
y5	667.3229	667.3297	0.0068	10.2	y10	1170.5063	1170.5161	0.0098	8.4
b6	716.3478	716.3461	-0.0017	2.4	b11	1236.5577	1236.563	0.0053	4.3
z6	752.3532	752.3587	0.0055	7.3	c11	1253.5815	1253.5896	0.0081	6.5
y6	768.3741	768.3668	-0.0073	9.5	z11	1267.5804	1267.5814	0.001	7.9
z7	823.3879	823.3958	0.0079	9.6	y11	1283.6198	1283.6002	-0.0196	15.0

Figure S5. Characterization of alkylation site in TEAS W273E by EThcD-MS/MS. (**A**) LC-MS analysis of nonalkylated tryptic peptide V[442-453]K of TEAS W273E after reaction with (*E*,*E*)-FPP. (**B**) EThcD-MS/MS analysis of nonalkylated tryptic peptide V[442-453]K of TEAS W273E after reaction with (*E*,*E*)-FPP**.**

C



D



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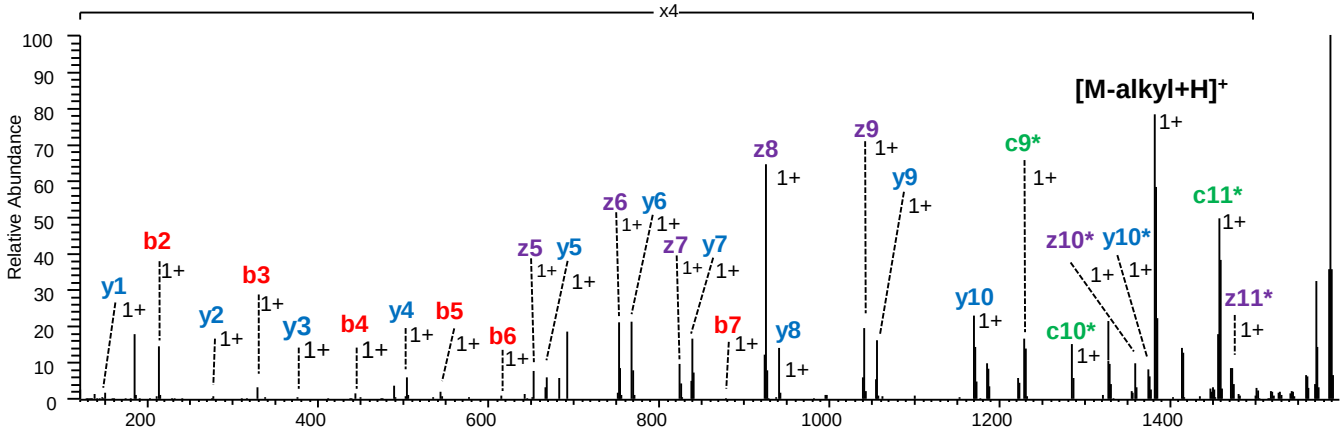


Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y1	147.1105	147.1128	-0.0023	15.6	y7	839.4076	839.4145	-0.0069	8.2
b2	213.1583	213.1598	-0.0015	7.0	b7	879.3975	879.4094	-0.0119	13.5
y2	276.1524	276.1554	-0.0030	10.9	z8	924.4350	924.4435	-0.0085	9.2
b3	328.1834	328.1867	-0.0033	10.0	y8	940.4560	940.4622	-0.0062	6.6
y3	375.2185	375.2238	-0.0053	14.1	z9	1039.4617	1039.4704	-0.0087	8.4
b4	443.2082	443.2136	-0.0054	12.2	y9	1055.4811	1055.4891	-0.0080	7.6
y4	504.2622	504.2664	-0.0042	8.3	y10	1170.5086	1170.5161	-0.0075	6.4
b5	544.2558	544.2613	-0.0055	10.1	c9*	1229.6514	1229.6666	-0.0152	12.4
b6	615.2963	615.2984	-0.0021	3.4	c10*	1328.7165	1328.735	-0.0185	13.9
z5	651.3153	651.3110	0.0043	6.6	z10*	1358.6763	1358.6854	-0.0091	6.7
y5	667.3265	667.3297	-0.0032	4.8	y10*	1374.6920	1374.7041	-0.0121	8.8
z6	752.3527	752.3587	-0.0060	8.0	$[M\text{-alkyl+H}]^+$	1382.6579	1382.6686	-0.0107	7.7
y6	768.3716	768.3668	0.0048	6.2	c11*	1457.7665	1457.7776	-0.0111	7.6
z7	823.3904	823.3958	-0.0054	6.6	z11*	1471.7610	1471.7694	-0.0084	5.7

* = +204.188Da

Figure S5. Characterization of alkylation site in TEAS W273E by EThcD-MS/MS. (**C**) LC-MS analysis of alkylated tryptic peptide V[442-453]K of TEAS W273E after reaction with (*E,E*)-FPP. (**D**) EThcD-MS/MS analysis of alkylated tryptic peptide V[442-453]K of TEAS W273E after reaction with (*E,E*)-FPP.

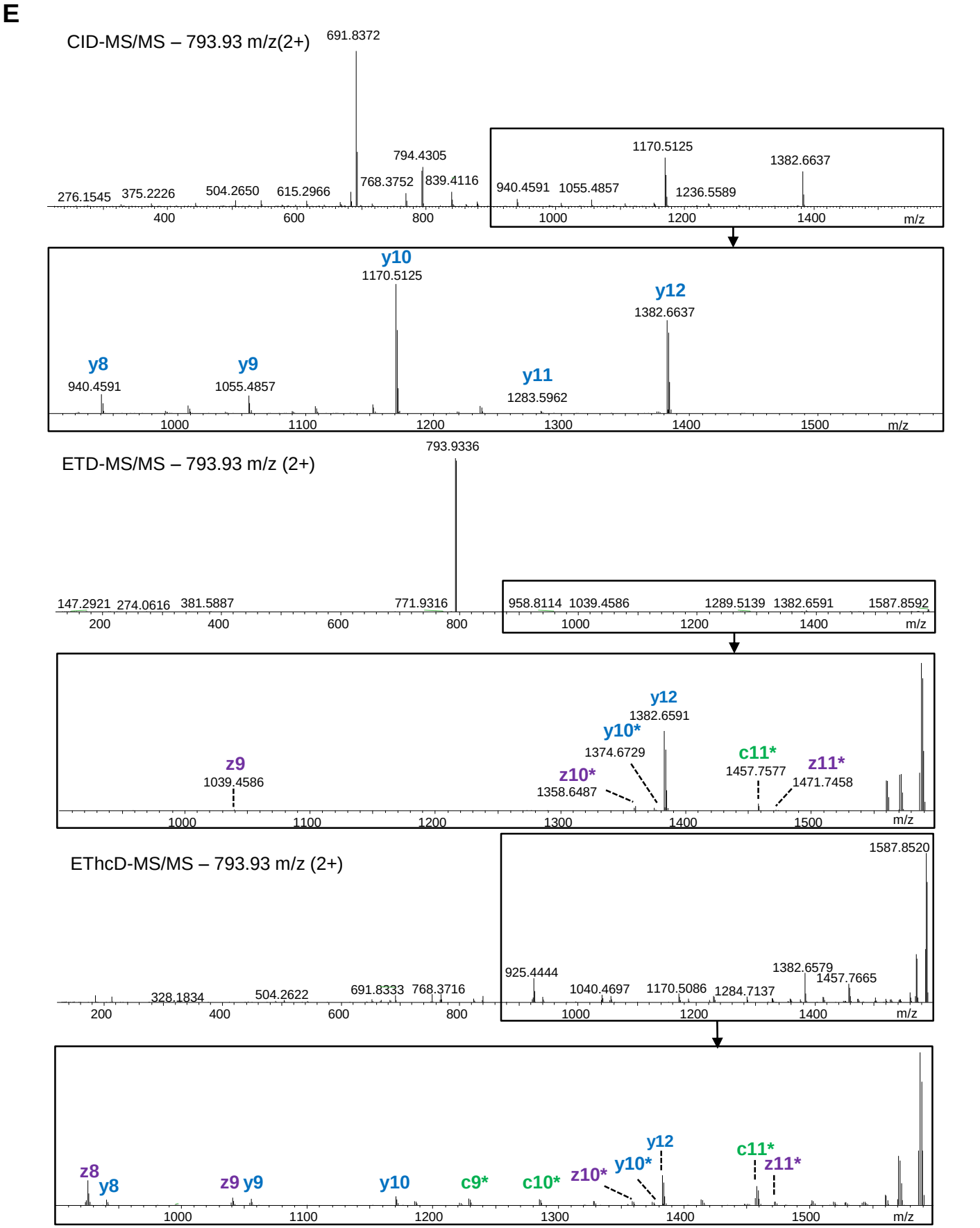
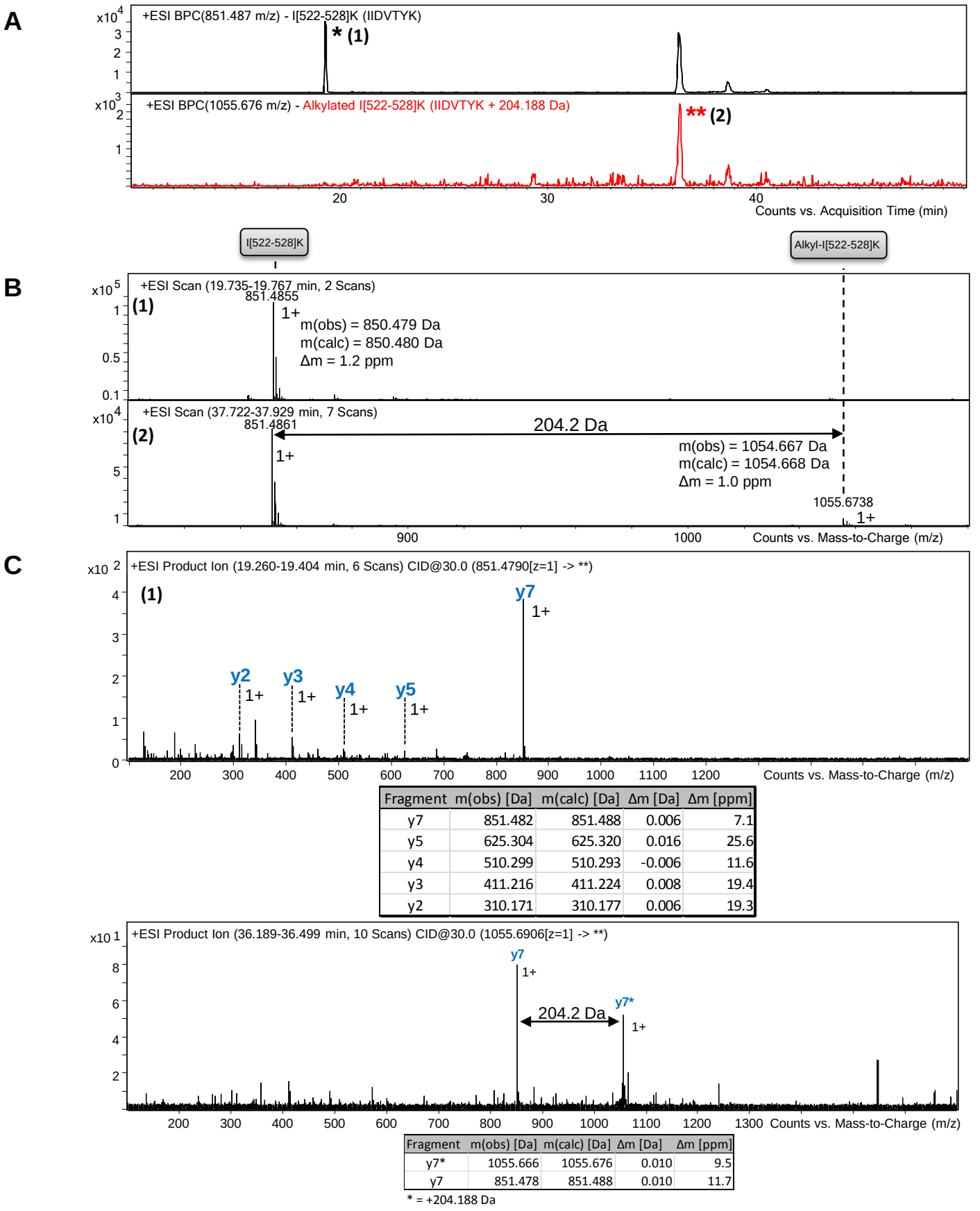


Figure S5. Characterization of alkylation site in TEAS W273E by EThcD-MS/MS. (**E**) Comparison of tandem mass spectrometry methods for characterization of alkylation modification on tryptic peptide V[442-453]K of TEAS W273E after reaction with (*E*,*E*)-FPP. Abbreviations: CID – collision induced dissociation, ETD – electron transfer dissociation, EThcD – electron-transfer and higher-energy collision dissociation, * - peptide fragment with alkyl mass shift (204.188 Da).



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y7	851.482	851.488	0.006	7.1
y5	625.304	625.320	0.016	25.6
y4	510.299	510.293	-0.006	11.6
y3	411.216	411.224	0.008	19.4
y2	310.171	310.177	0.006	19.3

Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y7*	1055.666	1055.676	0.010	9.5
y7	851.478	851.488	0.010	11.7

* = +204.188 Da

Figure S6. Characteriztion of second alkylation site in tobacco 5-*epi*-aristolochene synthase (TEAS) through alkylation analysis of TEAS homologue *Hyoscyamus muticus* premnaspirodiene synthase (HPS). (**A**) LC-MS analysis of alkylated I[522-528]K of HPS W280E after reaction with (*E,E*)-FPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. (**B**) MS analysis of nonalkylated (**1**) and alkylated (**2**) I[522-528]K of HPS W280E after reaction with FPP. (**C**) MS/MS analysis of nonalkylated (**1**) and alkylated (**2**) I[522-528]K of HPS W280E after reaction with FPP.

D

```
TEAS            MASAAVAN--YEEEIVRPVADFSPSLWGDQFLSFSIKNQVAEKYAKEIEALKEQTRNML- 57
HPS             MAPAIVMSNYEEEEIVRPVADFSPSLWGDRFHSFSVDNQVAEKYAQEIETLKEQTSTMLS 60
                **.* * .   ******************:* ***:.********:***:***** .**

TEAS            LATGMKLADTLNLIDTIERLGISYHFEKEIDDILDQIYNQN-----SNCNDLCTSALQFR 112
HPS             AACGTTLTEKLNLIDIIERLGIAYHFEKQIEDMLDHIYRADPYFEAHEYNDLNTSSVQFR 120
                 * * .*::.***** ******:*****:*:*:**:**. :      : *** **::***

TEAS            LLRQHGFNISPEIFSKFQDENGKFKESLASDVLGLLNLYEASHVRTHADDILEDALAFST 172
HPS             LLRQHGYNVSPNIFSRFQDANGKFKESLRSDIRGLLNLYEASHVRTHKEDILEEALVFSV 180
                ******:*:**:***:*** ******** **: ************** :****:**.**.

TEAS            IHLESAAPHLKSPLREQVTHALEQCLHKGVPRVETRFFISSIYDKEQSKNNVLLRFAKLD 232
HPS             GHLESAAPHLKSPLSKQVTHALEQSLHKSIPRVEIRYFIS-IYEEEEFKNDLLLRFAKLD 239
                 ************* :********.***.:**** *:*** **::*: **::********

TEAS            FNLLQMLHKQELAQVSRWWKDLDFVTTLPYARDRVVECYFWALGVYFEPQYSQARVMLVK 292
HPS             YNLLQMLHKHELSEVSRWWKDLDFVTTLPYARDRAVECYFWTMGVYAEPQYSQARVMLAK 299
                :********:**::*********************.******::*** ***********.*

TEAS            TISMISIVDDTFDAYGTVKELEAYTDAIQRWDINEIDRLPDYMKISYKAILDLYKDYEKE 352
HPS             TIAMISIVDDTFDAYGIVKELEVYTDAIQRWDISQIDRLPEYMKISYKALLDLYDDYEKE 359
                **:************* *****.**********.:*****:********:****.*****

TEAS            LSSAGRSHIVCHAIERMKEVVRNYNVESTWFIEGYTPPVSEYLSNALATTTYYYLATTSY 412
HPS             LSKDGRSDVVHYAKERMKEIVRNYFIEAKWFIEGYMPSVSEYLSNALATSTYYLLTTTSY 419
                **. ***.:* :* *****:**** :*:.****** *.***********:*** *:****

TEAS            LGMKSATEQDFEWLSKNPKILEASVIICRVIDDTATYEVEKSRGQIATGIECCMRDYGIS 472
HPS             LGMKSATKEHFEWLATNPKILEANATLCRVVDDIATYEVEKGRGQIATGIECYMRDYGVS 479
                *******::.****:.*******.. :***:** *******.********** *****:*

TEAS            TKEAMAKFQNMAETAWKDINEGLLRPTPVSTEFLTPILNLARIVEVTYIHNLDGYTHPEK 532
HPS             TEVAMEKFQEMADIAWKDVNEEILRPTPVSSEILTRILNLARIIDVTYKHNQDGYTHPEK 539
                *: ** ***:**: ****:** :********:*:** *******::*** ** ********

TEAS            VLKPHIINLLVDSIKI 548
HPS             VLKPHIIALVVDSIDI 555
                ******* *:****.*
```

Figure S6. Characteriztion of second alkylation site in tobacco 5-*epi*-aristolochene synthase (TEAS) through alkylation analysis of TEAS homologue *Hyoscyamus muticus* premnaspirodiene synthase (HPS). (**D**) Sequence alignment of TEAS and HPS with highlighted W→E mutation site (green) and the tryptic peptide (blue) with the putative second alkylation site (red).

