The Aroma Volatiles of Cannabis
How Terpene Synthases Create Chemical Diversity

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Cannabinoids are the primary determinants of cannabis potency

Terpenes may contribute to the full-spectrum effects of cannabis consumption

Terpenes are key contributors to the unique aroma of cannabis products

Cannabinoids and Terpenes: Chemically Diverse Classes of Natural Products

Lange & Zager (2022) *Phytochem. Rev.*

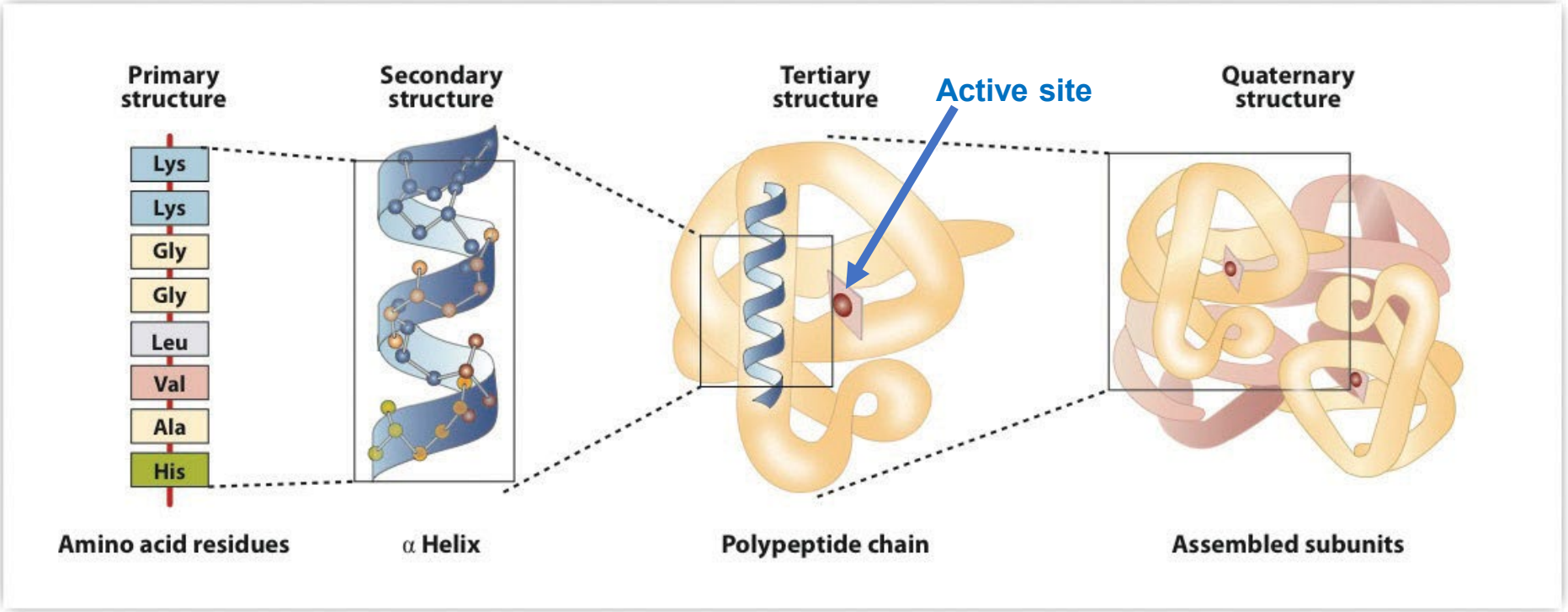
130 unique structures
(< 10 routinely analyzed)

Adams R.P. (2007) *Allured Publishing*

2,205 unique structures in GC-MS library
(< 20 routinely analyzed)

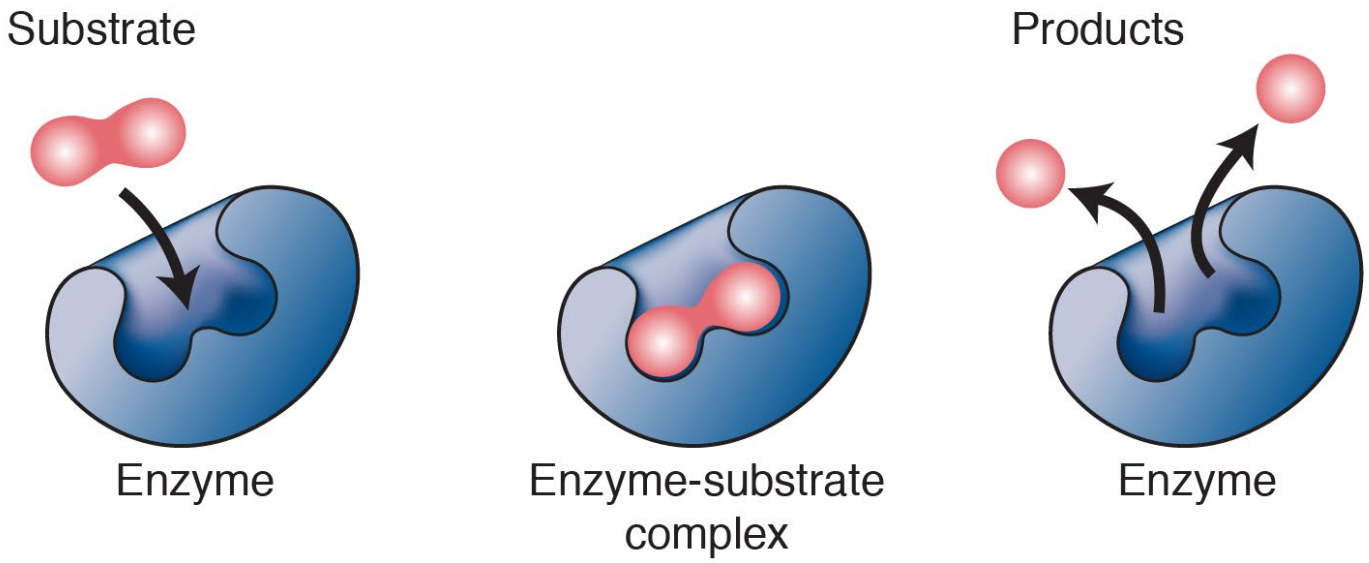
Terpene Biosynthesis: First Committed Step is Catalyzed by Terpene Synthases