A

#	Contig (GenBank)	Length [aa]	Protein sequence similarity/identity with TEAS [%/%]	Active site residues (wild-type TEAS positions)															M9 residues (wild-type TEAS positions)									Note
				273	277	301	305	401	404	407	441	444	448	452	512	520	525	527	274	291	372	402	406	436	438	439	516	
1	AWOK01001025	550	99/99	W	V	D	D	T	Y	L	R	D	T	E	L	Y	D	Y	A	V	V	T	Y	S	I	I	V	wild-type TEAS
2	AWOK01127688	548	97/95	W	V	D	D	T	Y	L	R	D	T	E	L	Y	D	Y	A	V	V	T	Y	S	I	I	V	
3	AWOK01250557	548	97/95	W	V	D	D	T	Y	L	R	D	T	E	L	Y	D	Y	A	V	V	T	Y	S	I	I	V	
4	AWOK01009162	555	91/82	W	V	D	D	T	Y	L	R	D	T	E	L	Y	D	Y	T	A	I	S	L	N	T	L	I	TEAS M9 mutant
5	AWOK01139108	548	90/82	W	V	D	D	T	Y	C	R	D	T	E	F	Y	D	Y	A	T	L	S	S	S	I	L	V	3 internal stop codons (R208*, R266*, W323*)
6	AWOK01416821	349*	96/95	W	V	D	D	T	Y	P	R	D	T	E	L	Y	D	Y	A	V	V	T	Y	S	I	I	V	N-terminal domain truncated (1-199), 2 internal stop codons (Y376*, Y520*)

B

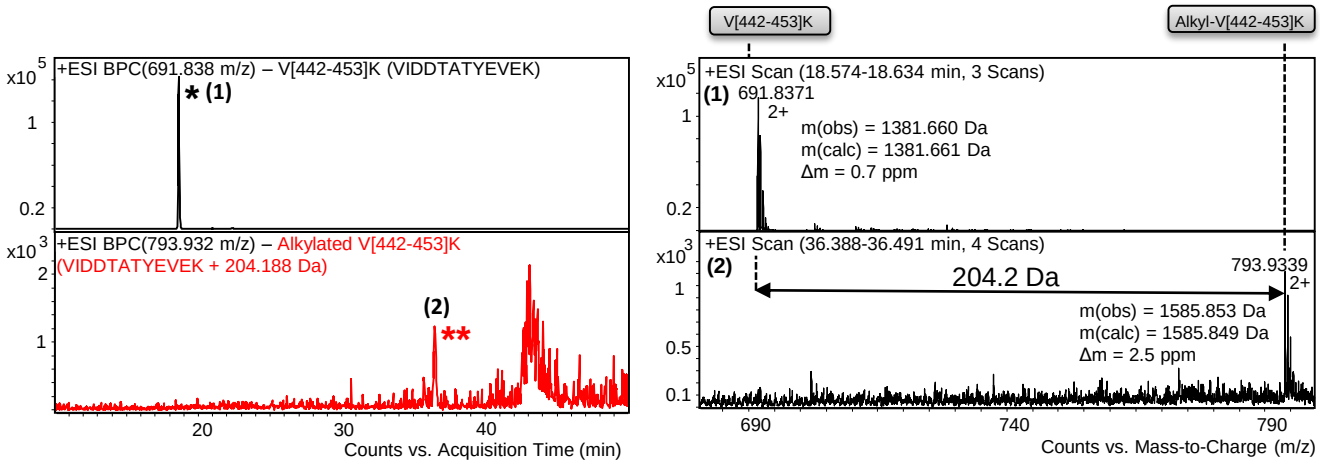
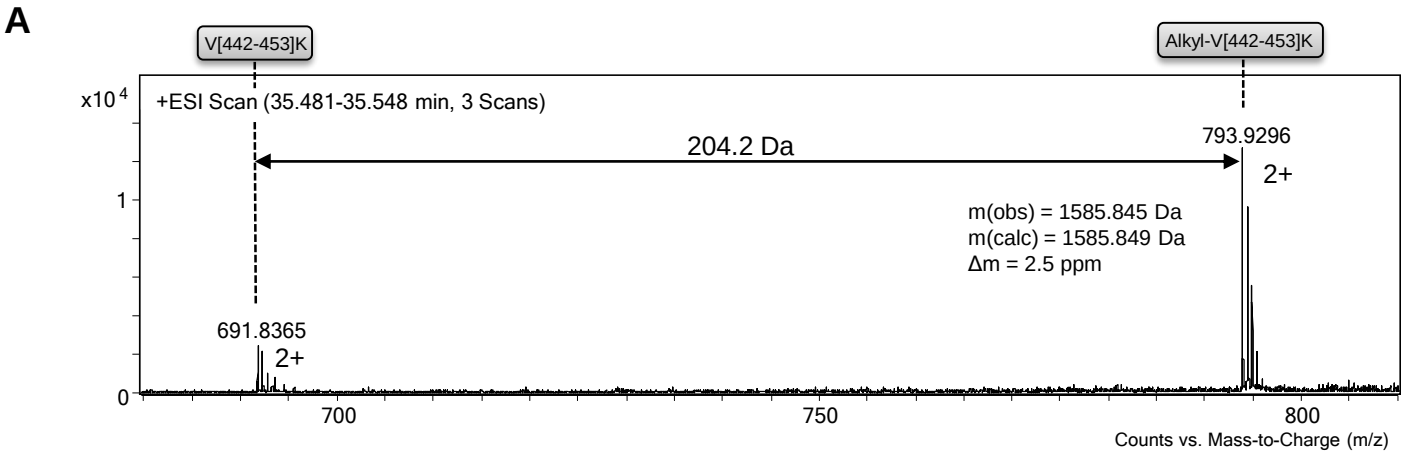
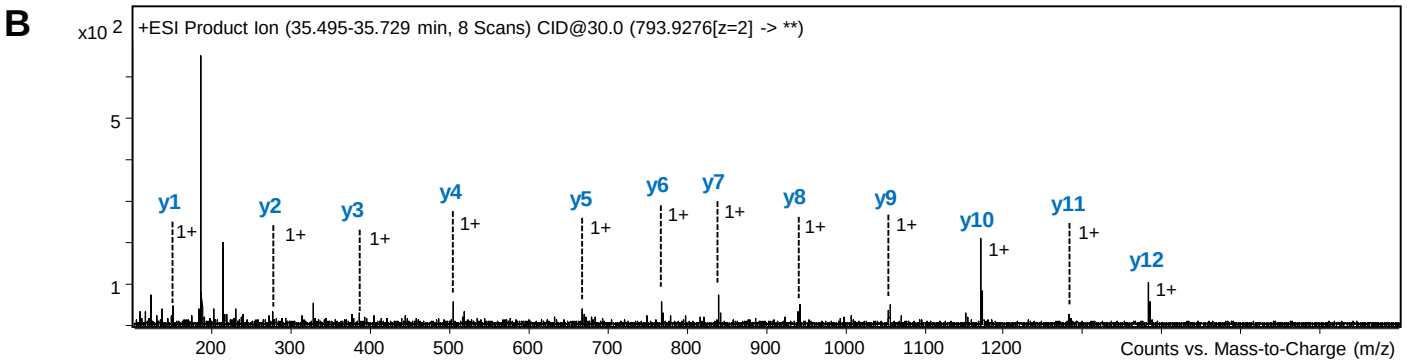


Figure S7. Genome mining of *Nicotiana tabacum* Basma Xanthi for self-alkylating TEAS gene homologues. (**A**) The genome search revealed a gene of a N-terminally truncated, inactivated TEAS homologue with an active site mutation that caused self-alkylation in the corresponding TEAS mutant (**B**). Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides.





Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y1	147.11	147.113	-0.003	20.4	y7	839.411	839.415	-0.004	4.8
y2	276.151	276.156	-0.005	18.1	y8	940.452	940.463	-0.011	11.7
y3	375.218	375.224	-0.006	16.0	y9	1055.468	1055.490	-0.022	20.8
y4	504.264	504.267	-0.003	6.0	y10	1170.515	1170.517	-0.002	1.7
y5	667.329	667.330	-0.001	1.5	y11	1283.575	1283.601	-0.026	20.3
y6	768.379	768.378	0.001	1.3	y12	1382.654	1382.669	-0.015	10.8

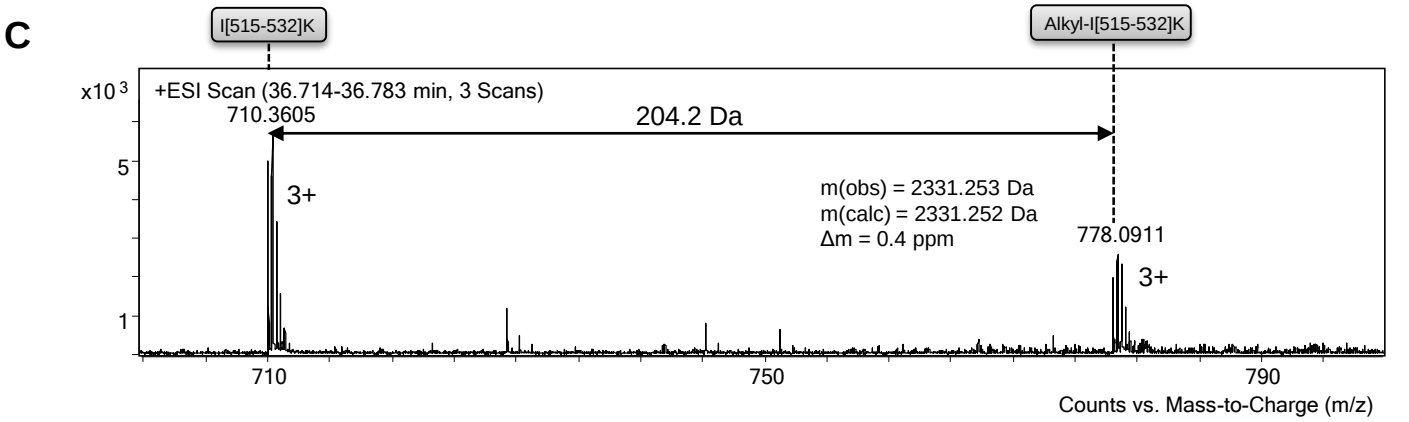
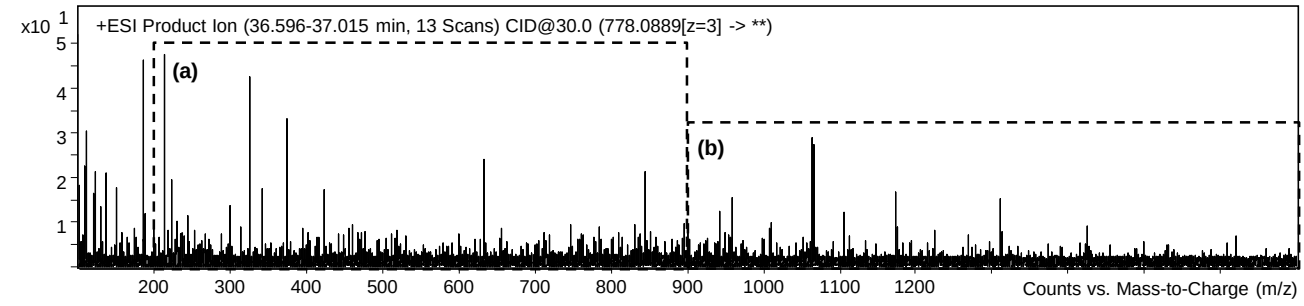
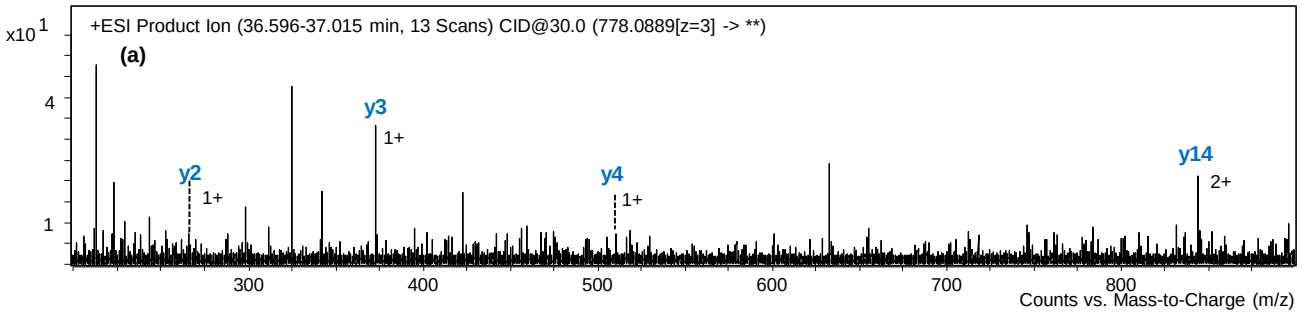
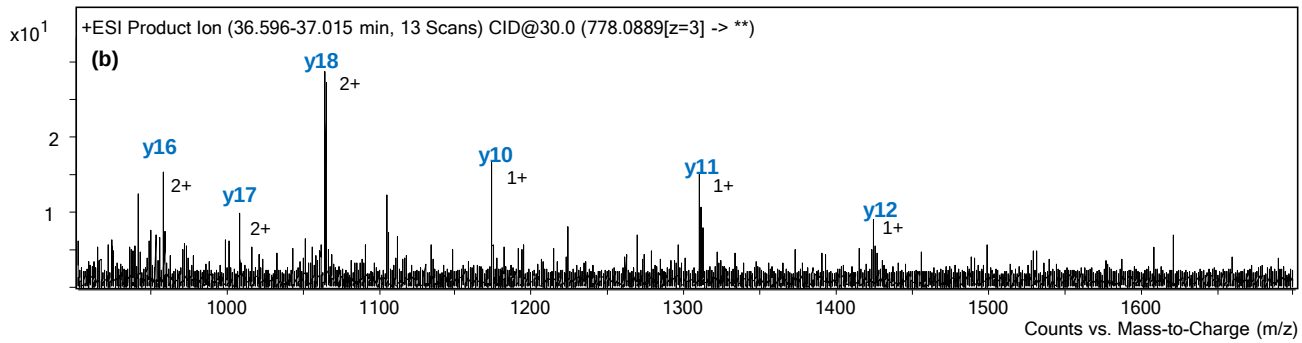


Figure S8. Alkylation analysis of wild-type TEAS with substrate analogues and with (*E,E*)-FPP at increased reaction temperatures. (**A**) MS spectrum of alkylated tryptic peptide V[442-453]K of wild-type TEAS after reaction with SPP. (**B**) MS/MS analysis of alkylated V[442-453]K of wild-type TEAS after reaction with sesquilavandulyl diphosphate (SPP). (**C**) MS spectrum of alkylated tryptic peptide I[515-532]K of wild-type TEAS after reaction with SPP.

D







Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y2	276.149	276.156	-0.007	26.8	y12	1423.686	1423.697	-0.011	8.1
y3	373.206	373.209	-0.003	7.8	y14	1687.812	1687.808	0.004	2.4
y4	510.262	510.268	-0.006	11.6	y16	1915.913	1915.919	-0.006	3.1
y10	1173.557	1173.554	0.003	2.6	y17	2015.029	2014.988	0.041	20.3
y11	1310.612	1310.613	-0.001	1.1	y18	2128.065	2128.072	-0.007	3.3

E

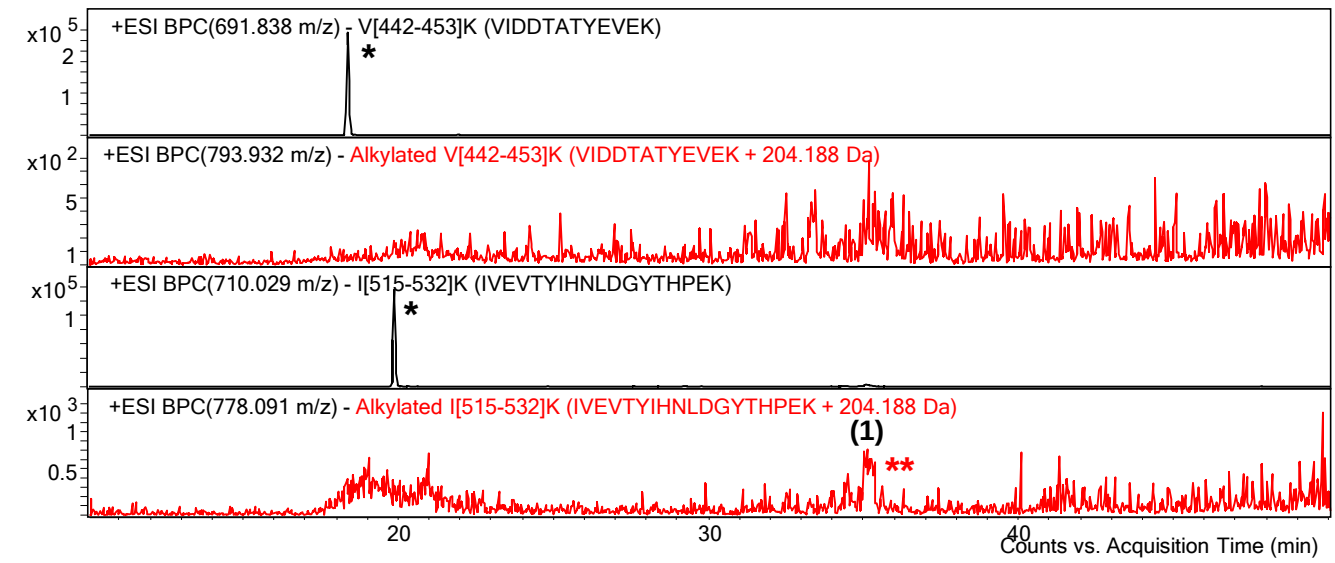


Figure S8. Alkylation analysis of wild-type TEAS with substrate analogues and with (*E,E*)-FPP at increased reaction temperatures. (**D**) MS/MS analysis of alkylated I[515-532]K of wild-type TEAS after reaction with SPP. (**E**) LC-MS analysis of non-alkylated and alkylated tryptic peptides V[442-453]K and I[515-532]K of wild-type TEAS after reaction with (*Z,E*)-FPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides.

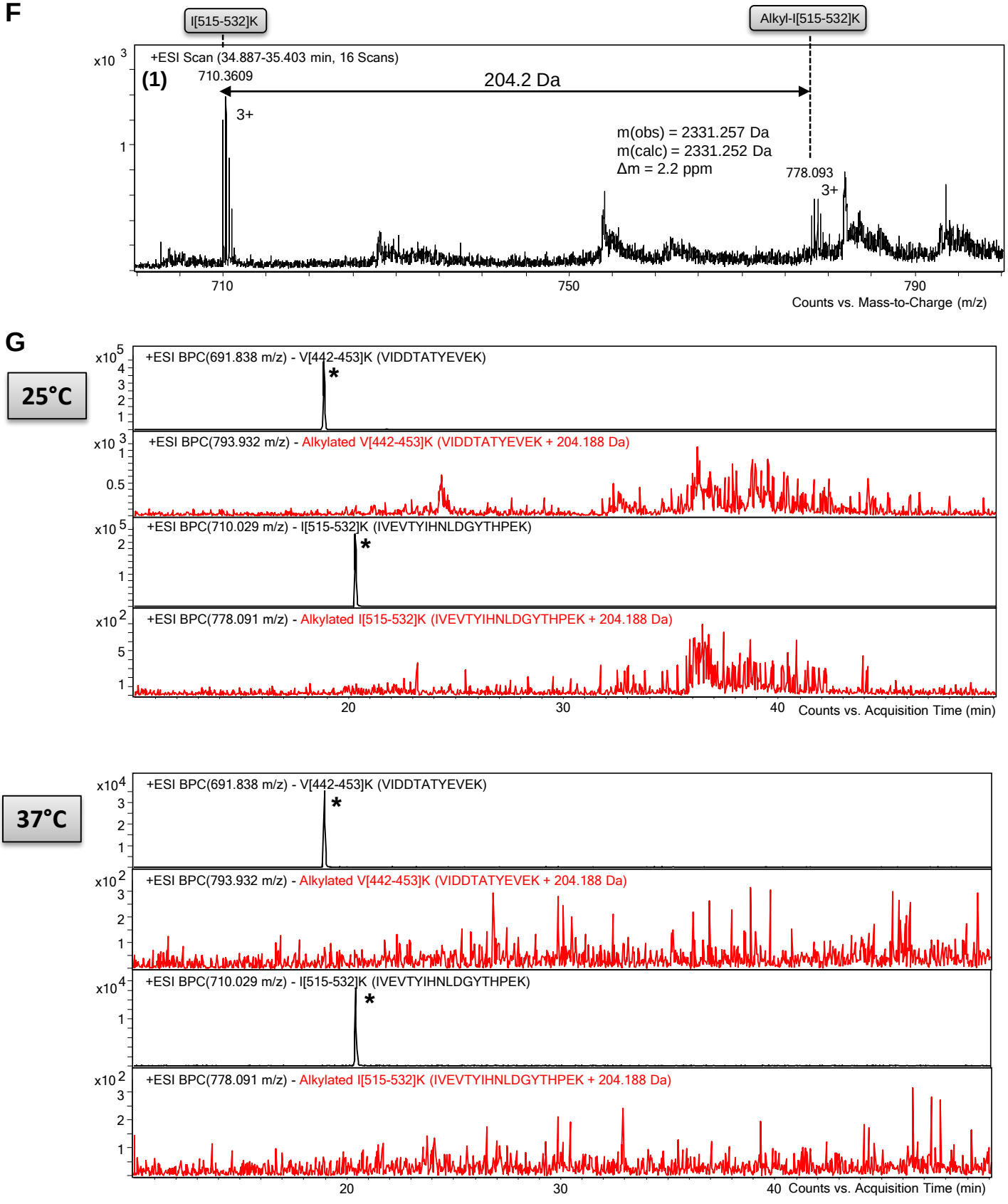
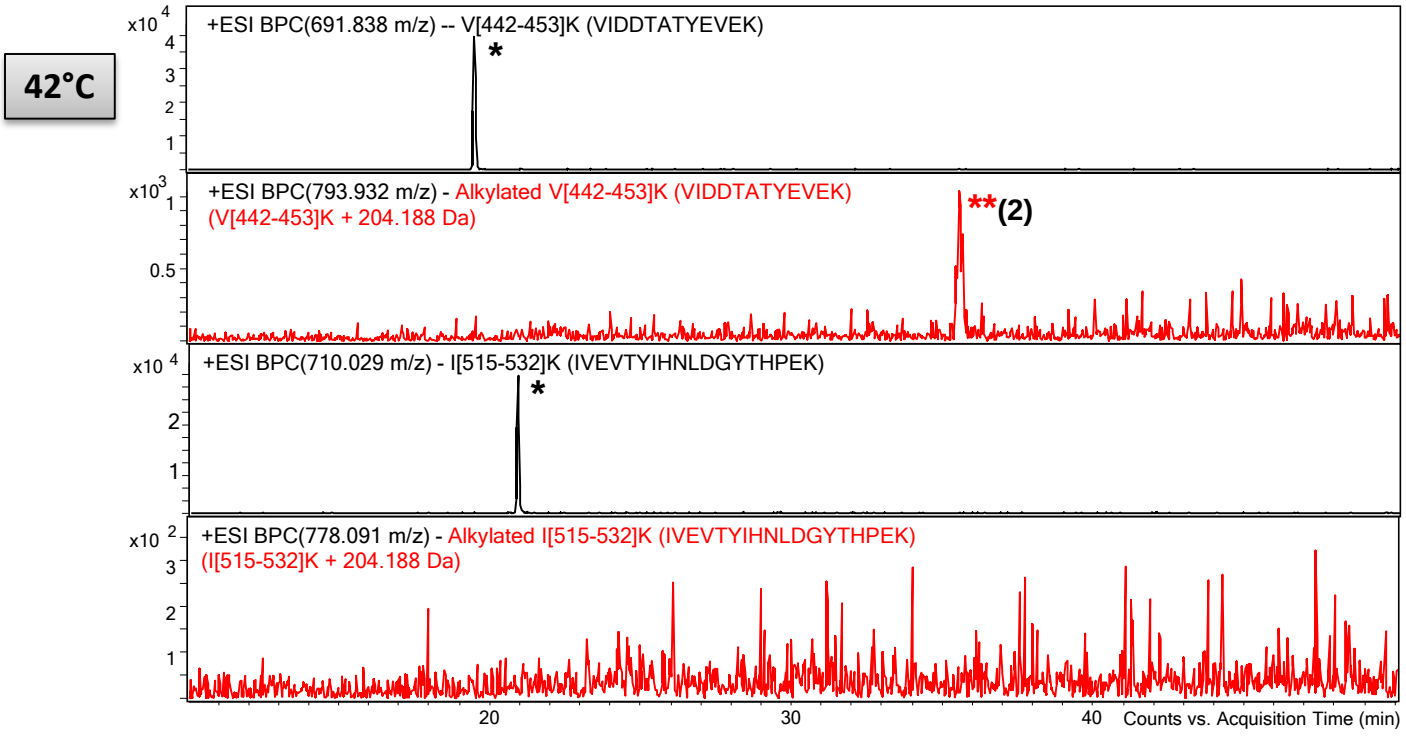