Terpene Synthase Catalysis:
Brief Introduction to Enzyme Function I

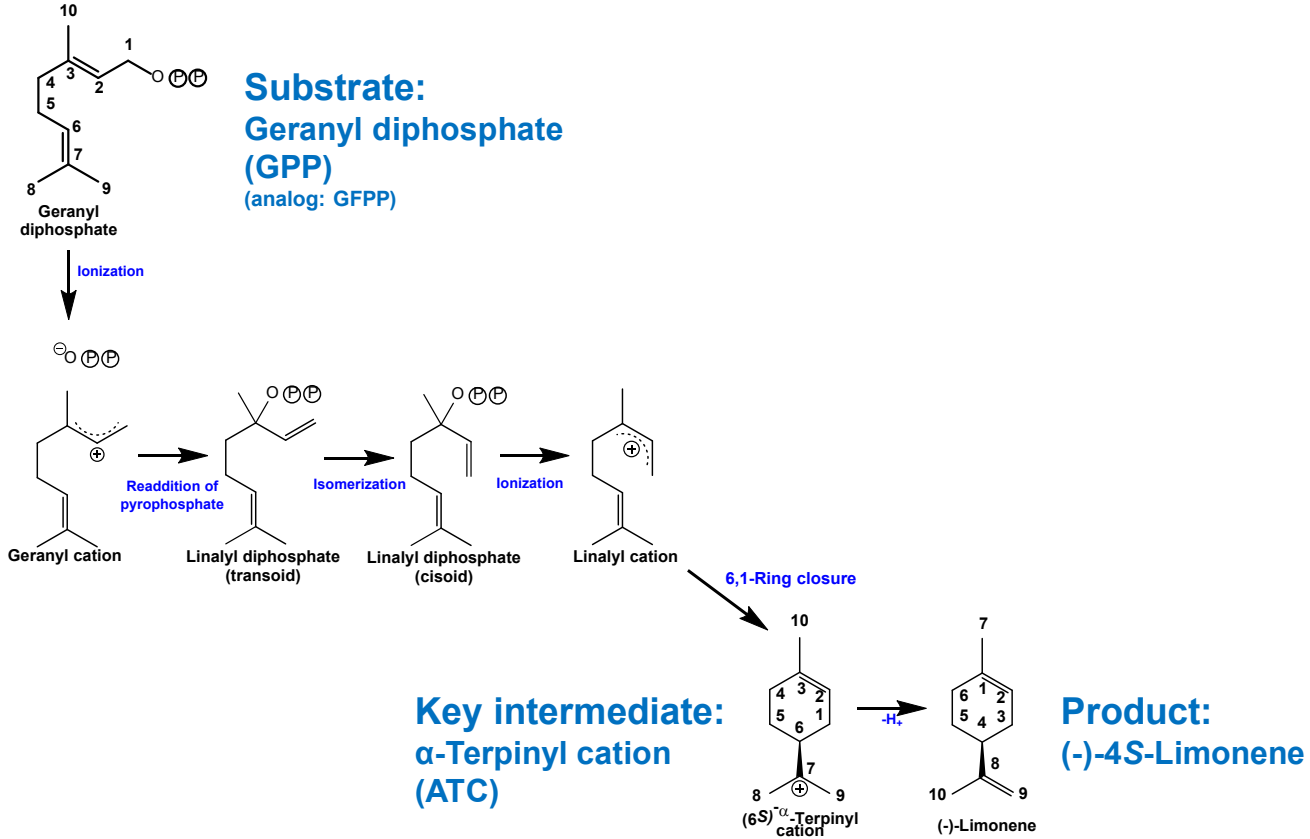


Terpene Synthase Catalysis:
Brief Introduction to Enzyme Function II

What Happens Inside an Enzyme's Active Site?



Monoterpene Synthase (MTS) Mechanism:
Spearmint (-)-4*S*-Limonene Synthase (LMNS) as an Example



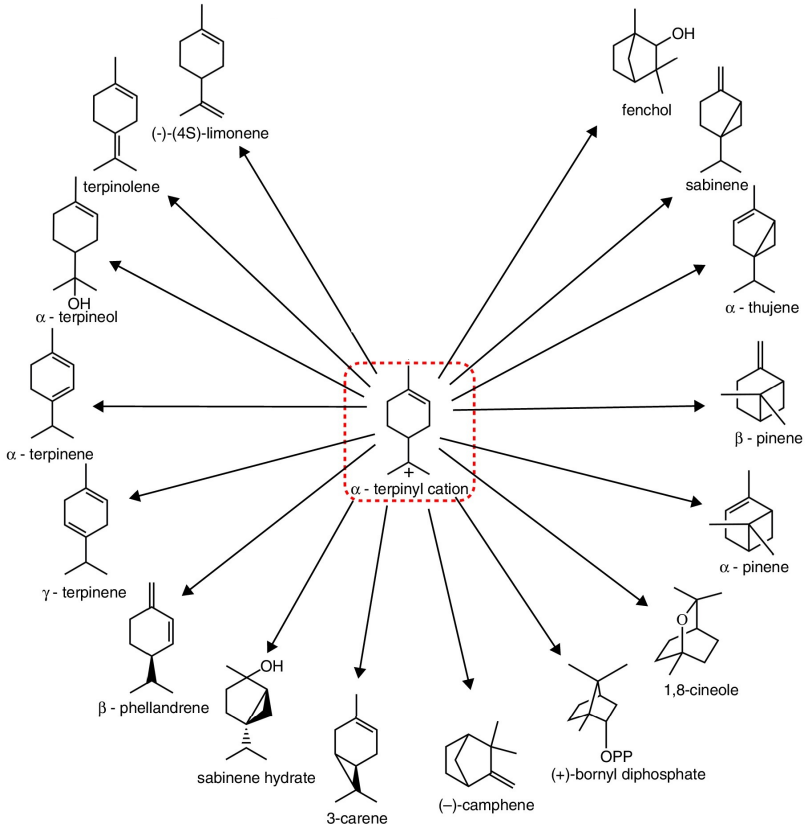
Substrate:
Geranyl diphosphate (GPP)
(analog: GFPP)

Key intermediate:
α-Terpinyl cation (ATC)

Product:
(-)-4*S*-Limonene

What are the Determinants of Specificity in Monoterpene Synthases?