Figure S8. Alkylation analysis of wild-type TEAS with substrate analogues and with (*E,E*)-FPP at increased reaction temperatures. (**F**) MS spectrum of alkylated I[515-532]K of wild-type TEAS (**1**) after reaction with (*Z,E*)-FPP. (**G**) LC-MS analysis of nonalkylated and alkylated V[442-453]K and I[515-532]K of wild-type TEAS after reaction with (*E,E*)-FPP at 25°C, at 37°C and at 42°C. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides.

G,
continued



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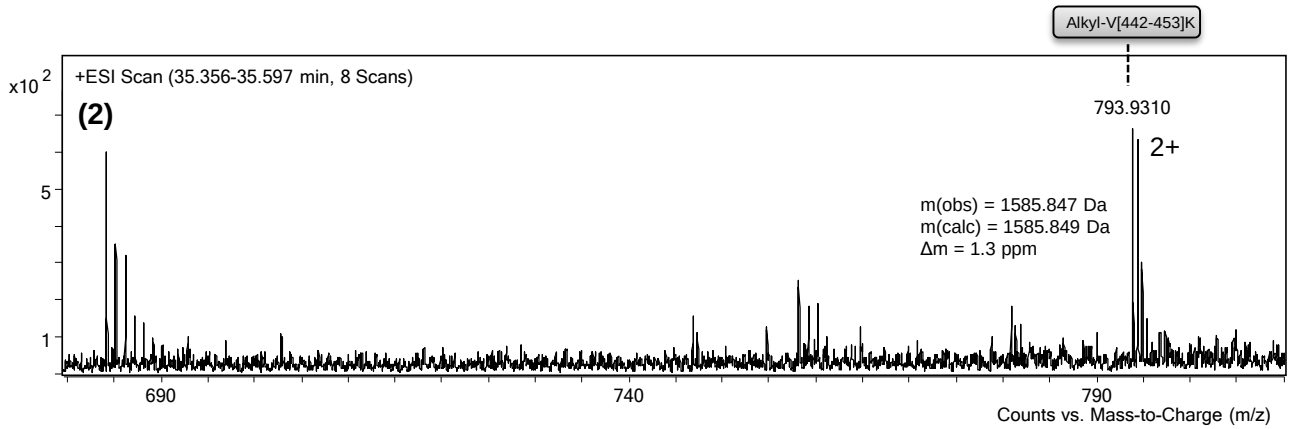


Figure S8. Alkylation analysis of wild-type TEAS with substrate analogues and with (*E,E*)-FPP at increased reaction temperatures. (**G, continued**) LC-MS analysis of nonalkylated and alkylated V[442-453]K and I[515-532]K of wild-type TEAS after reaction with (*E,E*)-FPP at 25°C, at 37°C and at 42°C. (**H**) MS spectrum of alkylated V[442-453]K of wild-type TEAS (**2**) after reaction with (*E,E*)-FPP at 42°C reaction temperature. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides.

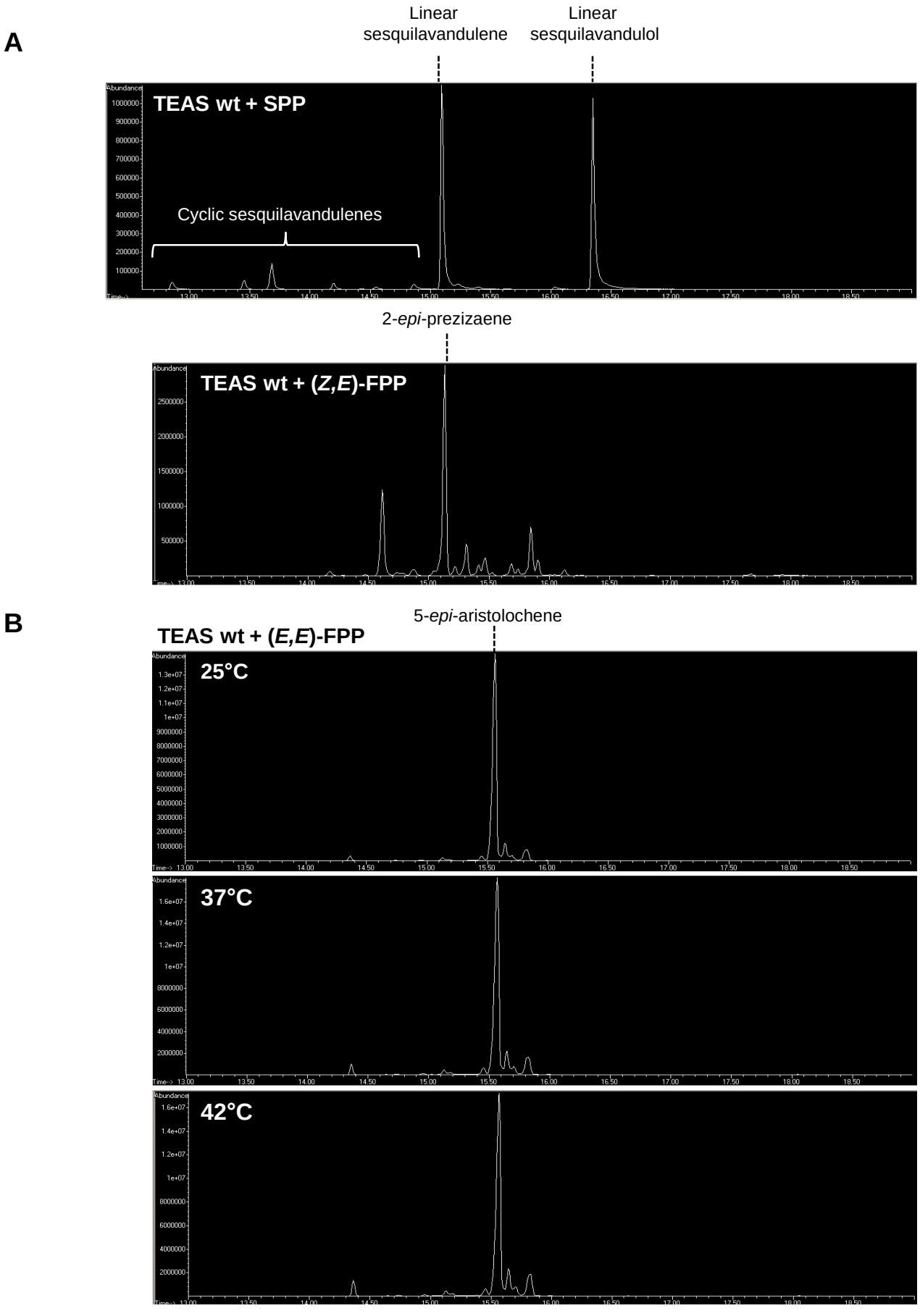


Figure S9. GC-MS analysis of wild-type TEAS activity depending on substrate or reaction temperature. (**A**) Wild-type TEAS product profile after reaction with substrate analogues sesquilavandulyl diphosphate (SPP) and (*Z*,*E*)-FPP for 12 h at 25°C. (**B**) Wild-type TEAS product profile after reaction with (*E*,*E*)-FPP for 12 h at 25°C, 37°C and 42°C.

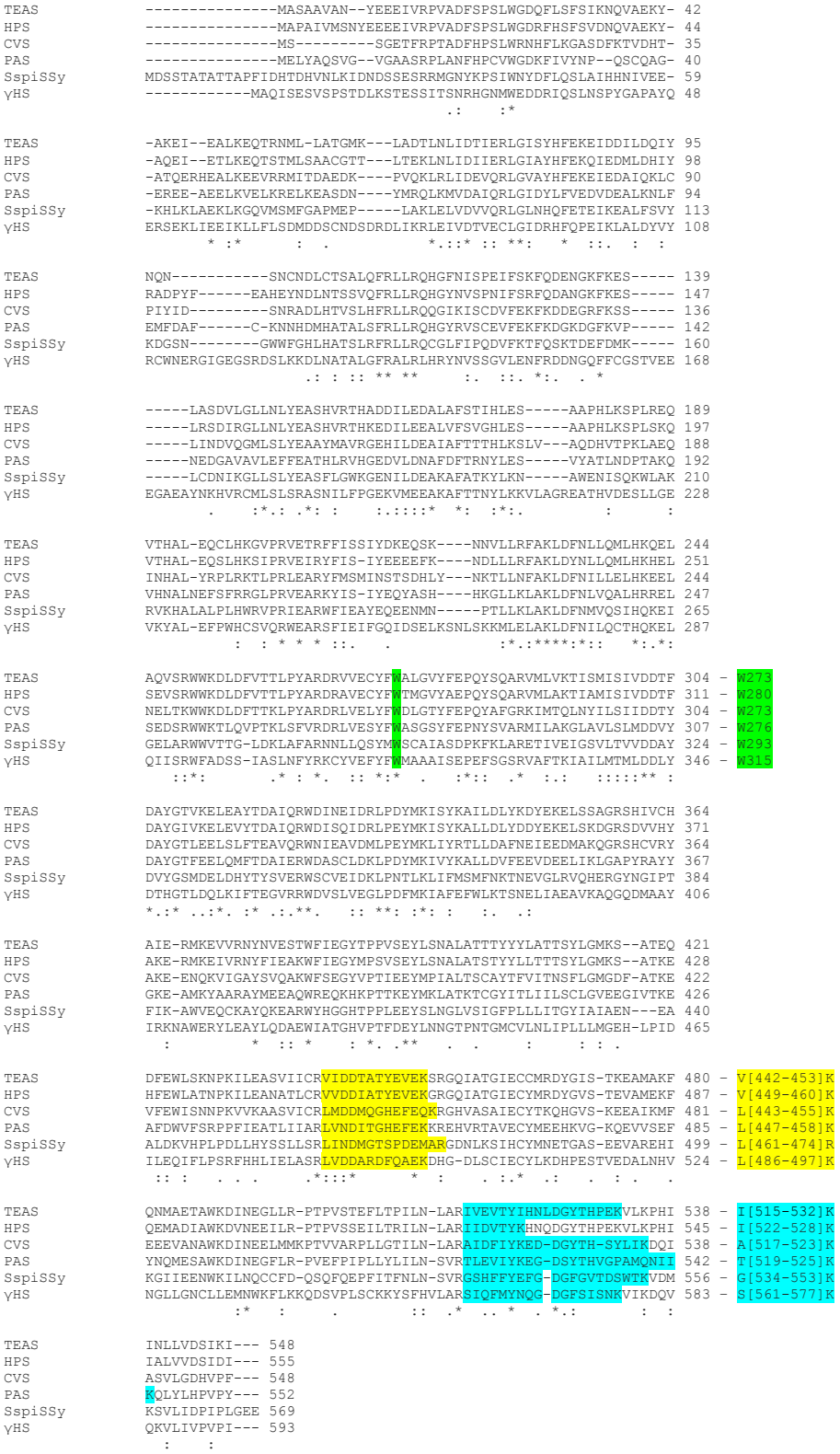


Figure S10. Multiple sequence alignment of sesquiterpene synthases investigated for self-alkylation. The active site tryptophan of TEAS and corresponding homologous tryptophan residues of other synthases are highlighted in green. Tryptic peptides of alkylation sites in TEAS and corresponding homologous peptides in other synthases are highlighted in yellow and blue. Abbreviations: TEAS – tobacco 5-*epi*-aristolochene synthase, HPS – *Hyoscyamus muticus* premnaspirodiene synthase, CVS – citrus valencene synthase, PAS – patchoulol synthase, SspiSSy – *Santalum spicatum* santalene synthase, γHS – γ-humulene synthase.

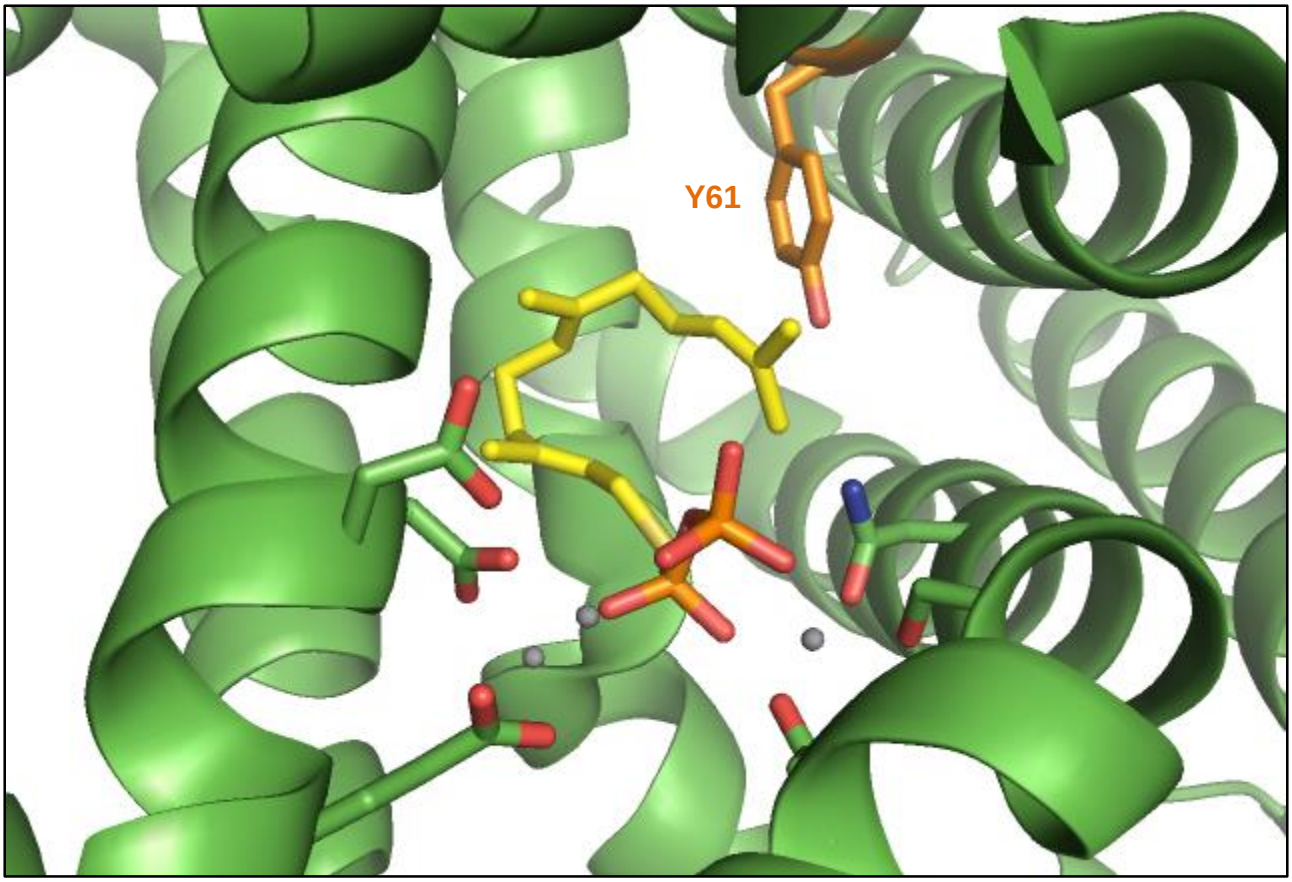


Figure S11. Active site model of *Aspergillus terreus* aristolochene synthase (ATAS, PDB: 4KUX) with substrate mimic farnesyl thiolodiphosphate (yellow). The residue Y61 (orange) was mutated for alkylation analysis due to its similar active site position as tryptophan 273 in tobacco 5-*epi*-aristolochene synthase (Figure 1A). Mg^{2+} cofactors are shown as grey spheres.