MONOCYCLIC



BICYCLIC

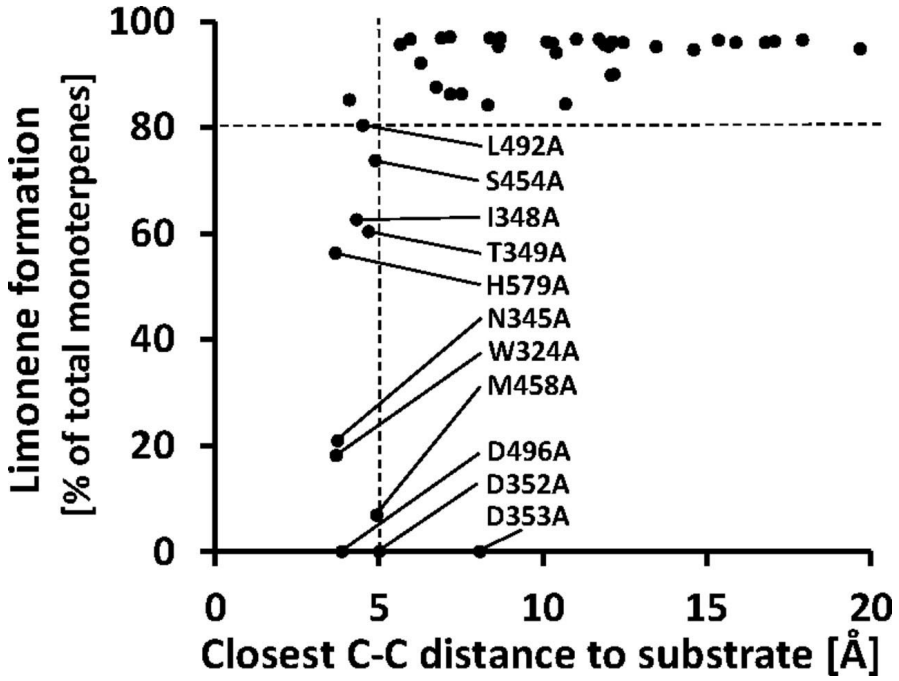
Defining the Active Site of a Terpene Synthase:
Spearmint (-)-4*S*-Limonene Synthase (LMNS) as an Example

Srividya et al. (2015) *Proc Natl. Acad. Sci. USA* 112, 3332-3337

Determine distances between substrate and putative active site residues based on crystal structure data

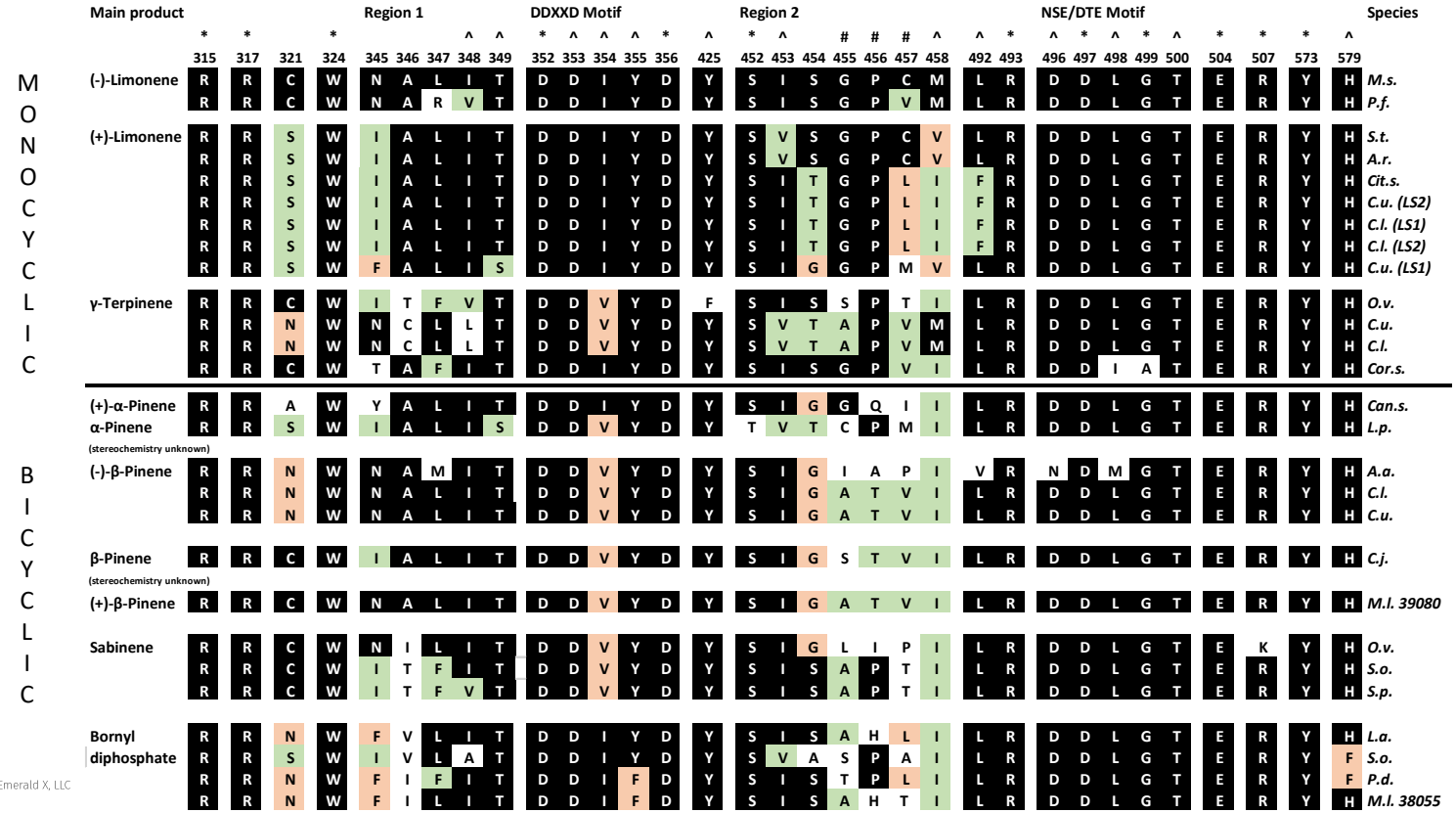
Mutate candidate active site residues to L-alanine

Characterize properties of enzyme mutants
- **express corresponding gene in *E. coli***
- **purify recombinant protein**
- **perform *in vitro* assays**
- **analyze products by GC-MS**



Comparing Active Site Residues Across Characterized Monoterpene Synthases (MTSs)

Kim et al. (2022) *ACS Catal.*

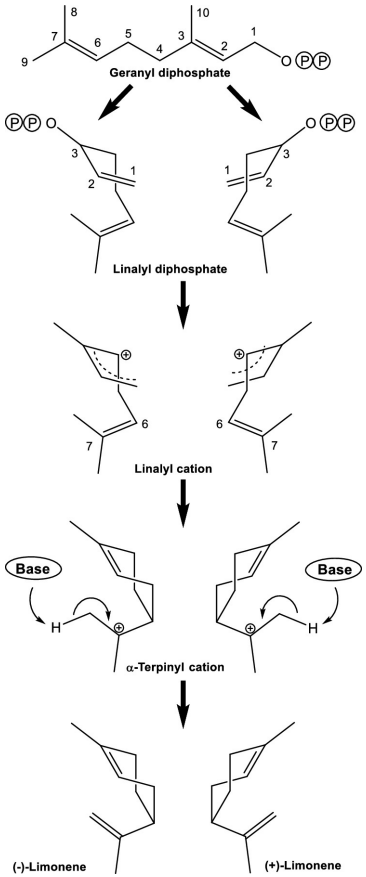


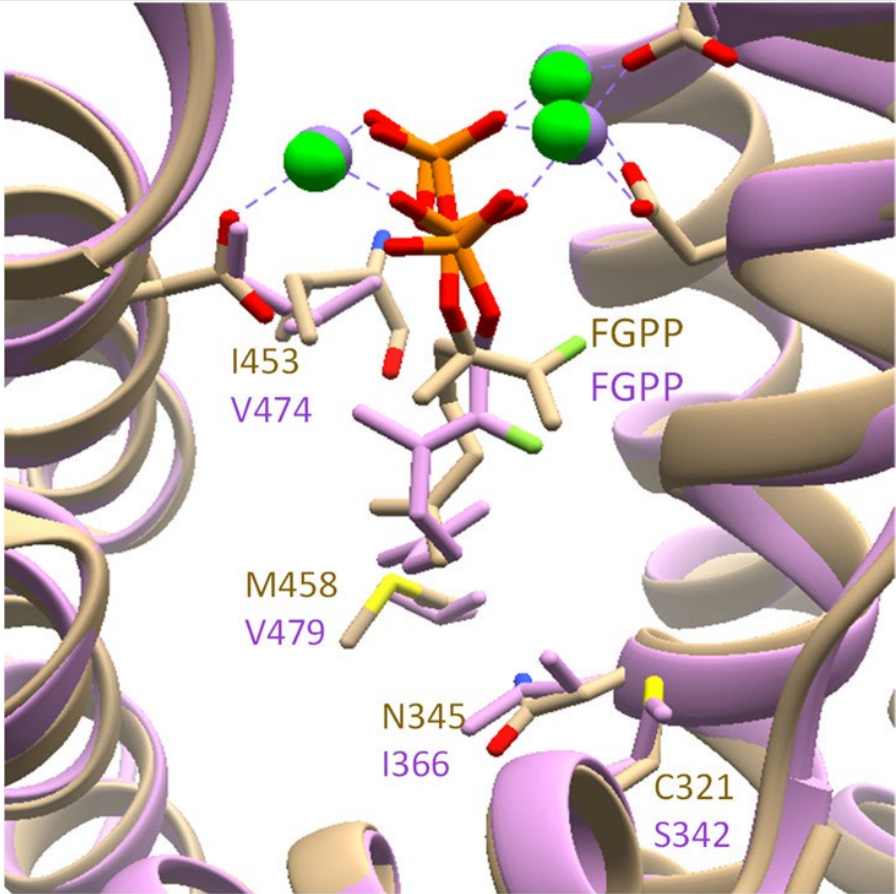
Identifying the Determinants of Enantioselectivity in Monoterpene Synthases (MTSs)

Srividya et al. (2020) *Biochemistry* 59, 1661-1664

(-)-4*S*-Limonene synthase (spearmint LMNS)
Color: sand

(+)-4*R*-Limonene synthase (Japanese catnip)
Color: purple





Identifying the Determinants of Enantioselectivity in Monoterpene Synthases (MTSs)

Srividya et al. (2020) *Biochemistry* 59, 1661-1664

Mutations to reverse the enantioselectivity of LMNS to produce (+)-limonene

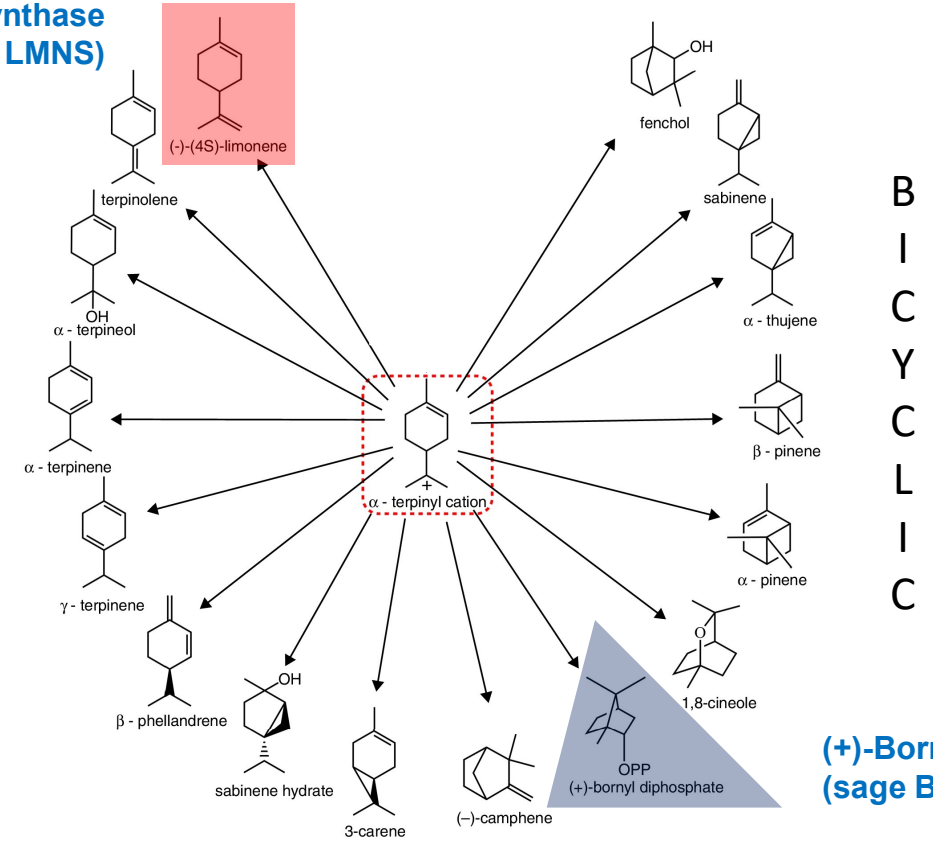
product	(−)-limonene synthase (*M. spicata*)	(+)-limonene synthase (*S. tenuifolia*)	C321S	N345I	I453V	M458I	M458V	N345I/ M458V	N345I/ M458I	N345I/ I453V/ M458V	C321S/N345I/ I453V/M458V
(−)-limonene	96.8	1.5	94.9	16.0	90.9	4.6	3.1	4.9	2.4	3.9	3.0
(+)-limonene	0.0	95.8	0.0	1.4	3.6	0.0	15.2	48.5	0.8	70.1	80.2
(+)-sabinene	0.0	0.3	0.0	18.5	0.0	6.4	52.2	23.2	0.0	15.6	10.5
(−)-sabinene	0.0	0.0	0.0	7.2	0.0	52.1	13.8	0.4	15.5	0.5	0.0
α-thujene	0.0	0.0	0.0	20.4	0.0	3.8	0.0	1.0	0.0	0.0	0.0
other	3.2	2.4	5.1	36.5	5.5	33.1	15.7	22.0	81.3	9.9	6.3