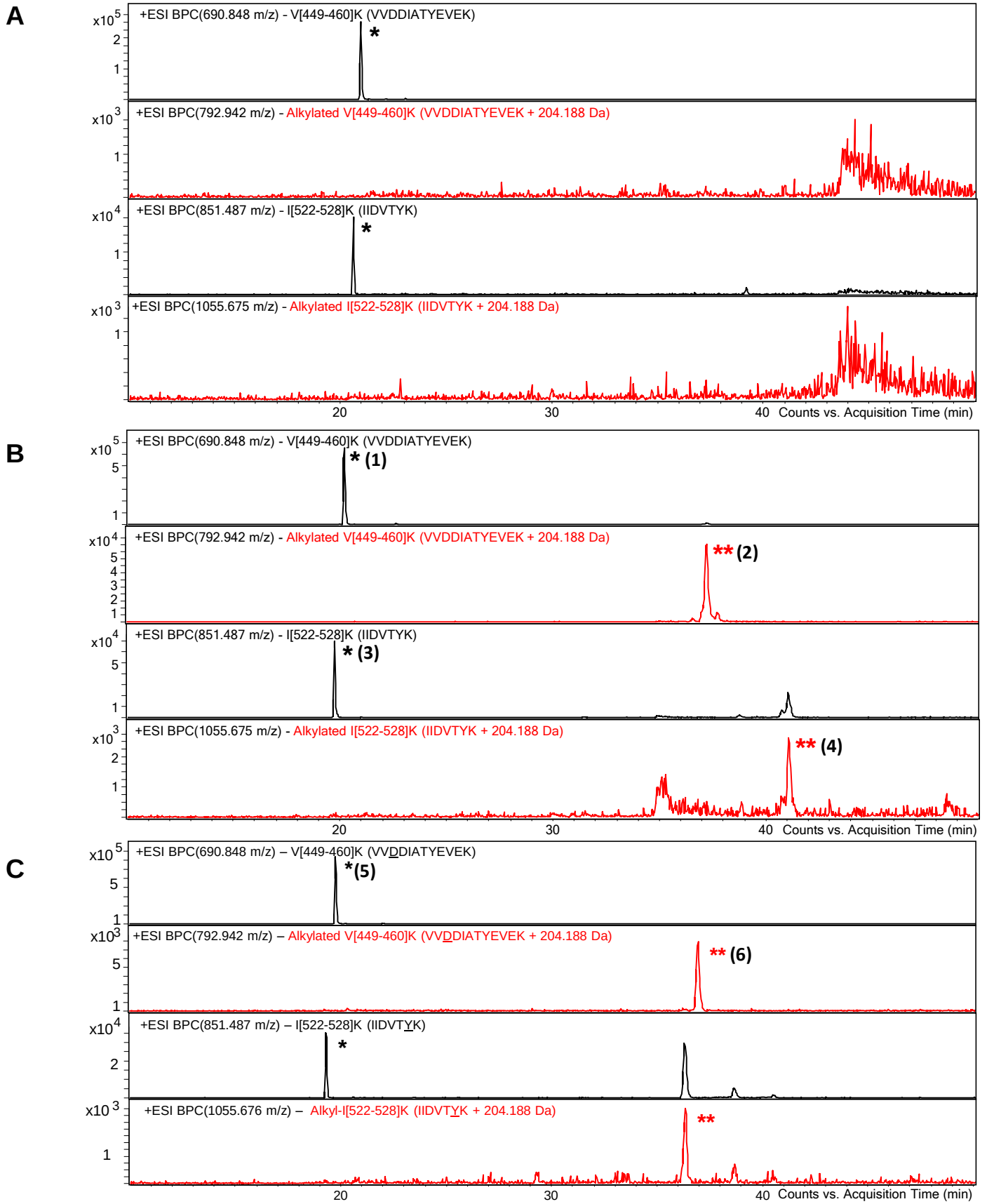
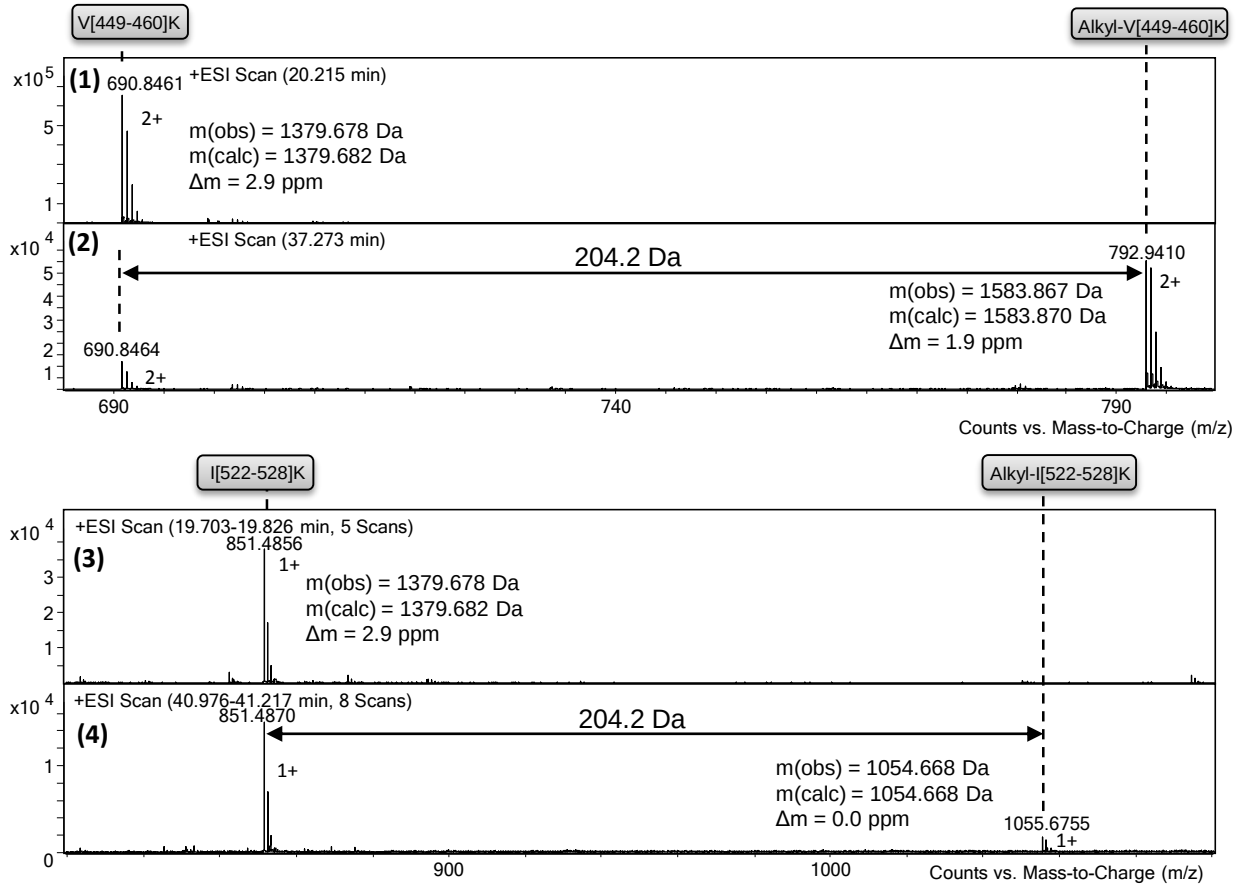
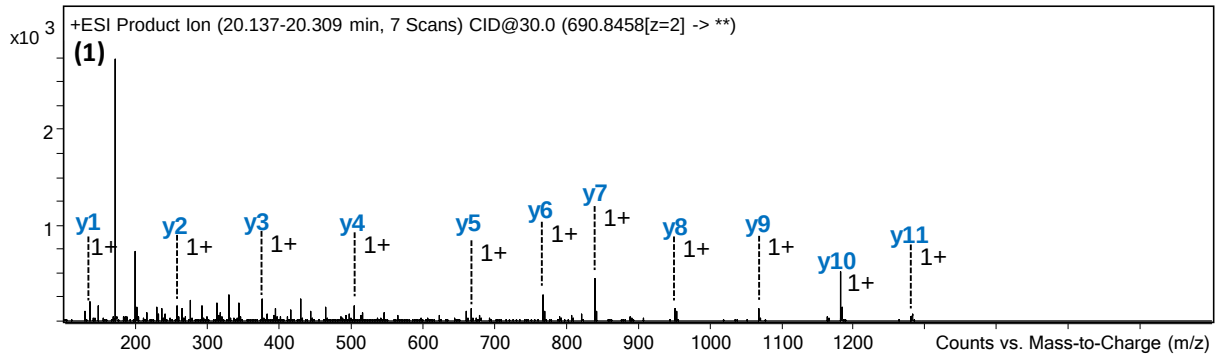


Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**A**) LC-MS analysis of nonalkylated and alkylated V[449-460]K and I[522-528]K of wild-type HPS after reaction with (*E*,*E*)-FPP. (**B**) LC-MS analysis of nonalkylated and alkylated V[449-460]K and I[522-528]K of wild-type HPS after reaction with SPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. (**C**) LC-MS analysis of nonalkylated and alkylated V[449-460]K and I[522-528]K of HPS W280E after reaction with (*E*,*E*)-FPP.

D



E

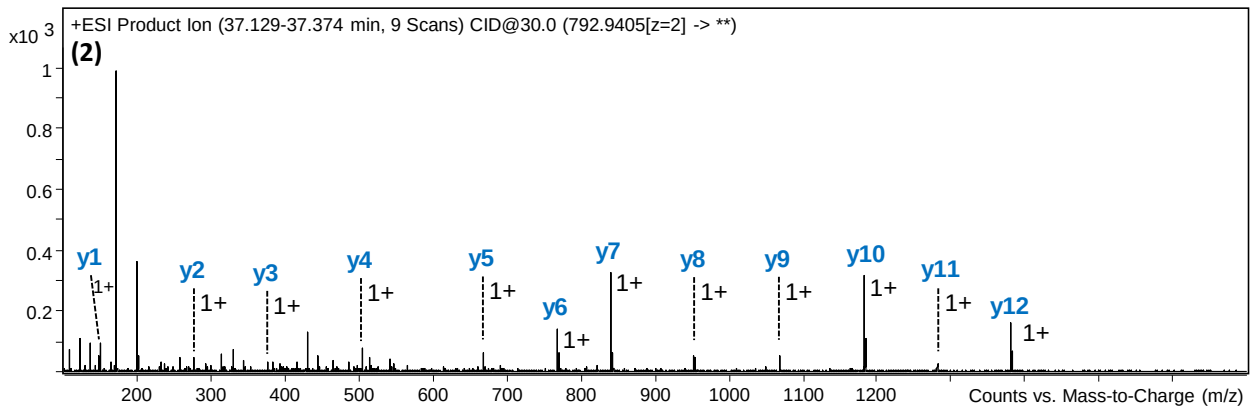


Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y11	1281.618	1281.622	-0.004	3.1
y10	1182.547	1182.533	0.014	11.8
y9	1067.515	1067.526	-0.011	10.3
y8	952.493	952.499	-0.006	6.3
y7	839.412	839.415	-0.003	3.6
y6	768.371	768.378	-0.007	9.1

Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y5	667.327	667.330	-0.003	4.5
y4	504.263	504.267	-0.004	7.9
y3	375.222	375.224	-0.002	5.3
y2	276.155	276.156	-0.001	3.6
y1	147.111	147.113	-0.002	13.6

Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**D**) MS analysis of nonalkylated (**1**) and alkylated (**2**) V[449-460]K and nonalkylated (**3**) and alkylated (**4**) I[522-528]K of wild-type HPS after reaction with SPP. (**E**) MS/MS analysis of nonalkylated V[449-460]K (**1**) of wild-type HPS after reaction with SPP.

F



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y12	1380.684	1380.690	-0.006	4.4	y6	768.372	768.378	-0.006	7.8
y11	1281.618	1281.622	-0.004	3.1	y5	667.326	667.330	-0.004	6.0
y10	1182.548	1182.533	0.015	12.7	y4	504.266	504.267	-0.001	2.0
y9	1067.537	1067.526	0.011	10.3	y3	375.229	375.224	0.005	13.3
y8	952.494	952.499	-0.005	5.3	y2	276.153	276.156	-0.003	10.9
y7	839.414	839.415	-0.001	1.2	y1	147.112	147.113	-0.001	6.8

G

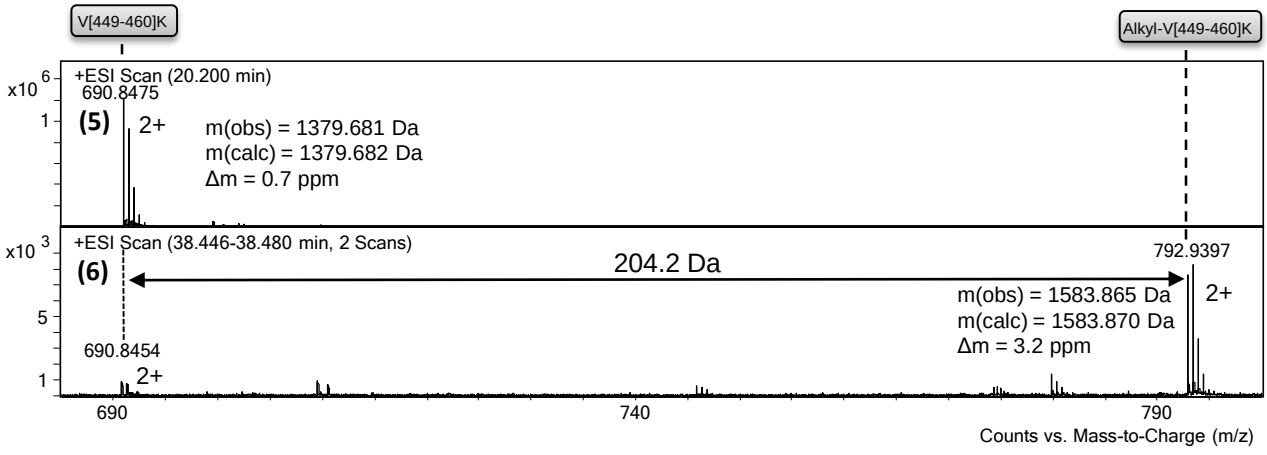
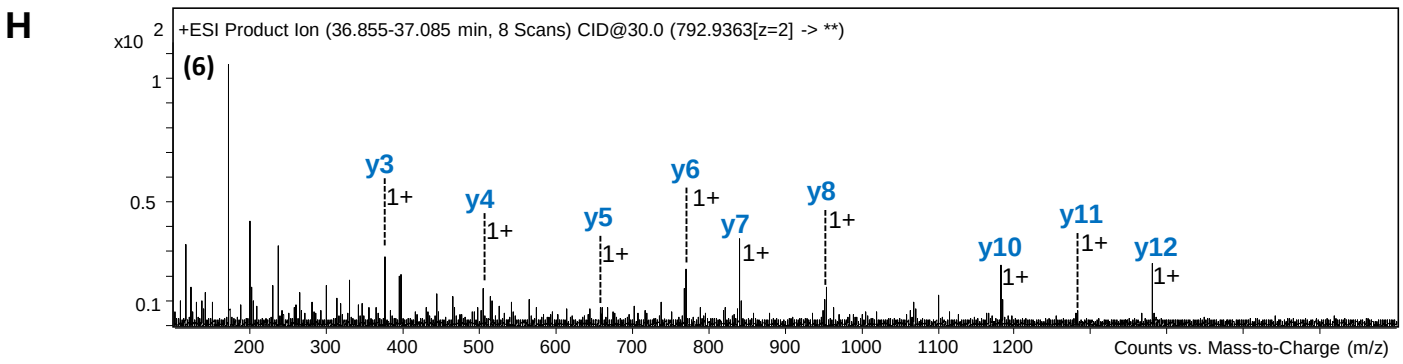
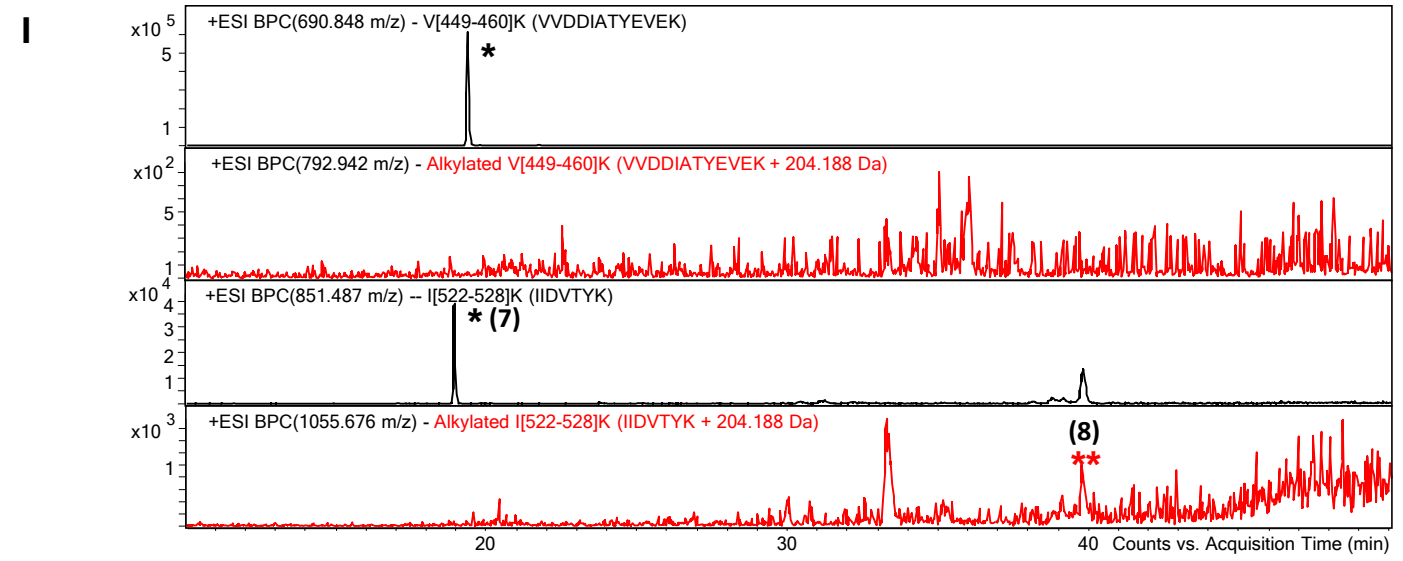


Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**F**) MS/MS analysis of alkylated V[449-460]K (**2**) of wild-type HPS after reaction with SPP. (**G**) MS analysis of nonalkylated (**5**) and alkylated (**6**) V[449-460]K and nonalkylated and alkylated I[522-528]K (see Figure S6) of HPS W280E after reaction with (*E,E*)-FPP.



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y12	1380.709	1380.690	-0.019	13.8
y11	1281.589	1281.622	0.033	25.8
y10	1182.536	1182.533	-0.003	2.5
y8	952.515	952.499	-0.016	16.8
y7	839.395	839.415	0.020	23.8

Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y6	768.375	768.378	0.003	3.9
y5	667.326	667.330	0.004	6.0
y4	504.267	504.267	0.000	0.0
y3	375.218	375.224	0.006	16.0



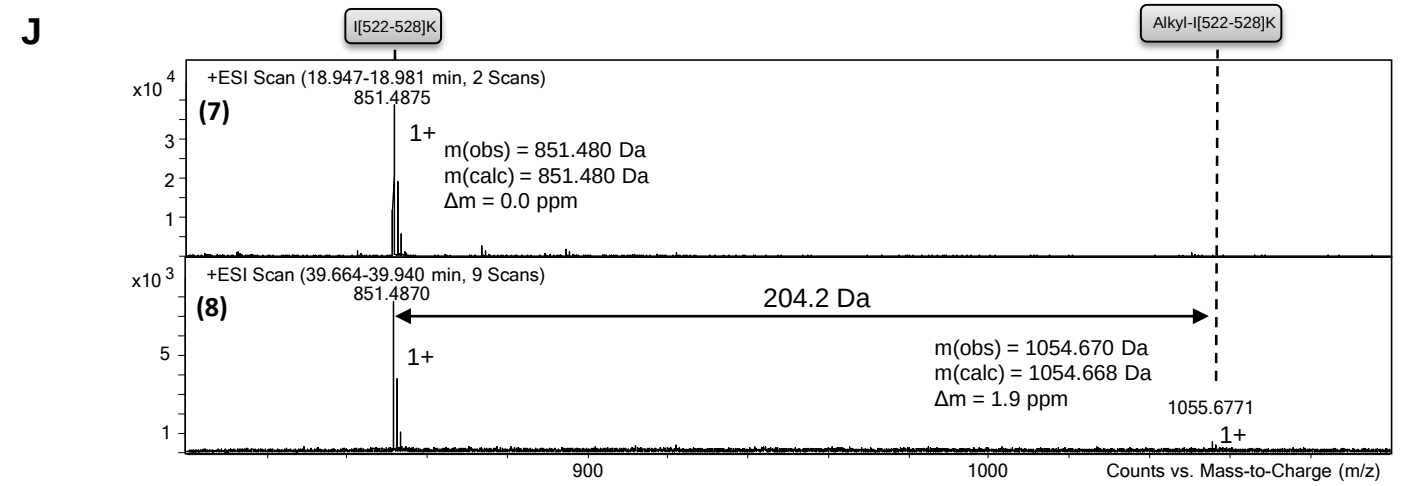


Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**H**) MS/MS analysis of alkylated V[449-460]K (**6**) of HPS W280E after reaction with FPP. (**I**) LC-MS analysis of nonalkylated and alkylated V[449-460]K and I[522-528]K of wild-type HPS after reaction with (*Z,E*)-FPP. (**J**) MS analysis of nonalkylated (**7**) and alkylated (**8**) V[449-460]K of wild-type HPS after reaction with (*Z,E*)-FPP.

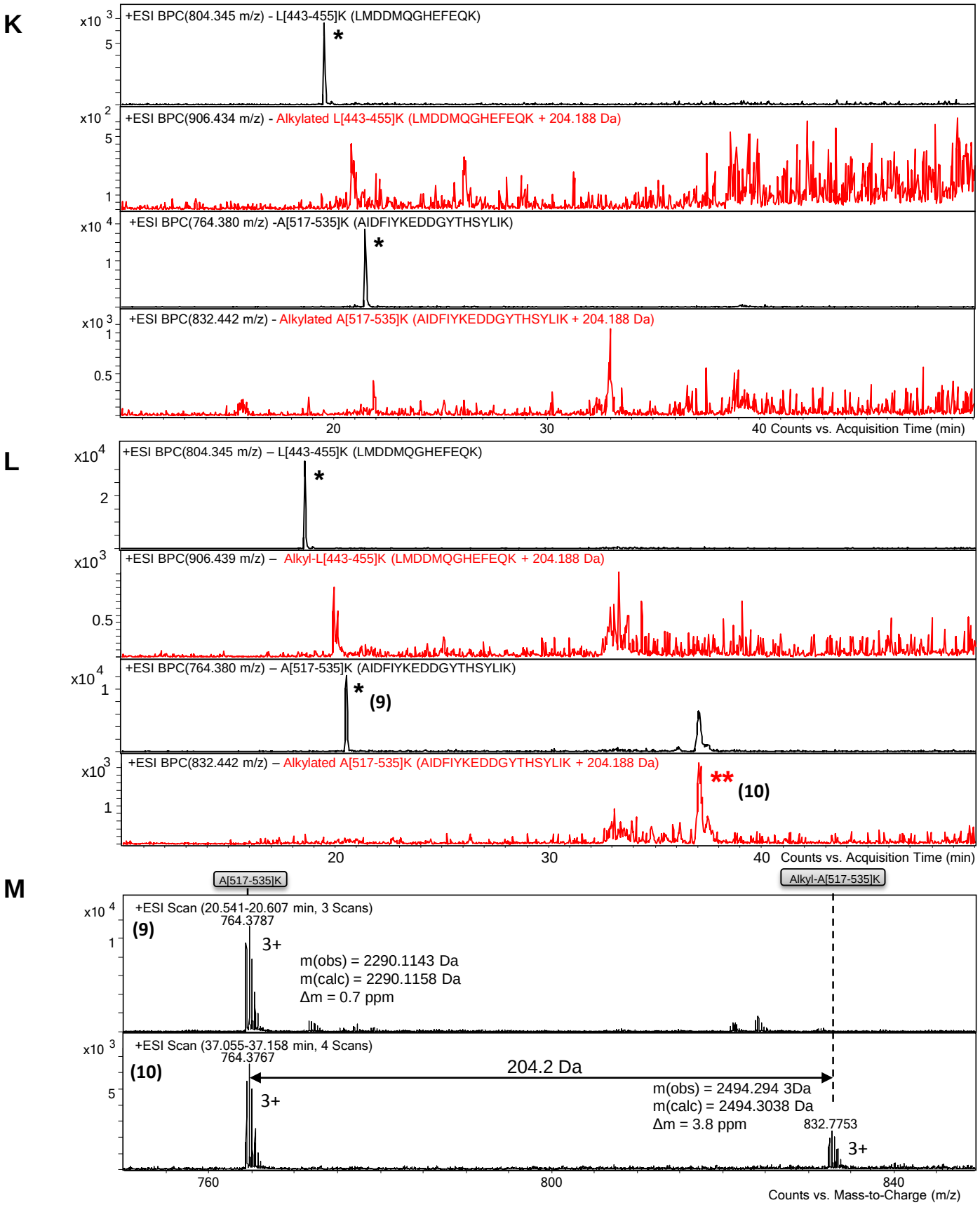
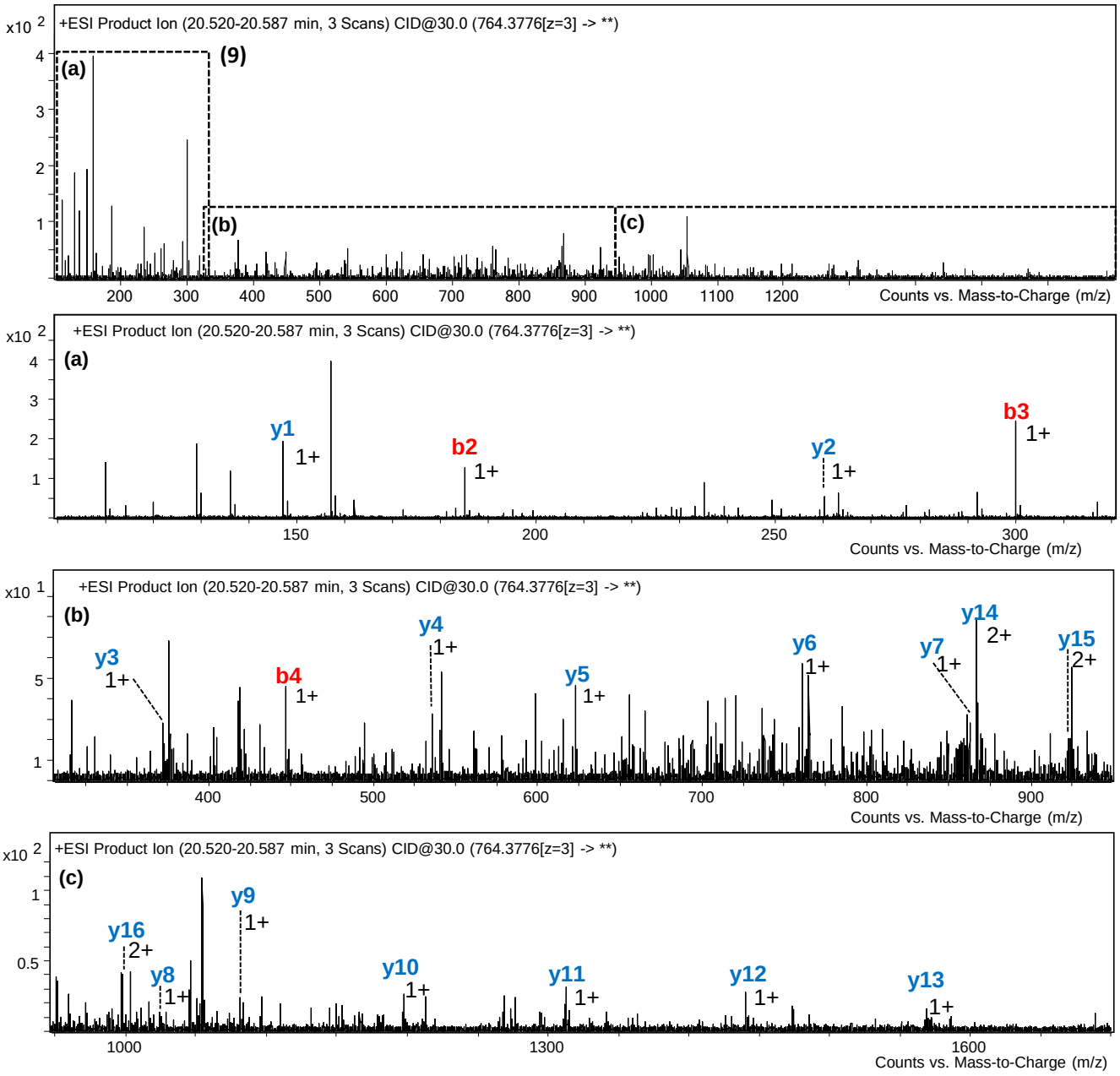


Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**K**) LC-MS analysis of nonalkylated and alkylated L[443-455]K and A[517-535]K of wild-type CVS after reaction with (*E*,*E*)-FPP. (**L**) LC-MS analysis of nonalkylated and alkylated L[443-455]K and A[517-535]K of CVS W273E after reaction with (*E*,*E*)-FPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. (**M**) MS analysis of nonalkylated (**9**) and alkylated (**10**) A[517-535]K of CVS W273E after reaction with (*E*,*E*)-FPP.

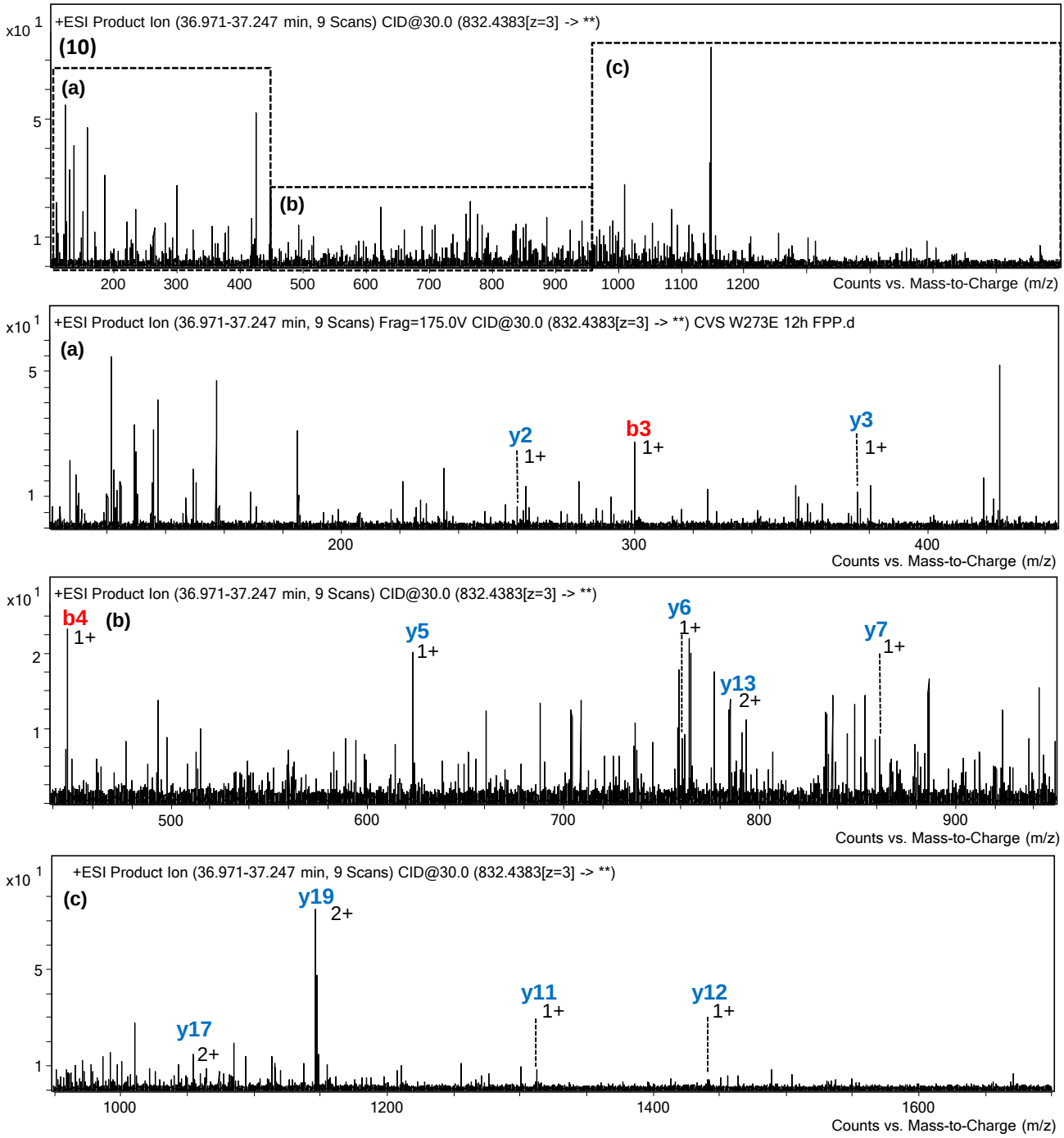
N



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y16	1992.004	1991.976	-0.028	14.1	y7	861.482	861.484	0.002	2.3
y15	1844.897	1844.907	0.010	5.4	y6	760.425	760.436	0.011	14.5
y14	1731.799	1731.823	0.024	13.9	y5	623.371	623.377	0.006	9.6
y13	1568.760	1568.760	0.000	0.0	y4	536.340	536.345	0.005	9.3
y12	1440.650	1440.665	0.015	10.4	b4	447.224	447.224	0.000	0.0
y11	1311.622	1311.608	-0.014	10.7	y3	373.278	373.282	0.004	10.7
y10	1196.602	1196.595	-0.007	5.9	b3	300.157	300.156	0.001	3.3
y9	1081.571	1081.568	-0.003	2.8	y2	260.201	260.198	-0.003	11.5
y8	1024.557	1024.549	-0.008	7.8	b2	185.129	185.129	0.000	0.0
					y1	147.113	147.112	-0.001	6.8

Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**N**) MS/MS analysis of nonalkylated (**9**) A[517-535]K of CVS W273E after reaction with (*E,E*)-FPP.

O



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y19	2291.089	2291.124	0.035	15.3	y6	760.431	760.436	0.005	6.6
y17	2107.019	2107.003	-0.016	7.6	y5	623.366	623.377	0.011	17.6
y13	1568.778	1568.760	-0.018	11.5	b4	447.223	447.224	0.001	2.2
y12	1440.674	1440.665	-0.009	6.3	y3	373.290	373.282	-0.008	21.4
y11	1311.648	1311.622	-0.026	19.8	b3	300.150	300.156	0.006	20
y7	861.465	861.484	0.019	22.1	y2	260.195	260.198	0.003	11.5

Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**O**) MS/MS analysis of alkylated A[517-535]K (**10**) of CVS W273E after reaction with (*E,E*)-FPP.

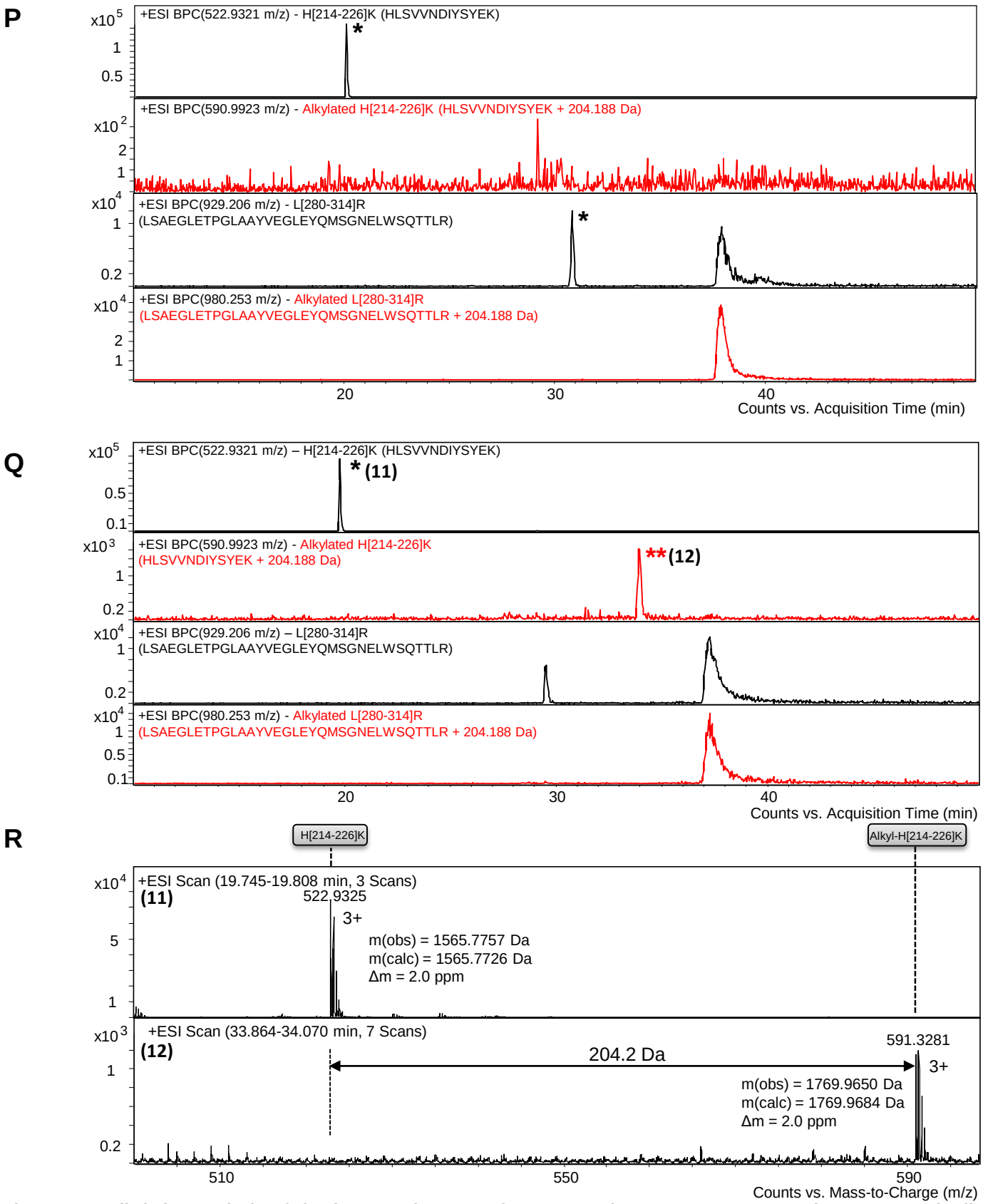
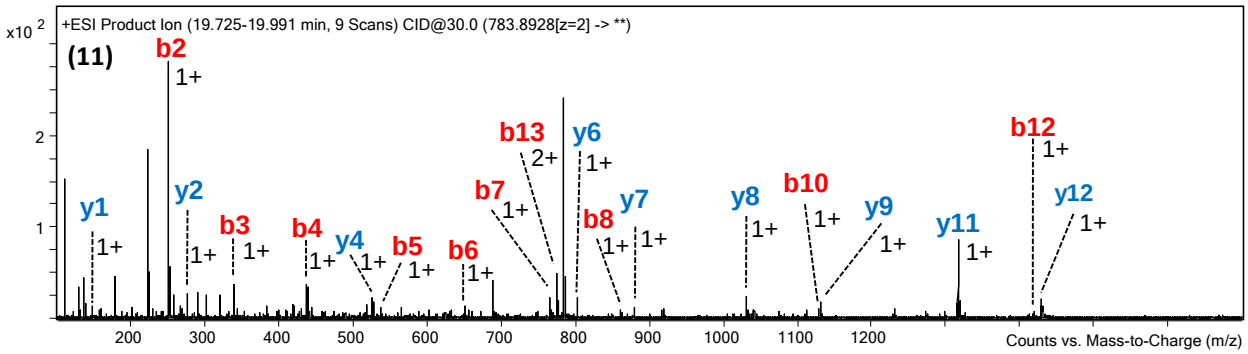


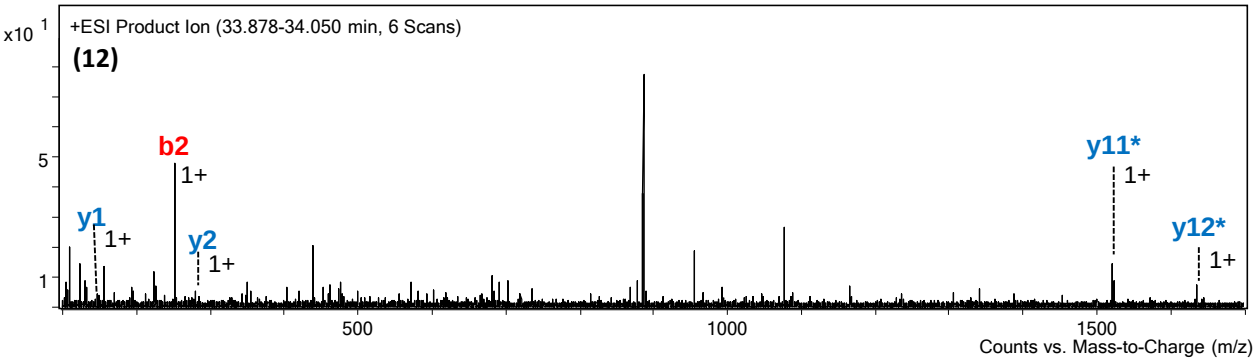
Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**P**) LC-MS analysis of nonalkylated and alkylated tryptic peptides H[214-226]K and L[280-314]R of wild-type ATAS after reaction with (*E,E*)-FPP. (**Q**) LC-MS analysis of nonalkylated and alkylated tryptic peptides H[214-226]K and L[280-314]R of wild-type ATAS after reaction with (*E,E*)-FPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. (**R**) MS analysis of nonalkylated (**11**) and alkylated (**12**) tryptic peptides H[214-226]K of ATAS Y61C after reaction with (*E,E*)-FPP.