Identifying the Determinants of the Ratio of Monocyclic to Bicyclic Monoterpenes

Kim et al. (2022) *ACS Catal.*

(-)-4*S*-Limonene synthase (spearmint LMNS)

MONOCYCLIC

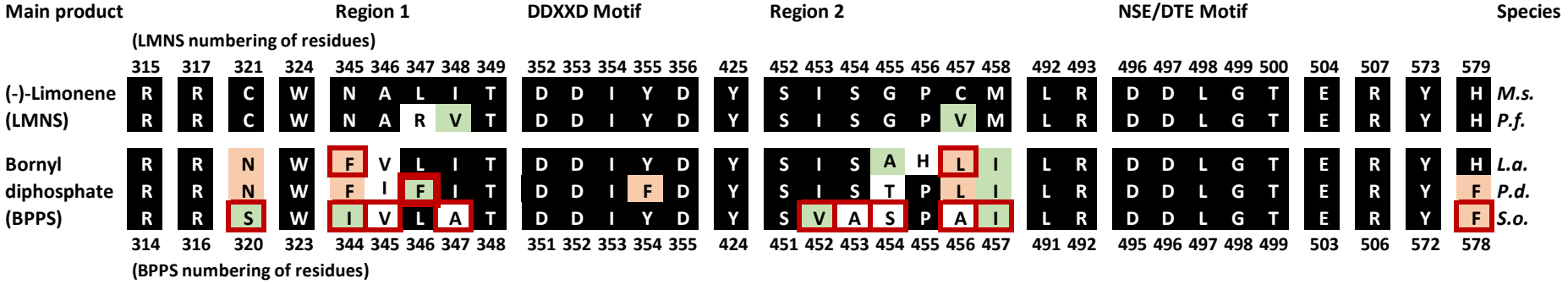


BICYCLIC

(+)-Bornyl diphosphate synthase (sage BPPS)

Variants of (-)-Limonene Synthase With Single Amino Acid Exchange Generate More Bicyclic Products

Kim et al. (2022) *ACS Catal.*

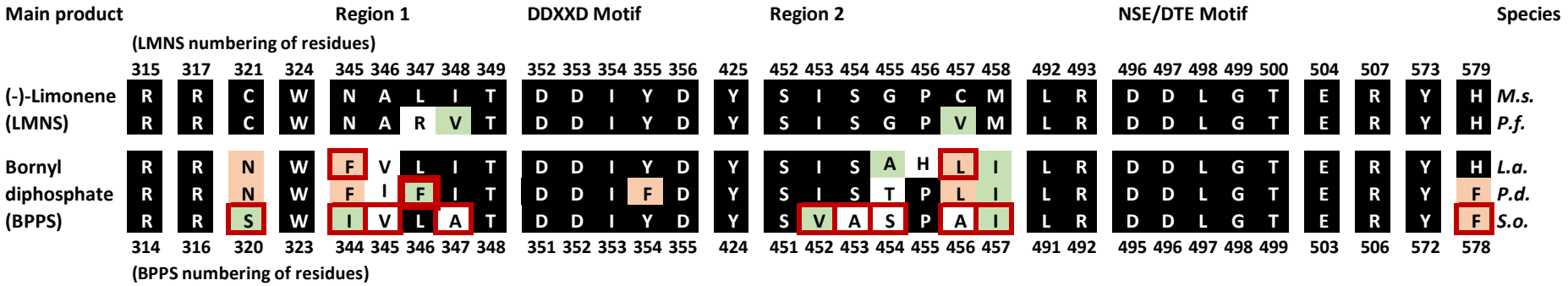


Product	Construct										
	Wild-type	C321N	C321S	N345I	N345F	A346V	L347F	I348A	I348V	I453V	S454A
Acyclic	0.9	1.4	1.1	19.1	23.2	3.5	1.1	15.0	1.3	1.5	2.3
Monocyclic	96.6	94.4	94.6	24.6	20.4	89.1	93.7	70.4	95.1	93.6	79.7
Bicyclic	2.5	4.1	4.3	56.3	56.4	7.5	5.2	14.6	3.7	4.8	18.0

	Construct								
	S454G	G455A	G455S	P456T	C457A	C457L	C457V	M458I	H579F
Acyclic	1.8	1.1	0.9	1.4	1.1	22.0	0.5	2.3	1.2
Monocyclic	59.7	94.9	88.4	92.8	96.3	78.0	96.3	8.4	87.5
Bicyclic	38.4	4.1	10.7	5.7	2.5	0.0	3.2	89.3	11.3

Variants of BPPS With Single Amino Acid Exchange Generate More Monocyclic Products

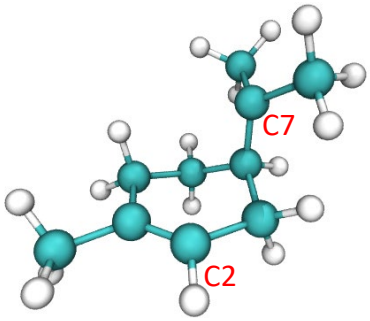
Kim et al. (2022) *ACS Catal.*



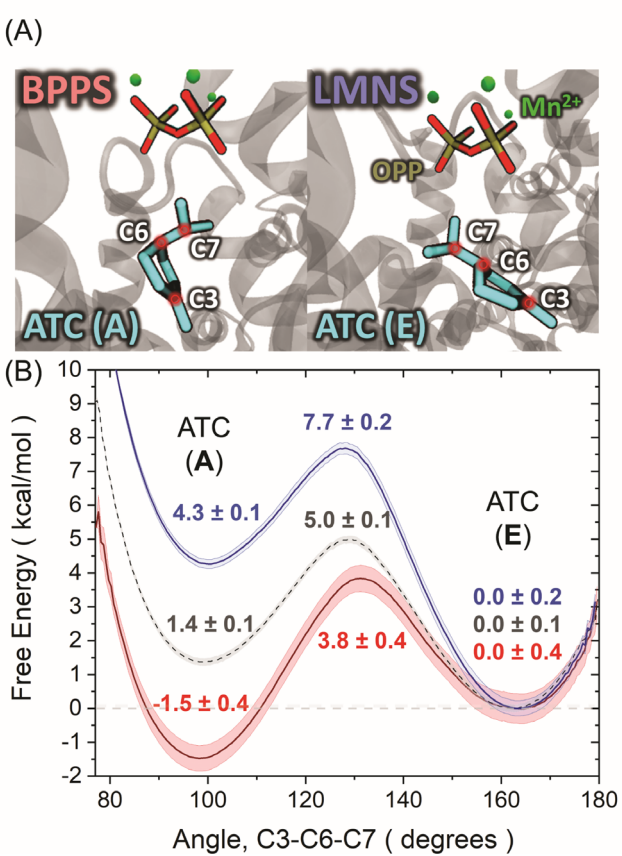
Product	Wild-type	I344N	V345A	A347I	V452I	A453S	S454G	A456C	I457M
Acyclic	2.1	3.4	1.6	1.5	6.3	1.5	5.8	1.6	3.8
Monocyclic	9.7	21.8	10.5	8.2	16.5	13.3	70.2	12.4	23.7
Bicyclic	88.2	74.8	88.0	90.4	77.3	85.3	24.0	83.6	72.6