S



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
b13	1548.750	1548.770	0.020	12.8	y6	802.3944	802.39874	0.004	5.4
y12	1429.710	1429.722	0.012	8.1	b7	765.3857	765.38957	0.004	5.1
b12	1420.662	1420.675	0.013	9.4	b6	650.3566	650.36263	0.00603	9.3
y11	1316.630	1316.637	0.008	5.7	b5	536.3247	536.3197	-0.005	9.3
y9	1130.530	1130.53702	0.008	6.7	y4	526.2505	526.25135	0.001	1.6
b10	1128.568	1128.56899	0.00099	0.9	b4	437.2513	437.25129	0.000	0
y8	1031.456	1031.469	0.013	12.7	b3	338.1787	338.18287	0.004	12.3
y7	917.4259	917.426	0.000	0.2	y2	276.1523	276.15599	0.004	13.4
b8	878.4812	878.474	-0.0076	8.6	b2	251.1487	251.15085	0.002	8.6
					y1	147.1151	147.1134	-0.0017	11.6

T



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y12*	1633.9395	1633.9095	-0.030	18.3
y11*	1520.8046	1520.8255	0.021	13.7
y2	276.1531	276.1560	0.003	10.5
b2	251.1491	251.1509	0.002	7.0
y1	147.1144	147.1134	-0.001	6.8

Figure S12. Alkylation analysis of dominant-product sesquiterpene synthases *Hyoscyamus muticus* premnaspirodiene synthase (HPS), citrus valencene synthase (CVS) and *Aspergillus terreus* aristolochene synthase (ATAS). (**S**) MS/MS analysis of nonalkylated tryptic peptide H[214-226]K (**11**) of ATAS Y61C after reaction with (*E*,*E*)-FPP. (**T**) MS/MS analysis of alkylated tryptic peptide H[214-226]K (**12**) of ATAS Y61C after reaction with (*E*,*E*)-FPP.

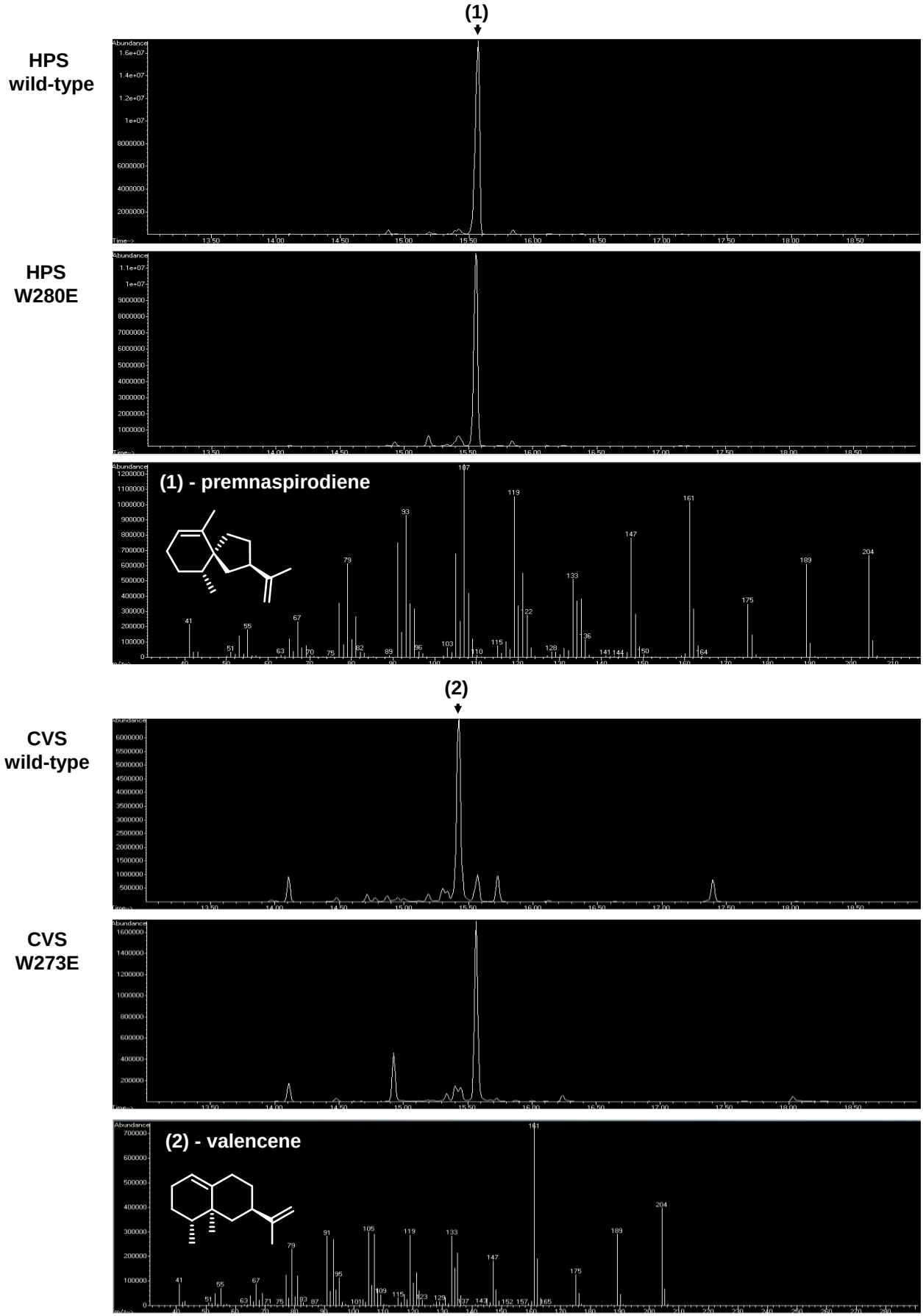


Figure S13. GC-MS analysis of product profiles of wild-type and active site mutants of sesquiterpene synthases after reaction with (*E,E*)-FPP. Total ion chromatograms of hexane extracts of corresponding enzyme reactions and GC-MS spectra of main products are shown.

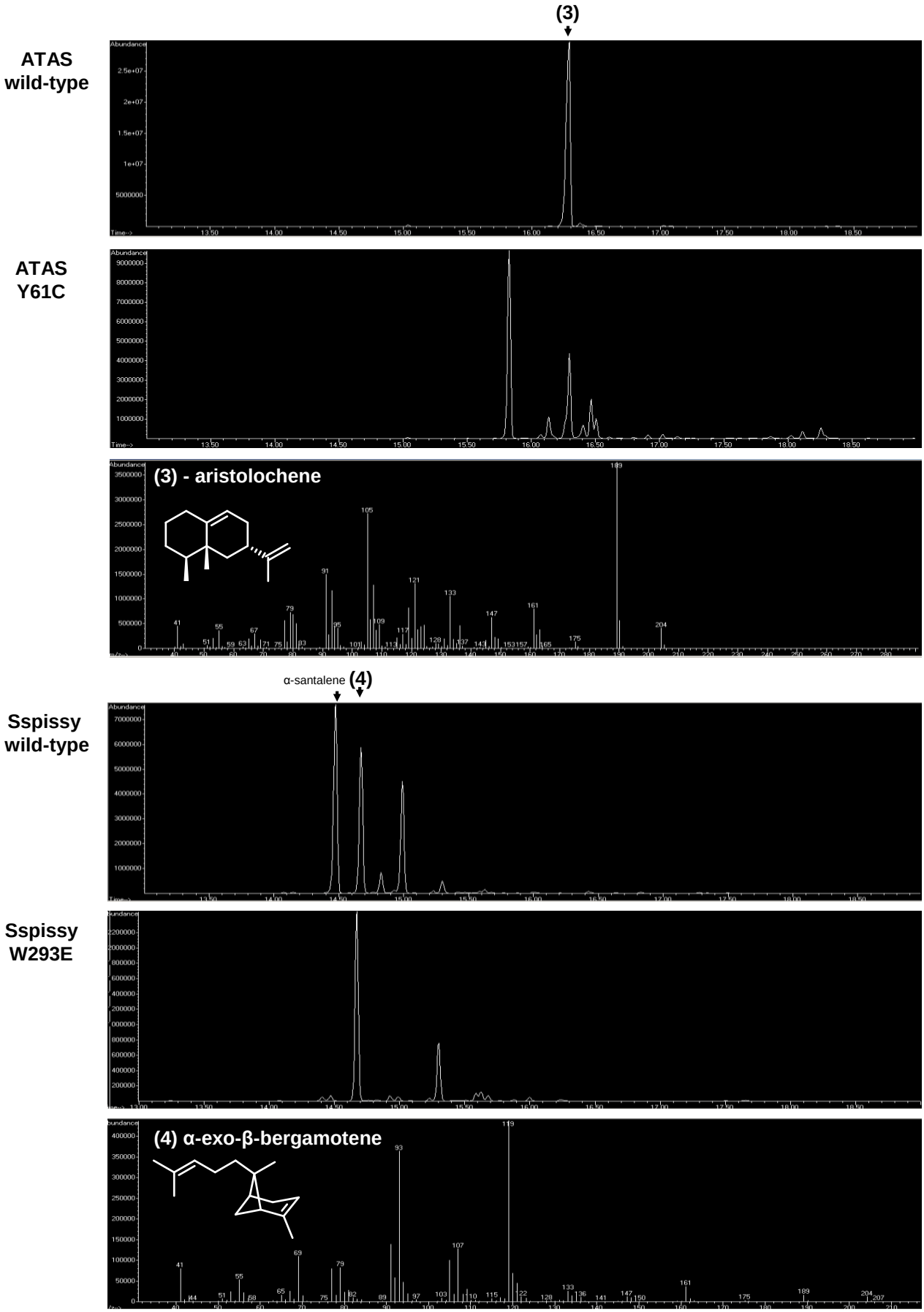


Figure S13. GC-MS analysis of product profiles of wild-type and active site mutants of sesquiterpene synthases after reaction with (*E,E*)-FPP. Total ion chromatograms of hexane extracts of corresponding enzyme reactions and GC-MS spectra of main products are shown.

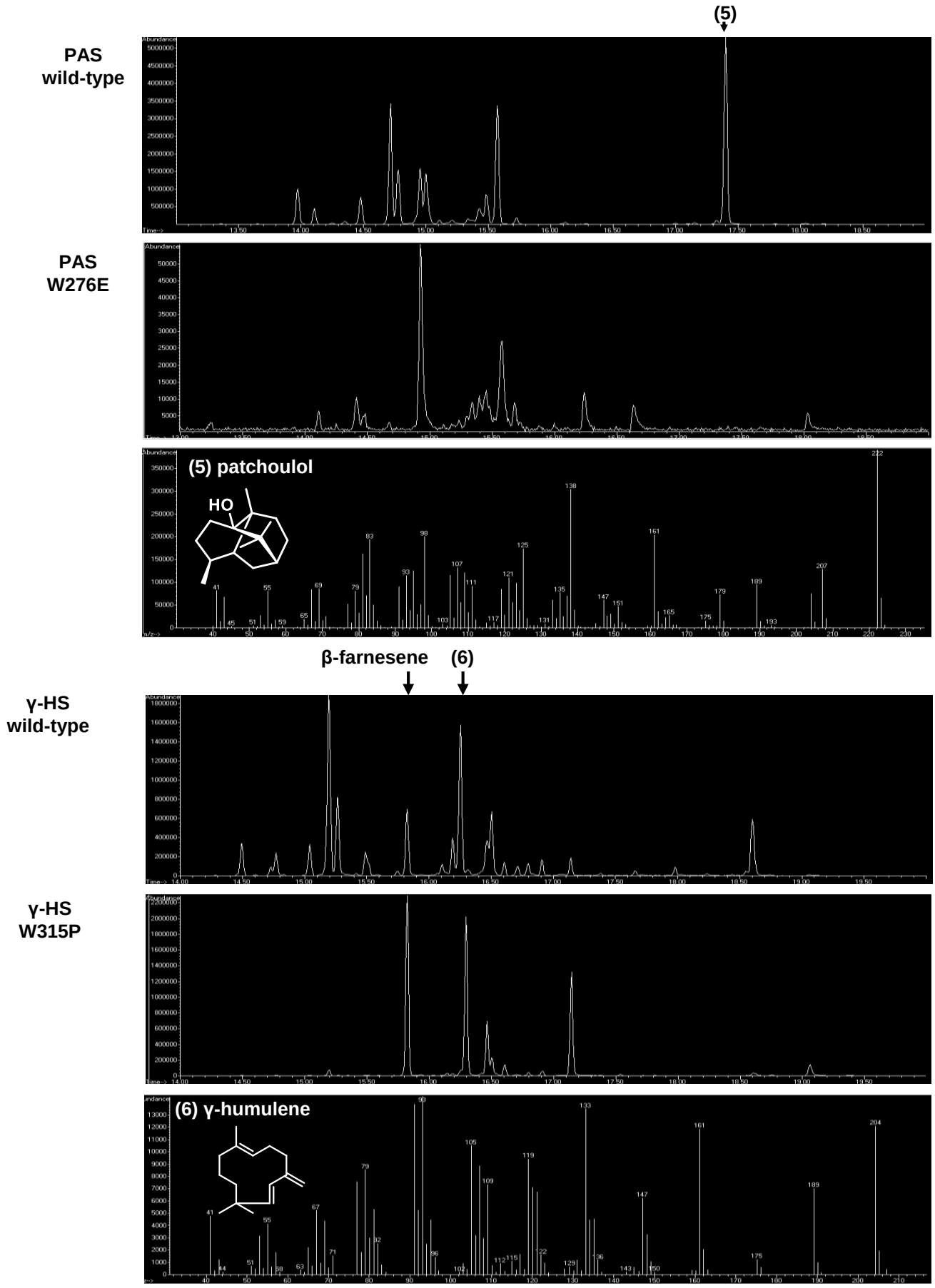


Figure S13. GC-MS analysis of product profiles of wild-type and active site mutants of sesquiterpene synthases after reaction with (*E,E*)-FPP. Total ion chromatograms of hexane extracts of corresponding enzyme reactions and GC-MS spectra of main products are shown.

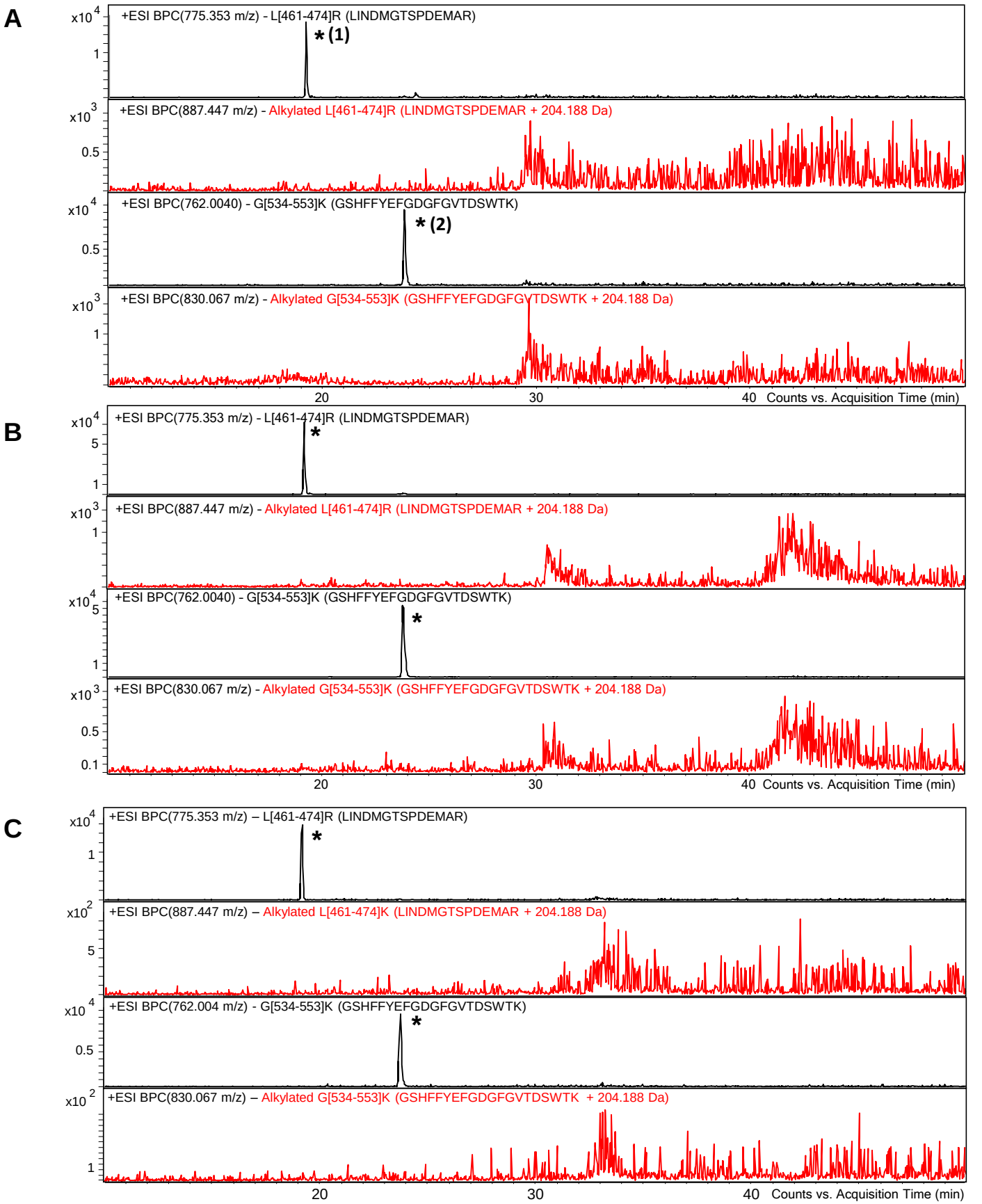
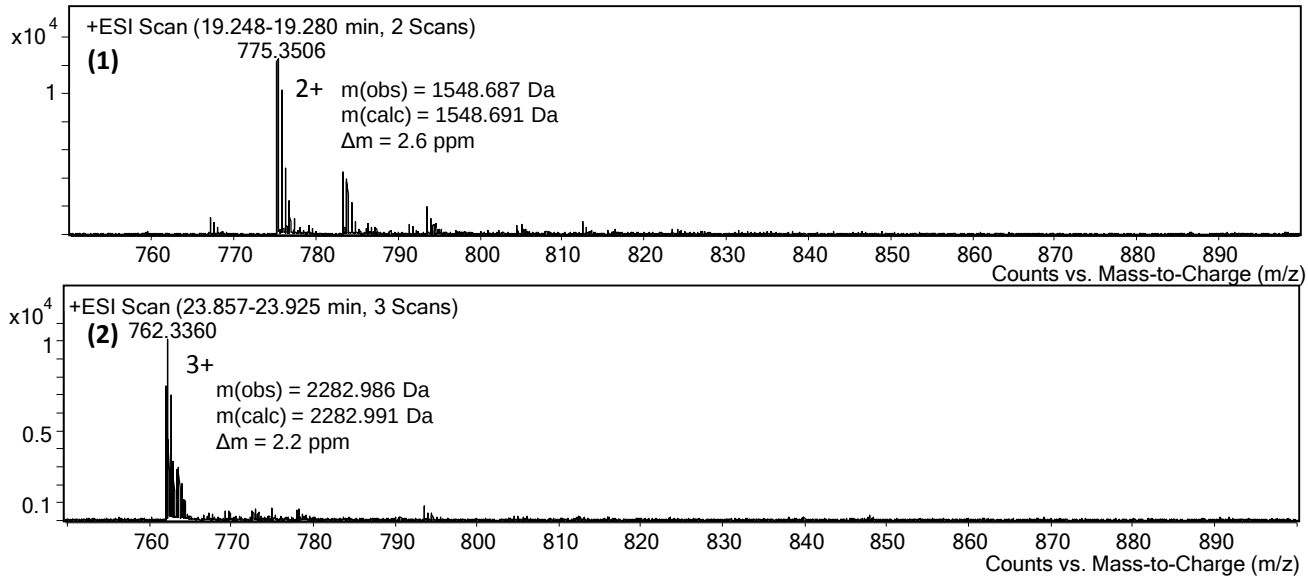
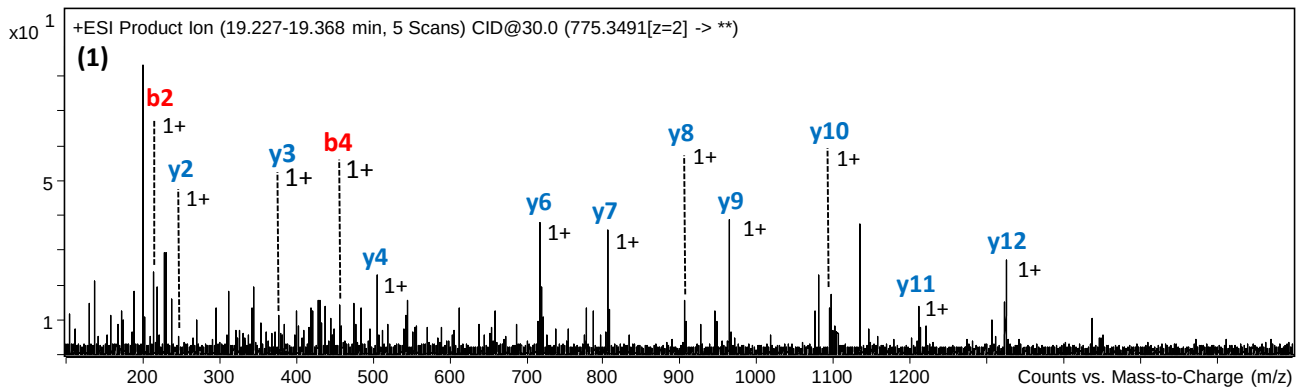


Figure S14. Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS). (**A**) LC-MS analysis of nonalkylated and alkylated L[461-474]R and G[534-553]K of wild-type SspiSSy after reaction with (*E,E*)-FPP. (**B**) LC-MS analysis of nonalkylated and alkylated L[461-474]R and G[534-553]K of wild-type SspiSSy after reaction with SPP. (**C**) LC-MS analysis of nonalkylated and alkylated L[461-474]R and G[534-553]K of SspiSSy W293E after reaction with (*E,E*)-PP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides.

D

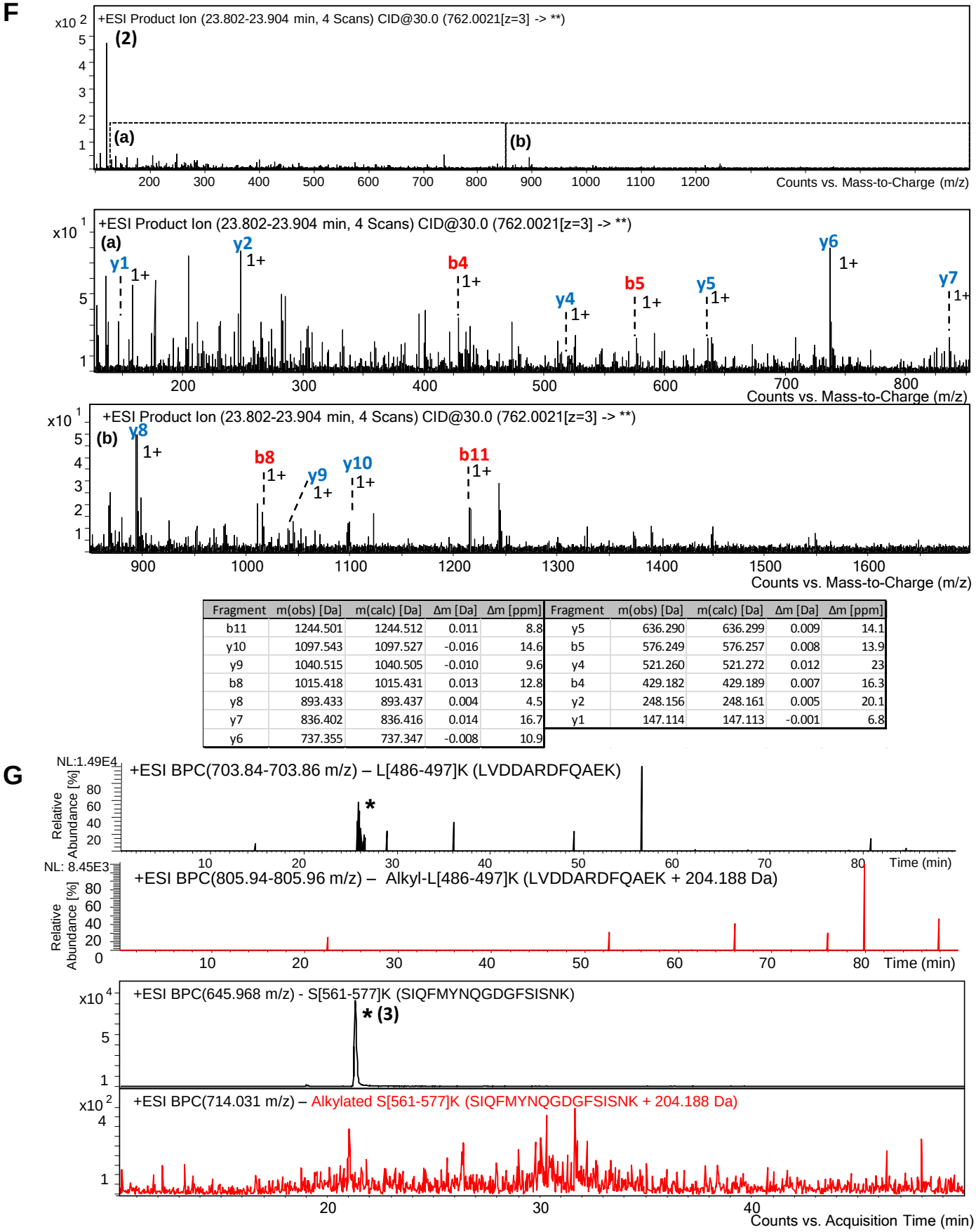


E



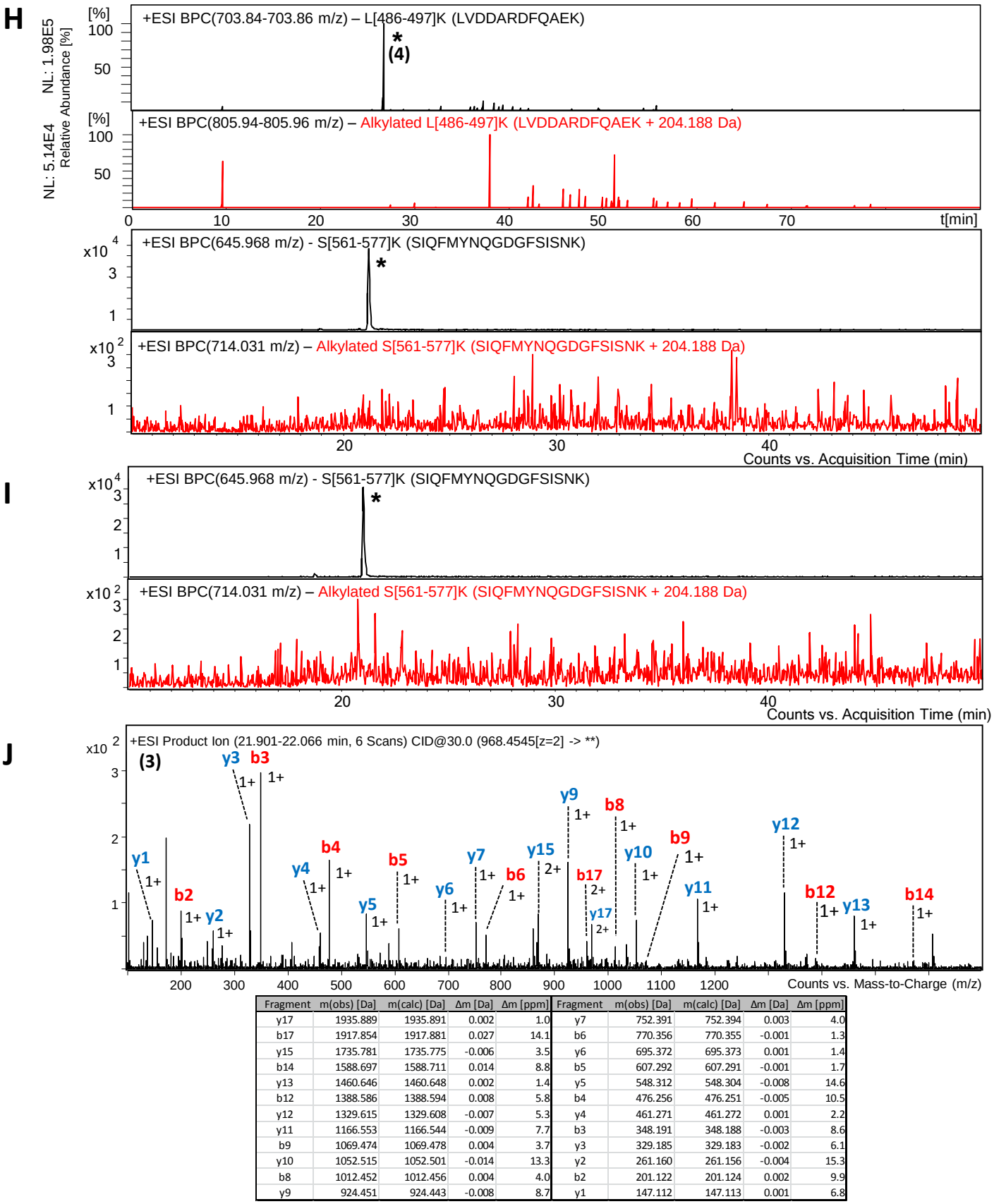
Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y12	1323.520	1323.531	0.011	8.3	y6	718.312	718.319	0.007	9.8
y11	1209.473	1209.488	0.015	12.4	y4	506.239	506.240	0.001	2.0
y10	1094.453	1094.461	0.008	7.3	y3	377.202	377.197	-0.005	13.3
y9	963.409	963.421	0.012	12.5	y2	246.157	246.157	0.000	0.0
y8	906.410	906.399	-0.011	12.1	b2	227.174	227.176	0.002	8.8
y7	805.348	805.352	0.004	5.0					

Figure S14. Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS). (**D**) MS analysis of nonalkylated L[461-474]R (**1**) and G[534-553]K (**2**) of wild-type SspiSSy after reaction with (*E*,*E*)-FPP. (**E**) MS/MS analysis of nonalkylated L[461-474]R (**1**) of wild-type SspiSSy after reaction with (*E*,*E*)-FPP.



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
b11	1244.501	1244.512	0.011	8.8	y5	636.290	636.299	0.009	14.1
y10	1097.543	1097.527	-0.016	14.6	b5	576.249	576.257	0.008	13.9
y9	1040.515	1040.505	-0.010	9.6	y4	521.260	521.272	0.012	23
b8	1015.418	1015.431	0.013	12.8	b4	429.182	429.189	0.007	16.3
y8	893.433	893.437	0.004	4.5	y2	248.156	248.161	0.005	20.1
y7	836.402	836.416	0.014	16.7	y1	147.114	147.113	-0.001	6.8
y6	737.355	737.347	-0.008	10.9					

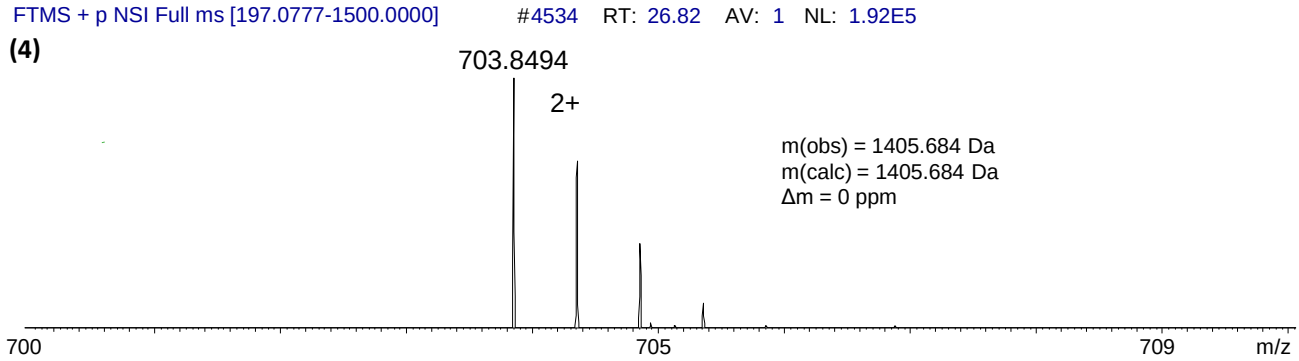
Figure S14. Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS). (**F**) MS/MS analysis of nonalkylated G[534-553]K (**2**) of wild-type SspiSSy after reaction with (*E*,*E*)-FPP. (**G**) LC-MS analysis of nonalkylated and alkylated L[486-497]K and S[561-577]K of wild-type γHS after reaction with (*E*,*E*)-FPP. Single asteriks mark BPC peaks of non-alkylated peptides, double asteriks mark BPC peaks of alkylated peptides.



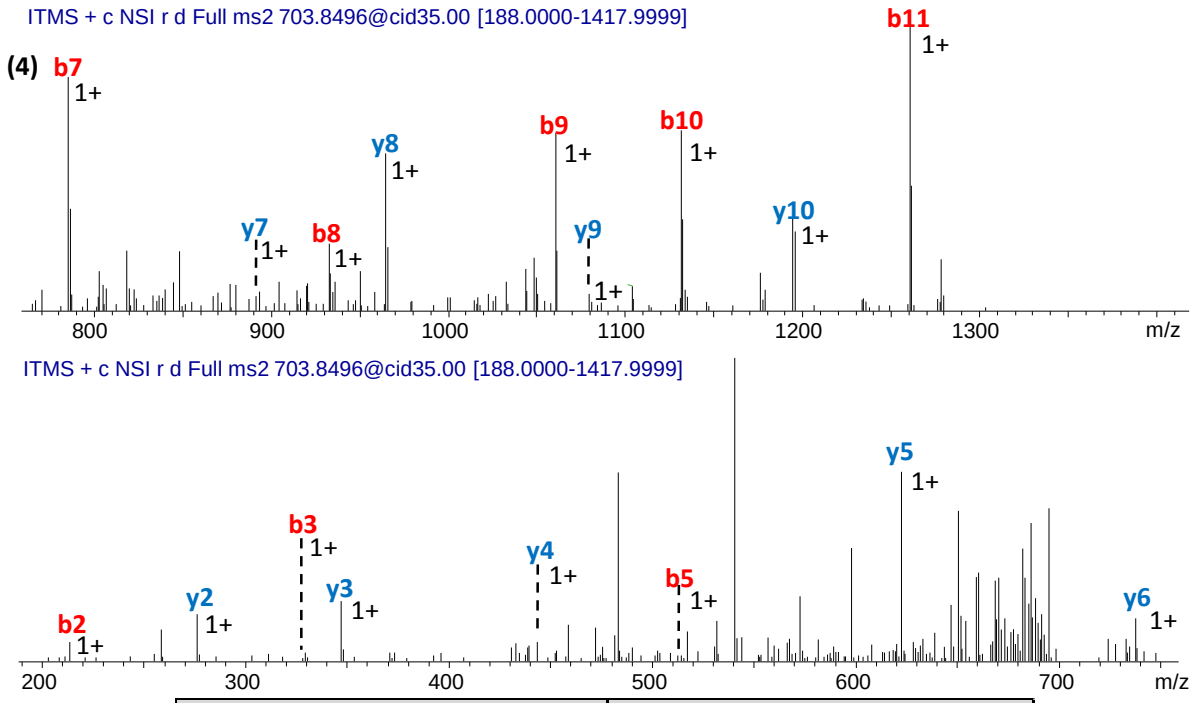
Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y17	1935.889	1935.891	0.002	1.0	y7	752.391	752.394	0.003	4.0
b17	1917.854	1917.881	0.027	14.1	b6	770.356	770.355	-0.001	1.3
y15	1735.781	1735.775	-0.006	3.5	y6	695.372	695.373	0.001	1.4
b14	1588.697	1588.711	0.014	8.8	b5	607.292	607.291	-0.001	1.7
y13	1460.646	1460.648	0.002	1.4	y5	548.312	548.304	-0.008	14.6
b12	1388.586	1388.594	0.008	5.8	b4	476.256	476.251	-0.005	10.5
y12	1329.615	1329.608	-0.007	5.3	y4	461.271	461.272	0.001	2.2
y11	1166.553	1166.544	-0.009	7.7	b3	348.191	348.188	-0.003	8.6
b9	1069.474	1069.478	0.004	3.7	y3	329.185	329.183	-0.002	6.1
y10	1052.515	1052.501	-0.014	13.3	y2	261.160	261.156	-0.004	15.3
b8	1012.452	1012.456	0.004	4.0	b2	201.122	201.124	0.002	9.9
y9	924.451	924.443	-0.008	8.7	y1	147.112	147.113	0.001	6.8

Figure S14. Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS). (**H**) LC-MS analysis of nonalkylated and alkylated L[486-497]K and S[561-577]K of γHS W315P after reaction with (*E,E*)-FPP. (**I**) LC-MS analysis of nonalkylated and alkylated S[561-577]K of wild-type γHS after reaction with SPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. (**J**) MS/MS analysis of nonalkylated S[561-577]K of wild-type γHS after reaction with (E,E)-FPP.

K



L



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]
b11	1260.621	1260.586	0.035	b7	785.339	785.379	-0.04
y10	1194.615	1194.539	0.076	y6	737.404	737.347	0.057
b10	1131.544	1131.539	0.005	y5	622.453	622.32	0.133
y9	1079.607	1079.512	0.095	b5	514.308	514.251	0.057
b9	1060.583	1060.506	0.077	y4	443.218	443.214	0.004
y8	964.485	964.549	-0.064	y3	347.09	347.193	-0.103
b8	932.485	932.448	0.037	b3	328.238	328.187	0.051
y7	893.622	893.448	0.174	y2	276.225	276.156	0.069
				b2	213.291	213.16	0.131

Figure S14. Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS). (**K**) MS analysis of nonalkylated L[486-497]K of γHS W315P after reaction with (*E,E*)-FPP. (**L**) MS/MS analysis of nonalkylated L[486-497]K of γHS W315P after reaction with (*E,E*)-FPP.