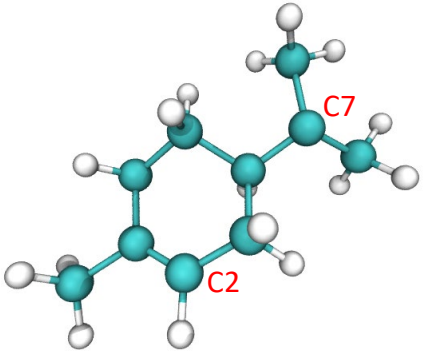
Free Energy Calculations Indicate That the ATC Assumes Different Conformations in LMNS and BPPS

Kim et al. (2022) *ACS Catal.*



ATC
(A)



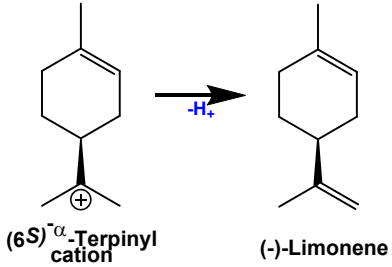


ATC
(E)

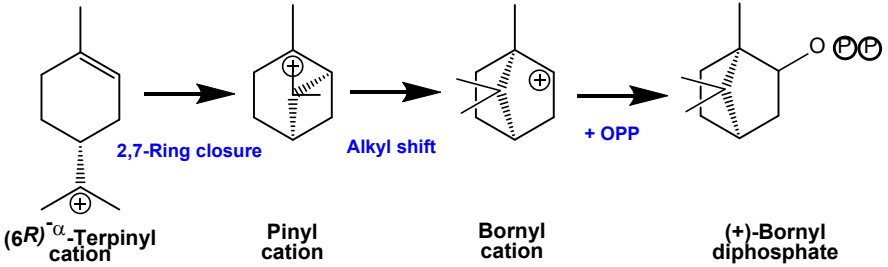
Formation of a Secondary Pinyl Cation is Energetically Favored in BPPS but Not in LMNS

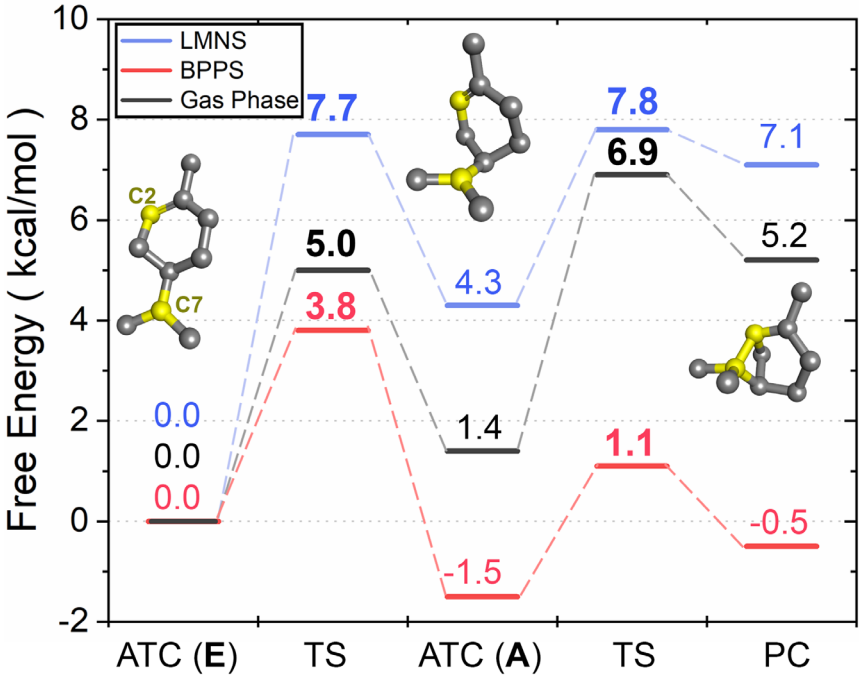
Kim et al. (2022) *ACS Catal.*

LMNS



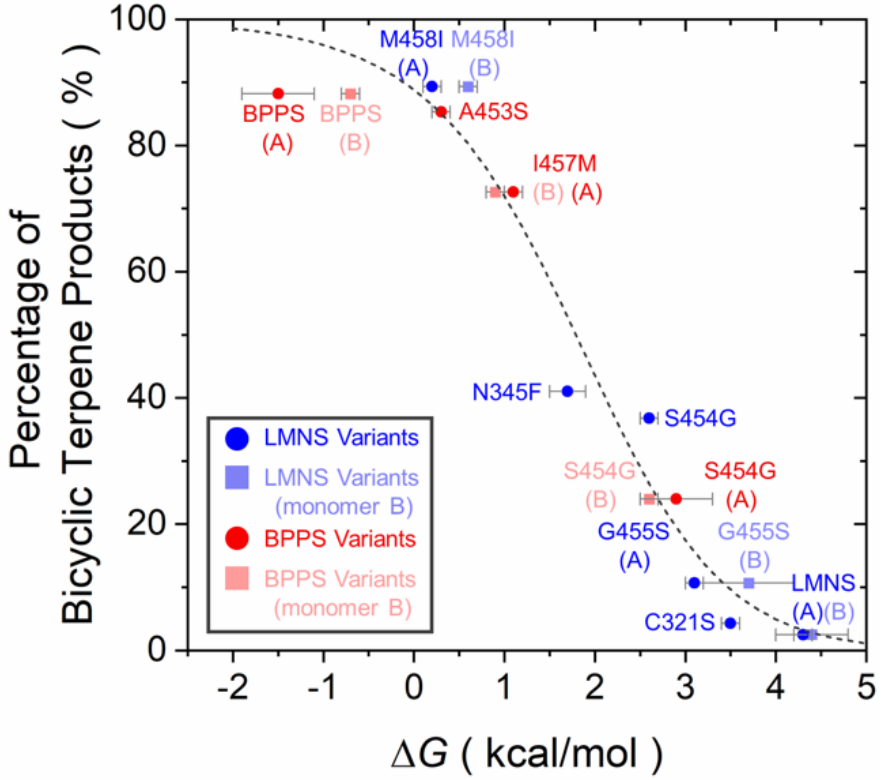
BPPS





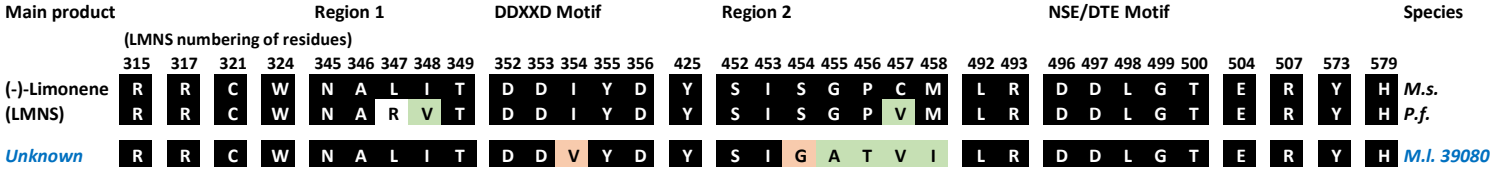
Free Energy Difference Between **A** and **E** Conformers of ATC Correlates With Product Formation