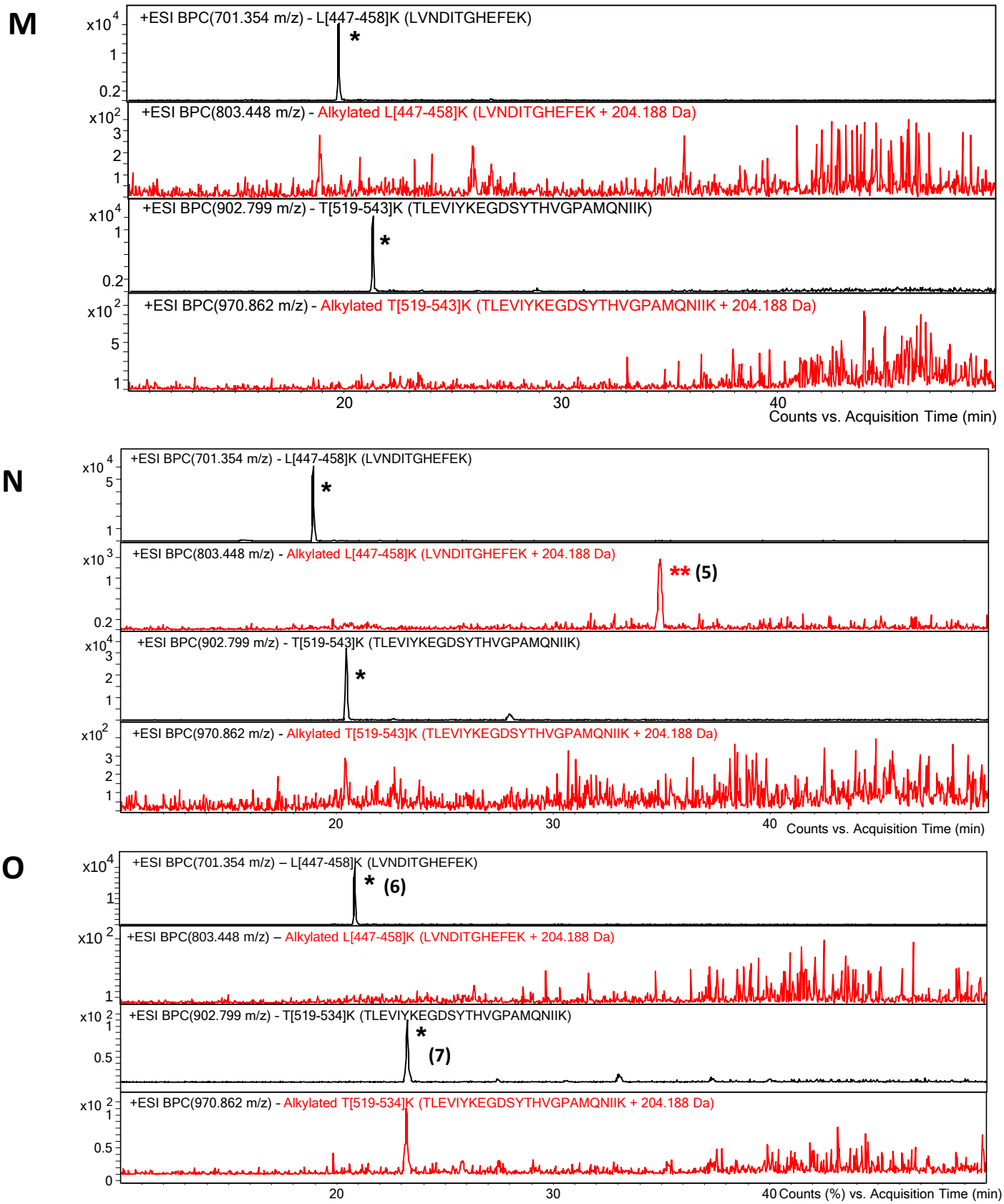
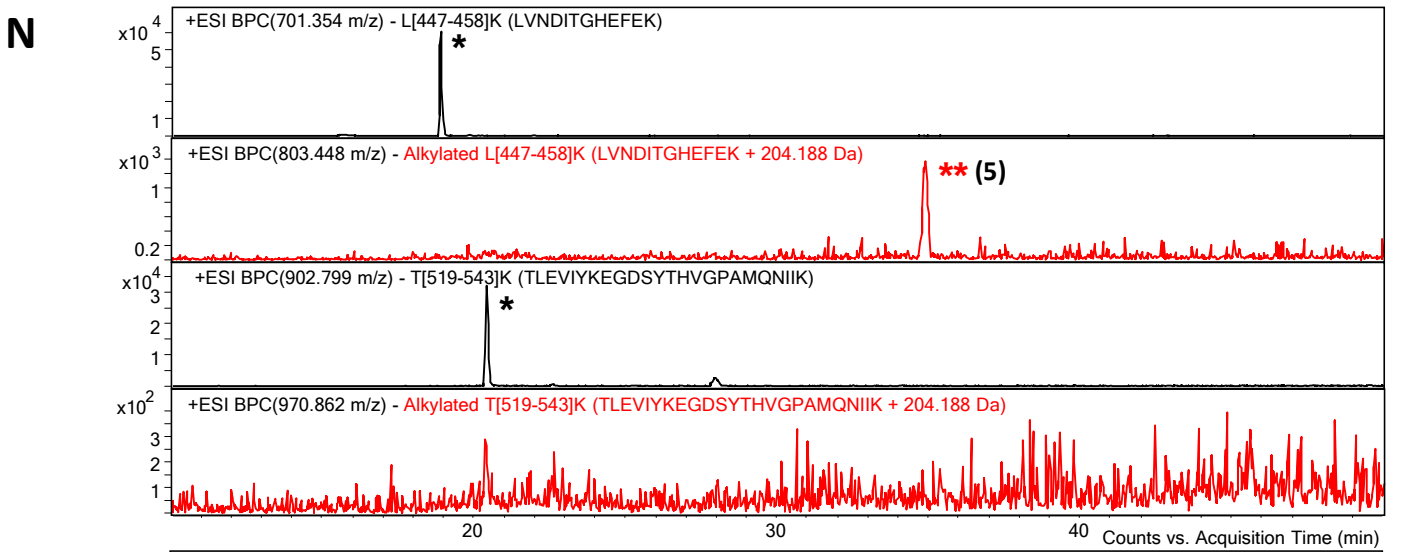
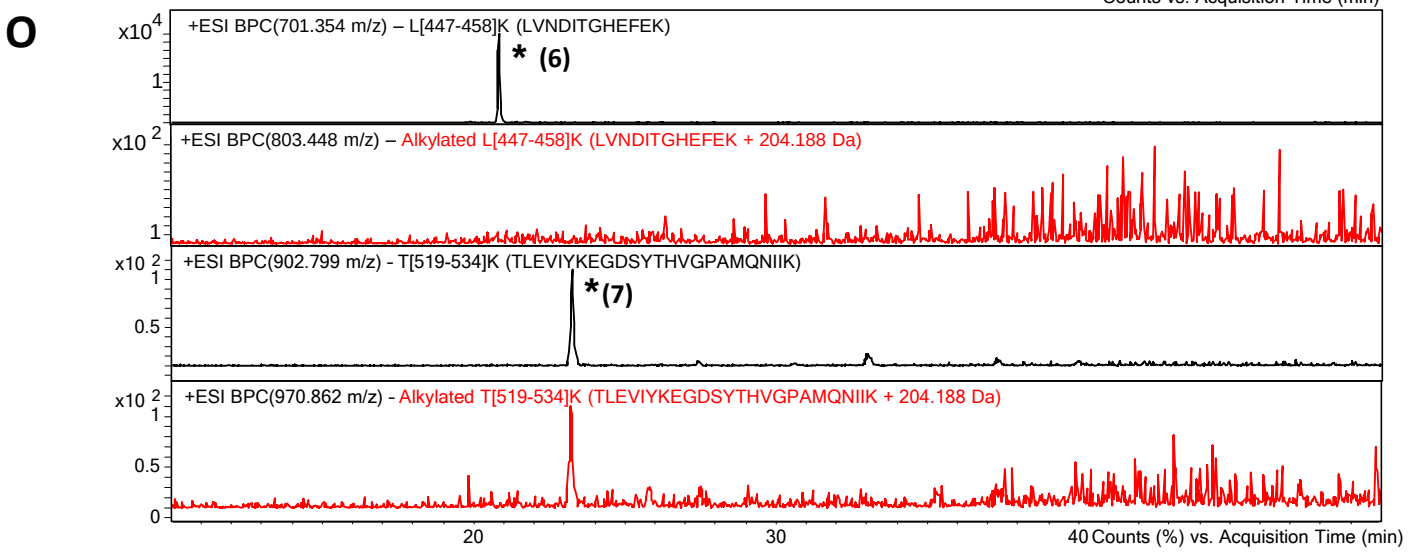
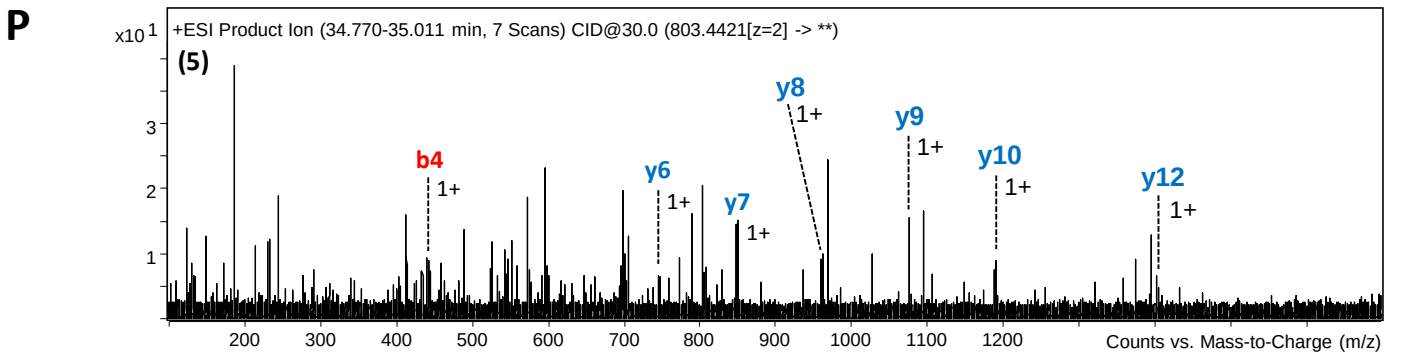


Figure S14. Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS). (**M**) LC-MS analysis of nonalkylated and alkylated L[447-458]K and T[519-543]K of wild-type PAS after reaction with (*E,E*)-FPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. (**N**) LC-MS analysis of non-alkylated and alkylated L[447-458]K and T[519-543]K of wild-type PAS after reaction with SPP. (**O**) LC-MS analysis of nonalkylated and alkylated L[447-458]K and T[519-543]K of PAS W276E after reaction with (*E,E*)-FPP. Single asteriks mark BPC peaks of non-alkylated peptides, double asteriks mark BPC peaks of alkylated peptides.



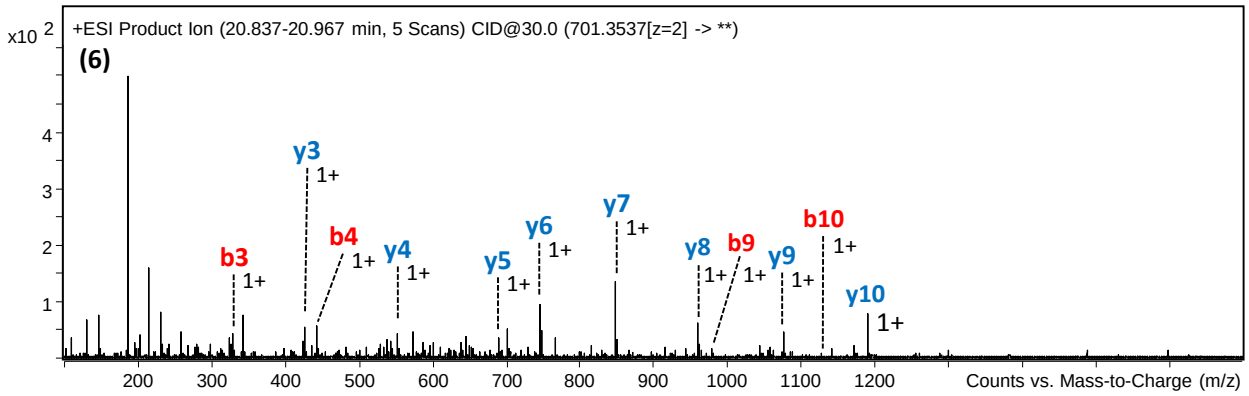




Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y12	1401.685	1401.702	0.017	12.1
y10	1189.535	1189.549	0.014	11.8
y9	1075.514	1075.506	-0.008	7.4
y8	960.471	960.479	0.008	8.3
y7	847.402	847.392	-0.010	11.8
y6	746.336	746.347	0.011	14.7
b4	442.225	442.230	0.005	11.3

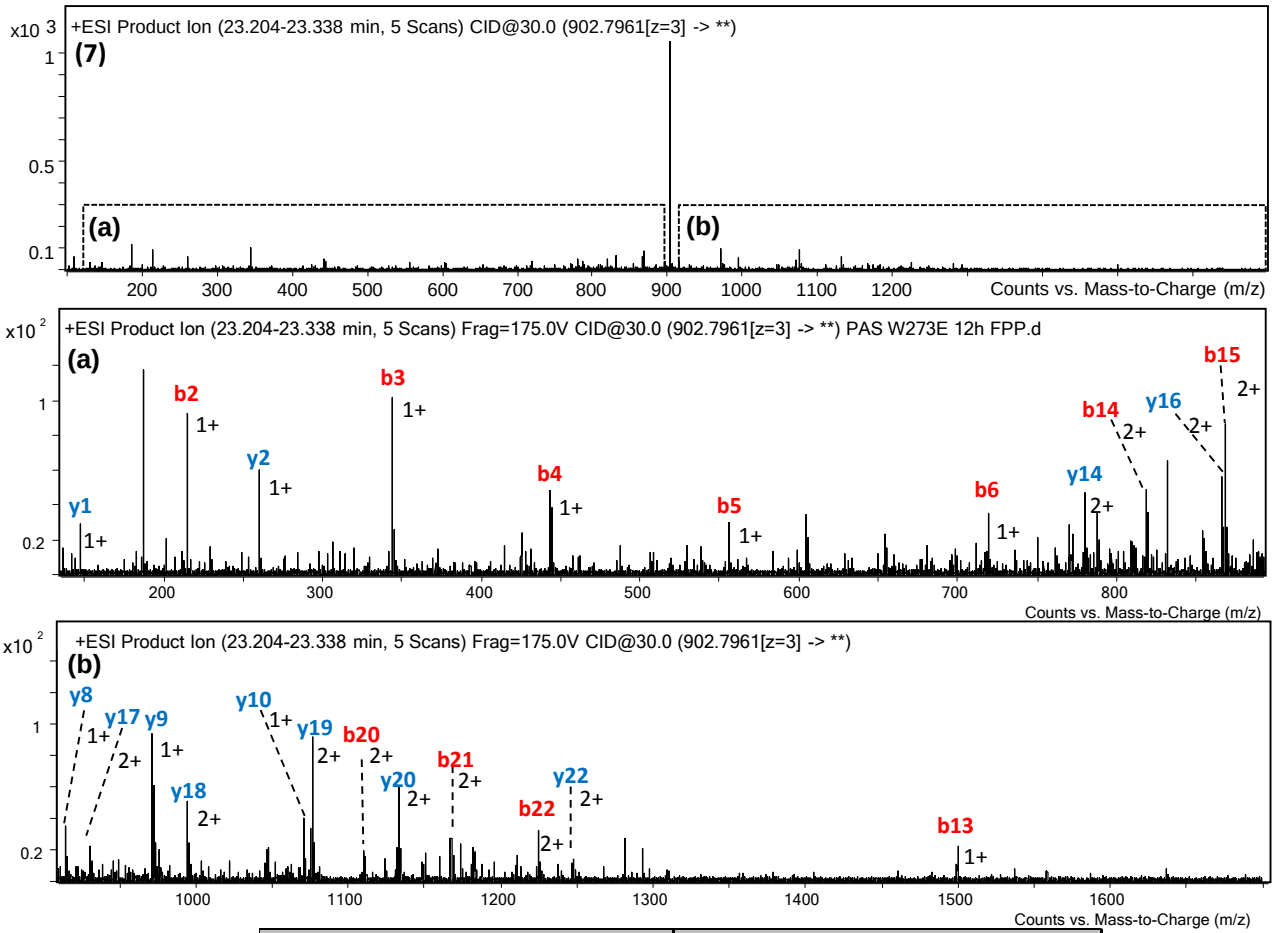
Figure S14. Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS).
(**N**) LC-MS analysis of nonalkylated and alkylated L[447-458]K and T[519-543]K of wild-type PAS after reaction with SPP. (**O**) LC-MS analysis of nonalkylated and alkylated L[447-458]K and T[519-543]K of PAS W276E after reaction with (*E,E*)-FPP. Single asteriks mark BPC peaks of nonalkylated peptides, double asteriks mark BPC peaks of alkylated peptides. (**P**) MS/MS analysis of alkylated L[447-458]K (**5**) of wild-type PAS after reaction with SPP.

Q



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y10	1189.533	1189.549	0.016	13.6	y5	689.337	689.326	-0.0113	16.4
b10	1126.543	1126.553	0.010	9.1	y4	552.265	552.267	0.002	4.2
y9	1075.502	1075.506	0.004	3.4	b4	442.227	442.230	0.004	8.2
b9	979.489	979.485	-0.0037	3.8	y3	423.218	423.224	0.007	16.1
y8	960.469	960.479	0.010	10.3	b3	327.204	327.203	-0.0006	1.9
y7	847.394	847.395	0.001	0.8	b2	213.157	213.160	0.004	17.6
y6	746.348	746.347	-0.0003	0.4	y1	147.112	147.113	0.001	8.8

R



Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]	Fragment	m(obs) [Da]	m(calc) [Da]	Δm [Da]	Δm [ppm]
y22	2492.282	2492.250	-0.0324	13	y14	1558.795	1558.805	0.011	6.9
b22	2447.190	2447.192	0.002	0.9	b13	1499.735	1499.727	-0.008	5.5
b21	2334.129	2334.108	-0.021	9	y8	914.502	914.513	0.011	12.3
y20	2264.166	2264.139	-0.0273	12.1	b6	719.390	719.398	0.008	11.7
b20	2220.040	2220.065	0.024	11	b5	556.335	556.330	-0.005	8.4
y19	2151.028	2151.055	0.027	12.3	b4	443.251	443.250	-0.001	1.4
y18	1987.978	1987.991	0.013	6.5	b3	344.184	344.182	-0.002	4.7
b15	1735.848	1735.854	0.006	3.7	y2	260.199	260.197	-0.002	7.1
y16	1730.862	1730.854	-0.0084	4.8	b2	215.139	215.140	0.000	1
b14	1636.783	1636.786	0.003	2.1	y1	147.114	147.113	0.000	2

Figure S14 | Alkylation analysis of multiproduct sesquiterpene synthases *Santalum spicatum* santalene synthase (SspiSSy), *Abies grandis* γ-humulene synthase (γHS) and *Pogostemon cablin* patchoulol synthase (PAS). (**P**) MS/MS analysis of alkylated L[447-458]K (**5**) of wild-type PAS after reaction with SPP. (**Q**) MS/MS analysis of nonalkylated L[447-458]K (**6**) of PAS W276E after reaction with (*E*,*E*)-FPP. (**R**) MS/MS analysis of nonalkylated T[519-543]K (7) of PAS W276E after reaction with (*E*,*E*)-FPP.