Kim et al. (2022) *ACS Catal.*



Combination of Sequence and Structural Comparisons Predicts Function of Previously Uncharacterized MTS

Kim et al. (2022) *ACS Catal.*



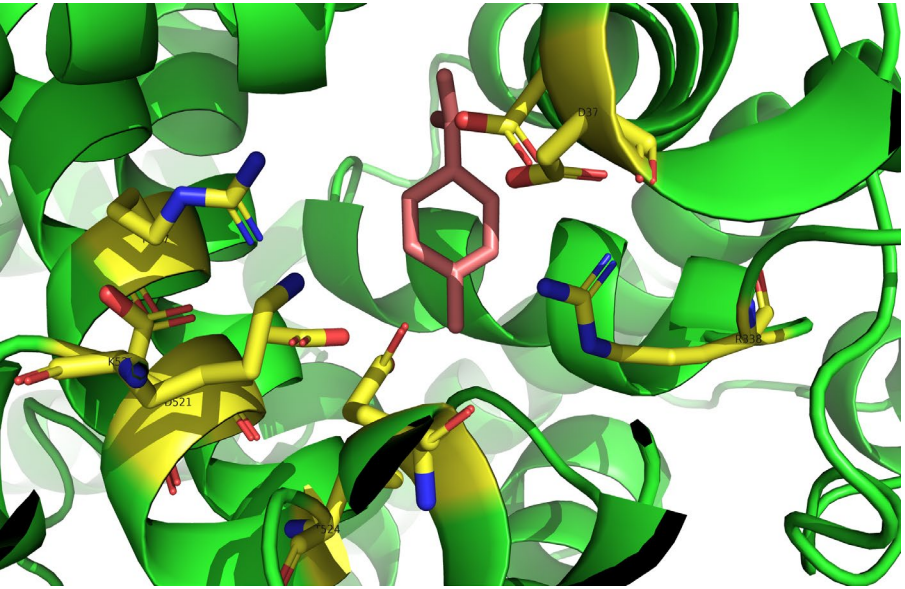
Product	Wild-type	C321N C457V M458I	S454G C457V M458I	C321N S454G C457V M458I	S454G G455A P456T C457V M458I	C321N S454G G455A P456T C457V M458I	*M.l.39080*
Acyclic							
β-Myrcene	0.9 ± 0.1	3.6 ± 0.3	2.5 ± 0.2	2.9 ± 0.2	5.7 ± 0.8	2.0 ± 0.1	1.6 ± 0.1
Monocyclic							
(-)-Limonene	96.6 ± 0.3	2.7 ± 0.4	7.7 ± 0.3	6.4 ± 0.6	2.3 ± 0.5	9.7 ± 0.9	1.4 ± 0.1
(+)-Limonene				0.9 ± 0.1		12.3 ± 2.0	
Terpinolene			1.3 ± 0.09	1.4 ± 0.1			
γ-Terpinene			1.6 ± 0.11	1.3 ± 0.3			
β-Phellandrene		4.7 ± 0.1	2.2 ± 0.15	2.2 ± 0.1			
1,8-Cineole			1.9 ± 0.1	0.5 ± 0.1			
(-)-α-Terpineol			3.4 ± 0.5	1.3 ± 0.4			
Bicyclic							
(-)-α-Pinene	0.6 ± 0.1	8.5 ± 0.6	12.4 ± 2.6	9.2 ± 0.2	13.3 ± 1.2	8.7 ± 2.3	7.6 ± 0.5
(+)-α-Pinene	0.6 ± 0.1	3.0 ± 0.3	1.1 ± 0.1	2.1 ± 0.3	3.0 ± 0.4		
(-)-β-Pinene	0.5 ± 0.1	45.1 ± 0.3	10.3 ± 0.2	38.3 ± 0.1	52.2 ± 3.2	52.1 ± 0.1	83.6 ± 2.1
(+)-β-Pinene	0.5 ± 0.1	4.3 ± 0.4	2.4 ± 0.2	3.6 ± 0.2	3.1 ± 0.7	2.7 ± 0.1	1.8 ± 0.1
(-)-Sabinene	0.3 ± 0.1	24.3 ± 0.3	46.8 ± 1.8	26.0 ± 0.9	18.1 ± 0.9		4.1 ± 0.2
(+)-Sabinene		3.8 ± 0.5	6.4 ± 0.7	3.3 ± 0.1	2.3 ± 0.7		

Combining Sequence and Structure Comparisons to Predict Cannabis Monoterpene Synthase Function

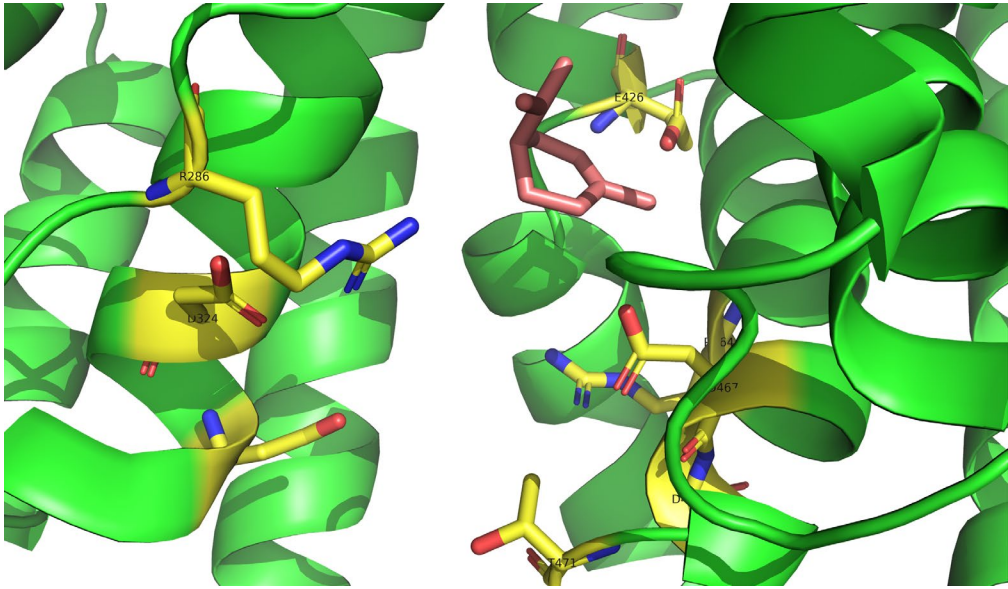
Zager et al. (2019) *Plant Physiol.* 180, 1877-1897

Annotation	Clade	Main product(s)	Region 1								DDXXD Motif						Region 2							NSE/DTE Motif								
			*	#		*			#	^	#	*	*	#	^	*	#	*							*	^	*	#	^	^	*	#	#	
		Reference: (-)-limonene synthase (spearmint)	315	317	321	324	345	346	347	348	349	352	353	354	355	356	425	452	453	454	455	456	457	458	492	493	496	497	498	499	500	504	507	573	579
		Acyclic monoterpenes																																	
CsTPS3FN	TPS-b2	β-myrcene	R	R	A	W	F	V	L	I	T	D	D	I	Y	D	Y	S	I	G	A	S	V	I	T	R	D	D	L	G	T	E	R	Y	H
CsTPS15CT *	TPS-b2	β-myrcene	R	R	A	W	F	V	L	I	T	D	D	I	Y	D	Y	S	I	G	A	S	V	I	T	R	D	D	L	G	T	E	R	Y	H
HlMTS2	TPS-b2	β-myrcene	R	R	A	W	F	V	L	I	T	D	D	I	Y	D	Y	S	I	G	G	P	V	I	V	R	D	D	F	G	T	E	R	Y	H
CsTPS23CH	TPS-b2	β-myrcene/linalool/limonene/terpinolene	R	R	C	W	Y	V	L	I	T	D	D	I	Y	D	Y	S	V	G	A	P	V	I	V	R	N	D	L	A	T	E	R	Y	H
CsTPS38FN	TPS-b2	(E)-β-ocimene	R	R	C	W	Y	V	L	I	T	D	D	I	Y	D	Y	S	V	G	A	P	I	I	V	R	N	D	L	A	T	E	R	Y	H
CsTPS6FN	TPS-b2	(E)-β-ocimene	R	R	C	W	F	E	L	V	T	D	D	I	Y	D	Y	S	V	G	A	P	V	I	L	R	N	D	L	A	T	E	R	Y	H
CsTPS5FN	TPS-b1	β-myrcene/α-pinene/limonene/sabinene	R	R	I	W	G	Q	L	V	T	D	D	I	F	D	Y	S	I	A	E	P	L	I	A	R	D	D	L	G	T	E	R	Y	V
CsTPS17BC *	TPS-b2	β-myrcene/linalool/geraniol	R	Q	W	W	I	S	L	V	Y	D	D	I	F	D	Y	S	S	G	V	P	V	V	L	R	D	D	L	G	S	E	N	Y	Q
		Monocyclic monoterpenes																																	
CsTPS37FN	TPS-b2	terpinolene	R	R	A	W	Y	V	L	I	T	D	D	I	Y	D	Y	S	V	G	G	P	I	I	L	R	D	D	L	G	T	E	R	Y	H
CsTPS33PK	TPS-b2	α-terpinene/γ-terpinene	R	R	C	W	Y	V	L	I	T	D	D	I	Y	D	Y	S	V	S	G	P	I	I	L	R	N	D	L	A	T	E	R	Y	H
CsTPS14CT *	TPS-b2	(-)-limonene	R	R	C	W	Y	V	L	L	T	D	D	M	H	D	Y	S	V	G	G	Q	V	I	L	R	D	D	L	G	T	E	R	Y	H
CsTPS1FN	TPS-b2	(-)-limonene	R	R	C	W	Y	V	L	L	T	D	D	M	H	D	Y	S	V	G	G	Q	V	I	L	R	D	D	L	G	T	E	R	Y	H
		Bicyclic monoterpenes																																	
CsTPS2FN	TPS-b2	(+)-α-pinene	R	R	A	W	Y	A	L	I	T	D	D	I	Y	D	Y	S	I	G	G	Q	V	I	L	R	D	D	L	G	T	E	R	Y	H
CsTPS2SK	TPS-b2	(+)-α-pinene	R	R	A	W	Y	A	L	I	T	D	D	I	Y	D	Y	S	I	G	G	Q	I	I	L	R	D	D	L	G	T	E	R	Y	H
		Monoterpene/sesquiterpene																																	
CsTPS31PK	TPS-b1	terpinolene/unknown sesquiterpene	R	R	I	W	G	L	L	L	T	D	D	I	Y	D	F	S	I	T	I	P	L	C	F	R	D	D	L	G	T	E	R	Y	F
CsTPS5PK	TPS-b1	α-pinene/bisabolol	R	R	T	W	G	Q	L	V	T	D	D	I	F	D	Y	S	I	A	E	P	L	I	A	R	D	D	L	G	T	E	R	Y	V
CsTPS32PK	TPS-b1	multiple monoterpenes and sesquiterpenes	R	R	T	W	G	Q	L	V	T	D	D	I	F	D	Y	S	I	A	E	P	L	I	A	R	D	D	L	G	T	E	R	Y	V
CsTPS35LS	TPS-b1	linalool/nerolidol	R	Q	W	W	I	S	L	V	Y	D	D	I	F	D	Y	S	S	G	V	H	V	V	L	R	D	D	L	G	S	E	N	Y	H
CsTPS18VF *	TPS-g	linalool/nerolidol	R	Q	W	W	I	S	F	V	Y	D	D	I	F	D	Y	S	S	G	V	H	V	V	L	R	D	D	F	G	S	E	E	Y	Q
CsTPS18Choco	TPS-g	linalool	R	Q	W	W	I	S	F	V	Y	D	D	I	F	D	Y	S	S	G	V	H	V	V	L	R	D	D	F	G	S	E	E	Y	Q
CsTPS19BL *	TPS-g	linalool/nerolidol	R	R	C	W	L	E	L	I	T	D	D	I	Y	D	Y	S	V	G	A	P	V	L	I	R	N	D	L	A	T	E	R	Y	H
CsTPS29BC *	TPS-g	linalool	R	R	N	W	V	C	I	L	T	D	D	V	Y	D	Y	S	V	G	G	H	A	A	T	R	D	D	L	G	T	E	R	F	I

Combining Sequence and Structure Comparisons to Predict Cannabis Monoterpene Synthase Function



CsTPS1 (limonene synthase)



CsTPS5 (α-pinene/bisabolol synthase)

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Anthony Smith, Kaycha Labs
Alireza Shaneh, Freelancer

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